Univerzita Karlova v Praze

1. lékařská fakulta

Studijní program: Biomedicína

Studijní obor: Fyziologie a patofyziologie člověka

MUDr. Jan Beneš

Regulace receptorů spřažených s G proteiny

Studie muskarinových a β–adrenergních receptorů u M_2KO myší

Regulation of G protein-coupled receptors

Study of muscarinic and β–adrenoceptors in M_2KO mice

Disertační práce

Školitel: prof. MUDr. Jaromír Mysliveček, Ph.D.

Konzultant: doc. MUDr. Vojtěch Melenovský, CSc.

Praha, 2014

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem řádně uvedl a citoval všechny použité prameny a literaturu. Současně prohlašuji, že práce nebyla využita k získání jiného nebo stejného titulu

Souhlasím s trvalým uložením elektronické verze své práce v databázi systému meziuniverzitního projektu Theses.cz za účelem soustavné kontroly podobnosti kvalifikačních prací.

V Praze, 2.1.2014

JAN BENEŠ

Poděkování

Rád bych poděloval především svému školiteli prof. MUDr. Jaromíru Myslivečkovi, Ph.D za odborné vedení a pomoc, kterou mi věnoval jak při pregraduálním tak postgraduálním studiu a při přípravě této práce.

Dále bych chtěl poděkovat svému školiteli konzultantovi doc. MUDr. Vojtěchu Melenovskému CSc. za veškerou pomoc, zejména pak za cenné rady a zkušenosti týkající se tvorby odborného textu.

Identifikační záznam

BENEŠ, Jan. *Regulace receptorů spřažených s G proteiny: Studie muskarinových a β–adrenergních receptorů u M_2KO myší. [Regulation of G protein-coupled receptors: Study of muscarinic and β–adrenoceptors in M_2KO mice.]* Praha, 2014, 93 s, 0 příl. Disertační práce (Ph.D.). Univerzita Karlova v Praze, 1. lékařská fakulta, Fyziologický ústav. Školitel Mysliveček, Jaromír, školitel konzultant Melenovský, Vojtěch.

Použité zkratky

AC ……. adenylyl cyclase

AR…….. adrenergic receptor, adrenoceptor

β-AR …….. β-adrenergic receptor, β-adrenoceptor

CaMKII…….. calmodulin activated protein kinase II

Df..... degree of freedom

EGF …… epidermal growth factor

EGFR ……. epidermal growth factor receptor

Epac …… exchange factor directly activated by cAMP

GPCR …….. G protein-coupled receptor

GRK …… G protein receptor kinase

HR …….. heart rate

IVSd…… interventricular septum thickness in diastole

JAK ……. Janus kinase

LVIDd ……. left ventricle end-diastolic diameter

MAPK ……. mitogen activated protein kinase

MMP…… matrix metalloproteinase

MR ……. muscarinic receptor

NOS …… NO synthase

NTS ……. nucleus tractus solitarii

PKC……. proteinkinase C

PLAX …….. parasternal long axis

PLB, PLC ……..... phospholipase B, C

PSAX …….. parasternal short axis

PTCH …….. patched

PYK2…… proline-rich tyrosine kinase

Rap ……... Ras-related protein

RTK ……. receptor tyrosine kinase

SMO …… smoothened

Smad……. intracellular proteins that transduce signals in TGFβ pathway

Src …… non-receptor protein tyrosine kinase (derived from the word „sarcoma“)

TGFβ ……... transforming growth factor β

Obsah

Souhrn (česky):

Cílem práce bylo podrobně analyzovat myši s vyřazeným M_2-receptorem (M_2KO) jednak za klidových podmínek a dále po působení stresového podnětu (chladový stres). Zaměřili jsme se zejména na roli antagonistických receptorů (adrenoceptorů) v srdci a jejich roli v adaptaci na chybějící M_2-receptor. Provedeny byly vazebné studie, studie genové exprese, echokardiografie, telemetrický monitoring srdeční činnosti, tělesné teploty a aktivity, analýza variability tepové frekvence, analýza biorytmů, analýza srdeční činnosti po podání agonistů a antagonistů muskarinových receptorů a adrenoceptorů, stanovení aktivity adenylyl cyklázy a NO syntázy, stanovení sérové koncentrace katecholaminů a genové exprese enzymů syntetizujících katecholaminy. Vyřazení M_2-receptorů vedlo ke kompenzatornímu snížení β_1 a β_2-adrenoceptorů doprovázené snížením jejich genové exprese, down-regulovány byly rovněž M_3-receptory. Chybějící M_2-receptory byly funkčně nahrazeny β_3-receptory, které byly upregulovány (nikoli kardioinhibičními M_4-receptory). Signalizace přes β_3-receptory vedla dále cestou adenylátcyklázy a nikoli NO syntázy. Všechny tyto změny byly zjištěny pouze v levé komoře, odpovědným mechanismem se tedy zdá být heterologní regulace. Mírně vyšší bazální frekvence a nižší tělesná teplota M_2KO myší podtrhuje důležitost telemetrické monitorace. I přes všechny pozorované rozdíly, celková srdeční funkce u M_2KO myší byla zachována.

