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Titel der Diplomarbeit

„The role of melatonin in blood pressure modulation - Characterization of specificity of anti-melatonin receptor 1 (MT_1) antibodies“

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Andreas Maier

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omnia mutantur, nihil interit

Everything changes, nothing perishes

(Ovid, Metamorphoses)

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1 Abstract

Background: The hormone melatonin is secreted by the mammalian pineal gland in the absence of light during the night. A major function of pineal-derived melatonin is the control of circadian and seasonal rhythmicities via the biological master clock, the suprachiasmatic nucleus. In addition, melatonin influences various body functions such the cardiovascular system. Studies in animals and humans indicate that melatonin impacts on blood pressure. A recent meta-analysis concluded that controlled-release melatonin significantly lowers systolic and diastolic blood pressure by a magnitude that is considered to be clinically relevant. Melatonin is therefore regarded as a putative antihypertensive agent. One of the mechanisms discussed for the anti-hypertensive effects of melatonin is a direct impact on the resistance of peripheral vessels via specific G protein-coupled melatonin receptors, MT_1 and/or MT_2. MT_1 and/or MT_2 expression was demonstrated in several rat vessels such as aorta, cerebral and caudal arteries, but mainly at mRNA level in total vessel tissue. Distribution and cell-type specific function of MT_1 and/or MT_2 protein in the diverse structural layers of the vessel walls, i.e. endothelial cells, smooth muscle cells or cells within tunica adventitia and perivascular fat tissue (PVAT) remain to be explored comprehensively to understand the relation between melatonin and peripheral blood pressure control. Our unpublished data have shown MT_1 mRNA expression in mesenteric arteries (MA) and their associated PVAT, which participate in blood pressure regulation and published studies suggested that melatonin influences the anti-contractile function of MA-associated PVAT via yet unknown mechanisms. MT_1 protein expression and localization in MA and PVAT, however, are currently unknown. The lack of information concerning melatonin receptor protein expression and localization is related to the fact that the available anti-MT_1 and MT_2 antibodies, especially those for rodents, are not well characterized. In this context, western blotting is not only the most important technique to evaluate protein expression in tissues but is also indispensable to show the specificity of antibodies.

Aim: This diploma thesis had two major aims. First, to determine specificity and cross-reactivity of three commercial anti-rat MT_1 antibodies obtained from the companies Biorbyt, Santa Cruz Biotechnology and Alomone Labs by western blotting. Second, applying the most specific antibody identified, confirm translation of MT_1 protein in MA and MA-associated PVAT.

Methods: Specificity of antibodies and protein expression was analysed by western blotting. Various tissues were derived from Wistar rats. Eye, brain and cerebellum (positive controls), blood plasma (negative control), as well as MA and MA-PVAT protein lysates were prepared

either with T-PER buffer for total, or with Mem-PER for membrane protein fractions. Proteins were separated by 12 % SDS-PAGE, blotted on PVDF membranes and processed with an indirect detection protocol, using secondary HRP-conjugated antibodies, and chemiluminescence detection.

Results: The three tested antibodies exhibited significant differences in their specificity. Anti-rat MT_1 from company Alomone Labs reacted with proteins in the expected molecular weight range of MT_1 (40-60 kDa) in all positive control tissues. Specificity of binding was assessed with a blocking peptide, which completely abolished the binding of the antibody. Cross-reaction of antibodies with albumin is an often observed and published phenomenon. The Alomone Labs antibody did neither cross-react with albumin nor IgG, two major serum proteins, which were detected in most tissue lysates. The antibody from Biorbyt reacted with proteins of molecular weight corresponding to MT_1, but additionally exhibited significant cross-reaction with albumin. The antibody from Santa Cruz reacted predominantly with albumin, and binding could not be reduced with the corresponding blocking peptide. Using the anti-rat MT_1 antibody from Alomone Labs, expression of MT_1 protein in MA and MA-PVAT was subsequently demonstrated by western blotting in total protein as well as membrane protein lysates, confirming translation of MT_1 mRNA in these tissues. The results of this thesis enabled subsequent investigation of localization of MT_1 in rat MA and MA-PVAT using the Alomone Labs antibody. MT_1 protein expression was shown in muscle cells of MA and in adipocytes of PVAT. Data demonstrating MT_1 mRNA and protein expression as well as localization in rat MA and surrounding PVAT were presented as an abstract and poster at the 5th retreat of the Center for Pathophysiology, Infectiology and Immunology in September 2014.

Summary and Outlook: Two out of three investigated antibodies exhibited significant cross reactivity with albumin. Cross-reactions with irrelevant proteins such as albumin have been described in literature and the results of this thesis underline the importance of antibody specificity testing prior to protein expression and localization studies. Presence of MT_1 protein in MA and MA-PVAT opens the way for future functional studies in *in vitro* systems that need to explore in more detail the interaction of melatonin with MT_1 and the subsequent cellular events that influence blood pressure.

2 Zusammenfassung

Einleitung: Melatonin ist ein körpereigenes Hormon, das in der Zirbeldrüse gebildet und ins Blut abgegeben wird. Die Produktion wird durch einen inneren Taktgeber, den Suprachiasmatischen Kern, gesteuert und findet nur bei Abwesenheit von Licht statt. Daher ist die Konzentration von Melatonin im Blut in der Nacht am höchsten. Neben seiner Hauptaufgabe, der Kontrolle von zirkadianen Rhythmen, besonders des Schlaf-Wach-Rhythmus, beeinflusst das Hormon viele andere Körperfunktionen, wie etwa den Blutdruck. Studien haben eine blutdrucksenkende Wirksamkeit sowohl bei Tieren als auch beim Menschen gezeigt. Eine rezente Meta-Analyse bestätigte, dass Präparate mit kontrollierter Freisetzung den systolischen und diastolischen Blutdruck signifikant und im klinisch relevanten Bereich senken können. Melatonin wird daher als potentielles blutdrucksenkendes Mittel angesehen, wobei die zugrunde liegenden Mechanismen nicht gut verstanden sind. Als eine Möglichkeit der Blutdruckregulation wird eine direkte Wirkung von Melatonin auf den Gefäßwiderstand über die spezifischen G-Protein-gekoppelte Melatonin-Rezeptoren, MT_1 und/oder MT_2 genannt. Beide Rezeptoren wurden in verschiedenen Rattengefäßen (Aorta, Rattenschwanz und Hirnarterien) nachgewiesen, allerdings nur auf mRNA Ebene in totalen Gewebslysaten. Die Verteilung und zellspezifische Funktionen von MT_1 bzw. MT_2 Proteinen in strukturellen Schichten der Gefäßwand, d.h. Endothelzellen, glatten Muskelzellen oder Zellen innerhalb der Tunica adventitia oder dem perivaskulären Fettgewebe (PVAT) müssen noch umfassend untersucht werden, um den Zusammenhang zwischen Melatonin und dessen blutdrucksenkender Wirkung zu verstehen. Unveröffentlichte Daten unserer Arbeitsgruppe zeigten MT_1 mRNA Expression in mesenterischen Rattenarterien (MA) und deren umgebenden PVAT. Publizierte Studien anderer Gruppen deuten darauf hin, dass die antikontraktilen Funktionen von MA assoziiertem PVAT durch noch unbekannte Mechanismen von Melatonin beeinflusst werden. Proteinexpression und Lokalisation von MT_1 in MA und dessen PVAT sind zurzeit unbekannt. Der Mangel an Informationen über die Melatonin Rezeptor Proteinexpression und -lokalisation im Rattengewebe beruht größtenteils auf der mangelhaften Charakterisierung kommerzieller MT_1 und MT_2 Antikörper. In diesem Kontext ist Western Blotting nicht nur die wichtigste Technik um Proteinexpression im Gewebe nachzuweisen, sondern auch unverzichtbar um die Spezifität eines Antikörpers zu untersuchen.

Ziele: Diese Diplomarbeit hatte zwei Hauptziele. Erstes Ziel war die Untersuchung der Spezifität von drei kommerziell erworbenen anti-Ratten MT_1 Antikörpern der Firmen, Biorbyt, Santa Cruz Biotechnology und Alomone Labs, mittels Western Blotting. Zweites Ziel war der Nachweis von MT_1 Proteinexpression in mesenterischen Rattenarterien (MA) und perivaskulären Fett (PVAT) mit dem spezifischsten der drei getesteten Antikörper.

Methoden: Die Spezifität der Antikörper und die Proteinexpression wurde mit Western Blotting Technik analysiert. Die verwendeten Gewebe stammten von Wistar Ratten. Aus Auge, Hirn und Kleinhirn (Positivkontrollen), Blutplasma (Negativkontrolle), sowie mesenterische Arterie (MA) und umgebendes perivaskuläres Fett (PVAT) wurden entweder mit T-PER Puffer totale Proteinlysate, oder mit Mem-PER Membranproteinlysate hergestellt. Diese wurden mit SDS-PAGE aufgetrennt, auf PVDF-Membranen geplottet und über indirekten Nachweis mit HRP-konjugierten sekundären Antikörpern und Chemilumineszenz Substraten entwickelt.

Resultate: Die drei getesteten Antikörper zeigten signifikante Unterschiede in ihrer Spezifität. Der anti-MT_1 Antikörper der Firma Alomone Labs reagierte mit Proteinen im erwarteten Molekulargewichtsbereich von MT_1 (40-60 kDa) in allen Positivkontrollen. Diese Signale waren spezifisch, da in Gegenwart des entsprechenden kompetitierenden Peptides, welches zur Herstellung des Antikörpers verwendet worden war, keine Signale mehr detektiert wurden. Außerdem zeigte dieser Antikörper weder eine Reaktion mit Albumin noch mit IgG. Beides sind Hauptproteine des Blutplasmas, ihre Gegenwart wurde aber auch in vielen Geweben nachgewiesen. Der Antikörper der Firma Biorbyt reagierte zwar mit Proteinen im Molekulargewichtsbereich von MT_1, zeigte aber auch eine signifikante Kreuzreaktion mit Albumin. Der letzte getestete Antikörper, von der Firma Santa Cruz, zeigte überwiegend Kreuzreaktionen mit Albumin, die Unspezifität des Antikörpers wurde in Experimenten mit kompetitierenden Peptid bestätigt. Eine Proteinexpression von MT_1 in MA und MA-PVAT konnte mit dem Antikörper von Alomone Labs eindeutig nachgewiesen werden. Die Resultate dieser Diplomarbeit ermöglichten in weiterer Folge die Lokalisation von MT_1 in Ratten MA und PVAT unter Verwendung des spezifischen Antikörpers von Alomone Labs. Dabei wurde MT_1 in Muskelzellen von MA und in den Adipozyten von MA-PVAT lokalisiert. Die Resultate wurden als Abstract und Poster am 5. Retreat des Zentrums für Pathophysiologie, Infektiologie und Immunologie im September 2014 präsentiert.

Zusammenfassung und Ausblick: Zwei von drei untersuchten kommerziellen Antikörpern gegen Ratten MT_1 zeigten signifikante Kreuzreaktionen mit Albumin. Kreuzreaktionen von Antikörpern mit irrelevanten Proteinen wie Albumin sind auch in der Literatur beschrieben und die Resultate dieser Arbeit unterstreichen die Wichtigkeit, Antikörper auf ihre Spezifität zu testen bevor Studien zur Expression und Lokalisation der Proteine durchgeführt werden. Der erfolgte Nachweis von MT_1 in MA und MA-PVAT in dieser Diplomarbeit war die Voraussetzung für zukünftige funktionelle Studien in isolierten Zellen der relevanten Gewebsabschnitte. Diese sollen zu einem besseren Verständnis der Interaktion von Melatonin mit MT_1 und den nachfolgenden zellulären Mechanismen führen, die auf die Blutdruckregulation einwirken.

3 Introduction

3.1 Melatonin

Melatonin, N-acetyl-5-methoxytryptamine, was first isolated from bovine pineal gland by the working group of Aaron Lerner [1-2]. Due to the fact that melatonin production and secretion by the pineal gland is restricted to the dark hours of the day, melatonin is known as the "hormone of darkness". The nocturnal melatonin secretion by the pineal gland enables chronobiotic synchronization of all body cells. Later on, synthesis was also demonstrated in extra-pineal tissues such as the gastrointestinal tract, skin, retina and others. Melatonin produced extrapineally, however, lacks a day-to-night variation and is generally not released into circulation. Local production of melatonin most likely provides protection for cells due to the anti-oxidative as well as the anti-inflammatory properties of the hormone [3].

A major function of pineal-derived melatonin is the control of circadian and seasonal rhythmicities. Melatonin, however, has turned out to contribute to the regulation of numerous other body functions. As one example, melatonin participates in the regulation of the cardiovascular system [4-5], including blood pressure [6], and is therefore regarded as a putative antihypertensive agent [7]. Age, as well as diverse neurodegenerative and cardiovascular diseases reduce melatonin production [8-10]. In turn, melatonin deficiency or dysfunction of melatonin signaling can induce and/or contribute to various pathologies such as hypertension [11-12] (see also Chapter 3.2).

3.1.1 Synthesis and metabolization of melatonin

The production of melatonin in the pineal gland is controlled by a circadian signal from the suprachiasmatic nucleus (SCN) [13], which is the biological master clock [14]. Melatonin also acts directly on melatonin receptors expressed in the SCN to modulate the clock itself. In the retina, light is transduced into a neural message. Via the retinohypothalamic tract, which connects the eye and the SCN, this „day"-message is passed to the SCN, resulting in inactivation of the superior cervical ganglia through a complex pathway involving the paraventricular nucleus and the upper thoracic intermediolateral cell column. From the superior cervical ganglia, postganglionic sympathetic noradrenergic fibers target the pineal gland. The onset of melatonin production in the pineal gland occurs with the decrease in SCN neuron firing rate in late day. This stimulates sympathetic activity and noradrenalin activates the β-receptors in the sympathetic postganglionic neurons of the pineal gland. The resulting increase of cAMP in the pinealocyts causes an up-regulation and activation of the enzyme arylalkylamine-N-acetyltransferase (AANAT), the first rate-limiting enzyme in the

biosynthesis of melatonin (see Figure 2). This circuit allows light to suppress the production and release of melatonin from the pineal gland and results in the nocturnal peak of the pineal melatonin production [15].

Pineal as well as extra-pineal melatonin biosynthesis in vertebrate cell is divided in two steps (Figure 1 and Figure 2) and starts with the essential amino acid tryptophan, which is hydroxylated through the enzyme tryptophan-5-hydroxylase (TPH) and then converted to serotonin via aromatic L-amino acid decarboxylase (AADC; Figure 1).

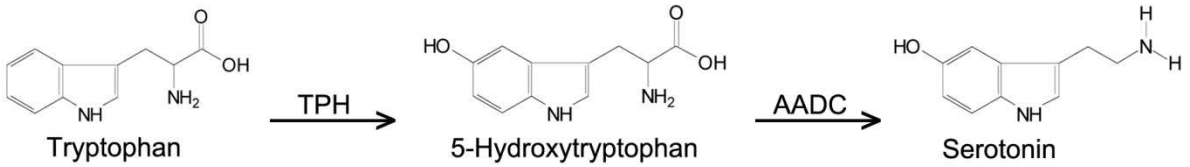

Figure 1: First step of biochemical synthesis of melatonin via serotonin.
Tryptophan is hydroxylated via tryptophan-5-hydroxylase (TPH) and decarboxylated by aromatic L-amino acid decarboxylase (AADC) to Serotonin (created according to data from [15-17]).

Serotonin is an indolamine and *per se* a high potent neurotransmitter, but it is also the precursor for melatonin. The enzyme arylalkylamine-N-acetyltransferase (AANAT) produces an intermediate product, N-Acetylserotonin (see Figure 2). Finally, acetylserotonin *O*-methyltransferase (ASMT; formerly named hydroxyindole-*O*-methyltransferase; HIOMT) converts N-Acetylserotonin to melatonin. It is mainly AANAT, which is referred to as the rate-limiting enzyme of melatonin synthesis in vertebrates, but ASMT may also play a role [15, 17].

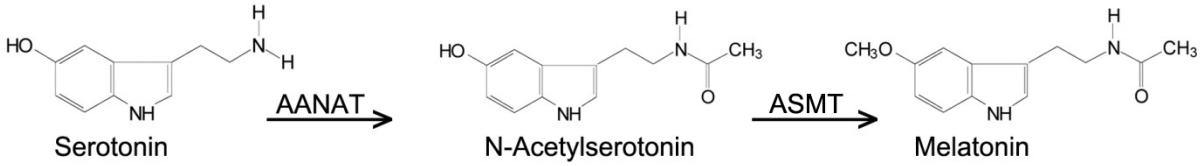

Figure 2: Second and final step of melatonin biosynthesis.
Serotonin is acetylated by arylalkylamine-N-acetyltransferase (AANAT) and finally processed via acetylserotonin *O*-methyltransferase (ASMT) to melatonin (created according to data from [15-17]).

Once formed, pineal melatonin is immediately secreted, reaching night time concentrations up to 0.5 nM in human blood. Local concentrations in tissues with endogenous melatonin production, such as the gut, can even exceed blood plasma levels by several 100-folds [9]. Melatonin as well as many of its metabolites such as 6-hydroxymelatonin, N_1-acetyl-N_2-formyl-5-methoxykynuramine (AFMK) and N_1-acetyl-5-methoxykynuramine (AMK) exhibit direct free radical scavenger activity. As melatonin and the generated metabolites successively scavenge free radicals, they build up a so-called „free radical scavenging cascade“, which causes the high efficiency of melatonin in protecting organisms from oxidative stress (see 3.1.2) [17-18]. In addition to these direct anti-oxidative effects,

circulating melatonin can bind to several receptors, including plasma membrane-located membrane receptors (see 3.1.3). Via these G-protein coupled receptors, various signal transduction pathways can be activated in diverse target cells, which impact on multiple regulatory processes. Among other factors, the effects of melatonin depend on the melatonin dose and the type of receptor expressed in the cell [19-20].

The metabolization of melatonin is more complex and involves enzyme-dependent as well as enzyme-independent processes. Enzyme-independent metabolization is the result of interaction with reactive oxygen species (ROS) or reactive nitrogen species (RNS) and occurs extra- and intracellular. Circulating melatonin is mainly metabolized enzymatically in the liver. Various cytochrome P_{450} isoforms, such as CYP1A2, CYP1A1 or CYP1B1 can all catalyse hydroxylation in position 6. Thereafter, sulfate- or glucuronic acid- conjugates are formed and excreted into the urine. Alternative metabolization routes include CYP2C19 or CYP21A2, which can demethylate melatonin to *N*-acetylserotonin, while diverse other enzymes oxidize melatonin. As a consequence of the very short half-life - less than 1 h - of melatonin in plasma, the blood levels of this hormone correlate closely with the pineal production rate [8, 9, 17].

3.1.2 Melatonin and oxidative stress

Oxidative stress

Oxidative stress results from an imbalance between the generation of ROS and RNS and their removal. ROS and RNS include molecules such as superoxide (O_2^-), hydroxyl radical (HO•), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), nitric oxide (NO), and hypochlorous acid (HOCl), which are side products of regular metabolic pathways and usually scavenged by endogenous and exogenous antioxidant molecules such as melatonin, glutathione as well as antioxidant enzymes or Vitamin C, respectively. An excess production of ROS/RNS results in oxidative stress, which finally leads to cell and organ damage [21].

Oxidative stress is involved in the pathophysiology of many diseases including CVDs, Type 2 diabetes, cancer, and Alzheimer diseases. It is also a key player in the pathogenesis of hypertension [22-28]. Human hypertension is associated with an increase in oxidative stress and reduced levels of anti-oxidative enzymes such as catalase and/or superoxide dismutase as well as reduced levels of ROS and RNS scavengers such as glutathione, and vitamins C and E [29]. Angiotensin II stimulates non-phagocytic NADPH oxidase, which leads to an accumulation of hydrogen peroxide, superoxide, and peroxynitrite. These ROS are involved in the role of angiotensin-II-induced hypertension [30]. The bioavailability of NO is reduced,

which is released by endothelial cells of the vascular system and is a vasodilator. NO is rapidly degraded by superoxide anion, which itself acts as vasoconstrictor [31].

Antioxidant activity of melatonin

Melatonin acts in two ways to lower oxidative stress. It can scavenge ROS/RNS, which requires high millimolar concentrations of melatonin and it stimulates the activity and expression of antioxidant enzymes (see Figure 3).

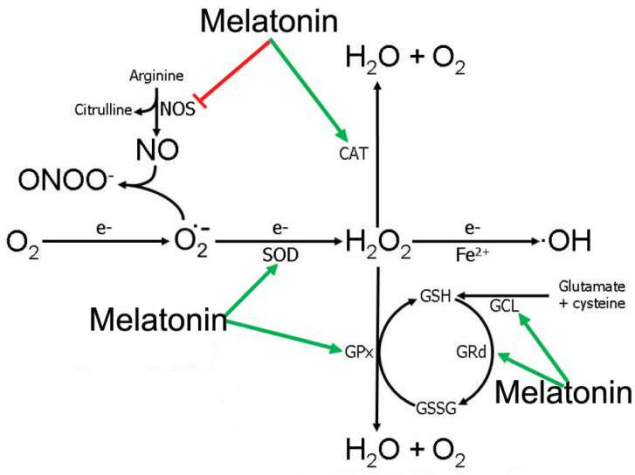

Figure 3: Melatonin is a potent antioxidant hormone. Green arrows show stimulating effects on different antioxidant enzymes, the red arrow means inhibition of the pro-oxidative enzyme NOS.
Abbreviations : e^- - electron, Fe^{2+} - ferrous iron, GCL - glutamate-cysteine ligase, GPx - glutathione peroxidase, GSH - glutathione, GRd - glutathione reductase, GSSG - glutathione disulfide, H_2O_2 - hydrogen peroxide, NO - nitric oxide, $ONOO^-$ - peroxynitrite, NOS - nitric oxide synthase, O_2 - molecular oxygen, $O_2^{\bullet-}$ - superoxide anion radical, ·OH - hydroxyl radical (modified after [32]).

Melatonin and its metabolites have high antioxidant potential. The potent scavenging effect of melatonin can be explained by the chemical structure. As a lipophilic molecule melatonin can migrate easily into tissues and cells. This extends the protective action to all subcellular structures. Furthermore, as an indolamine it can better stabilize the negative load of radicals. Acting as a direct scavenger, melatonin is able to reduce the level of both oxygen and nitrogen species, neutralizing different free radicals, such as singlet oxygen, superoxide anion radical, hydroperoxide, hydroxyl radical, lipid peroxide radical and highly toxic peroxynitrite anion [17]. High levels of oxidative stress cause a rapid drop of circulating melatonin levels due to rapid metabolization of melatonin by interaction with ROS/RNS.

Among the indirect antioxidant actions of melatonin is the stimulation of gene expressions and activation of superoxide dismutase (SOD), catalase and glutathione peroxidase [33] or the reduction of electron leakage from the mitochondrial electron transport chain [34]. Table 1 lists the effect of melatonin on some ROS/RNS and antioxidant enzymes.

The observed antihypertensive effects of melatonin are partly the result of its antioxidant properties [35]. Melatonin is able to preserve endothelial function, to increase NO production as well as reduces its degradation, which leads to vasodilation and reduction of blood pressure.

Table 1: An overview of melatonin effects on ROS/RNS and counterbalancing antioxidative enzymes. Abbreviations: ↓ - down regulation, ↑ - up regulation of enzymes, RNS - reactive nitrogen species, ROS - reactive oxygen species (created according to data from [36]).

ROS / RNS	Chemical structure	Melatonin effect	Antioxidative enzymes	Abbreviation	Melatonin effect
Hydrogen peroxide	H_2O_2	↓	Superoxid dismutase	SOD	↑
Hydroxyl radical	$^{\bullet}OH$	↓	Catalase	CAT	↑
Superoxide anion radical	$O_2^{\bullet-}$	↓	Glutathione peroxidase	GPx	↑
Nitric oxide	NO	↓	Glutathione reductase	GRd	↑
Peroxynitrite	$ONOO^-$	↓	Glucose-6-phosphate dehydrogenase	G6PD	↑

3.1.3 Melatonin receptors

Many physiological and pharmacological actions of melatonin including circadian entrainment [37] are mediated through melatonin receptors [38]. Originally, mammalian melatonin receptors were classified into ML_1 and ML_2 types, based on kinetic and pharmacological differences of 2-[^{125}I]iodomelatonin binding [39].

