Potential mechanisms behind blood pressure modulation by melatonin: expression analysis of melatonin receptors MT₁ and MT₂ in the rat aorta
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Edel sei der Mensch,
Hilfreich und gut!
Denn das allein
Unterscheidet ihn
Von allen Wesen,
Die wir kennen.

J. W. v. Goethe
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1 ABSTRACT / KURZFASSUNG

English

Introduction: Melatonin, known as the hormone of darkness, is a versatile substance produced in the pineal gland from its precursor substance serotonin. Melatonin plays a role in many processes including regulation of the body's internal clock, appetite, sleep, radical scavenging, and supposedly in behavior, tumor suppression, blood pressure and many more. Because of its sleep promoting effect, melatonin is used as a mild hypnotic, even though the effects of orally administered melatonin are limited. During the course of its metabolization, melatonin can scavenge a great number of reactive oxygen species, which is the cause for the extremely potent antioxidative capabilities of this substance. Apart from numerous other binding sites, two G-protein coupled receptors (GPCRs), MT₁ and MT₂, exist in mammals which recognize melatonin as ligand and mediate many of its functions. Both receptors are coupled to Gi-proteins, but while MT₁ is almost ubiquitously expressed in many tissues, MT₂ is restricted to few regions of the body, like eye and brain. These GPCRs show circadian expression patterns in some tissues. Importantly, MT₁ and MT₂ are expressed in the cardiovascular system, however these data are conflicting and the influence of the GPCRs on, and their expression and location in the cardiovascular system are still not completely understood and remain to be fully characterized. It was suggested that MT₁ mediates vasoconstriction through direct activation of smooth muscle cells, while MT₂ mediates vasodilation through activation of nitric oxide synthase in the endothelium of the blood vessels. In any case, exogenous melatonin lowers blood pressure, both of healthy and hypertensive animals and humans. The mechanisms involved in this blood pressure regulating properties include melatonin's antioxidative capabilities, a dampening influence of melatonin on the central nervous system and the melatonin GPCRs. Aims: 1) Establish methods for RT-PCR and RT-qPCR experiments for investigation of MT₁ and MT₂ mRNA expression levels. 2) Investigate whether there is expression of MT₁ and MT₂ mRNA in the rat aorta. 3) Investigate the difference between melatonin GPCR mRNA expression levels in aortas from rats of two different time points. 4) Investigate the difference between melatonin GPCR mRNA expression levels in aortas from spontaneously hypertensive rats (SHR) and control rats. 5) Establish and perform immunofluorescence (IF) stainings to investigate the location of the melatonin GPCRs in the rat aorta. Methods: RNA was extracted and reversely transcribed using standard protocols. PCR with diverse primer pairs either retrieved from literature or custom designed was performed for MT₁ and MT₂. RT-qPCR experiments were performed using commercially available assays for rat MT₁ and MT₂. For method establishment, pre-frozen rat tissue samples of various organs were used.
16 aorta samples from two different time points – eight from day and eight from the night – were investigated to assess the difference in melatonin GPCR mRNA expression levels depending on the circadian phase state. 16 aorta samples of SHR and control rats – eight each – were investigated to evaluate the difference in melatonin GPCR mRNA expression levels between normotensive and hypertensive animals. Paraffin embedded sections and cryo-sections were used for localization of MT₁ by IF-staining, which was done using two different antibodies directed against MT₁. Two mouse tissues, brain and intestine, were used as positive controls. **Results:** Observed expression levels of MT₁ and MT₂ in the rat organs did not exactly correlate with the levels in the literature, indicating rat-strain or interindividual differences. MT₁ mRNA was present in almost all aorta samples and showed a high interindividual variability in expression levels, while MT₂ mRNA was not present in aortas at all. There was no significant difference in MT₁ mRNA levels between the daytime and nighttime groups, though a trend toward higher expression in the nighttime group might be observed with a larger sample number. However, a significant difference in expression was found between aortas from SHR, which exhibited about 4 times (0–8) more mRNA for MT₁, and the aortas from control rats, supporting a function of melatonin in blood pressure reduction. Immunofluorescence showed weak staining for MT₁, but surprisingly in the tunica adventitia of the aortas, while a strong, region specific, staining in the positive controls was found. This was in contrast to the expected localization of MT₁ in the tunica media. **Conclusion:** We could demonstrate that MT₁ is indeed expressed in the rat aorta as model for the vascular system, while MT₂ is not present at all. It seems that the expression levels for MT₁ mRNA are not dependent on the time of day, although the sample size was too low and the number of time points too few for any final statement. We found a significant difference in expression of MT₁ between normotensive and hypertensive animals, supporting a function of MT₁ in the blood pressure modulating capabilities of melatonin. MT₁ seemed to be mainly located in the tunica adventitia of the aorta and not, as expected, in the tunica media. This localization of MT₁ and the absence of MT₂, however, are in conflict with the current hypothesis on the influence of the melatonin GPCRs on blood pressure regulation by melatonin and ask for further investigation.
Deutsch

Einleitung: Melatonin ist eine sehr vielseitige Substanz, die in der Zirbeldrüse aus Serotonin entsteht und auch als „das Hormon der Dunkelheit“ bezeichnet wird. Zu den vielfältigen Einflussbereichen von Melatonin im Körper gehören zum Beispiel die Regulation der inneren Uhr, des Appetites und des Schlafs sowie das Abfangen freier Radikale. Vermutet wird auch ein Einfluss auf Verhalten, Tumorsuppression, Blutdruck und andere Funktionen des Körpers. Wegen seines schlaufvermittelnden Effektes wird Melatonin als mildes Hypnotikum eingesetzt, allerdings ist seine Effektivität bei oraler Anwendung begrenzt. Im Rahmen des Abbaus von Melatonin im Körper können eine große Menge freier Radikale gebunden und somit unschädlich gemacht werden, was die starke antioxidative Wirkung von Melatonin erklärt. Für Melatonin gibt es mehrere Bindungsstellen; zwei davon, MT₁ und MT₂, sind G-Protein gekoppelte Rezeptoren, die auch in Säugetieren vorkommen. Obwohl beide Rezeptoren vom Gi-Typ sind, ist MT₁ fast in allen Teilen des Körpers exprimiert, wohingegen MT₂ nur in bestimmten Geweben, wie z.B. Auge oder Gehirn, zu finden ist. Die Expression dieser Rezeptoren zeigt in manchen Geweben einen zirkadianen Rhythmus. Zwar sind MT₁ und MT₂ auch in Blutgefäßen vorhanden, die Datenlage zu deren Lokalisation, Funktion und Expression im vaskulären System ist allerdings widersprüchlich und noch abzuklären.

2 INTRODUCTION

2.1 General information about melatonin

Melatonin (chemical name N-acetyl-5-methoxytryptamine or N-[2-(5-methoxy-1H-indol-3-yl)-ethyl]-ethanamide, see Figure 1) is commonly known as the “darkness hormone” because melatonin levels are highly dependent on lighting conditions, being low at day and high at night [1-2]. Melatonin is (in vertebrates) produced in the pineal gland [3] but also in various other areas such as the suprachiasmatic nucleus (SCN) [4], leucocytes [5] the retina, the bone marrow, the skin, the gastro intestinal tract, the skin, and probably others [6-7]. It was first discovered in the year of 1958 by Lerner et al. [8]. Melatonin was named after its ability to lighten the color of melanocytes in certain animals and its precursor serotonin [7-9].

![Figure 1: Structure of melatonin.](image)

Melatonin has a variety of roles, like control of circadian rhythms and sleep by playing an essential part in the body’s internal clock. Thereby, the main function of melatonin is to inform various parts of the body about photoperiods (= day length), so that changes in pigmentation, appetite, sleep etc. can be organized. Other functions of melatonin include vascular and blood pressure (BP) modulation, scavenging of free radicals and reduction of oxidative stress, stimulation of the immune system, regulation of seasonal reproduction and body temperature, inhibition of tumor proliferation, osteolysis inhibition, behavior regulation and others [6-7, 10-12].

Because of this multitude of functions and because of numerous other facts – melatonin’s non receptor-dependent antioxidative capability, synthesis in organs other than the pineal gland, and that melatonin can be taken up with food supplementing the endogenous production – Tan et al. [7] point out that melatonin could not only be described as a hormone, but as a vitamin, too.

Melatonin not only occurs in animals, but also in unicellular organisms and fungi [13] as well as in plants, where it exhibits a similar circadian rhythm like in animals, although the data on its function is yet not very conclusive. Various roles, such as that
of an antioxidant, have been hypothesized. The first definite job of melatonin in plants seems to be that of a growth promoter [9].

2.2 Melatonin biosynthesis

Melatonin is synthesized from serotonin in two steps (see Figure 2). The first step is acetylation of serotonin to the intermediate N-acetylserotonin by the aryalkylamine-N-transferase (AANAT). The acetyl group is supplied by AcCoA. The second step is methylation of N-acetylserotonin to N-acetyl-5-methoxy-tryptamine (melatonin) by the hydroxyindole-O-methyltransferase (HIOMT) [1]. The methyl group is carried over from S-adenosyl-methionine [2]. Melatonin synthesis is highly dependent on environmental light and therefore follows a circadian rhythm. In low light conditions, melatonin levels are greatly increased, with correspondent increase of AANAT activity. HIOMT activity, on the other hand, is not increased or, at most, to a lesser extent. Nocturnal melatonin synthesis also rapidly diminishes again after exposure to light (see Figure 2) [1, 14].

![Figure 2: Daily rhythm of indoles in the rat pineal gland. Molecular changes are highlighted with red rectangles / lines. Periods of darkness are indicated by gray shading. Nocturnal increased activity of AANAT results in decreased serotonin and increased NAS-levels. HIOMT activity is also increased during nighttime, but to a much lesser extent. As a result, melatonin concentration is greatly elevated during the night. Modified after [1].](image-url)
2.2.1 Adrenergic control of melatonin biosynthesis

Melatonin synthesis in the pineal gland is strongly controlled by adrenergic stimulation via the SCN. The SCN is the main controller of all circadian rhythms in mammals and synchronizes the various more or less autonomous “clocks” in mammals [15]. It is the driving element of the circadian rhythm of melatonin. In the rat, the SCN is largely controlled by lighting conditions [16], but can also function autonomously, when left in complete darkness. Nerves emerging from the SCN ultimately activate sympathetic fibers which in turn innervate the pineal gland [1].

Stimulation of α1-adreno-receptors activates protein kinase C (PKC) via increased Ca^{2+} and diacylglycerol (DAG) concentrations in the pineal cells (see Figure 3).

![Figure 3](image)

Figure 3: Adrenergic regulation of melatonin synthesis. Norepinephrine (NE) binds to α1 or β1 receptors. α1 stimulation potentiates the effect of β1 stimulation by activating protein kinase C (PKC) which in turn activates the adenylate cyclase (AC) of the β1 receptor. The AC produces cyclic AMP (cAMP) which activates protein kinase A (PKA). PKA has now two different methods of raising AANAT-activity: 1) by phosphorylization of cAMP-response-element-binding-protein (CREB) which then promotes transcription of the AANAT-gene, and 2) by phosphorylization of AANAT which leads to a complex of AANAT with 14-3-3, rendering AANAT insensitive to proteasomal proteolysis. Modified after [1].

While stimulation of α1 alone has no effect on AANAT activity [17], the increased Ca^{2+} influx potentiates the effect of protein kinase A (PKA). PKA is activated by cAMP generated through β1-stimulation, therefore acting as an amplifier for the β1-adrenergic signal [18]. A cAMP-responsive element binding protein (CREB) has been identified [19], which, together with an adjacent 5-base-box function, acts as a promoter for the
AANAT-gene. Furthermore, PKA acts via direct phosphorylation of AANAT [17], leading to the formation of an AANAT-complex with a 14-3-3 protein, a kind of "shielding protein" which protects the AANAT from proteolysis [20].

In summary, there are two mechanisms for regulation of AANAT activity: increased transcription of AANAT-mRNA and inhibition of preoteolysis by coupling with 14-3-3 proteins [14].

2.2.2 Melatonin metabolism

Melatonin is metabolized mainly in the liver via the cytochromes CYP1A2 and CYP1B2. Metabolic pathways are 6-hydroxylation to 6-hydroxy-melatonin with subsequent sulfatation and excretion, O-demethylation to N-acetyl-5-hydroxy-tryptamine and non-enzymatic deacetylation to 5-methoxytryptamine [21]. This last, non-enzymatic way for metabolism of melatonin is also called kynuric pathway. It is essential for melatonin's antioxidative abilities. In its course, free radicals such as O₂⁻, CO₃⁻, NO⁻ or H₂O₂ are scavenged [6] (see Figure 4).

![Figure 4: Simplified kynuric pathway of melatonin metabolism. After [6].](image)

2.3 Melatonin receptors and other melatonin binding proteins

2.3.1 MT₁ and MT₂ – G-protein coupled receptors

In 1994 the first melatonin receptor Mel₁C was found in Xenopus laevis (African clawed frog) and discovered to be a G-protein coupled receptor (GPCR) of the Gi-type, therefore reducing cAMP levels by inhibiting the adenylyl cyclase. It was shown that the receptor was highly affine to melatonin and had a structure of 420 amino acids grouped in 7 hydrophobic elements [22], typical of GPCRs [23]. However, Mel₁C is not expressed in mammals, but only in fish, amphibiae or birds. Two other melatonin receptors have been found shortly afterwards, which were originally called Mel₁A and Mel₁B, respectively, but then re-named melatonin receptor 1 (MT₁) and Melatonin receptor 2 (MT₂) [24].

MT₁ was first discovered in sheep and humans. [25]. The receptor has a size of 350 amino acids (in rats: 353 amino acids [26]) with 7 transmembranic domains [27]
and is also a G-protein coupled receptor of the G_i-type. The expression of MT_1 is, at least partly, regulated by circadian rhythms controlled by the SCN [24].

One year later, MT_2 was found in humans. The receptor comprises 362 amino acids (in rats: 364 amino acids [26]) and is, again, a G-protein coupled receptor of the G_i-type [28].

Depending on the cell type in which the receptors are expressed, they also seem to exhibit coupling with G_q-proteins, indicating possible parallel signaling pathways for these receptors [27]. See Figure 5 for an overview of the proposed signaling pathways of the melatonin receptors.

![Figure 5: Melatonin GPCR signaling cascade. Modified after [27]. Q. v. for detailed information.](image)

The human MT_1 and MT_2 receptors show a different affinity for melatonin (K_i 80.7 vs 383 pM) [29] and other compounds. Some of these substances are important for studies on receptor-mediated melatonin effects. Luzindole (2-benzyl-N-acetyltryptamine), an unselective antagonist for both melatonin GPCRs, was identified by Dubocovich [30] and is often used in studies to suppress receptor-mediated melatonin effects. 4P-ADOT (4-phenyl-2-acetamidotetraline) and 4P-PDOT (4-phenyl-2-propionamidotetralin) serve as relatively selective antagonists of MT_2 [31]. More recently, N-butanoyl-5-methoxy-1-methyl-β,β-trimethylene-tryptamine, which is an antagonist at MT_1, but an agonist at MT_2 and N-butanoyl-5-methoxy-1-methyl-β,β-tetramethylene-tryptamine, which is an antagonist at MT_1 not showing any interaction with MT_2, thus being selectively antagonistic at MT_1 [32], have been identified and will allow further studies on the function of melatonin’s GPCRs.
**Distribution and functions**

The distribution of melatonin GPCRs has been investigated in many studies. For humans, studies on mRNA expression via RT-PCR as well as protein expression via western blot or immunohistochemical methods revealed MT$_1$ and, to a lower extent, MT$_2$ to be present in regions of the brain, ovary, kidney, intestine and various other organs and cell types [33]. However, while well characterized antibodies are available against human MT$_1$ and MT$_2$, no reliable antibodies for other species have been reported yet. Therefore, most studies concerning melatonin GPCR expression in other species than human, e.g. rat, were performed on the mRNA level [24].

Earlier studies investigating MT$_1$ and MT$_2$ mRNA expression in the rat used only incomplete sequences of the receptor’s mRNA as template for primer design [34-36]. Addressing these issues, Ishii et al. [26] identified the gene structures including the whole mRNA sequence for both rat MT$_1$ and MT$_2$. Using newly designed exon-spanning primers, designed from the identified mRNA sequences for both receptors, they demonstrated occurrence of MT$_1$ and MT$_2$ mRNA – without the risk of false positives through genomic DNA – in a great variety of rat tissues, like lung, kidney and intestine (see Figure 6).

*Figure 6*: RT-PCR analysis of MT$_1$, MT$_2$ and β-actin expression in various rat tissues. Both MT$_1$ and MT$_2$ were detected in all tissues. β-actin is used as control. Modified after [26].
Melatonin exhibits an enormous multitude of functions via its known G-protein-coupled-receptors. An extensive discussion of all the effects of the melatonin receptors would go beyond the scope of this introduction. An overview can be seen in Figure 7, details can be found e.g. in [33], [37] or [6]. The expression, distribution and functionality of MT\textsubscript{1} and MT\textsubscript{2} in the cardiovascular system will be discussed in 2.6.

![Diagram](image-url)

**Figure 7:** MT\textsubscript{1}, MT\textsubscript{2} and MT\textsubscript{3} (see 2.3.2) responses in the CNS and peripheral tissue [37]. Q.v. for detailed information.

**Structure and functionality**

Both the human and rat MT\textsubscript{1} gene consist of two exons. The rat MT\textsubscript{2} gene consists of three exons, whereas the human MT\textsubscript{2} gene contains only two. This suggests that regulation of MT\textsubscript{2} receptor expression might be species specific [26].

All melatonin GPCRs, Mel\textsubscript{1C}, MT\textsubscript{1} and MT\textsubscript{2}, show a high degree of homology with each other: 60% for the complete sequences, 73% within the transmembrane sections [28]. The rat receptors exhibit 84.1% and 78.3% identity with their human counterparts [26]. As of yet there are no x-ray crystallographic structures available for any of these receptors. The predicted membrane topologies of human MT\textsubscript{1} and MT\textsubscript{2} are shown in Figure 8.
The C-terminal tails of both MT<sub>1</sub> and MT<sub>2</sub> are necessary for interaction with the G-protein and receptor internalization [27, 39]. For details see [40] and [39]. The C-terminal tail of MT<sub>1</sub> also interacts with the protein MUPP1 amongst others, which joins MT<sub>1</sub> to cAMP regulation and nitric oxygen synthase (NOS). This could explain the fact that NO production is inhibited by melatonin [24]. However, at least in endothelial cells, not the melatonin GPCRs seem to be involved in the reduction of nitric oxide (NO) production [41-42], but interaction with intracellular Ca<sup>2+</sup> mobilization [41] or NF-κB inhibition [42]. In addition to the G-proteins, MT<sub>1</sub> and MT<sub>2</sub> interact with a number of other proteins, like the actin binding protein filamin A, and insulin receptor substrate 4 (IRS4), proteins commonly associated with other GPCRs [24].

GPCRs generally tend towards di- or oligomerization [23] and the melatonin GPCRs are no exception [24]. It was demonstrated that the relative propensity of the MT<sub>1</sub> / MT<sub>2</sub> heterodimer and the MT<sub>1</sub> / MT<sub>1</sub> homodimer is similar, but for the MT<sub>2</sub> / MT<sub>2</sub> dimer it is three to four times lower. Heterodimers also interact with selective ligands for MT<sub>1</sub> and MT<sub>2</sub>, resulting in a possible problem with data interpretation of ligand affinities or receptor expression patterns [43].

In 1996, G protein-coupled receptor 50 (GPR50), an orphan GPCR belonging to the melatonin receptor family (45% identical to MT<sub>1</sub> and MT<sub>2</sub>), but not interacting with melatonin or any other known ligand, was discovered. It has an unusually long C-terminal tail consisting of over 300 amino acids, but no N-linked glycosylation sites [44]. See Figure 9 for predicted membrane topology.

**Figure 8:** Predicted membrane topology of MT<sub>1</sub> and MT<sub>2</sub> with deduced amino acid structures. Y = potential N-linked glycosilation site. Solid dots = identical amino acids in MT<sub>1</sub> and MT<sub>2</sub>. MT<sub>1</sub>: [38]; MT<sub>2</sub>: [28].
Ten years later, it was shown that GPR50 is as likely to engage in homodimers as in heterodimers with MT₁ or MT₂ but not with β₂-adrenoreceptors or CC chemokine receptors (CCR). Heterodimers of GPR50 with MT₂ did not change the activity of MT₂, but dimers with MT₁ strongly inhibit receptor function. It seems that the C-terminal tail inhibits the interaction of MT₁ with the G-protein and β-arrestin [24]. Since MT₂ dimers with GPR50 show no decreased activity, MT₂ could act as an endogenous antagonist for GPR50, thus inhibiting the inhibition of MT₁ by GPR50 [45]. For an overview of melatonin GPCR dimerizations see Figure 10.

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**Figure 9**: Predicted membrane topology of GPR50, with deduced amino acid structure. Modified after [44].

**Figure 10**: Melatonin GPCR dimerizations. Modified after [27].

### 2.3.2 Other melatonin binding sites

Another protein binding and reacting to melatonin is MT₃, originally named ML2 and discovered in 1988 [24, 46]. Compared to MT₁ and MT₂, it exhibits a relatively low affinity for melatonin and fast kinetics [24, 47]. In the year 2000, MT₃ could be identified
as the quinone reductase 2 (QR2), a well known enzyme with oxidoreductive properties [48]. X-ray structural analysis of the melatonin / QR2 complex proved the interaction between melatonin and QR2 [49]. QR2 seems to increase cytotoxic effects caused by reactive quinones, seeing that, for example, QR2 knockdown cells are 42–48 % less sensitive to menadione poisoning [50]. While the mechanisms of QR2 and its regulation are still somewhat unclear [27], it seems very probable that some of the beneficial aspects of melatonin such as its antioxidative capability are connected with its binding affinity to the QR2 binding site [24].

In addition to the GPCRs MT₁ and MT₂ as well as QR2, melatonin interacts with numerous other targets. In 1993, Benitez-King et al. [51] demonstrated affinity of melatonin for calmodulin, thus modulating intracellular Ca²⁺ functions. It was proved recently that melatonin has a very significant influence on calcium / calmodulin-dependent protein kinases in rat INS-1 cells [52]. Calreticulin, another calcium binding protein, has high affinity for melatonin, too. Calreticulin is a protein of the endoplasmatic reticulum with various functions like chaperon activity or integrin function. There are speculations that melatonin might influence calreticulin’s internalization in the nucleus [53].

Melatonin has an (albeit low) affinity for nuclear receptors such as Retinoid-related orphan receptor alpha (RORα) 1, 2, and retinoid Z receptors (RZR). While the RORs are probably involved in regulating effects on leucocytes, little is known about RZR [6]. For more details on these nuclear receptors see [27].

Finally, melatonin seems to have some influence on mitochondria; while it seems to modulate electron flux and leakage [27], it also inhibits the mitochondrial transition pore (mtPTP), which plays a role in the development of ischemia. Melatonin reduces infarct volume and neuron loss, so that the inhibition of mtPTP might be a very important feature of the anti-apoptotic effect of melatonin [54].

### 2.4 Antioxidative effects of melatonin

One of melatonin’s most prominent features is its antioxidative capacity. Melatonin is most efficient in scavenging free radicals with almost no pro-oxidant side effects [6]. It was discovered that the O-methyl and N-acetyl groups (see Figure 1) are most important for melatonin’s extraordinarily strong direct radical scavenging ability [55]. During the metabolism of melatonin to AFMK (N¹-acetyl-N²-formyl-5-methoxykynuramine), the metabolite apparently most important for radical scavenging, up to four free radicals can be eradicated (see Figure 4). Other metabolites of melatonin or their reactions of formation and degradation accordingly exhibit radical scavenging properties as well [6]. Additional to this antioxidant mechanism are indirect effects via
inhibition of NO production [42], up-regulation of antioxidant enzymes like catalase [56] and down-regulation of pro-oxidant enzymes like myeloperoxidase [57], inducible NO-synthase [56] or superoxide dismutase [58].

Various in vivo studies have confirmed melatonin’s antioxidative capacity and capability to reduce oxidative stress. For example, it was demonstrated that melatonin reduces renal damage from oxidative stress in rats with artificially induced chronic renal failure [59]. Another example is reduced damage, caused by alcohol induced oxidative stress, in the aorta of melatonin treated rats [60].

Further examples for and information on the oxidative stress related effects of melatonin can be found in a variety of reviews, e.g. [6, 61-63].

2.5 Pharmacological aspects of melatonin

As a medication, melatonin has phase shifting effects for sleep or body temperature. This might be beneficial for e.g. jet lag or shift work [64], although the observed effect of oral melatonin has been relatively slim [65]. A large meta-analysis showed an average increase in sleep duration of 12.8 minutes and reduced sleep onset by 4.0 minutes [66]. However, a recent study performed on autistic children showed greater benefits of melatonin on sleep onset (47 minutes) as well as sleep duration (52 minutes) when compared to placebo [67]. Melatonin also shows positive effects when treating depression [64].

Another interesting application for melatonin might be the treatment of poisoning with – or toxic side effects of – various substances like sulfur mustard [68], arsenite [69], mercuric chloride [70-71], nickel [72] or carbon tetrachloride [73], the chemotherapeutic drugs adriamycin [74] and doxorubicin [75] as well as the immunosuppressant cyclosporine A [76]. For doxorubicin, simultaneous treatment with melatonin seems not only to ameliorate the cytotoxic side effects of the doxorubicin, but melatonin also increases the drug’s apoptosis inducing effect in hepatoma cells in vitro [77].

One more field of application for melatonin might be the treatment of glaucoma [78]. While melatonin does not decrease intraocular pressure, it reduces the destructive effects of ocular hypertension [79].

