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ACTA UNIVERSITATIS OULUENSIS

*Pirkko Sallinen*

# MYOCARDIAL INFARCTION

## *ASPECTS RELATING TO ENDOGENOUS AND EXOGENOUS MELATONIN AND CARDIAC CONTRACTILITY*


*FACULTY OF SCIENCE,*
*DEPARTMENT OF BIOLOGY,*
*UNIVERSITY OF OULU*

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*PIRKKO SALLINEN*

# MYOCARDIAL INFARCTION

Aspects relating to endogenous and exogenous melatonin and cardiac contractility

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Docent Seppo Saarela

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Professor Eero Mervaala

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Faculty of Science, Department of Biology, University of Oulu, P.O.Box 3000, FI-90014 University of Oulu, Finland
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## *Abstract*

Melatonin is an important modulator of several physiological and behavioural processes, and it influences the function of many different tissues. Melatonin has effective antioxidative properties, but some of its actions in mammals are also mediated through the $MT_1$ and $MT_2$ melatonin receptors. Antioxidative properties are seen especially when the melatonin concentration is high (≥ nM), and melatonin's affinity for its receptors appears at lower concentrations (pM).

Recently, the involvement of melatonin in protecting the heart against cardiac diseases, including myocardial infarction (MI), has been brought out. MI alters the structure and function of myocardium, attenuating for example cardiac contractility by affecting the amount and function of the essential $Ca^{2+}$ handling proteins, dihydropyridine receptor (DHPR), ryanodine receptor ($RyR_2$) and sarco-endoplasmic reticulum (SR) $Ca^{2+}$-ATPase2 (SERCA2). MI also evokes many adaptive responses in organisms, such as elevated production of atrial and brain natriuretic peptides (ANP and BNP).

In this thesis, the expression of $MT_1$ and $MT_2$ receptor mRNAs was investigated in several rat tissues. Furthermore, the effect of MI and exogenous melatonin on the rat endogenous melatonin and on the expression of cardiac $MT_1$, $MT_2$, DHPR, $RyR_2$ and SERCA2 proteins was evaluated. The concentrations of ANP and BNP were also measured after post-MI melatonin administration.

The results show the expression of $MT_1$ and/or $MT_2$ receptor mRNAs in the hypothalamus, retina, small intestine, liver and heart, which indicates that at least some effects of melatonin could be mediated through the receptors in these tissues. Melatonin synthesis in the pineal gland increased rapidly in response to MI, supporting an important role of endogenous melatonin in protecting the heart after MI. Furthermore, exogenous melatonin altered the mRNA expression of DHPR, $RyR_2$ and SERCA2 after MI, suggesting that melatonin might contribute to the post-infarction cardiac contractile function. The results also revealed a novel, positive relationship between melatonin and ANP, and thereby bring out one more possible way of melatonin to protect the heart against MI-induced injuries.

Taken together, the present thesis (i) supports the notion that melatonin is an important endogenous protective agent of the organism, and (ii) extends our knowledge of melatonin's post-infarction cardioprotective actions.

*Keywords:* dihydropyridine receptor (DHPR), melatonin receptor, $MT_1$, $MT_2$, natriuretic peptides, ryanodine receptor ($RyR_2$), sarco-endoplasmic reticulum $Ca^{2+}$-ATPase2 (SERCA2)

**Sallinen, Pirkko, Melatoniinin merkitys sydänlihaksen supistumisessa infarktin jälkeen**
Luonnontieteellinen tiedekunta, Biologian laitos, Oulun yliopisto, PL 3000, 90014 Oulun yliopisto
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Oulu

## *Tiivistelmä*

Melatoniini osallistuu monien fysiologisten toimintojen ja käyttäytymisen säätelyyn sekä vaikuttaa useiden eri kudosten toimintaan. Melatoniini on tehokas antioksidantti, mutta jotkut sen vaikutuksista välittyvät myös $MT_1$ ja $MT_2$ melatoniini reseptorien kautta. Antioksidatiiviset vaikutukset tulevat esiin erityisesti silloin, kun melatoniinin pitoisuus on korkea (≥ nM). Alhaisemmilla pitoisuuksilla (pM) on puolestaan havaittavissa melatoniinin sitoutuminen reseptoreihinsa.

Viime aikoina on tullut esille melatoniinin mahdollinen suojavaikutus sydänsairauksia, kuten sydäninfarkteja, vastaan. Sydäninfarkti muuttaa sydänlihaksen rakennetta ja toimintaa esimerkiksi vaikuttamalla supistuksen kannalta välttämättömien proteiinien, dihydropyridiini reseptorin (DHPR), ryanodiini reseptorin ($RyR_2$) ja sarko-endoplasmakalvoston $Ca^{2+}$-ATPaasi2:n (SERCA2) lukumääriin ja toimintaan, ja sitä kautta muun muassa heikentää sydämen supistuvuutta. Sydäninfarkti laukaisee elimistössä myös monia adaptiivisia vasteita, kuten eteispeptidin (ANP) ja aivojen natriureettisen peptidin (BNP) lisääntyneen erityksen.

Tässä väitöstyössä tutkittiin $MT_1$ ja $MT_2$ reseptorien mRNAn ilmentymistä useissa rotan eri kudoksissa. Lisäksi tutkittiin sydäninfarktin ja eksogeenisen melatoniinin vaikutuksia rotan endogeeniseen melatoniiniin sekä sydämen $MT_1$, $MT_2$, DHPR, $RyR_2$ ja SERCA2 proteiinien ekspressioon. Myös ANP ja BNP pitoisuudet mitattiin.

Tulokset osoittivat $MT_1$ ja/tai $MT_2$ reseptori mRNAn ilmentymisen hypotalamuksessa, silmän verkkokalvolla, ohutsuolessa, maksassa ja sydämessä, minkä perusteella ainakin osa melatoniinin vaikutuksista saattaisi olla reseptorivälitteisiä näissä kudoksissa. Tulosten mukaan käpyrauhasen melatoniinisynteesi lisääntyi nopeasti sydäninfarktin jälkeen, mikä tukee käsitystä endogeenisen melatoniinin tärkeästä roolista infarktin jälkeisessä sydämen suojauksessa. Lisäksi eksogeeninen melatoniini muutti DHPR:n, $RyR_2$:n ja SERCA2:n mRNA ekspressiota infarktin jälkeen, mikä voisi merkitä, että melatoniini saattaa vaikuttaa infarktin jälkeiseen sydämen supistuvuuteen. Tulosten osoittama positiivinen riippuvuus melatoniinin ja ANP:n välillä tuo puolestaan esille yhden uuden mahdollisen keinon, jonka kautta melatoniini voisi suojata sydäntä infarktin aiheuttamia vaurioita vastaan.

Yhteenvetona voidaan todeta, että tämä väitöstyö (i) tukee käsitystä, että endogeenisella melatoniinilla on tärkeä merkitys elimistön suojaamisessa, ja (ii) laajentaa tietämystämme infarktin jälkeisistä melatoniinin sydäntä suojaavista vaikutuksista.

*Asiasanat:* dihydropyridiini reseptori (DHPR), melatoniini reseptori, $MT_1$, $MT_2$, natriureettinen peptidi, ryanodiini reseptori ($RyR_2$), sarko-endoplasmakalvoston $Ca^{2+}$-ATPaasi2 (SERCA2)

*To my beloved family,*
*Tarmo*
*Heidi*
*Sara*
*Niko*
*Juho*
*Milja*

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Oulu, February 2008 Pirkko Sallinen

# Abbreviations

| | |
|---|---|
| AAAD | aromatic-L-amino-acid decarboxylase |
| AA-NAT | arylalkylamine N-acetyltransferase |
| AMK | $N^1$-acetyl-5-methoxy-kynurenine |
| ANP | atrial natriuretic peptide |
| ATPase | adenosine triphosphatase |
| BNP | brain natriuretic peptide |
| β-AR | β-adrenergic receptor |
| β-ARK | β-adrenergic receptor kinase |
| cAMP | cyclic adenosine monophosphate |
| CAT | catalase |
| cGMP | cyclic guanosine monophosphate |
| DHPR | dihydropyridine receptor |
| ECC | excitation-contraction coupling |
| FKBP | FK-506 binding protein |
| GPCR | G-protein coupled receptor |
| GPx | glutathione peroxidase |
| GSH | glutathione |
| HIOMT | hydroxyindol-O-methyltransferase |
| $IP_3$ | inositol 1,4,5-trisphosphate |
| LV | left ventricle |
| LTCC | L-type $Ca^{2+}$ channel |
| MI | myocardial infarction |
| NA | noradrenaline |
| NO | nitric oxide |
| NT-proANP | aminoterminal fragment of proatrial natriuretic peptide |
| $ONOO^-$ | peroxynitrite |
| PAF | platelet-activating factor |
| PBS | phosphate-buffered saline |
| PKA | protein kinase A |
| PLB | phospholamban |
| PT | pars tuberalis |
| rBNP | rat brain natriuretic peptide |
| RIA | radioimmunoassay |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |

| | |
|---|---|
| RT-PCR | reverse transcription-polymerase chain reaction |
| RyR | ryanodine receptor |
| SA | sinoatrial |
| SCN | suprachiasmatic nucleus |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SEM | standard error of mean |
| SERCA | sarco-endoplasmic reticulum $Ca^{2+}$-ATPase |
| SOD | superoxide dismutase |
| SR | sarco-endoplasmic reticulum |
| TM | transmembrane |
| TPH | tryptophan hydroxylase |

# List of original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I Sallinen P, Saarela S, Ilves M, Vakkuri O & Leppäluoto J (2005) The expression of $MT_1$ and $MT_2$ melatonin receptor mRNA in several rat tissues. Life Sci 76: 1123-1134.

II Sallinen P, Mänttäri S, Leskinen H, Ilves M, Vakkuri O, Ruskoaho H & Saarela S (2007) The effect of myocardial infarction on the synthesis, concentration and receptor expression of endogenous melatonin. J Pineal Res 42: 254-260.

III Sallinen P, Mänttäri S, Leskinen H, Ilves M, Ruskoaho H & Saarela S (2007) Time course of changes in the expression of DHPR, $RyR_2$ and SERCA2 after myocardial infarction in the rat left ventricle. Mol Cell Biochem 303: 97-103.

IV Sallinen P, Mänttäri S, Leskinen H, Vakkuri O, Ruskoaho H & Saarela S (2008) Long-term post-infarction melatonin administration alters the expression of DHPR, $RyR_2$, SERCA2 and $MT_2$ and elevates the ANP level in the rat left ventricle. J Pineal Res (in press).

# Contents

# 1 Introduction

The pineal secretory product, melatonin, is a versatile molecule of the organism which has an influence on a wide variety of physiological and behavioural processes. Melatonin's different characteristics and effects have been studied since it was first identified by Lerner *et al.* in 1958. In the past few years, a potential therapeutic effect of melatonin on treating different diseases, among them cardiac diseases (Reiter *et al.* 2002), has been brought out. In particular, the free radical scavenging properties of melatonin have been suggested to mediate these protective effects (Chen *et al.* 2003, Wang *et al.* 2005). However, the involvement of melatonin receptors, for example in cardioprotective actions of this indole, has also been suggested recently (Tomas-Zapico & Coto-Montes 2005).

Myocardial infarction (MI), on the other hand, is one of the most frequent causes that lead to heart failure (Hasenfuss & Pieske 2002). MI is known to impair cardiac contractility and intracellular $Ca^{2+}$ cycling (Holt *et al.* 1998), among other deleterious effects on the morphology and function of the heart (St. John Sutton & Sharpe 2000). Since heart failure develops when the heart is not capable of pumping a sufficient amount of blood needed for metabolic functions of the tissues, assuring proper cardiac contractility seems to be essential in preventing the onset of heart failure (Houser & Margulies 2003). Furthermore, dihydropyridine receptor (DHPR), ryanodine receptor ($RyR_2$) and sarco-endoplasmic reticulum (SR) $Ca^{2+}$-ATPase2 (SERCA2) are fundamental $Ca^{2+}$ handling proteins, which have a major role in the cardiac excitation-contraction coupling (ECC) (Barry & Bridge 1993). Therefore, changes in the density or function of these proteins might cause contractile dysfunction in the failing heart (Houser *et al.* 2000), and studies elucidating the factors that could possibly protect DHPR, $RyR_2$ and SERCA2 against MI-induced injuries are needed.