ML_1 includes the high affinity plasma membrane receptors MT_1 (formerly Mel1a), and MT_2 (formerly Mel1b), which are both G protein-coupled receptors, cloned in the mid-1990s. Human MT_1 protein (see Figure 4) consists of 350 amino acids [40], while human MT_2 protein contains 362 amino acids [41]. The human MT_1 and MT_2 proteins have 60% sequence homology and exhibit affinities for melatonin in the picomolar range (MT_1: Kd = 20–40 pmol/l, MT_2: Kd = 160 pmol/l [42-43]. The rat MT_1 and MT_2 cDNAs encode proteins of 353 and 364 amino acids, respectively and show 78-93% sequence homology with the human and mouse counterparts [44]. A third high-affinity receptor, Mel 1c, has been found only in fish, amphibians and birds [45]. GPR50 is a protein expressed in eutherian mammals. Although it shares about 45 % homology with other melatonin receptors, melatonin is not bound by the receptor. It is therefore regarded as an orphan G protein-coupled receptor whose function and ligands remain to be discovered [45-46].

ML_2 comprises low affinity receptors with Kds in the nanomolar range. The contribution of these proteins to melatonin signaling remains incompletely understood [38, 45]. The nuclear receptors retinoid related orphan nuclear hormone receptor (ROR-α), the retinoid Z receptor (RZR), calmodulin and calreticulin belong to this group. Another binding site for melatonin, originally termed MT_3, was later identified as quinone reductase 2. MT_3 has low affinity for 2-[^{125}I]iodomelatonin (Kd = 3–9 nM) and is not a G protein-coupled receptor [47].

Central and peripheral expression of melatonin receptors MT_1 and MT_2

The presence of high affinity melatonin receptors (MT_1 and MT_2) in tissues was originally revealed by [^{3}H]-melatonin. Later, the receptor expression was studied at mRNA level (in situ hybridization, reverse transcription-PCR experiments) as well at the protein level using either radioligand-binding or immunohistochemical techniques. However, detection of melatonin receptors at the protein level is hampered because of low expression levels on the one hand and the lack of specific high affinity antibodies, particularly for rodent receptors, on the other hand. Consequently most studies investigating MT_1 or MT_2 expression in membrane preparations, cells or tissues were performed with 2-[^{125}I]iodomelatonin radioligand.
Using these techniques, the mammalian membrane receptors have not only been identified in the central nervous system, but also in many peripheral organs. In summary, MT_1 receptors are expressed in the brain, cardiovascular system, immune system, male and female reproductive organs, skin, liver, kidney, adrenal cortex, retina, pancreas and spleen. In the brain, the receptor is found in regions such as the cerebellum, the hypothalamus, or the SCN. MT_2 has been detected in the immune system, brain, retina, pituitary, blood vessels, testes, kidney, gastrointestinal tract, mammary glands, adipose tissue, and the skin [38, 43, 48-50].

Expression and localization of human MT_1 or MT_2 protein has been investigated using characterized antibodies [51-53]. MT_1 was detected in human hypothalamus and cerebellum [52], in the hippocampus [54-55], the eye [56-57], pineal gland and occipital cortex [58], breast [59], human and mouse brain [60] and SCN [61]. Expression of MT_2 protein was demonstrated in hippocampus [62], pineal gland and occipital cortex [58].

The pattern of expression of melatonin receptors in other species has so far been mainly determined at mRNA level using either the reverse transcription-PCR (RT-PCR) or *in situ* hybridization. Using these techniques, the MT_1 mRNA was for instance expressed in the majority of the central and peripheral tissues studied in rats [44, 63]. Although protein expression and localization of rat melatonin receptors has been investigated in several studies, the applied commercial antibodies lacked sufficient characterization [64-70].

Table 2 summarizes some information on melatonin receptor expression and function in different tissues [50].

Table 2: Distribution of G protein-coupled melatonin receptors MT_1 and MT_2 and the role of melatonin in various tissues (according to [50]).

Tissues	Melatonin receptors expressed	Role of melatonin
SCN	MT2	Regulate circadian rhythm
Retina	MT2, RORα	Decrease dopamine release
Vasculature	MT1, MT2	MT1: vasoconstrictor MT2: vasodilator
Immune system	MT1, MT2, RORα	Inhibit leukotriene rolling Promote immune cell proliferation Stimulates IL2 and IL6 production
Reproducive system	MT1, MT2	Decreases GnRH, LH, FSH release
Pancreas	MT1, MT2, RORα	Decrease insulin release
Skin	MT1, MT2, RORα	Regulate hair growth, and functions of epidermis
GI tract	MT1, MT2	Decrease gastric contraction, peristalsis, and sertonin's actions Increases bicarbonate, amylase, and CCK release
Bone	MT1, RORα	Increases osteoblastic activity and decrease osteoclastic activity
Kidneys	MT1, MT2	Protects from inflammation, regulate glomerular filtration
Placenta	MT1, MT2, RORα	ROS scavenger, decrease apoptosis
Uterus	MT1, MT2	Myometrial contractility
Endometrium	MT1, MT2	Trophoblast invasion in early pregnancy

Expression of melatonin receptors MT_1 and MT_2 in vessels

There is evidence that part of melatonin´s anti-hypertensive properties are caused by interaction with specific receptors MT_1 or MT_2 localized in peripheral vessels or in blood pressure modulating structures of the central nervous system [6, 7, 71]. In line with an impact of melatonin on peripheral vessels, several studies have demonstrated presence of MT_1 and/or MT_2 in diverse vessels and a role of melatonin on vessel contraction or dilation.

Using 2-[^{125}I]iodomelatonin radioligand, specific melatonin binding sites were detected in smooth muscle layer of rat cerebral arteries and the caudal arteries [72]. Using isolated caudal and cerebral arteries, it was observed that melatonin could potentiate contraction of these arteries [72-74]. Later, the binding sites for melatonin in the tail artery and cerebral arteries of rats were identified as being MT_1 using RT-PCR [75-78]. Expression of MT_1 mRNA was also confirmed in bovine cerebral arteries [79] and in human cerebral arteries [80]. An expression of MT_2 mRNA in cerebral arteries could not be demonstrated [77], while contradictory data of MT_2 mRNA expression were reported from caudal arteries [75, 78]. However, based on experiments with isolated rat caudal artery segments, the expression and opposite function of MT_1 (vasoconstricitive) and MT_2 (vasodilative) in caudal arteries has been suggested [81].

In the rat aorta, expression and function of MT_1 was suggested based on observed effects of melatonin on rat aortic rings *in vitro* [82]. While original studies failed to demonstrate mRNA expression [77] and 2-[^{125}I]iodomelatonin radioligand binding [72], the expression of MT_1

mRNA and protein was confirmed more recently [66, 69] including the demonstration of a predominant localization of MT_1 in the adventitia of rat aorta [66]. MT_2 mRNA, on the other hand, was not detected in rat aorta [66, 77], while expression of MT_2 mRNA was shown in human aorta [83].

In most cases, the demonstration of mRNA expression of melatonin receptors has not been accompanied by a proof of protein expression. Likewise, the localization of melatonin receptors to the diverse structural layers of vessels is still missing, but urgently required to better understand the function of melatonin receptors in blood pressure modulation.

Signal transduction of melatonin receptors MT_1 and MT_2

Both melatonin receptors, MT_1 and MT_2, are G protein-coupled receptors (GPCRs) and have seven transmembrane domains (Figure 4). MT_1 and MT_2 bind melatonin at their N-terminal (extracellular) domain, while their C-terminal (intracellular) domain binds the signalling factors required to mediate the intracellular effects (Figure 5).

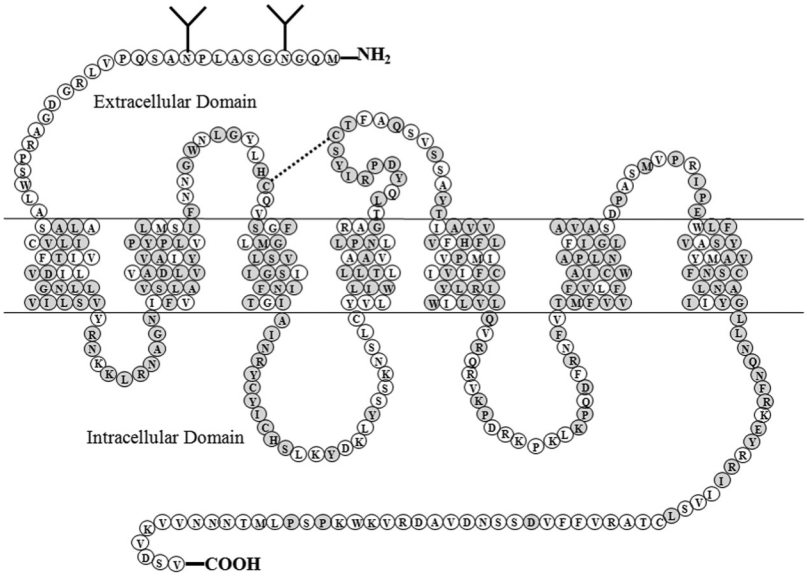

Figure 4: Membrane topology of the human MT_1 receptor.
Grey coloured amino acids show the sequence homology to human MT_2, which is 60% identical with MT_1 (according to [84, 142]).

MT_1 and MT_2 receptors activate several signaling pathways, with few receptor-subtype specific differences detected so far. The receptors can form homodimers or higher-order oligomers. Strong evidence for formation of MT_1/MT_2 heteromers has been obtained recently in mouse retina cells. Both receptors as well as MT_1/MT_2 heteromers can activate the G_i/cAMP pathway, but also the G_q/PLC/Ca^{2+} pathway as shown in Figure 5. Receptor subtype-specific differences have been reported for instance for the MT_2 receptor, which can inhibit cGMP formation through the soluble guanylyl cyclase pathway. However, a full and detailed picture of G-protein coupled signaling via melatonin receptors is still lacking [43, 85].

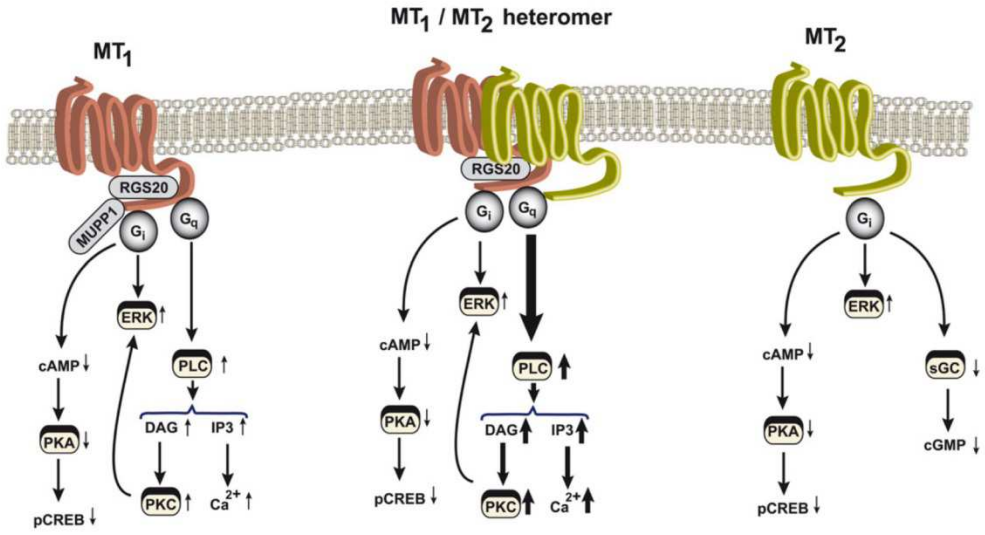

Figure 5: Coupling of melatonin receptors MT_1 and MT_2 to their G-protein (G_q/G_i) and their major intracellular signalling pathways (for more detailed information see [85]).

Activation of these signaling cascades in blood vessels was suggested to contribute to blood pressure regulation [71]. Vasoconstriction by melatonin might be explained by receptor activation in vascular smooth muscle cells. Activation of the Gq signal pathway, for example, may activate phosphoinositide phospholipase C (PLC), which then could result in increasing intracellular Ca^{2+} levels. On the other hand, MT_2 receptors on endothelial cells could – via activation of PKC - increase cytosolic Ca^{2+} in endothelial cells. This may result in production of NO, which then stimulates production of guanylat cyclase in vascular smooth muscle cells leading to vasodilation [71]. Whether these pathways are indeed activated by melatonin in the diverse cell types of vessels and to which extend, has not been investigated so far.

3.2 Cardiovascular diseases and melatonin

3.2.1 Cardiovascular diseases

According to the WHO (world health organisation), cardiovascular diseases (CVD) are disorders of heart and blood vessels that include hypertension, heart attack, stroke, peripheral vascular disease, cardiomyopathies as well as congenital and rheumatic heart diseases. CVDs are the number one cause of death globally, especially in the western world. [86]. While currently 18% of deaths are due to CVDs, this number is projected to rise to 24% in 2030, when appropriate actions remain undone. A major risk factor for CVDs and also a cause of chronic kidney disease is hypertension [87]. Worldwide, hypertension, already affects more than one billion people. It is estimated that nine million people die from hypertension every year [88].

3.2.2 Hypertension

Within the body, blood circulates in blood vessels. This circulation is driven by the pumping heart, which creates a blood pressure by pushing the blood against the vessel walls. High blood pressure or hypertension is a persistently elevated pressure, which in turn causes the heart to pump harder. Untreated, it can cause enlargement of the heart, heart attack and eventually heart failure. Another consequence can be blood leakage in the brain causing a stroke. Moreover, elevated blood pressure can cause kidney failure, blindness, rupture of blood vessels and cognitive impairment.
The American Heart Association defines a normal, healthy, blood pressure (BP) for an adult, (≥ 20 years) as a systolic pressure of 120 mmHg (millimetre of mercury) and a diastolic blood pressure of 80 mmHg. Hypertension is diagnosed when values are higher than normal BP, ranging from stage 1, which is 140/90 mmHg BP up to hypertensive crisis with higher than 180/110 mmHg BP, in which emergency care is needed [89].

In most cases, hypertension results from a complex interaction of genetic, environmental, and demographic factors. Social determinants are factors such as ageing, income or education, which impact on a variety of behavioral risk factors for high blood pressure, e.g. unhealthy diet, physical inactivity, constant elevated stress level as well as smoking and harmful use of alcohol. When combined with certain metabolic risk factors including obesity and diabetes, the risk to develop CVDs is further increased [90].

More than 90% of affected individuals suffer from essential hypertension, also known as hypertension of unknown cause. Among the multiple factors involved in the pathophysiology of essential hypertension are for example increased sympathetic nervous system activity, unbalanced salt intake, increased production of sodium-retaining hormones and vasoconstrictors, deficiencies of vasodilators, elevated renin secretion with consecutive increased production of angiotensin II and aldosterone, and many others. The renin angiotensin aldosterone system (RAAS; see Figure 6) is one of the central blood pressure regulatory mechanisms and main target of current antihypertensive drugs [91].

Renin secretion is activated in the juxtaglomerular cells of the kidney when blood pressure is low. Alternatively, the sympathetic nervous system can stimulate renin release, thereby explaining why a high stress level over the time can cause high BP. The enzyme renin cleaves the liver product angiotensinogen to angiotensin I. Angiotensin converting enzyme (ACE), mainly produced by the endothelial cells of the lung, then converts angiotensin I to the potent peptide angiotensin II , which causes vasoconstriction resulting in blood pressure increase. Moreover, angiotensin II (AT II) induces a release of aldosterone - a hormone from

the adrenal cortex - and the antidiuretic hormone (ADH, or vasopressin) from the neurohypophysis. Both act on kidneys and increase the reuptake of sodium and water, which causes the feeling of thirsty and again an increase of blood pressure. Insulin, a peptide hormone produced in the pancreas, also suppresses sodium loss into the urine. This is of great significance for people who suffer to diseases with elevated levels of this hormone, i.e. Type 2 diabetes or obesity, which is associated with metabolic syndrome. Although they are metabolic resistant to insulin, they are not resistant to retain sodium [144].
The sympathetic nervous system is generally a mediator for stress situations (fight-or-flight). One important role of the sympathetic nervous system is controlling the blood pressure by influencing the RAAS system. It also controls the release of melatonin from the pineal gland via the SCN (see chapter 3.1) and in return melatonin modulates the autonomic nervous system. This fact was proven in the model of the pinealectomized rats, which have increased catecholamine values, which could be lowered with melatonin application [6].

In addition, high blood pressure is associated with elevated peripheral vascular resistance because of alterations in structure and function of small arteries. Remodeling of these vessels, in turn, contributes to high blood pressure. Research over the last years has even suggested that structural and functional abnormalities in the vasculature precede hypertension and contribute to the pathogenesis. Among factors contributing to vessel abnormalities are endothelial dysfunction, increased oxidative stress, vascular remodeling, and decreased compliance. Whether antihypertensive agents that normalize resistance vessel structure such as ACE inhibitors and others are more effective in preventing CVD than agents that lower blood pressure without affecting vascular remodeling remains to be determined [92].

3.2.3 Treatment of hypertension

Life style change

The central point in treatment as well as prevention of hypertension is the adoption of life style parameters. This is used as first intervention for low risk patients as monotherapy or in addition to pharmacological therapy independent to the stage of hypertension. According to the European Society of Hypertension (ESH) guidelines 2013 [93] this includes healthy diet with high consumption of vegetables and fruits, moderation of alcohol consumption, quitting smoking, regular physical exercise, salt restriction and weight reduction.

Pharmacology intervention

Not all patients diagnosed with hypertension require medication. Patients at medium to high risk, however, need one or more of the current essential medicines to lower their

cardiovascular risk. Among these pharmacological substances are AT II receptor antagonists or angiotensin receptor blockers (ARB), ACE inhibitors, which prevent formation of angiotensin II, long-acting calcium channel blockers (CCB), which lower contractility of smooth muscle cells, alpha blocker, diuretics, beta blockers, which target the heart, and renin antagonists. Blocking of ACE has a strong antihypertensive effect. ACE-inhibitors are often used as first line therapy (stage 1 and 2 hypertension) or in combination with thiazide diuretics or other drug classes. The main side effects are a consequence of the pharmacological profile. Angioedema, dry cough and hypotension can be caused by a higher bradykinin level, as bradykinin is degraded by ACE under physiological conditions. Diuretics are used on the one hand to treat oedema and renal failure, on the other hand they are an important antihypertensive drug with long term controlling effects. They increase the volume of urine due to blocking of various electrolyte transporters. Mainly, thiazide diuretics with the principal agent hydrochlorothiazide as well thiazide-like diuretics (e.g. chlorthalidone, indapamide) are used for treatment of hypertension. Usually, they are combined with other antihypertensive drug classes (see Figure 6). Side effects are typically caused by the higher loss of electrolytes and water, like hypokalaemia, hypovolemia and dizziness. 10% of patients with hypertension do not even respond to a combination of three antihypertensives (typically diuretic, CCB plus ACE inhibitor or ARB) and maybe treated by adding the aldosterone antagonist spironolactone to the treatment regimen [91].

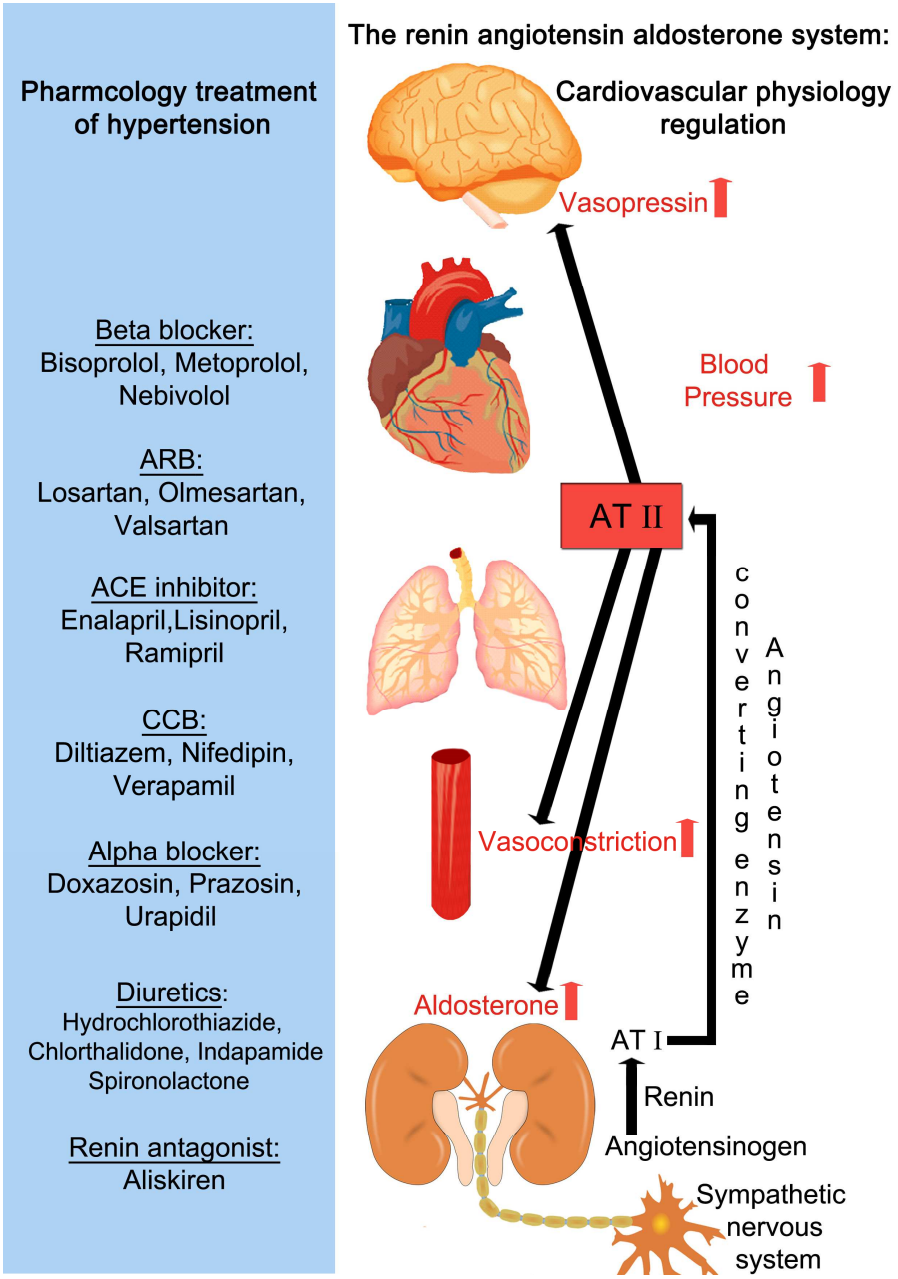

Figure 6: Selection of the main classes of hypertensive medications (on the left side). RAAS (on the right side), the physiological blood pressure regulation, with key factors in developing hypertension especially focused on AT II. Red arrows mark effect of high levels of AT II (created according to data [94, 95, 143]).

3.2.4 Melatonin and blood pressure

Numerous studies have shown a link between melatonin and blood pressure regulation. First results on this topic were obtained from pinealectomized rats in the 1970s. Removing the pineal gland reduced melatonin levels in serum and elevated the blood pressure by 20 mm Hg compared to the control group [96-97].

This initiated studies in diverse rat models for hypertension such as the spontaneously hypertensive rats (SHR), L-NAME-induced hypertensive rats, or pinealectomized rats, where chronic treatment with melatonin was able to reduce blood pressure [98-100]. Subsequent

studies in humans showed that melatonin given to healthy men could reduce blood pressure [101], that nighttime melatonin supplementation reduced nocturnal blood pressure in otherwise untreated hypertensive men [102], non-dipping women [103], or patients with nocturnal hypertension [104]. In this context, it was also observed that non-dipping hypertensive patients have a reduced nocturnal melatonin secretion when compared to dipping hypertensive patients [105-106].