Many melatoninerrous substances have been developed [80], however, few are available for human use. Melatonin itself, available as over-the-counter-medicine in the US and available on prescription in the European Union, is probably safe in low dosages [64]. Agomelatine, an agonist at MT₁ and MT₂ and an antagonist at 5-HT₂C receptors, is used primarily as an antidepressant with good efficacy even in severely depressed patients, and exhibits relatively few adverse effects [81]. Ramelteon, a
selective MT₁ and MT₂ agonist, is effective in promoting sleep in animals and patients with insomnia by regulating the body’s natural clock. Unlike other hypnotics, it does not cause withdrawal symptoms or dependence and has only mild side effects [82]. Finally, another new compound, Tasimelteon, a high affinity MT₁ and MT₂ agonist, has proved in clinical trials to improve sleep quality, onset and duration in patients with transient insomnia. Adverse effects were similar for Tasimelteon and placebo [83].

A very promising field for melatonin treatment might be the prevention or alleviation of damage from cardiovascular diseases. Various studies have shown that in patients with acute myocardial infarction [84], coronary heart disease [85-86] or cardiac syndrome X [87], nighttime melatonin levels are reduced. Therefore, it was investigated, whether exogenous melatonin has a beneficial effect on these conditions. Indeed, on artificially ischemic rodents, exogenous melatonin exhibits dramatic effects: animals pre-treated with melatonin 10 minutes before artificially induced ischemia exhibit more than 50 % reduction of the total number of premature ventricular contractions, duration of ventricular fibrillation and ventricular tachycardia. Infarct size was reduced as well. Mortality decreased from 59 % in rats without melatonin treatment to 0–10 % in rats with pre-occlusion melatonin administration. All these effects were attributed to melatonin’s antioxidative capabilities [57]. The protective action of melatonin has been demonstrated by similar studies (e.g. [58]) or studies performed on different organs in similar ischemic conditions (e.g. [88]). Because of these promising results, the phase II MARI A trial is being undertaken to confirm the cardioprotective capability of melatonin for patients with acute myocardial infarction undergoing primary angioplasty [89-90].

For more information about melatonin and its protective influence on the cardiovascular system see [91] or [92].

2.6  Melatonin and blood pressure (BP) regulation

2.6.1  General overview on BP regulation

The regulatory mechanisms of BP are manifold. Three parameters influence BP: heart activity (heart rate and contractile force), blood volume and peripheral resistance of the arterioles and capillaries. Through vasoconstriction of arterioles, BP is elevated, while vasodilation reduces BP[93]. To describe all these parameters and the mechanisms that influence them would go beyond the scope of this work. A simplified overview is given in Figure 11. The reader is directed to the pertinent literature for details [94].
In humans, hypertension is defined as a BP of > 140 (systolic) or > 90 (diastolic) mm Hg. Hypertension is a key risk factor for development of cardiovascular events like myocardial infarction, edema, ischemia or other diseases. Over 20 % of the adult population of the USA take antihypertensive drugs [95]. Hypertension can be caused by a variety of factors, but in most cases the cause is unknown – this condition is referred to as essential hypertension.

2.6.2 The aorta

In contrast to the capillaries and arterioles, the aorta contributes very little to BP formation [93]. Its smooth muscles mainly provide stiffness and elasticity. Nevertheless, the aorta is often used as a model system for all blood vessels, since the arterioles and capillaries are almost impossible to prepare because of their sheer minuteness.

The aorta is the largest blood vessel in the mammalian body, and is very similar in structure among all mammalian species [96]. The aorta, like all blood vessels, consists of three layers or tunicas. The innermost layer (nearest to the lumen of the vessel) is called tunica intima, or tunica interna. It consists of the endothelium of the vessel and connective tissue. The outer border of the tunica intima is the internal elastic lamina, which is the first of many elastic fibers, alternating with layers of smooth muscle cells. Together, they form the second tunica, the tunica media. The outermost “ring” of elastic fibers delimits it from the tunica adventitia, or tunica externa, which is comprised mainly of connective tissue and, as the name suggests, connects the aorta to the surrounding tissue [97-98]. See Figure 12 for a schematic of a blood vessel and a histological section of an aorta.
An important function of the endothelium in blood vessels is the production of nitric oxide (NO), which was identified as the endothelial derived relaxing factor. NO is produced in endothelial cells via NO-synthase and then diffuses in gaseous form in the tunica media where it activates the guanylyl cyclase, which leads to subsequent relaxation of the smooth muscles [100]. Activation of the NO-synthase can be mediated through a variety of factors, including melatonin [61].

In contrast, vasoconstriction is mediated only through direct activation of receptors on the smooth muscle cells. Examples for these substances include norepinephrine or angiotensin II [101], but probably also melatonin [61].

2.6.3 Reduced melatonin levels lead to hypertension

Melatonin's physiological function in BP regulation is revealed by the fact that in pinealectomized rats BP increases significantly. Interestingly, the effect is only temporary, as the elevated BP returns to normal values 90 days after the operation [102]. A possibly BP related side effect of pinealectomy is reduced release of vasopressin (a peptide hormone secreted by the pituitary gland regulating water retention and vasoconstriction). Vasopressin levels are significantly lower in pinealectomized rats than in sham operated animals. The osmotic threshold for vasopressin release remains unchanged and only the quantity of released hormone is reduced. This indicates that it is not the actual release of vasopressin that is mediated by melatonin, but the magnitude of the response to altered osmotic conditions, possibly...

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Figure 12: Anatomical structure of the aorta. Left panel: schematic depiction of the aorta. Right panel: Histological section of the aorta. TI: tunica intima, end: endothelium, el: elastic lamellae, n: smooth muscle tissue, TM: tunica media, TA: tunica adventitia. Left panel modified after [97], right panel modified after [99].
via some central mechanism not yet discovered [103]. However, since vasopressin action seems to be lower in rats with less melatonin, this fact cannot add to the explanation of the elevated BP in pinealectomized rats.

In patients with nocturnal hypertension, melatonin levels are decreased [104]. This adds to the hypothesis that inhibited melatonin secretion leads to elevated BP, thus suggesting a BP reducing effect of melatonin. Because of this, it seems irritating at first that in other studies, patients with essential hypertension exhibited significantly increased melatonin levels. However, treatment with lacidipine, an antihypertensive drug, not only reduces BP, but the elevated melatonin levels, too [105]. Taken together with the observation that melatonin restores contractile and relaxing functionality in the aorta [106-108], this suggests that BP is not elevated because of the elevated melatonin levels, but in spite of them. Natural melatonin secretion might be increased in hypertensive patients to counteract the elevated BP.

Because of these findings, the use of exogenous melatonin to combat hypertension seems promising.

### 2.6.4 Influence of melatonin on animal models of hypertension

Exogenous melatonin exerts a profound influence on hypertension in animals. However, the mechanism of this influence on the BP remains somewhat unclear. As discussed already, melatonin exerts direct influence on smooth muscle tone in blood vessels – and therefore possibly peripheral resistance –, although the data on this influence is conflicting (see 2.6.6).

Apart from this direct influence, melatonin may play a role in mediating central BP regulation as well. Baroreflex responses are improved under melatonin treatment as sympathetic output is decreased. Inhibition via GABAergic fibers from the SCN of areas in the central nervous system responsible for BP regulation might be a possible explanation [61, 109]. In a study performed on rats with stress induced hypertension by electric shocks for two weeks, pre-treatment with melatonin reduced caudal arterial pressure almost to control values. Associated with this reduction was a decrease of angiotensin II levels which was possibly caused by GABA_A signaling, since the BP and angiotensin II reducing effect of melatonin was nullified when given in combination with bicuculine, a GABA_A antagonist [109]. This central regulatory mechanism might be receptor-mediated, as another study on the same model of hypertension showed. Under melatonin influence GABA levels in the central nervous system were elevated, while luzindole nullified the effect of melatonin completely [110]. The angiotensin II dependent decrease of BP mentioned before was also observed in rats with angiotensin II levels artificially increased by means of a renal artery clip. Melatonin
treatment reduced the elevated BP and improved the angiotensin II dependent reduced cardiac function [111].

Another factor in melatonin’s BP reducing activity might be its antioxidative capability, which prevents structural damage in blood vessels, therefore helping to retain their dilative capabilities [61]. In spontaneously hypertensive rats (SHR; see below), melatonin treated animals exhibit decreased aortic [76] and cardiac [112] collagen content (as opposed to “normal” development of untreated SHR [113]) and thus heart fibrosis is prevented. The apoptosis rate of blood vessel cells is elevated, too, which might be an indicator for increased remodeling of vasculature in response to melatonin [114].

Melatonin treatment of these genetically predisposed hypertensive rats also results in greatly decreased BP in the melatonin treated group (systolic: 149 vs 195 mm Hg in the untreated group). As expected, superoxide and NF-κB levels are reduced [115] in treated animals. Additionally, melatonin improves maximum response of mesenteric arteries of SHR to endothelium dependent vasodilating stimuli like acetylcholine [116].

SHR are a model for essential hypertension; they were originally bred from normal Wistar-Kyoto rats by Okamoto [117]. After reaching adulthood, the systolic BP of SHR reaches 180–200 mm Hg, while after about 40–50 weeks, SHR develop hypertension associated cardiovascular diseases [118]. The cause for this development of hypertension is believed to be of higher central origin [119].

SHR as model of hypertension were also used in the thesis at hand to study the difference in melatonin receptor expression levels in the vascular system between normotensive and hypertensive animals.

### 2.6.5 Influence of melatonin on the BP of humans

Orally administered melatonin significantly reduced BP together with norepinephrine levels without affecting the heart rate in healthy men [120] and reduced BP in women by about 9 mm Hg [121]. Similar studies on healthy subjects confirmed these findings [122-123]. Prolonged nocturnal melatonin treatment did not change diurnal BP variations [123].

In patients with essential nocturnal hypertension, repeated melatonin treatment at bedtime reduced nocturnal BP by 6 (systolic) and 4 (diastolic) mm Hg. The reduction is most significant during the night (see Figure 13) [124].
Similar results were obtained in a study performed on so-called “non-dipper” patients who exhibit an abnormal circadian BP profile, manifesting in elevated BP during the night, but normal BP during the day. Starting from the observation that melatonin levels are decreased during the night in non-dippers [104], 5 mg of melatonin were administered daily before sleep. 35 % of the patients showed complete pattern normalization (vs 15 % in the control group). This effect was not only caused by nighttime BP reduction, but also by daytime BP elevation. Because of these results, the authors of the study recommend close monitoring of the circadian BP profile when using melatonin as antihypertensive medication to avoid the risk of induced arterial hypertension [125]. Another dilemma with long time, high dose melatonin treatment adds to this problem: chronic treatment leads to reduced ROS levels in the blood, but in a long term view this is not only beneficial, since ROS also play important regulatory functions. Reduced ROS levels might lead to reduced NO-synthetase induction and therefore reduced blood vessel dilation, thus again leading to elevated BP [126].
2.6.6 Contribution of MT\textsubscript{1} and MT\textsubscript{2} to BP regulation

Expression of MT\textsubscript{1} and MT\textsubscript{2} in the vasculature

MT\textsubscript{1} and MT\textsubscript{2} were detected in the human vascular system using RT-PCR and western blot [127-128]. MT\textsubscript{1} receptor expression was shown to be highly dependent on circadian rhythm, with the lowest values at night and the highest values in the afternoon [129].

The data on the rat vascular system is conflicting. Pharmacological studies indicated that MT\textsubscript{1} is indeed expressed in the rat's cardiovascular system. Lartaud et al. [56] demonstrated a clear effect of melatonin on rat aortic rings. Melatonin prevented incubation induced loss of contraction which was reversible by the melatonin receptor antagonist luzindole, therefore suggesting present and functional receptors. Similarly, melatonin effects direct vasoconstriction in rat cerebral arteries [130], and the vasoconstriction effect can be reversed by luzindole in rat arterioles. This clearly points to melatonin GPCRs as the responsible element for vasoconstriction in these arteries [131].

Then again, Chucharoen et al. [132] could detect neither MT\textsubscript{1}, nor MT\textsubscript{2} mRNA in rat cerebral arteries, while Masana et al. [36] reported MT\textsubscript{1} and MT\textsubscript{2} mRNA to be present in the rat caudal artery; however, because the primers used in both studies were located only on one exon, it is possible that amplification of genomic DNA influenced the findings. To address this problem, experiments with exon spanning primers are necessary, so that false positives due to amplification of genomic DNA can be excluded.

If MT\textsubscript{1} is indeed expressed in the vasculature of the aorta, the circadian expression pattern observed in humans [129] seems to be organ and / or species specific, as MT\textsubscript{1} expression levels in the rat heart and aorta do not exhibit a diurnal rhythm, as observed on the protein level by Benova et al. [108]. In this study, MT\textsubscript{1} was identified in the aorta of Wistar rats on the protein level by western blot, but of course the problem with antibody specificity [24] remains, putting the results into perspective. Investigations on the mRNA level are necessary to confirm or discard these results on circadian expression of MT\textsubscript{1} and were performed in the course of this thesis.

Mechanisms of melatonin influence on the vasculature

There have been many different reports on melatonin’s influences on vasculature and the pathways explaining these influences.

It was suggested that melatonin effects vasoconstriction through blockage of BK\textsubscript{Ca} channels, probably via MT\textsubscript{1} [61], as blockage of these channels inhibited further
constriction by melatonin but not by compounds using a different mechanism of action like the NO-synthase inhibitor L-NAME (N-nitro-L-arginine-methyl-ester) [131].

In contrast to MT₁, MT₂ seems to mediate vasodilation: Doolen et al. [133] noted that isolated rat caudal segments showed increased constriction in presence of the MT₂ antagonist 4-phenyl-2-acetamidotetraline.

These findings were confirmed in 2002 by Masana et al. [36] by comparing the contractile responses of the rat arteries in presence of melatonin alone, melatonin compared with 6-chloro-melatonin (10-fold less sensitive to MT₁ than MT₂) and melatonin plus 4-Phenyl-2-propionamidotetralin, an antagonist for MT₁ and MT₂, which is selective for MT₂ in concentrations of about 10 nM. 6-chloro-melatonin (MT₂ agonist) was 10 times less effective in potentiating vasoconstriction than melatonin (Figure 14, left panel), suggesting that MT₁ and not MT₂ is responsible for potentiation of exogenously induced vasoconstriction (in this case by phenylephrine). Selective antagonistic concentrations of 4-Phenyl-2-propionamidotetralin for MT₂ (10 nM) markedly increased the contractile response to melatonin (Figure 14, right panel). This strongly suggests that MT₂ attenuates MT₁ mediated vasoconstriction. Nevertheless, the vasodilating influence of MT₂ is covered by the contracting effect of MT₁, as melatonin alone had a contracting effect.

Figure 14: Left graph: effect of low affinity MT₁ agonist 6-chloro-melatonin (6-Cl-melatonin) on contractile response compared to melatonin, demonstrating MT₁ dependent increase in contractile force. Right graph: effect of melatonin in presence of a MT₂ selective concentration of 4-Phenyl-2-propionamidotetralin (4P-PDOT) compared to melatonin alone, demonstrating generally elevated contractile force and increased maximum response to melatonin which suggests an attenuating influence of MT₂ on MT₁. Modified after [36].

So, in summary, MT₁ mediates vasoconstriction and MT₂ vasodilatation, but probably to a lesser extent. It has been proposed that vasoconstriction is triggered through G_q mediated Ca^{2+} efflux from the sarcoplasmatic reticulum directly into smooth muscle cells. Vasodilation on the other hand is mediated indirectly via melatonin induced activation of NOS in endothelial cells of the blood vessels (see Figure 15).
Details can be found in [61]. See chapter 2.6.2 for basic information on blood vessels. For understanding of these proposed pathways, it is essential to know whether both melatonin receptors are present in the cardiovascular system. The thesis at hand confronts this question.

**Figure 15** Left panel: potential pathway mediating melatonin induced vasoconstriction in vivo. Melatonin activates $MT_1$ (and $MT_2$) directly on smooth muscle cells which both activate a $G_q$-protein, leading to $Ca^{2+}$ efflux from the sarcoplasmatic reticulum and subsequent muscle contraction. At the same time $MT_1$ activates a $G_i$-protein which inhibits the formation of cAMP and subsequent muscle relaxation. Right panel: potential pathway mediating melatonin induced vasodilatation in vivo. Melatonin activates $MT_2$ on endothelial cells, which leads to activation of NOS through increased $Ca^{2+}$ concentrations. At the same time, melatonin scavenges ROS that normally intercepts NO in the tissue. Both modified after [61]. Q. v. for abbreviations, literature and detailed information.
3 AIMS

Melatonin is known to influence BP and this influence is, at least partly, mediated by the G-protein coupled receptors (GPCRs) MT₁ and MT₂ [61].

Because the vascular system plays an integral role in BP regulation and melatonin is known to influence vascular tone, both dilating and contracting (e.g. [36, 56, 108, 131, 134]), it is of interest to know how these responses are mediated. The data on melatonin GPCR expression in the vascular system in general and the aorta as a model is conflicting. Both MT₁ and MT₂ have been found as well as not found in various studies with various vascular models including the aorta [56, 108, 127, 132, 135]. Most of these experiments were performed on the protein level or via pharmacological studies and it has to be noted that the antibodies for rat melatonin GPCRs are not well characterized [24]. The few RT-PCR studies [36] were performed with intra-exon primers designed from non-complete sequences of the mRNA of both receptors. Recently, Ishii et al. [26] demonstrated the expression of rat MT₁ and MT₂ mRNA in many rat tissues by RT-PCR using exon spanning primers derived from the complete mRNA sequences. Starting from the findings and methods of that study [26], in the thesis at hand the expression of MT₁ and MT₂ in the rat aorta were to be characterized, pursuing the following aims.

1) Establishment of molecular biological methods for demonstrating MT₁ and MT₂ mRNA expression

The first aim of the thesis was to establish the RT-PCR and RT-qPCR protocols for detection of rat MT₁ and MT₂ mRNA. To achieve this goal, we had to establish the method for RNA extraction and reverse transcription and test the published RT-PCR method by Ishii et al. [26] and an RT-qPCR method on various rat organs as well as adjust the methods as necessary to achieve consistent results.

2) Investigating the expression of MT₁ and MT₂ in the rat aorta

Following the establishment of the methods, RT-PCR experiments to investigate the expression of MT₁ and MT₂ mRNA in the rat aorta as a model system for arterial blood vessels were performed.
3) **Quantification of MT<sub>1</sub> and MT<sub>2</sub> mRNA expression difference in the rat aorta depending on the time of day**

The melatonin GPCRs are known to exhibit circadian expression patterns in some organs [136]. Because BP is subjected to high diurnal variations [137] and the regulating influence of melatonin on BP is known [61], it was of interest to assess whether the expression levels of MT<sub>1</sub> and / or MT<sub>2</sub> were different in the vascular system depending on the time of day. The data on this is conflicting, as a diurnal variation has been demonstrated for human coronary arteries [129], while in the rat aorta, no circadian rhythms were observed at the protein level [108]. Consequently, as a third aim of the thesis, we performed RT-PCR and RT-qPCR experiments to evaluate a possible difference in receptor expression at the mRNA level between rat aortas of two time groups.

4) **Quantification of MT<sub>1</sub> and MT<sub>2</sub> mRNA expression difference in the rat aorta depending on BP**

Because the mechanisms of BP regulation by melatonin are still somewhat unclear [61], it is of interest to know whether there is an expression difference of melatonin GPCRs – if they are present – in the vascular system between normotensive and hypertensive animals. As of yet, the only data available is from L-NAME induced hypertensive rats, where no difference in expression was found on the protein level [108]. Therefore, as a fourth aim of the thesis, we performed RT-PCR and RT-qPCR experiments on aortas of control and spontaneously hypertensive rats (SHR) to assess if there was such a difference at the mRNA level.

5) **Localization of MT<sub>1</sub> and MT<sub>2</sub> protein in the rat aorta**

If the melatonin GPCRs can be found on the mRNA level because of the proposed different signaling and regulating mechanisms of MT<sub>1</sub> and MT<sub>2</sub> [61], it is of interest to know the allocation of MT<sub>1</sub> and / or MT<sub>2</sub> within the aorta. Some studies on the localization of the melatonin GPCRs have been performed [135, 138], but so far only on human and chicken samples and using immunohistochemistry and radio assays respectively. To better understand the effects of melatonin on MT<sub>1</sub> and MT<sub>2</sub> in blood vessels, immunofluorescence (IF) stainings to localize MT<sub>1</sub> and MT<sub>2</sub> in the structure of the rat aorta were performed as the fifth aim of the thesis.
4 MATERIALS AND METHODS

4.1 Samples

4.1.1 Various rat organs for PCR method establishment

Various rat organs (brain, eye, heart, intestine, kidney, liver, lung, skeletal muscle, testes and thymus) have been prepared and frozen in 2008 by M. Svoboda (Department of Pathophysiology, Medical University Vienna). These tissues were used for establishing the methods for RNA-isolation, RT-PCR, RT-qPCR.

4.1.2 Rat aortas from two different time points

Sixteen prepared aortas of normal (Wistar) rats were kindly provided by Professor M. Zeman, Comenius University of Bratislava, Slovakia. Prior to dissection, the animals were kept in a 12/12 day/night rhythm (lights off at 22:00 hours and lights on at 10:00 hours). Half of the aortas were obtained in operations 2 hours before lights on (nighttime group; A75, A76, A77, A79, A81, A82, A83, A88) and 2 hours before lights off (daytime group; A31, A32, A33, A34, A35, A36, A37, A38), respectively. The aortas were then frozen at -80 °C and finally transferred to Vienna on dry ice.

These aortas were used to examine whether melatonin receptor expression follows a circadian rhythm in the rat aorta by usage of PCR experiments.

The names of the aortas are taken from the nomenclature used by the Comenius University of Bratislava and kept for better traceability of the samples.

4.1.3 Rat aortas from SHR and control rats

Sixteen prepared aortas of SHR (ASHR 01–ASHR 08) and control (Wistar; ACTRL 01–ACTRL 08) rats were kindly provided by Professor M. Zeman, Comenius University of Bratislava, Slovakia. After dissection, a part of each aorta was fixated in HOPE® solution (DCS, Hamburg, Germany) and transferred to Vienna on blue ice, while the other part was frozen at -80 °C and transferred to Vienna on dry ice. The HOPE® fixated parts of the aortas were then paraffin-sectioned by H. Uhrova (Department of Pathophysiology, Medical University Vienna), while the other part was used for RNA extraction and the subsequent PCR experiments.

These aortas were used to examine whether the expression of MT₁ and MT₂ is different in the aortas of hypertensive and normotensive rats by usage of PCR and IF experiments with paraffin-sections.
4.1.4 Additional rat aortas for IF experiments

These aortas were prepared for cryo-sectioning in 2008 by M. Benova (Comenius University of Bratislava, Slovakia; during a research stay at Department of Pathophysiology, Medical University Vienna) and stem from SHR (A_cryo4–A_cryo6) and control (Wistar; A_cryo1–A_cryo3) rats. These tissues were used for IF method establishment to examine the difference in melatonin receptor expression by usage of IF experiments with cryo-sections.

4.1.5 Mouse organs for IF experiments

The organs (brain and intestine) were prepared by H. Uhrova, R. Stumberger and C. Brünner-Kubath and then HOPE® fixated and paraffin-sectioned by H. Uhrova (all Department of Pathophysiology and Allergy Research, Medical University Vienna). These organs were used as positive controls in some IF experiments.

4.2 RNA isolation from frozen tissues

4.2.1 Background

After [139].

In order to demonstrate the occurrence of specific mRNA, the first step is to isolate the total RNA of all tissues that are to be investigated. The total RNA of a tissue is isolated by extracting it from deep frozen or freshly prepared tissue samples, which are first pulverized and then subjected to peqGOLD TriFast™ treatment. PeqGOLD TriFast™ treatment is an optimized guanidineisothiocyanate / phenol method, suitable for RNA, DNA and protein extraction. After extraction and homogenization with PeqGOLD TriFast™, chloroform is added to the mixture, where the DNA and protein fraction will enrich, while the more hydrophilic RNA will stay in the aqueous phase. The RNA can then be precipitated using isopropanol, washed with ethanol and finally solved in DNase, RNase, DNA free water (“PCR water”). The isolated and purified RNA can then be stored over a long period of time at -80 °C.
4.2.2 Materials

- Box with ice
- Chloroform (Merck, 2445)
- Coolable Centrifuge (Eppendorf, Centrifuge 5415R)
- DNase, RNAse, DNA free distilled water, “PCR water”, (Gibco, 0977035)
- DNase, RNAse, DNA free pipette tips
  - 1250 µL (Biozym, Safe seal tips® professional, 770600)
  - 200 µL (Biozym, 770280)
- DNase, RNAse, DNA free tubes 1.5 mL (Biozym, 710310)
- Ethanol 70% (diluted from Ethanol absolute, VWR Prolabo, 20821.310)
- Forceps
- Gloves for cryogenic work (handling of liquid N₂, Tempshield, Cryogloves®)
- Heating block (Eppendorf, Thermomixer 5436)
- Homogenizer (IKA® Werke, T10 basic Ultra Turrax®, 3420000)
- Isopropanol (Merck, 1.09634.1011)
- Latex gloves (Hartmann, Peha-Soft® powderfree, 942162)
- Liquid N₂
- Mortar + pistil
- Paper towels
- peqGOLD Trifast™ (peqLAB, 30-20XX)
- Polystyrene (styrofoam) boxes (two) with lids
- Polystyrene rack
- Protective glasses
- Small flasks (Greiner bio-one, scintillation vial 27.0/60.0 mm, 619301; saw off neck)
- Vortexer (Labino, L24)
4.2.3 Method

1. Preparation
   a. Fill the Styrofoam boxes with liquid N\textsubscript{2}
   b. Put the Styrofoam rack in the box
   c. Put the small flasks in the styrofoam rack
   d. Put the sample(s) (from -80 °C cooling unit) in the styrofoam box
   e. Put the mortar into the second styrofoam box and fill mortar and box with some liquid N\textsubscript{2}

2. Sample preparation
   a. Put 50–100 mg or about 5x5x1 mm of tissue (break if necessary) into the mortar (with liquid N\textsubscript{2})
   b. Pulverize the tissue with the pistil and avoid complete evaporation of the N\textsubscript{2} in the mortar
   c. Transfer the crushed tissue plus some liquid N\textsubscript{2} into a flask (scintillation vial with sawed off neck)
   d. Keep the flask containing the sample in the Styrofoam box with liquid N\textsubscript{2} and avoid drying out of the liquid N\textsubscript{2} in the flask

3. TriFast™ utilization
   a. When all samples are homogenized, let the N\textsubscript{2} in the flask evaporate and add 1 mL of Trifast™ reagent (per max. 100 mg of tissue) to the flask (one flask at a time!)
   b. Transfer the flask from the Styrofoam box to ice
   c. Homogenize for 30 seconds with UltraTurrax® (avoid excess heat generation)
   d. Transfer the homogenated tissue into one 1.5 mL tube (per 1 mL Trifast™) and ...
   e. ... put the tube immediately on ice and incubate for 5 min
   f. Incubate for 5 min at rt – from now on, work in a bench designated for RNA extraction (storage is now possible at -80 °C)

4. RNA isolation
   a. Add 200 µL chloroform
   b. Vortex for 15 sec
   c. Incubate for 5 min at rt
   d. Centrifuge for 15 min at 12,000 x g / 4 °C → phase separation
   e. Transfer the upper aqueous phase (containing RNA) to a fresh tube
   f. Centrifuge again for 15 min at 12,000 x g / 4 °C, meanwhile …

5. RNA precipitation
4. Materials and methods

a. … prepare a fresh tube and fill it with 500 µL isopropanol

b. Transfer the aqueous phase (containing RNA) to the tube with isopropanol

c. Vortex for 10 sec

d. Incubate for 10 min on ice

e. Centrifuge for 15 min at 12,000 x g / 4 °C

f. Remove the isopropanol from the precipitate

6. RNA washing

a. Wash the pellet with 1 mL ethanol 70 % (v / v) by vortexing

b. Centrifuge for 10 min at 12,000 x g / 4 °C

c. Remove the ethanol thoroughly from the pellet

d. Repeat steps 6a through 6c once, then continue to step 7a

7. Solving and storing

a. Dry the pellet in a heating block at 55 °C for 1 (max. 3) min with opened lid

b. Add 20 µL PCR water

c. Pipette up and down a few times until the RNA is dissolved

d. Incubate at 55 °C for 5 min

e. Store RNA at -80 °C

4.3 Quantification and purity assessment of RNA and DNA

4.3.1 Background

The quantity of RNA or DNA is then determined by UV/VIS spectrophotometry. The ratio of the samples' absorbance at 260 nm and 280 nm ($A_{260} / A_{280}$) is used to assess its purity. For extracted RNA, the PeqGOLD TriFast™ instruction manual [139] specifies an achievable ratio of 1.60–2.00, depending on the type of tissue and certain other factors.