In order to increase our knowledge relating to melatonin and MI, the present thesis focused mainly on the effects of MI and exogenous melatonin on endogenous melatonin and on the post-MI contractile function, as evaluated by the expression of DHPR, $RyR_2$ and SERCA2.

# 2 Review of the literature

Myocardial infarction (MI) initiates the post-infarction left ventricular remodelling (LV remodelling) which leads to the functional decline of the left ventricle (LV) and finally to heart failure. MI has, for example, an impact on the expression of different types of cardiac membrane receptors and ion channels. Melatonin, the main secretory product of the pineal gland, is, on the other hand, known to protect the heart against MI injuries. Therefore, the following review of the literature summarizes the basic characteristics of melatonin, MI and LV remodelling. It also deals with the sarcolemmal dihydropyridine receptor (DHPR), ryanodine receptor (RyR) and sarco-endoplasmic reticulum (SR) $Ca^{2+}$-ATPase2 (SERCA2) since they play a major role in the contractility of the heart and were thereby of interest in this thesis. Furthermore, an update is given on what is presently known about the effects of melatonin on the heart after MI.

## 2.1 Melatonin

Melatonin is an ancient, indole-derived neurosecretory product that exists in all living organisms. It is synthesized in the pineal gland in a circadian manner and secreted into the blood during the hours of darkness. Melatonin is also synthesized in the retina, the Harderian gland, the gastrointestinal tract and possibly some other extrapineal sites as well (Reiter 1991b, Tijmes *et al.* 1996). However, the circulating melatonin in mammals is primarily derived from the pineal gland (Reiter 1991b), and the melatonin synthesized in other organs is suggested to act mainly locally (Vanecek 1998).

The history of the pineal gland goes back to the 17$^{th}$ century when the French philosopher Descartes suggested that the pineal controlled “the flow of the soul”. By the end of the 19$^{th}$ century the role of the pineal in the mammalian endocrinology was discovered. (Drijfhout 1996.) Modern experimental studies on melatonin started in 1917, when McCord & Allan discovered that the extracts of the pineal gland had a blanching effect on tadpole skin (see Morgan *et al.* 1994). Furthermore, Lerner *et al.* (1958) succeeded in isolating the bioactive compound and they elucidated the chemical structure of this compound as N-acetyl-5-methoxytryptamine (Lerner *et al.* 1959). (Fig. 1)

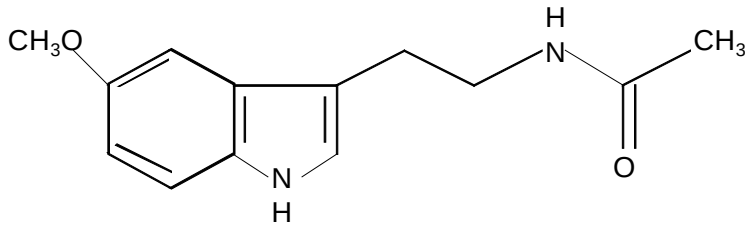


**Fig. 1. The chemical structure of melatonin.**

Melatonin is usually portrayed as a hormone, and many physiological functions are ascribed to its hormonal properties (Tan *et al.* 2003). In mammals, these include for example involvement in various photic regulations, such as adaptation to light intensity and regulation of the daily and seasonal behaviour and physiology (Vanecek 1998). Enhancement of the function of the immune system (Maestroni 1998), vasoregulatory activity (Ting *et al.* 1999), inhibition of cancer growth (Blask *et al.* 1999) and the effect on treating circadian rhythm sleep disorders (Cajochen *et al.* 2003) are also based on the hormonal effects of melatonin. However, several features of melatonin distinguish it from classical hormones. These include melatonin's characteristics as an autocoid and paracoid, lack of storage mechanisms and actively regulated secretion, its alternative sources (e.g. vegetables, fruits and rice) and its non-receptor mediated free radical scavenging activity (Tan *et al.* 2003). Potential therapeutic effect on Alzheimer's disease (Wang *et al.* 2005) and at least some of melatonin's cardioprotective actions (e.g. Lee *et al.* 2002, Chen *et al.* 2003, Sahna *et al.* 2005), among others, are believed to be related to the antioxidant action of melatonin. Furthermore, it has been suggested that the primary function of melatonin is to act as a free radical scavenger and broad-spectrum antioxidant and that the receptor-mediated functions of this indoleamine may have been acquired during evolution (Tan *et al.* 2007).

The rhythm of melatonin synthesis in the pineal gland is generated by the biological clock in the hypothalamic suprachiasmatic nuclei (SCN). The activity of the SCN is synchronized by the daily light-dark cycle through a direct neuronal pathway from the ganglion cells of the retinae to the SCN (retinohypothalamic fibres). At night, in the absence of light stimulating the retinae, the electrical activity of the SCN is increased and a neural message is sent from the SCN through the multisynaptic pathway to the pineal gland. (Reiter 1991a.) Increased neural activity causes the release of the neurotransmitter noradrenaline (NA) from

the postganglionic nerve endings. The released NA, in turn, regulates the activity of arylalkylamine N-acetyltransferase (AA-NAT), the limiting enzyme for the synthesis of melatonin (Reiter 1991c). During the day, light suppresses the electrical activity of the SCN and therefore diminishes the synthesis of melatonin (Reiter 1991a).

Melatonin is synthesized from tryptophan in a four-step enzymatic pathway (Fig. 2). Tryptophan is first hydroxylated and then decarboxylated, resulting in the formation of serotonin (5-hydroxytryptamine). Serotonin is converted to N-acetylserotonin by AA-NAT, and in the last step N-acetylserotonin is methylated by hydroxyindol-O-methyltransferase (HIOMT) to form melatonin. (Axelrod 1974.) There are two main pathways in the catabolism of melatonin (Fig. 2). About 60% of melatonin is hydroxylated to 6-hydroxymelatonin, which undergoes further conjugation to form either 6-sulfomelatonin or 6-hydroxymelatonin glucuronide. Furthermore, about 15% is metabolized to the $N^1$-acetyl-5-methoxy-kynurenine (AMK), while about 25% of melatonin remains unchanged. (Boutin *et al.* 2005.) All the metabolites are excreted into urine and, as in melatonin synthesis, there is a circadian rhythm in the excretion, with higher rates during darkness (Reiter 1991c).

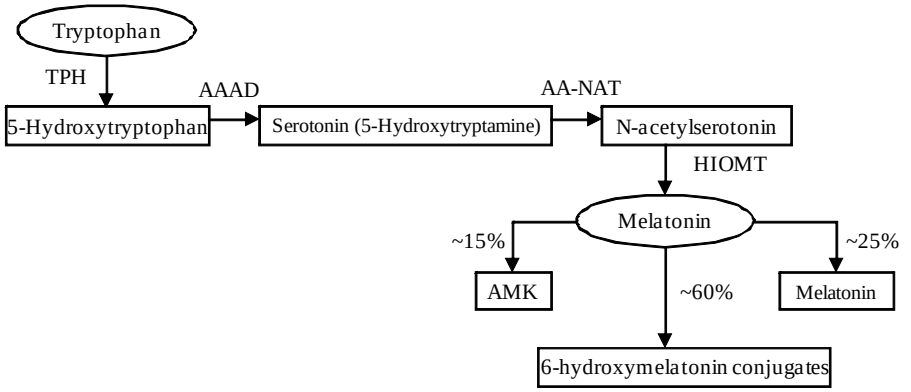


**Fig. 2. The synthesis and catabolism of melatonin. AAAD, aromatic-L-amino-acid decarboxylase; AMK, $N^1$-acetyl-5-methoxy-kynurenine; AA-NAT, arylalkylamine N-acetyltransferase; HIOMT, hydroxyindol-O-methyltranferase; TPH, tryptophan hydroxylase.**

### 2.1.1 Melatonin receptors

The first melatonin receptor gene, expressed in *Xenopus laevis* melanophores, was cloned in 1994 (Ebisawa *et al.* 1994). After that many receptors and receptor fragments have been cloned from different animal classes. The length of melatonin receptor proteins is 346-420 amino acids and their molecular weights are 39-47 kDa (Kokkola & Laitinen 1998).

The cloning of melatonin receptor genes has revealed that this family of receptors belongs to the superfamily of G-protein coupled receptors (GPCRs). Melatonin receptors have the major structural characteristics of GPCRs, i.e. they consist of a single polypeptide chain, which has seven transmembrane (TM) domains connected by intra- and extracellular loops, an extracellular N-terminus and an intracellular C-terminus (Fig. 3). In addition, there are few amino acid residues that are conserved throughout the GPCR-family. However, some specific sequence motifs found in other GPCRs are not present in melatonin receptors. These include the D/ERY (aspartic acid/glutamic acid – arginine – tyrosine) motif at the end of TM3 of other GPCRs which is replaced with a NRY (asparagine – arginine – tyrosine) in the melatonin receptors. Also, the conserved proline residue within the NP_ _Y motif in TM7 of almost all the other GPCRs is replaced by an alanine residue giving the NA _ _Y motif in melatonin receptors. (Kokkola & Laitinen 1998.)

Since melatonin receptors are located in the cell membrane, melatonin regulates the function of the cell through G-protein-regulated effectors such as ion channels and enzymes generating second messengers. In most cases it has been reported that melatonin decreases the intracellular cyclic adenosine monophosphate (cAMP) concentration (e.g. Carlson *et al.* 1989). Melatonin has also been observed to regulate other second messengers such as cyclic guanosine monophosphate (cGMP) (Vanecek & Vollrath 1989), intracellular $Ca^{2+}$ concentration (Vanecek & Klein 1992), protein kinase C (McArthur *et al.* 1997), diacylglycerol and arachidonic acid (Vanecek & Vollrath 1990). Furthermore, it has been suggested that melatonin may regulate the activity of transcription factors. It is, for example, known to be involved in regulating the expression of antioxidant enzymes (Rodriguez *et al.* 2004), and melatonin might also regulate the gene expression in the cells of the SCN and pars tuberalis (PT) (Vanecek 1998).

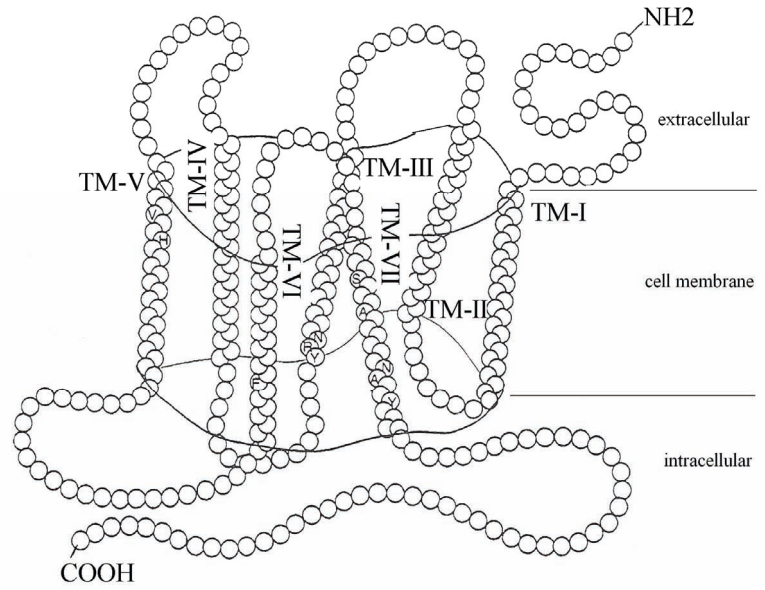


**Fig. 3. Schematic structure of the melatonin receptor (Kokkola & Laitinen 1998, figure modified by the author). TM, transmembrane.**

### *Melatonin receptor subtypes*

Based on their DNA and amino acid sequences, the melatonin receptors can be divided into three subtypes. Two of these, $MT_1$ and $MT_2$, are expressed in mammals[1], and the third, $Mel_{1c}$, is expressed in birds, amphibians and fish (Kokkola & Laitinen 1998). Two of these melatonin receptors ($MT_1$ and $Mel_{1c}$) have similar pharmacological specificity: 2-iodomelatonin > melatonin > 6-chloromelatonin > 6-hydroxymelatonin > N-acetyl-5-hydroxytryptamine > serotonin. $MT_2$ receptors differ only in that the affinity of 2-iodomelatonin, melatonin and 6-chloromelatonin is equal for this receptor. (Reppert *et al.* 1996.)