In summary, these observations led to the assumption that melatonin could be used as an anti-hypertensive drug [7, 71, 107]. Several studies meanwhile evaluated the effects of melatonin on nocturnal blood pressure, originally with inconsistent results. But a recent meta-analysis on the results of seven studies came to the conclusion, that melatonin, when applied as controlled-release melatonin, can significantly improve blood pressure, while fast-release melatonin has no effect. Controlled-release melatonin could lower systolic blood pressure by 6.1 mmHg and diastolic blood pressure by 3.51 mmHg. This mean reduction in nocturnal blood pressure is of a magnitude that is considered to be clinically relevant. Most important, no serious adverse events due to melatonin treatment were reported in all studies included in the meta-analysis. Due to the fact that the included studies were of small size and performed only over short-periods, the beneficial effects of controlled-release melatonin on blood pressure still have to be proven in long term studies of appropriate size. Overall, however, these results suggest that melatonin can improve nocturnal blood pressure control. Melatonin may especially be useful for high-risk patients with nocturnal hypertension. Normal day production but reduced melatonin production at night is seen in severely hypertensive patients and in hypertensive patients with non-dipping blood pressure patterns [108].

Nevertheless, it is currently unclear how melatonin exerts its anti-hypertensive effects. Several mechanisms are discussed. First, melatonin has an anti-oxidant effect, mentioned in chapter 3.1.2, which would preserve endothelial function and lead to vasodilation. Second, melatonin infers with the autonomic system, shifting the balance from the adrenergic to the cholinergic nervous system. Third, melatonin may directly impact on local blood pressure regulation in peripheral vessels via the specific receptors MT_1 and/or MT_2. This last mechanism is specifically unclear. Blood vessels are composed of several structural layers (explained in 3.3.). The effect of melatonin on blood vessels (dilation, contraction) would not only depend on the type of receptor expressed but also on the cellular location of the expressed receptor [81]. What is currently missing, are expression and localization studies of melatonin receptor MT_1 and MT_2 proteins using well-defined antibodies to further explore the role of melatonin binding to individual cell-types in blood vessels involved in blood pressure regulation.

3.3 Blood vessels and the surrounding perivascular adipose tissue (PVAT)

3.3.1 Vessel structure

The general structure of blood vessels is shown in Figure 7. It consists of several layers with varying thickness, depending on the function and location of the blood vessel. The *tunica intima* is the most inner and thinnest layer of the vessel, adjacent to the lumen. It is a single layer of endothelial cells surrounded by a thin layer of connective tissue, the *internal elastic lamina*. Capillaries, which mediate exchange of blood and tissue, are usually composed only of this cell layer and some connective tissue. Endothelial cells secrete multiple substances that, among other functions, have an impact on dilation or constriction of vessels by influencing the vascular smooth muscle cells (VSMC). VSMC are located in the *tunica media*, and mediate vessel constriction and relaxation. In addition, it contains elastic fibers and connective tissue and is surrounded by the *external elastic lamina*. In arteries, the tunica media is the thickest layer. The *tunica adventitia* or *tunica externa* consists mainly of connective tissue, where nerves and small capillaries (vasa vasorum) are embedded. In veins, the tunica externa is the thickest layer. The perivascular adipose tissue (PVAT) is found throughout the body, around most arteries and veins with a diameter >50 µm. It consists of adipocytes, fibroblasts, stem cells, mast cells, and nerves. Depending on the anatomical region and vessel caliber, PVAT may be more abundant as seen for the aorta, or even absent as observed in cerebral arteries or in the microcirculation [109].

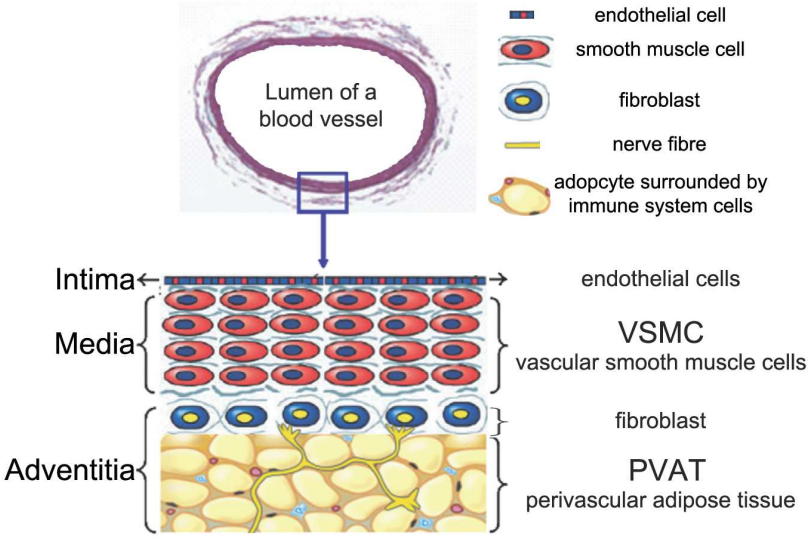

Figure 7: General structure of a blood vessel, stratified in three layers: *Tunica intima*, *media* and *adventitia*, listed from inner to outside (modified after [110]).

3.3.2 PVAT function

Classically, PVAT was supposed to offer mainly mechanical protection for the vessels. PVAT seems to have an additional role in thermoregulation. Moreover, it also functions as an active endocrine organ, which influences vessel functions, for instance the contraction, proliferation and migration of VSMCs. PVAT, at different locations, has been shown to secrete a wide variety of molecules, including hormones, cytokines, fatty acids, and oxygen radicals. Of importance, PVAT also releases vasoactivators, contracting as well as dilating factors. PVAT around the aorta and MA of rats has been shown to exert a direct relaxing effect on VSMCs, mediated by one or more adventitia/adipocyte-derived relaxing factors (ADRFs). Therefore, PVAT contributes to blood pressure regulation [111-113].

3.3.3 PVAT and melatonin receptors

Previously, we demonstrated expression of MT_1, but not MT_2 mRNA in rat aortas [66] as well as MA (Molcan, Ellinger, Zeman, unpublished results). Surprisingly, expression of MT_1 mRNA was found in the PVAT co-isolated with the vessels (Molcan, Ellinger, Zeman, unpublished results). While evidence for MT_1 and MT_2 mRNA expression in adipocytes isolated from rat inguinal and epididymal fat [114] and MT_1 function in adipocytes derived from epididymal fat has been obtained [115], the expression and function of MT_1 in PVAT has never been investigated before. Recent studies implicated that melatonin impacts on the anti-contractile function of MA-associated PVAT [116]. It might therefore be speculated that melatonin, via melatonin receptor MT_1, causes secretion of ADRFs from PVAT promoting vessel dilation.

4 AIMS

Cardiovascular diseases are the number one cause of death globally [117]. Increased blood pressure is among the most important risk factors and therapeutic treatment of hypertension reduces both morbidity and mortality. Melatonin was shown to be effective in blood pressure reduction in hypertensive animals and humans. Coevally displaying minimal side effects, it is considered as an anti-hypertensive drug [6-7]. However, the role of melatonin in blood pressure modulation is still incompletely understood; current data suggest that melatonin has peripheral as well as central effects, and functions through receptor-dependent as well as -independent actions [11].

Peripheral, receptor-dependent hypotensive effects of melatonin are probably mediated by the G protein-coupled receptors MT_1 and MT_2, but this is not well characterized. Receptor expression was demonstrated in various vessel types in rats [66, 69, 72, 76-78, 118], cattles [79] and humans [80, 83]. Most of these studies investigated solely MT_1 and/or MT_2 mRNA expression in total vessel tissue. Distribution and function of MT_1 and/or MT_2 protein in the structural layers of the vessel walls remain to be explored comprehensively, but so far only very few studies addressed receptor subtype localization [54, 66, 69]. The lack of information concerning melatonin receptor protein expression/localization is related to the fact that the available anti-MT_1 and MT_2 antibodies, with few human exceptions [51, 53], are not well characterized.

Antibodies are extremely important tools for the study of receptor structure, function and localization, but the quality of the results depends mainly on the quality (i.e. specificity) of the primary antibody employed. Specific antibodies allow for investigation of the molecular weight or post-translational modification such a protein glycosylation via western blotting (WB). They are a prerequisite to investigate the localization of the receptor in tissues and cells by immunohistochemistry. Moreover, by immune-precipitation, antibodies can enable the dissection of receptor signal transduction pathways. Therefore, in order to investigate expression, localization and function of melatonin receptor proteins, specific primary antibodies must be identified. WB is not only the most important technique to evaluate protein expression in tissues but is also necessary to show the specificity of the antibodies [119].

Previously, we have demonstrated expression of MT_1 but not MT_2 in rat aortas [66] as well as mesenteric arteries (MA) (Molcan, Ellinger, Zeman, unpublished results). Expression of MT_1 mRNA was also found in PVAT co-isolated with the vessels (Molcan, Ellinger, Zeman, unpublished results). While adipose tissue in general functions as a highly active endocrine

organ, PVAT around the aorta and MA of rats has been shown to exert a direct relaxing effect on VSMCs, mediated by one or more adventitia/adipocyte-derived relaxing factors (ADRFs) [109, 112]. Recent studies suggest that melatonin impacts on the anti-contractile function of MA-associated PVAT [116]. This may occur via melatonin receptors, but the expression of MT_1 in PVAT has never been investigated before.

This thesis was initiated to enable further exploration of MT_1 protein expression, localization and function in rat vessels (specifically MA) with their associated PVAT. The two major aims of this thesis were:

Aim 1: Determining specificity and cross-reactivity of commercial available anti-rat MT_1 antibodies

Three commercial antibodies - selected from the companies Biorbyt, Santa Cruz Biotechnology and Alomone Labs - were evaluated by WB for specificity and cross-reactivity on total lysates of tissues with published MT_1 expression (brain, cerebellum and eye).

Aim 2: Demonstration of MT_1 protein expression in MA and MA-associated PVAT

Using the most specific antibody identified in aim 1, MT_1 protein expression in total lysates of MA and MA-PVAT of normotensive rats was explored by WB in order to confirm protein translation of recently demonstrated MT_1 mRNA expression in these tissues.

5 Materials and Methods

5.1 Biological specimens

5.1.1 Rat organs and blood serum

The majority of rat tissues as well as rat serum samples were kindly provided by Prof. Michal Zeman, PhD, Comenius University Bratislava, Slovakia in 2013. Tissues were derived from male normotensive Wistar rats obtained from Anlab Praha (Czech Republic) at the age of 16 weeks. Animals were sacrificed during the light phase, the following tissues were rapidly sampled and immediately frozen at -80°C by Lubos Molcan, PhD (Group Prof. Zeman): blood serum, total brain, cerebellum, eye, mesenteric arteries (free from perivascular fat tissue), mesenteric artery perivascular fat tissue (MA-PVAT) as well as thoracic perivascular fat tissue. Clotted blood serum samples were centrifuged with 2000 x g at 4°C for 15 minutes (min), the supernatant was collected and frozen at -80°C.

Furthermore, perivascular fat tissue of rat aorta, rat epididymal fat tissue and fat tissue associated with rat kidneys were a kind gift from Prof. Heinz Redl, PhD, Ludwig Boltzmann-Institute for Experimental and Clinical Traumatology, Vienna. These tissues were derived from Sprague-Dawley rats and immediately frozen at -80°C in 2014.

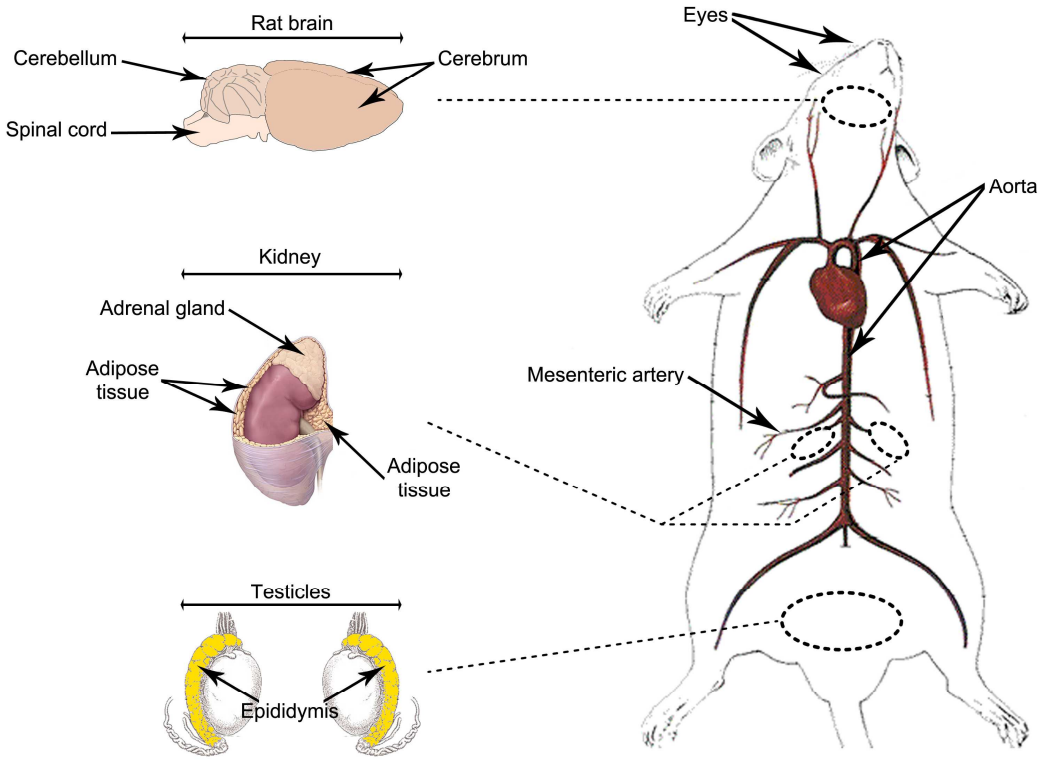

Figure 8: Overview and localisation of rat tissues used in this thesis [120-121].

5.2 Protein isolation from frozen tissues

Background

In order to perform immunological detection by western blotting, proteins must be extracted and solubilized from the frozen biological specimens. This requires initial sample pulverization by e.g. mortar and pestle to break up the extracellular matrix and increase surface area. Subsequently, extraction and solubilisation of total proteins or, alternatively, enriched membrane fractions, is performed with appropriate buffers such as T-PER (Tissue Protein Extraction Reagent for total protein fractions) or Mem-PER (Membrane Protein Extraction Reagent, both from Thermo Fisher Scientific Inc.) buffer, respectively. T-PER is a ready to use physiological buffer solution (pH 7.6) containing a mild, non-specified detergent. The Mem-PER kit contains three components, which have to be applied consecutive as described in 5.2.4 in order to enrich integral membrane proteins and membrane associated proteins. Tissue protein lysates prepared with both T-PER as well as Mem-PER buffer are compatible with immunoassays such as western blot [122-123].

5.2.1 Materials

- Analytical balance (Mettler AT200)
- Box with ice
- Coolable Centrifuge (Eppendorf, 5415R)
- Disposable Plastic Pasteur Pipettes, 3 ml
- Forceps
- Gloves for cryogenic work (handling of liquid N_2, Tempshield, Cryo gloves®)
- Latex gloves (PraxiMed, Meditrade, Gentle Skin Sensitive, REF 1221)
- Mortar and pestle
- Paper towels
- Pipette (Thermo Scientific, Finnpipette F2)
- Pipette tips (Thermo Scientific, Finntip Flex 200)
- Polystyrene Rack
- Reaction tubes: transparent 2 ml and 650 µl (Biozym 710190, VWR 211-0024)
- Safety glasses
- Scalpel
- Small plastic container, thick enough to stand temperature of liquid N_2 (fill level ~10 ml) e.g.: Greiner bio-one, scintillation vial 27.0/60.0 mm, 619301, saw off neck
- Styrofoam Box
- Thermomixer Comfort (needed only for total protein fraction) (Eppendorf, Germany)

- Vortexer (Scientific Industries, Vortex Genie 2, Model G560E)
- Water bath (needed only for membrane fraction needed)

5.2.2 Reagents

- Aqua bidestillata, (Aqua bidest.) prepared with ELGA Centra, Lab water
- Hydrochloric acid 37%, HCl (Merck, 1003171000)
- Liquid Nitrogen (N_2)
- Mem-PER Eukaryotic Membrane Protein Extraction Kit (Thermo Scientific, 89826) for membrane protein fractions
- Protease Inhibitor Cocktail (Thermo Scientific, Halt Protease Inhibitor Cocktail, 87786)
    - Protease Inhibitor Cocktail (100X stock solution), 1mL
    - EDTA Solution, 0.5M (100X stock solution), 1mL
- Sodium chloride, NaCl (Merck, 1064040500)
- TBS Buffer
    - 6.05 g Tris
    - 8.76 g NaCl
    - Dissolve with Aqua bidest. (~800 ml)
    - Adjust pH to 7.5 with HCl
    - Fill up to a final volume of 1000 ml Aqua bidest.
- T-PER Tissue Protein Extraction Reagent (Thermo Scientific, 78510) for total protein extraction
- Tris =Trometamol=Tris(hydroxymethyl)aminomethane (Trizma® base, Sigma, T1503)

5.2.3 Method for total protein isolation with T-PER [122]

1) Preparation:
    a) Add liquid N_2 to Styrofoam box (fluid level about 20% of height)
    b) Put the mortar and the pestle into the Styrofoam box and also fill mortar with liquid N_2
2) Sample preparation:
    a) Cut the tissue into small pieces using a clean scalpel and choose a tissue sample between 50 mg – 100 mg (determine weight on analytical balance)
    b) Place this specimen in mortar and crush it with pestle until the consistency is pulverulent like sand
    c) Transfer this sample with some liquid N_2 to a small plastic container
3) T-PER Buffer:
    a) After complete evaporation of the nitrogen, add the respective amount of T-PER buffer (1 ml buffer per 50 mg tissue)

b) Add both components (EDTA and protease inhibitor) of protease inhibitor cocktail (dilute 1:100, i.e.1 µl per 100 µl)
c) This mixture is transferred into a 2 ml reaction tube

4) Protein isolation:
a) Disrupt cells by shaking the reaction tube for 10 min at 4°C in Thermomixer at 1400 rpm
b) Transfer sample on ice to a precooled centrifuge (4°C)
c) Centrifuge for 15 min at 4°C with 16.000 x g
d) Collect supernatant with a pipette without touching the pellet
e) Discard the pellet
f) The supernatant contains the total protein fraction (No sub-fractionation into membranes, cytoplasm or any other cell component)
g) Split supernatant in aliquots (e.g. 650 µl) and store at -80°C

5.2.4 Method for membrane protein isolation with Mem-PER [123]

1) Preparation:
a) Add liquid N_2 to Styrofoam box (fluid level about 20% of height)
b) Put the mortar and the pestle into the Styrofoam box and fill mortar with liquid N_2

2) Sample preparation:
a) Cut the tissue into small pieces using a very clean scalpel and choose a tissue sample between 50 mg – 100 mg (determine weight on analytical balance)
b) Place this specimen in mortar and crush it with pestle until the consistency is pulverulent like sand
c) Transfer this sample with some liquid N_2 to a small plastic container

3) Mem-PER Buffer :
a) After complete evaporation of nitrogen, add 200 µl TBS Buffer for each 20 mg tissue and wash it by vortexing briefly
b) Transfer to a reaction tube and centrifuge at 1.000 x g at 4°C for 5 min
c) Discard supernatant and resuspend pellet in 150 µl of Reagent A per 20 mg tissue
d) Incubate 10 min at room temperature (r.t.) with occasional vortexing, while white, flocculent debris appear after adding
e) Place lysed cells on ice
f) Mix 2 parts Reagent C with 1 part Reagent B, making sufficient mixture for each sample to obtain 450 µl [123] (Note: round up and add 10% to the result!)
g) Keep Reagent B and C on ice at all times
h) Add 450 µl of this mix to the suspension with Reagent A and vortex
i) Incubate reaction tubes on ice for 30 min, vortexing every 5 min

4) Protein isolation:
   a) Centrifuge samples at 10.000 x g for 5 min at 4°C
   b) Transfer supernatant to new 2 ml reaction tubes
   c) Incubate 20 min in 37°C water bath to separate the membrane fractions
   d) Centrifuge tubes r.t. for 3 min at 10.000 x g
   e) Do the next step (f) quickly, because the separation disappears with time
   f) Carefully remove hydrophilic phase (top layer) from the hydrophobic protein phase (bottom layer) and safe it in new tube
   g) Place separated fractions on ice
   h) Majority of membrane proteins (e.g. MT_1 receptor) will be in the lower hydrophobic fraction, while cytoplasmic proteins are found in the hydrophilic phase
   i) Aliquot samples and store at -80°C

5.3 Determination of the protein concentration using the bicinchoninic acid (BCA) assay

Background

A variety of spectrophotometric methods enable the determination of the protein concentration in protein lysates. The BCA-assay is one of the most popular methods for protein quantification. It is compatible with up to 5% detergent in the sample, and therefore compatible with many protein extraction protocols that use detergents to extract proteins (5.2). The BCA-assay is a two-step procedure. In a first step, proteins react with alkaline cupric sulfate in the presence of tartrate, thereby reducing Cu^{2+} to Cu^{1+}. A tetradentate copper complex forms from four peptide bonds and one atom of copper. This is known as “Biuret reaction” [124], which was extended to the BCA-Assay by Smith and co-workers [125]. In the subsequent second step, BCA chelates the cuprous ion. An intense purple colored complex results, which absorbs light between 550 nm to 570 nm. The intensity of the colour is proportional to the amount of protein present in the sample.

5.3.1 Materials

- Box with ice
- Incubator, set to 37°C
- Infinite M200 PRO (multimode microplate reader device), Tecan
- Latex gloves (PraxiMed, Meditrade, Gentle Skin Sensitive, REF 1221)
- Paper Towels
- Pipette (Thermo Scientific, Finnpipette F2)
- Pipette tips (Thermo Scientific, Finntip Flex 200)

- Polystyrene Rack
- Reaction tubes, 2 ml, transparent (Biozym, 710190)
- 96-well plate, flat transparent bottom (Greiner Bio-One)

5.3.2 Reagents

- BCA Protein Assay Kit (Pierce Biotechnology, 23227)
    - Reagent A (500 ml, 23228)
    - Reagent B (25 ml, 23224)
    - BSA-standard Solution Ampule, 2 mg/ml (Bovine Serum Albumin, 10 x 1 ml)
- T-PER Tissue Protein Extraction Reagent (Thermo Scientific, 78510) for total protein extraction, or Mem-PER Eukaryotic Membrane Protein Extraction Kit (Thermo Scientific, 89826) for membrane protein fractions

5.3.3 Method [145]

1) Preparation:
    a) The BSA-standard solution (2 mg/ml) included in the BCA-Assay is diluted with T-PER buffer: 1:1 (1+1), 1:5 (1+4), 1:10 (1+9), 1:25 (1+24), 1:50 (1+49) and 1:100 (1+99). These dilutions can be stored at -20°C and be reused [1, 145]
    b) This standard is used to set up a standard curve
2) Determination of protein concentration:
    a) Apply 3 x 25 µl of pure T-PER buffer (blank) to the 96-well plate (25 µl/well)
    b) Apply 3 x 25 µl of each BSA-standard dilution to the plates
    c) The protein concentration of the samples should be in the range of the standard curve (effective range of the BCA-assay is 100-1500 µg/µl). As the protein concentration is unknown, at least 3 different dilutions per sample should be prepared using T-PER buffer (e.g. undiluted, 1:5, 1:10)
    d) Transfer 25 µl from each dilution into a well of the 96-well plate – apply 3 repetitions per sample
    e) Mix Reagent A (BCA Protein Assay Kit) with Reagent B (BCA Protein Assay Kit) immediately before use in a ratio of 50:1 [145]
    f) Add 200 µl to each well containing a sample, standard or blank. Calculate the appropriate amount of reagent mixture (e.g. 26 wells filled, round up to 30 wells. Multiply with x 200 µl = 6.000 µl Reagent A, 120 µl Reagent B) [1, 145]
    g) Shake plate for 10 sec in microplate reader, Infinite M200 PRO, and incubate for 30 min at 37°C
    h) Read out samples absorbance in microplate reader at wavelength 562 nm and store outcome as excel sheet [145]

i) Subtract mean of the blank absorption values from all standard and sample absorption values (to account for absorption of buffer alone). A standard curve can then be calculated with the results of the BSA standard dilutions (X-axis is protein concentration, Y-axis is absorption). Using linear regression, the measured absorbance of the samples is then transformed into the protein concentration per sample (µg/µl) taking the individual sample dilutions into consideration.