4.3.2 Materials

- Box with ice (for transportation of samples)
- DNase, RNase, DNA free pipette tips
  - 10 µL (Biozym, 770010)
  - 20 µL (Peqlab, 81-1020)
- Paper towels
- Soft paper wipes (Kleenex®)
4 Materials and methods

- Solvent, 10 µL (here: DNase, RNase, DNA free distilled water, “PCR water”; Gibco, 0977035) for blank measurements
- Spectrophotometer (Thermo Fisher Scientific, NanoDrop™ 1000)
- Vortexer (Labinco, L24)

4.3.3 Method

1. Start the ND-1000 program
2. Choose the appropriate user name from the drop down menu (enter a password if necessary) and then choose the button “Nucleic Acid”
3. The software prompts to load a water sample
4. Sample loading procedure
   a. Open the instrument’s arm
   b. Apply a volume of the sample (1–2 µL) to the lower measurement pedestal
   c. Carefully close the instrument’s arm (do not use force)
5. Click “OK”
6. Load a water sample (PCR water)
7. After initialization of the instrument is complete, wipe the residue from the upper and lower pedestals with a soft wipe.
8. Select the appropriate calculation method (DNA-50 or RNA-40) for the type of sample (DNA or RNA) using the drop down menu
9. Load a blank sample (here: 1 µL PCR water)
10. Click “Blank”
11. Clean the pedestals
12. Load 1 µL of sample
13. Click “Measure”
14. Take down the readings (concentration, $A_{260} / A_{280}$ ratio)
15. Clean the pedestals
16. Repeat steps 11–14 for all samples
17. Load a blank sample (here: 1 µL PCR water)
18. Click “Measure” – the spectrum should exhibit a relatively flat bottom line
19. Clean the pedestals and do not forget to close the sample arm
20. Click “Exit”, then “Exit” again to close the program
4.4 Agarose gel-electrophoresis (GE)

4.4.1 Background

GE is employed to separate nucleic acids like RNA and DNA by their size. As the name suggests, the gels employed in this method are made of dissolved and re-solidified agarose in TRIS-acetic-acid-EDTA (TAE) buffer. The samples are mixed with loading dye, which contains dyes that migrate at the same speed as nucleotides of a certain length and also serves the purpose of increasing the samples’ specific weight, so it will not be washed out of the gel pocket. The sample-loading dye mixtures are then loaded in the pockets of the gel, which is submerged in TAE buffer. After the loading procedure, a current is applied along the gel. Because RNA and DNA are negatively charged, they will be drawn to the anode. The gel retains this migration, larger nucleic acid molecules being more strongly retained than smaller ones. After a certain time, the current is switched off and the separated nucleic acids appear as bands along the migration route.

To make these bands visible, the gel contains ethidium bromide, which intercalates with DNA or RNA molecules. This intercalation results in orange fluorescence if the molecule complex is subjected to UV light. Therefore, while (trans-)illuminating the gel with UV light, nucleic acid bands can be seen and photographed.

When used for testing the intactness of isolated total RNA, two distinct bands will appear for the 28S and 18S rRNA (ribosomal RNA, accounting for over 98 % of the total RNA in a cell) if the RNA is not degraded. If the RNA is degraded – through contamination with RNase – GE will show a smear of low molecular weight bands.

When used for analysis of (RT-) PCR products, a marker, containing a defined amount of DNA fragments of several defined lengths, should be used to assess the size of the PCR product.

4.4.2 Buffers and solutions

0.5 M EDTA solution

Materials

- Aqua bidest.
- EDTA, Na-salt (Merck, 1.08418.1000)
- NaOH (Merck, 1.06498.1000)
- pH-meter (Metrohm, pH-Lab 827)
4 Materials and methods

Method
1. Solve 93.05 g EDTA, Na-salt in 400 mL aqua bidest.
2. Adjust the pH to 8.0 with NaOH
3. Adjust the volume to 500 mL with aqua bidest.
4. Store at rt

TAE stock solution (50x)

Materials
- 0.5 M EDTA solution (see above)
- Aqua bidest.
- Glacial acetic acid (Merck, 8.18755.2500)
- Tris (Merck, 1.08382.1000)

Method
1. Solve 242 g of Tris in 750 mL aqua bidest.
2. Add 57.1 mL of glacial acetic acid
3. Add 100 mL of 0.5 M EDTA
4. Fill to 1000 mL with aqua bidest.
5. Store at rt

1x TAE buffer

Materials
- TAE stock solution (50x) (see above)
- Aqua bidest.

Method
1. Dilute one part TAE stock solution (50x) with 49 parts aqua bidest. (1 : 50)
2. Store at rt

Ethidium bromide solution 10 mg / mL

Materials
- Aqua bidest.
- Ethidium bromide (Amresco, 0492-56)
- Latex gloves (Hartmann, Peha-Soft® powderfree, 942162)
- Nitrile gloves (Meditrade Medicare, Senso Nitril®, 1421I)
Materials and methods

**Method**

1. Solve 10 mg ethidium bromide in 1 mL aqua bidest.
2. Store at rt

**6x loading dye**

**Materials**

- Bromphenolblue (Amresco, 0449)
- Xylene cyanol FF (Amresco, 0819)
- Glycerol (Merck, 1.04094.0500)
- DNAse, RNAse, DNA free distilled water, “PCR water” (Gibco, 0977035)
- Latex gloves (Hartmann, Peha-Soft® powderfree, 942162)

**Method**

1. Mix 0.25 % (w / v) of bromphenolblue
2. 0.25 % (w / v) xylene cyanol FF
3. 30 % (v / v) glycerol
4. and 69.5 % (v / v) PCR water
5. Store in aliquots at -20 °C

**4.4.3 Preparation of agarose gels**

**Materials**

- 1x TAE buffer (see 4.4.2)
- Agarose (Invitrogen, 10975-035)
- Combs and walls fitting for the GE chamber
  - Wall (Peqlab, 41-1325-Wall)
  - Comb 28 well (Peqlab, 41-1325-28D)
  - Comb 20 well (Peqlab, 41-1325-20D)
  - Comb 16 well (Peqlab, 41-1325-16D)
- Ethidium bromide solution 10 mg / mL (see 4.4.2)
- GE chamber (Peqlab, 41-1325)
- Latex gloves (Hartmann, Peha-Soft® powderfree, 942162)
- Microwave oven (Moulinex® Micro-Chef Mo 55)
- Nitrile gloves (Meditrade Medicare, Senso Nitril®, 14211)
- Saran wrap
4 Materials and methods

Method

1. Prepare the GE chamber (combs and side walls)
2. According to the desired concentration of the gel, mix the respective amount of agarose with 1x TAE buffer (e.g. 2.625 g agarose in 175 mL 1x TAE buffer for a 1.5% gel)
3. Microwave the solution until the first boiling appears
4. Shake the solution and let it cool down until it is not steaming any more
5. Add 0.007% of ethidium bromide solution (10 mg/mL; e.g. 12.5 µL in 175 mL gel solution)
6. Pour the gel into the prepared GE chamber and let the gel solidify
7. Add walls to the GE chamber if applicable
8. Remove the gel(s) and store them in plastic foil at 4°C

4.4.4 Electrophoresis

Materials

- GE chamber (Peqlab, 41-1325)
- GE power supply (Peqlab, EV231)
- Agarose gel with ethidium bromide (see 4.4.3)
- UV transillumination imaging equipment (Biozym, ChemiImager 4400)
- Latex gloves (Hartmann, Peha-Soft® powderfree, 942162)
- Ladder (Fermentas, GeneRuler™, SM0323)
- Nitrile gloves (Meditrade Medicare, Senso Nitril®, 1421I)
- DNAse, RNAse, DNA free pipette tips
  - o 10 µL (Biozym, 770010)
  - o 20 µL (Peqlab, 81-1020)
  - o 100 µL (ART®, 100E, 2065E)
- DNAse, RNAse, DNA free distilled water, “PCR water” (Gibco, 0977035)
- 6x loading dye (see 4.4.2)
- Sterile 96 well plates (Greiner Labortechnik, PS-Mikrotiterplatte, 96K, kobaltsteril, 655161)
- Aqua bidest.
Method

1. Gel-electrophoresis
   a. Mix a volume of sample (depending on the size of the gel pockets) with one sixth of loading dye in a well of a 96 well plate (pipette up and down to mix)
   b. Repeat step a. for all samples
   c. Load the gel
   d. Ensure correct alignment of the gel in the GE rack and close the lid
   e. Connect the GE electrodes to the GE power supply
   f. Run the gel (suggested conditions: 150 V, 1:30 h; check the propagation of the loading dye during the run)

2. Analysis of the gel
   a. Open the hatch of the transilluminator and place the gel on the surface for UV illumination
   b. Start the chemi-Imager 4400 program
   c. Click on “Acquire”
   d. Activate epi-white, ensure that the aperture is not set too low
   e. Align the gel and magnification so that it fills the preview completely
   f. Click on “Exposure Preview”
   g. Close the hatch, deactivate epi-white and activate UV
   h. Change the exposure time and aperture so that the gel is correctly exposed
   i. Click on “Capture Image”
   j. After the image is taken, deactivate UV and click “Print”
   k. Exit the program
   l. Open the hatch, remove the gel, clean the UV surface with aqua bidest. and close the hatch again
4.5 Reverse Transcription

4.5.1 Background

After [140-141].

Because RNA itself is not suitable for PCR, it has to be converted to DNA first. This is done using reverse transcription, where RNA, e.g. total isolated RNA extracted from tissue, is reversely transcribed to single stranded complementary DNA (cDNA). This is done by adding dNTPs, random primers and reverse transcriptase, an enzyme gained from retro viruses, to the RNA sample. RNAse inhibitor can be added, too, to prevent the degradation of the template RNA due to minute contamination with RNAses. To avoid mix-ups and pipetting error, all components except for the samples are pre-mixed in a mastermix and added to the separate samples just before reaction.

The reaction mixture is warmed to 37 °C for two hours, during which the random primers bind to the RNA and the reverse transcriptase synthesizes a complementary strand of cDNA in 3' direction. After the two hours, the mixture is heated shortly to 85 °C to permanently inactivate the enzyme. The cDNA can then be stored over a long period of time at -20 °C and used for subsequent PCR or qPCR experiments.

4.5.2 Materials

- -20 °C storage box (for reverse transcriptase and RNAse inhibitor)
- Box with ice
- DNAse, RNAse, DNA free distilled water, "PCR water" (Gibco, 0977035)
- DNAse, RNAse, DNA free pipette tips
  - 10 µL (Biozym, 770010)
  - 20 µL (Peqlab, 81-1020)
  - 100 µL (ART®, 100E, 2065E)
- DNAse, RNAse, DNA free tubes with flat lid 0.2 mL (Biozym, 710920)
- Latex gloves (Hartmann, Peha-Soft® powderfree, 942162)
- RT Kit (Applied Biosystems, “High capacity cDNA reverse transcription kit”, 4368814), contains:
  - 10x RT buffer
  - 10x RT random primers
  - 25x dNTP Mix (100 mM)
  - Multiscribe™ Reverse Transcriptase 50 U / µL
  - RNAse inhibitor
- Thermal cycler (Eppendorf mastercycler personal, 53332 000.014)
- Vortexer (Labinco, L24)
4.5.3 Method

1. Preparation of 10 µL of the RT 2x mastermix (per 20 µL reaction)
   a. Let the components thaw slowly on ice, do not take the enzymes out of the refrigerator yet!
   b. Calculate the necessary amounts of reagent according to the number of samples plus one aliquot in excess
   c. Prepare the RT 2x mastermix according to the following table (add the enzymes last, take them out of the refrigerator only in the -20 °C storage box and put them back in the refrigerator as soon as possible!)

   Table 1: Recipe for 10 µL 2x reverse transcription mastermix using the high capacity cDNA reverse transcription kit [142].

<table>
<thead>
<tr>
<th>Vol [µL]</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>10x RT buffer</td>
</tr>
<tr>
<td>0.8</td>
<td>10x dNTP mix</td>
</tr>
<tr>
<td>2.0</td>
<td>10x RT random primers</td>
</tr>
<tr>
<td>3.2</td>
<td>PCR water</td>
</tr>
<tr>
<td>1.0</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>1.0</td>
<td>RNAse inhibitor</td>
</tr>
</tbody>
</table>

   d. Vortex the RT 2x mastermix for a short while and put it on ice

2. Preparation of the samples
   a. Pipette an aliquot of RNA sample (containing 2 µg of RNA) in a 0.2 mL tube
   b. Add PCR water for a total volume of 10 µL
   c. Add 10 µL of RT mastermix and pipette up and down a few times to mix
   d. Keep the prepared sample on ice
   e. Repeat steps a–d for all samples

3. Reverse transcription
   a. Put all the samples in the thermal cycler
   b. Use the following temperature program:
4 Materials and methods

Table 2: Temperature program for reverse transcription using the high capacity cDNA reverse transcription kit [142].

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>T [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 min</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>120 min</td>
<td>37</td>
</tr>
<tr>
<td>3</td>
<td>5 s</td>
<td>85</td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
<td>4</td>
</tr>
</tbody>
</table>

c. Run the program
d. After the program is finished, store the transcription products (cDNA) at -20 °C

4.6 PCR – Polymerase chain reaction

4.6.1 Background

After [143].

DNA or cDNA can be amplified using polymerase chain reaction (PCR). PCR was invented in 1983 and is now a fundamental tool in the life sciences [141]. dNTPs, target specific primers for the nucleotide sequence of interest, and the enzyme Taq-polymerase (from the bacterium thermus aquaticus) are added to the (c)DNA of the sample. To stabilize the enzyme, MgCl₂ is added to the reaction mixture. To avoid mix-ups and pipetting error, all components except for the samples are pre-mixed in a mastermix and added to the separate samples just before reaction.

Initially, the reaction mixture is heated to about 95 °C for a few minutes to completely denaturize all double strands of DNA.

The samples are then subjected to three steps of different temperatures for different periods of time: 1) Denaturation: 95 °C for about 30 seconds to one minute for complete denaturation of the double strands of the DNA. 2) Annealing: a reaction-specific annealing temperature (often about 55–60 °C) for about 30 seconds. At this temperature, the primers will anneal to the specific complement area of the target DNA strand. After the primer has annealed to its target, the Taq-polymerase will bind to the primer / target-complex and starts elongation of the template strand's complement in 3' direction. The annealing temperature depends on the primers and should be about 5 °C lower than the lowest melting temperature (= temperature where the primer denaturizes from the template) of the primer pair's two primers. If the temperature is too low, the PCR will become unspecific and multiple strands of DNA will be amplified, because the primers can bind to non-perfect complements. If the temperature is too high, the primers will not be able to anneal to anything at all, resulting in no
amplification whatsoever. 3) Elongation: the temperature is raised to about 72 °C for about 30 seconds. At this temperature, the Taq-polymerase will work most efficiently, so that (at least in theory) the target sequence is doubled at the end of the elongation step. Then, the cycle starts over with denaturation again for a specified number of times, which depend on the amount of original template DNA and have to be determined empirically. After the last cycle, the sample is again incubated at 72 °C for a few minutes of final elongation and then stored at 4° C until removed from the thermocycler.

After PCR, the amplified DNA can be examined by applying GE or stored at -20 °C over a long period of time.

When amplifying cDNA obtained through RT, the complete procedure is consequently called RT-PCR. When performing RT-PCR, it is essential to keep the RNA and subsequent cDNA free from contaminating genomic DNA, which would cover up the presence or lack of cDNA obtained through RT. One way of ensuring that only cDNA is amplified is the use of specially designed primer pairs, where both primers lie on two different adjoining exons (exon spanning primers). When using such primers, only the amplicon from cDNA templates will be of the correct size, because the introns, which are present in the genomic DNA, will be spliced out in the mRNA and thus the respective cDNA.

Because interpretation of GE can lead to false identifications of the bands (in case of unspecific amplification, amplicons can appear that seem to be at the same length as the target of interest), there are ways of ensuring an observed band's identity.

One possible method is the use of a so called “nested” PCR, where two PCRs are performed consecutively: the primer pairs for both PCRs lie, as the name suggests, nested in each other. The “outer” primers amplify their specific product. After this “outer” reaction is complete, a small aliquot of the reaction mixture is re-amplified using the “inner” primers, but with much fewer cycles. Because the target of the inner primers is the amplicon from the “outer” PCR, it should be greatly enriched if the amplification was specific. Therefore the “inner” PCR will result in a smaller product of high quantity. If both reactions were specific, GE of the “inner” product will show a band at a shorter length than GE of the “outer” product. If both products are of the expected size, it is highly probable that the amplification was specific. Another advantage of the nested PCR is that PCR products with very low yield can be amplified to a much greater extent, making bands visible that were not or hardly before [144].

Other ways to ensure specificity of a PCR is sequencing the respective PCR product (see 4.8) or restriction enzyme digestion (not performed in this thesis).
To ensure correct interpretation of the results from a PCR experiment, there are three types of control samples that can be included in every PCR reaction batch. The first type is a blind sample, containing no sample, but only PCR mastermix and water, to exclude that any observed bands derive from contamination of the reagents. This type of control should be included every time. The second type is the -RT sample, containing non-reversely transcribed RNA. This control sample is best prepared by performing an RT reaction with all components except the enzyme reverse transcriptase. It should be included in methods using intra-exon primers, to exclude amplification of genomic DNA and can be included in other methods as well. The third type of control sample is a positive control, which contains cDNA of the desired target in high and reliable concentration. This is very helpful if there is none or very little template in the samples of interest, so that one can ensure the success of the PCR.

4.6.2 General procedure

Materials

- -20 °C storage box (for the Taq-polymerase)
- 10 mM dNTP Mix (Fermentas, R0192)
- Box with ice
- DNAse, RNAse, DNA free distilled water, “PCR water” (Gibco, 0977035)
- DNAse, RNAse, DNA free pipette tips
  - 10 µL (Biozym, 770010)
  - 20 µL (Peqlab, 81-1020)
  - 100 µL (ART®, 100E, 2065E)
  - 200 µL (Biozym, 770280)
- DNAse, RNAse, DNA free tubes with convex lid 0.2 mL (Biozym, 710900)
- Forward and reverse primer (see 4.6.3)
- Latex gloves (Hartmann, Peha-Soft® powderfree, 942162)
- Taq-Polymerase (Fermentas, Taq DNA Polymerase (recombinant), EP0404), supplied with:
  - 10x PCR buffer (Taq Buffer with KCl or Taq Buffer with (NH₄)₂SO₄)
  - 25 mM MgCl₂
- Thermal cycler (Eppendorf Mastercycler personal, 53332 000.014)
- Vortexer (Labinco, L24)
Method

1. Preparation of PCR 2x mastermix (per reaction)
   a. Let the components thaw slowly on ice, do not take the enzymes out of the refrigerator yet!
   b. Calculate the necessary amounts of reagent according to the number of samples plus one aliquot in excess
   c. Prepare the PCR 2x mastermix (add the enzymes last, take them out of the refrigerator only in the -20 °C storage box and put them back in the refrigerator as soon as possible!). See 4.6.3 for the mastermix recipes.
   d. Vortex the PCR 2x mastermix for a short while and put it on ice

2. Preparation of the samples
   a. Pipette an aliquot of cDNA sample (containing the desired amount of corresponding RNA)
   b. Add PCR water to the desired total volume
   c. Prepare a blind sample (PCR water only)
   d. Add the appropriate amount of PCR 2x mastermix and pipette up and down a few times to mix
   e. Store the prepared sample on ice
   f. Repeat steps a–e for all samples (prepare only one blind!)

3. Polymerase chain reaction
   a. Put all the samples in the thermal cycler
   b. Use the appropriate temperature program for the experiment (see 4.6.3)
   c. Run the program
   d. After the program is finished, store the PCR products at -20 °C

4.6.3 Primers, mastermixes and temperature programs

PCR method for MT<sub>1</sub>, MT<sub>2</sub> and β-actin according to Ishii et al. [26]

The sequences of the primers, the respective mastermix recipe and temperature program were taken from Ishii et al. [26]. These methods were originally supposed to suffice for all RT-PCR experiments in the course of this work. The primers are designed as exon spanning and therefore amplification of genomic DNA should be excluded. These primer pairs will be henceforth referred to as “Ishii primers”.

Primers were custom synthesized by Eurofins, MWG operon (Ebersberg, Germany)
4 Materials and methods

Primers

Table 3: PCR primers according to Ishii et al. [26].

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat MT1 fwd.</td>
<td>5’-ATGGCCCTGGCTGTGCTGCGTAAG-3’</td>
<td>316 bp</td>
</tr>
<tr>
<td>rat MT1 rev.</td>
<td>5’-TAAGTATAGACGTCAGCGCAAAGGAAATG-3’</td>
<td></td>
</tr>
<tr>
<td>rat MT2 fwd.</td>
<td>5’-GGAGCGCCCCCAAACCGAGT-3’</td>
<td>390 bp</td>
</tr>
<tr>
<td>rat MT2 rev.</td>
<td>5’-GGATCTCCCCAAAGCTACCCACCCT-3’</td>
<td></td>
</tr>
<tr>
<td>rat β-actin fwd.</td>
<td>5’-GTCCACACCGCCACCTCGG-3’</td>
<td>496 bp</td>
</tr>
<tr>
<td>rat β-actin rev.</td>
<td>5’-CTTCCCGGTCCATCATCAAT-3’</td>
<td></td>
</tr>
</tbody>
</table>

Mastermix recipe

25 µL reactions are used in this method, therefore, 12.5 µL mastermix are prepared per sample. All three primer pairs are used with the same temperature program and mastermix recipes.

Table 4: Recipe for 12.5 µL of 2x PCR mastermix for PCR experiments according to the method described by Ishii et al. [26].

<table>
<thead>
<tr>
<th>C</th>
<th>Vol [µL]</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 mM</td>
<td>0.5</td>
<td>10 mM dNTP mix</td>
</tr>
<tr>
<td>0.2 µM</td>
<td>0.05</td>
<td>forward primer</td>
</tr>
<tr>
<td>0.2 µM</td>
<td>0.05</td>
<td>reverse primer</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>10x buffer with KCl</td>
</tr>
<tr>
<td>1.5 mM</td>
<td>1.5</td>
<td>25 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>6.9</td>
<td>PCR water</td>
</tr>
<tr>
<td>1 U</td>
<td>1.0</td>
<td>Taq-Polymerase</td>
</tr>
</tbody>
</table>
**Modified mastermix recipe for MT₂**

This mastermix recipe was only used in one experiment (see 5.1.2).

**Table 5:** Modified recipe for 12.5 µL of 2x PCR mastermix for one PCR experiment concerning MT₂ according to the method described by Ishii et al. [26].

<table>
<thead>
<tr>
<th>C</th>
<th>Vol [µL]</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 mM</td>
<td>0.5</td>
<td>10 mM dNTP mix</td>
</tr>
<tr>
<td>0.2 µM</td>
<td>0.05</td>
<td>forward primer</td>
</tr>
<tr>
<td>0.2 µM</td>
<td>0.05</td>
<td>reverse primer</td>
</tr>
<tr>
<td>2.5</td>
<td></td>
<td>10x buffer with (NH₄)₂SO₄</td>
</tr>
<tr>
<td>2.0 mM</td>
<td>2.0</td>
<td>25 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>6.4</td>
<td>PCR water</td>
</tr>
<tr>
<td>1 U</td>
<td>1.0</td>
<td>Taq-Polymerase</td>
</tr>
</tbody>
</table>

**Temperature program**

**Table 6:** Temperature program for PCR experiments according to the method described by Ishii et al. [26]. This temperature program was used for β-actin, MT₁ and MT₂ in initial experiments, but was for MT₂ replaced by the one described in below because of unsatisfactory results (see 5.1.2).