Full-length $MT_1$ receptors or their fragments have been cloned for example from man, sheep, rodents, birds, fish and *Xenopus laevis* (see Kokkola & Laitinen 1998). The $MT_1$ melatonin receptor subtype is thought to mediate the reproductive and circadian effects of melatonin, because it is abundantly

[1] The nomenclature and classification of melatonin receptors used here has been approved by the Nomenclature Committee of International Union of Pharmacology (IUPHAR) (Dubocovich *et al.* 1998).

expressed in mammalian brain (e.g. Mazzucchelli *et al.* 1996), SCN (e.g. Weaver & Reppert 1996) and PT (e.g. Reppert *et al.* 1994). Furthermore, it has been reported that *Phodopus sungorus* does not have functional $MT_2$ receptors, but it still shows normal seasonal reproductive and circadian responses to melatonin. Therefore, $MT_1$ receptors must mediate the above-mentioned effects of melatonin in this species (Weaver *et al.* 1996). $MT_1$ receptors are presumably also involved in many other physiological processes regulated by melatonin, because in addition to the different regions of the brain, these receptors are found in several peripheral tissues (Li *et al.* 1998, Fujieda *et al.* 1999, Ting *et al.* 1999, Peschke *et al.* 2000, Zhao *et al.* 2000, Woo *et al.* 2001, Chucharoen *et al.* 2003, Poirel *et al.* 2003, Aust *et al.* 2004, among others).

Full-length $MT_2$ receptors or their fragments have been cloned for example from man, rodents, chicken, fish and *Xenopus laevis* (see Kokkola & Laitinen 1998). $MT_2$ receptors have been implicated, for example, in retinal physiology (Reppert *et al.* 1995), dilating cardiac vessels (Doolen *et al.* 1998), inflammatory responses in microcirculation (Lotufo *et al.* 2001), and possibly in the cardioprotective actions of melatonin (Lochner *et al.* 2006).

$Mel_{1c}$ receptor subtype has been cloned in full length from chicken and *Xenopus laevis*, and a fragment has been cloned from zebrafish (Kokkola & Laitinen 1998). An additional receptor, which is over 40% identical to the melatonin receptors, has been cloned, but it does not bind melatonin and is often called melatonin-related receptor. Furthermore, an additional melatonin receptor, MT3 (ML2), has been proposed, but this protein belongs to the family of quinone reductases, and the affinity for melatonin of this receptor is lower than that of $MT_1$ or $MT_2$ receptors. (Barrett *et al.* 2003.) The physiological significance of this melatonin binding activity is not clear, but it might be related to the protection against oxidative stress, as quinone reductases generally prevent electron transfer reactions of quinones and through this reduce the oxidative stress (Pandi-Perumal *et al.* 2006).

In addition to membrane melatonin receptors, melatonin appears to be a ligand for the nuclear RZR/ROR receptors[2]. These receptors are suggested to mediate nuclear melatonin signalling and they are related to the transcriptional regulation effects and immunomodulator effects of melatonin (Tomas-Zapico & Coto-Montes 2005).

[2] The orphan nuclear RZR/ROR receptors form a subfamily within the superfamily of nuclear hormone receptors (Pozo *et al*. 2004)

### *Regulation of melatonin receptors*

Melatonin is released from the pineal gland in a circadian manner, and the nocturnal peak of melatonin secretion persists for hours. The density of melatonin receptors changes depending on the time of the day, and also following melatonin exposure (Vanecek & Vollrath 1989, Morgan *et al.* 1994). Prolonged exposure to melatonin is reported to result in the desensitization of $MT_1$ and $MT_2$ receptors and therefore, it is thought that receptor desensitization is one of the main factors regulating the functional, receptor-mediated, effects of melatonin (Witt-Enderby *et al.* 2003). However, the mechanisms that regulate melatonin receptor expression and receptor desensitization are complex. Receptors can be regulated in a homologous or heterologous manner. In homologous regulation, melatonin itself affects the receptor expression, and for example G-protein uncoupling, internalization or receptor down-regulation may be involved in receptor regulation. In heterologous regulation, other stimuli, such as photoperiod, regulate the receptors. (Witt-Enderby *et al.* 2003.) Studies showing that the developmental stage affects the density of melatonin receptors and observations on regulation of $MT_1$ mRNA expression independent of rhythmic melatonin secretion furthermore reveal the complexity of the mechanisms regulating melatonin receptor expression (Vanecek & Vollrath 1989, von Gall *et al.* 2002).

## *2.1.2 Free radicals and melatonin*

Free radicals, molecules with an unpaired valence electron, are very unstable and highly reactive. Among these are reactive oxygen species (ROS), which are able to damage for example lipids, proteins and DNA, and therefore induce functional and structural alterations of the cells (Tan *et al.* 1993). Antioxidants, on the other hand, are molecules that protect cells by reacting with and neutralizing free radicals (Reiter *et al.* 1997). ROS are formed in the organism during many metabolic processes and cells have an antioxidant defence system with enzymatic and nonenzymatic mechanisms. Classic enzymes involved in protection against ROS include superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GPx). Nonenzymatic antioxidants, free radical scavengers, are electron donors and can therefore neutralize ROS toxicity by removing them directly. (Tomas-Zapico & Coto-Montes 2005.)

Melatonin is known to be a highly efficient scavenger of free radicals, even more efficient than the well-known antioxidants vitamin E, ascorbic acid and

glutathione (GSH) (Pieri *et al.* 1995). Because of its lipophilicity, melatonin enters easily every cell compartment and actively reduces oxidative damage to molecules such as nuclear DNA (Tan *et al.* 1994), lipids (Longoni *et al.* 1998) and proteins (Reiter 2000). Melatonin is able to remove directly many ROS, among them the hydroxyl radical (·OH), the most reactive and toxic of the free radicals. Melatonin's interaction with free radicals forms metabolites, some of which are also effective free radical scavengers. In addition, 6-hydroxymelatonin, the hepatic metabolite of melatonin, is reported to be able to effectively scavenge free radicals. These reactions significantly increase the efficacy of melatonin to protect the cells against oxidative damage. Besides ROS, melatonin is able to neutralize and scavenge non-radical, but toxic, reactive nitrogen species (RNS), e.g. nitric oxide (NO·) and peroxynitrite ($ONOO^-$). (Reiter & Tan 2003.) These antioxidant effects of melatonin have been primarily observed using pharmacological doses of melatonin ($\geq$ nM), but there are also studies showing the relevance of melatonin as a physiological antioxidant. For example, removal of the pineal gland, which reduces endogenous melatonin levels, has been reported to exaggerate oxidative damage after treatment of rats with free radical generating agents (Reiter 1998).

Melatonin also functions as an indirect antioxidant by acting synergistically with other antioxidants and stimulating the synthesis of the major antioxidant enzymes including SOD, CAT and GPx (Reiter *et al.* 1997). Melatonin receptors are suggested to be involved in the activation of these enzymes (Tomas-Zapico & Coto-Montes 2005). The concentrations of melatonin required to activate membrane melatonin receptors (20-160 pM) are in the range of blood melatonin levels (Reiter 2000), which indicates that the physiological melatonin levels contribute to the total free radical scavenging capacity of the organism (Reiter 1998). Furthermore, this antioxidant is able to down-regulate pro-oxidant enzymes, for example NO synthases (Pandi-Perumal *et al.* 2006). It may also prevent free radical formation by reducing the electron leakage in the mitochondrial respiratory chain (Reiter & Tan 2003).

## 2.2 Cardiac muscle contraction

Calcium, the sarcolemmal L-type $Ca^{2+}$ channel (LTCC), $Ca^{2+}$ release channel (RyR) and SERCA2 are essential to the contractile function of the heart. They are fundamental in the process of excitation-contraction coupling (ECC), the process from electrical excitation of the myocyte to contraction and relaxation of the heart

(Bers 2002). The cardiac LTCC consists of $\alpha_{1C}$, β and $\alpha_2$-δ subunits. The $\alpha_{1C}$ subunit (dihydropyridine receptor, DHPR) contains the pore of the channel, and is alone capable of allowing voltage-gated calcium influx (Mukherjee & Spinale 1998). In mammals, three forms of RyRs are expressed, of which $RyR_2$ is the major $Ca^{2+}$ release channel in the heart (Marks 2000), while SERCA has five different isoforms, SERCA2a being the one expressed in the cardiac muscle (Hasenfuss 1998).

Depolarization of the cell membrane initiates the ECC by opening the DHPRs and allowing an influx of calcium, a diffusible second messenger, into the myocyte (Barry & Bridge 1993) (Fig. 4). The increase in the intracellular $Ca^{2+}$ activates the calcium-sensitive $RyR_2$s, which are located in close proximity to the DHPRs (Cheng *et al.* 1993). There is about 1 DHPR for every 5-10 $RyR_2$ channels in the cardiac muscle, and the activated $RyR_2$s release calcium from SR, leading to a massive increase in the intracellular calcium concentration (Fill & Copello 2002). Released calcium then binds to the myofilament protein troponin C and through this activates the contractile proteins and initiates the contraction (systole) (Barry & Bridge 1993).

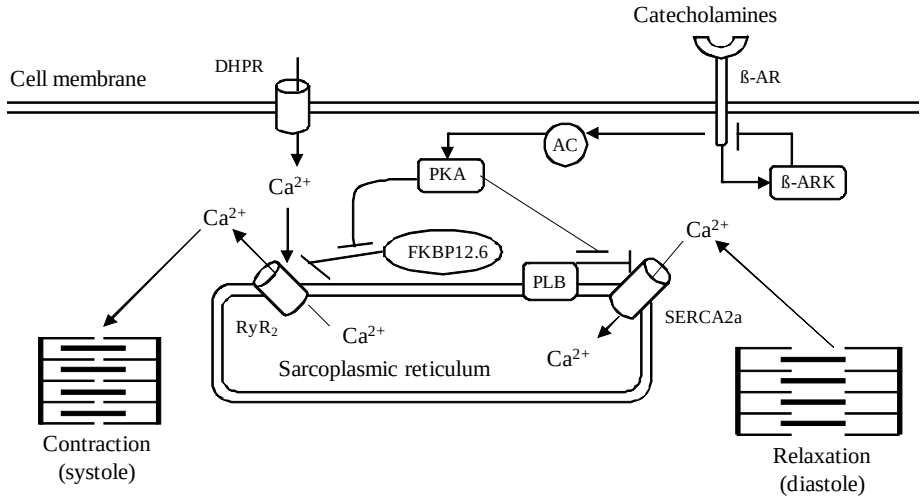


**Fig. 4. Calcium signalling during cardiac contraction (Olson 2004, figure redrawn and modified by the author). AC, adenylate cyclase; β-AR, β-adrenergic receptor; β-ARK, β-adrenergic receptor kinase; DHPR, dihydropyridine receptor; FKBP12.6, FK-506 binding protein (calstabin2); PKA, protein kinase A; PLB, phospholamban; $RyR_2$, ryanodine receptor; SERCA2a, sarco-endoplasmic reticulum $Ca^{2+}$-ATPase2.**

Signalling by adrenergic agonists also stimulates cardiac performance. $RyR_2$ is associated with several regulatory proteins, including calstabin2 (FK-506 binding protein FKBP12.6), which stabilizes the channel in a closed state, reduces its activity and prevents aberrant calcium leak from the SR (Wehrens *et al.* 2005). Phosphorylation of the $RyR_2$ by protein kinase A (PKA) results in dissociation of FKBP12.6 and increased sensitivity to calcium-induced activation. PKA, on the other hand, is activated by the elevated intracellular cAMP concentration resulting from the activation of adenylate cyclase (AC) by the signalling from the β-adrenergic receptor (β-AR). The β-adrenergic receptor kinase (β-ARK) is also activated in response to β-AR signalling and it creates a negative feedback loop. (Olson 2004.)