Klíčová slova: M_2KO myši, adrenoceptory, muskarinové receptory, srdce, stres, adaptace

Abstract (in English):

The aim of the work was to perform in-depth analysis of M_2KO mice both at baseline and upon a challenge with a cold stress and to explore the role of opposing receptors (i.e. adrenoceptors) in adaptation to lacking M_2-receptors in the heart. We have performed receptor binding studies, study of receptor gene expression, echocardiography, telemetric monitoring of heart rate, body temperature and activity, heart rate variability and biorhythm analysis, analysis of heart rate response to the application of drugs (carbachol, atropine, isoprenaline, propranolol), assessment of adenylyl cyclase and NO synthase activity, measurement of catecholamine blood concentration and gene expression of catecholamine-synthesizing enzymes. We have found that the disruption of M_2-receptor gene caused a compensatory decrease of cardiostimulatory β_1-adrenoceptors and β_2-adrenoceptors with corresponding down-regulation of the gene expression, M_3-receptors were down-regulated as well. Missing M_2-receptors were functionally replaced by the main cardioinhibitory β_3-adrenoceptors that were up-regulated, not by cardioinhibitory M_4-receptors. β_3-adrenoceptors were found to signal through adenylyl cyclase instead of NO synthase. All these changes were found in the left ventricle only, so heterologous regulation is likely to be the responsible mechanism. Slightly higher basal heart rate and lower basal body temperature of M_2KO animals underscore the importance of telemetric measurement to avoid biased recordings. Despite all the changes found the overall cardiac function was unaltered.

Key words: M_2KO mice, adrenoceptors, muscarinic receptors, heart, stress, adaptation

1. Introduction

1.1. Cellular signalization

In a multicellular organism, all cells are involved in cooperation, which requires mechanisms for cell communication. There is either a direct cell-to-cell signaling or cells communicate at the longer distances through „signaling molecules“ that control metabolic processes within cells, the synthesis and processing of proteins, the growth and differentiation of tissues and the composition of both intracellular and extracellular environment.

Signaling molecules are synthesized and released by signaling cells and either influence just neighboring cells (paracrine signaling, synaptic transmission) or travel throughout the body (hormones) but induce a specific response only in target cells that have appropriate receptors. Substances used for intercellular signaling encompass a huge variety of items including small molecules (derivatives of amino acids, lipids, acetylcholine, steroids), oligopeptides (vasopressin) or large proteins (growth hormone, TSH etc.).

Principally, there are two different receptor systems. Receptors can be localized either intracellularly (in this case, they usually bind hydrophobic molecules such as steroids that diffuse spontaneously through the plasma membrane) or they are located in the plasma membrane. Ligands for these receptors bind to a structurally complementary site on the extracellular or membrane-spanning domains of the receptor, which causes conformational changes in the cytosolic domain of the receptor, which subsequently induces specific changes within the cell - a process called signal transduction. Signals from outside the cell can induce either changes in the activity or function of preexisting proteins or they can induce a de-novo production of proteins (proteosynthesis). Although there are many signaling pathways that enable the cell to accomplish a myriad of cellular functions there are principally only a few major classes cell surface receptors. G protein-coupled receptors comprise the largest family (with a several variants represented by hedgehog and Wnt receptors), catalytic receptors (cytokine receptors, TGFβ receptors, receptor tyrosine kinases) and Notch receptors (Alexander et al., 2011). These are depicted in Fig. 1.1.