<table>
<thead>
<tr>
<th>Step</th>
<th>Cycles</th>
<th>Time</th>
<th>T [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2 min</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>β-actin: 25</td>
<td>30 s</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>MT₁: 40</td>
<td>20 s</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>MT₂: 40</td>
<td>30 s</td>
<td>72</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>5 min</td>
<td>72</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>Hold</td>
<td>4</td>
</tr>
</tbody>
</table>
4 Materials and methods

**Modified temperature program for MT$_2$**

*Table 7: Modified temperature program for PCR experiments according to the method described by Ishii et al. [26].*

<table>
<thead>
<tr>
<th>Step</th>
<th>Cycles</th>
<th>Time</th>
<th>T [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2 min</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>30 s</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>20 s</td>
<td>57</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>30 s</td>
<td>72</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>5 min</td>
<td>72</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>Hold</td>
<td>4</td>
</tr>
</tbody>
</table>

**PCR method for MT$_2$ (modified after Sugden et al. [34])**

The sequences of the primers, the respective mastermix recipe and temperature program were taken from [34]. The primers are designed for a nested PCR and as both pairs lie in one exon, amplification of contaminating genomic DNA is possible. The primer pairs will be henceforth referred to as “Sugden primers” in general or as “Sug out MT2” for the outer primers of the nested PCR and “Sug nes MT2” for the inner primers of the nested PCR. See 5.4.3 for the primer selection process. Primers were custom synthesized by Eurofins, MWG operon (Ebersberg, Germany).

**Primers**

*Table 8: Primers for experiments conducted according to the method described by Sugden et al. [34].*

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sug out MT2 fwd.</td>
<td>5’-ATCTGTACAGTGGCACCTACC-3’</td>
<td>292 bp</td>
</tr>
<tr>
<td>Sug out MT2 rev.</td>
<td>5’-TTCTCTCAGCTTTGGCTTCAC-3’</td>
<td></td>
</tr>
<tr>
<td>Sug nes MT2 fwd.</td>
<td>5’-AGCCTCATCTGGCTTCAC-3’</td>
<td>154 bp</td>
</tr>
<tr>
<td>Sug nes MT2 rev</td>
<td>5’-TGGAAGAGGAAGTGATG-3’</td>
<td></td>
</tr>
</tbody>
</table>
Mastermix recipe

20 µL reactions are used in this method, therefore, 10 µL mastermix are prepared per sample. Both primer pairs are used with the same temperature program and mastermix recipes.

**Table 9:** Recipe for 10 µL of 2x PCR mastermix for PCR experiments according to the method described by Sugden et al. [34]

<table>
<thead>
<tr>
<th>C</th>
<th>Vol [µL]</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mM</td>
<td>0.2</td>
<td>10 mM dNTP mix</td>
</tr>
<tr>
<td>0.5 µM</td>
<td>0.1</td>
<td>forward primer</td>
</tr>
<tr>
<td>0.5 µM</td>
<td>0.1</td>
<td>reverse primer</td>
</tr>
<tr>
<td>2.0</td>
<td>2.0</td>
<td>10x buffer with KCl</td>
</tr>
<tr>
<td>1.5 mM</td>
<td>1.2</td>
<td>25 mM MgCl₂</td>
</tr>
<tr>
<td>5.4</td>
<td>5.4</td>
<td>PCR water</td>
</tr>
<tr>
<td>1 U</td>
<td>1.0</td>
<td>Taq-Polymerase</td>
</tr>
</tbody>
</table>

**Temperature program**

**Table 10:** Temperature program for PCR experiments according to the method described by Sugden et al. [34]

<table>
<thead>
<tr>
<th>Step</th>
<th>Cycles</th>
<th>Time</th>
<th>T [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2 min</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1 min</td>
<td>94</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1 min</td>
<td>55</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>2 min</td>
<td>72</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>10 min</td>
<td>72</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>Hold</td>
<td>4</td>
</tr>
</tbody>
</table>

**PCR method for MT₂ (self-designed)**

See 5.4.5 for the primer design process. The primer pairs will be henceforth referred to as MS1_rMT2 and MS2_rMT2. Both custom designed primer pairs share the same reverse primer. Primers were custom synthesized by Eurofins, MWG operon (Ebersberg, Germany). The mastermix recipe and temperature program were both custom designed, following the recommendations in the manufacturer’s instructions of the Taq-Polymerase [143].
4 Materials and methods

Primers

*Table 11:* Self designed primers for rat MT2 amplification. MS1_rMT2 rev. and MS2_rMT2 rev. are identical.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS1_rMT2 fwd.</td>
<td>5’- TAGCACTTGCTGGGCGGGGA-3’</td>
<td>540 bp</td>
</tr>
<tr>
<td>MS1_rMT2 rev.</td>
<td>5’- AGGCTCGGTGGTAGGTCGCA-3’</td>
<td></td>
</tr>
<tr>
<td>MS2_rMT2 fwd.</td>
<td>5’- GCCCATCTATCCCGGGCTGC-3’</td>
<td>483 bp</td>
</tr>
<tr>
<td>MS1_rMT2 rev.</td>
<td>5’- AGGCTCGGTGGTAGGTCGCA-3’</td>
<td></td>
</tr>
</tbody>
</table>

Mastermix recipe

*Table 12:* Self-designed recipe for 12.5 µL of 2x PCR mastermix for use with the self designed primers for rat MT2.

<table>
<thead>
<tr>
<th>C</th>
<th>Vol [µL]</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 mM</td>
<td>0.5</td>
<td>10 mM dNTP mix</td>
</tr>
<tr>
<td>0.2 µM</td>
<td>0.05</td>
<td>forward primer</td>
</tr>
<tr>
<td>0.2 µM</td>
<td>0.05</td>
<td>reverse primer</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>10x buffer with KCl</td>
</tr>
<tr>
<td>1.5 mM</td>
<td>1.5</td>
<td>25 mM MgCl2</td>
</tr>
<tr>
<td></td>
<td>6.9</td>
<td>PCR water</td>
</tr>
<tr>
<td>1 U</td>
<td>1.0</td>
<td>Taq-Polymerase</td>
</tr>
</tbody>
</table>

Temperature program

*Table 13:* Self-designed temperature program for use with the self-designed primers for rat MT2.

<table>
<thead>
<tr>
<th>Step</th>
<th>Cycles</th>
<th>Time</th>
<th>T [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>3 min</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>1 min</td>
<td>57</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1 min</td>
<td>72</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>10 min</td>
<td>72</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>Hold</td>
<td>4</td>
</tr>
</tbody>
</table>
4.7 DNAse treatment of RNA samples

4.7.1 Background

To avoid amplification of genomic DNA when using non exon spanning primers in PCR, the RNA is treated before the RT with DNase, which should degrade present DNA contaminations in the isolated RNA samples but leaving the RNA intact.

4.7.2 Materials

- -20 °C storage box (for DNase)
- DNAse I, Amplification Grade (Invitrogen, 18068-015), supplied with:
  - 10x DNAse I Reaction buffer
  - 25 mM EDTA (pH 8.0)
  - DNAse I, Amp Grade, 1 U / µL
- DNAse, RNAse, DNA free distilled water, “PCR water” (Gibco, 0977035)
- DNAse, RNAse, DNA free pipette tips
  - 10 µL (Biozym, 770010)
- DNAse, RNAse, DNA free tubes with flat lid 0.2 mL (Biozym, 710920)
- Latex gloves (Hartmann, Peha-Soft® powderfree, 942162)
- Thermal cycler (Eppendorf mastercycler personal, 53332 000.014)

4.7.3 Method

According to manufacturer’s instruction [145].

1. Pipette an aliquot of RNA sample in a 0.2 mL tube containing 1 µg of RNA
2. Add 1 µL of DNAse I reaction buffer
3. Add 1 µL DNAse I, Amp Grade, 1 U / µL
4. Fill with PCR water to 10 µL
5. Incubate for 15 minutes at rt
6. Add 1 µL of 25 mM EDTA solution
7. Heat for 10 minutes at 65 °C (thermocycler)
8. Add the RT mastermix (minus 1 µL of PCR water per sample) for direct RT or store at -20 °C

Note that the total volume of the DNAse treated samples is 11 µL. The volume of PCR water in the RT-mastermix has therefore to be reduced by 1 µL (from 3.2 to 2.2 µL per reaction). See 4.5 for information about reverse transcription.
4.8 DNA extraction and purification from agarose gels

4.8.1 Background

After [146]. The length of the PCR product alone is not enough to safely determine the identity of observed bands from a PCR experiment, because a different fragment of similar size might be mistaken for the expected one. Therefore, in addition to performing a nested PCR, sequencing of the isolated amplicon is a safe way to ensure its identity.

In order to sequence an isolated DNA band from an agarose gel, the band has to be excised from the gel, the gel has to be dissolved and the DNA extracted from the mixture, using a DNA gel extraction kit (QIAquick®, Gel Extraction Kit).

The principle of the kit’s method of operation is a centrifugeable column filled with a silica membrane, which adsorbs the DNA, but not impurities and contaminations. The gel slice is first dissolved in lysis buffer, which contains a pH indicator to enable easy checking of the optimal pH (< 7.5). If the pH is too basic, the DNA will not adsorb on the silica membrane. Residual buffer and impurities are then washed away using washing buffer. Because adsorption of the DNA to the silica matrix occurs only at acidic pH, the DNA can be eluted from the column using pure water or 5 mM Tris.HCl (pH 8.5) buffer.

To ensure the success of the extraction, the concentration of the DNA is quantified using spectrophotometry (see 4.3) and a small amount of the DNA solution can be checked for intactness via GE (see 4.4).

4.8.2 Materials

- Centrifuge (Eppendorf, Centrifuge 5417R)
- DNase, RNAse, DNA free distilled water, “PCR water” (Gibco, 0977035)
- DNase, RNAse, DNA free pipette tips
  - 100 µL (ART®, 100E, 2065E)
- DNase, RNAse, DNA free tubes 1.5 mL (Biozym, 710310)
- Heating block (Eppendorf, Thermomixer 5436)
- Isopropanol (Merck, 1.09634.1011)
- Latex gloves (Hartmann, Peha-Soft® powderfree, 942162)
- Pipette tips
  - 1000 µL (Biozym, 721000)
  - 200 µL (Biozym, 720230)
- QIAquick® Gel Extraction Kit (Qiagen, 28704), contains:
  - Buffer EB
Materials and methods

- Buffer PE
- Buffer QG
- Spin columns + 2 mL collection tubes
- Na-acetate (Merck, 6268)
- Vortexer (Labinco, L24)

4.8.3 Method

1. Excise the DNA fragment(s) from the agarose gel (minimize excess gel)
2. Weigh the gel slice(s) (approx.)
3. Add 3 volumes of Buffer QG to per volume of gel (100 µg equals 100 µL)
4. Note: If the concentration of the agarose gel is 2% or higher, use 6 volumes of buffer QG
5. Note: One spin column can take up to 400 mg of gel. If the weight of the gel slice(s) exceed(s) 400 mg, divide it in appropriate parts and use multiple spin columns
6. Incubate at 50 °C for 10 min and vortex every 2–3 min until the gel slice(s) is (are) dissolved
7. If the color of the mixture is orange or red, add 10 µL of 3 M Na-acetate (pH 5.0)
8. Add 1 gel volume of isopropanol to the sample and vortex
9. Place a QIAquick® spin column in a 2 mL collection tube
10. Apply the sample to the column and centrifuge for 1 min at 17,900 x g
11. If the volume of the sample exceeds 800 µL, centrifuge twice using the same column
12. Discard the flow-through and place the column back in the collection tube
13. Add 0.5 mL of Buffer QG to the column and centrifuge for 1 min at 17,900 x g
14. Discard the flow-through and place the column back in the collection tube
15. Add 0.75 mL of Buffer PE to the column and centrifuge for 1 min at 17,900 x g
16. Discard the flow-through and centrifuge again for 1 minute at 17,900 x g
17. Place the column in a DNAse, RNAse, DNA free 1.5 mL tube (cut off cap!)
18. Add 50 µL of Buffer EB or PCR water to the center of the column membrane and centrifuge for 1 minute at 17,900 x g
19. Note: alternatively, 30 µL might be used – the column should then stand for 1 minute prior to centrifugation
20. Transfer the eluate (with a DNAse, RNAse, DNA free 100 µL pipette tip) from the tube with the cut off cap to a fresh DNAse, RNAse, DNA free tube.
21. Store at -20 °C.
4.9 DNA Sequencing and sequence comparison

After extraction and purification from agarose gels (see 4.8) the DNA is sent to MWG Eurofins Operon (Ebersberg, Germany) for sequencing. The sequencing results are then analyzed by the BLASTn program from the National Center for Biotechnology Information [147-148].

4.10 Real time PCR (qPCR)

4.10.1 Background

After [149].

Real-time PCR, abbreviated as “qPCR” (for “quantitative PCR”) to avoid mix-ups with RT-PCR, is a technique to quantify the amount of amplicon accumulated during the PCR process. Therefore, the amplification can be tracked “live” (thus the name “real-time”), in contrast to qualitative PCR, where the result of the amplification can be analyzed only at the end of the complete reaction. Also, real-time PCR is much more sensitive, because the increase in amplicon is measured through increasing fluorescence by the machine and not through manual optical inspection of a gel, as in conventional qualitative PCR.

With real-time PCR the concentration of the template can be measured as absolute (as copies of the template) or relative (compared to a standard gene). Real time PCR can of course also be used in conjecture with reverse transcription (RT-qPCR).

As mentioned before, the amplification is detected through the increase in fluorescence of the sample. This is achieved through one of the following two methods: One possibility is the exploitation of unspecific intercalation of a fluorescent dye with the DNA (e.g. SYBR® green I). The second option is the usage of sequence specific DNA probes (e.g. Taq-Man®) which will bind to the DNA and release a fluorescent dye (reporter) from the vicinity of a quencher after the polymerase (exhibiting exonuclease activity) “cleaves” the probe off (see Figure 16). This increase in fluorescence is then measured by photodiodes in the real-time PCR machine. Because of the sensitivity of the method, the components of the reaction mixture have to be very carefully balanced, so normally pre-fabricated mastermixes and primer kits, as well as standardized reaction plates, are used.
4.10.2 Materials

- Box with ice
- Centrifuge (Sigma 4K15C, 10742)
- DNase, RNase, DNA free distilled water, “PCR water” (Gibco, 0977035)
- DNase, RNase, DNA free pipette tips
  - 10 µL (Biozym, 770010)
  - 100 µL (ART®, 100E, 2065E)
  - 20 µL (Peqlab, 81-1020)
- DNase, RNase, DNA free tubes with flat lid 0.2 mL (Biozym, 710920)
- Fast 96-well reaction plate 0.1 mL (Applied Biosystems, 346907)
- Latex gloves (Hartmann, Peha-Soft® powderfree, 942162)
- MicroAmp™ Optical Adhesive Film (Applied Biosystems, 4311971)
- StepOnePlus™ Real Time PCR System with Tower Computer (Applied Biosystems, 4376599)
- Taq-Man® Gene Expression Assay(s) (20x Mix)
- Taq-Man® Gene Expression Master Mix (Applied Biosystems, 4369016)
- Vortexer (Labinco, L24)
4 Materials and methods

4.10.3 Method

20 µL reactions were used in this thesis. For other volumes (10–50 µL are possible), the respective volumes of the reaction components have to be adjusted accordingly.

Mastermix

*Table 14: Mastermix recipe for 20 µL qPCR reactions (after [150]).*

<table>
<thead>
<tr>
<th>Vol [µL]</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Taq-Man® 2x Master Mix</td>
</tr>
<tr>
<td>1</td>
<td>Taq-Man® Assay 20x</td>
</tr>
<tr>
<td>4</td>
<td>PCR water</td>
</tr>
</tbody>
</table>

Gene expression assays

*Table 15: Gene expression assays used in qPCR experiments in this thesis. VIC: Taq-Man® VIC; FAM: 6-Carboxyfluorescein.*

<table>
<thead>
<tr>
<th>Target</th>
<th>Assay</th>
<th>Reporter</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB (β-actin)</td>
<td>Rat ACTB (Applied Biosystems, 4352340E-0805007)</td>
<td>VIC</td>
</tr>
<tr>
<td>MT₁</td>
<td>Rat Mtnr1a (Applied Biosystems 626218 G10)</td>
<td>FAM</td>
</tr>
<tr>
<td>MT₂</td>
<td>Rat Mtnr1b (Applied Biosystems 626217 G5)</td>
<td>FAM</td>
</tr>
</tbody>
</table>

Instructions

For detailed instructions consult the manufacturer’s manual [151].

1. Theoretical preparation
   a. Select the samples, targets of interest and number of repetitions.
      Include negative (-RT) and blind samples (water only) as well.
   b. Prepare a clearly laid out plate layout
   c. Calculate the amount of cDNA necessary for the sum of all respective wells of all targets. Add one aliquot of cDNA to compensate for pipetting errors.

2. Practical preparation (work on ice)
   a. Prepare dilutions of the samples (the aspired amount of cDNA per sample should be contained in 5 µL of PCR water)
   b. Prepare the mastermixes for all targets; always add 1–2 aliquots to compensate for pipetting error
c. Pipette all diluted samples (and blinds / negatives) in their respective wells (use a fresh tip for each well to ensure volume conformity) in a 96 well reaction plate

d. Add the mastermixes to the respective wells

e. Seal the reaction plate with the optical adhesive film

f. Remove the opaque cover foil from the back of the film

g. Place the film centered on the plate

h. Use a scraper to flatten the film

i. Remove the holding flaps of the film by holding down the film on the plate with the scraper with one hand and pulling on the flap with the other hand

j. Make sure that the seal between plate and film is tight

k. Centrifuge the plate at 2000 x g for 1 minute

l. Remove dust from the bottom of the plate by blowing or a brush

3. Real time PCR

a. Insert the plate into the instrument and close the hatch

b. Start the program “StepOne Software v2.0”

c. Click on “Advanced Setup”

d. Choose “Standard (~2 hours to complete a run)” under “rampspeed”

e. Choose “Quantitation – Comparative CT (ΔΔCT)”

f. Name the experiment

g. Go to “Plate Setup”

h. Name all targets and choose the appropriate reporter (specified in the assays data sheet)

i. Name all samples

j. Click on the tab “Assign Targets and Samples”

k. Select all wells of one target and activate the checkbox next to the respective target name

l. Select the blind samples of the target and click on “N” next to the sample name

m. Repeat steps k–l for all targets

n. Select all wells of a sample and activate the checkbox next to the respective sample name

o. Repeat step n for all samples

p. Select the reference sample and the endogenous control

q. Go to “Run Method”

r. Set the reaction volume per well to 20 µL
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4. Click on “START Run”

5. Wait for the instrument to complete the run

6. After completion of the run, take the plate out of the instrument and close the hatch again

4. Subsequent work after the run is completed

a. Select “Analysis”

b. Go to “Amplification Plot”

c. Select “Graph Type: Log”

d. Check “Show threshold”

e. Select a target

f. Uncheck “Auto” and set the threshold within the exponential phase of the amplification (drag the line with the mouse)

g. Repeat steps e–f for all targets

h. Click on “Export” and save the data to an appropriate location (e.g. USB-stick)

i. Close the program, when asked to save the unsaved data, click “yes” and save at an appropriate location

4.10.4 Analysis of results

After [149, 151]. The amount of quantified DNA can be expressed in multiple ways. One of the most common ones is the $\Delta$CT value. The CT (cycle threshold) value is the number of PCR cycles necessary for a template in a sample to become detectable (so the fluorescence of the sample exceeds the threshold of the background fluorescence). The $\Delta$CT value then specifies the difference between the CT of the template in question and a reference gene (e.g. beta actin) which is expressed equally in all the samples. Since the relative ratio of the standard and the template in question does not change, this difference (the $\Delta$CT value) is used to “normalize” the CT values and account for discrepancies in pipetting, sample concentration, etc. The $\Delta$CT values can be converted from base 2 to base 10 using the formula from Figure 17.

$$E_n = 2^{-\Delta CT}$$

Figure 17: Conversion of the expression difference of a target to a the reference gene from the base 2 $\Delta$CT values to the n-fold ($E_n$; base 10) value.

The $\Delta$CT can then again be measured against other $\Delta$CT values, e.g. a placebo group against a verum group. This value is then called $\Delta\Delta$CT and specifies the
difference in gene (or mRNA in case of qRT-PCR) expression between the two groups. This difference can then be converted from a base 2 system (CT) to a base 10 system using the formula from Figure 18.

\[
E_n = 2^{-\Delta\Delta CT}
\]

**Figure 18**: Conversion of the expression difference of two targets from the base 2 $\Delta\Delta CT$ values to the $n$-fold ($E_n$; base 10) value.

Of course, this simple conversion works only if the expression efficiency of the amplification reaction is (near) 100 % and the same for all targets. For short amplicon lengths (< 150 bp, true for all TAQMan® Assays), these requirements are met (Applied Biosystems, User Bulletin #2: Relative Quantitation of Gene Expression (Applied Biosystems Inc., Part Number 4303859B)).

A standard sample (of all measured samples) can be chosen to act as a reference. The expression of various samples can then be indicated as $E_n$-fold expression of the reference.

All samples should be measured at least in duplicates (better are triplicates or even quadruplicates) to increase the accuracy of the method. The $\Delta CT$ values are then indicated as mean $\Delta CT$ of the repeats. To ensure primer specificity and correct amplification, -RT and negative samples should be included for all template groups.

### 4.11 Immunofluorescence (IF)

#### 4.11.1 Background

IF is a method used to visualize the location or occurrence of proteins in tissues or cells by use of fluorescence microscopy.

Staining of tissue sections is done in two steps: first, the primary antibody is applied to the tissue. The primary AB recognizes specific antigens (e.g. smooth muscle actin or the melatonin MT₁ receptor) and binds to them. It is created in a host species and can be either poly- or monoclonal. After the primary antibody has bound to its target, a secondary antibody is applied, which recognizes the primary antibody based on the primary AB’s host species. This secondary antibody is conjugated with a fluorescent dye, which glows during excitation with light of a specific frequency.

To exclude that the observed fluorescence stems from unspecific binding of the secondary antibody to the tissue, a negative control, with only the secondary antibody applied, should be included in each experiment.
Before the staining, the tissue has to be fixated. This can be done using a variety of methods; examples are the usage of formaldehyde (PFA) or the new HOPE®-solution (DCS, Hamburg, Germany). For sectioning, the tissue needs to be either frozen (in liquid N\textsubscript{2}) or embedded in paraffin. Accordingly, the tissue is then either cryo-sectioned or paraffin sectioned. Afterwards different steps are necessary to prepare it for staining depending on the method used for sectioning. In case of a cryo-sections the tissue only needs to be rehydrated (using PBS) and then bathed in an ammonium-chloride (50 mM) solution to remove any excess PFA remaining in the tissue. With paraffin sections the procedure is more complex, because the paraffin has to be removed and the rehydration has to be done using a descending alcohol series. HOPE®-fixated paraffin sections are somewhat easier to rehydrate, as only two isopropanol and acetone steps are necessary. Also, if a tissue was embedded in paraffin, normally antigen retrieval has to be done, because paraffin in conjecture with PFA can mask antigens. This is done by boiling the sections in citrate buffer, citraconic anhydride, or by using proteases.

After rehydration, the tissue has to be permeabilized to ensure that the primary antibody can “reach” its target and is not hindered by membranes, etc. Also, unspecific binding sites have to be blocked using blocking serum (serum from the animal the second antibody was derived from, or, if this is not possible, with a protein dissolved in buffer such as bovine serum albumin (BSA)) to reduce the background fluorescence. These two steps are normally done at the same time by incubating the tissue with serum containing small amounts of a detergent (Saponin, Triton-X 100 or Tween 20).

If more than one protein, or if the relative positions of two proteins in a tissue, are of interest, double, triple, or even quadruple stainings are possible. The primary antibodies against the different proteins have to be derived from different species, of course. This can be achieved using two different approaches, which both have specific advantages and disadvantages.

The first approach is parallel staining, where the primary and secondary antibodies, respectively, are mixed and applied together. The advantage is that the amount of time for the procedure is the same as for a single staining and that all primary antibodies can be applied overnight, so staining should be strong for all targets. However, the primary antibodies (and secondary antibodies, respectively) may influence each other if they are dissolved in the same solution.

The second approach is sequential staining, where the staining procedure is repeated for each primary / secondary AB combination. The advantage is that the antibodies have less chance of influencing each other and the stainings are usually more reliable. The disadvantage is a proportional increase in time necessary for each
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Further target, since all washing and incubation steps have to be repeated for each antibody duo.

After the staining procedure with all the antibodies is completed, the tissue is usually dyed for nucleic acids, therefore making the nuclei of the cells visible. This is done with chemicals which intercalate in DNA, which results in fluorescence. An example for such a “nucleic acid dye” is 4’,6-diamidino-2-phenylindole (DAPI).

When all dyeing steps are completed, the tissue is embedded in a mounting medium which is pervious for a broad range of light frequencies, ideally for the whole UV/VIS/IR spectrum. An example for such a medium is Mowiol.

4.11.2 Tissue sectioning

General remarks

For IF staining, thin sections of tissue (1–15 µm) are necessary. This can be done by cryo-sectioning, where the tissue is cut in a frozen state. The tissue is embedded in TissueTek™, a substance based on polyvinyl alcohol, which is liquid at rt, but freezes to a solid, sliceable mass at low temperatures. All tissues cut and used for cryo-sectioning in this work were fixated and frozen in TissueTek™ in 2008 by M. Benova (Institute for Pathophysiology, Medical University Vienna). Therefore, the method for fixating the tissue prior to initial freezing will not be discussed in this work.