5.4 Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis (SDS-PAGE)

Background

SDS-PAGE was first applied by U. K. Laemmli [126]. Gel-electrophoresis is a procedure used to separate proteins contained in tissue or cell samples according to their electrophoretic mobility (i.e. rate of migration/electric field strength in a given medium). This depends on length/molecular weight, protein charge, and conformation of the molecule. SDS (sodium dodecyl sulfate) is an anionic detergent and is used to denature proteins and to apply a negative charge over each protein in proportion to its mass. Linearization of proteins is further supported by addition of reducing agents such as ß-mercaptoethanol to the sampling buffer, which break disulfide bonds (see 5.4.2) as well as boiling (95°C) of the sample. As a result, proteins will be fractionated strictly by their molecular weight (MW). Polyacrylamide (PA) gels are synthetic, transparent, relative chemically inert and can be prepared from acrylamide and the cross-linker bisacrylamide (after addition of ammonium persulfate and TEMED to initiate cross-linking) over a wide range of concentrations (3-30%), i.e. pores size. The gels function as a molecular filter in which bigger proteins get stuck in the upper part and smaller ones can reach the end of the gel.

In this thesis, 12% PA gels were used, which provide an optimal separation for proteins in the range of 20-150 kDa. To increase protein resolution, discontinuous SDS-PAGE was applied. In this method, separation gel and stacking gel have different pore sizes and contain different ions (discontinuous buffer system). These conditions enhance the sharpness of protein bands [127].

5.4.1 Materials

- Box with ice
- Capillary pipet tips for gel loading (Biozym, 728205)
- Coolable centrifuge (Eppendorf, 5415R)
- Filter papers with various width and length

- Gel electrophoresis apparatus (PeqLab, PerfectBlue Twin S), including glass plates, ceramic plates, spacers and combs (0.8 mm) and casting base
- Graduated cylinder 1000ml, 2000 ml
- Latex gloves (PraxiMed, Meditrade, Gentle Skin Sensitive)
- Laboratory bottles, 2000 ml, 1000 ml (Schott, Duran)
- Magnetic stirrer (Framo-Gerätetechnik, M21/1)
- Paper towels
- Pen, water-resistant
- pH-Meter (Metrohm, pHLab 827)
- Power supply for electrophoresis apparatus (PeqLab, EV231)
- Pipette (Thermo Scientific, Finnpipette F2)
- Pipette tips (Thermo Scientific, Finntip Flex 200)
- Polystyrene Rack
- Saran wrap
- Scale (Mettler, PJ3000)
- Thermomixer Comfort (Eppendorf, Germany) adjusted to 95°C
- Reaction tubes: transparent 2 ml, 1,6 ml and 650 µl (Biozym 710190, Biozym 710310, VWR 211-0024)
- Vacuum pump
- Vortexer (Scientific Industries, Vortex Genie 2, Model G560E)

5.4.2 Reagents

- Acetone (Merck, 1000142511)
- Acrylamide Bis solution 40%, 37.5:1 (Bio-Rad, 161-0148) for separation gel
- Acrylamide Bis solution 40%, 19:1 (Bio-Rad, 161-0144) for stacking gel
- APS, Ammonium persulfate (Sigma, A3678-25G)
- Aqua bidest. (prepared with ELGA Centra, LabWater)
- Bromophenol blue (Sigma, B0126)
- Ethanol 70%
- Glycerol 85% (Merck, 1040940500)
- Glycine (Merck, 1041691000)
- Hydrochloric acid 37%, HCl (Merck, 1003171000)
- Isopropanol (2-Propanol Merck, 1070222511)
- ß-Mercaptoethanol (Sigma, M6250)
- Page Ruler Prestained Protein Ladder (Thermo Scientific, 26616), containing 10 stained recombinant proteins (170, 130, 100, 70 (red), 55, 40, 35, 25, 15, 10 (green))

- SDS, Sodium dodecyl sulfate (Sigma, L3771-100G)
- TEMED, Tetramethylethylendiamin (Bio-Rad, 161-0801-MSDS)
- Tris =Trometamol=Tris(hydroxymethyl)aminomethane (Trizma® base, Sigma, T1503)
- Tween 20 for electrophoresis (Merck, 8221840500)

5.4.3 Preparation of buffers and solutions for electrophoresis

- 10 % (w/v) APS solution [145]
    - Dissolve 1 g APS in a final volume of 10 ml Aqua bidest.
    - Aliquote (1 ml) and store at -20°C

- 10x running buffer (stock solution) [145]
    - 30 g Tris (0.25 M)
    - 144 g Glycine (1.92 M)
    - 10 g SDS (1% w/v)
    - Dissolve and bring to a final volume of 1000 ml Aqua bidest.
    - Store at r.t.

- 1x running buffer [145]
    - Mix 1 vol. 10x stock solution and 9 vol. Aqua bidest.
    - Store at r.t.
    - Final concentration
        - Tris 25 mM
        - Glycine 192 mM
        - SDS 0.1% (w/v)

- 10 % (w/v) SDS solution
    - Dissolve 10 g SDS in a final volume of 100 ml Aqua bidest.
    - Store at r.t.

- 4x SDS-PAGE sample buffer (stock solution) [145]
    - Mix and dissolve by stirring
        - 4.8 ml 0.5 M Tris-HCl, pH 6.8 (240 mM Tris-HCl)
        - 4 ml Glycerol (40% v/v)
        - 0.5 ml β-mercaptoethanol (5% v/v)
        - 0.8 g SDS (8% w/v))
        - 4 mg Bromophenol blue (0.04% w/v)
    - filter and store in aliquots at -20°C

- 1x SDS-PAGE sample buffer (working solution)
    - Mix 1 vol. 4x stock solution and 3 vol. Aqua bidest.
    - Final concentration
        - Tris 60 mM
        - Glycerol 10 % (v/v)
        - β-mercaptoethanol 1,25 % (v/v)
        - SDS 2 % (w/v)
        - Bromophenol blue, 0.1% (w/v)

- 3 M Tris-HCl, pH 8.8 (500 ml) [145]
    - Dissolve 181.7 g Tris in Aqua bidest. (~450 ml)
    - Adjust pH 8.8 with HCl
    - Adjust to a final volume of 500 ml

- 0.5 M Tris-HCl, pH 6.8 (100 ml) [145]
    - Dissolve 6.1 g Tris in Aqua bidest. (~80 ml)
    - Adjust pH 6.8 with HCl
    - Adjust to a final volume of 100 ml

5.4.4 Methods

5.4.4.1 Preparation of separation gel

1. All materials used (plates, combs, spacers) must be clean: washed with detergent and water, followed by 70 % ethanol
2. Fix the amount of gels required for the experiment
3. Assemble the apparatus: ceramic plates, spacers (0.8 mm) and glass plates to build the casting stand
4. Before pouring the gel, test the tightness of the apparatus: fill it up with water and wait 5 min to verify absence of chamber leakage
5. If this test is ok, empty the apparatus and dry it accurately
6. All materials must be absolutely dry before going on
7. Mark the filling level for the separation gel (draw a line at around two-thirds of the total gel height with a water-resistant pen)
8. Mix first 4 ingredients listed in Table 3 and add APS and TEMED immediately before pouring the gel, as they induce the gel polymerization [145]

9. Pour gel-solution into casting stand (up to the marked line) leaving enough place for stacking gel (~2 cm)
10. Overlay each gel with isopropanol immediately after filling to ensure vertical surfaces
11. Wait at least 1 h at r.t. in order to let the gel polymerize
12. Gels, which are not used immediately can be wrapped in saran wrap, placed in a wet-chamber and stored for a maximum of 2 weeks at 4°C

Table 3: Recipe for two 12 % separation gels

Number of Gels	Gel final %	Acrylamide and Bis Acrylamide solution 40 % 37.5 : 1	Tris-Hcl (3M, pH 8.8)	SDS (10%)	Aqua bidest.	APS (10%)	TEMED
2	**12%**	**3.75 ml**	**1.25 ml**	**100 µl**	**6.95 ml**	**100 µl**	**10 µl**

5.4.4.2 Preparation of stacking gel

1. Remove isopropanol
2. Any remaining fluid on the surface of the separation gel has to be absorbed carefully with filter paper. Be aware not to destroy the gel
3. Mix the first 4 ingredients listed in Table 4 and add APS and TEMED immediately before pouring the gel, as they induce the gel polymerization [145]
4. Overlay the separation gel with this stacking gel-solution, fill it to the brim of the apparatus and immediately afterwards insert a clean comb (10 or 12 wells) carefully avoiding air bubbles!
5. Wait at least 30 min at r.t. in order to let the gel polymerize
6. Mark the location of the comb with a water-resistant pen to be able to find the loading wells more easily once they are filled with running buffer
7. Remove comb
8. Do a suction cleaning of the wells with vacuum
9. Fill the electrophoresis apparatus with 1x running buffer in such a way that the gel is completely bathed from above and has a reservoir at bottom
10. Now the gel is ready to be loaded with samples and to start the SDS-PAGE

Table 4: Recipe for two 4% stacking gels

Number of Gels	Gel final %	Acrylamide and Bis Acrylamide solution 40 % 19 : 1	Tris-Hcl (0.5M,pH 6.8)	SDS (10%)	Aqua bidest.	APS (10%)	TEMED
2	**4%**	**0.75 ml**	**0.95 ml**	**75 µl**	**5.80 ml**	**75 µl**	**10 µl**

5.4.4.3 Preparation of samples

1. Keep all samples on ice to avoid protein denaturation

2. In order to load defined protein amounts per well of the gel, the desired protein amount is divided by the protein concentration of the sample. The proteins in the calculated volume of the sample are precipitated with 10 vol. ice cold acetone for 30 min on ice, vortexing every 5 min
3. Proteins are collected as a pellet by centrifugation for 10 min at 14.000 x g at 4°C
4. The supernatant is removed – invert open tubes on a paper towel and allow pellet to dry for 5 min
5. The pellet is resolved in 20 µl 1x SDS-PAGE sample buffer (acetone must not be contained!)
6. All samples are heated to 95°C for 5 min in Thermomixer
7. Centrifuge for 5 sec with 3.000 x g to collect the fluid at the bottom of the tube
8. The samples can either be immediately loaded to perform electrophoresis or stored at -20°C.
9. If they are stored at -20°C, repeat step 6 and 7 before loading of samples on the gel

5.4.4.4 SDS-PAGE

1. Load samples, 20 µl each, and 6 µl from PageRuler marker into the slots of the gel
2. Fill any empty sample well with 1x SDS-PAGE sample buffer
3. Close lid of the apparatus and connect it to power supply
4. Adjust power supply to a voltage of 150 V, maximal current (1000 mA) and maximal electrical power (150 W)
5. Run the gel until the dye (Bromophenol blue) in SDS-PAGE sample buffer has reached the bottom of the gel (about 90 min)
6. (Approximately 20 min before finishing, start with preparation for Western Blot)
7. Stop power supply, unplug and disassemble electrophoresis apparatus and continue with Western Blot

5.5 Western Blot (WB)

Background

Western blotting (Immunoblotting), developed by Towbin et al [128], describes a quantitative electrophoretic transfer technique from polyacrylamide gels to nitrocellulose or, especially for proteins used, PVDF (polyvinylidene fluoride) membranes. Current flow is used to pull proteins from the gel onto a PVDF or nitrocellulose membrane. As the result of the blotting process, the proteins are finally exposed on the surface of the membrane maintaining the organization they had within the gel [1, 145]. Both, PVDF and nitrocellulose membranes, bind proteins non-specifically by hydrophobic as well as charged interactions. Nitrocellulose is

more fragile, but cheaper than PVDF membranes [145]. The transfer process can be evaluated by reversible Ponceau S staining. Ponceau S is a sodium salt of a diazo dye, which is used as rapid, non-permanent red staining of protein bands on PVDF and nitrocellulose membranes [129]. At the end of the experiment a permanent staining, Amido black, is applied to compare for protein loading [130].

5.5.1 Materials

- Ballpoint pen
- Blotting apparatus, Semi-Dry Electro Blotter (PeqLab, PerfectBlue)
- Centrifuge tubes 15 ml, transparent (TPP, 91017)
- Centrifuge tubes 50 ml, transparent (TPP, 91051)
- Disposable Plastic Pasteur Pipettes 3 ml
- Extra Thick Blot Paper, Miniblot size 7 x 8.4 cm (Bio-Rad, #170-3966)
- Freezer bags
- Graduated cylinder, 1000 ml, 2000 ml
- Graduated pipettes 20 ml, 5 ml
- Laboratory bottles, 2000 ml, 1000 ml (Schott, Duran)
- Latex gloves (PraxiMed, Meditrade, Gentle Skin Sensitive)
- Magnetic stirrer (Framo-Gerätetechnik, M21/1)
- Paper towels
- Pipette controller (PIPETBOY acu 2, Integra)
- pH-Meter (Metrohm, pHLab 827)
- Power supply for Western blotting apparatus (PeqLab EV231)
- PVDF transfer membranes, 0.45 µm (Pall Corp., BioTrace P/N 66543)
- Scale (Mettler, PJ3000)
- Scanner (Canon CanoScan LiDE 700F)
- Shaker (Scientific industries, Roto-Shake Genie, SI-1102)
- Vacuum bag sealer (AudioTon)

5.5.2 Reagents

- Acetic acid (Glacial acetic acid, Merck, 100063)
- Amido black 10 B (Amido Black Staining Solution 2X, Sigma A8181)
- Aqua bidest., prepared with ELGA Centra, LabWater
- Dry milk powder (instant milk powder, Maresi)
- Glycine (Merck, 1041691000)
- Hydrochloric acid 37%, HCl (Merck, 1003171000)

- Isopropanol (2-Propanol, Merck, 1070222511)
- Methanol, MeOH (Merck, 1060022500)
- Ponceau S (Sigma, P3504)
- Potassium chloride, KCl (Merck, 104933)
- Potassium dihydrogen phosphate, KH_2PO_4 (Merck, 104877)
- Sodium chloride, NaCl (Merck, 1064040500)
- Sodium dodecyl sulfate, SDS (Sigma, L3771-100G)
- Sodium phosphate dibasic, Na_2HPO_4 (Merck, 106585)
- Tris =Trometamol=Tris(hydroxymethyl)aminomethane (Trizma® base, Sigma, T1503)
- Tween 20 for electrophoresis (Merck, 8221840500)

5.5.3 Preparation of buffers and solutions for WB

- Amido black staining solution 2x (Sigma, A8181-1EA)
    - Dilute Amido Black Staining Solution with aqua bidest in a ratio 1:1

- Amido black destaining solution
    - 25 % (v/v) isopropanol
    - 10 % (v/v) acetic acid
    - 65 % (v/v) Aqua bidest.

- Blotto (= blocking buffer) [145]
    - 25 g dry milk powder (5 % (w/v))
    - Dissolve and bring to a final volume of 500 ml PBS (use sterile PBS to extend life time)
    - Add 0.1 % (v/v) Tween 20
    - Prepare aliquots in 50 ml tubes
    - Store aliquots at -20°C

- Ponceau red destaining solution [145]
    - 5 ml acetic acid (1% (v/v))
    - Dissolve and bring to a final volume of 500 ml Aqua bidest.

- Ponceau red staining solution [145]
    - 0.5 g Ponceau S (0.1 % (w/v))
    - 25 ml acetic acid (5 % (v/v))
    - Dissolve and bring to a final volume of 500 ml Aqua bidest.

- 1x PBS [145]
    - Dissolve the following chemicals in 800 ml Aqua bidest:
        - 8.0 g NaCl
        - 0.2 g KCl
        - 1.44 g Na_2HPO_4
        - 0.24 g KH_2PO_4
    - Adjust pH to 7.4 with HCl
    - Adjust volume to 1000 ml with Aqua bidest.

- Transfer buffer [145]
    - 5.8 g Tris (48 mM)
    - 2.93 g Glycine (39 mM)
    - 0.375 g SDS (0.0375 % (w/v))
    - 200 ml MeOH (20% (v/v))
    - Dissolve and bring to a final volume of 1000 ml A.bidest.
    - Store at r.t.

- Wash buffer
    - 1x PBS
    - add 0.1% (v/v) Tween 20 (i.e. 1 ml Tween 20 + 999 ml PBS)

5.5.4 Methods

5.5.4.1 Preparation and procedure of WB

1. Start with preparations about 20 min before the end of the SDS-PAGE (see 5.4.4.4 (6))
2. Per gel, prepare 1 piece of PVDF membrane, and 2 pieces of extra thick blot paper with the same size as the gel (approximate dimensions: 85 mm width and 55 mm length)
3. Submerge PVDF membranes first in MeOH (100%) for 1 min, then in Aqua bidest. for 1 min and finally keep it in transfer buffer until the WB is assembled. This procedure is necessary, because the PVDF membrane is extremely hydrophobic and will not wet in aqueous solutions until pre-wetted with MeOH
4. After electrophoresis, dissemble apparatus and submerge the gels in transfer buffer for around 1 min

5. Assemble blotting apparatus in the following way:
   Anode – extra thick blot paper – PVDF – gel – extra thick blot paper - Cathode
6. This staple should be moistened with transfer buffer, but there should not be excess fluid
7. To avoid air bubbles, which is very important while blotting, use a 15 ml centrifuge tube and role it once and softly over the blot paper before closing the apparatus with the cathode
8. Connect to power supply and adjust to 3 mA/cm^2 blot (constant current!), maximal voltage, and maximal electrical power. Run the transfer for 90 min (i.e.: one blot ~ 47 cm^2 -> 140 mA is used, 280 mA for 2 blots)

5.5.4.2 Ponceau red staining

1. All subsequent steps are performed at r.t.
2. After transfer, dissemble blotting apparatus and immediately clean electrodes with clear water
3. Transfer PVDF membrane to a clean plastic tray of appropriate size and wash for 5 min with wash buffer (discard gel)
4. Stain proteins on PVDF membrane with Ponceau red solution, shaking for 5 min
5. Remove excess dye by using Ponceau distaining solution, shaking for 5 min
6. Wrap blots in saran wrap and scan blots to preserve protein pattern, safe as .tif-file
7. Thereafter, wash membrane with wash buffer for 10 min shaking to completely remove Ponceau red staining from membrane

5.5.4.3 Blocking unspecific binding sites on the membrane

Before incubation with primary antibody, it is necessary to block unspecific binding sites to reduce background labelling. Different blocking buffers can be used, which are ranging from fat-free dry milk powder to purified proteins such as BSA. For this thesis dry milk powder at a concentration of 5 % in PBS/Tween 20 (blotto) was chosen for blocking.

1. Take blotto (5.5.3) out of the -20°C device and let it completely defrost. To speed up thawing, put the tube in warm water
2. Prepare a freezer bag with slightly larger dimensions than the blot
3. Take the blot out of wash buffer (recipe see 5.5.3), put it in the freezer bag and heat seal three sites with the vacuum bag sealer
4. Add 20 ml of blotto to the bag and blot and heat seal the last site
5. Put membrane in bag on shaker for 1 h at r.t.

5.6 Immunodetection and membrane stripping for reprobing

Background

After blocking nonspecific binding sites, binding of specific antibodies allows detection proteins of interest. Detection of bound antibodies is performed either via antibody-conjugated enzymes or fluorescent probes. The latter can be visualized with a fluorescent imaging system. Enzymes must be combined with an appropriate substrate to generate a chromogenic precipitate on the membrane. Alternatively, in the most sensitive detection method, the enzyme is combined with a chemiluminescence substrate. This results in light production, which is captured by a film or a CCD camera. In direct immunodetection, it is the primary antibody, which is coupled to an enzyme or is fluorescently tagged. In indirect detection, the blot is incubated with a primary antibody to detect a specific protein followed by a labelled secondary antibody, which is directed against the primary antibody. As direct conjugation of the primary antibody may influence its immunoreactivity, the indirect protocol is most often used. Indirect detection offers several additional advantages such as signal amplification or availability of multiple labelled secondary antibodies.

In this thesis, the indirect detection was used. After incubation with a primary and a horseradish peroxidase (HRP)-conjugated secondary antibody, the membrane is incubated with a chemiluminescence substrate as described in 5.6.3.3. Then, the emitted light can be detected by exposing the blot to a chemiluminescence / X-ray film. After film development, blackened lines will indicate the position of proteins, which have reacted with HRP-linked antibodies.
When using chemiluminescence for detection, all antibodies can subsequently be stripped (removed) from the membranes allowing for several subsequent re-incubations (reprobing) of the membranes with other primary/secondary antibody combinations. The removal of antibodies from the membranes often requires a combination of detergents, reducing agents, heat and/or low pH. In this thesis, a stripping-puffer containing sodium-laurylsulfate and ß-mercaptoethanol in combination with increased temperature (60°C) was applied.

5.6.1 Materials

- Chemiluminescence film (GE Healthcare Amersham™ Hyperfilm™ ECL, 45-001-505)
- Disposable plastic Pasteur pipettes 3 ml
- Film development chamber with darkroom lamp (Ilford, E14,15 w)
- Film exposure cassette (Sigma, Du Pont Cronex Lightning Plus)
- Film-developing machine (Agfa GP 1000)
- Freezer bags

- Box with ice
- Latex gloves (PraxiMed, Meditrade, Gentle Skin Sensitive)
- Paper towels
- Plastic vessel to wash the blot
- Saran wrap
- Scanner (Canon CanoScan LiDE 700F)
- Scissors
- Shaker (Scientific Industries, Roto-Shake Genie)
- Vacuum bag sealer (AudioTon)

5.6.2 Reagents

- Blotto (see 5.5.3)
- Chemiluminescent Substrate (SuperSignal West Pico, 34078)
- Primary goat anti-mouse serum albumin antibody, α-albumin (Abcam, ab19194)
- Primary goat anti-rat IgG (H+L) antibody, α-IgG (Alexa Fluor® 488, A-11006)
- Primary anti-rat MT_1 antibodies (see chapter 6.1, Table 5)
    - Alomone labs, amr-031
        - provided with a blocking peptide
    - Biorbyt, orb11085
    - Santa Cruz, sc-13186
        - Santa Cruz blocking peptide, sc-13186 P
- Secondary antibodies (see chapter 6.1, Table 6)
    - Anti-goat-IgG-HRP, sc-2020
    - Anti-rabbit-IgG-HRP, sc-2004
- Stripping buffer (Roti®-Free Stripping-Puffer 2.0, Carl Roth, 3319.1)
- Wash buffer (see 5.5.3)

5.6.3 Methods

5.6.3.1 Incubation of the membrane with the primary antibody

1. Prepare the primary antibody in the desired concentration diluted in blotto and keep on ice. Prepare 4 ml solution per blot (for example, to prepare a 1:200 dilution, combine 20 µl antibody with ~4 ml blotto). Continue with (3).
2. Optional step: When testing for antibody specificity with a blocking peptide, split the prepared antibody solution equally in two tubes and label them (“blocking” and “control”). Add the blocking peptide provided with the primary antibody to the

"blocking" tube. For SC antibody, the amount of blocking peptide should be twice as much as the total amount of primary antibody in this tube (e.g. to 1 µg primary antibody, add 2 µg blocking peptide). For AL antibody, the amount of blocking peptide should be the same as the amount of primary antibody (e.g. to 1 µg primary antibody, add 1 µg blocking peptide).

Add an equivalent amount of blotto to the "control" tube. Incubate both tubes at r.t. for 30 min or overnight at 4°C, constantly shaking. Thereafter, continue with (3).