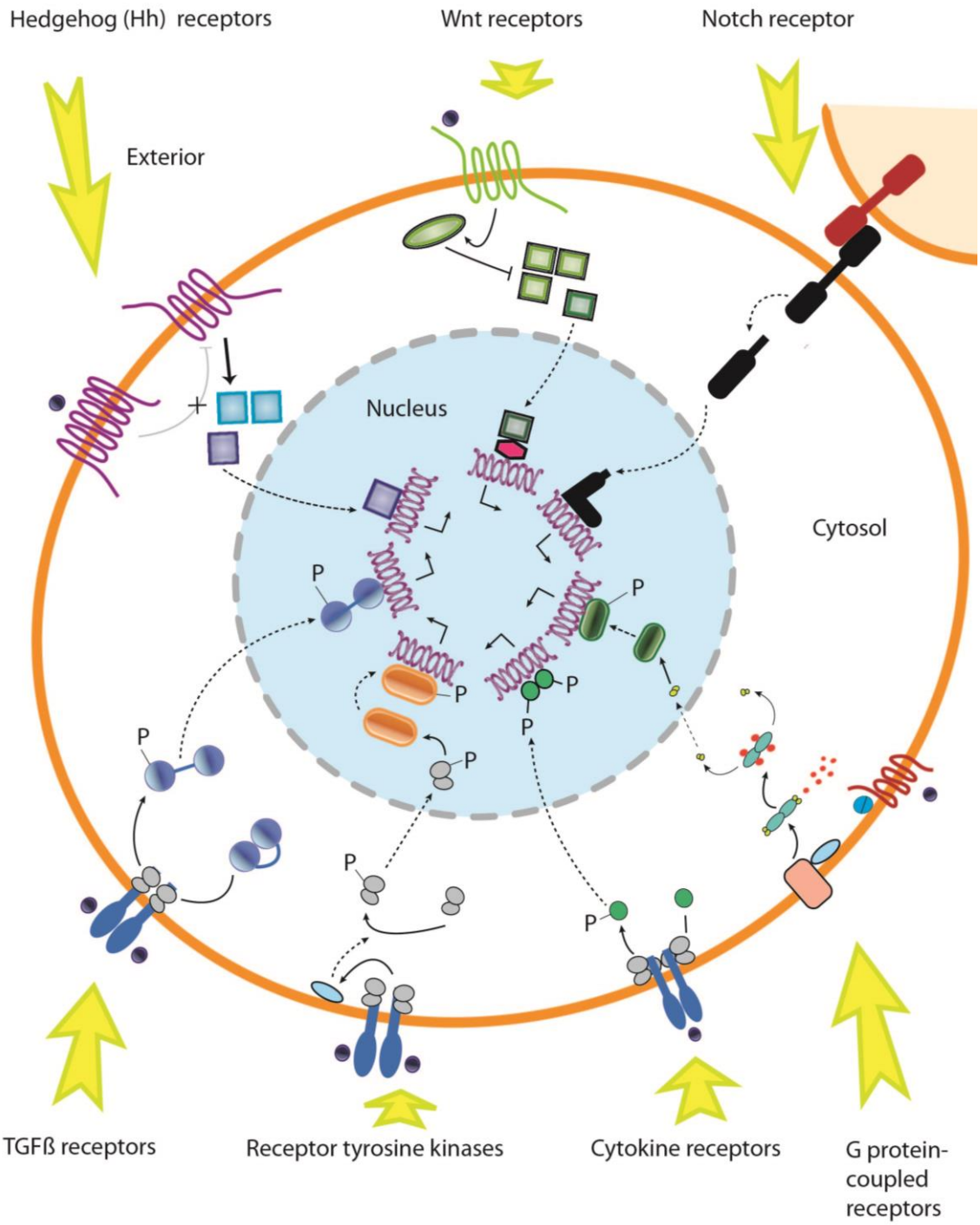

Fig. 1.1. The overview of major types of surface receptors

Major classes of cell-surface signaling receptors are depicted.

Hedgehog pathway is shown in the upper left corner. Binding a ligand (e.g. sonic hedgehog) on a receptor (Patched-1, PTCH1) leads to the inhibition of Smoothened (SMO), which releases the inhibition of SMO upon GLI transcription factors. GLI then migrates into the

nucleus where it regulates the expression of target genes. Hedgehog pathway is particularly important in development.

Wnt pathway is activated by binding of Wnt-protein ligand to a Frizzled family receptor, which passes the signal to the Dishevelled protein inside the cell. There are three Wnt pathways: the canonical Wnt pathway (using β-catenin signaling protein) and two noncanonical pathways (planar cell polarity pathway, Wnt/calcium pathway) that work independently of β-catenin. Wnt pathways are crucial in embryonic development, axis patterning, cell fate specification, cell proliferation, cell migration and insulin sensitivity.

Notch signaling usually occurs between neighboring cells. One expresses the Notch ligand (e.g. Delta), the adjacent one then Notch receptor. Binding of the ligand promotes two steps involving proteolysis; as a result, intracellular domain is liberated and enters the nucleus where it regulates the gene expression. Notch signaling is important in multiple processes including neuronal and cardiovascular development.

TGFβ signaling pathway begins with binding a ligand to TGFβ type II receptor (sometimes type III receptor that presents the ligand to the type II receptor). Type II receptor then recruits and phosphorylates type I receptor, which releases the inhibition of type I receptor kinase activity. Activated type I receptor phosphorylates Smad protein. Two phosphorylated Smad proteins associate with Co-smad protein and enter the nucleus, where they regulate the expression of target genes.