Alternatively, paraffin-sections, where the tissue is embedded in paraffin and cut at rt can be used. All paraffin sections used for IF experiments in this work were fixated, embedded and cut in 2010 by H. Uhrova (Institute of Pathophysiology and Allergy Research, Medical University Vienna). Therefore, the methods for paraffin embedding and sectioning will not be discussed in this work.

Cryo-sectioning

Materials

- Acetone (Merck, 1.00014.2500)
- Cryomicrotome (Microm, 500 O-M)
- Cryomicrotome blade (Microm Sec 35, 152200)
- Latex gloves (Meditrade Gentle Skin sensitive, 1221)
- Paper towels
- Positively precharged slides (Thermo Scientific Superfrost® Plus Gold, K5800AMNZ)
- Scalpel, brush, forceps
- TissueTek™ (Sakura, 4583)
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Method

1. Switch on the microtome by activating “Light”, “Motor”, and “Object”
2. Mount the TissueTek™ embedded tissue on one of the stamps in the instrument by applying TissueTek™ on the stamp, letting it solidify for a moment, then adding the embedded tissue
3. Check the orientation of the tissue, correct by thawing and reorientation if necessary
4. Let the tissue with the TissueTek™ freeze completely inside the microtome
5. Apply more TissueTek™ on the stamp so that a large area around the embedded tissue is covered with TissueTek™; avoid protruding parts of the tissue
6. Repeat steps 3–5 for all samples
7. Insert a microtome blade in the blade holder
8. Insert a stamp with embedded tissue in the stamp-holder and fasten the holding screw
9. Adjust the angle of the blade and / or the stamp, if necessary
10. Move the stamp away from the blade by using the [↑] button
11. Set the section thickness to 40 µm
12. Use the motor to approach the blade with the section
13. Slice off TissueTek™ at 40 µm until the tissue is cut together with the TissueTek™
14. Set the section thickness to 1–15 µM (7 µM for aorta sections)
15. Slice off a few sections to adapt the microtome to the new thickness
16. Carefully cut a section off using the hand-wheel (clockwise) – while the section is being cut, gently pull the section away from the blade using a brush; try to keep a flat, undisrupted section
17. Lower a positively precharged slide parallel over the section – at about 10–5 mm, the section will be “sucked” onto the slide
18. Keep the slide with the tissue at rt for about 10 min
19. Store the slide at -20 °C
20. Repeat steps 16–19 for all sections of the sample
21. Clean the blade every few sections with acetone
22. Move the stamp away from the blade by using the [↑] button and remove the stamp after releasing the holding screw
23. Remove the remaining tissue (still embedded in TissueTek™) from the stamp using the scalpel
24. Store the remaining tissue at -80°C (still embedded in TissueTek™)
25. Repeat steps 8–24 for all samples
26. Remove the microtome blade and store or discard it
27. Clean the microtome using brushes and acetone soaked towels
28. Switch off the microtome by deactivating “Light”, “Motor” and “Object”, but do not deactivate “Box”

4.11.3 Buffers, solutions and reagents

PBS (phosphate buffered saline)

Materials

- KCl (Merck, 4936.1000)
- NaCl (Merck, 1.06404.1000)
- KH₂PO₄ (Merck, 1.04873.1000)
- Na₂HPO₄·2H₂O (Merck, 1.06580.1000)
- Aqua bidest.
- Osmometer (Osmomat 030, Gonotec)
- pH-meter (Metrohm, pH-Lab 827)

Method

1. Solve in 800 mL aqua bidest
   a. 0.2 g KCl
   b. 8.01 g NaCl
   c. 0.21 g KH₂PO₄
   d. 1.44 g Na₂HPO₄·2H₂O
2. Adjust pH to 7.4 with HCl or NaOH
3. Fill to 1000 mL with aqua bidest.
4. Osmolarity should be between 0.280–0.305 osmol / kg
5. Store at 4 °C

50 mM NH₄Cl in PBS

Materials

- NH₄Cl (Merck, 1.01142.1000)
- PBS (see above)

Method

1. Dissolve 2.67 g of NH₄Cl in 1000 mL aqua bidest.
2. Store at rt
4 Materials and methods

10 mM citrate buffer

**Materials**
- Aqua bidest.
- Citric acid monohydrate (Merck, 244)
- pH-meter (Metrohm, pH-Lab 827)
- Tri-sodium citrate dihydrate (Sigma C-8532)

**Method**
1. Mix
   a. 8.5 mL of a 0.1 M solution of citric acid monohydrate:
      i. 21.06 g citric acid monohydrate
      ii. 1000 mL aqua bidest.
   b. 41.5 mL of a 0.1 M solution of tri-sodium citrate dehydrate:
      i. 29.41 g tri-sodium citrate dihydrate
      ii. 1000 mL aqua bidest.
2. Adjust to 500 mL with aqua bidest.
3. Adjust to pH 6 with solution a or b
4. Store at 4 °C

Mowiol mounting medium

**Materials**
- Centrifuge (Rotixa, Hettich)
- Centrifuge tubes 50 mL (VWR, 50 ml Super Clear® Centrifuge tubes, 525-0307)
- Disposable sterile syringe filters, 25 mm, 0.20 Micron, cellulose acetate membrane acrylic (IWAKI 2052-025)
- Glycerol 87 % (Merck 1.04094.0500)
- Hydrochloric acid, fuming 37 % (Merck 1.00314.1000)
- Magnetic hot plate stirrer
- Mowiol 4-88 (FLUKA 81381)
- NaN₃ (Sigma S-8032)
- Syringes, single use, 10 mL (B. Braun, Melsungen AG H4606108V)
- Tris (Merck 1.08382.1000)
- Tubes 1.5 mL (Biozym, 710160)
- Waterbath (GFL, 1083)
Method

1. Mix in a 50 mL tube
   a. 6 g glycerol
   b. 2.4 g Mowiol 4-88
2. Let the mixture disperse for 1 h at rt under frequent shaking
3. Add 6 mL aqua bidest.
4. Stir for 1 h at rt
5. Add 12 mL 0.2 M Tris/HCl pH 8.5 + 0.02 % (v / w) NaN₃
6. Incubate for 2 h in the waterbath at 50 °C and stir every 20 min
7. Centrifuge at 5,000 x g for 15 min
8. Filtrate through a 0.2 µm syringe filter
9. Prepare 1 mL aliquots
10. Store at -20 °C

4.11.4 Staining procedures for cryo-sections

Materials

- Aluminum foil
- Blocking serum (sera) from host animal of secondary AB(s) (5 % in PBS + 0.05 % saponin; see 4.11.6)
- Antibodies (see 4.11.6)
- Box with ice
- Coolable Centrifuge (Eppendorf, Centrifuge 5415R)
- Coverslips (Menzel-Gläser)
- DakoPen (Dako, S2002)
- DAPI (4',6-diamidino-2-phenylindole; Roche 10236276001) 1:10,000 in PBS
- Dyeing trays (plastic or glass)
- Forceps
- Latex gloves (Meditrade Gentle Skin sensitive, 1221)
- Mowiol (Roth, 07031)
- pipette tips
  - 10 µL (Biozym, 720031)
  - 200 µL (Biozym, 720230)
  - 1000 µL (Biozym, 721000)
- Solutions and buffers
  - 50 mM NH₄Cl (see 4.11.3)
  - PBS (see 4.11.3)
4 Materials and methods

- Tubes 1.5 mL (Biozym, 710160)
- Vortexer (Labconco, L24)
- Wet chamber

Notes

✓ Washing steps are performed in dyeing trays and always for 5 minutes unless otherwise stated
✓ Incubation steps are always performed in a wet chamber, the solution the tissue is incubated with is applied directly on the tissue with a pipette
✓ Always take the slides out of the trays very slowly to avoid detaching of the sections
✓ Always keep the tissue wet
✓ Antibodies (primary and secondary) are always diluted in blocking buffer (5 % animal serum of the respective secondary AB + 0.05 % saponin in PBS or 1 % BSA + 0.05 % saponin in PBS). Use the serum from the host animal of the secondary antibody.
Single-staining procedure

1. Tissue preparation (rehydration and blocking)
   a. Wash once for 10 min in 50 mM NH$_4$Cl
   b. Wash 3 times in PBS
   c. Draw a circle around the tissue(s) with the Dako-Pen
   d. Incubate with blocking buffer for 1 h at rt

2. Primary antibody
   a. Centrifuge the antibody at 14,000 x g for 5 min
   b. Dilute the antibody in blocking buffer
   c. Incubate with primary antibody in a wet chamber for 1 h–24 h
   d. Wash 5 times in PBS

3. Secondary antibody (work in the dark!)
   a. Centrifuge the antibody at 14,000 x g for 5 min
   b. Dilute the antibody in blocking buffer
   c. Incubate with secondary antibody in a wet chamber for 1 h

4. Nuclei staining
   a. Wash twice in PBS
   b. Incubate for 10 min with DAPI
   c. Wash twice in PBS
   d. Rinse once in aqua bidest. (in and out)

5. Embedding
   a. Apply an appropriate volume of Mowiol on the tissue and the slide
   b. Cover with coverslip
   c. Dry for 24 h in the dark at rt

6. Store the slides at 4 °C in the dark
Double-staining procedures

Parallel staining

1. Tissue preparation (rehydration and blocking)
   a. Wash once for 10 min in 50 mM NH₄Cl
   b. Wash 3 times in PBS
   c. Draw a circle around the tissue(s) with the Dako-Pen
   d. Incubate with blocking buffer (use 1% BSA in PBS + 0.05% saponin, if the host animals for the secondary ABs are not identical)

2. Primary antibodies
   a. Centrifuge the antibodies at 14,000 x g for 5 min
   b. Dilute the antibodies in blocking buffer (use 1% BSA in PBS + 0.05% saponin, if the host animals for the secondary ABs are not identical) and mix them
   c. Incubate with primary antibody mixture in a wet chamber for 1 h–24 h
   d. Wash 5 times in PBS

3. Secondary antibodies (from now on, work in the dark!)
   a. Centrifuge the antibodies at 14,000 x g for 5 min
   b. Dilute the antibodies in blocking buffer (use 1% BSA in PBS + 0.05% saponin, if the host animals for the secondary ABs are not identical) and mix them
   c. Incubate with secondary antibody mixture in a wet chamber for 1 h

4. Nuclei staining
   a. Wash twice in PBS
   b. Incubate for 10 min with DAPI
   c. Wash twice in PBS and rinse once in aqua bidest. (in and out)

5. Embedding
   a. Apply an appropriate volume of Mowiol on the tissue and the slide
   b. Cover with coverslip and dry for 24 h in the dark at rt

6. Store the slides at 4 °C in the dark
Sequential staining

1. Tissue preparation (rehydratization and blocking)
   a. Wash once for 10 min in 50 mM NH₄Cl
   b. Wash 3 times in PBS
   c. Draw a circle around the tissue(s) with the Dako-Pen
   d. Incubate with blocking buffer (use the serum of the host animal for the first secondary antibody; if this is not possible because of cross reactions with the other antibodies, use 1% BSA in PBS + 0.05% saponin)

2. First primary antibody
   a. Centrifuge the antibody at 14,000 x g for 5 min
   b. Dilute the antibody in blocking buffer (use the serum of the host animal for the first secondary antibody; if this is not possible because of cross reactions with the other antibodies, use 1% BSA in PBS + 0.05% saponin)
   c. Incubate with primary antibody in a wet chamber for 1 h–24 h
   d. Wash 5 times in PBS

3. First secondary antibody (from now on, work in the dark!)
   a. Centrifuge the antibody at 14,000 x g for 5 min
   b. Dilute the antibody in blocking buffer (use the serum of the host animal for the second secondary antibody; if this is not possible because of cross reactions with the other antibodies, use 1% BSA in PBS + 0.05% saponin)
   c. Incubate with secondary antibody in a wet chamber for 1 h
   d. Wash 5 times in PBS

4. Second primary antibody
   a. Centrifuge the antibody at 14,000 x g for 5 min
   b. Dilute the antibody in blocking buffer (use the serum of the host animal for the first secondary antibody; if this is not possible because of cross reactions with the other antibodies, use 1% BSA in PBS + 0.05% saponin)
   c. Incubate with primary antibody in a wet chamber for 1 h–24 h
   d. Wash 5 times in PBS

5. Second secondary antibody
   a. Centrifuge the antibody at 14,000 x g for 5 min
   b. Dilute the antibody in blocking serum
   c. Incubate with secondary antibody in a wet chamber for 1 h
4 Materials and methods

6. Nuclei staining
   a. Wash twice in PBS
   b. Incubate for 10 min with DAPI
   c. Wash twice in PBS
   d. Rinse once in aqua bidest. (in and out)

7. Embedding
   a. Apply an appropriate volume of Mowiol on the tissue and the slide
   b. Cover with coverslip
   c. Dry for 24 h in the dark at rt

8. Store the slides at 4 °C in the dark

4.11.5 Staining procedures for HOPE®-fixated paraffin sections

Materials
- 10 mM citrate buffer, pH 6 (see 4.11.3)
- Acetone (Merck, 1.00014.2500)
- Aqua bidest.
- Dyeing trays (plastic and glass)
- Isopropanol (Merck, 1.09634.1011)
- Oven (Mammert, TV15u), kept at 60 °C
- PBS (see 4.11.3)
- Refrigerator
- Steamer (Braun, MultiGourmet Type 3-216)
- Further materials: see 4.11.4

Method
1. Deparaffination
   a. Incubate the slides with the sections for 10 min in warm isopropanol (60 °C)
   b. Wash with fresh, warm isopropanol (60 °C)

2. Rehydration
   a. Incubate 2x for 10 min in 70 % cold (4 °C) acetone (keep in refrigerator)
   b. Wash 2x with aqua bidest.

3. Incubate for 5 min in aqua bidest.

4. Antigen retrieval
   a. Fill a plastic dyeing tray with 10 mM citrate buffer and put it in the steamer
   b. Pre-heat the citrate buffer for 20 min
c. Incubate the slides for 15 min in the hot citrate buffer in the steamer
d. Take the tray out of the steamer and let it cool to RT
e. Wash twice in PBS (5 min each)

5. The further procedure is identical with the ones for single staining or double staining of cryo-sections (see 4.11.4)

4.11.6 Antibodies and blocking buffers

The respective antibodies used in the experiments are given in the results chapter (5.5). As blocking buffer, always 5 % animal serum of the respective secondary AB + 0.05 % saponin in PBS is used. Santa Cruz is abbreviated as SC.

Primary antibodies

Table 16: Primary antibodies used in this thesis. All dilutions of the antibodies are given, as well as information about the origin of the respective antibody (modified after the datasheets of the respective antibodies [152-155]).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilutions</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat Anti MT1 (SC, sc-13186)</td>
<td>1:50, 1:20, 1:10</td>
<td>Affinity purified goat polyclonal antibody raised against a peptide mapping near the C-terminus of MT$_2$ of rat origin; 200 µg / mL</td>
</tr>
<tr>
<td>Rabbit Anti MT1 (Abbiotec, 250761)</td>
<td>1:50, 1:10</td>
<td>Affinity purified rabbit polyclonal antibody raised against a KLH-conjugated synthetic peptide encompassing a sequence within the center region of MT$_1$ of human origin</td>
</tr>
<tr>
<td>Rabbit Anti Pecam-1 (SC, sc-1506)</td>
<td>1:50</td>
<td>Affinity purified rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of PECAM-1 of mouse origin; 200 µg / mL</td>
</tr>
<tr>
<td>Mouse Anti SM Actin (Sigma, A2547)</td>
<td>1:1000</td>
<td>Mouse monoclonal antibody raised against a KLH-conjugated synthetic decapeptide encompassing the N-terminus of α-smooth muscle actin</td>
</tr>
</tbody>
</table>
Secondary antibodies

Table 17: Secondary antibodies used in this thesis. All dilutions of the antibodies are given, as well as information about the respective antibody (modified after the data sheets of the respective antibodies[156-158]). All secondary antibodies are mixed 1:1 with glycerol (Merck, 1.04094.0500).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey Anti Goat Alexa Fluor® 568 (Invitrogen, A11057)</td>
<td>1:1000</td>
<td>Affinity purified donkey antibodies against goat IgG heavy chains and all classes of Ig light chains, conjugated to Alexa Fluor® 568 dye; mixed 1:1 with glycerol</td>
</tr>
<tr>
<td>Donkey Anti Goat Alexa Fluor® 647 (Invitrogen, A21447)</td>
<td>1:1000</td>
<td>Affinity purified donkey antibodies against goat IgG heavy chains and all classes of Ig light chains, conjugated to Alexa Fluor® 647 dye; mixed 1:1 with glycerol</td>
</tr>
<tr>
<td>Goat Anti Mouse Alexa Fluor® 568 (Invitrogen, A21042)</td>
<td>1:1000</td>
<td>Affinity purified goat antibodies against mouse IgG heavy chains and all classes of Ig light chains, conjugated to Alexa Fluor® 568 dye; mixed 1:1 with glycerol</td>
</tr>
<tr>
<td>Goat Anti Rabbit Alexa Fluor® 568 (Invitrogen, A11011)</td>
<td>1:1000</td>
<td>Affinity purified goat antibodies against rabbit IgG heavy chains and all classes of Ig light chains, conjugated to Alexa Fluor® 568 dye; mixed 1:1 with glycerol</td>
</tr>
<tr>
<td>Goat Anti Rabbit Alexa Fluor® 647 (Invitrogen, A21244)</td>
<td>1:1000</td>
<td>Affinity purified goat antibodies against rabbit IgG heavy chains and all classes of Ig light chains, conjugated to Alexa Fluor® 647 dye; mixed 1:1 with glycerol</td>
</tr>
</tbody>
</table>

Blocking buffers

Materials

- Animal serums / BSA
  - Donkey (Jackson Immuno Research, 017-000-121)
  - Goat (Jackson Immuno Research, 005-000-121)
- PBS (see 4.11.3)
- Saponin (Sigma S-1252)
- 50 mL tubes (VWR, 50 ml Super Clear® Centrifuge tubes, 525-0307)

Method

1. Mix
   a. 25 mL animal serum
   b. 400 mL PBS
   c. 250 µL saponin
2. Adjust to 500 mL with PBS
3. Prepare 50 mL aliquots
4. Store at -20 °C
4.11.7 Photography of IF-stained sections

**Materials**

- **ZEISS Axio Imager Z1 microscope equipped with TissueFAXS, TissueGnostics GmbH (TF)**
  - **Objectives**
    - EC-Plan Neofluar 2.5 x / 0.07
    - EC-Plan Neofluar 20 x / 0.5
  - **Camara (PCO Pixelfly)**
- **Ethanol 70 % (v / v), diluted from Ethanol 96 % (v / v) (Merck, 1.00983.1000)**

**Method**

1. Clean the slides using Ethanol 70 % (v / v)
2. Insert the slides in the slide holder
3. Insert the slide holder into the stage
4. Start the TissueFaxs application
5. Acquire all areas of interest*
6. Remove the slides from the slide holder and store them at 4 °C

*To describe the process of using the TF image acquisition system in detail would go beyond the scope of this work. See the instructions of the TF for detailed information.

**Notes**

- Images are saved as PNG
- All parameters for image acquisition (exposure time, lamp intensity, thresholds, gain …) are recorded for each channel and each experiment and are stated with the respective image series in the results section.
- The tonality of all IF-images in this work have been adjusted by the same amount for better clarity in print (10 / 1.25 / 200) in Adobe® Photoshop®
- If not specified otherwise, all pictures consist of the overlay of the respective channel(s) of the secondary AB(s) and the DAPI channel
- All images are captured using the 20 x objective
4.11.8 Experiment parameters

Single stainings for SM-actin and pecam-1

Table 18: Experiment parameters for single staining of aorta sections stained for SM-actin with Alexa Fluor® 568. Blocking buffer and ABs: see 4.11.6, incubation times and dilution(s) are stated; o/n: overnight; Tex: name of the channel for acquisition of 568 stained images; LT: lower threshold (parameter to reduce background in the TF software).

<table>
<thead>
<tr>
<th>Type</th>
<th>Single staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking buffer</td>
<td>5 % goat serum + 0.05 % saponin in PBS</td>
</tr>
<tr>
<td>Primary AB</td>
<td>o/n 1:500 Mouse Anti SM Actin</td>
</tr>
<tr>
<td>Secondary AB</td>
<td>1 h 1:1000 Goat Anti Mouse Alexa Fluor® 568</td>
</tr>
<tr>
<td>Acquisition parameters</td>
<td>DAPI: 30 ms, Tex: 150 ms + LT 250</td>
</tr>
</tbody>
</table>

Table 19: Experiment parameters for single staining of aorta sections stained for pecam-1 with Alexa Fluor® 568. Blocking buffer and ABs: see 4.11.6, incubation times and dilution(s) are stated; o/n: overnight; Tex: name of the channel for acquisition of 568 stained images; LT: lower threshold (parameter to reduce background in the TF software).

<table>
<thead>
<tr>
<th>Type</th>
<th>Single staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking buffer</td>
<td>5 % goat serum + 0.05 % saponin in PBS</td>
</tr>
<tr>
<td>Primary AB</td>
<td>o/n 1:50 Rabbit Anti Pecam</td>
</tr>
<tr>
<td>Secondary AB</td>
<td>1 h 1:1000 Goat Anti Rabbit Alexa Fluor® 647</td>
</tr>
<tr>
<td>Acquisition parameters</td>
<td>DAPI: 30 ms, Tex: 150 ms + LT 250</td>
</tr>
</tbody>
</table>
Double stainings for SM-actin + pecam-1

Table 20: Experiment parameters for double staining of aorta sections stained for SM-actin + pecam-1 with Alexa Fluor® 568 and Alexa Fluor 647. Blocking buffer and ABs: see 4.11.6, incubation times and dilution(s) are stated; o/n: overnight; Tex: name of the channel for acquisition of 568 stained images; Cy5: name of the channel for acquisition of 647 stained images; LT: lower threshold (parameter to reduce background in the TF software); G: gain (parameter for signal amplification in the TF software).

<table>
<thead>
<tr>
<th>Type</th>
<th>Double staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking buffer</td>
<td>5 % goat serum + 0.05 % saponin in PBS</td>
</tr>
<tr>
<td>1st Primary AB</td>
<td>o/n / 1h 1:500 Mouse Anti SM Actin</td>
</tr>
<tr>
<td>1st Secondary AB</td>
<td>1 h 1:1000 Goat Anti Mouse Alexa Fluor® 568</td>
</tr>
<tr>
<td>2nd Primary AB</td>
<td>o/n / 1h 1:50 Rabbit Anti Pecam</td>
</tr>
<tr>
<td>2nd Secondary AB</td>
<td>1 h 1:1000 Goat Anti Rabbit Alexa Fluor® 647</td>
</tr>
<tr>
<td>Acquisition parameters</td>
<td>DAPI: 30 ms, Tex: 150 ms + LT 250, Cy5: 450 ms + G + LT 150</td>
</tr>
</tbody>
</table>

Single stainings for MT₁ protein expression on cryo-sections

Table 21: Experiment parameters for single staining of aorta sections stained for MT₁ using the Abbiotec AB with Alexa Fluor® 568. Blocking buffer and ABs: see 4.11.6, incubation times and dilution(s) are stated; o/n: overnight; Tex: name of the channel for acquisition of 568 stained images.

<table>
<thead>
<tr>
<th>Type</th>
<th>Single staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking buffer</td>
<td>5 % goat serum + 0.05 % saponin in PBS</td>
</tr>
<tr>
<td>Primary AB</td>
<td>o/n 1:200 Rabbit Anti MT1 (Abbiotec)</td>
</tr>
<tr>
<td>Secondary AB</td>
<td>1 h 1:1000 Goat Anti Rabbit Alexa Fluor® 568</td>
</tr>
<tr>
<td>Acquisition parameters</td>
<td>DAPI: 50 ms, Tex: 400 ms</td>
</tr>
</tbody>
</table>

Table 22: Experiment parameters for single staining of aorta sections stained for MT₁ using the SC AB with Alexa Fluor® 568. Blocking buffer and ABs: see 4.11.6, incubation times and dilution(s) are stated; o/n: overnight; Tex: name of the channel for acquisition of 568 stained images.

<table>
<thead>
<tr>
<th>Type</th>
<th>Single staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking buffer</td>
<td>5 % donkey serum + 0.05 % saponin in PBS</td>
</tr>
<tr>
<td>Primary AB</td>
<td>o/n 1:50 Goat Anti MT1 (SC)</td>
</tr>
<tr>
<td>Secondary AB</td>
<td>1 h 1:1000 Donkey Anti Goat Alexa Fluor® 568</td>
</tr>
<tr>
<td>Acquisition parameters</td>
<td>DAPI: 50 ms, Tex: 400 ms</td>
</tr>
</tbody>
</table>
4 Materials and methods

Table 23: Experiment parameters for single staining of aorta sections stained for MT1 using the SC AB with Alexa Fluor® 647. Blocking buffer and ABs: see 4.11.6, incubation times and dilution(s) are stated; o/n: overnight; Cy5: name of the channel for acquisition of 647 stained images; G: gain (parameter for signal amplification in the TF software).

<table>
<thead>
<tr>
<th>Type</th>
<th>Single staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking buffer</td>
<td>5% donkey serum + 0.05% saponin in PBS</td>
</tr>
<tr>
<td>Primary AB</td>
<td>o/n 1:50, 1:20  Goat Anti MT1 (SC)</td>
</tr>
<tr>
<td>Secondary AB</td>
<td>1 h 1:1000  Donkey Anti Goat Alexa Fluor® 647</td>
</tr>
<tr>
<td>Acquisition parameters</td>
<td>DAPI: 20 ms, Cy5: 400 ms + G</td>
</tr>
</tbody>
</table>

Single stainings for MT1 protein expression on paraffin sections

Table 24: Experiment parameters for single staining of aorta and control paraffin sections stained for MT1 using the Abbiotec AB in two different concentrations with Alexa Fluor® 647. Blocking buffer and ABs: see 4.11.6, incubation times and dilution(s) are stated; o/n: overnight; Cy5: name of the channel for acquisition of 647 stained images; G: gain (parameter for signal amplification in the TF software).