3. After blocking of membrane for 1 h as described in 5.5.4.3, open the bag and transfer membrane into a new freezer bag adjusted to the same size as before.
4. Again, heat seal three sites with the vacuum bag sealer
5. Add the antibody solution and seal the bag
6. Let the antibody incubate at 4°C on shaker over night
7. On the next day open bag and put membrane in wash buffer (5.5.3) at r.t.
8. Change the wash buffer 4 times and perform each washing step at least 10 min on the shaker

5.6.3.2 Incubation of the membrane with the secondary antibody

1. Prepare secondary antibody the same way as described for the primary antibody (5.6.3.1)
2. Use again 4 ml per blot, but dilute 1:2000
3. NOTE: the secondary antibody has to be directed against the host of the primary, otherwise no signal will be detected!
4. Place membrane in a fresh freezer bag, apply secondary antibody solution and seal the bag
5. Let this antibody bind to membrane for 1 h at r.t. on the shaker
6. Afterwards, wash the blot with wash puffer 4 times on the shaker, each washing step should last for at least 10 min

5.6.3.3 Incubation with chemiluminescence substrate, film exposure and film development

1. Mix equal parts of both components of the chemiluminescence substrate to obtain a final volume of 0,1 ml/cm^2 membrane
2. Put the membrane on a saran wrap, cover with this solution and incubate for 5 min (switch off room lamps during this incubation)
3. Wrap the membrane in a fresh piece of saran wrap and gently smooth out air bubbles
4. Place and fix the membrane with the protein side up in a film exposure cassette

5. Proceed in dark room
6. NOTE: Open the box containing chemiluminescence films ONLY at light from special darkroom lamp (otherwise, films are ruined!)
7. Adjust and place a sheet of chemiluminescence film on top of membrane
8. Close cassette and expose for individual times (exposure times depend on the antibodies used and proteins investigated)
9. Develop the film immediately using a film developing machine
10. Scan all results (the developed films) and save as .tif- files

5.6.3.4 Membrane stripping for reprobing

In order to reuse a blot, i.e. to incubate it with another set of antibodies, Roti®-Free Stripping-Puffer 2.0 can be applied.

1. Use 10 ml of stripping buffer per membrane
2. Heat buffer in tube up to 60°C
3. Apply it to the blot, sealed in a freezer bag
4. Let it shake for 40 min at 60°C
5. Apply washing buffer and change the buffer 4 times
6. Each washing step should last for at least 15 min on the shaker
7. Afterwards, start again incubation with blocking buffer (see 5.5.4.3) and immunodetection (see 5.6.3.1 - 5.6.3.3)

5.6.3.5 Amido black staining

Following immunodetection, the permanent Amido black 10 B staining is done. This staining dyes protein in a black-blue colour, providing a comparison for protein loading.

1. Place blot in a plastic vessel
2. Incubate with Amido black staining solution 1x for only 1 min on the shaker
3. Afterwards put the solution back in the storage bottle (it can be reused a few times)
4. Wash the blot with Amido black destaining solution, cover it with saran wrap, in order to prevent evaporation
5. Shake for 30 min at r.t.
6. Then wash the blot with Aqua bidest. for 1 min
7. Wrap blot in saran wrap and scan blots to preserve protein pattern, safe as .tif-file
8. Throw blot away

6 Results

6.1 Selection of antibodies for detection of rat MT_1 by WB

In this study, three commercially available primary antibodies against rat MT_1 were tested for cross-reactivity and specificity by WB. Table 5 summarizes suppliers and relevant product information. Important inclusion criteria were rat species reactivity and a demonstrated application in WB as well as in immunohistochemistry/immunofluorescence microscopy on fixed, paraffin-embedded tissue to be able to explore MT_1 localization in future experiments. Furthermore, antibodies raised against peptide antigens were selected to reduce the probability of cross-reactivity, which is more often seen for antibodies raised against protein antigens. Antibody sc-13186, from Santa Cruz Biotechnology was included since it has been used in several published studies [64-69]. Among the other 9 suppliers of MT_1 antibodies, Abnova, Origene and MyBioSource were excluded because they used proteins instead of peptides to generate the antibodies and antibodies-online was not recommending the antibody for IHC application. From the remaining 5 companies (Abbiotec, Alomone Labs, Biorbyt, Bioss Inc. and Novus Biologicals), which fulfilled all inclusion criteria, the Biorbyt antibody, orb11085, was selected based on previous good experiences of our laboratory with the company's antibody products. Finally, anti-rat MT_1 antibody from Alomone Labs, amr-031, was selected since a blocking peptide was provided with the antibody. Although the supplier does not explicitly recommend this antibody for immunohistochemistry/immunofluorescence microscopy, a tissue staining by immunofluorescence microscopy is provided on the homepage [131].

For signal detection in WB, an indirect protocol was chosen, where an HRP-labeled secondary antibody directed against the primary antibodies was applied. A major advantage of this protocol over direct detection is the signal amplification (melatonin receptors exhibit low expression levels). Furthermore, labeling of the secondary antibodies excludes that the immune-reactivity of the primary antibodies is affected. Affinity purified secondary antibodies were used in this study to increase sensitivity and reduce background caused by nonspecific antibodies. The product information of the secondary antibodies is summarized in Table 6.

Table 5: Relevant product information of the primary anti-rat MT_1 antibodies used in this thesis Abbreviations: C-Cow, Ch-Chicken, D-Dog, H-Human, M-Mouse, P-Pig, R-Rat, Sh-Sheep, ELISA – Enzyme-linked immunosorbent assay, IHC – Immunohistochemistry, WB - Western blot

Company	SANTA CRUZ	BIORBYT	ALOMONE LABS
Abreviation in Thesis	SC	BB	AL
Catalog Number	sc-13186	orb11085	amr-031
Anitbody Type	Polyclonal	Polyclonal	Polyclonal
Host	Goat	Rabbit	Rabbit
Species Reactivity	H,M,R	H,C,Ch,D,M,P,R,Sh	H,M,R
Guaranteed Applications	WB, IHC, ELISA	WB, IHC, ELISA	WB
Suggested Dilutions	WB:1:100-1:1000	WB: 1:100-1:500	WB: 1:200
Blocking Peptide	sc-13186 P	not avaliable	provided with antibody
Immunogen	Near the C-terminus of MEL-1A-R of rat origin	Unspecified	Amino acid residues 223-236 of mouse origin

Table 6: Relevant product information of the secondary, HRP-conjugated antibodies used in this thesis Abbreviations: α-goat: anti-goat-IgG - HRP conjugated secondary antibody, α-rabbit: anti-rabbit-IgG - HRP conjugated secondary antibody, HRP- Horseradish peroxidase, WB - Western blot

Company	SANTA CRUZ	SANTA CRUZ
Product Identification	Anti-Goat IgG-HRP	Anti-Rabbit IgG-HRP
Abreviation in Thesis	α-goat	α-rabbit
Catalog Number	sc-2020	sc-2004
Host	Donkey	Goat
Suggested Dilutions	WB:1:500 - 1:10000	WB:1:500 - 1:10000
Used Dilution	WB: 1:2000	WB: 1:2000

6.2 Preparation of tissue lysates used as MT_1 positive controls in WB

Expression of rat MT_1 mRNA in a variety of rat tissues has been investigated previously [44, 66, 132] and along with other sites was found in brain [44, 66] as well as in eye [66, 132]. Therefore total protein lysates of rat eye and brain were prepared as positive control lysates. First, because MT_1 protein expression in total tissue lysates by WB had already been successfully demonstrated [67, 133] and second, to verify minimal cross-reactivity with non-MT_1 proteins in total tissue, where subsequent localization studies need to be done. Rat eye and brain and, later on, also cerebellum tissues were collected as described in chapter 5.1.1 and total protein lysates were prepared with T-PER buffer (see chapter 5.2.3). Protein concentration was determined according to chapter 5.3.3.

Rat MT_1 is composed of 353 amino acids [44, 134] and consequently the unglycosylated rat MT_1 protein has a proposed MW of 40 kDa, which might shift to a higher MW upon glycosylation. Indeed, a MW of 37-40 kDa has been demonstrated by WB for MT_1 in several studies [67, 68, 135, 133], though not all [66]. As MT_1 contains two consensus sites for N-terminal asparagine-linked glycosylation [44], proteins with higher MW (40-60 kDa) might therefore be expected as well. For human MT_1, a MW of 60 kDa has been shown by WB [51]. To facilitate identification of 40-60 kDa proteins, this MW range was marked in all WB figures of this thesis with brackets.

6.3 Determining specificity and cross-reactivity of SC and BB anti-MT_1 antibodies on positive control lysates

6.3.1 SC and BB anti-MT_1 antibody specificity tested on total protein lysates from brain and eye

Varying amount of protein (10-60 µg protein/lane) were loaded on one 12 % gel and separated under reducing and denaturing conditions by SDS-PAGE (Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis) as described in chapter 5.4.4 in order to resolve the proteins strictly by size. Melatonin receptors contain cysteine residues in the extracellular domain and might form intramolecular disulfide bridges [136], which may influence the electrophoretic separation, resulting in additional bands or bands of unexpected MW. Following separation, proteins were transferred to a PVDF membrane (see chapter 5.5.4). Subsequently, immunoblotting was performed as described in chapter 5.6.3. The blot was first incubated with SC antibody and the corresponding secondary HRP-linked antibody (Figure 10). Following development of the blot, the membrane was stripped (as described in

chapter 5.6.3.4) from antibodies and incubated with BB antibody and the corresponding secondary antibody. This result is shown in Figure 11.

Figure 9 compares removable Ponceau red staining, which was done directly after blotting, and permanent Amido black staining, that was applied after the last antibody, here BB. Both dyes were applied to control for loaded protein amounts and to ensure efficient transfer of proteins during blotting. As seen in Figure 9, the two dyes gave comparable results. Therefore, from hereon, only Ponceau red staining is shown for all experiments.

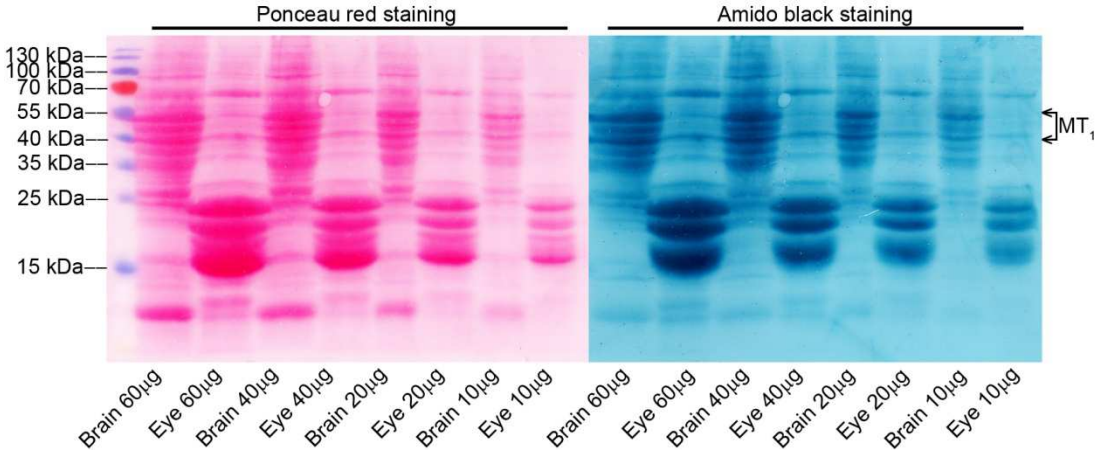

Figure 9: Comparing Ponceau red with Amido black staining from proteins on WB membranes. Left blot: Ponceau red staining, right blot: Amido black staining

As shown in Figure 10, SC antibody did not detect any band at 40 kDa. Prominent bands were detected mainly in eye below 25 kDa. Less intense bands were found at 55 kDa and 70 kDa in eye. Some faint signals at this MW were also found in brain.

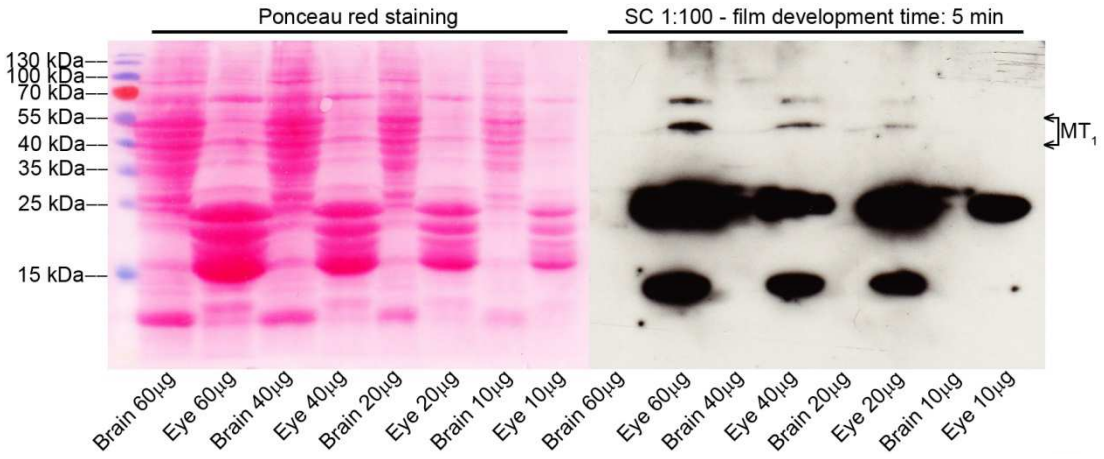

Figure 10: WB analysis of MT_1 expression in total protein lysates of brain and eye using SC antibody. Left blot: Ponceau red staining, right blot: incubation with SC 1:100 - film development time: 5 min

The result of the incubation with BB antibody is shown in Figure 11. A band at 40 kDa was found in the brain fractions, but several other bands of higher MW (70-100 kDa) and lower MW (25 kDa) were also detected. In the eye sample, this antibody did not react with proteins in the range of 40-60 kDa, but resulted in very prominent bands at 25 and 15 kDa. The identity of these proteins could not be determined in this thesis.

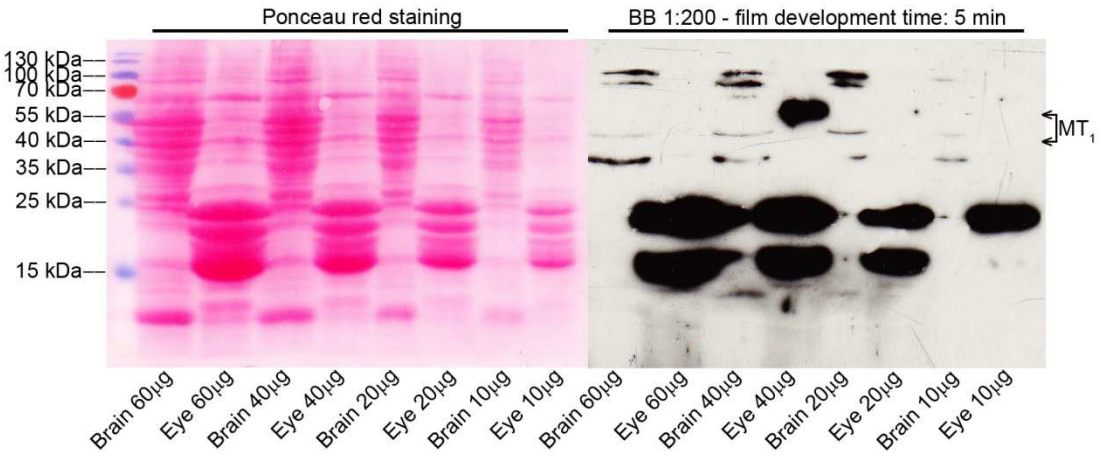

Figure 11: WB analysis of MT_1 expression in total protein lysates of brain and eye using BB antibody. Left blot: Ponceau red staining, right blot: incubation with BB 1:200 - film development time: 5 min

To summarise this first experiment, SC anti-MT_1 antibody did not detect a protein of 40 kDa in the positive control samples rat eye and brain, but detected proteins of higher MW in the eye sample, which could represent a glycosylated MT_1 form and prominent proteins of lower MW. BB antibody reacted with a protein of 40 kDa mainly in brain, but detected also many other proteins of higher (brain) or lower (eye) MW.

6.3.2 Determination of secondary antibody unspecific cross-reactivity on total protein lysates of brain and eye

As seen from the first experiment, various proteins of unexpected sizes (i.e. <40-60 kDa and >40-60 kDa) were detected with two anti-MT_1 antibodies. To test for non-specific reactivity of the employed secondary antibodies, the next experiment was performed. Different amount of brain samples (50 and 100 µg/lane) were separated by 12 % SDS-PAGE and proteins transferred to a PVDF membrane. Following transfer, the membrane was cut in four parts. Two parts were incubated with BB and SC 1:100 and their respective secondary antibodies, the other parts were incubated with the respective secondary antibodies alone (α-goat and α-rabbit, 1:2000). The results are shown in Figure 12.

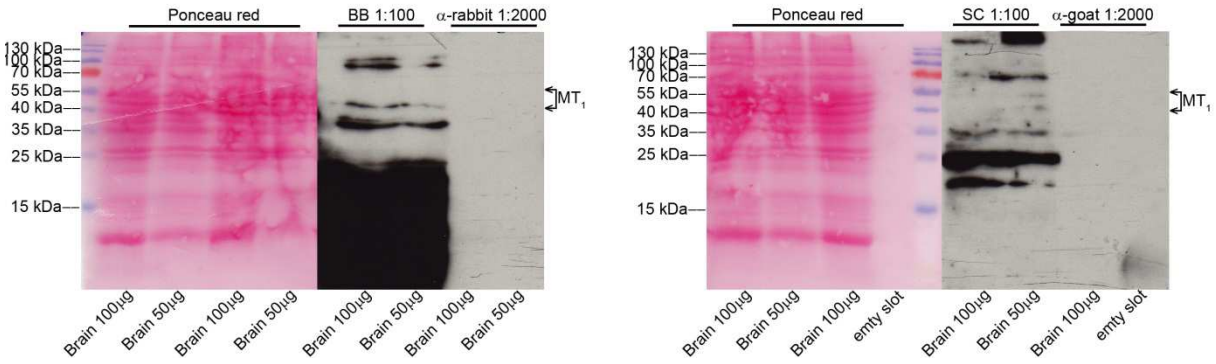

Figure 12: WB analysis of MT_1 expression in total protein lysates of brain using two different primary antibodies and determination of secondary antibody cross-reactivity.
Left blot: Ponceau red compared with BB 1:100 combined with secondary antibody and α-rabbit 1:2000 alone, right blot: Ponceau red compared with SC 1:100 combined with secondary antibody and α-goat 1:2000 alone - film development time: 20 min

As previously seen in Figure 11, BB antibody gave a band at 40 kDa in brain, but also reacted with other proteins at higher and lower MW. The reason for the black staining of the film from 25 kDa down to the end of this blot was unique and remained unclear. In analogy to Figure 10, SC gave no signal at 40 kDa. Again, however, the antibody reacted with proteins of higher and lower MW. A longer film exposure time compared to Figure 10 and Figure 11 was chosen to test the specificity/cross-reactivity of the secondary antibodies. No signal was detected when only secondary antibodies were used, thereby demonstrating that any observed band was caused by BB and SC anti-MT_1 antibody.

6.3.3 SC and BB anti-MT_1 antibody specificity on totally lysates of brain, cerebellum and eye

In the next experiment, we introduced total lysate prepared from rat cerebellum as an additional positive control. Expression of mRNA had been demonstrated there, but also evidence for functional expression of MT_1 in rat cerebellar granule cells had been obtained [137-138]. Following preparation of total protein lysate from cerebellum with T-PER buffer and determination of protein concentration, eye, brain and cerebellum samples (50 and 100 µg/lane each) were separated by 12 % SDS-PAGE and proteins were transferred to a PVDF membrane (see chapters 5.4.4, 5.5.4).

The membrane was first probed with BB antibody and its corresponding secondary antibody. The lowest dilution i.e. the highest antibody concentration recommended by the company (1:100) was used. Figure 13 and Figure 14 show the results of the WB, obtained after film development times of 30 sec and 1 min, respectively.

In analogy to Figure 11 and Figure 12, BB antibody detected proteins with MW of about 40 kDa in all brain and cerebellum samples. As expected the intensity of the signal was stronger

in cerebellum. Upon prolonged incubation (>1 min) and at high protein concentrations (100 µg/lane), bands at this MW were also seen in the eye sample. Unfortunately, even in cerebellum, many other proteins were detected under the applied conditions.

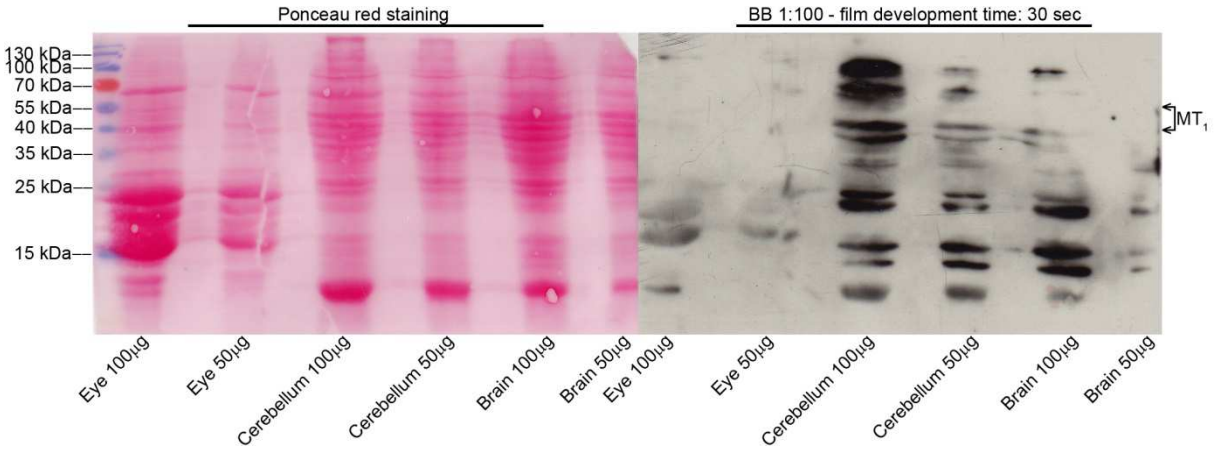

Figure 13: WB analysis of MT_1 expression in total protein lysates of eye, cerebellum and brain using BB antibody. Left blot: Ponceau red staining, right blot: BB 1:100 - film development time: 30 sec

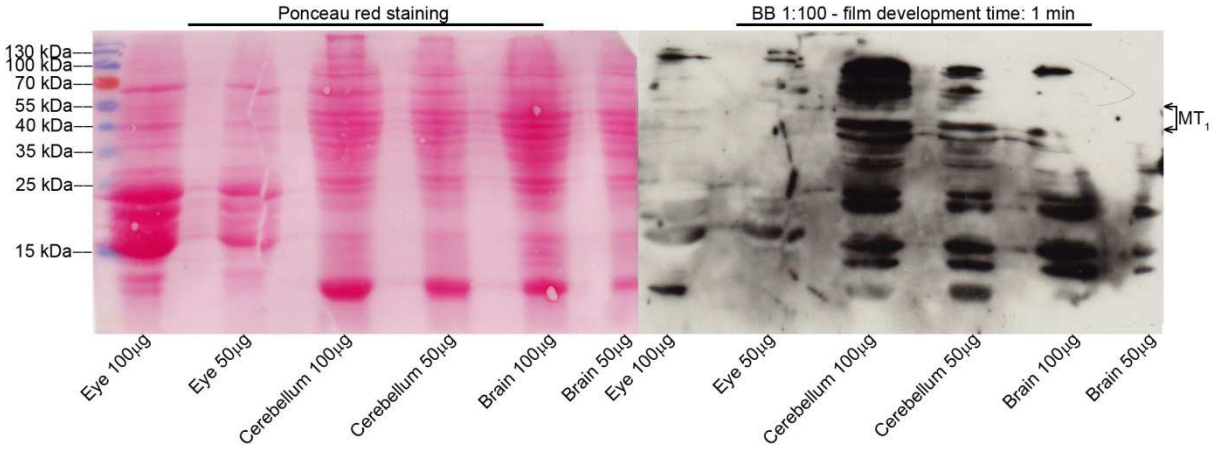

Figure 14: WB analysis of MT_1 expression in total protein lysates of eye, cerebellum and brain using BB antibody. Left blot: Ponceau red staining, Right blot: BB 1:100 - film development time: 1 min

Following stripping (chapter 5.6.3.4) of antibodies, the same blot was reused for incubation with SC antibody and its corresponding secondary antibody. Again, the lowest recommended dilution (1:100) was used (Figure 15). As seen in the previous blots (Figure 10 and Figure 12), SC antibody did not react with a 40 kDa band in any of the samples (except for a low signal in cerebellum 50 µg). The blot revealed bands at higher (70 kDa) and lower MW (25 kDa) as seen before, but not all of the previously detected additional protein bands (e.g. 15 kDa) were deserved in this experiment.