In order for cardiac muscle relaxation (diastole) to occur and the $Ca^{2+}$ stores to be replenished for the next contraction, most of the intracellular $Ca^{2+}$ must be removed. Phospholamban (PLB) regulated SERCA2a has main responsibility for the reuptake of $Ca^{2+}$ from cytosol back into the SR in the cardiac muscle (Bers 2002). When unphosphorylated, PLB inhibits calcium uptake by SERCA2a, but phosphorylation by PKA diminishes its inhibitory activity, thus allowing the enhanced function of SERCA2a (Olson 2004).

## 2.3 Myocardial infarction

Acute MI leads to a lack of oxygen in cardiac muscle and has deleterious effects in cardiac tissue. LV remodelling resulting from MI has a major role in the progression to heart failure and death (Gaballa & Goldman 2002).

### *2.3.1 LV remodelling*

The acute loss of myocardium induces a process of LV remodelling which alters the structure and function of the infarcted area, the border zone and remote non-infarcted myocardium. Post-infarction remodelling has been arbitrarily divided into an early phase (within 72 hours from MI) and a late phase (beyond 72 hours). (St. John Sutton & Sharpe 2000).

Early remodelling is characterized by increased loading conditions and the infarct expansion, which results in wall thinning and the dilatation of the ventricle and causes increased wall stresses. During the early phase of the remodelling many adaptive responses are also triggered. These include, for example, sympathetic activation, the activation of the renin-angiotensin-aldosterone system

and elevated production of atrial and brain natriuretic peptides (ANP and BNP). (St. John Sutton & Sharpe 2000). ANP and BNP are suggested to have many beneficial, post-infarction compensatory effects in the organism, for example against ventricular overload (Kuroski de Bold 1998). These peptides also have an effect on the blood pressure (Tamamori *et al.* 1997) and they might regulate the formation of collagen scar after MI (Magga *et al.* 2004). In addition, ANP and BNP are commonly used as diagnostic markers of heart failure (Ruskoaho 2003).

During the first hours after MI, oedema and inflammation also develop in the infarcted regions (Pfeffer & Braunwald 1990). Furthermore, MI triggers production and release from the heart of several negative inotropic mediators, such as ROS, cytokines, platelet-activating factor (PAF), arachidonic acids, and NO (Stangl *et al.* 2002). An enormous production of ROS enhances the oxidation of cellular molecules, including lipids, DNA, proteins and amino acids. Many essential proteins, e.g. ion channels, are particularly sensitive to oxidative modifications (Chakraborti *et al.* 2007).

Late remodelling is associated with time-dependent dilatation, scar formation in the infarcted area, deterioration of the contractile function and hypertrophy of the non-infarcted region (St. John Sutton & Sharpe 2000). Hypertrophy develops lengthwise with series replication of sarcomeres (Holt *et al.* 1998) and it is an adaptive response of the cardiac muscle to compensate for the increased load, progressive dilatation and decreased contractility (St. John Sutton & Sharpe 2000). However, the changes in the structure and function of the left ventricle lead to significant systolic and diastolic dysfunction, which the non-infarcted myocardium cannot sufficiently compensate to prevent the eventual development of heart failure (Goldman & Raya 1995).

### 2.3.2 The expression of DHPR, $RyR_2$ and SERCA2 after myocardial infarction

Cardiac contractility, intracellular $Ca^{2+}$ cycling and muscle relaxation are impaired in the failing heart after MI (Holt *et al.* 1998, Gomez *et al.* 2001). For example, reduced SR $Ca^{2+}$ storage and release and slow removal of $Ca^{2+}$ from the cytoplasm appear to contribute to the reduced contractility and prolongation of systole that are typical features of the failing heart (Chen *et al.* 2002). The sympathetic activation, the activation of the renin-angiotensin system and the down-regulation of the β-AR expression and function, which are seen during the LV remodelling, are also related to abnormalities in the contractility of the heart

(Wehrens *et al.* 2005, Yano *et al.* 2005). Furthermore, MI-induced free radical generation causes membrane lipid peroxidation, leads to oxidation of structural proteins and enzymes, and induces mitochondrial damage and apoptotic cell death. These changes are believed to alter membrane permeability and attenuate the functional activity of the proteins. In addition, the oxidized proteins are often susceptible to proteolytic cleavage, which may decrease the amount of the proteins. (Stangl *et al.* 2002, Chakraborti *et al.* 2007.) Since DHPR, $RyR_2$ and SERCA2a have a major role in the contractility of the heart, and changes in the expression of these proteins may be important regulators of the LV function after MI, the effect of MI on these proteins is reviewed below.

There are contradictory results in previous reports regarding the effect of MI on the expression of LTCC/DHPR. Bersohn *et al.* (1997) reported a decrease in the abundance of LTCCs after ischaemia/reperfusion *in vitro*, whereas Zucchi *et al.* (1995) found no change in the LTCC density. However, in long-term *in vivo* ischaemia studies with permanent left anterior coronary artery ligation, the amount of DHPR is generally reported to be decreased (Dixon *et al.* 1990, 8-wk ligation; Gopalakrishnan *et al.* 1991, 4-wk ligation; Zhang *et al.* 1995, 3-wk ligation). In addition, altered β-adrenergic signalling after MI modulates the function of the DHPRs by hyperphosphorylating LTCCs (Chen *et al.* 2002), which, in turn, augments $I_{Ca}$ (Yamaoka & Kameyama 2003).

$RyR_2$ protein and mRNA levels have been reported to be decreased in the ischaemic-reperfused heart (Temsah *et al.* 1999) as well as 7 (Guo *et al.* 2003, Shao *et al.* 2005) and 8 weeks (Netticadan *et al.* 2000) after MI induced by coronary artery ligation. On the other hand, Ren *et al.* (2004) found no change in the $RyR_2$ mRNA expression 40 weeks after MI. The function of $RyR_2$ is also altered after MI, because hyperphosphorylation of the $RyR_2$ causes FKBP12.6 to dissociate from the $RyR_2$ complex, resulting in uncoupling of multiple $RyR_2$s and disturbing the simultaneous opening of $RyR_2$s during systole and closing during diastole (Chakraborti *et al.* 2007). Furthermore, the dissociation of FKBP12.6 increases diastolic $Ca^{2+}$ leak from the SR, which may trigger arrhythmias (Wehrens *et al.* 2005).

Decreased SERCA2a mRNA and protein levels in the ischaemic-reperfused heart and after permanent coronary artery ligation have been observed in several earlier studies (Zarain-Herzberg *et al.* 1996, Iijima *et al.* 1998, Temsah *et al.* 1999, Netticadan *et al.* 2000, Guo *et al.* 2003, Prunier *et al.* 2005, Shao *et al.* 2005). As an exception to these results, Ren *et al.* (2004) found no change in the SERCA2 mRNA expression in their long-term ischaemia study (40-wk ligation).

However, a decrease in the expression of SERCA2a mRNA and protein is reported to be associated with impaired SERCA2a activity (Hasenfuss 1998). The hypophosphorylation of PLB in heart failure and oxidative protein fragmentation have also been suggested to be related to the deteriorating SERCA2a function (Moreau *et al.* 1998, Wehrens *et al.* 2005). Functional decline of the SERCA2a leads to increased cytosolic $Ca^{2+}$ concentrations, resulting in diastolic and systolic dysfunction (Olson 2004).

### *2.3.3 The effect of melatonin after myocardial infarction*

Heart failure following MI is associated with an increased myocardial oxidative stress and antioxidant deficit (Hill & Singal 1996). The generation of free radicals is believed to be an important factor causing several deleterious MI induced injuries in cardiac tissue and in heart contractility (Chen *et al.* 2003). Previous studies have provided evidence that antioxidant agents may play an important role in preventing post-ischaemic contractile failure, for example (Stangl *et al.* 2002).

Interestingly, nocturnal secretion of the body's natural antioxidant, melatonin, has been reported to be decreased in patients with coronary heart disease (Brugger *et al.* 1995) or acute MI (Dominguez-Rodriguez *et al.* 2002). In addition, the observed circadian rhythm of sudden death caused by heart disease correlates inversely with the rhythm of circulating melatonin, so that the rate of sudden cardiac death is highest in the early morning hours when plasma melatonin levels are at their lowest (Muller *et al.* 1987, Brzezinski 1997). Furthermore, previous reports have demonstrated that melatonin has protective effects against MI injuries (e.g. Tan *et al.* 1998, Lagneux *et al.* 2000, Lee *et al.* 2002). Most of the effects are believed to be mediated through the antioxidant action of melatonin, but melatonin receptors are also suggested to be involved in these processes (Tomas-Zapico & Coto-Montes 2005).

Mainly acute myocardial post-ischaemic effects of melatonin have been studied. Several previous experiments show that melatonin, both at pharmacological and physiological concentrations, attenuates rhythm disturbances, reduces the incidence of ventricular fibrillation and tachycardia, reduces the infarction size and improves the functional recovery after short ischaemia-reperfusion (Tan *et al.* 1998, Kaneko *et al.* 2000, Lagneux *et al.* 2000, Szarszoi *et al.* 2001, Sahna *et al.* 2002b, Dobsak *et al.* 2003, Sahna *et al.* 2005, Lochner *et al.* 2006). These beneficial effects of melatonin might, at least partly, be a consequence of the observations that melatonin enhances the activity of the

rat sarcolemmal $Ca^{2+}$ pump (Chen *et al.* 1993), increases the high-voltage activated $Ca^{2+}$ currents in chick embryonic heart cells (Mei *et al.* 2001) and reduces the hypoxia-induced intracellular $Ca^{2+}$ accumulation in rat cardiomyocytes (Salie *et al.* 2001). Melatonin is also reported to possess anti-adrenergic effect in the rat papillary muscle (Abete *et al.* 1997), and it may suppress sympathetic nerve function as well as decrease catecholamine release (Tan *et al.* 1998). Due to its antioxidant features, pharmacological concentrations of melatonin has been shown to decrease lipid peroxidation, improve the antioxidant capacity of the heart (Sahna *et al.* 2005) and suppress the promotion of apoptosis after ischaemia-reperfusion (Dobsak *et al.* 2003).

As seen here, melatonin has many beneficial effects against cardiac injuries induced by short ischaemia-reperfusion. However, the exact role of melatonin in the pathophysiology of MI and the long-term post-infarction effects of melatonin are still unclear.

# 3 Aims of the study

Melatonin, as a ubiquitous substance of the organism, is known to have an impact on the function of many different tissues. Recently, there has been an arising interest in the possible cardioprotective actions of melatonin. However, it is not always clear how these putative actions of melatonin are mediated. Melatonin has effective antioxidant properties, but it also mediates some of its effects through receptors. On the other hand, myocardial infarction has deleterious effects on the cardiac tissue and on the contractility of the heart. For example, MI-induced oxidative stress may depress the activity of the LTCC, $RyR_2$ and SERCA2a, proteins essential for proper heart muscle contraction. In addition, MI activates many adaptive responses of the organism, including the elevated production of ANP and BNP. However, there are no previous studies providing information about the effect of MI on the endogenous melatonin or the effect of exogenous melatonin on the expression of the above-mentioned fundamental cardiac proteins after MI. The post-infarction effects of melatonin on ANP and BNP levels have not been reported so far either, and the expression of melatonin receptors in the heart was also unclear. Therefore, the objectives of the present thesis were to:

1. Investigate the expression of $MT_1$ and $MT_2$ melatonin receptor mRNA in the heart and several other rat tissues (I);
2. Study the effects of MI on the synthesis and levels of endogenous melatonin as well as on the expression of $MT_1$ and $MT_2$ melatonin receptors (II);
3. Evaluate the effects of MI on the expression of DHPR, $RyR_2$ and SERCA2 during left ventricular remodelling (III);
4. Explore the effect of exogenous melatonin on the endogenous melatonin and on the expression of $MT_1$, $MT_2$, DHPR, $RyR_2$ and SERCA2 protein as well as on the concentrations of ANP and BNP after MI (IV).