<table>
<thead>
<tr>
<th>Type</th>
<th>Single staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking buffer</td>
<td>5% goat serum + 0.05% saponin in PBS</td>
</tr>
<tr>
<td>Primary AB</td>
<td>o/n 1:50, 1:10  Rabbit Anti MT1 (Abbiotec)</td>
</tr>
<tr>
<td>Secondary AB</td>
<td>1 h 1:1000  Goat Anti Rabbit Alexa Fluor® 647</td>
</tr>
<tr>
<td>Acquisition parameters</td>
<td>DAPI: 40 ms, Cy5: 500 ms + G</td>
</tr>
</tbody>
</table>

Table 25: Experiment parameters for single staining of aorta and control paraffin sections stained for MT1 using the SC AB in two different concentrations with Alexa Fluor® 647. Blocking buffer and ABs: see 4.11.6, incubation times and dilution(s) are stated; o/n: overnight; Cy5: name of the channel for acquisition of 647 stained images; G: gain (parameter for signal amplification in the TF software); Trans: transmitted light.

<table>
<thead>
<tr>
<th>Type</th>
<th>Single staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking buffer</td>
<td>5% donkey serum + 0.05% saponin in PBS</td>
</tr>
<tr>
<td>Primary AB</td>
<td>o/n 1:50, 1:20  Goat Anti MT1 (SC)</td>
</tr>
<tr>
<td>Secondary AB</td>
<td>1 h 1:1000  Donkey Anti Goat Alexa Fluor® 647</td>
</tr>
<tr>
<td>Acquisition parameters</td>
<td>DAPI: 20 ms, Cy5: 400 ms + G, Trans: 75 ms / 5.9 V</td>
</tr>
</tbody>
</table>
5 RESULTS

5.1 Establishment of molecular biological methods to demonstrate expression of MT$_1$ and MT$_2$ mRNA in rat tissues

In order to achieve the primary goal of this thesis, i.e. the confirmation of melatonin receptor mRNA expression in the rat aorta, the methods for RNA isolation, purity assessment, reverse transcription and PCR experiments had to be established. Ishii et al. [26] have recently demonstrated the occurrence of MT$_1$ and MT$_2$ mRNA in a variety of rat tissues.

Accordingly, several rat organs where expression of either MT$_1$ or MT$_2$ had been established were used as positive control (see 4.1.1). The RNA from these organs was extracted (see 5.1.1), transcribed into cDNA (see 5.1.2) and MT$_1$ as well as MT$_2$ mRNA expression was demonstrated by subsequent PCR (see 5.1.2) and qPCR (see 5.1.3).

5.1.1 RNA-Isolation

The RNA of ten different rat organs (see 4.1.1) was isolated (see 4.2). Photometric measurement of all samples (see 4.3) revealed acceptable purity ($A_{260}/A_{280}>1.6$) of the isolated RNAs. Yields of total RNA (see Table 26) varied, even though the same amount of tissue from each organ was used for RNA extraction (about 70 mg). GE (see 4.4) of the isolated RNA showed that it was intact for all organs and exhibited no signs of degradation (see Figure 19).

*Table 26: Concentration, purity and yield of extracted RNA from various rat organs. Skeletal m.: skeletal muscle.*

<table>
<thead>
<tr>
<th>Organ</th>
<th>C [ng / µL]</th>
<th>$A_{260}/A_{280}$</th>
<th>RNA yield [µg]</th>
</tr>
</thead>
<tbody>
<tr>
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<td>827.1</td>
<td>1.68</td>
<td>16.54</td>
</tr>
<tr>
<td>Eye</td>
<td>1212.5</td>
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<td>24.25</td>
</tr>
<tr>
<td>Heart</td>
<td>1529.4</td>
<td>1.80</td>
<td>30.59</td>
</tr>
<tr>
<td>Intestine</td>
<td>3926.7</td>
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<td>264.68</td>
</tr>
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<td>Kidney</td>
<td>3116.2</td>
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<td>Liver</td>
<td>1880.6</td>
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<td>188.06</td>
</tr>
<tr>
<td>Lung</td>
<td>1012.1</td>
<td>1.80</td>
<td>20.24</td>
</tr>
<tr>
<td>Skeletal m.</td>
<td>1978.8</td>
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<td>39.58</td>
</tr>
<tr>
<td>Testes</td>
<td>4050.8</td>
<td>1.90</td>
<td>133.55</td>
</tr>
<tr>
<td>Thymus</td>
<td>545.5</td>
<td>1.65</td>
<td>10.91</td>
</tr>
</tbody>
</table>
5 Results

Figure 19: Integrity control of total RNA from all extracted organs by GE. The isolated RNA of all organs is intact as judged by intact 28 S and 18 S rRNA bands.

5.1.2 RT-PCR experiments on the isolated RNA

Reverse transcription was performed (see 4.5). The obtained cDNAs were used for all following PCR experiments addressing β-actin, MT₁ and MT₂ expression in the rat organs.

RT-PCR for β-actin mRNA expression to prove successful RT

The first PCR experiment was executed for β-actin to confirm the success of the reverse transcription. β-actin is a so-called “housekeeping gene”, i.e. a gene that is common in all tissue and expressed ubiquitously in high concentrations. Primers and protocol were selected according to Ishii et al. [26]. 200 ng cDNA or water (no cDNA) were subjected to the PCR reaction. See 4.6.2 for the general protocol and 4.6.3 for experiment specific conditions. The experiment shows successful amplification of β-actin mRNA in all samples (see Figure 20), confirming success of the RT and – once more – integrity of the isolated RNA. Strengths of the observed bands were similar.

Figure 20: β-actin mRNA expression in various rat organs analyzed by RT-PCR, confirming the success of the reverse transcription. Expected length of the PCR product was 496 bp.
**RT-PCR for MT₁ mRNA expression**

The next step was amplification of MT₁ mRNA in the rat organs, following the procedure from Ishii et al. [26]. However, using identical settings, only the eye and intestine samples showed very weak bands at the expected size. For all other organs (including e.g. testes, liver or kidney, which exhibited strong bands for MT₁ in the original experiment by Ishii et al. [26]) there were no bands visible at all (data not shown).

Because of this weak amplification, we conducted a new PCR experiment, using double the amount of template cDNA (400 ng instead of 200 ng; RNA equivalent) per sample, and more cycles (increased from 35 to 40). See 4.6.2 for the general protocol and 4.6.3 for experiment specific conditions. The results of this modification can be seen in Figure 21, which shows bands of varying intensity for MT₁ in all tissues, with exception of the liver. Congruent with the results of the last experiment, eye and intestine samples show the strongest bands.

![Figure 21: MT₁ mRNA expression in various rat organs analyzed by RT-PCR. Expected length of the PCR product was 316 bp.](image)

**RT-PCR for MT₂ mRNA expression**

All rat organs were next tested for MT₂ expression, following exactly the procedure described by Ishii et al. [26]. 400 ng of cDNA (RNA equivalent) or water (no cDNA) were subjected to PCR. See 4.6.2 for the general protocol and 4.6.3 for experiment specific conditions.

Unexpectedly, no bands appeared for any organ (data not shown). To exclude methodical error, the procedure was repeated and yielded the same negative results (data not shown).

To address this problem, the protocol was modified using another PCR reaction buffer with NH₄SO₄, which should provide higher primer specificity and product yields [143]. To conserve material, this experiment was only performed on three selected samples (kidney, eye and intestine). However, this experiment again failed to
demonstrate MT$_2$ expression in any of the three tested organs, as a PCR product was detected neither at the expected length of 390 bp nor at any other length (data not shown).

As a final measure to verify MT$_2$ mRNA expression in brain and eye [24, 26], in the next experiment the annealing temperature of the PCR was reduced from 60 °C to 57 °C. See 4.6.2 for the general protocol and 4.6.3 for experiment specific conditions.

![Figure 22: MT$_2$ mRNA expression in rat brain and eye analyzed by RT-PCR. Expected length of the PCR product was 390 bp.](image)

As shown in Figure 22 by the appearance of a major band at 390 bp, this time, amplification was successful for the eye and brain samples. Probably due to the decreased annealing temperature, some other (but much weaker) bands appeared for both samples. Nevertheless, since the main bands were clearly at the correct length and of great intensity, the adapted method with the decreased annealing temperature was chosen to demonstrate MT$_2$ mRNA expression in the aorta.
5.1.3 RT-qPCR for ACTB, MT₁ and MT₂ mRNA expression

Next, the method for qPCR was established according to the procedure described in 4.10. Using qPCR, it is possible to discern the expression levels of various targets at a much greater sensitivity than with conventional “qualitative” PCR.

ACTB (β-actin) was used as reference gene, as it seems to be evenly expressed in all tissues (see Figure 20). Both MT₁ and MT₂ were analyzed in this experiment.

In addition to a negative sample (no cDNA), a -RT sample (intestine RNA treated like the other samples, except that no reverse transcriptase was added to the reaction mixture) was included as well. If the observed amplifications had been due to amplification of genomic DNA, the -RT sample would have shown the same or similar CT values as the reversely transcribed samples.

All samples, each containing 100 ng of the cDNA synthesized in 5.1.2, were prepared in triplicates. The mean of all CT values was calculated (CTm) and used as base for the following data analysis.

With respect to ACTB, the experiment went well for all samples except the eye, where ACTB did not show any expression. This can probably be attributed to a preparation error. Because of this, the ΔCT values for the eye could not be calculated, while CT values for all other samples are shown in Table 27.

According to the calculation, MT₁ mRNA expression was found in all samples at varying levels, highest in brain, lowest in lung (see Figure 23), correlating quite well with the expression levels observed in the qualitative PCR (see Figure 21). MT₂ mRNA expression was found in brain, kidney, testes and thymus, while the other samples did not exhibit amplification. The highest CT value for MT₂ was found in the eye, confirming MT₂ mRNA expression, but as noted before, no ΔCT values can be given because of the failed ACTB amplification.

It is obvious that the CTm values for all samples were very high, especially for MT₂, indicative of rather low expression of the receptors. They also exhibit relatively high standard deviations (CTSd) – therefore the comparison of expression levels is to be interpreted with caution. The -RT and negative samples showed amplification for ACTB as well, indicating some trace contamination. However, the difference in expression levels between them and the positively reverse-transcribed samples was so high (15–19 CT) that the contaminants’ influence on the result is negligible. For MT₁ and MT₂, the -RT and negative samples showed no amplification.
Table 27: ACTB (β-actin), MT1, and MT2 mRNA expression in various rat organs analyzed by RT-qPCR. Amplification failed for ACTB in the eye sample, therefore no ΔCT values could be calculated. The respective values are printed in gray. Empty cells indicate failed amplification. CTm: mean CT value from three repeats; CTmSd: standard deviation for CTm; ΔCTm: mean ΔCT value based on the ACTB CTm values as reference; ΔCTmSd: cumulative standard deviation for ΔCTm; D(ACTB): decimal difference in expression related to ACTB (ΔCTm converted from base 2 to base 10, assuming a linear amplification efficiency of 100 %); ΔΔCTm: mean ΔΔCT value for MT2 related to MT1 (difference ΔCTm for MT1 and MT2); ΔΔCTmSd: cumulative standard deviation for ΔΔCTm; D(MT1): decimal difference in expression related to MT1 (ΔΔCTm converted from base 2 to base 10, assuming a linear amplification efficiency of 100 %), only given for MT2 expressing samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Target</th>
<th>CTm</th>
<th>CTmSd</th>
<th>ΔCTm</th>
<th>ΔCTmSd</th>
<th>D(ACTB)</th>
<th>ΔΔCTm</th>
<th>ΔΔCTmSd</th>
<th>D(MT1)</th>
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<td>Brain</td>
<td>ACTB</td>
<td>17.07</td>
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<tr>
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<tr>
<td>Skeletal m.</td>
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</tbody>
</table>
Figure 23: The diagram shows the mean ΔCT values of MT₁ and MT₂ mRNA expression in relation to ACTB (β-actin) mRNA expression in various rat organs as calculated from the results of RT-qPCR. The lines on the top of the bars indicate the respective standard deviation; the indicators are missing in case of only one of the three repeats succeeding. Higher bars mean less expression. The missing bars for MT₂ in many organs indicate failed amplification. ΔCTₘ: mean ΔCT value based on the ACTB CTₘ values as reference.

For those samples where amplification for MT₁ and MT₂ was successful the ΔΔCT values for MT₂ related to MT₁ were calculated as reference. The difference in expression between MT₁ and MT₂ ranged from 2.7 to 3.7 CT (or 0.15–0.076 converted to base 10, assuming a linear amplification efficiency of 100 %), indicating an about ten-fold difference in expression on average (see Figure 24).

Figure 24: The diagram shows the logarithmic difference in MT₂ expression in relation to MT₁ from RT-qPCR analysis of ACTB (β-actin), MT₁ and MT₂ mRNA expression in various rat organs. Base MT₁ expression is 1.00. D(MT₁): decimal difference in expression related to MT₁ (ΔΔCTₘ converted from base 2 to base 10, assuming a linear amplification efficiency of 100 %).

To summarize, methods (RT-PCR, RT-qPCR) for demonstration of MT₁ and MT₂ mRNA in rat tissues had been successfully established.
5.2 Preparation of rat aorta samples for subsequent PCR experiments

Two different groups of aortas with two subgroups each were investigated (see 4.1.2 and 4.1.3). The two groups each served as basis for the different aims (see chapter 3) of the thesis.

5.2.1 RNA isolation

Aortas from two different time points

The RNA was extracted and measured by photometry in two batches (batch 1: A31, A32, A33, A75, A76 and A77, batch 2: A34, A35, A36, A37, A38, A79, A81, A82, A83 and A88) according to the described procedures (see 4.2 and 4.3). Because the amount of tissue was in some cases very small, the concentrations and total isolated amounts of RNA were rather low (see Table 28). Five samples were below the specified range for the $A_{260} / A_{280}$ ratio (1.6–2.0; A32, A33, A75, A76 and A77). 300 ng RNA were used for GE (see 4.4) to prove intactness of all samples’ RNA (see Figure 25).

Table 28: Concentration, purity and yield of RNA extracted from rat aortas from two different time points. Daytime group (orange): A31–A38; nighttime group (blue): A75–A88.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Group</th>
<th>C [ng / µL]</th>
<th>$A_{260} / A_{280}$</th>
<th>Isolated amount [µg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A31</td>
<td>Daytime</td>
<td>730.1</td>
<td>1.82</td>
<td>14.60</td>
</tr>
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<td>Daytime</td>
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<td>Daytime</td>
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</tr>
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<td>A35</td>
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<td>1.72</td>
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<td>Nighttime</td>
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<td>1.88</td>
<td>12.46</td>
</tr>
</tbody>
</table>
Figure 25: Integrity control of total RNA of rat aortas from two different time points by GE. The isolated RNA of all samples is intact as judged by intact 28 S and 18 S rRNAs. Because the RNA was extracted and analyzed in two batches (see main text), their respective lanes show different saturations. Daytime group (orange): A31–A38; nighttime group (blue): A75–A88.

Aortas from SHR and control rats

The RNA was extracted and measured by photometry according to the described procedures (see 4.2, 4.3 and 4.4). Because the amount of tissue was in some cases very small, the concentrations and total isolated amounts of RNA were so low that for four samples (ASHR 04, ASHR 08, ACTRL 01 and ACTRL 04), too little isolated RNA remained for further experiments (see Table 29). Therefore, they could not be used in any of the subsequent procedures concerning the SHR and control aortas.

With exception of ASHR 07, all samples that were being used for further experiments lay within the specified $A_{260}/A_{280}$ range (1.6–2.0). To conserve RNA, only 100 ng were used for GE (see 4.4), which proved intactness of all samples’ RNA (see Figure 26).
Table 29: Concentration, purity and yield of RNA extracted from aortas of SHR and control rats. For four samples (ASHR 04, ASHR 08, ACTRL 01 and ACTRL 04) the total isolated amount was too low for further analysis (see main text). The respective entries in the table are printed in gray.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Group</th>
<th>C [ng / µL]</th>
<th>$A_{260} / A_{280}$</th>
<th>Isolated amount [µg]</th>
</tr>
</thead>
<tbody>
<tr>
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<td>99,0</td>
<td>1,6</td>
<td>1,98</td>
</tr>
<tr>
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<td>SHR</td>
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<td>1,62</td>
<td>5,15</td>
</tr>
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<td>SHR</td>
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<td>1,6</td>
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</tr>
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<td>1,46</td>
<td>0,35</td>
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<tr>
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<td>1,73</td>
<td>3,50</td>
</tr>
<tr>
<td>ASHR 06</td>
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<td>1,59</td>
<td>1,07</td>
</tr>
<tr>
<td>ASHR 07</td>
<td>SHR</td>
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<td>1,55</td>
<td>0,95</td>
</tr>
<tr>
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<td>SHR</td>
<td>3,8</td>
<td>1,23</td>
<td>0,08</td>
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<tr>
<td>ACTRL 01</td>
<td>Control</td>
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<td>1,76</td>
<td>0,78</td>
</tr>
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<td>ACTRL 02</td>
<td>Control</td>
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<td>1,67</td>
<td>3,29</td>
</tr>
<tr>
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<td>1,71</td>
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<td>3,70</td>
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<td>Control</td>
<td>157,8</td>
<td>1,79</td>
<td>3,16</td>
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</table>

Figure 26: Integrity control of total RNA of aortas from SHR (red) and control (green) rats by GE. The isolated RNA of all samples was intact as judged by intact 28 S and 18 S rRNAs.
5.2.2 Reverse transcription

All samples of the aortas from two different time points were reversely transcribed according to the described procedure (see 4.5). Reverse transcription was performed in two batches (see 5.2.1 for the break-up). All samples from the SHR and control rats, excepting those with too little isolated RNA (see 5.2.1), were reversely transcribed according to the described procedure (see 4.5).

5.2.3 RT-PCR for β-actin mRNA expression

To demonstrate the success of the RTs, 50 ng of all aorta samples or water (no cDNA) were tested for β-actin mRNA expression. See 4.6.2 for the general protocol and 4.6.3 for experiment specific conditions. The experiment shows successful amplification of β-actin mRNA in all samples (see Figure 27 and Figure 28). To make sure that the observed amplicons were not caused by genomic DNA (which is thought to be impossible since the primers are exon spanning [26]), -RT samples (A35 and ASHR 02, respectively) were included in the experiments as well. As shown in the respective figures, the -RT sample exhibited no amplification.

![Figure 27](image_url)

**Figure 27**: β-actin mRNA expression in rat aortas from two different time points analyzed by RT-PCR, confirming the success of the reverse transcription. Expected length of the PCR product was 496 bp. Daytime group (orange): A31–A38; nighttime group (blue): A75–A88.

![Figure 28](image_url)

**Figure 28**: β-actin mRNA expression in aortas of SHR (red) and control (green) rats analyzed by RT-PCR, confirming the success of the reverse transcription. Expected length of the PCR product was 496 bp.
5 Results

5.3 Expression of MT₁ mRNA in rat aortas

5.3.1 Aortas from two different time points

RT-PCR for MT₁ mRNA expression

Next, aortas from different time points were examined for the presence of MT₁ mRNA in the rat aorta and for an overview of expression levels between the two aorta groups. 400 ng of cDNA (RNA equivalent) per sample or water (no cDNA) were subjected to PCR. A -RT sample (A35) was included as well. See 4.6.2 for the general protocol and 4.6.3 for experiment specific conditions.

![Figure 29: MT₁ mRNA expression in aortas from two different time points analyzed by RT-PCR. Expected length of the PCR product was 316 bp. Daytime group (orange): A31–A38; nighttime group (blue): A75–A88.](image)

Bands of the expected length for MT₁ were present in all samples but one (A32). Taken together, the bands in the daytime group (A31–A38) appeared less intense than those in the nighttime group (A75–A88). Especially A83 and A88 (both of the nighttime group) exhibited very strong bands (Figure 29). It has to be noted that the varying intensities and absences could have resulted from outside factors as well, such as pipetting errors, varying cDNA concentrations after RT, contaminations, etc. To confront these possible sources of error, a qPCR was performed at a later stage (see below).

Because there were no bands present in the -RT sample, amplification of genomic DNA could be excluded. The blind sample exhibits no bands either, excluding contamination of the reagents.

To confirm the identity of the observed bands they were cut out of the gel and processed for sequencing.

Sequencing of MT₁ RT-PCR-products

To extract a sufficient amount of DNA for sequencing, the excised bands of A33, A34, A38, A75, A83 and A88 were pooled and the DNA was extracted and purified according to the described procedure (see 4.8). Concentration measurement of the
eluated DNA by photometry (see 4.3) revealed a concentration of 8.8 ng / µL. 5 µL of the eluate were analyzed using GE to determine the extracted DNA’s purity. Only a single band at the expected length of 316 bp was visible (not shown). The eluate was then sent to MWG Eurofins Operon (Ebersberg, Germany) for sequencing (see 4.9) together with aliquots containing 30 pmol of each primer (rat_MT1_fwd and rat_MT1_rev, see 4.6.3).

<table>
<thead>
<tr>
<th></th>
<th>169</th>
<th>TGGCCCTGGCTGTGCTGCGGTAAGTCACCCAGGGGACCATGAAGGGCAATGTCAGCGAGC 228</th>
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<tr>
<td>fwd</td>
<td>1</td>
<td>AAATGCAGCGGAG 13</td>
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<tr>
<td>rev</td>
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<td>TGGCCCTGGCTGTGCTGCGGTAAGTCACCCAGGGGACCATGAAGGGCAATGTCAGCGAGC 215</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>fwd</td>
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<tr>
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<tr>
<td>fwd</td>
<td>254</td>
<td>TGACGTCTATACTTAA 269</td>
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</table>

**Figure 30:** Comparison of the sequencing results from the MT1-RT-PCR products and the published sequence for MT1 mRNA (RefSeq: NM_053676.1). Combined length of the sequencing results of both primers: 315 bp. E-values: fwd = 6E-145, rev = 1E-147; fwd: rat_MT1_fwd; rev: rat_MT1_rev.

Sequencing worked well and comparison with the published sequence for the rat MT1 mRNA ([26], RefSeq: NM_053676.1) showed 100 % identity over a length of 315 bp (expected: 316 bp). This result confirmed the identity of the observed amplicons as MT1.

**RT-qPCR for quantification of MT1 mRNA expression**

Having proven that MT1 mRNA is indeed expressed in the rat aorta, a real-time PCR experiment was performed to investigate the suggestion from the qualitative PCR (see above) that there might be a difference in expression levels between aortas collected at different time points.

RT-qPCR was executed after the described method (see 4.10) using 100 ng of cDNA (RNA equivalent) per sample. The samples were prepared in duplicates (ACTB) and triplicates (MT1). A positive control (eye) from the various rat tissues already investigated (see 5.1.2) was also included as reference. No -RT samples were included
because the previous RT-qPCR experiment (see 5.1.3) had already proven that the assays are specific for cDNA.

The blind samples were clean for MT1, but showed an expression of ACTB at very high CT levels. However, the difference in expression levels in comparison was so big that the contamination did not affect analysis of the data (see Table 30).

Table 30: ACTB (β-actin), MT1 and MT2 mRNA expression in rat aortas from two different time points and rat eye as positive control analyzed by RT-qPCR. Empty cells indicate failed amplification. CTm: mean CT value from two (ACTB) or three (MT1) repeats; CTmSd: standard deviation for CTm; ΔCTm: mean ΔCT value based on the ACTB CTm values as reference; ΔCTmSd: cumulative standard deviation for ΔCTm; Δ(CTm): decimal difference in expression related to ACTB (ΔCTm converted from base 2 to base 10, assuming a linear amplification efficiency of 100 %); ΔΔCTm: mean ΔΔCT value for MT1 related to eye as positive control (difference ΔCTm for MT1 in the aortas and MT1 in the eye); ΔΔCTmSd: cumulative standard deviation for ΔΔCTm; D(Eye): decimal difference in expression related to MT1 expression in the eye (ΔΔCTm converted from base 2 to base 10, assuming a linear amplification efficiency of 100 %).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Target</th>
<th>CTm</th>
<th>CTmSd</th>
<th>ΔCTm</th>
<th>ΔCTmSd</th>
<th>D(CTB)</th>
<th>ΔΔCTm</th>
<th>ΔΔCTmSd</th>
<th>D(Eye)</th>
</tr>
</thead>
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<td>0.055</td>
<td></td>
<td></td>
<td>1.5E-07</td>
<td>4.953</td>
<td>1.015</td>
<td>3.2E-02</td>
</tr>
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<td>0.064</td>
<td></td>
<td></td>
<td>1.8E-07</td>
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<td>1.2E-02</td>
</tr>
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<td></td>
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<td></td>
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Calculated (see 4.10) decimal differences between MT<sub>1</sub> expression in the eye and the samples can be seen in Figure 31. All aorta samples showed expression of MT<sub>1</sub>, the amount of expression varying strongly between the individual samples. The same observation had been made before in the course of the qualitative PCR. However, the individual discerned MT<sub>1</sub> expression levels of the aortas did not correlate with the results from the qualitative PCR. The positive control (eye) showed the highest expression of MT<sub>1</sub>, which is again congruent with the results from the qualitative PCR (see above). As in the previous qPCR experiment, the CT values for MT<sub>1</sub> in the aortas were very high (35–38), indicating low expression of MT<sub>1</sub> mRNA.