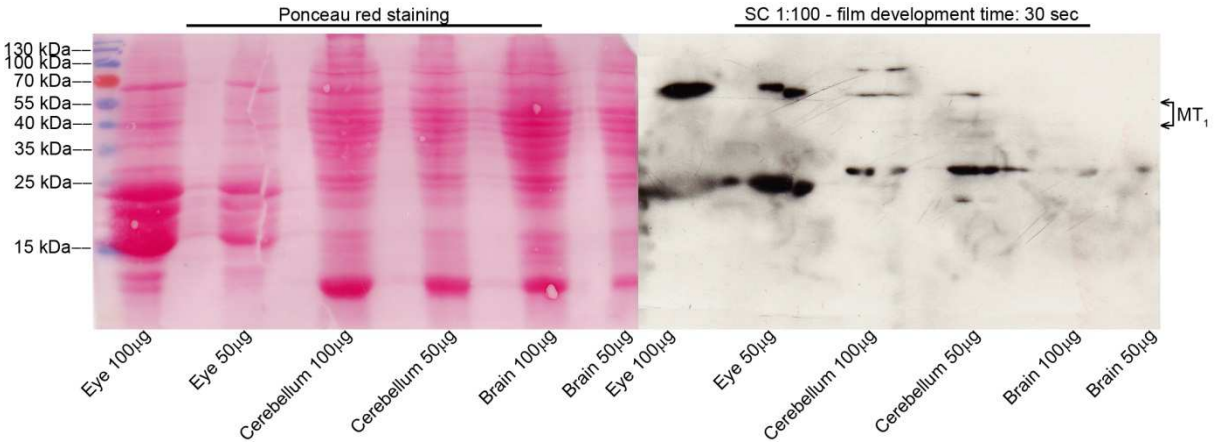

Figure 15: WB analysis of MT_1 expression in total protein lysates of eye, cerebellum and brain using SC antibody. Left blot: Ponceau red staining, right blot: SC 1:100 - film development time: 30 sec

In summary of these three initial experiments, only BB antibody detected a protein of 40 kDa (the MW of unglycosylated MT_1) in total brain and, more pronounced in cerebellum lysate. Both, BB as well as SC antibody reacted with several proteins of smaller (<25 kDa) and larger (>60 kDa) MW. On the one hand, these proteins may represent degradation products or glycosylated and/or oligomerized MT_1 proteins. Protein degradation was unlikely as tissue samples had been rapidly frozen after sampling. Moreover, lysates were prepared in the presence of protease inhibitors, were aliquoted to avoid degradation due to repeated freezing and thawing and were always kept on ice during sample preparation. In principle, protein oligomerization should be prevented due to reducing and denaturing conditions, however, especially with GPCRs, irreversible oligomerization may occur in SDS micelles. Another explanation for the observed bands could be nonspecific interaction of the antibodies with MT_1 unrelated proteins.

6.3.4 Optimization of WB results by modification of SC and BB antibody dilutions and wash conditions

To reduce any non-specific binding of the antibodies to the PVDF membranes, the previous experiment was repeated but with 1) reduced protein amounts, 2) modified antibody dilutions and 3) prolonged washing conditions: 25 µg as well 100 µg/lane of eye, brain and cerebellum

samples were loaded on two 12 % SDS-PAGE gels. Proteins were separated and transferred under identical conditions to PVDF membranes. One blot was incubated with BB antibody and the other with SC antibody, both antibodies were diluted 1:500, which is the highest dilution recommended for BB antibody. After incubation with secondary antibodies, the films were developed and the results are displayed in Figure 16 (BB antibody) and Figure 17 (SC antibody).

At a dilution of 1:500, the number and intensity of the bands was dramatically reduced for both antibodies, including those previously visible in the MW range of MT_1 (40-55 kDa). Consequently, this antibody dilution is not applicable to detect MT_1.

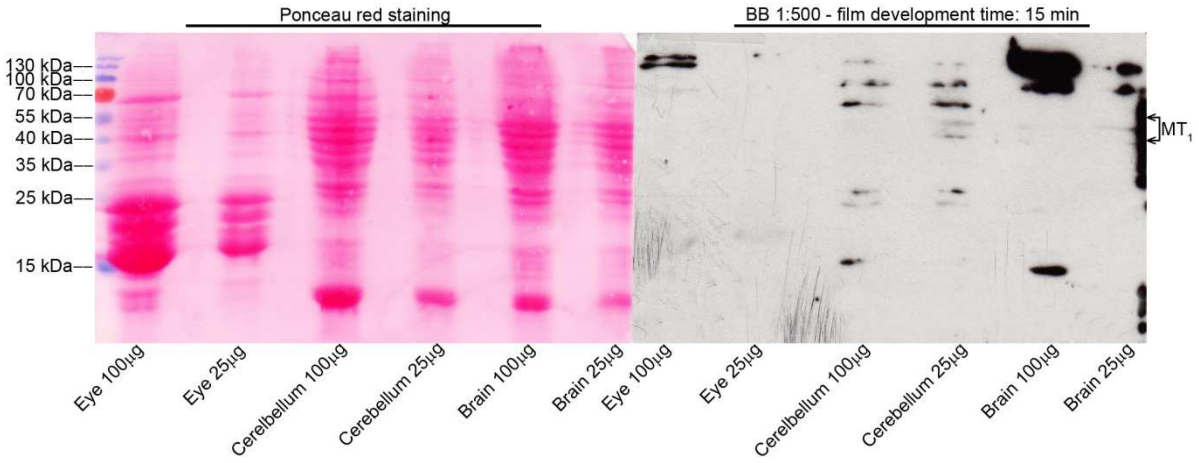

Figure 16: WB analysis of MT_1 expression in total protein lysates of eye, cerebellum and brain using BB antibody. Left blot: Ponceau red staining, right blot: BB 1:500 - film development time: 15 min

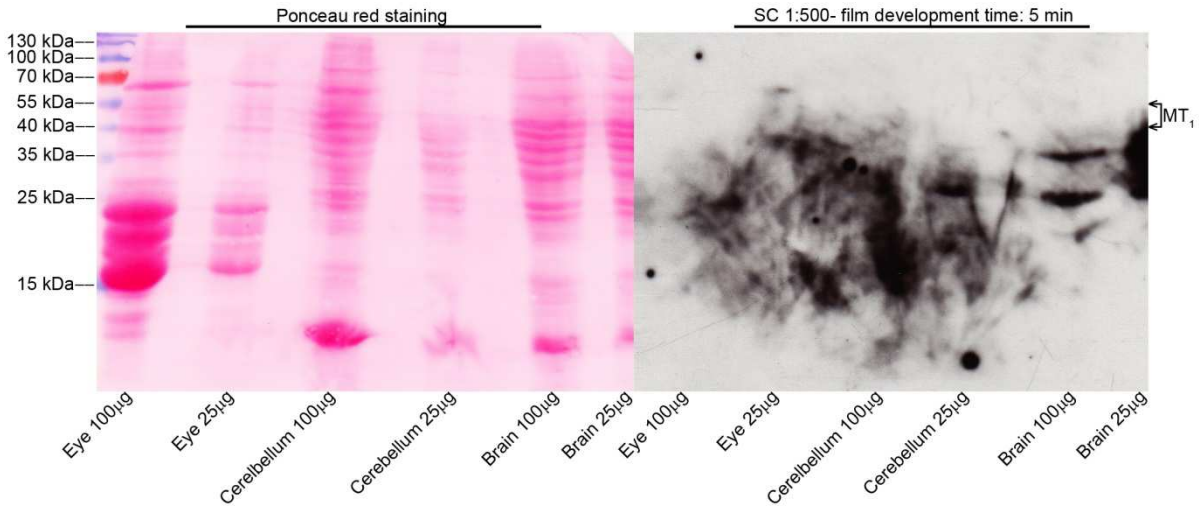

Figure 17: WB analysis of MT_1 expression in total protein lysates of eye, cerebellum and brain using SC antibody. Left blot: Ponceau red staining, right blot: SC 1:500 - film development time: 5 min

Thereafter, membranes were stripped (chapter 5.6.3.4) and the blots reincubated with the same antibodies at a higher concentration (1:250). Usually, the blots were washed 4 times for 10 min after incubation with the secondary antibody. Blots incubated with BB and SC

antibody and washed for this regular period are shown in Figure 18 (BB antibody) and Figure 20 (SC antibody). Following film development, the blots were then washed for 1 week at 4°C in wash buffer (see chapter 5.5.3 : PBS containing 0.1% detergent Tween 20) to minimize non-specific interactions. These results are depicted in Figure 19 (BB antibody) and Figure 21 (SC antibody).

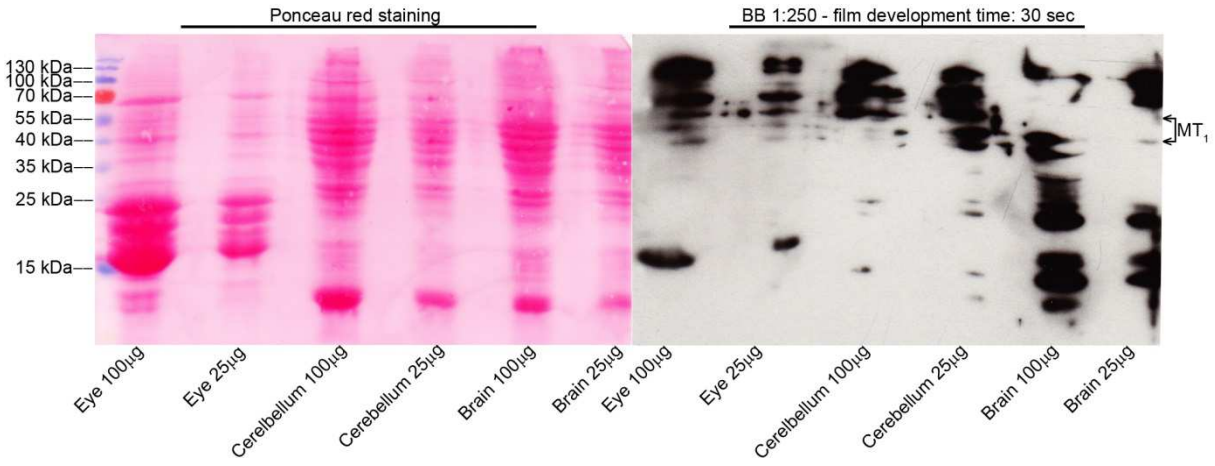

Figure 18: WB analysis of MT_1 expression in total protein lysates of eye, cerebellum and brain using BB antibody. Left blot: Ponceau red staining, right blot: BB 1:250 - film development time: 30 sec

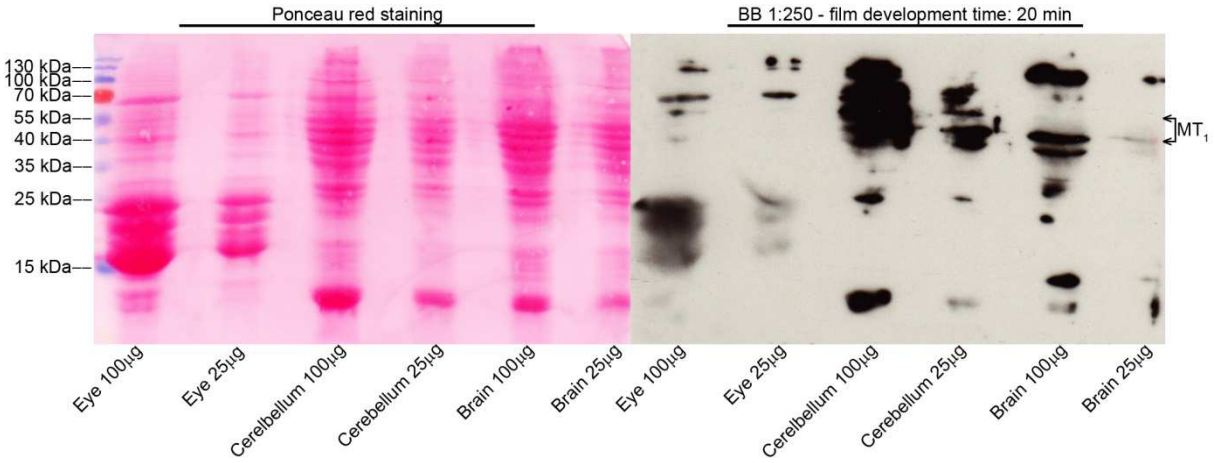

Figure 19: WB analysis of MT_1 expression in total protein lysates of eye, cerebellum and brain using BB antibody. Left blot: Ponceau red staining, right blot: BB 1:250 after washing with wash buffer for one week at 4°C - film development time: 20 min

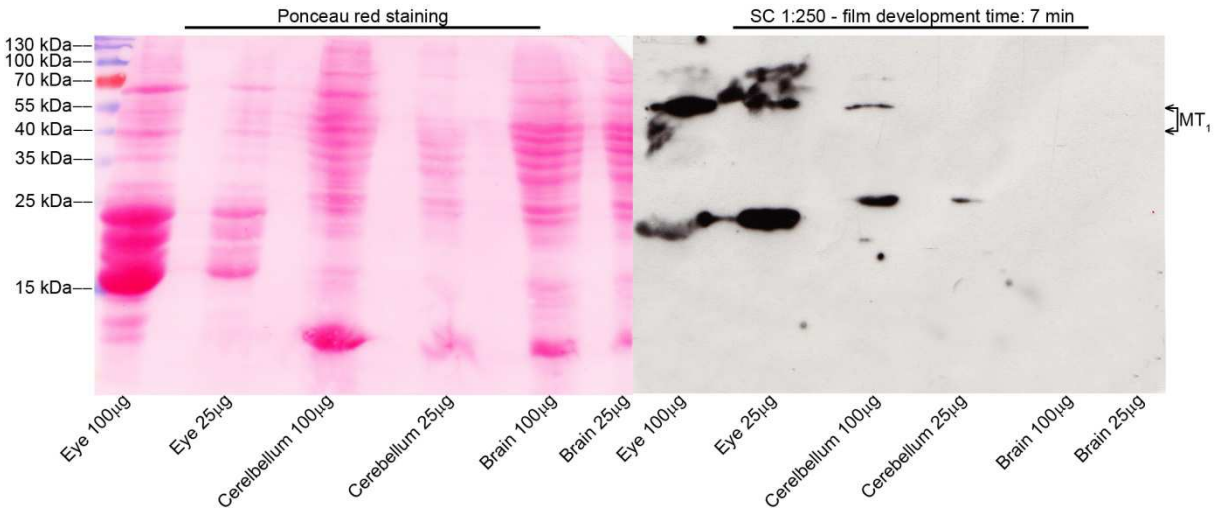

Figure 20: WB analysis of MT_1 expression in total protein lysates of eye, cerebellum and brain using SC antibody. Left blot: Ponceau red staining, right blot: SC 1:250 - film development time: 7 min

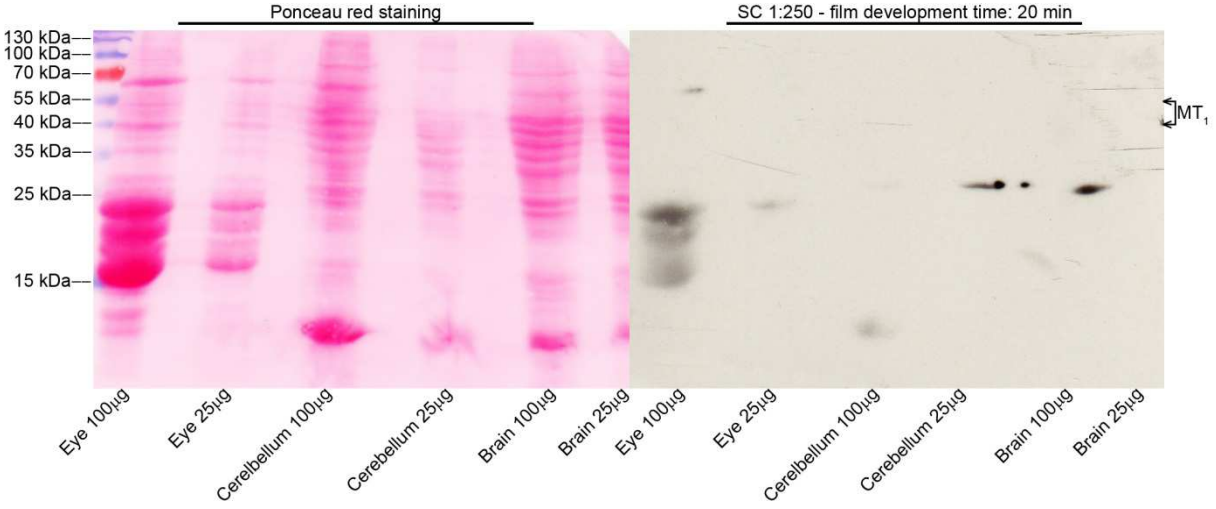

Figure 21: WB analysis of MT_1 expression in total protein lysates of eye, cerebellum and brain using SC antibody. Left blot: Ponceau red staining, right blot: SC 1:250 after washing with wash buffer for one week at 4 °C - film development time: 20 min

In the case of BB antibody, a dilution of 1:250 still enabled detection of proteins in the range of 40-60 kDa. Prolonged washing improved the detection of these proteins in brain and cerebellum samples, while at the same time the reaction with proteins of a smaller MW was reduced. Although a dilution of 1:250 appeared to be better than 1:100 or 1:500, the antibody still detected several proteins in total cell lysates.

The SC antibody, when used at a 1:250 dilution, did not detect proteins of the MW of interest (compare Figure 15). Prolonged washing reduced antibody reactivity even further (compare Figure 20 and Figure 21).

In summary, optimization of BB antibody dilution (rising from 1:100 to 1:250) and prolongation of the final washing step improved the performance of this antibody in WB.

Proteins in the MW range of MT_1 were detected in brain and cerebellum, while reaction with other protein was partly reduced. Nevertheless several proteins were detected with BB antibody. None of the SC antibody dilutions resulted in detection 40-60 kDa proteins of interest. Even under this condition both antibodies reacted with a protein at MW of 65-70 kDa.

6.3.5 Testing for SC and BB anti-MT_1 antibody cross-reactivity with albumin contained in total protein lysates of blood serum as well as cerebellum and eye

The aim of the next experiment was to find out, if the 65-70 kDa band observed on most blots was the result of antibody cross reaction with rat albumin, which is a 65 kDa protein [139]. Cross reaction of polyclonal antibodies with albumin have been described in literature [140] and have also been observed in our laboratory (Isabella Ellinger, unpublished observations). As albumin constitutes about 50 % of the blood proteins, rat blood serum was prepared as described in chapter 5.1.1 and added as a positive control to detect cross reactivity of BB and SC antibodies with albumin. 100 µg/lane total protein lysates of cerebellum and eye samples as well as 25-100 µg of blood serum were loaded on two 12 % SDS-PAGE gels and proteins were separated simultaneously. After transfer to PVDF membranes, one blot was incubated with BB antibody (1:250), while the other blot was incubated with SC antibody (1:250). After incubation with secondary antibodies, films were developed. Subsequently, the blots were stripped, and then incubated with an antibody against serum albumin at a dilution of 1:1000 (see chapter 5.6.2). The results are shown for BB antibody in Figure 22 and for SC antibody in Figure 23.

A protein of about 65 kDa was one of the major proteins in all blood plasma samples as shown by Ponceau red staining. Furthermore this protein also reacts with the albumin antibody. Anti-albumin antibody also reacts with 65-70 kDa protein in rat cerebellum and eye samples confirming the presence of this protein in the tissue samples. BB antibody (Figure 22) and even more pronounced SC antibody (Figure 23) reacted with the albumin protein in rat blood serum, which ran at the same MW as the 65-70 kDa protein in the tissue samples, suggesting that this band represents indeed cross reaction of anti-MT_1 antibodies with albumin. Moreover, BB and SC antibody cross reacted with low MW proteins (<25 kDa) in blood serum, underpinning the low specificity of both antibodies.

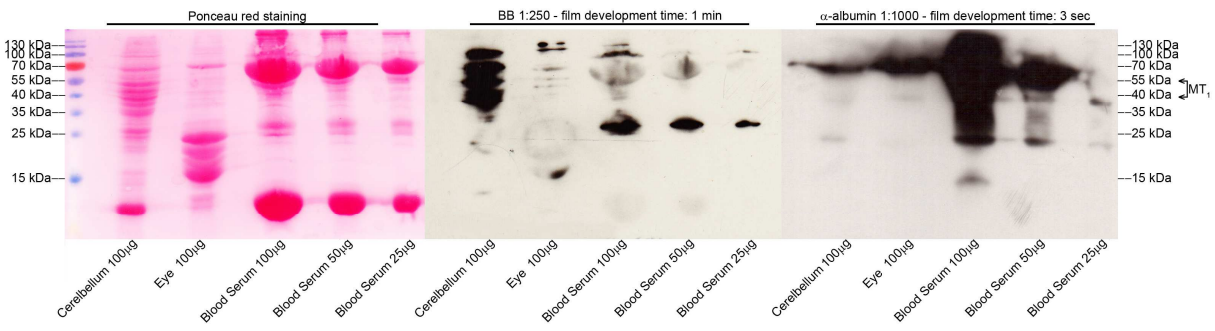

Figure 22: WB analysis of MT_1 expression in total protein lysates of cerebellum, eye, and blood serum using BB antibody (middle blot) and α-albumin antibody (right blot).
Left blot: Ponceau red staining, middle Blot: BB 1:250 - film development time: 1 min, right blot: α-albumin 1:1000 - film development time: 3 sec
Abbreviation: α-albumin: goat anti-serum albumin antibody from company Abcam (ab19194)

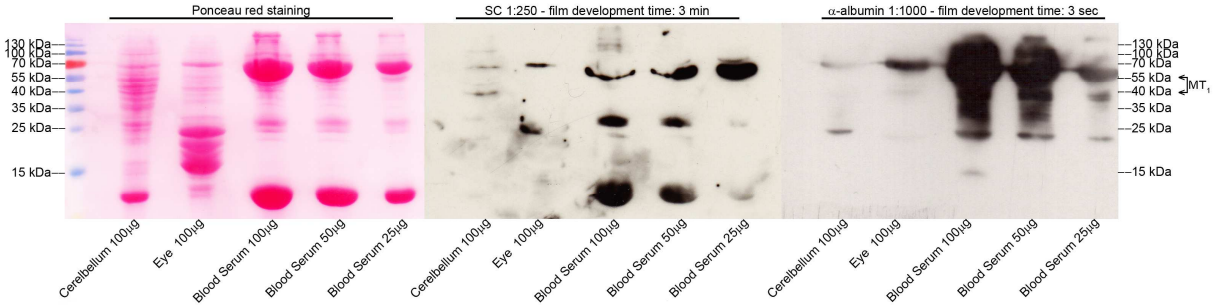

Figure 23: WB analysis of MT_1 expression in total protein lysates of cerebellum, eye, and blood plasma using SC antibody (middle blot) and α-albumin antibody (right blot).
Left blot: Ponceau red staining, middle Blot: SC 1:250 - film development time: 3 min, right blot: α-albumin 1:1000 - film development time: 3 sec
Abbreviation: α-albumin: goat anti-serum albumin antibody from company Abcam (ab19194)

In summary, both tested anti-MT_1 antibodies (BB and SC) exhibited significant cross reactivity with albumin, the major blood protein as well as several other proteins of smaller MW in blood serum.

6.3.6 AL anti-MT_1 antibody specificity on total protein lysates of cerebellum, eye, epididymal fat and mesenteric artery

Due to the fact that only Biorbyt (BB) antibody showed some reactivity with proteins in the MT_1 MW range (40-55 kDa), but BB as well as Santa Cruz (SC) antibody exhibited significant cross reaction with other proteins such as albumin, a third anti-MT_1 antibody was introduced and tested.