# 4 Materials and methods

The materials and methods described here summarize the experimental procedures used in the studies. Detailed information is given in the original papers.

## 4.1 Animals and tissue preparations

Male (I-IV) and female (I) Sprague-Dawley rats, obtained from the Centre of Experimental Animals at the University of Oulu, were used in the experiments. Animals received food and water *ad libitum* and were maintained on a 12:12 LD cycle. In paper I samples of the heart, hypothalamus, Harderian gland, eye, small intestine, liver and blood were collected from the animals, half of which were killed at midnight (24.00 h, dim red light) and the others at noon (12.00 h). In papers II-IV MI was induced under anaesthesia by ligation of the left anterior descending (LAD) coronary artery. The sham-operated rats underwent the same surgical operation without the ligation of LAD. In paper IV, vehicle or melatonin (4.50 mg/kg per day) was administered by subcutaneous Alzet Osmotic Pumps (model 2002, Scanbur Bk Ab, Sweden) continuously for two weeks after MI. Animals were killed in the morning (9.00-10.00 h) one day (II & III), two (II-IV) or four weeks (II & III) after MI. Transthoracic echocardiography was performed for the sedated animals before decapitation, after which LV, pineal gland and blood samples were collected for further analyses. LV was washed with saline to remove blood from the tissue. All the experimental procedures were approved by the Animal Use and Care Committee of the University of Oulu (I-IV) and Oulu County Government (II-IV).

## 4.2 RNA analysis

Total RNA was isolated from the tissue samples using a QuickPrep Total RNA Extraction Kit (Amersham Pharmacia Biotech, USA; I, pineal RNA in II, MI-samples in IV), the guanidine thiocyanate-CsCl method (Magga *et al.* 1994; LV RNA in II and III) or E.Z.N.A. RNA isolation Kits (Omega Bio-tek, USA; Sham-samples in IV). For the mRNA analysis, 0.4-1.0 μg of total RNA was converted into cDNA using a First Strand cDNA Synthesis Kit (MBI Fermentas, Lithuania). Real-time quantitative RT-PCR was carried out with a TaqMan® 1000 Rxn Gold/Buffer pack reagent Kit (Applied Biosystems, USA; I) or an Absolute

QPCR ROX Mix Reagent Kit (ABgene, GB; II & III) and an ABI Prism 7700 Sequence Detection System (Applied Biosystems). In experiment IV, the analysis was done with the Roche LightCycler® 2.0 Instrument using a LightCycler® TaqMan® Master Kit (Roche Diagnostics, Germany). TaqMan probes and primers were designed with the aid of the Primer Express program (Applied Biosystems); the sequences for the primers and probes are shown in Table 1. In studies I-III each reaction contained 5 µl RT reaction product as a template and 20 µl reaction mixture (including specific primers and probe). In the amplification protocol, denaturation for 10 minutes at 95°C was followed by 40 cycles with 15 s at 95°C, 1 min annealing and extension at 60°C. In experiment IV, each reaction contained 5 µl of template and 15 µl reaction mix with specific primers and probe. The template was initially pre-incubated for 10 min at 95°C, followed by a 45-cycle program with 10 s at 95°C, 30 s at 60°C and 1 s at 72°C.

To ensure the reliability of the RT-PCR method used, a sample without RT reaction was used as a negative control during the first PCR runs. The results of the RT-PCR experiments are presented as relative mRNA ratios (gene/18S rRNA to control). Because the control sample, which was given the ratio value 1.0, was not the same in all the PCR runs, the reported relative values of the studied mRNA appearances in the different papers are not directly comparable to each other.

**Table 1. Primers and probes used in the PCR analyses.**

| Target (used in) | Primer / probe | Sequence | Accession number |
|---|---|---|---|
| AA-NAT (II, IV) | Forward | 5´-CCGCTGCCTCACGCC-3´ | U40803 |
| | Reverse | 5´-TGAGATAAAGGCTTCGCGCT-3´ | |
| | Probe | 5´-AGGATGCCACCAGTGCGTTTGAGATT-3´ | |
| DHPR (III, IV) | Forward | 5'-TTGACAATGTTCTGGCAGCC-3' | M59786 |
| | Reverse | 5'-TCTGGCCACCCCTCGA-3' | |
| | Probe | 5'-TGATGGCCCTCTTTACCGTCTCCACC-3' | |
| $MT_1$ (I, II, IV) | Forward | 5´-CAGTACGACCCCCGGATCTA -3´ | AF130341 |
| | Reverse | 5´-GGCAATCGTGTACGCCG-3´ | |
| | Probe | 5´-TCCTGTACCTTCACCCAGTCCGTCAGC-3´ | |
| $MT_2$ (I, II, IV) | Forward | 5´-ATGTTCGCAGTGTTTGTGGTTT-3´ | AF141863 |
| | Reverse | 5´-ACTGCAAGGCCAATACAGTTGA -3´ | |
| | Probe | 5´-CGCCATATGCTGGGCCCCC-3´ | |
| $RyR_2$ (III, IV) | Forward | 5'-TGCTGCGAGCCGGG-3' | AF363960 |
| | Reverse | 5'-TGGCGGTGGCGTAGGA-3' | |
| | Probe | 5'-ACTATGACCTGCTGATTGACATCCACCTCA-3' | |
| SERCA2a (III, IV) | Forward | 5'-CAGCCATGGAGAACGCTCA-3' | NM017290 |
| | Reverse | 5'-TCGTTGACCCCGAAGTGG-3' | |
| | Probe | 5'-ACAAAGACCGTGGAGGAGGTGCTGG-3' | |
| 18S rRNA (I-IV) | Forward | 5'-TGGTTGCAAAGCTGAAACTTAAAG-3' | V01270 |
| | Reverse | 5'-AGTCAAATTAAGCCGCAGGC-3' | |
| | Probe | 5'-CCTGGTGGTGCCCTTCCGTCA-3' | |

## 4.3 Western blotting

Total protein concentration of the LV samples from MI and sham rats was measured according to Bradford (1976) (II-IV). Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was carried out as previously described by Laemmli (1970) using a 0.75 mm thick 7.5% separating gel and a 3.5% stacking gel. The proteins were separated electrophoretically and transferred to nitrocellulose membrane according to Towbin *et al.* (1979). Membranes were first incubated with a primary antibody and then with a secondary antibody (Table 2), and the protein bands in the membranes were analysed densitometrically with the FluorS MultiImager program (Bio-Rad, USA).

The identification of the bands was based on the specificity of the commercial antibodies used and the molecular weights of the proteins studied. In addition to the bands of the expected molecular weights, the antibodies for $MT_1$,

$MT_2$ and SERCA2 produced another band with higher molecular weight ($MT_1$ and $MT_2$ 37 kDA vs. 50 kDA, SERCA2 100 kDa vs. 250 kDa). Precision Protein Standards (Bio-Rad) was used as a standard in each experiment.

**Table 2. List of antibodies used.**

| Antibody | Source | Used in |
|---|---|---|
| Primary antibody | | |
| Anti-Ryanodine Receptor | Sigma-Aldrich Inc, USA | III, IV |
| L-type $Ca^{++}$ CP α1C | Santa Cruz Biotechnology Inc, USA | III, IV |
| MEL-1A-R | Santa Cruz Biotechnology Inc, USA | II, IV |
| MEL-1B-R | Santa Cruz Biotechnology Inc, USA | II, IV |
| SERCA2 | Santa Cruz Biotechnology Inc, USA | III, IV |
| Secondary antibody | | |
| AP conjugated IgG, goat anti-mouse | Bio-Rad, USA | III, IV |
| AP conjugated IgG, goat anti-rabbit | Bio-Rad, USA | IV |
| AP conjugated IgG, rabbit anti-goat | Bio-Rad, USA | II-IV |

## 4.4 Radioimmunoassay (RIA)

The concentration of melatonin in plasma and different tissues was measured radioimmunologically as described earlier (Vakkuri *et al.* 1984; I, II, IV). In brief, samples were extracted with chloroform; the chloroform phase was washed with water, dried overnight and diluted into PBS (phosphate-buffered saline) for the MT-RIA. Duplicate samples of 100 µl were incubated with melatonin antiserum and 2-[$^{125}$I]-iodomelatonin and the immunocomplexes were precipitated with 2.5 M ammonium sulfate.

ANP and BNP levels were measured by RIAs from the extracted plasma and LV samples (IV). The plasma samples were extracted by SepPak cartridges (Vuolteenaho *et al.* 1992), and the eluates were redissolved in 0.5 ml of RIA buffer. ANP and BNP were extracted from the tissues by homogenizing LV samples with 40 mM HCl containing acetic acid (2 M). Homogenates were centrifuged for 30 min at 3000 rpm, the supernatants were lyophilized and reconstituted with 1.0 ml of the RIA buffer. After that, the samples for the ANP-RIA were incubated in duplicates of 25 µl with the specific rabbit antiserum and $^{125}$I-labelled $Tyr_0$-NT-pro$ANP_{79-98}$ overnight at 4°C. The bound and free fractions were separated with double antibody in the presence of polyethylene glycol. The extracted samples for the BNP-RIA were incubated in duplicates of 100 µl with the specific goat antiserum overnight at 4°C. Then, $^{125}$I-labelled r$BNP_{22-42}$ was

added and after incubation for another night, the immunocomplexes were precipitated with double antibody in the presence of polyethylene glycol.

## 4.5 Statistical analyses

Depending on the distribution of the data, differences between groups were evaluated by Student's t-test (I, III) one-way analysis of variance (ANOVA) with Tukey's post-hoc test (III) or non-parametric Kruskall-Wallis analysis and Mann-Whitney U-test (II, IV). The results are presented as mean ± SEM and the data were statistically analysed with SPSS software for Windows. Differences at the 95% level ($p<0.05$) were considered significant.

# 5 Results

This chapter summarizes the main results obtained from the previously described series of studies. Complete results are presented in the original papers.

## 5.1 The expression of $MT_1$ and $MT_2$ melatonin receptors (I, II, IV)

In paper I, $MT_1$ and $MT_2$ melatonin receptor mRNAs were shown to be expressed in several rat tissues at noon and midnight (Fig. 5). Of the tissues studied, a clearly detectable expression of both $MT_1$ and $MT_2$ receptor mRNAs was seen in the hypothalamus, retina and small intestine. Furthermore, a low expression of $MT_2$ mRNA was observed in the liver and heart sinoatrial (SA) node. The appearance of both melatonin receptor mRNAs in the heart apex and Harderian gland and the expression of $MT_1$ mRNA in liver and heart SA node was under the detection limit (I).