![Figure 31: Diagram showing the logarithmic difference in MT<sub>1</sub> mRNA expression in the aortas from two different time points in relation to MT<sub>1</sub> mRNA expression in the eye. Base MT<sub>1</sub> expression of the eye is 1.00. The lines on the lower ends of the bars indicate the respective standard deviations. Lower reaching bars mean less expression. Groups: bars of samples belonging to the daylight group are printed in orange, bars of samples belonging to the nighttime group are printed in blue; D(Eye): decimal difference in expression related to MT<sub>1</sub> in the eye (ΔΔCTm converted from base 2 to base 10, assuming a linear amplification efficiency of 100 %).](image)

Analysis of the mean expression levels shows a tiny elevation in MT<sub>1</sub> mRNA expression in the nighttime group compared to the daytime group of ΔΔCTm 21.60 (Sd 0.82) vs 21.84 (Sd 0.84). See Figure 32 for a graphical representation. As a trend, this is indeed what was observed in the qualitative PCR, but the difference is minute and the standard deviations of the means are many times bigger.
5 Results

![Diagram showing the mean ΔCTm values of the aortas from the two investigated time points. The lines on top of the bars indicate the respective standard deviations. ΔCTm: mean ΔCT value based on the ACTB CTm values as reference.]

In summary, MT₁ mRNA is expressed in the rat aorta; however, there is no significant difference in expression levels between aortas from animals sacrificed at daytime or nighttime.

5.3.2 Aortas from SHR and control rats

RT-PCR for MT₁ mRNA expression

The experiments with the rat aortas from different time points had already established that MT₁ is present in the rat aorta, and there was no significant difference in MT₁ mRNA expression found in the aorta that depends on the diurnal phase state. The remaining question was whether MT₁ mRNA expression is different between hypertensive and normotensive rats. To investigate this issue, an RT-PCR was performed for MT₁ mRNA expression in the SHR and control rat aortas.

As the RNA yield from these samples was very low (see Table 29), only a small amount of cDNA was available for experiments. Therefore, 200 ng cDNA (RNA equivalent) per sample or water (no cDNA) were subjected to PCR. A -RT sample (ASHR 02) was included as well. See 4.6.2 for the general protocol and 4.6.3 for experiment specific conditions.
The majority of bands in the SHR group was much stronger than the bands in the control group (see Figure 33). Amplification succeeded in all SHR samples, with a particularly strong band for the ASHR 05 sample, but failed in ACTRL 06 and ACTRL 07. These findings were confirmed by a repeat of the experiment, which yielded the same results (not shown). The variations in expression levels concur with the findings from the aortas from two different time points (see 5.3) and emphasize the possibility of interindividual differences in MT₁ expression levels. This could also explain the observed difference in MT₁ and MT₂ mRNA expression between our experiment with various rat organs (see 5.1.2) and the experiment by Ishii et al. [26]. To further investigate this difference in mRNA expression between SHR and control rats, a qPCR was performed next.

![Figure 33: RT-PCR analysis of MT₁ mRNA expression in aortas from SHR and control rats. Expected length: 316 bp.](image)

**RT-qPCR for M₁ mRNA expression in aortas**

RT-qPCR was executed after the described method (see 4.10) using only 50 ng of cDNA (RNA equivalent), because of the very low yield of the RNA extraction already discussed.

All samples were prepared in triplicates. A positive control (brain) from the various rat aortas already investigated (5.1.2) was used as reference. Also, -RT samples were included from both the SHR (ASHR 02) and control group (ACTRL 03).
5 Results

Table 31: ACTB (β-actin), MT₁, and MT₂ mRNA expression in aortas from SHR and control rats and rat brain as positive control analyzed by RT-qPCR. Empty cells indicate failed amplification. CTm: mean CT value from three repeats; CTmSd: standard deviation for CTm; ΔCTm: mean ΔCT value based on the ACTB CTm values as reference; ΔΔCTmSd: cumulative standard deviation for ΔCTm; D(ACTB): decimal difference in expression related to ACTB (ΔCTm converted from base 2 to base 10, assuming a linear amplification efficiency of 100 %); ΔΔCTm: mean ΔΔCT value for MT₁ related to brain as positive control (difference ΔCTm for MT₁ in the aortas and MT₁ in the brain); ΔΔCTmSd: cumulative standard deviation for ΔΔCTm; D(Brain): decimal difference in expression related to MT₁ expression in the brain (ΔΔCTm converted from base 2 to base 10, assuming a linear amplification efficiency of 100 %).

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<th>Sample</th>
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<th>CTmSd</th>
<th>ΔCTm</th>
<th>ΔCTmSd</th>
<th>D(ACTB)</th>
<th>ΔΔCTm</th>
<th>ΔΔCTmSd</th>
<th>D(Brain)</th>
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</table>

Like in the other RT-qPCR experiments (see 5.1.3 and 5.3.1), a signal for ACTB occurred even in the blind and -RT samples, although again at much higher CT values (35–38 versus 16–17 in the positive groups), which means the contamination will not affect analysis of the data. No signal was detected for MT₁ in the -RT and blind samples (see Table 31).

The control samples exhibited generally higher ΔCT values than the SHR samples. The biggest difference is evident between the aorta samples and the brain sample, where the concentration of mRNA is several orders of magnitude higher. Calculated
(see 4.10) decimal differences between MT₁ expression in the eye and the samples can be seen in Figure 34. The band intensity observed in the qualitative RT-PCR correlates nicely with data from the RT-qPCR (see above).

**Figure 34:** The diagram shows the logarithmic difference in MT₁ mRNA expression in the aortas of SHR and control rats in relation to MT₁ mRNA expression in the eye. Base MT₁ expression of the eye is 1.00. The lines on the bottom ends of the bars indicate the respective standard deviations. Lower reaching bars mean less expression. Groups: bars of samples belonging to the control group printed in green, bars of samples belonging to the SHR group are printed in red; D(Eye): decimal difference in expression related to MT₁ in the brain (ΔΔCTm converted from base 2 to base 10, assuming a linear amplification efficiency of 100%).

Analysis of the mean MT₁ mRNA expression levels showed a distinct elevation in MT₁ mRNA expression in the SHR group compared to the control group of ΔCTm 19.76 (Sd 1.12) vs 21.76 (Sd 0.97). A graphical representation can be seen in Figure 35. Also, the observed mean expression level and standard deviation of the control group are comparable with the observed mean expression levels and standard deviations for the aortas from two different time points (control vs nighttime vs daytime): ΔCTm 21.76 (Sd 0.97) vs 21.60 (Sd 0.82) vs 21.84 (Sd 0.84).
Figure 35: The diagram shows the mean ΔCTm values of the aortas from the two investigated time points. The lines on top of the bars indicate the respective standard deviations. ΔCTm: mean ΔCT value based on the ACTB CTm values as reference.

By calculating the ΔΔCT values for the two groups and conversion to the decimal system (see 4.10), the mean difference between the SHR and control rats turned out to be about four-fold (4.01). However, the standard deviation is very high (4.19, relative standard deviation 104%). Nevertheless, taking the standard deviation into account, the difference in expression ranges from about zero- to eight-fold. This means that the expression of mRNA in aortas of SHR is zero to eight times higher than in normotensive rats (see Figure 36). A RT-qPCR experiment with a bigger number of samples would probably show the difference in mRNA expression to a greater extent.

It should also be noted that the amplification succeeded in all of the SHR samples’ replicates, but not in all replicates of the control samples (18 SHR vs 9 controls). This fact can also be attributed to the observed difference in expression levels: because the levels of mRNA in the control rats’ aortas are lower than in the SHR’s aortas, amplification might easily fail, since the observed CT values are already extremely high (CT 36–39; see Table 31).

Figure 36: The diagram shows the n-fold expression of MT₁ mRNA in SHR aortas compared to control aortas.

In conclusion, we proved significantly higher expression of MT₁ mRNA in SHR compared to control rat aortas.
5.4 Expression of MT$_2$ mRNA in rat aortas

5.4.1 RT-PCR for MT$_2$ mRNA expression according to the established method

The first RT-PCR experiment concerning MT$_2$ mRNA expression in the aorta was performed on the aortas from two different time points (see 4.1.2) according to the method by Ishii et al. [26] modified as described in 5.1.2. 400 ng of cDNA (RNA equivalent) per sample or water (no cDNA) were subjected to PCR. A negative sample (no cDNA), a -RT sample (A35) and a positive control (brain) were also included in the experiment. See 4.6.2 for the general protocol and 4.6.3 for experiment specific conditions.

In the positive control (brain), amplification succeeded and yielded a very strong and distinctive band at the expected length. Under close inspection, bands at the expected length of 396 bp seemed also to be present in some of the aorta samples (A32, A38 and A77). However, bands of other lengths were present too, some of them much stronger (A38) than the alleged MT$_2$ bands (see Figure 37). The experiment was repeated with similar results (not shown).

![Figure 37](image)

**Figure 37**: MT$_2$ mRNA expression in aortas from two different time points analyzed by RT-PCR. Expected length of the PCR product was 390 bp. Daytime group (orange): A31–A38; nighttime group (blue): A75–A88.

The bands from the brain sample were excised from the gel and processed for sequencing according to 5.4.2.

5.4.2 Sequencing of the MT$_2$ PCR product from the brain sample

The DNA from the gel slices was extracted and purified according to the described procedure (see 4.8). Concentration measurement of the eluated DNA by photometry (see 4.3) revealed a concentration of 5.2 ng / µL. 5 µL of the eluate were analyzed using GE to determine the extracted DNA’s purity. Only a singular band at the expected length of 390 bp was visible (not shown). The eluate was then sent to MWG Eurofins Operon (Ebersberg, Germany) for sequencing (see 4.9) together with aliquots containing 30 pmol of each primer (rat_MT2_fwd and rat_MT2_rev, see 4.6.3).
5 Results

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**Figure 38:** Comparison of the sequencing results from the MT2 RT-PCR products of the brain sample and the published sequence for MT2 mRNA ([26], RefSeq: NM_001100641.1). Combined length of the sequencing results of both primers: 390 bp. E-values: fwd = 0.0, rev = 0.0; fwd: rat_MT2_fwd; rev: rat_MT2_rev.

Sequencing worked well and comparison with the published sequence for the rat MT2 mRNA ([26], RefSeq: NM_001100641.1) showed 99 % identity over a length of 390 bp (expected: 390 bp). This result confirmed the identity of the observed amplicons in the brain sample as MT2 and proved that the PCR method by Ishii et al. (2009) worked to detect MT2 mRNA.

### 5.4.3 Search for and selection of alternative PCR protocols

Because of the unsatisfactory results with the Ishii primers with regard to MT2 in the aorta (sequencing proved its efficacy for the brain sample; 5.4.2), a search in the literature for other primers for rat MT2 was performed. The published sequence of MT2 ([26], RefSeq: NM_001100641.1) was used to compare the positions of the varying primer pairs. See Table 32 for the results of the search.

**Table 32:** Primer pairs for rat MT2 available in the literature. The primers published in the respective papers are highlighted in the same way as the corresponding sequence in the drawing of the sequence further below. For the reverse primers, the corresponding reverse complements are denoted in brackets.
### Results

<table>
<thead>
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<th>Source</th>
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<th>Product</th>
<th>Notes</th>
</tr>
</thead>
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<td>5'-GGAGCGCCCAAGCAGTG-3'</td>
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<td></td>
<td>rev.</td>
<td>(ATGACGTGGTTATGGGAATT)</td>
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<td></td>
<td></td>
<td>5'-GGATCTCCCCAAAATGAGCT-3'</td>
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<tr>
<td></td>
<td></td>
<td>(TCACTTTCTGTGGCTGCACT)</td>
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<tr>
<td>Sallinen et al. [35]</td>
<td>fwd.</td>
<td>5'-ATGTCACAGTCTGCT-3'</td>
<td>65 bp for qPCR, equiv. to</td>
</tr>
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<td></td>
<td>rev.</td>
<td>(ATGACGGTTGGGTACTTGGGGAGATCC)</td>
<td>[159] and [160]</td>
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<td>(GAAGGCCATCTCTCTTCCAA)</td>
<td>“outer” primers of a nested PCR</td>
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<tr>
<td>Sugden et al. [34]</td>
<td>fwd.</td>
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<td>154 bp</td>
</tr>
<tr>
<td></td>
<td>rev.</td>
<td>(CATCCACACTCCCTTCCCTAA)</td>
<td>“inner” primers of a nested PCR</td>
</tr>
<tr>
<td>Masana et al. [36]</td>
<td>fwd.</td>
<td>5'-CCCTCCTACAGCTCCTCA-3'</td>
<td>264 bp</td>
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<tr>
<td></td>
<td>rev.</td>
<td>(TACCGCTGAGACCCAGCCTTGATTT)</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 39:** Sequence of rat MT$_2$, showing the position of various primer pairs. Exon 1 is printed in blue, exon 2 is printed in bordeaux, exon 3 is printed in dark green. See Table 32 for highlight color mapping.

Except for the primers designed by Ishii et al. [26], all primer pairs are located exclusively in exon 2 (see Figure 39). This is problematic because not only reversely transcribed mRNA, but also traces of genomic DNA in the samples will be amplified in the course of the PCR. However, two primer pairs (outer and inner) used by Sugden et al. [34] were selected for further investigation of MT$_2$ mRNA expression, because a
nested PCR provides a method of amplifying very weak products with high specificity (see 4.6.1).

5.4.4 RT-PCR for MT₂ mRNA expression according to the Sugden protocol

A PCR experiment using the selected primers from Sugden et al. [34] was set up. To conserve cDNA of the aorta samples, the cDNA from the various rat organs (see 5.1.2) was used to test the new primers. 400 ng of cDNA (RNA equivalent) per sample or water (no cDNA) were subjected to PCR. A -RT sample (intestine) was included as well. See 4.6.2 for the general protocol and 4.6.3 for experiment specific conditions.

Unfortunately, all samples exhibited strong bands at the expected length of 292 bp. in addition to other bands of varying lengths appearing in many samples, the -RT sample exhibited a band of similar strength as did the rest of the samples at the expected length (Figure 40). This proved that the intra-exon primers from Sugden et al. [34] amplified traces of genomic DNA left after the RNA extraction procedure. Also, when compared with the results gained by usage of the method and primers by Ishii et al. [26] (see 5.4.1), the ubiquitous presence of very strong bands, with almost no difference between the samples, seemed rather improbable.

![MT₂ mRNA expression in various rat organs analyzed by RT-PCR according to Sugden et al. [34]. Expected length of the PCR product was 390 bp.](image)

The nested PCR was performed nevertheless with 1 µL PCR product from the outer PCR to test the primers for function. It produced bands of high intensity in all samples at the expected length of 154 bp (not shown).

To address the problem of genomic contamination in the extracted RNA, DNAse treatment was performed according to the described method (see 4.7). DNAse digestion should degrade DNA contaminations in the samples of extracted RNA, leaving the RNA intact. After the procedure was completed, the RNA was reversely transcribed following the described method (see 4.5). After DNAse treatment, amplification using the intra-exon Sugden primers would be expected to happen only in
samples containing cDNA (and not genomic DNA) for MT₂, thus providing evidence for
MT₂ receptor expression in the respective samples. -RT samples of all DNAs treated
RNA samples were prepared as well to check if DNAs treatment was successful.

The method was used on RNA samples of brain and eye from the various rat
organs gained in 5.1.1., and aliquots of the aorta samples from two different time points
(see 5.2) with the highest particular total amount of isolated RNA (A35, A36, A37, A81,
A83 and A88).

Intactness of the cDNA after reverse transcription was checked via a PCR for
β-actin according to the established method (see 5.1.2) and proved that the RT worked
and DNAs treatment did not result in RNA degradation, as correct amplification
occurred in all samples (data not shown).

The following PCR experiments for MT₂ in the eye and liver according to the
method after Sugden et al. [34] indicated, however, that some of the reaction
components were contaminated, because amplification occurred also in the negative
controls with no cDNA (data not shown). After a series of experiments (data not
shown), the outer primers of the nested PCR were identified as the source of the
contamination. Therefore, only the inner primers were used for the following PCR for
MT₂ mRNA expression in the rat aortas.

In this experiment, six DNAs treated aorta samples from the aortas of two
different time points were used in a PCR experiment with the inner Sugden primers
only. The experiment was immediately done with the more “valuable” aorta samples
instead of the samples from the rat organs, to minimize the risk of another
contamination. The cycles for this inner PCR had to be changed from 20 to 40 to
account for the reduced amount of template (because of the lack of a preceding outer
PCR). A positive control sample (eye), as well as -RT samples of all investigated
samples were included in the reaction setup. 400 ng cDNA or water (no cDNA) were
subjected to the PCR reaction. See 4.6.2 for the general protocol and 4.6.3 for
experiment specific conditions.

The reaction went well and distinctive bands of the expected length of 154 bp were
visible in all samples. The blind sample showed no bands, indicating that no
contamination had occurred. However, all -RT controls still exhibited bands of 154 bp
length as well, albeit of smaller intensity than their respective +RT counterpart (see
Figure 41). Also, the bands from the aorta samples were as strong as the band from
the eye sample, which seemed rather unlikely after the results for MT₁ (see 5.3.1).
**Figure 41:** MT$_2$ mRNA expression in rat aortas from two different time points and rat eye as positive control analyzed by RT-PCR according to Sugden et al. [34] using the protocol for the inner PCR only. Expected length of the PCR product was 154 bp. Daytime group (orange): A35–A37; nighttime group (blue): A81–A88.

These results suggest that DNase treatment does not work efficiently enough for total degradation of genomic DNA in the samples. Therefore, no further experiments were conducted using this method.

### 5.4.5 Design of new exon spanning primers for MT$_2$

Based on the described results, new sets of exon spanning primers were designed following standard guidelines for primer design [161] using the primer design software tool of the National Center for Biotechnology Information [162]. The published sequence of MT$_2$ ([26], RefSeq: NM_001100641.1) was used to compare the positions of the varying primer pairs (see Figure 42). From a wide selection of possible candidates (not shown), the primer pairs MS1$_r$MT2 and MS2$_r$MT2 (see 4.6.3 and Table 33) were chosen and custom-synthesized by MWG Eurofins Operon (Ebersberg, Germany).

**Table 33:** Self-designed primer pairs for rat MT$_2$ (MS1$_r$MT2 and MS2$_r$MT2) and the Ishii primers for comparison (rat_MT2). MS1$_r$MT2 rev. and MS2$_r$MT2 rev. are identical. The primers are highlighted in the same way as the corresponding sequence in the schematic of the sequence (Figure 42). For the reverse primers, the corresponding reverse complements are denoted in brackets.

<table>
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<td>5′-AGGCTCGTGAGTTGGTGAGTCGC-3′</td>
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<td></td>
<td>(TGCGACCTACCCCGAGCCT)</td>
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Figure 42: Sequence of rat MT$_2$, showing the position of the self-designed primer pairs and the Ishii primers (see Table 33 for highlight color mapping). Exon 1 is printed in blue, exon 2 is printed in bordeaux, exon 3 is printed in dark green. (Sequence: modified after [26], NM_001100641.1).

The mastermix recipe and temperature program for the primers’ respective PCR procedures was designed according to the described method (0).

These particular two primer pairs were selected because they allow combination with the Ishii primers, if necessary, or the execution of a nested PCR, using the Ishii primers as inner primers, when using MS1_rMT2 as outer primers. Also, because the melting temperatures of these three primer pairs are relatively similar, PCR experiments using all three primer pairs could be conducted in one batch.

5.4.6 RT-PCR for MT$_2$ mRNA expression with the newly designed primers

Assessment of primer functionality and effectiveness

Three simultaneous PCRs were carried out on the confirmed MT$_2$ expressing brain sample (see 5.4.2) from the various rat organs (see 4.1.1) to assess the effectiveness of the new primers by comparing their respective band intensities with those derived from the Ishii primers. The experiments were performed in one batch. All the PCRs worked and produced single, defined bands at the respective expected lengths for MT$_2$. The band for the MS2_rMT2 primer was extremely weak, whereas the bands for the Ishii and MS1_rMT2 primers were about equal in intensity (data not shown). Therefore, the primer pair MS1_rMT2 was used for the next experiment on the aorta samples.
Results

RT-PCR for MT$_2$ mRNA expression in rat aortas from two different time points

Six samples of the aortas from two different time points (A31, A35, A36, A37, A81, A83 and A88; see 4.1.2) and the positive controls brain and eye (see 4.1.1) were investigated. 400 ng cDNA or water (no cDNA) were subjected to the PCR reaction using the self-designed primers MS1_rMT2. See 4.6.2 for the general protocol and 4.6.3 for experiment specific conditions.

Singular bands of the expected length of 540 bp were visible in the eye and brain positive controls. In the aorta samples weaker bands at lengths of 500 bp and / or 600 bp, but not at 540 bp were observed (data not shown). The bands of both lengths from the aorta samples and the singular bands of the expected length from the eye and brain samples were excised from the gel and processed for sequencing.

Sequencing of alleged MT$_2$ RT-PCR products

The excised bands were pooled for a higher DNA yield after extraction: the first pool included the bands from the aorta samples with a length of 600 bp; the second pool included the bands from the aorta samples with a length of 500 bp; the third pool included the bands with a length of 540 bp from the eye and brain positive controls.

The DNA from the gel slices was extracted and purified according to the described procedure (see 4.8). Concentration measurement of the eluated DNAs by photometry (see 4.3) revealed concentrations of 5.9 ng / µL (first pool), 7.0 ng / µL (second pool) and 8.3 ng / µL (third pool). 5 µL of the eluates were analyzed using GE to determine the extracted DNA’s purity. Only singular bands at the expected length for each pool were visible (not shown). The eluates were then sent to MWG Eurofins Operon (Ebersberg, Germany) for sequencing (see 4.9) together with aliquots containing 30 pmol of each primer (MS1_rMT2_fwd and MS1_rMT2_rev, see 4.6.3).

Unfortunately, sequencing worked only for pool three (brain and eye samples). Comparison with the published sequence for the rat MT$_2$ mRNA ([26], RefSeq: NM_001100641.1) showed 100 % identity over a length of 540 bp (expected: 540 bp). This result confirmed the identity of the observed amplicons from the brain and eye sample as MT$_2$ and proves that the new method with the self-designed primers is able to amplify MT$_2$ in rat tissue correctly. However, for pools one and two (all aorta samples), sequencing failed, producing no results.
Results

<table>
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<td>T</td>
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</table>

Figure 43: Comparison of the sequencing results from pool three (brain and eye) and the published sequence for MT2 mRNA (RefSeq NM_001100641.1) Combined length of the sequencing results of both primers: 540 bp; E-values: fwd = 0.0, rev = 7E-150; fwd: MS1_rMT2 fwd; rev: MS1_rMT2 rev.

To ensure that a methodical error was not the cause for the failed sequencing of the aorta bands, the experiment was repeated. Similar results as in the experiment above were obtained after RT-PCR and sequencing of the bands from the aorta samples failed again (data not shown).

Nested RT-PCR for MT2 mRNA expression in rat aortas from two different time points

The previous experiments already indicated that MT2 mRNA was not expressed in the rat aorta. To confirm this hypothesis, a nested PCR, using the self-designed primers as outer primers and the Ishii primers as inner primers, was performed. The nested PCR should enrich a possibly very faint MT2 amplicon in the rat aorta samples and show selectively only single bands of the expected product.

Again, 400 ng cDNA (RNA equivalent) of the six samples of the aortas from two different time points (A31, A35, A36, A37, A81, A83 and A88; see 4.1.2) and the positive controls brain and eye (see 4.1.1) were subjected to the outer RT-PCR with
the self-designed primers MS1_rMT2. See 4.6.2 for the general protocol and 4.6.3 for experiment specific conditions. The two positive controls (brain and eye) were diluted 1:5 and the eye sample was additionally diluted 1:10 to account for the low amount – if any – of MT2 mRNA expected to be present in the aorta samples. A 1:10 dilution of the brain sample was also prepared but not included in the experiment because of spillage. Analysis of the outer RT-PCR showed again bands at varying lengths and intensities in the aorta samples. In contrast to previous PCR (0), almost all aorta samples (except for A35) exhibit bands at the observed lengths of 600 and 500 bp.

Figure 44: MT2 mRNA expression in rat aortas of two different time points and rat brain and eye as positive controls analyzed by RT-PCR according to the self-designed PCR protocol as outer PCR. Expected length of the PCR product was 540 bp. Daytime group (orange): A35–A37; nighttime group (blue): A81–A88.

The inner PCR was performed with 1 µL of the PCR products of the outer PCR used as template according to the method by Ishii et al. [26] modified as described in 5.1.3. See 4.6.2 for the general protocol and 4.6.3 for experiment specific conditions. The number of cycles was reduced from 40 to 20 to accommodate the use of the protocol as an inner PCR.

Figure 45: MT2 mRNA expression in rat aortas of two different time points and rat brain and eye as positive controls analyzed by RT-PCR according to the modified method by Ishii et al. [26] as inner PCR. Expected length of the PCR product was 390 bp. Daytime group (orange): A35–A37; nighttime group (blue): A81–A88.
Amplification occurred only in the three positive controls. The bands were very intense and at the expected length of 390 bp. In addition to these bands, a single low intensity band occurred at approximately 500 bp. No bands whatsoever were visible in the aorta samples. The experiment was repeated, yielding the same results (not shown).

From this experiment it was concluded that MT$_2$ mRNA is not expressed in the rat aorta. Therefore, no further experiments (aortas from SHR and control rats, qPCR, IF) were performed for MT$_2$.

5.5 Expression analysis of MT$_1$ protein in the rat aorta by IF

Aortas of control and SHR rats were investigated for MT$_1$ protein expression using IF experiments. As mentioned above, because of the results from the mRNA level (no MT$_2$ mRNA is present in the rat aorta), experiments for MT$_2$ were not performed. For information on antibodies and blocking buffers, see the respective materials and methods sections (4.11.6).

Because of the postulated mechanism of MT$_1$’s influence on blood vessels, where MT$_1$ acts through direct activation of smooth muscle cells, causing vasoconstriction [61], we first established IF localization of the smooth muscle cell marker α-smooth muscle-actin (SM-actin) and the endothelial cell marker pecam-1 in single and double stainings for use in possible co-localization experiments with MT$_1$.

5.5.1 Single stainings for SM-actin and pecam-1 protein expression

The first IF experiments were performed on frozen rat aortas from SHR and control rats (prepared by M. Benova, Department of Pathophysiology, Medical University Vienna, in 2008; see 4.1.4). The aortas were cryo-sectioned according to the described procedure (see 4.11.2).