Receptor tyrosine kinases (RTKs) are receptors that have intrinsic protein tyrosine kinase activity in their cytosolic domain. Upon binding a ligand they dimerize, which causes the phosphorylation of cytosolic receptor tyrosine residues. Phosphorylated tyrosine residues then act as substrates for other proteins (e.g. Son-of-sevenless, Sos) that transmit the signal further in the cell. Growth factors (epidermal, fibroblast, vascular endothelial) and insulin typically signal through RTKs.

Cytokine receptors share many similarities with RTKs. Upon binding a ligand, cytokine receptor dimerizes, which brings Janus kinases (JAKs) located in the intracellular portion of the receptor to the close proximity. They phosphorylate the inner portion of the receptor leading to the activation of STAT proteins that dimerize and enter the nucleus, where they control a transcription of target genes.

Signaling through **G protein-coupled receptors** will be thoroughly reviewed in the following sections. Adapted after (Brivanlou and Darnell, 2002).

1.2. G protein-coupled receptors

G protein-coupled receptors (GPCR) are one of the major receptor systems in organisms. They transfer various messages as different as photons, organic odorants, nucleosides, nucleotides, peptides, lipids and proteins. There are at least six families of G protein-coupled receptors showing no similarity in their sequence. Similarly, considerable differences exist concerning the receptor binding sites. Adrenoceptors belong to the group Ib (Bockaert and Pin, 1999).

According to their name, these receptors signal through G proteins. From the structural point of view all G protein-coupled receptors contain seven membrane-spanning regions having their N-terminal segment on the extracellular side and their C-terminal segment on the cytosolic side of the plasma membrane. The structure of the GPCR is in Fig. 1.2.

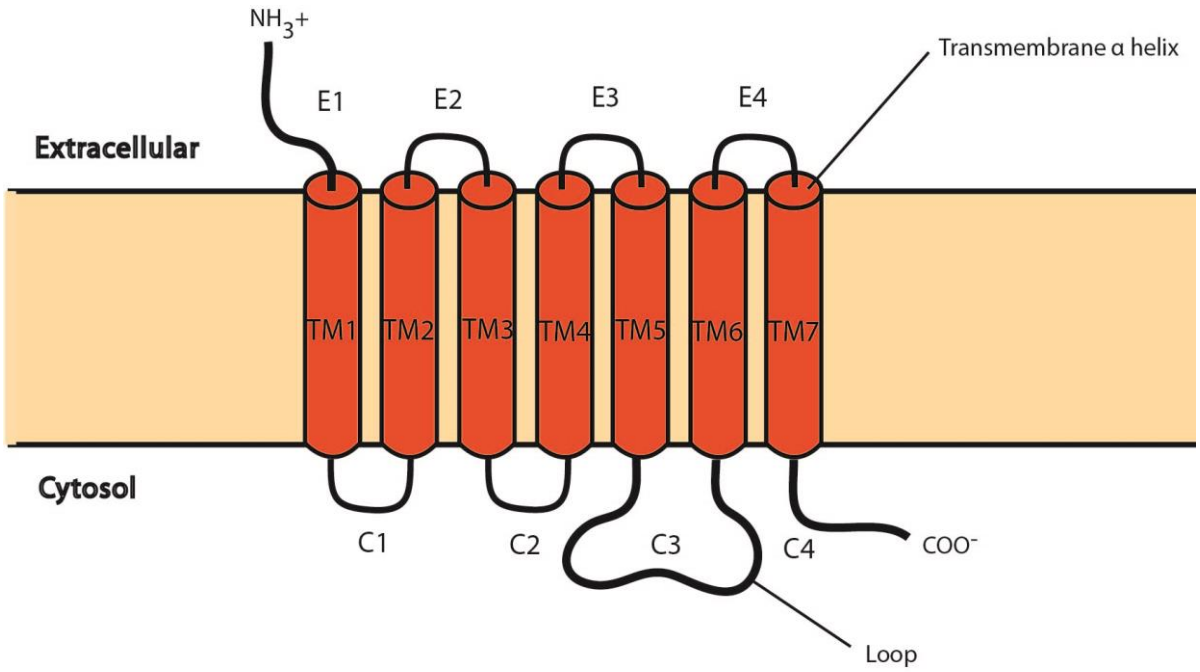

Fig 1.2.: The structure of the typical G protein coupled receptor

G protein-coupled receptors have four extracellular segments (E1-E4) seven transmembrane domains (TM1-TM7) and four cytosolic segments (C1-C4). The carboxy-terminal segments C4 and C3 loop (in some receptors also C2 loop) are involved with the interaction with G protein. Adapted after (Lodish H, 2000).