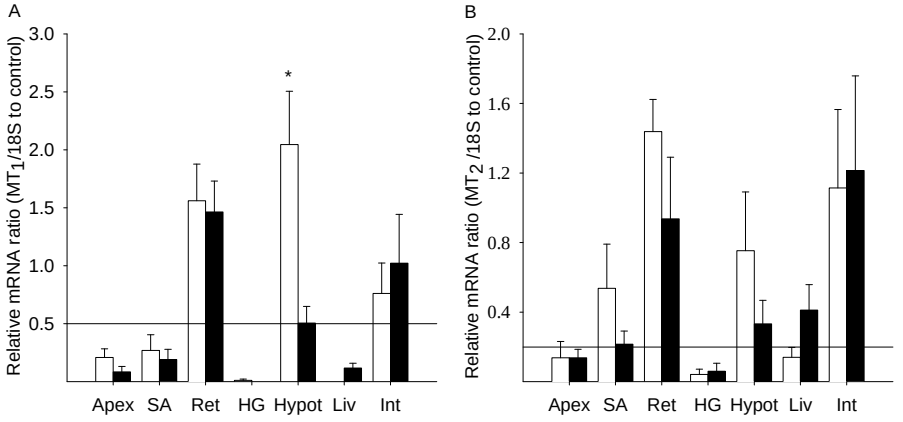


**Fig. 5. Mean relative values of the $MT_1$ (A) and $MT_2$ (B) melatonin receptor mRNAs in the studied rat tissues at noon (white bars) and at midnight (black bars). Each bar represents the mean (±SEM) of 5 to 8 animals. Tissues with no detectable mRNA expression were defined as those with a ratio value below 0.5 for $MT_1$ mRNA and 0.2 for $MT_2$ mRNA. The relative values of the $MT_1$ and $MT_2$ receptor mRNA appearances are not directly comparable to each other due to the difference between pools. *P < 0.5 versus midnight. Apex indicates heart apex; SA = heart SA node; Ret = retina; HG = Harderian gland; Hypot = hypothalamus; Liv = liver; Int = small intestine. Figure modified from paper I.**

However, in studies II and IV, the expression of both $MT_1$ and $MT_2$ receptors in the rat left ventricle was confirmed by Western blotting (proteins) and real-time quantitative RT-PCR (mRNAs) (II: Fig. 2 and Fig. 3, IV: Fig. 2).

A significant difference ($P < 0.05$) in the expression of the $MT_1$ receptor mRNA was shown in the hypothalamic tissue between noon and midnight, whereas no changes were observed in the other tissues in the expression of either receptor mRNA between day and night (Fig. 5) (I).

## 5.2 Echocardiography (II-IV)

In papers II-IV, echocardiography was used to characterize the effect of MI on the LV structure and function one day (II, III), two weeks (II-IV) and four weeks (II, III) after coronary artery ligation (II: Table 1, III: Table 1, IV: Table 2). Serious post-infarction systolic dysfunction of the LV was seen since LV ejection fraction and fractional shortening of MI rats were significantly decreased after MI compared with the control hearts (II-III: $P < 0.001$, IV: $P < 0.01$). The observed thinning of the anterior wall and dilatation of the LV of the MI hearts indicated further the remodelling process of the LV after MI (II-III: $P < 0.001$, IV: $P < 0.01$).

## 5.3 The effect of myocardial infarction on endogenous melatonin (II)

To investigate the effect of MI on endogenous melatonin, the time course of changes in the synthesis, concentration and receptor expression of melatonin was examined one day, two weeks and four weeks after MI. Experimentally induced MI was shown to increase pineal melatonin synthesis significantly, as evaluated by the expression of AA-NAT mRNA (the rate-limiting enzyme for the synthesis of melatonin) (II, Fig. 1A). On the first post-infarction day, the AA-NAT mRNA expression was 4.3-fold ($P < 0.001$) in the pineals of MI rats compared with sham-animals. Two weeks after the infarction, the difference was still 1.7-fold ($P < 0.05$). The elevated AA-NAT mRNA expression was associated with the increase in the plasma and LV melatonin concentrations (II, Fig. 1C-D). In the LVs of MI rats, the levels of melatonin were 1.4 times ($P = 0.01$) and in plasma 1.6 times ($P < 0.001$) higher than in sham-operated animals one day after MI. No statistical differences were seen in the pineal melatonin concentrations between MI and sham-operated animals in the experiment (II, Fig 1B). In addition, the

melatonin synthesis and the concentration in LV and plasma of MI rats returned to the normal level during the four-week follow-up period.

The expression of both $MT_1$ and $MT_2$ melatonin receptors was altered after MI (Fig. 6).

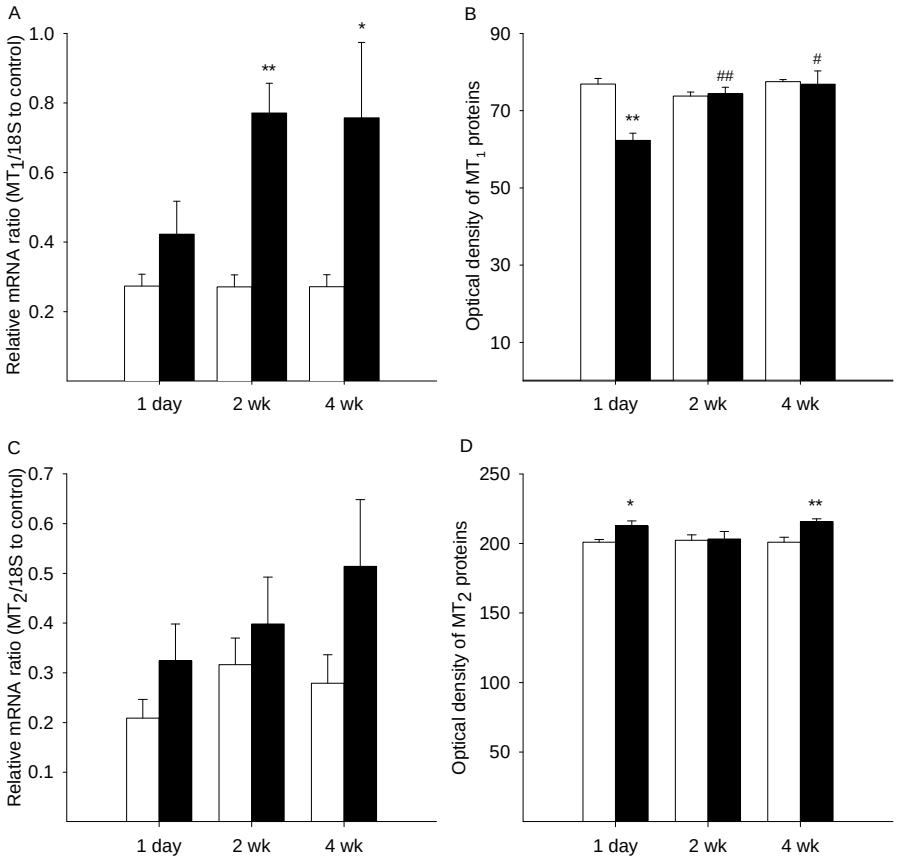


**Fig. 6. Mean relative values of the $MT_1$ receptor mRNA (A), optical density of $MT_1$ receptor proteins from Western blots (B), $MT_2$ receptor mRNA (C) and optical density of $MT_2$ receptor proteins from Western blots (D) of the rat left ventricle 1 day, 2 weeks and 4 weeks after MI. White bars represent samples of sham-operated hearts and black bars MI hearts. Each bar represents the mean (±SEM) of 5 to 8 animals. *P < 0.05 versus sham, **P < 0.01 versus sham, #P < 0.05 versus MI 1 day, ##P < 0.01 versus MI 1 day. Figure modified from paper II.**

$MT_1$ mRNA levels of the MI LVs were 2.8 times ($P < 0.01$) higher than in control LVs two weeks after MI, and a significant difference was also seen at four weeks (2.3-fold, $P > 0.05$) (Fig. 6A). The amount of $MT_1$ proteins, on the other hand, was decreased by 19% ($P < 0.01$) in MI LVs compared with control LVs one day after infarction, followed by recovery during the next two weeks (Fig. 6B). In the expression of $MT_2$ mRNA, a slight upward trend was seen in the MI LVs during the four-week follow-up period after MI, but the differences between MI and sham-operated groups were not statistically significant (Fig. 6C). As opposed to the amount of $MT_1$ receptors, $MT_2$ receptor proteins in MI LVs were observed to be increased one day ($P < 0.05$) and four weeks ($P < 0.01$) after MI when compared with the control LVs (Fig. 6D).

## 5.4 The effect of myocardial infarction on the expression of DHPR, $RyR_2$ and SERCA2 (III)

The time course of changes in the expression of LV DHPR, $RyR_2$ and SERCA2 one day, two weeks and four weeks after MI was investigated in order to evaluate the effect of MI on the expression of these proteins. MI was shown to be associated with a transiently reduced mRNA expression followed by recovery toward control values during the four-week follow-up period (III, Fig. 1). One day after the infarction, the expression of DHPR mRNA was decreased by 67% ($P < 0.001$), $RyR_2$ mRNA by 54% ($P < 0.01$) and SERCA2a mRNA by 55% ($P = 0.06$) in the MI LVs compared with control LVs. For the DHPR mRNA, the difference between MI and sham hearts was still significant ($P = 0.001$) at two weeks (III, Fig. 1A), and for $RyR_2$ mRNA the difference was observed even four weeks after MI ($P < 0.01$) (III, Fig. 1B). However, the post-infarction recovery in the mRNA expression of each protein in the MI LVs was remarkable; DHPR mRNA expression was increased to 2.9-fold ($P < 0.01$), $RyR_2$ mRNA to 1.7-fold ($P < 0.01$) and SERCA2a mRNA to 2.4-fold ($P < 0.05$) at four weeks compared to the expression seen at one day after MI (III, Fig. 1).

In addition to the changes in mRNA levels of the above-mentioned proteins, MI was associated with a decreased amount of SERCA2 proteins in MI LVs (III, Fig. 2C). At the time of four weeks, the quantity of these proteins in the MI group was significantly lower ($P < 0.05$) than that observed on the first post-infarction day. The amount of DHPR proteins in MI LVs also tended to decrease during the myocardial remodelling after MI; the amount being 34% ($P = 0.06$) lower at four weeks compared to one day after MI (III, Fig. 2A). Despite the observed changes

in the $RyR_2$ mRNA, MI did not seem to have an effect on the amount of $RyR_2$ proteins (III, Fig. 2B).

## 5.5 The effects of exogenous melatonin after myocardial infarction (IV)

To examine the post-infarction effects of exogenously given melatonin on the expression of LV DHPR, $RyR_2$, SERCA2, $MT_1$ and $MT_2$ proteins, on endogenous melatonin as well as on the levels of plasma and LV ANP and BNP, subcutaneous pumps were used to administer melatonin continuously for two weeks after MI. As presumed, melatonin pumps multiplied plasma and LV melatonin concentrations compared to rats with vehicle pumps (IV, Table 3). Exogenous melatonin was also shown to alter the mRNA expression of DHPR, $RyR_2$ and SERCA2a, since the expressions were significantly lower in the MI LVs of melatonin-treated rats compared with vehicle rats two weeks after MI (IV, Fig. 1A). For the expression of DHPR mRNA, the difference between MI LVs of these groups was 74% ($P < 0.01$), for $RyR_2$ mRNA it was 83% ($P < 0.05$) and for SERCA2a the difference was 69% ($P < 0.05$). Contrary to the altered mRNA expression, the effect of exogenously given melatonin was not seen in the amount of these proteins two weeks after MI (IV, Fig. 1B).

Relating to the expression of melatonin receptors, the post-infarction effects of two weeks' melatonin supply were only seen in the amount of $MT_2$ receptor proteins, which was 1.9 times higher ($P < 0.05$) in the LVs of melatonin-treated MI rats compared to vehicle MI rats (IV, Fig. 2B).

Furthermore, the concentrations of ANP and BNP were significantly elevated in the LVs of MI animals compared with sham-operated rats two weeks after MI ($P < 0.05$ for BNP in both groups and for ANP in MI + vehicle group; $P < 0.01$ for ANP in MI + melatonin group) (IV, Fig. 3). In addition, exogenously given melatonin elevated the MI LVs' ANP levels to over 5-fold ($P < 0.05$) compared with MI LVs of vehicle rats (IV, Fig. 3A). The BNP concentration in the MI LVs or either NP concentration in the plasma of the MI rats was not significantly affected by two weeks' post-infarction melatonin administration, even though a slight upward trend might have been seen compared with MI + vehicle rats.

# 6 Discussion

An experimental model of MI, induced by ligation of the left anterior descending coronary artery of the rat, was used in the present study to investigate some aspects of the wide-ranging field relating to melatonin and MI. The role of endogenous melatonin and the expression of $MT_1$ and $MT_2$ melatonin receptors after MI were studied. In addition, the possible effect of melatonin on the post-infarction contractile function of the heart was examined by evaluating the expression of DHPR, $RyR_2$ and SERCA2 after MI. Furthermore, the relationship between melatonin and the widely used diagnostic markers of heart failure, ANP and BNP, was assessed.