The aorta sections were stained either for SM-actin or pecam-1 and photographed according to the described procedures (see 4.11.4 and 4.11.7). Negative samples using the secondary ABs only were prepared as well. See chapter 4.11.8, Table 18 and Table 19 for experiment parameters.

Both single stainings worked well, as shown in Figure 46: There was bright staining in the tunica media showing staining for SM-actin. Staining for pecam-1 highlighted only the endothelial cells of the tunica intima, as expected. In both cases, the negative controls showed no fluorescence, indicating that the observed stainings was due to specific binding of the primary ABs only.
5.5.2 Double staining for SM-actin + pecam-1 protein expression

Since the single stainings went well, the same antibodies and samples were used for establishment of double staining protocols.

The aorta sections were stained and photographed according to the described procedures for sequential and parallel double staining (see 4.11.4 and 4.11.7). For the sequential staining, two groups of samples were investigated: in one group the first primary AB was for SM-actin, in the other group the first primary AB was for pecam-1. Because the incubation with the primary AB was done overnight, this setup was chosen to determine if the duration of the incubation (overnight vs 1 h) with either of the two primary antibodies has an effect on the staining. In the parallel experiment, both primary ABs were applied overnight simultaneously. Because both primary antibodies are detected with secondary antibodies gained from goat, the same blocking buffer was used. See chapter 4.11.8, Table 20 for experiment parameters.

Negative samples using the secondary ABs only were prepared, as well as cross negatives, containing a primary antibody and the respective other secondary antibody to exclude false positive detections of the secondary antibodies.
All staining procedures went well, and the antibodies were selective for their respective targets (see Figure 47).

Figure 47: IF analysis of double stainings for SM-actin + pecam-1 protein localization in rat aorta sections. A: SM-actin overnight, pecam-1 1h; B: pecam-1 cross negative; C: SM-actin 1h, pecam-1 overnight; positive; D: SM-actin cross negative; E: parallel staining positive; F: parallel staining negative; secondary antibodies: Alexa Fluor® 647 (pecam-1) and Alexa Fluor® 568 (SM-actin) TA: tunica adventitia; TM: tunica media; TI: tunica intima.

There is a striking – yet not unexpected – difference between the three different groups: while the pecam staining was not very strong when the primary AB was only applied for 1 hour, the staining was vibrant when the section was incubated with the AB
overnight. The actin staining was strong even when the AB was only applied for 1 hour. The adventitia exhibited some background fluorescence.

The cross negatives showed that the secondary antibodies are selective for their respective targets. No staining of the “wrong” primary antibody or of the tissue in general occurs. The same was observed in the negatives (not shown).

The parallel staining procedure produced a very nicely stained section, with less staining of the adventitia than in the sequential approaches. Since both primary antibodies were applied overnight (together), the stainings of both types were very vibrant. There was little staining visible in the negative (mostly in the media).

5.5.3 Single stainings for MT₁ protein expression on cryo-sections

The first IF experiment for MT₁ protein expression in the rat aorta was performed on the cryo-sectioned aortas. The aorta sections were stained for MT₁ using the Abbiotec 25076 and SC sc-13186 antibodies and were photographed according to the described procedures (see 4.11.4 and 4.11.7). Negative samples using the secondary ABs only were prepared as well. See chapter 4.11.8, Table 21 and Table 22 for experiment parameters.

All stained sections (representatives are shown in Figure 48) showed some background fluorescence, mainly in the elastic lamellae in the tunica media. The staining was brighter with the Abbiotec AB (Figure 48, panel A) than with the SC AB (Figure 48, panel C). The negative controls showed no staining. However, it has to be noted that the exposure time was very high (400 ms) for the red channel (for pecam-1 or SM-actin, a brighter and much more defined staining was captured at only 150 ms; see 5.5.1 and 5.5.2); therefore the specificity of these findings was questionable.
Figure 48: IF analysis of stainings for MT1 protein localization in rat aorta cryo-sections. A: Abbiotec positive; B: Abbiotec negative; C: SC positive; D: SC negative; secondary antibody: Alexa Fluor® 568; TA: tunica adventitia; TM: tunica media; TI: tunica intima.

Because the staining achieved with the SC AB was especially weak, another experiment, using a different secondary antibody and an additional higher concentration of the primary antibody, was performed. See chapter 4.11.8, Table 23 for experiment parameters.
Figure 49: IF analysis of stainings for MT₁ protein localization in rat aorta cryo-sections using the SC AB in two different concentrations and Alexa Fluor® 647 as secondary AB. A: SC 1:50; B: SC 1:20; C: SC negative; TA: tunica adventitia; TM: tunica media; TI: tunica intima.

There was some staining visible in the sections (mainly in the tunica adventitia; a representative is shown in Figure 49) and it was a little stronger in the sections stained with the 1:20 diluted antibody (Figure 49, panel B), than with the 1:50 diluted antibody (Figure 49, panel A). Grain-like noise was observed scattered over the tissue, in the positive sections as in the negative sections, but stronger in the positive sections. However, the staining of the adventitia observed in the positive sections is not present in the negative section.

5.5.4 Single stainings for MT₁ protein expression on paraffin sections

After the unsatisfactory results from the cryo-sections, the staining for MT₁ was conducted on the paraffin sections. Aorta sections prepared by H. Uhrova (Department of Pathophysiology and Allergy Research, Medical University Vienna; see 4.1.3) were used for the following experiments. Because these aortas are the same ones that were analyzed for MT₁ mRNA expression in 5.3.2, it was already known that the SHR group expressed more mRNA than the control group. Therefore it seemed obvious to conduct
the experiments on the SHR aortas, because the expression (if any) of MT₁ protein in the cryo-sections was extremely low.

In the paraffin section group, positive controls were available: because of the results from the MT₁ mRNA expression experiments in various rat organs (see 5.1.2 and 5.1.3), we knew what organs contained mRNA for MT₁ in rats. Rats were not available at the time, therefore mice were used. All antibodies are selective for proteins from human, rat or mouse origin, so the use of mouse organs was not a problem. R. Stumberger, C. Brünner-Kubath and H. Uhrova (all Department of Pathophysiology and Allergy Research, Medical University Vienna) prepared a mouse (mus musculus) intestine and brain. H. Uhrova did the HOPE® fixation and paraffin-embedding, as well as the sectioning of these organs.

**Stainings using the Abbiotec anti MT₁ Antibody**

Sections of mouse brain and intestine were stained together with the aorta sections (SHR aortas) for MT₁ using the Abbiotec 25076 antibody. The aortas were stained in different concentrations of 1:50 and 1:10, while the organs were stained only 1:10. Staining and photography was done according to the described procedures (see 4.11.5 and 4.11.7). Negative samples using the secondary ABs only were prepared as well. See chapter 4.11.8, Table 24 for experiment parameters.

At 1:50 staining was visible in the aortas (a representative is shown in Figure 50) which became much more pronounced at 1:10. It seems that the antibody’s target is primarily located in the adventitia and also – but to a lesser extent – in the cells of the media and intima. The negative control exhibited no staining.
Figure 50: IF analysis of stainings for MT; localization in rat aorta paraffin sections using the Abbiotec AB in two different concentrations and Alexa Fluor® 647 as secondary AB. A: Abbiotec 1:50; B: Abbiotec 1:10; C: Abbiotec negative; TA: tunica adventitia; TM: tunica media; TI: tunica intima.

In the brain, staining with the Abbiotec AB produced a marked amount of tissue background fluorescence (in contrast to the negative control; Figure 51) as well as stained fibers in the brain, and nuclei and meninges of the cerebellum (Figure 52, red arrows).
Figure 51: IF analysis of the negative control for MT₁ localization in mouse brain paraffin sections, using the Abbiotec AB and Alexa Fluor® 647 as secondary AB. The framed parts are magnified. The pictures of the middle panel correspond to 200 x magnification.
Figure 52: IF analysis of staining for MT β localization in mouse brain paraffin sections using the Abbiotec AB and Alexa Fluor® 647 as secondary AB. The framed parts are magnified. The pictures of the middle panel correspond to 200 x magnification. Red arrows mark areas of interest (see main text).
In the staining of the intestine, the tissue showed extremely high tissue background fluorescence and again staining of the tissue borders. The negative tissue exhibited no fluorescence (see Figure 53).

**Figure 53**: IF analysis of staining for MT₁ localization in mouse intestine paraffin sections using the Abbiotec AB and Alexa Fluor® 647 as secondary AB. A: Abbiotec positive; B: Abbiotec negative.

**Stainings using the SC anti MT₁ antibody**

Sections of both organs were stained with the aorta sections (ASHR 02, ASHR 03 and ASHR 06) for MT₁ using SC sc-13186 antibody. The aortas were stained in different concentrations of 1:50 and 1:20, while the organs were stained only 1:20. Staining and photography was done according to the described procedures (4.11.5 and 4.11.7). Negative samples using the secondary ABs only were prepared as well. See chapter 4.11.8, Table 25 for experiment parameters. A new batch of antibody was used for this experiment, therefore concentrations of 1:50 and 1:20 (and not 1:10) were used because it was probable that the staining with the new antibody would be stronger than with the old batch.
Like with the Abbiotec AB and the cryo-sections, the (weak) staining found was mainly located in the tunica adventitia (TA). Also, there was a lot of grain-like noise visible in and around the tissue. The staining in the 1:20 (Figure 54, panel B) treated sections was a little bit stronger than in the 1:50 treated ones (Figure 54, panel A).

There was no staining visible in the negatives (Figure 54, panel C). However, some grain-like noise can still be seen scattered over the tissue (similar to the – stronger – pattern in the cryo-sections, see Figure 49).

**Figure 54:** IF analysis of stainings for MT₁ localization in rat aorta paraffin sections using the SC AB in two different concentrations and Alexa Fluor® 647 as secondary AB. A: SC 1:50; B: SC 1:20; C: Abbiotec negative; TA: tunica adventitia; TM: tunica media; TI: tunica intima.
A lot of background was visible in the negative control of the brain sample (outer layer of cerebellum; Figure 55).

**Figure 55:** IF analysis of the negative control for MT₁ localization in mouse brain paraffin sections, using the SC AB and Alexa Fluor® 647 as secondary AB. The framed parts are magnified. The pictures of the middle panel correspond to 200 x magnification.
Like in the brain sample stained with the Abbiotec anti MT₁ AB (Figure 52), fibers in the brain tissue and the meninges of the cerebellum were stained the strongest (Figure 56, red arrows). Like in the aorta samples, there was a lot of grain-like noise on and around the tissue.

![Image of IF analysis of staining for MT₁ localization in mouse brain paraffin sections using the SC AB and Alexa Fluor® 647 as secondary AB. The framed parts are magnified. The pictures of the middle panel correspond to 200 x magnification. Red arrows mark areas of interest (see main text).]

In contrast to the staining with the Abbiotec AB (Figure 53), the stained regions of tissue (Paneth cells?) in the intestine were much more defined and restricted to specific clusters of cells (Figure 58, red arrows). There was no staining in the negative sample. There is also notably less noise than in the other negative samples (Figure 57).
Figure 57: IF analysis of the negative control for MT₁ localization in mouse intestine paraffin sections, using the Abbiotec AB and Alexa Fluor® 647 as secondary AB. The framed parts are magnified.

Figure 58: IF analysis of staining for MT₁ localization in mouse brain paraffin sections using the SC AB and Alexa Fluor® 647 as secondary AB. The framed parts are magnified. The bottom right picture shows an additional overlay with the respective transmission image. Red arrows mark areas of interest (see main text).
In conclusion, we showed that specific and strong stainings for MT₁ protein expression with the available antibodies from SC and Abbiotec are possible (mouse brain and intestine). In contrast, the stainings observed in the aorta sections, both paraffin as well as cryo-sectioned, were very weak and did not look very specific. The best results were obtained with the Abbiotec antibody on paraffin sections, where a dilution of 1:10 produced a moderate staining for MT₁ in the tunica adventitia. Because of this weak expression in tissue layers other than the expected (tunica adventitia), no co-localization experiments with pecam-1 (for the tunica intima) or SM-actin (for the tunica media) were performed.
6 DISCUSSION

The results are discussed according to the order of the aims of the thesis.

1) Establishment of molecular biological methods for demonstration of MT₁ and MT₂ mRNA expression

The establishment of the methods for RNA extraction and reverse transcription worked well and provided no difficulty. RT-PCR method establishment for MT₁ worked without problems as well, however, for the MT₁ RT-PCR, the cycles had to be adjusted from 35 to 40 and the template cDNA (RNA equivalent) from 200 to 400 ng compared to the described method by Ishii et al. [26]. This could indicate worse transcription efficiency of our polymerase or reverse transcriptase.

The observed amplifications for MT₁ mRNA in the rat organs are not congruent with the results from Ishii et al. [26], as e.g. testes, liver and kidney, which showed the strongest expression of all organs in [26], only exhibited weak bands for MT₁ in our experiment. These results were confirmed by the qPCR, where the observed ΔCT values were for the most part congruent with the band intensities from the qualitative PCR. In our experiment, the intestine, brain and eye showed the highest expression for MT₁. The expression in these tissues is well established already [35]. A possible explanation of this discrepancy could be variation of expression between different rat strains, or expression dependent on the circadian phase state (at least in some tissues). However, diurnal variations in MT₁ expression have only been reported for humans [129] and not for the rat [108]. Of course it is also possible that the observed differences in melatonin GPCR mRNA expression are the result of interindividual variations, like the ones found for MT₁ mRNA expression in the aorta.

The method for MT₂ RT-PCR experiments had to be changed initially, since the original protocol according to [26] did not produce any results even for tissues where MT₂ occurrence is well established, e.g. brain or eye [6]. In contrast to Ishii et al. [26], who observed MT₂ mRNA expression in most of the investigated tissues (see Figure 6), RT-qPCR analysis showed that MT₂ mRNA was only expressed in 4 out of 10 tissues (the eye sample was not included in the RT-qPCR experiment) and at very low levels. Sallinen et al. [35] also tested various rat organs for MT₂ mRNA and demonstrated expression in the intestine, the liver and the heart, where no amplification was observed in our experiment. Nevertheless, the positive results from brain [26, 163], testes [26], kidney [26, 159] and thymus [159] are confirmed in the literature for the respective rat tissues. The reasons for the observed differences in expression of MT₂ might be the same as for MT₁ expression.
2a) Expression of MT₁ mRNA in the rat aorta

In experiments performed with aortas from the two different time points, MT₁ mRNA expression was confirmed in the rat aorta, which is in line with the findings of other studies on the rat aorta [108] and other rat [36] and human [127, 129] blood vessels. MT₁ mRNA was detected in all samples, though at varying intensities. The identity of the bands was confirmed by sequencing of the PCR product. The absence of a band in one sample and the different intensities in the various samples are interesting because they indicate large interindividual differences in MT₁ receptor expression between the animals. RT-qPCR did indeed confirm these great interindividual differences of MT₁ mRNA expression at the same time of day.

2b) Expression of MT₂ mRNA in the rat aorta

Concerning MT₂ mRNA, different methods were tried to find an expression of its mRNA in the aorta, since various studies reported an expression of MT₂ mRNA in the vascular system of humans [128] and rats alike [35-36], even if the data is — as discussed in the introduction, see 2.6.6 — conflicting.

The only method that yielded amplification of MT₂ in the aorta samples was the nested PCR method from Sugden et al. [34], using inter-exon primers. Unfortunately, the bands for MT₂ persisted even in DNase treated -RT samples and as long as the -RT samples exhibit any bands at all, the results for the normal (+RT) samples are not valid, thus indicating presence of genomic DNA. Additionally, with this method, the observed bands for MT₂ were much stronger than those for MT₁. Even if a direct comparison is illicit, it seems rather improbable that MT₂ would be so readily detectable when the aorta samples exhibit only weak MT₁ bands, or even, in one case, none at all and MT₁ is usually higher expressed than MT₂ [24].

The results from the nested PCR using the exon spanning self-designed and Ishii primers performed on the rat aortas from two different time points then clearly proved that no mRNA for MT₂ is present in the rat aorta. Our results support the findings of other studies which showed no expression of MT₂ mRNA in rat cerebral arteries [132]. Preparation or methodical error can be excluded because amplification of MT₂ mRNA in the positive controls brain and eye worked and was confirmed by sequencing. Based on the absence of MT₂ mRNA, we did not perform a RT-qPCR or IF staining for MT₂ on any of the aorta samples.

3) Quantification of MT₁ mRNA expression difference in the rat aorta depending on the time of day

Maybe as a result of those large interindividual differences, this experiment showed no significant difference in expression levels of MT₁ mRNA between the two time points.
Therefore, it cannot be concluded that MT₁ mRNA expression in the aorta is related to the time of day, or if it is, the sample size and number of time points in this experiment were far too small and too few to detect it with any significance. This confirms the results of Benova et al. [108], who did not find any difference in MT₁ expression in the rat aorta at the protein level and emphasizes the organ specificity of the circadian rhythms of this receptor, because in other organs, a circadian expression pattern was indeed observed [129, 136].

4) Quantification of MT₁ mRNA expression difference in the rat aorta depending on BP

In contrast to this result, both RT-PCR and RT-qPCR experiments showed a significant increase (about 4-fold) in MT₁ receptor mRNA in SHR when compared to control rats. This difference proves that there is indeed some kind of link between hypertension and melatonin GPCR expression. However, it seems to occur only in genetically predisposed hypertensive animals, since L-NAME induced hypertension did not increase MT₁ protein expression in the aorta [108]. Then again, even though changes in MT₁ expression levels were observed as a result of the genetic Alzheimer’s disease [135], they were also observed as a result of artificially created ischemia [160], putting this hypothesis of genetic influence in perspective. Therefore, other additional mechanisms might account for the observed differences.

Because exogenous melatonin reduces BP in spontaneously hypertensive rats [115], and MT₂, supposed to have vasodilating effects [61], seems not to be present in the aorta, the BP reducing effects of melatonin might be attributed rather to melatonin’s non receptor-mediated antioxidative capability [61] or GABAergic influence on the central nervous system [109] than effects on the vasculature mediated via GPCRs.

5) Localization of MT₁ protein in the rat aorta

Although expression of MT₁ mRNA was demonstrated in this study for the rat aorta and expression of MT₁ protein by western blotting (Prof. M. Zeman, Comenius University of Bratislava, Slovakia; personal communication; and [108]), the location of MT₁ protein in the various layers of the aorta remains questionable.

The functioning of the staining procedures was confirmed by the stainings for pecam-1 and α-SM-actin, which worked fine in single as well as sequential and parallel double stainings. These stainings proved that the aorta sections were still antigenic and that the endothelium was intact (positive staining with pecam-1).

When staining for MT₁, however, only very weak cell-associated fluorescence, but high background noise was observed. These grain-like speckles were only observed with the anti-MT₁ AB from SC, using Alexa Fluor® 647 as secondary AB, but not with
the primary anti-MT$_1$ AB from Abbiotec using the same secondary AB, or the SC primary AB with Alexa Fluor® 568 as secondary AB. Therefore, it might be possible that this background was the result of formation of unspecific aggregates of the SC AB with the Alexa Fluor® 647 secondary antibody.

Though the combination of the SC antibody with Alexa Fluor® 568 did not result in background noise, the observed staining was also very weak. It seems likely that it is the result of unspecific adhesion of the primary AB to the tissue, rather than target specific binding. The anti-MT$_1$ Abbiotec antibody generally produced stronger stainings with less background noise. With both antibodies, the staining was stronger in the tunica adventitia than in the other layers of the aorta. This was observed in paraffin sections as well as – to a lesser extent – in cryo-sections. However, this staining pattern was only seen using Alexa Fluor® 647 as secondary antibody. When used with Alexa Fluor® 568, only some extremely faint staining of the tunica media could be seen.

From the proposed vasoconstrictive function of MT$_1$, a localization of the receptor in smooth muscle cells was expected. Surprisingly, the only staining for MT$_1$ in aortas was associated with the tunica adventitia. However, a similar situation has been reported in the literature. Immunohistochemical staining of cerebral vessels from Alzheimer patients showed MT$_1$ localization only in the adventitia, too [135]. The applied anti-human-MT$_1$ AB is described as being well characterized. Since no other studies exist that have investigated MT$_1$ localization in blood vessels, it is possible that MT$_1$ indeed is only localized in the tunica adventitia.

It has to be noted that paraffin embedding is known to mask antigens and the tissue we used for cryo-sectioning had already been frozen for more than a year. It is possible that the receptor protein, even more so since the expression is very low, had already been degraded and was therefore not detectable any more by IF methods. Furthermore, the reliability of the available antibodies remains somewhat questionable, because other studies indicate that at least a SC AB against human MT$_1$ is rather unspecific (Ramezanian and Ellinger, unpublished data).
Nonetheless, we can exclude procedural error or degraded antibodies, because staining of the positive controls (mouse brain and intestine) worked nicely and produced quite interesting results. To our knowledge, no studies on the localization of MT\textsubscript{1} in the intestine have been performed, and our experiments show highly selective staining of what may be Paneth cells at the bottom of the intestinal villi. In the brain, specific staining of fibers of yet undetermined nature was shown. One might speculate that these fibers are capillaries, which would be of great interest with respect to the aims of this thesis. Both of these occurrences of MT\textsubscript{1} have to be investigated in a later work.

\section{Conclusion and outlook}

The principal aim of this thesis was to prove the expression of the melatonin GPCRs MT\textsubscript{1} and MT\textsubscript{2} in the rat aorta on mRNA level, thereby helping to understand the mechanism by which melatonin contributes to BP regulation.

We confirmed MT\textsubscript{1} is indeed expressed in the rat aorta. We observed no differences in expression between samples collected at night and samples collected at day, but the sample size (eight from each group) might have been too small for a definitive conclusion. However, an about 4-fold increase in mRNA levels was observed in aortas from SHR compared to control rats, adding to the evidence of melatonin’s BP regulating activity. On the other side, we proved that MT\textsubscript{2} mRNA is not expressed in the rat aorta.

Since our results from IF indicate that MT\textsubscript{1} is not expressed in the smooth muscle cells of the tunica media, and MT\textsubscript{2} was shown not to be present in the rat aorta at all, the proposed mechanism of action of melatonin’s BP modulating properties \cite{61} might have to be reviewed, even if the increase in mRNA in SHR emphasizes a role of MT\textsubscript{1} in BP regulation.

In future studies, the investigation of a difference in mRNA expression levels of MT\textsubscript{1} could be redone, using a larger population of samples and perhaps more than two time points. Additionally, we discovered very specific localization of MT\textsubscript{1} in the mouse brain and intestine, which has to be investigated further in additional studies.
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ΔΔCT</td>
<td>Difference between two ΔCT values</td>
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<td>ΔCT</td>
<td>Difference between two CT values</td>
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<td>AANAT</td>
<td>Arylalkylamine-N-transferase</td>
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<td>AB(s)</td>
<td>Antibody(ies)</td>
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<td>AC</td>
<td>Adenylate cyclase</td>
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<td>ACTB</td>
<td>β-actin (in context of qPCR)</td>
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<td>AGA-GE</td>
<td>Agarose gel electrophoresis</td>
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<td>approx.</td>
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<td>BP</td>
<td>Blood pressure</td>
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<td>bp</td>
<td>Base pairs</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>C</td>
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<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CCR</td>
<td>CC chemokine receptors</td>
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<td>CHS</td>
<td>Coronary heart syndrome</td>
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<td>CREB</td>
<td>cAMP-response-element-binding-protein</td>
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<td>CT</td>
<td>Cycle threshold</td>
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<td>DAG</td>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>GABA&lt;sub&gt;A&lt;/sub&gt;</td>
<td>Gamma-aminobutyric-acid A receptor</td>
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<td>NO</td>
<td>Nitric oxide</td>
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<td>o/n</td>
<td>Overnight</td>
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<td>Polymerase chain reaction</td>
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<td>Paraformaldehyde</td>
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<td>Protein kinase A</td>
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<td>PKC</td>
<td>Protein kinase C</td>
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<td>qPCR</td>
<td>Real time (quantitative) polymerase chain reaction</td>
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<td>Santa Cruz</td>
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<td>Suprachiasmatic nucleus</td>
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<td>Spontaneously hypertensive rats</td>
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9 REFERENCES


80. Mor, M., Rivara, S., Pala, D., Bedini, A., Spadoni, G., and Tarzia, G., Recent advances in the development of melatonin MT(1) and MT(2) receptor agonists. Expert Opin Ther Pat, 2010. ahead of print.


9 References


10 CURRICULUM VITAE

Personal details
Name Martin Wolfgang Schepelmann
Date and place of birth November 10th, 1983; Vienna
Nationality Austria

Education and career
1990–1994 Elementary school (Maria Regina, 1190 Vienna)
1994 – 2002 Secondary school (Döblinger Gymnasium, 1190 Vienna)
05/2002 Graduation (Matura)
2003–2004 Training as paramedic (graduation with honors) in the context of the
Austrian compulsory community service (Austrian Red Cross)
2004–2010 Study of Pharmacy at the University of Vienna, Austria
06/2007 Leading a workshop for microscopic plant anatomy in two tenth and
one ninth grade biology classes at a secondary school
(Billrothgymnasium, 1190 Vienna)

Awards
04/1998 1st place at the Austrian finals of CyberSchool98
(students develop IT-projects; www.cyberschool.at)
05/2001 1st place at the Austrian finals of Business@School
(students develop business plans; initiated by the Boston Consulting
Group; www.business-at-school.de)
06/2001 3rd place at the Austrian-German finals of Business@School (see
above) in Munich, Germany
01/2008 Merit scholarship of the University of Vienna (StudFG WS2008) for
excellent academic achievements in the year 2007

Special skills
Languages German (native language), English (fluent)
IT Microsoft Office (Word, Excel, Powerpoint)
Adobe Photoshop, Adobe Premiere, system assembling and
maintenance, operating systems (DOS, Windows 3.11, 95, 98, ME,
NT, 2000, XP, Vista, 7), basic network administration
ECDL (European Computer Driving License; acquired at the Austrian
Computer Society)
Driving license B

Hobbies Movies, reading, IT, classical music, microscopy, history