Total protein lysates of MT_1-positive tissue samples, eye and cerebellum (100 µg each) as well as blood serum (25 µg) to control for albumin cross reaction, were used in a first experiment. Furthermore total protein lysates prepared from rat epididymal fat and mesenteric artery (without perivascular fat), 100 µg each, were included. All samples were

separated on two 12% SDS-PAGE gels, proteins were transferred to PVDF membranes and subjected to immunodetection. Blot 1 was incubated with AL antibody (dilution 1:200), while blot 2 was incubated with BB antibody (dilution 1:250) to compare these two antibodies.

Figure 24 depicts the result with the new anti-MT_1 antibody (AL). In all tissue samples, major proteins bands were found in the 40-55 kDa MW range. The only exception was cerebellum, where several proteins of smaller MW were detected too. Reactivity in fat and mesenteric artery was lower than observed in cerebellum and eye. No cross reaction of AL antibody was observed with rat blood serum.

The obtained result for BB antibody is shown in Figure 25 and both antibodies are directly compared in Figure 26. Although BB antibody also detects proteins in the MW range of MT_1, it exhibits a clear cross reactivity with rat blood serum and causes more bands than AL antibody.

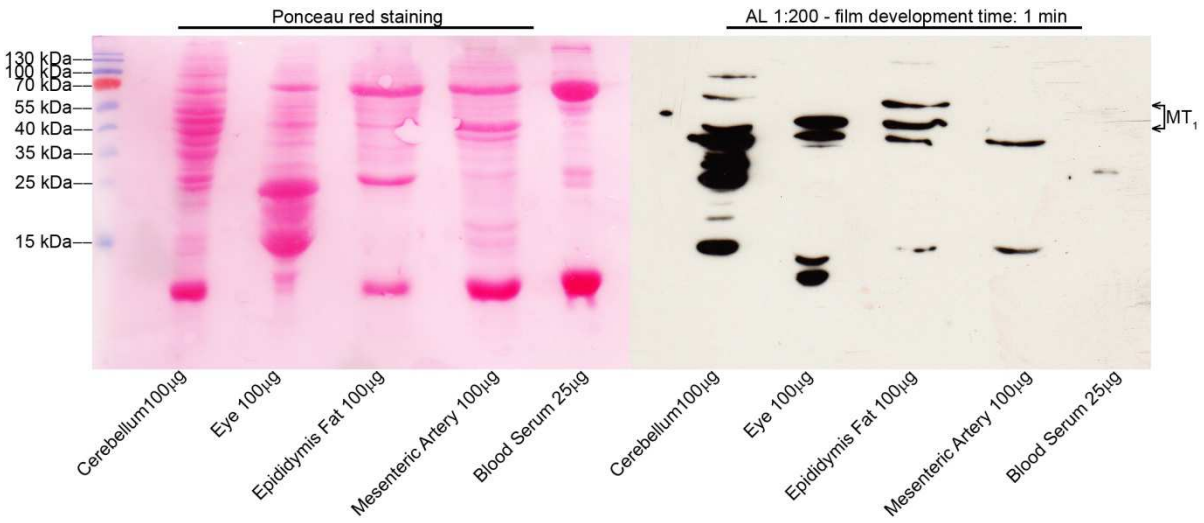

Figure 24: WB analysis of MT_1 expression in total protein lysates of cerebellum, eye, epididymis fat, MA and blood serum using AL antibody.
Left blot: Ponceau red staining, right blot: AL 1:200 - film development time: 1 min
Abbreviations: AL - Alomone primary anti-rat MT_1 antibody (amr-031), MA - mesenteric artery

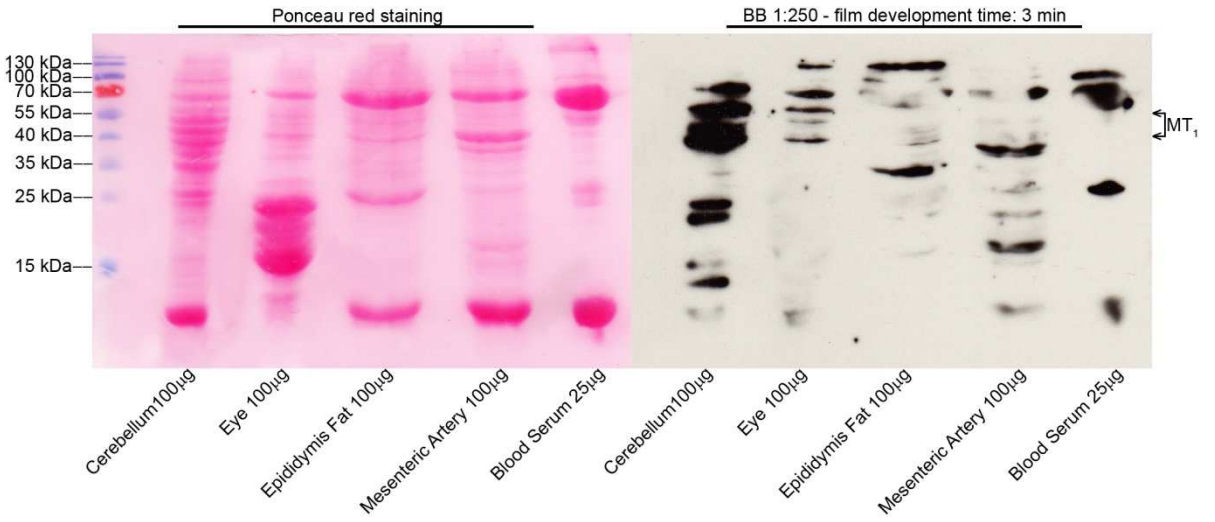

Figure 25: WB analysis of MT_1 expression in total protein lysates of cerebellum, eye, epididymal fat, MA and blood serum using BB antibody.
Left blot: Ponceau red staining, right blot: BB 1:250 - film development time: 3 min

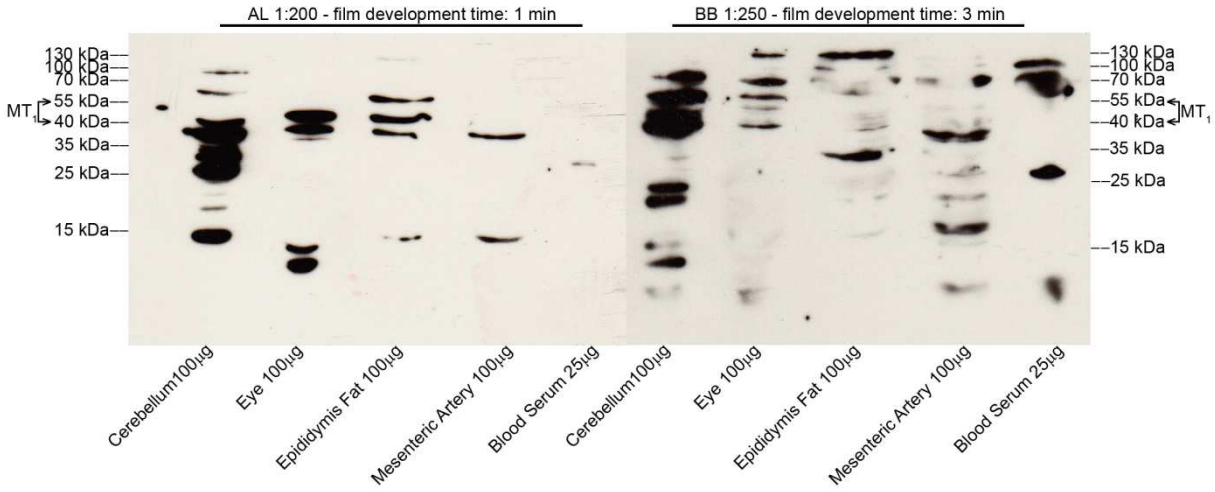

Figure 26: WB analysis of MT_1 expression in total protein lysates of cerebellum, eye, epididymal fat, MA and blood serum - a comparison of AL antibody (left) and BB antibody (right).
Left blot: AL 1:200 - film development time: 1 min, right blot: BB 1:250 - film development time: 3 min
Note: MW marker over 70 and lower 40 kDa at this figure are not congruent, because the right blot, which was incubated with BB antibody, was slightly bigger.

Next, the AL blot was stripped from anti-MT_1 antibody and was reincubated with antibody that allowed detection of IgG heavy (55 kDa) and light chain (25 kDa, Figure 27) and albumin (65 kDa, Figure 28) to detect any cross reaction with these prominent serum proteins. When these blots are directly compared to initiate AL blot (Figure 24), it is obvious that AL antibody does not cross react with the major serum proteins, IgG (Figure 27) and albumin (Figure 28), which are present in several tissue samples due to the presence of blood.

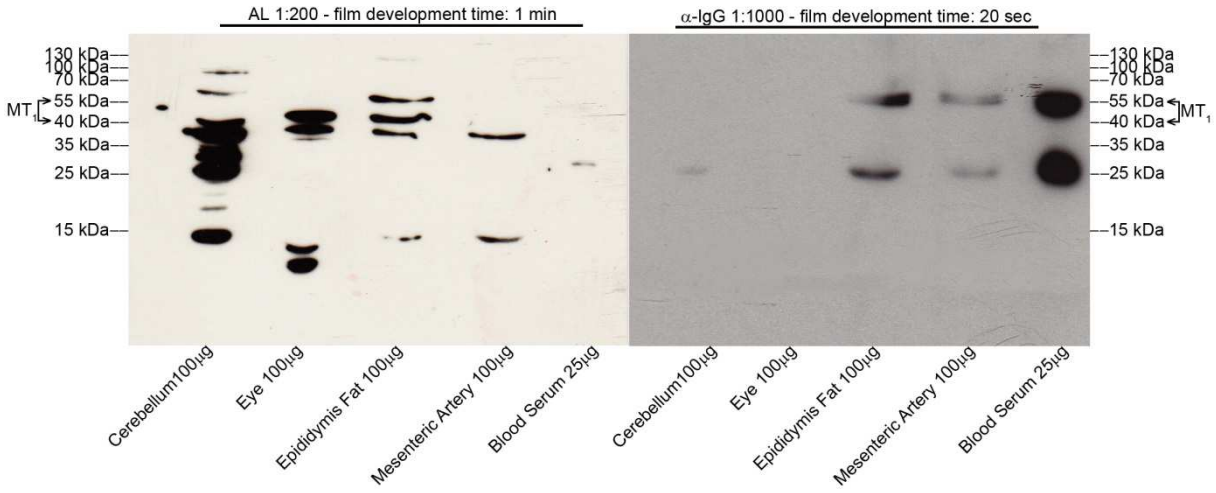

Figure 27: Comparing WB analysis of total protein lysates of cerebellum, eye, epididymal fat, MA and blood serum using AL and α-IgG antibody.
Left blot: AL 1:200 - film development time: 1 min, right blot: α-IgG 1:1000 - film development time: 20 sec
Abbreviation: α-IgG: goat anti-rat Immunoglobulin G (H+L) antibody, A-11006; MA - mesenteric artery

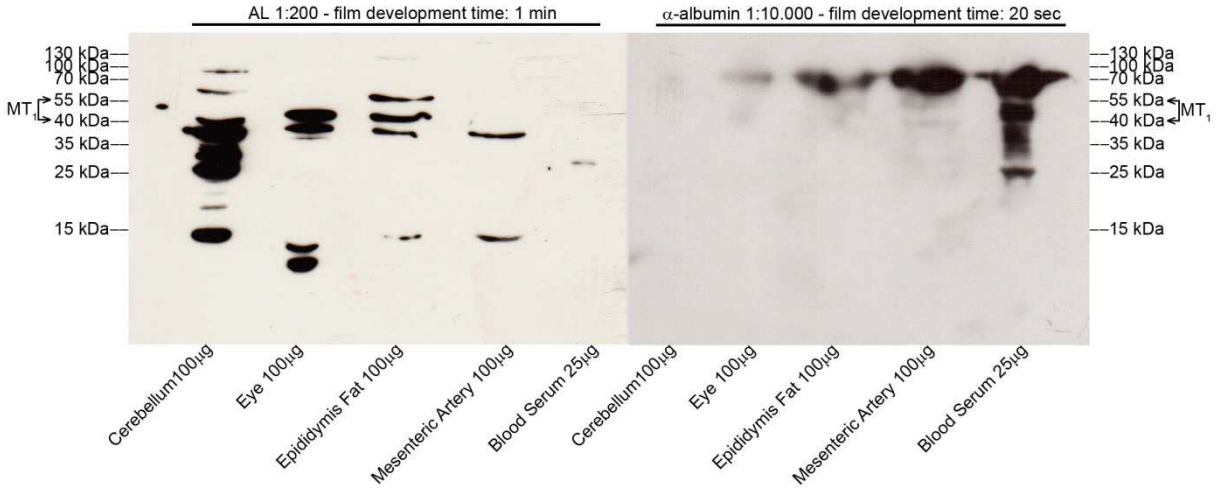

Figure 28: Comparing WB analysis of total protein lysates of cerebellum, eye, epididymal fat, MA and blood serum using Al and α-albumin antibody.
Left blot: AL 1:200 – film development time: 1 min, right blot: α-albumin 1:10.000 - film development time: 20 sec. Abbreviation: α-albumin: goat anti-serum albumin antibody, ab19194; MA - mesenteric artery

6.3.7 Validation of AL MT_1 antibody-specificity in comparison to SC MT_1 antibody-specificity using blocking peptides

Having confirmed that AL antibody exhibits low cross reactivity with abundant serum proteins such as albumin and IgG, it was tested whether the detected proteins in the range of 40-55 kDa MW were indeed MT_1. For this experiment, all protein samples used in the previous experiment were loaded twice on two 12 % reducing SDS-Gels (25 µg/lane). Following separation and transfer of proteins, each blot was divided in two equal parts. For each of the antibodies, SC and AL, the dilution 1:200 was prepared with a volume of 4 ml. Afterwards this was divided in half and 2 ml from each antibody solution was mixed with the blocking

peptide according to chapter 5.6.3.1 (2). The left side of the two blots was incubated with AL and SC antibody alone, while the right side of the two blots was incubated with AL and SC antibody pre-treated with the blocking peptide. The blocking peptide is identical to the peptide used to generate the anti-MT_1 antibody. Ideally, preincubation of the antibody with the blocking peptide shall reduce/abolish binding of the antibody to MT_1 present in the lysates of the membrane.

We could indeed observe this reduction of AL binding to the blot as shown in Figure 29. By stripping and reincubation of this blot with anti-albumin antibodies we verified that the bands observed with the AL anti-MT_1 antibodies are not caused by albumin cross reaction (Figure 30). When this experiment was done with the SC antibody, it demonstrated mainly cross reactivity with albumin, preincubation with the blocking peptide did not result in any significant reduction of the signal (Figure 31). This is not surprising as from the previous experiments we already suspected cross reaction with albumin. The cross reaction with albumin was confirmed by stripping and reincubation of the blot with α-albumin (Figure 32).

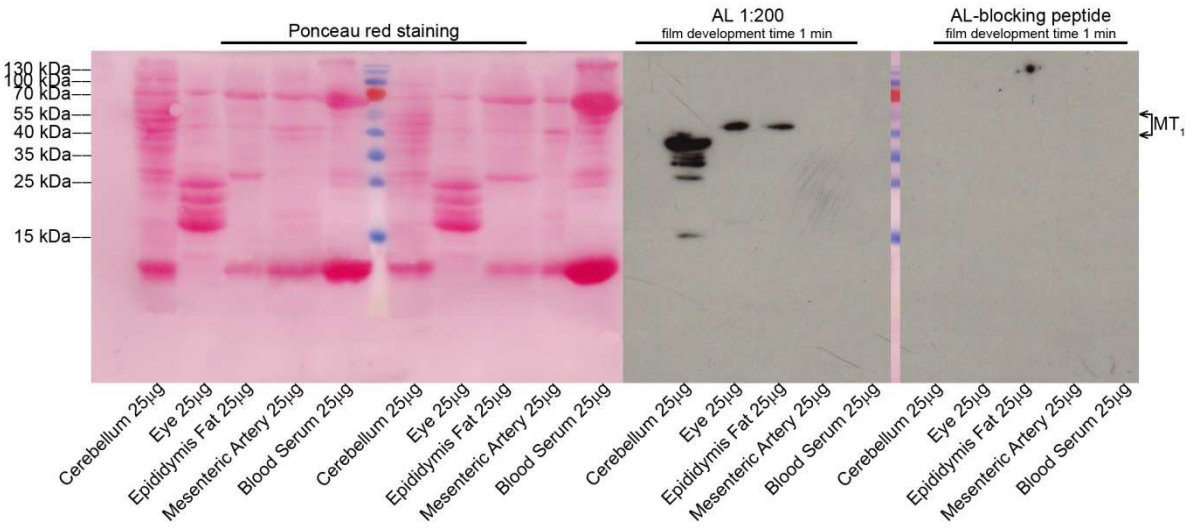

Figure 29: WB analysis of MT_1 expression in total protein lysates of cerebellum, eye, epididymal fat, MA, and blood serum using AL antibody alone and combined with AL-blocking peptide.
Left blot: Ponceau red staining, right blot: AL 1:200 compared to AL with blocking peptide - film development time: 1 min

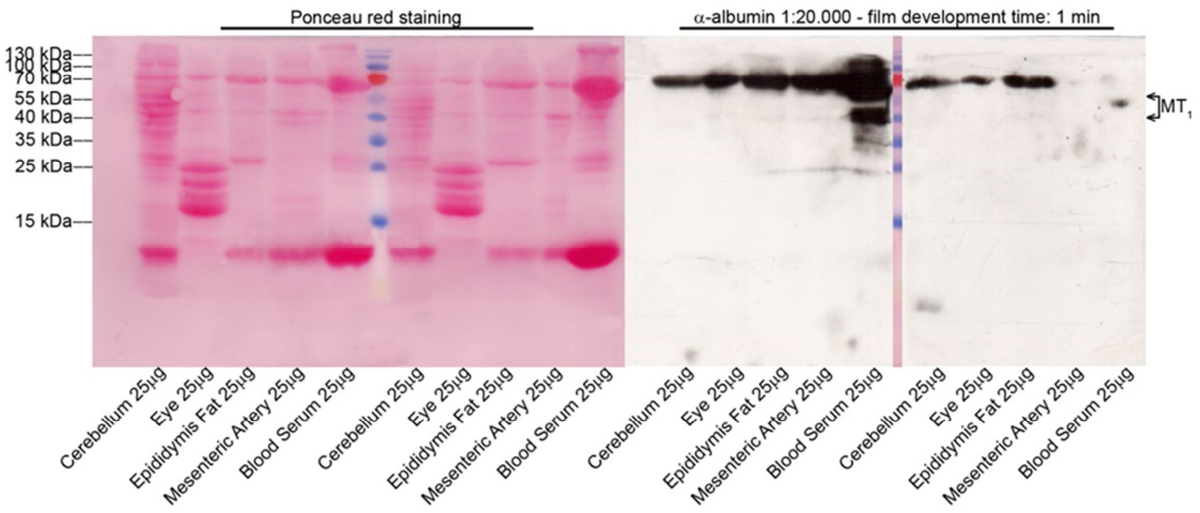

Figure 30: WB analysis of MT_1 expression in total protein lysates of cerebellum, eye, epididymal fat, MA, and blood serum using α-albumin antibody.
Left blot: Ponceau red staining, right blot: both parts of the blot were incubated with α-albumin 1:20.000 - film development time: 1 min

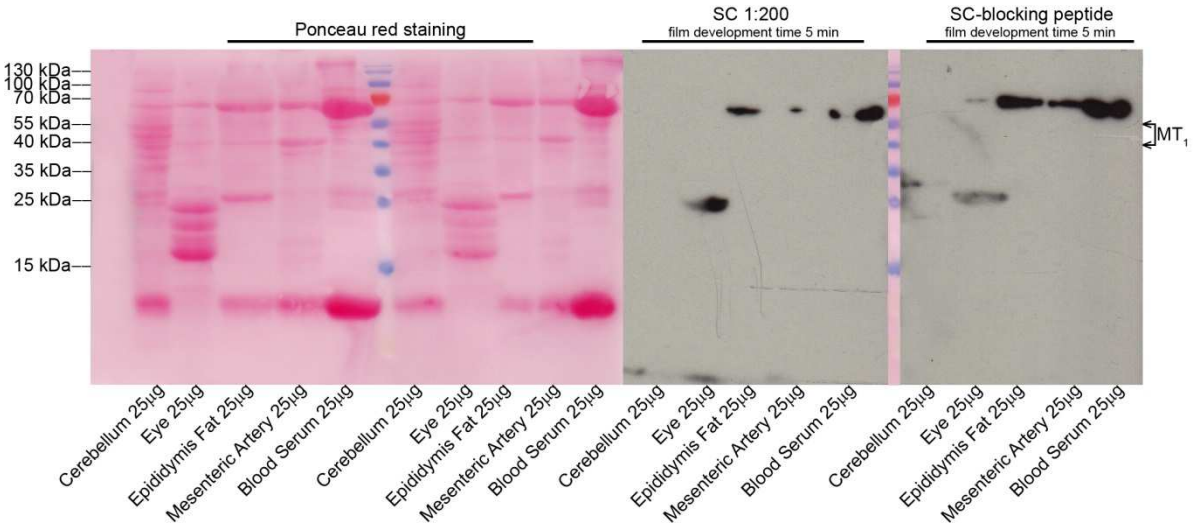

Figure 31: WB analysis of MT_1 expression in total protein lysates of cerebellum, eye, epididymal fat, MA, and blood serum using SC antibody alone and combined with blocking peptide.
Left blot: Ponceau red staining, right blot: SC 1:200 compared to SC with blocking peptide - film development time: 5 min

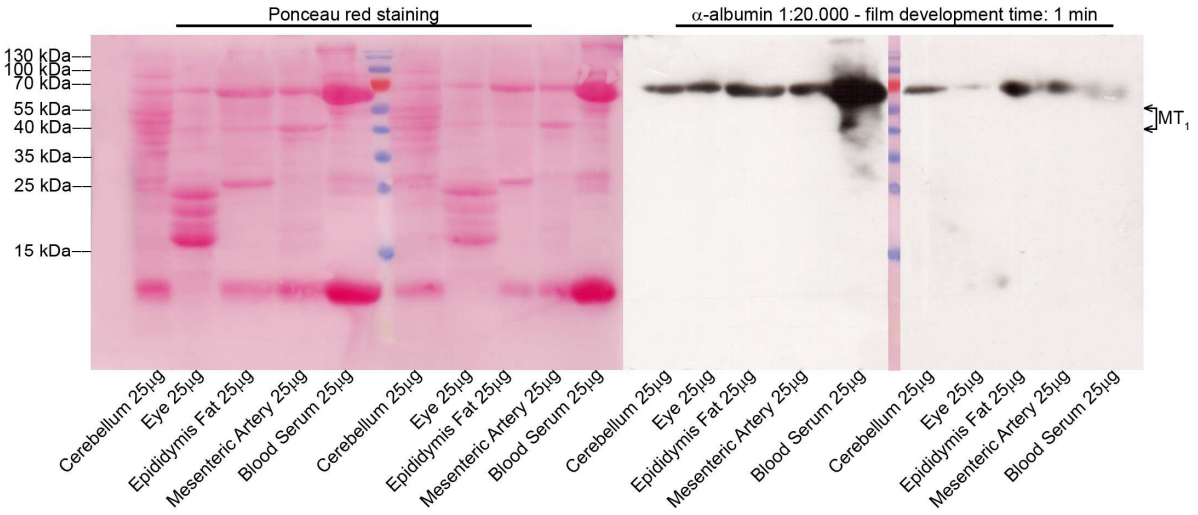

Figure 32: WB analysis of MT_1 expression in total protein lysates of cerebellum, eye, epididymal fat, MA, and blood serum using α-albumin antibody.
Left blot: Ponceau red staining, right blot: both parts of the blot were incubated with α-albumin 1:20.000 - film development time: 1 min

In summary, anti-MT_1 antibody from Alomone Labs was found to be a specific anti-MT_1 antibody, which is able to detect MT_1 proteins in total protein lysates of positive control tissues (eye, cerebellum) as well as other rat tissue samples, where MT_1 mRNA expression has been previously observed.