In order to perform the above-mentioned analyses, the real-time quantitative RT-PCR method was used to determine the expression of mRNAs, and the amount of proteins was defined by Western blotting. In addition, transthoracic echocardiography was used to characterize the post-infarction structural and functional changes of the heart. Melatonin and natriuretic peptide concentrations were measured by RIAs.

## 6.1 Endogenous melatonin

The rhythmically released, lipophilic pineal melatonin is known to be involved in various physiological functions depending on the circadian rhythm (Vanecek 1998), but it is also known to affect the function of many different tissues, including the heart (Wiechmann *et al.* 1988, Antolin *et al.* 1996, Poon *et al.* 1997, Kaneko *et al.* 2000, Ohta *et al.* 2000, Hunt *et al.* 2001). In the recent years the possible cardioprotective actions of melatonin have received increasing attention. In the majority of these studies, pharmacological concentrations of melatonin have been used, e.g. (Tan *et al.* 1998, Lagneux *et al.* 2000, Sahna *et al.* 2005), but Sahna *et al.* (2002a, 2002b) showed that also physiological melatonin concentrations have beneficial effects on the morphology and function of the post-ischaemic heart.

The results of the present study (II), support the idea that endogenous melatonin has an important role in protecting the heart against MI-induced injuries. Melatonin synthesis, as evaluated by the expression of the pineal AA-NAT mRNA, was rapidly elevated in response to the MI, being already significantly higher in MI rats compared with sham-operated animals on the first post-infarction day. This acute increase in the mRNA of the penultimate enzyme

of melatonin biosynthesis was associated with elevated concentrations of melatonin in the plasma and LV of MI rats. The observation that melatonin seems to be a naturally protective agent against injuries due to MI is noteworthy, especially in the case of older people, as plasma melatonin levels have been observed to decrease with age (Waldhauser *et al.* 1998). Since free radicals have been suggested to be one of the main factors causing the MI-induced cardiac damage (Dhalla *et al.* 2000) and melatonin is known to have efficient antioxidant properties (Tan *et al.* 1993), low melatonin concentrations might predispose to myocardial diseases by diminishing the beneficial protective effects of melatonin.

Melatonin has several features that distinguish it from the 'classic' antioxidants, such as vitamin C, vitamin E and GSH, and arouse interest in possible usage of melatonin as a cardioprotective agent. Besides being a more efficient antioxidant (Pieri *et al.* 1995), melatonin is able to diffuse easily through the cell membranes due to its lipophilic and hydrophilic character, and therefore mediate its antioxidant effects directly to the cytosolic and nuclear structures in all compartments of the cell (Reiter 1991c). In addition, the antioxidative mechanisms of melatonin seem to differ from other antioxidants, for example from those of vitamins C and E, and GSH. As these are electron donors, they may prevent oxidation as well as promote it. Melatonin, on the other hand, is an electron-rich molecule, which does not promote oxidation, but interacts with free radicals via an additive reaction to form several stable end products. In addition, some metabolites, such as AMK and 6-hydroxymelatonin, generated by oxidation of melatonin, are regarded effective free radical scavengers. Thereby, a sort of scavenging cascade reaction is formed. (Tan *et al.* 2000.) Furthermore, melatonin acts synergistically with other antioxidants (Reiter *et al.* 1997), stimulating the expression of a number of antioxidative enzymes (Rodriguez *et al.* 2004). Based on these beneficial properties, and considering melatonin's low toxicity and the fact that it is inexpensive to produce, melatonin deserves attention as a potential post-ischaemic therapeutic agent (Reiter *et al.* 2002).

In addition to myocardial injuries, melatonin might be a naturally protective agent anywhere in the organism against stress and tissue damage. This is suggested by the observations made in papers II and IV, where melatonin concentrations in plasma and LV of sham-operated animals were unpredictably high. This might be a consequence of possible tissue damage, even inflammatory response, produced by the sham operation and the implantation of subcutaneous pumps. Therefore, the generation of free radicals might be increased in the surgical areas, and this might lead to elevated melatonin concentrations.

Furthermore, melatonin is reported to have anti-inflammatory (Wu *et al.* 2001) as well as antiapoptotic (Dobsak *et al.* 2003) effects, which are important mechanisms for protecting the organism against tissue damage, including the MI-induced injuries in the heart.

## 6.2 The expression of $MT_1$ and $MT_2$ melatonin receptors

Due to the strong evidence that free radicals are involved in incurring the cardiac injuries after MI (Chen *et al.* 2003), and because the expression of melatonin receptor subtypes in the rat heart has been unclear, it is not surprising that it is mainly the free radical scavenging properties of melatonin that have been suggested to explain its post-ischaemic protective effects, e.g. (Kaneko *et al.* 2000, Lee *et al.* 2002, Chen *et al.* 2003, Sahna *et al.* 2005). The possibility of melatonin receptor involvement in the cardioprotective actions of this indole has been brought out in some studies only recently (Tomas-Zapico & Coto-Montes 2005, Lochner *et al.* 2006, Rezzani *et al.* 2006).

In view of the uncertainty regarding the mechanism that mediates the effects of melatonin in the heart and other tissues, the expression of both $MT_1$ and $MT_2$ receptor mRNAs was examined in several rat tissues in the paper I. The observed expression of $MT_1$ and/or $MT_2$ receptor mRNA in the hypothalamus, retina, small intestine and liver confirmed and extended the results of previous studies (Fujieda *et al.* 1999, Sugden *et al.* 1999, Poon *et al.* 2001, Poirel *et al.* 2003), indicating that membrane receptors might, at least partly, be involved in mediating the effects of melatonin in these tissues. Regardless of this, the results of study I could not thoroughly clarify the prevailing ambivalence relating to the expression of $MT_1$ and $MT_2$ receptor mRNAs in the heart. $MT_2$ mRNA expression was found in the heart SA node, but the expression of $MT_1$ receptors was not detectable in either heart apex or SA node at midnight and noon. However, this issue was clarified further in studies II and IV, where the expression of both receptor subtypes in the rat LV was proved by showing the presence of proteins and mRNAs of $MT_1$ and $MT_2$ melatonin receptors using Western blotting and real-time quantitative RT-PCR method.

When studying the effect of MI (II) and post-infarction exogenous melatonin administration (IV) on the expression of LV $MT_1$ and $MT_2$ melatonin receptors, it was observed that MI altered the expression of both receptor subtypes, but subcutaneous melatonin supply after MI only seemed to have an impact on the amount of $MT_2$ receptor protein. The findings that, in contrary to $MT_1$ receptors,

the amount of LV $MT_2$ receptor proteins seemed to be maintained right after MI (II), and that exogenously given post-infarction melatonin even increased the amount of these receptors (IV), could indicate a more important role of $MT_2$ receptors in the protection of the heart against MI-induced injuries compared with $MT_1$ receptors. In the literature, there are very few previous studies considering the role and possible differences in the function of $MT_1$ and $MT_2$ melatonin receptor subtypes in the heart after MI. However, our hypothesis relating to the importance of functional cardiac $MT_2$ receptors after MI would agree with Chen *et al.* (2003), who used $MT_1$ melatonin receptor-deficient mice in their study, reporting that the post-infarction cardioprotective effects of melatonin are not dependent on $MT_1$ receptors. In addition, Lochner *et al.* (2006) and Rezzani *et al.* (2006) showed that luzindole, a melatonin receptor antagonist, which has a higher affinity for the $MT_2$ than for the $MT_1$ receptor subtype (Dubocovich *et al.* 1997), interfered with the protective actions of melatonin in the rat heart. However, in the present thesis, it was also shown that $MT_1$ melatonin receptors are expressed in the heart after MI, and that their decreased post-infarction expression was recovered two weeks after MI. Thereby, according to the present knowledge, the possible role of $MT_1$ receptors in the protection of the heart against post-infarction injuries cannot not be ruled out.

To date, when considering their cardioprotective actions, melatonin receptors are proposed to mediate particularly melatonin's potential to stimulate the major antioxidant enzymes (Mayo *et al.* 2002, Tomas-Zapico & Coto-Montes 2005). Tomas-Zapico & Coto-Montes (2005) suggested that a nuclear transcription factor, RORα, which is activated by $Ca^{2+}$/calmodulin (CaM)-dependent kinases, downregulates the antioxidant gene expression of the cell. On the other hand, RORα is a nuclear receptor for melatonin, and melatonin seems to be able to block the RORα activity through direct interaction with calmodulin or through $MT_1$ and/or $MT_2$ melatonin receptors. Therefore, via restraining the RORα transcriptional activity, melatonin may modulate antioxidant enzyme production, and its membrane receptors are probably involved in this. (Tomas-Zapico & Coto-Montes 2005.) However, additional investigations are needed to clarify further the post-infarction importance of both melatonin receptor subtypes.

## 6.3 The expression of DHPR, $RyR_2$ and SERCA2

A progressive depression in cardiac contractility and relaxation are typical features of the heart failure caused, for example, by MI. Contractile dysfunction is suggested to be a result of the post-ischaemic abnormalities in $Ca^{2+}$ regulation and $Ca^{2+}$ signalling (Houser & Margulies 2003). Alterations in the abundance or activity of the fundamental cardiac calcium handling proteins, DHPR, $RyR_2$ and SERCA2, might cause abnormal $Ca^{2+}$ transients in the failing heart (Houser *et al.* 2000). Therefore, a sufficient amount and function of these proteins, necessary for proper contractility of the heart, seems to be essential for the body after MI. In addition, MI-induced increased oxidative stress is observed to modify the cellular proteins (Chakraborti *et al.* 2007) and to result in contractile dysfunction (Dhalla *et al.* 2000). Since melatonin is known to be a highly efficient antioxidant of the organism (Tan *et al.* 1993), one interest of this thesis was to investigate the effect of MI, and especially the effect of exogenous melatonin administered after MI, on the contractility of the heart as evaluated by the expression of DHPR, $RyR_2$ and SERCA2 in the rat LV.

The LV mRNA expression of these proteins was demonstrated to decrease immediately after MI, but the expression levels recovered toward control values during the four-week follow-up period (III). Despite the mRNA recovery, a decreasing trend in the amounts of DHPR and SERCA2 proteins was seen four weeks after MI. Interestingly, when melatonin was given subcutaneously for two weeks after MI, the mRNA expression of DHPR, $RyR_2$ and SERCA2 of these LVs was even lower than that in the LVs of vehicle-treated MI animals, but the amount of the studied proteins in the LVs of both groups was at the same level (IV). These results support the previous reports that MI alters the expression of DHPR, $RyR_2$ and SERCA2, e.g. (Gopalakrishnan *et al.* 1991, Shao *et al.* 2005), and also bring out a new aspect for melatonin's cardioprotective properties; this indoleamine might contribute to the post-infarction contractile function of the heart by regulating the expression of DHPR, $RyR_2$ and SERCA2.

Proper cardiac contractility requires successful, consecutive processes by the studied $Ca^{2+}$ handling proteins, and therefore, alterations in one or more of these processes probably have effects on the cardiac function after MI. It has been reported that the amount of DHPRs seem to decrease after MI, e.g. (Zhang *et al.* 1995, III), and MI may also modulate the function of this $Ca^{2+}$ channel (Gomez *et al.* 2001). These changes would probably deteriorate cardiac contractility by reducing the influx of $Ca^{2+}$ into the myocyte, and thereby decreasing the SR $Ca^{2+}$

release. In addition, the observed post-ischaemic changes in the abundance, e.g. (Shao *et al.* 2005), and function (Wehrens *et al.* 2005) of $RyR_2$s further promote the failure of ECC, and may lead to the increased cytosolic $Ca^{2+}$ during diastole by causing an abnormal $Ca^{2+}$ leak from the SR (Yano *et al.* 2005). Furthermore, the level of functional SERCA2 proteins in the SR is suggested to be one of the fundamental determinants of cardiac contractility, and a direct correlation between SERCA2 level and contractile state of the heart has been reported (Periasamy & Huke 2001). The decline in the amount and function of the SERCA2 proteins in cardiac disease reported in earlier studies (Zarain-Herzberg *et al.* 1996, Wehrens *et al.* 2005, III) results in decreased SR $Ca^{2+}$ uptake, SR $Ca^{2+}$ load, and through this in attenuated contractile function. Taken together, all of these $Ca^{2+}$ handling proteins have an important role in the contraction of the heart, and it has also been suggested that there may be cross-talk or functional dependence between these proteins (Periasamy & Huke 2001). Therefore, the importance of studying the factors that could protect the DHPR, $RyR_2$ and SERCA2 proteins against post-MI damages, and possibly ensure sufficient contractile function after MI, is notable.