1.3. G proteins

G protein-coupled receptors belong to a family of proteins that transmit the signal through binding of GTP and its subsequent hydrolysis to GDP and phosphate (GTPase superfamily).

GTP hydrolyzing proteins are either monomeric (e.g. Ras protein) or trimeric, consisting of α, β, and γ subunit (G proteins). Both monomeric and trimeric GTPase superfamily proteins exist in two distinct forms – an inactive form with bound GDP and active form with bound GTP.

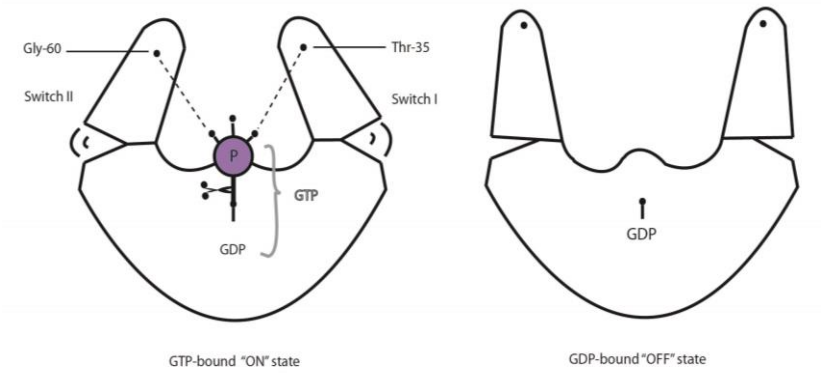

Fig. 1.3.1: Switching mechanism for GTPase superfamily proteins

In order to be able to transduce the signal, G protein needs to be able to switch between two states (GTP-bound "on" state, GDP-bound "off" state). In the active ("on") state, two domains (named switch I and II, respectively) interact with the terminal γ-phosphate of the bound GTP through the interactions with conserved threonine and glycine residues. Upon GTP hydrolysis, γ-phosphate disappears leading to the different conformation of both switch I and II- inactive ("off) state. Adapted after (Vetter and Wittinghofer, 2001)**.**

G proteins are localized at the intracellular side of cytoplasmic membrane. All G proteins contain three subunits designated as α, β and γ. β and γ subunits remain bound together and are referred to as Gβγ subunit. The Gα subunit is a GTPase switch protein that cycles between an inactive (off) and active (on) state. Upon binding a ligand, a conformational changes in the receptor occurs, which leads to the binding of activated receptor to G protein (Gα subunit). This leads to a conformational changes in Gα and is followed by a dissociation of GDP and binding of GTP (that is more abundant than GDP in the cell). Gα and Gβγ subunits then dissociate and activated Gα (in some cases also Gβγ) transmit the signal further on other messengers. It is due to 3D-conformation when the third phosphate of GTP causes a conformational change and two domains (termed switch I and switch II) interact with the terminal γ-phosphate of GTP. Subsequently, intrinsic GTPase activity causes the hydrolysis of GTP to GDP and Pi, which leads to the release of γ-phosphate and to a conformational change and a separation of both switch I and II with the loss of the ability to transmit the signal. Both

subunits (Gα and Gβγ) then reassemble and are ready for the next round of signal transmission (Fig. 1.3.2).