6.4 Demonstration of MT_1 protein expression in MA and MA-associated PVAT as well as additional fat tissue samples derived from aorta, kidney and epididymis and the positive control lysates, cerebellum and eye

We had previously observed expression of MT_1 but not MT_2 in rat MA as well as PVAT (Molcan, Ellinger, Zeman, unpublished results). Other groups had demonstrated MT_1 (and MT_2) mRNA expression in adipocytes isolated from epididymal fat [114]. In our last experiment we addressed aim 2 of this thesis. We applied the most specific anti-MT_1 antibody (Alomone; AL) to investigate MT_1 protein expression by WB in these tissues. Moreover, we included PVAT from the rat aorta and kidney-associated fat in the analysis to explore MT_1 protein expression in these tissues. From all samples, total protein lysates (ToP) prepared as described in 5.2.3, were used. Additionally, we prepared membrane-enriched protein lysates (MeP) from eye, MA-PVAT and aorta-PVAT, using Mem-PER buffer as described in 5.2.4.

Two 12% SDS-PAGE gels were prepared as described in 5.4.4 and total (ToP) or enriched membrane protein (MeP) lysates were loaded as detailed in Figure 33 and Figure 35. Proteins were separated and blotted simultaneously on two membranes (chapter 5.5.4). Both

blots were incubated with AL antibody (1:200). After developing the films, the blots were washed for one week at 4°C in wash buffer (PBS with 0.1% Tween) to reduce non-specific binding, followed by another round of chemiluminescence-based detection of bound antibodies.

On the first gel, total protein lysates from cerebellum (20 µg) and eye (50 µg) were loaded as positive controls. Total protein (ToP) lysates from MA as well as MA-PVAT, aorta-PVAT and fat tissue from kidney and epididymis was loaded with 75 µg/lane. The corresponding immunoblot of this gel obtained after incubation with AL antibody (diluted 1:200) is shown in Figure 33, the result after intensive washing of the blot (1 week, 4°C) is depicted in Figure 34.

We also prepared membrane-enriched protein lysates (MeP; as described in 5.2.4). MeP of PVAT from MA and (thoracic) aorta were loaded on the second gel (100 as well as 50 µg/lane). MeP from eye (50 µg) was loaded as a positive control. Finally, this gel also contained ToP from cerebellum (20 µg; positive control) and MA (50 µg). A corresponding WB of this gel resulting from incubation with AL antibody (diluted 1:200) is shown in Figure 35, the result after intensive washing of the blot (1 week, 4°C) is depicted in Figure 36.

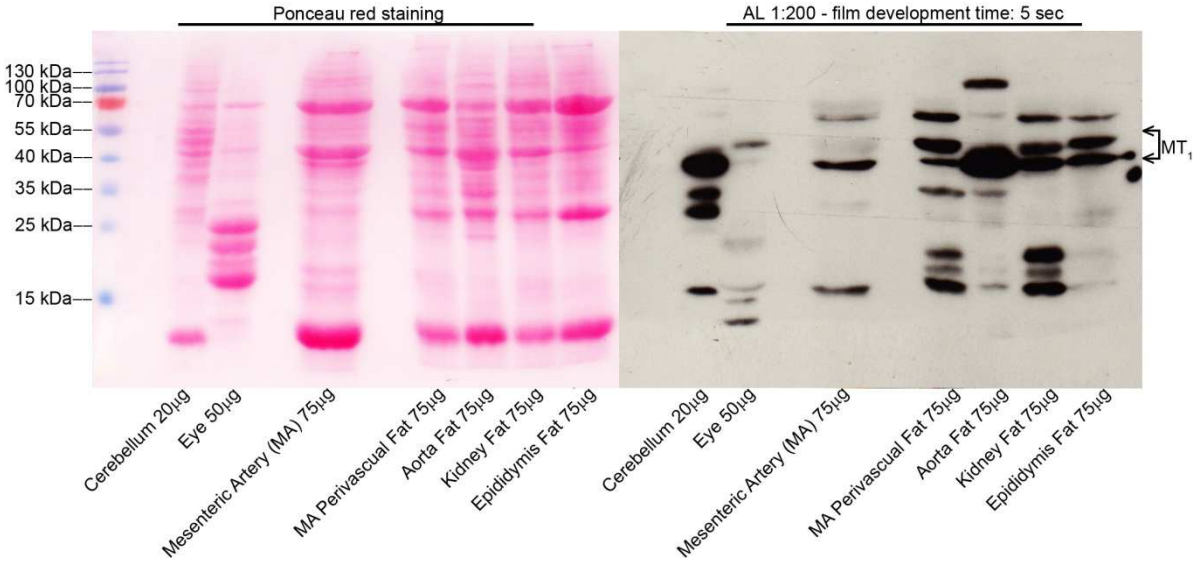

Figure 33: WB analysis of MT_1 expression in total protein lysates of cerebellum, eye, MA and PVAT of MA, aorta, kidney fat, epididymal fat using AL antibody.
Left blot: Ponceau red staining, right blot: AL 1:200 - film development time: 5 sec
Abbreviations: AL - Alomone antibody, MA - mesenteric artery

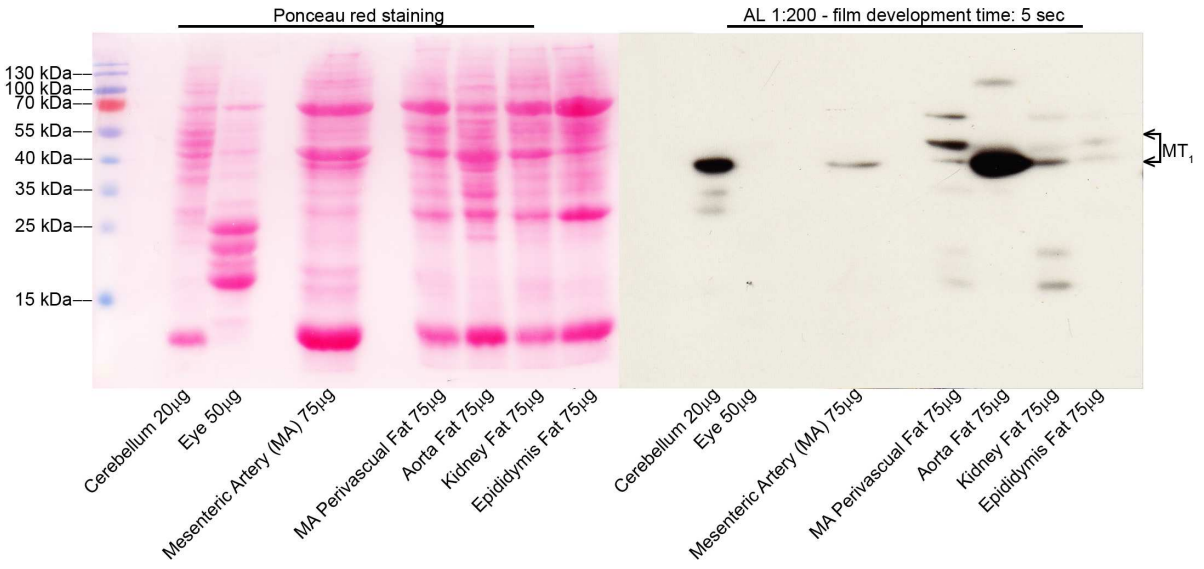

Figure 34: WB analysis of MT_1 expression in total protein fractions of cerebellum, eye, MA and PVAT of MA, aorta, kidney and epididymis using AL antibody.
Left blot: Ponceau red staining, right blot: AL 1:200 after 1 week washing with wash buffer at 4°C - film development time: 5 sec

As shown in Figure 33, AL antibody reacted in all tissues with proteins ranging from 40-60 kDa. Some reaction occurred in all tissues with proteins between 15 and 25 kDa. However, a comparison of Figure 33 and Figure 34 (extended wash) reveals that especially the proteins of 40 kDa (all samples) and 50-55 kDa (MA-PVAT) give signals even after an extensive washing period of 1 week, which argues for the most specific antibody-antigen interaction at this MW. A band of around 70 kDa was also visible in some of the samples (MA-PVAT, kidney and epididymal fat), which was most prominent in MA-PVAT. Detection of MT_1 protein in the various tissues required loading of a minimal amount of protein. In the course of this thesis, four different protein amounts of the same MA sample (ToP) were tested by WB with AL anti-MT_1 antibody, namely 100 µg (Figure 28), 75 µg (Figure 33), 50 µg (Figure 35) and 25 µg (Figure 29). While neither 25 µg nor 50 µg gave a signal, 100 µg and 75 µg resulted in similar signal intensities, indicating that about 75 µg of total protein lysate from MA is required to detect a protein of about 40 kDa (unglycosylated MT_1). Similarly, we tested 25 µg, 50 µg and 100 µg of total protein lysates of eye with AL antibody (1:200). Only when 100 µg of the eye sample were loaded, we were able to detect a 40 kDa protein and a larger (50-55 kDa) protein. The smaller one was hardly detected in samples containing less protein. The strongest signal was obtained from cerebellum, where we tested 20 µg as well as 100 µg of the total protein lysate. Even 20 µg of protein gave an intense signal at 40 kDa. Interestingly, we could not detect a protein of larger size in this tissue, which is comparable to results observed for MA. Among the various fat tissues, from all of which comparable protein amounts were loaded, aorta-PVAT showed the highest reactivity with AL anti-MT_1 antibody. As seen in MA and cerebellum samples, it was predominantly a 40 kDa protein

which reacted in aorta-PVAT while all other fat tissues loaded displayed bands of 40 kDa as well as of larger MW.

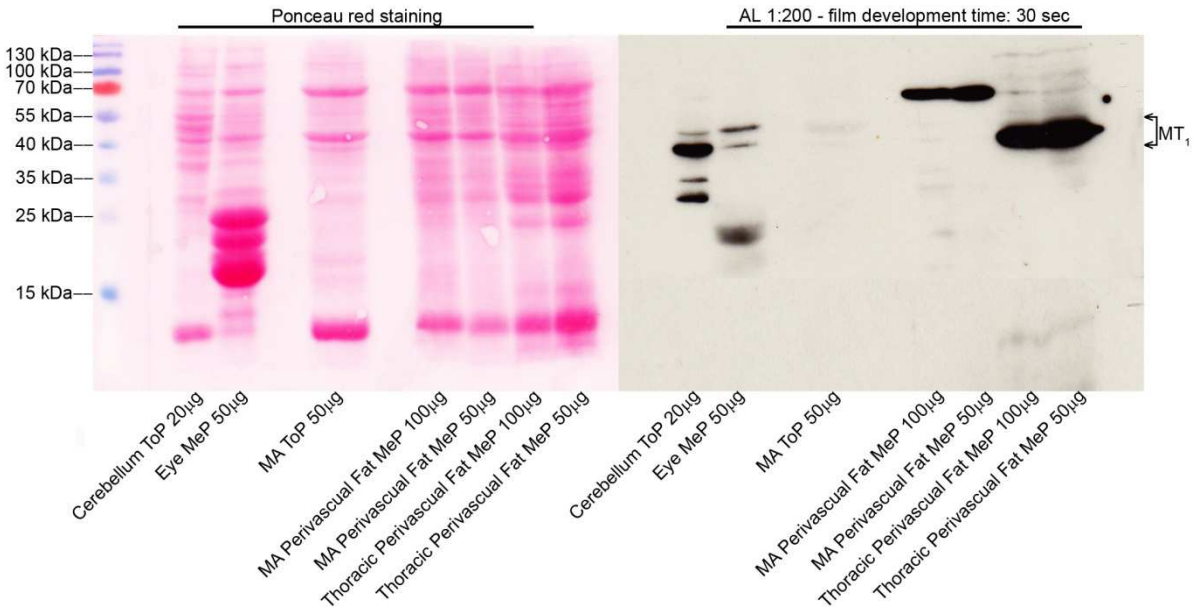

Figure 35: WB analysis of MT_1 expression in membrane (MeP) and total (ToP) protein lysates of different tissues using AL antibody.
Left blot: Ponceau red staining, right blot: AL 1:200 - film development time: 30 sec
Abbreviations: MA - mesenteric artery, MeP - membrane protein, ToP - total protein

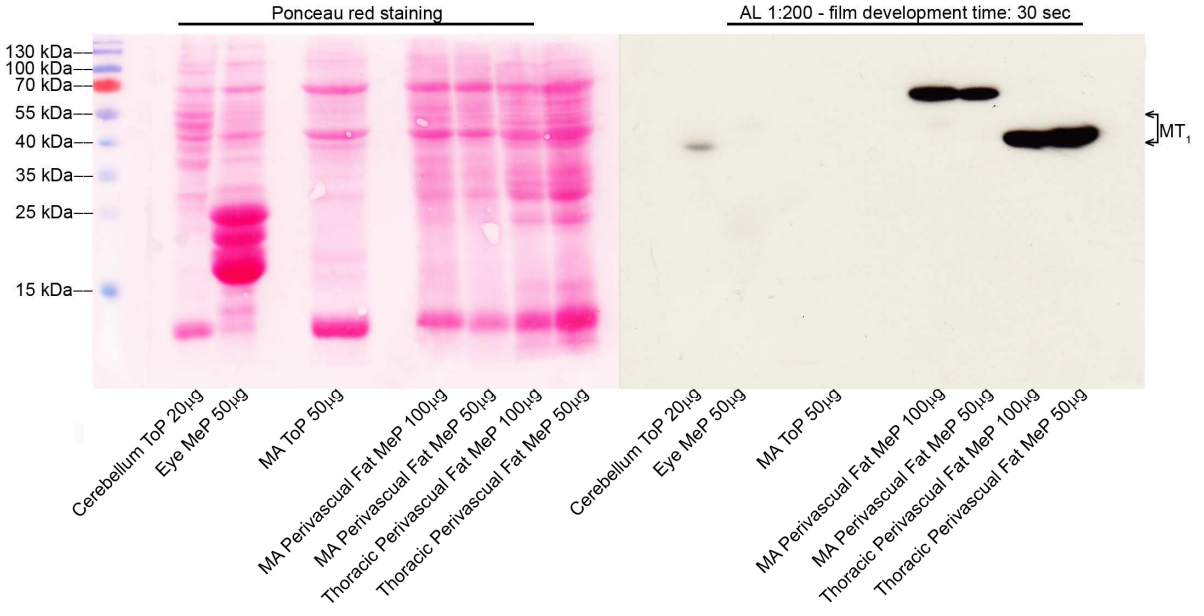

Figure 36: WB analysis of MT_1 expression in membrane (MeP) and total (ToP) protein lysates of different tissues using AL antibody. Left blot: Ponceau red staining, right blot: AL 1:200 after 1 week washing with wash buffer at 4°C - film development time: 30 sec
Abbreviations: MA - mesenteric artery, MeP - membrane protein, ToP - total protein

The results obtained from probing of protein lysates prepared with Mem-PER buffer with AL antibody are displayed in Figure 35 and Figure 36. Mem-PER buffer should enrich membrane-spanning or-associated proteins over soluble proteins. Eye, MA-PVAT and aorta-

PVAT samples prepared with this buffer reacted with AL antibody and resulted in blots were predominantly proteins in the range of 40-70 kDa were detected. Prolonged washing of the blot (as shown in Figure 36) confirmed antibody specificity. Of interest, the major protein detected in eye and aorta-PVAT was of 40 kDa while the major protein in MA-PVAT prepared with Mem-PER buffer was of around 70 kDa. Future experiments will have to reveal whether the difference between these molecules is due to different glycosylation.

In summary, using the specific AL anti-MT_1 antibody we could show that the rat tissues MA, MA-PVAT, aorta-PVAT, as well as epididymal and kidney fat express MT_1 protein. While a non-glycosylated (40 kDa) protein was detected in all tissues, the antibody also detected several proteins of higher MW (50-55 kDa and 70 kDa), which may represent glycosylated MT_1 proteins, but were not found in all tissues investigated.

7 Discussion

7.1 Determination of antibody specificity of three commercially available anti-rat MT_1 antibodies

Three antibodies against rat-MT_1 were tested in this study and an antibody from Alomone Lab was found to be the most specific. In the tissue lysates serving as positive controls, i.e. brain and cerebellum [44, 66] as well as eye [66, 132], the antibody reacted with proteins in the expected MW range of rat MT_1 (40-60 kDa). This reaction was specific since in the presence of blocking peptides, the binding of the antibody was completely abolished. The antibody did neither cross react with albumin nor IgG, two major serum proteins. Antibody cross reactivity with albumin has been found in other laboratories [140] and has also been observed for several antibodies tested previously in our laboratory. We confirmed abundant presence of albumin and IgG in blood samples by WB using anti-albumin and anti-IgG antibodies and presence of minor amounts in most tissues investigated, but found no cross-reaction of Alomone antibody with these proteins.

The exact molecular size of MT_1 protein expressed in various tissues and species is unknown and may vary. Rat MT_1 comprises 353 amino acids [44, 134] and therefore the unglycosylated rat MT_1 protein has 40 kDa. Indeed, a MW of 37-40 kDa has been demonstrated by WB for MT_1 in several studies [67, 68, 133, 135], though not all [66]. As MT_1 contains two consensus sites for N-terminal asparagine-linked glycosylation [44], higher MW proteins (40-60 kDa) might also be expected. Few well-characterized antibodies against the membrane receptor MT_1 exist, most generated against the human MT_1 [51, 53]. When used in WB experiments these antibodies detected 60 kDa proteins (the expected MW of the glycosylated form of the receptor), suggesting that human MT_1 is predominantly glycosylated. MT_1 antibodies used to detect rodent MT_1 are mainly commercial antibodies, which lack sufficient characterization. One of the first commercially available anti-rat MT_1 antibody was antibody sc-13186 from Santa Cruz Biotechnology which has been used in several published studies [64, 66, 67,69]. In these studies, rat MT_1 was described to have a MW corresponding to 37 kDa [67] or 60 kDa [66, 69], in some studies the MW of the detected bands was not reported [64]. In the present study, we investigated the specificity of the antibody from Santa Cruz Biotechnology in more detail. We observed strong cross-reaction with albumin (65-70 kDa) but no detection of 40 kDa proteins (MT_1). We therefore do not recommend this antibody for future MT_1 protein expression studies. Using a self-developed antibody against mouse MT_1, and confirming the specificity in wild type and MT_1 knock out mice, the MT_1 protein in mouse retina was recently described to be a 40 kDa protein [141]. Although rat and mouse MT_1 show more than 90% sequence homology [44], future studies *in vitro* and *in situ*

will have to clarify in more detail whether rat and mouse MT_1 proteins are predominantly expressed as unglycosylated proteins (40 kDa) or glycosylated molecules (60 kDa). In this context it was interesting to note that MT_1 protein detected by Alomone antibody in all total protein lysates was mainly a 40 kDa protein, while the protein detected in enriched membrane fractions of MA-PVAT was of higher MW (>60 kDa, see Figure 35). It is currently unclear whether this could represent a glycosylated receptor or a receptor dimer.

Finally, the antibody from Biorbyt showed cross-reaction with albumin but also reacted with proteins of MT_1 size.

7.2 Demonstration of MT_1 protein expression in MA and MA-associated PVAT

Previously, we found expression of MT_1, but not MT_2 mRNA in rat aortas [66] as well as MA (Molcan, Ellinger, Zeman, unpublished results). Using the most specific antibody identified in aim 1 (Alomone Labs), MT_1 protein expression in total lysates of MA and MA-PVAT of normotensive rats was explored by WB in order to confirm protein translation of MT_1 mRNA in these tissues. Using Alomone antibody, we could detect MT_1 protein in MA and MA-PVAT for the first time. Likewise, we also detected MT_1 in PVAT associated with TA and in epididymal fat. Expression in the latter is in line with literature, where expression of MT_1 mRNA in adipocytes isolated from epididymal fat has been observed [114]. Furthermore, evidence for MT_1 function in adipocytes derived from epididymal fat was obtained [115]. Subsequent analysis of MT_1 localization in MA and MA-PVAT by microscopy (Kuhn, Seidelmann, Ellinger, unpublished results) confirmed the presence of the receptor in VSMCs and adipocytes, suggesting various modes of interaction of melatonin with cell structures in rat MA (see Figure 29).

7.3 Outlook

Recent studies implicated that melatonin impacts on the anti-contractile function of MA-associated PVAT [116]. The presence of MT_1 protein in MA-PVAT, which was demonstrated in this study, may allow hypothesizing that melatonin, via melatonin receptor MT_1, causes secretion of ADRFs from PVAT promoting vessel dilation. In order to understand the complex mechanism of blood pressure modulation by melatonin, future studies will have to analyze the effects of melatonin on release of ADRFs from isolated adipocytes derived from PVAT.

8 List of abbreviations

- A - Ampere
- ACE - Angiotensin converting enzyme
- ADRF - Adipocyte derived relaxing factor
- AL - Alomone Labs primary anti-rat MT_1 antibody, amr-031
- ARB - Angiotensin II receptor blockers
- AT II - Angiotensin II
- BB - Biorbyt primary anti-rat MT_1 antibody, orb11085
- BP - Blood pressure
- C - Cow
- cAMP - Cyclic adenosine monophosphate
- CCB - Calcium channel blockers
- CVD - Cardiovascular disease
- D - Dog
- EDTA - Ethylenediaminetetraacetic acid
- ELISA - Enzyme-linked immunosorbent assay
- g - Measurement unit for gravity of earth
- GPCR - G protein-coupled receptor
- H - Human
- h - Hour
- HRP - Horseradish peroxidase
- IgG - Immunoglobulin G
- IHC - Immunohistochemistry
- Kd - Dissociation constant
- kDa - Kilodalton
- L-NAME - N^{ω}-Nitro-L-arginine methyl ester
- M - Mouse
- MA - Mesenteric artery without perivascular fat
- MA-PVAT - Mesenteric artery perivascular fat tissue
- Mem-PER - Membrane Protein Extraction Kit, company Thermo scientific
- MeP - Membrane protein
- mRNA - Messenger Ribonucleic acid
- MT_1 - Melatonin receptor 1a
- MW - Molecular weight
- N_2 - Nitrogen
- NO - Nitric oxide

- P - Pig
- PA - Polyacrylamide
- PKC - Protein kinase C
- PLC - Phospholipase C
- PVAT - Perivascular adipose tissue
- PVDF - Polyvinylidene fluoride
- R - Rat
- RAAS - Renin angiotensin aldosterone system
- RNS - Reactive nitrogen species
- ROS - Reactive oxygen species
- RT-PCR - Reverse transcription polymerase chain reaction
- r.t. - Room temperature
- SC - Santa Cruz primary anti-rat MT_1 antibody, sc-13186
- SCN - Suprachiasmatic nucleus
- SDS-PAGE - Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis
- sec - Second
- Sh - Sheep
- T-PER - Tissue Protein Extraction Reagent, company Thermo scientific
- TeP - Total protein
- V - Volt
- vol. - Volume
- VSMC - Vascular smooth muscle cell
- W - Watt
- WB - Western blot
- α-albumin - Abcam primary anti-mouse serum albumin antibody, ab19194
- α-goat - Anti-goat-IgG-HRP conjugated secondary antibody, sc-2020
- α-IgG - Alexa Fluor® 488 Goat Anti-Rat IgG (H+L) Antibody, A-11006
- α-rabbit - Anti-rabbit-IgG-HRP conjugated secondary antibody, sc-2004

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10 Curriculum Vitae

Personal details

Name	Andreas Rudolf Maier
Year of Birth	1984
Nationality	Austria

Education and career

1990-1994	Elementary School
1994-2002	Secondary School (BG/BRG Franklinstraße 21, 1210 Vienna)
2002	Graduation (Matura)
2003-2004	Training and graduation as paramedic in the context of the Austrian compulsory community service (Austrian Red Cross)
2004-2014	Study of Pharmacy at the University of Vienna, Austria
07/2014	Assistant at the workshop “Kinderuni” of the Medical University of Vienna
09/2014	Poster at 5th Retreat of the Center for Pathophysiology, Infectiology and Immunology (Medical University of Vienna)

Practical Experience

Since 2007	Employed at St. Richard Apotheke, Bisamberg