The results of the present thesis (IV) could indicate that melatonin may be one of these factors. A reason for the observed difference in the mRNA expression, but not in the protein amounts, of DHPR, $RyR_2$ and SERCA2 between melatonin- and vehicle-treated MI rats, might be melatonin's protective effect on these $Ca^{2+}$ handling proteins after MI. This, in turn, would reduce the demand for new protein synthesis. The mechanism for this suggested effect of melatonin was not investigated, but it might be, at least partly, mediated through the free radical scavenging activity of melatonin, since free radicals are reported to decrease the amount and alter the function of the cardiac DHPRs (Guerra *et al.* 1996), $RyR_2$s (Holmberg *et al.* 1991) and SERCA2s (Moreau *et al.* 1998). Some of the possible protective effects might also be mediated through the cardiac membrane melatonin receptors by stimulating the synthesis of the other antioxidant enzymes, as described earlier.

In addition, at least one more hypothesis relating to melatonin's possible contribution to the cardiac contractility after MI could be proposed. Left ventricular remodelling following MI is associated with increased, chronic sympathetic activation and alterations in β-adrenergic signalling (St. John Sutton & Sharpe 2000). This, on the other hand, causes, for example, PKA-mediated hyperphosphorylation of the LTCCs (Chen *et al.* 2002), $RyR_2$s (Yano *et al.* 2005) and PLBs (Wehrens *et al.* 2005), which attenuates the cardiac contractile function.

Blockade of β-adrenergic receptors has been reported to restore the cardiac function and to reduce the mortality rate in patients with heart failure (Bristow 2000) by, for example, reducing intracellular cAMP levels and decreasing the activity of PKA (Wehrens *et al.* 2005). In turn, Abete *et al.* (1997) showed that melatonin possesses receptor-mediated anti-adrenergic effects in cardiac muscle, and they suggested that melatonin may act through a reduction of cAMP accumulation. In addition, melatonin may decrease catecholamine release and suppress sympathetic nerve function (Tan *et al.* 1998). Furthermore, antioxidants are reported to protect against the alterations in β-adrenergic signalling due to ischaemia-reperfusion (Persad *et al.* 1997). Thereby, it could be assumed that melatonin might improve the cardiac contractility after MI also by normalizing the altered β-adrenergic signalling through its receptor-mediated as well as antioxidant actions. This hypothesis would, however, need more investigation to be confirmed.

## 6.4 ANP and BNP concentrations

Cardiac ANP and BNP synthesis and secretion are elevated after MI. This increase is generally seen as a cardioprotective mechanism, given the many beneficial, compensatory effects of these peptides on the function of the heart. ANP and BNP are suggested to act as circulating hormones, but also as autocrine and/or paracrine factors (Nishikimi *et al.* 2006). They have, for example, an important role in regulating blood pressure and blood volume through natriuretic, diuretic, and vasodilatory properties and inhibition of the renin-angiotensin-aldosterone system (McGrath *et al.* 2005). In addition, ANP and BNP suppress the activity of the sympathetic nervous system (McGrath *et al.* 2005), and they inhibit cardiac fibroblast proliferation (Tamamori *et al.* 1997) and myocyte hypertrophy (Horio *et al.* 2000). In ischaemia-reperfusion related injuries, exogenously given ANP was shown to reduce infarct size (Okawa *et al.* 2003) and to improve the recovery of the cardiac function (Sangawa *et al.* 2004) in isolated rat hearts. Furthermore, Kasama *et al.* (2007) reported that ANP prevented LV remodelling in patients with AMI. As seen, ANP and BNP affect the post-ischaemic morphology and function of the heart in several ways.

In the present study (IV), it was shown for the first time that post-MI administered melatonin even increased the already elevated LV ANP level, and tended to increase the LV BNP level. The finding reveals one more possible mechanism by which melatonin might protect the heart against MI-induced

injuries. By contributing to the LV concentrations of these peptides, melatonin probably reinforces the beneficial autocrine and/or paracrine effects of cardiac ANP and BNP after MI, because the effect of exogenous melatonin on the circulating plasma ANP and BNP levels in MI rats was not clearly seen (IV). It would be interesting to study further the observed, novel positive relationship between melatonin and ANP, and to investigate the undefined mechanism through which this interaction is mediated, since both melatonin and natriuretic peptides are suggested to be promising therapeutic agents for the treatment of cardiac diseases (Reiter *et al.* 2002, Nishikimi *et al.* 2006).

## 6.5 Considerations of the experimental methods used

In the present thesis, the real-time quantitative RT-PCR method was used to measure the mRNA levels of the genes studied. This technique has many advantages over conventional RT-PCR; it simplifies and accelerates the quantification process as well as combines high sensitivity with reliable specificity (Bustin 2000). However, there might also appear some problems, for example with RNA quality, detection specificity and data normalizing, which are important to take into account. Therefore, in these studies, care was taken to carry out all the work from tissue sampling to performing PCR in sterile conditions. Also, in addition to the primers, target-specific TaqMan probes were used to ensure specificity. For normalizing the data, 18S rRNA was chosen, because of its abundance and inertness to external stimuli. For example, Bas *et al.* (2004) showed that 18S rRNA is a stable housekeeping gene and normalization to 18S rRNA gave a result that reflected target gene mRNA expression levels of the cell.

Furthermore, it can be argued that detected mRNA levels may not always necessarily reflect the levels of corresponding proteins produced by the cell (Gygi *et al.* 1999). This is true, since many kinds of post-transcriptional modulation occur. However, measuring the mRNA expression levels gives significant information of possible up- or downregulation of the protein synthesis, for example after MI. Nevertheless, the actual amount of proteins is also important to evaluate, as was done in the present thesis (II-IV).

Tissue and plasma melatonin concentrations were measured using a well-validated RIA (Vakkuri *et al.* 1984, Vakkuri *et al.* 1985). In study I, the tissues were not washed with saline after preparation, and therefore, some of the detected melatonin might have been derived from the blood. However, in papers II-IV, prepared hearts were rinsed with saline to diminish the blood contamination.

To mimic human heart failure, MI was induced by coronary artery ligation (II-IV). This is a widely used rat model of heart failure, since the progression of myocardial failure seen in this model has many features that are similar to the clinical syndrome of heart failure after MI in people. In addition, the response to the pharmacological therapy in rats seems to predict what will happen in humans given the same treatment. (Goldman & Raya 1995.) Of course, we always have to be careful when extrapolating the results from animal studies to people due to the possible differences between species physiology. However, these studies are often a prerequisite when developing effective treatments for human heart failure, since they provide important information, including data about myocardial changes and the effects of different treatments during the progression of LV remodelling and heart failure.

# 7 Conclusions and prospects

Two aspects relating to melatonin and MI were the main focus in the studies of this thesis. First, the synthesis, concentration and receptor expression of endogenous melatonin were evaluated to deepen the knowledge concerning the role of melatonin as a protective agent against MI injuries. Secondly, the expression of DHPR, $RyR_2$ and SERCA2 was explored in order to investigate the possible beneficial effects of melatonin on the post-MI contractile function. Based on the results of this thesis and previous studies, a simplified, hypothetical model of certain relationships between MI, melatonin and cardiac contractility is presented in Fig.7.

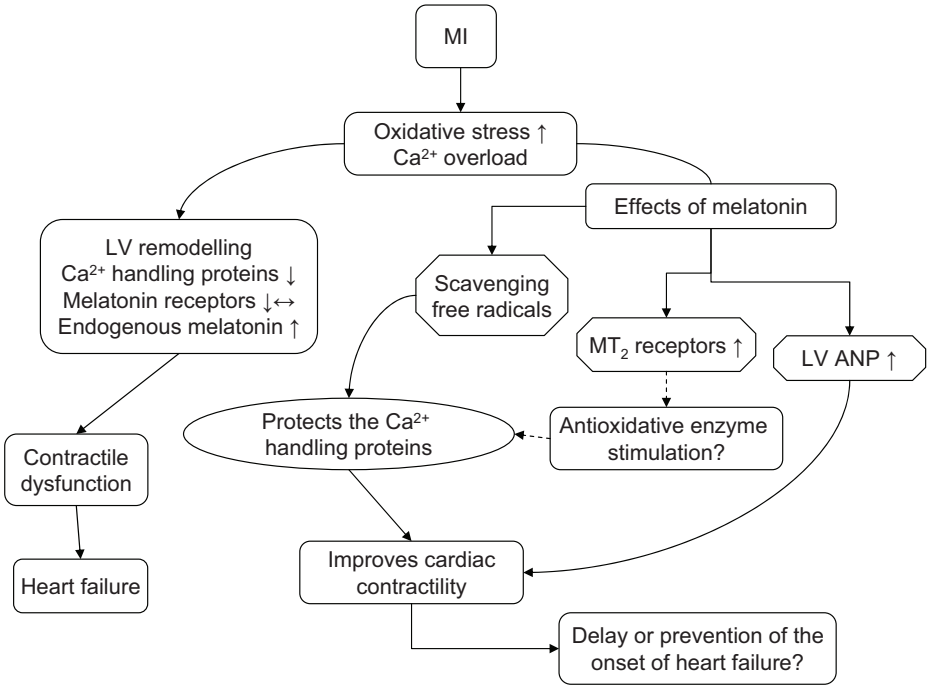


**Fig. 7. A simplified, hypothetical model of certain relationships between myocardial infarction, melatonin and cardiac contractility. MI indicates myocardial infarction, LV = left ventricle, ANP = atrial natriuretic peptide, ↑ = increasing levels, ↓ = decreasing levels, ↔ = no changes.**

The present findings confirm the previously suggested importance of endogenous melatonin in protecting the heart after MI. Furthermore, the results regarding the $MT_1$ and $MT_2$ receptor expression help to unravel the mechanism through which melatonin mediates its effects in different tissues. Since melatonin receptors were observed to be expressed in several rat tissues, and MI as well as exogenous melatonin altered this expression in the heart, it is proposed that in addition to melatonin's free radical scavenging properties, melatonin receptors could have a role in mediating the actions of melatonin in these tissues.

Furthermore, melatonin was shown to have an impact on the expression of the essential $Ca^{2+}$ handling proteins; DHPR, $RyR_2$ and SERCA2 after MI. This observation indicates indirectly, for the first time, that melatonin might protect these proteins against MI-induced injuries, and therefore contribute to the post-infarction contractile function of the heart. In addition, a novel finding of the positive relationship between melatonin and LV ANP concentration reveals one more possible way for melatonin to protect the heart after MI.

Even if the present results add new information to the knowledge of melatonin's role and effects after MI, they also show that there are still areas that need to be clarified in order to understand better the role of melatonin in the pathophysiology of MI. Additional studies are needed to specify further the post-infarction importance of different melatonin receptor subtypes to elucidate the mechanisms that mediate the cardioprotective effects of melatonin. Since proper contraction of the heart is essential for life, and the recovery of cardiac function is vital after MI, the effects of melatonin, particularly on the function of the $Ca^{2+}$ handling proteins, also deserve further investigation. For example, different binding or activity studies might confirm the indirect evidence of melatonin's protective effect on the post-infarction cardiac contractility received in this study. In addition, studies on the observed interaction between melatonin and ANP would be important to widen the understanding of the broad spectrum of melatonin's functions in the organism.

In summary, it is not difficult to see melatonin as a promising therapeutic agent in cardiac diseases, and further studies, including human ones, are reasonable.

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