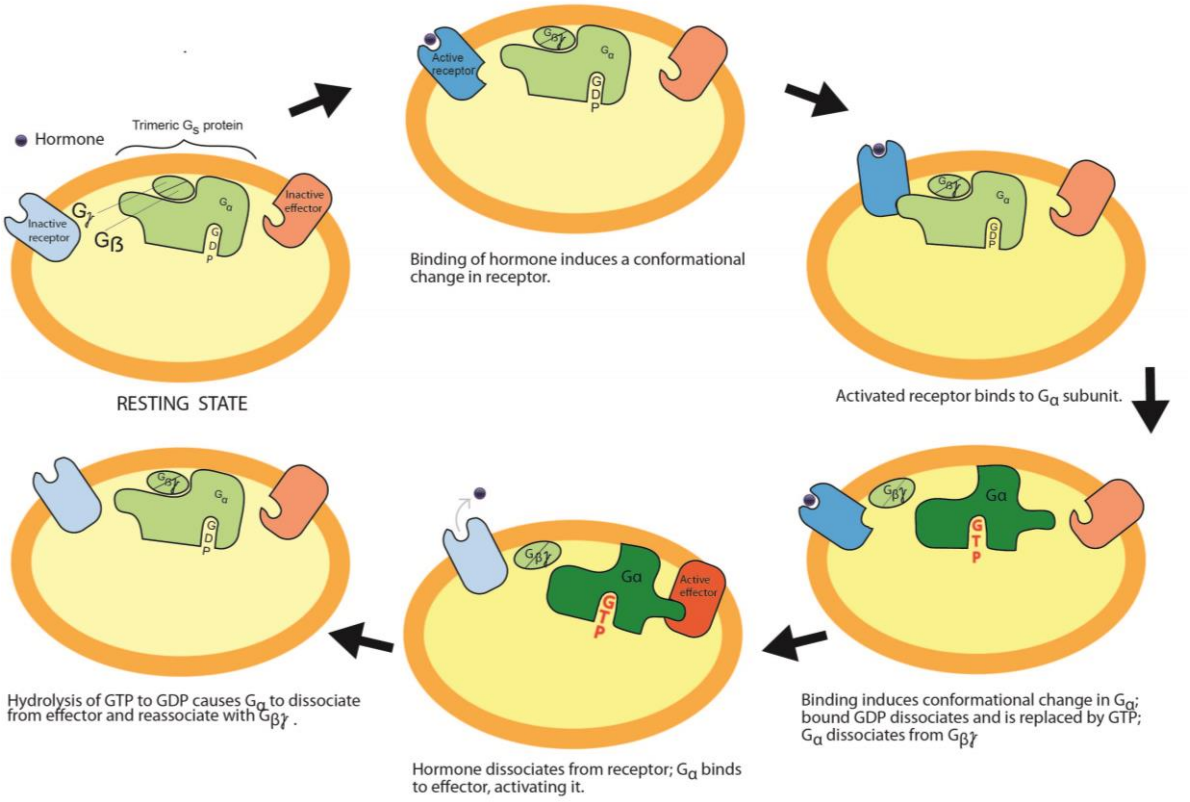

Fig. 1.3.2. Signal transduction through G protein

Adapted according to the (Lodish H, 2000).

Signal transduction occurs at multiple levels (receptor ligand, receptor, G protein, adenynyl cyclase, cAMP, protein kinase A, activated enzymes) and at all levels the signal is usually greatly amplified. For example, a single molecule of a ligand activates one hundred molecules of G protein, leading to several hundred molecules of cAMP, each activating protein kinase A that subsequently activates several hundred molecules of the target protein. Thus, the plasmatic adrenaline concentration as low as 10^{-10} mmol.l^{-1} can induce substantial biological effects. This cascade leads not only to a mere amplification of the signal, but also allows an entire group of proteins involved in the same biological process to be regulated in a coordinated manner.

There are multiple G proteins encoded in genomes of organisms. Among the 3 subunits (α, β and γ) only α subunits directly interact with G protein-coupled receptors. The most important for the purpose of this thesis is Gs that activates adenylyl cyclase, Gi that inhibits it and Gq

that activates phospholipase C. Compared to the enormous number of GPCR the number of genes encoding α subunit of G protein is very small (Hurowitz et al., 2000). Based on their sequence morphology, it has been long thought that there were only 4 major classes of Gα proteins (Gs, Gi, Gq and G_{12}) (Downes and Gautam, 1999). Each class can be subdivided into 2-4 families: the Gs class contain $G\alpha_s$ and $G\alpha_{olf}$; Gi comprises $G\alpha_t$, $G\alpha_o$, $G\alpha_i$ and $G\alpha_z$; Gq encompasses $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{15/16}$; and G_{12} contains $G\alpha_{12}$ and $G\alpha_{13}$ (Downes and Gautam, 1999). Only recently the fifth class of Gα proteins (Gv) was discovered (Oka et al., 2009). This fifth type was lost in many lineages including mammals.

1.4. Types of G protein-coupled receptors (relevant for the topic of the thesis)

1.4.1. Adrenoceptors

Adrenoceptors are membrane receptors located in both neuronal and non-neuronal tissue. They are responsible for mediating response to endogenous catecholamines noradrenaline (NA) and adrenaline (A). Noradrenaline is released from the noradrenergic postganglionic nerve terminals and both NA and A are secreted from the adrenal medulla. They are responsible for controlling many important physiological functions (cardiovascular, respiratory, neuronal, pupil contraction and dilatation, digestive, energetic metabolism, endocrine) (Calzada and de Artinano, 2001).

An observation that there must be more than one type of adrenoceptors was published in 1948, when Ahlquist observed differences in responses evoked by synthetic amines. He concluded that these differences must be due to the presence of different receptors, which he termed α and β (Ahlquist, 1948). α-adrenoceptors have mostly excitatory effects (vasoconstriction, uterine musculature contraction, urethra contraction, pupil dilation etc.) with the exception of intestinal relaxation. On the contrary, β-adrenoceptors have mostly inhibitory effects (vasodilatation, uterine musculature relaxation, bronchodilatation etc.) with the exception of cardiac stimulation (Calzada and de Artinano, 2001).

1.4.1.1. α-adrenoceptors

α-adrenoceptors divide into α_1 and α_2 subtypes. Both subtypes have other three subtypes (α_{1A}, α_{1B}, α_{1D} for α_1 and α_{2A}, α_{2B} and α_{2C} for α_2-subtype). Formerly, α_{1C} was also described but it was shown later that this receptor is encoded by the same gene as α_{1A}-AR and now it is considered as low affinity subtype of α_{1A}-AR (Hiraizumi-Hiraoka et al., 2004, Muramatsu et al., 2005).

All α_1 subtypes are activated by both adrenaline and noradrenaline and blocked by the α_1-adrenoceptor inhibitor prazosine. The subtypes vary in their amino acid sequence, tissue distribution and signaling pathways they activate. Recent findings on adrenoceptor signaling revealed many features that have challenged a classical linear paradigm of G protein-coupled receptor signaling. Adrenoceptors were proved to couple to multiple G proteins and even signal through G protein independent pathways, switch their signaling pathways over time and interact within or between their receptor subfamilies (Xiao et al., 2006). Thus paradigm of linear G protein-coupled signaling has gradually yielded to the model of „signalome“ where an individual adrenoceptor associates with multiple signaling pathways in time and spatially specific manner (Park and Palczewski, 2005). Moreover, adrenergic receptors were found to possess constitutive activity (Kjelsberg et al., 1992) and to form both homo- and heterodimers (Hague et al., 2004, Uberti et al., 2005). Having this in mind, it can be stated that according to the general paradigm, α_1-AR couple to Gq protein and activate phospholipase Cβ_1, which leads to diacylgylcerol (DAG) and inositoltriphosphate (IP_3) production and subsequent activation of protein kinase C (PKC). The role of calcium release due to increased IP_3 concentration in cardiac myocytes is controversial. The α_{1A}-subtype was shown to mediate cardioprotection through the activation of ERK. Under certain circumstances, α_{1B}-AR can couple to Gi protein (Olshansky et al., 2008).

α_2-AR couple to Gi protein and inhibit adenylyl cyclase (Gibson and Gilman, 2006). The expression and function of both α_1 and α_2 adrenoceptors in the heart will be discussed in a separate chapter. Both α_1 and α_2 subtypes are present postsynaptically in blood vessels, where they mediate vasoconstriction (Langer and Hicks, 1984). α_1-adrenoceptors are expressed in the prostate gland and are used as a therapeutic target in treating symptoms with benign prostate hyperplasia (Roehrborn and Schwinn, 2004). α_2-adrenoceptors are widely expressed throughout the central nervous system and the periphery. In the central nervous system, the area with a major density of α_2-adrenoceptors is the locus coeruleus, a region that contains the cell bodies for the ascending and descending noradrenergic neurons (MacDonald and Scheinin, 1995).

α_{2A}-subtypes are present in the prefrontal cortex and α_{2A} agonists are therapeutically used in the treatment of attention deficit hyperactivity disorder (Childress and Sallee, 2012). α_{2A}-subtype plays a major role in regulating vegetative nervous system; they are present on presynaptic noradrenergic nerve terminals where they inhibit the release of noradrenaline and adrenaline and thus inhibiting sympathetic nerve output (Hein et al., 1999). α_{2A}-adrenoceptors were also found in platelets (Shekim et al., 1994), kidney and gastric mucosa (Gyires et al.,

2007).

1.4.1.2. β-adrenoceptors

As already mentioned, the presence of distinct adrenergic receptors (termed α and β) was first described in 1948 (Ahlquist, 1948). In 1967, the evidence about distinct types of β-adrenoceptors was given. The first subtype mediated heart rate response to catecholamines; the second one then mediated bronchodilatation and vasodepression (Lands et al., 1967). Later on, the third subtype of β-adrenoceptor was found (Emorine et al., 1989). Moreover, at the end of 20th century, a fourth β-adrenoceptor was described (β_4-AR) (Kaumann and Molenaar, 1997) but similarly as in the case of α_{1C} it was found later that this β_4-AR is only a low affinity state of β_1-AR (Granneman, 2001, Kaumann et al., 2001). However, the role of this receptor both in physiological processes as well as in pathology is estimated (Myslivecek et al., 2003, Mallem et al., 2004).

β-adrenoceptors play multiple roles in the organism. Both β_1- and β_2-adrenoceptors are expressed in blood vessels; the proportion of both receptor subtypes varies in different vessel types (Chruscinski et al., 2001). β_2- and β_3-AR are expressed in bone marrow stroma cells where they have specific functions and collaborate during progenitor mobilization (Mendez-Ferrer et al., 2010). The presence of β-adrenoceptors has been suggested on nerves, β-adrenoceptors have been revealed in perivascular sympathetic nerves near the adventitial-medial border (Briones et al., 2005), which is consistent with a nurturing function of catecholamines in the repair and maintenance of nerves. β_2-AR are widely expressed in bronchial tissues, but the exact localization of them has been challenged recently (Davies, 2009). It has been long assumed that β_2-AR are on smooth muscle cells and they are responsible for removing asthma-induced contraction and mediate bronchodilatation. However, other cell types including the pulmonary airway epithelium have been proposed as central factors in airway reactivity and remodeling (Daly and McGrath, 2011). β–adrenoceptors also play an important role in mediating various metabolic processes; they play a role in insulin secretion from the pancreatic β-cells as adrenergic tone is one of the mechanisms regulating insulin secretion: β_2-AR activation stimulates insulin secretion and α-AR activation inhibits it. This effect, is however species specific. Human β-cells express β-adrenoceptors but β-cells of a rat do not (Lacey et al., 1990). β-adrenoceptors are also expressed in the liver where they play a critical role in the regulation of blood sugar and glycogen metabolism. Adrenaline and β-AR are the most important mediators of glycogen regulation (Nonogaki, 2000). β_2-adrenoceptor stimulation induces glycogen breakdown and

accelerates the rate of glycolysis. Moreover, it also stimulates a glucagon release (Shulman, 1999). Effects of β-agonist on liver beyond the glycogen regulation have been described. β_2-stimulation was shown to be protective against hepatocyte apoptosis; an effect that was mitigated by β_2-AR inhibition (Andre et al., 1999). β_2-agoinsts were also shown to improve muscle glucose transport in rat model of obesity and diabetes (Pan et al., 2001). The β_2-AR response has been showed to be critical in mediating hypoglycaemia awareness. Defective tissue response to glucagon and adrenaline is responsible for this syndrome leading to β_2-AR insensitivity. After a careful avoidance of hypoglycaemia, the restored sympathetic response occurs primarily by improved β_2-AR sensitivity (Fritsche et al., 2000). β_2-adrenoceptors have been also proved to play a role in mediating a parathyroid hormone action on bone formation. Intermittent parathormone treatment increases bone mass formation, but for a proper functioning of this axis the presence of β_2-receptors is critically important (Hanyu et al., 2012). Recently, it has been found that β_2-adrenoceptors (through both Gs-PKA and β-arrestin pathways) trigger DNA damage and suppress p53 levels and thus mediate the effect of chronic stress (Hara et al., 2011).

Adipocytes are another cell population largely influenced by β-adrenoceptors. β_3-adrenoceptors have been identified as critical receptor system involved in mediating adrenergic action upon adipocytes (Lipworth, 1996). Among other, β_3-adrenoceptors are playing a dominant role in regulating the release of leptin from the adipocytes. Noradrenaline and β_3-agonists inhibit a leptin release out of the adipocytes (Nonogaki, 2000).

1.4.2. Muscarinic receptors

Similarly to β-adrenoceptors, muscarinic receptors (MR) are metabotropic receptors that are coupled to G proteins. There are five subtypes of MR, which are distinct gene products. Analogously to β-adrenoceptors, recent research on MR signaling revealed the amazing complexity of signaling pathways originating on MR (Nathanson, 2000). However, according to their prevailing signaling pattern, muscarinic receptors can be divided into two major classes: odd numbered subtypes (M_1-, M_3-, and M_5-receptors) and even numbered subtypes (M_2- and M_4-receptors). Odd numbered subtypes generally activate phospholipase C (PLC), employing pertussis toxin-insensitive G proteins of the Gq superfamily, and do not inhibit adenylyl cyclase. Even numbered subtypes inhibit adenylyl cyclase through pertussis toxin-sensitive G proteins of the Gi family. However, both M_2- and M_4-MR can potentially activate PLC when expressed at high levels in certain cell types due to the release of βγ subunits (Katz et al., 1992). Under certain circumstances, both Gi and Gq-specific MR can

be potentially coupled to Gs and stimulate adenylyl cyclase activity (Migeon and Nathanson, 1994) but it is not the only way how MR can activate adenylyl cyclase. By increasing intracellular calcium levels, Gq-coupled MR can activate calmodulin-sensitive adenylyl cyclase and thus increase the level of cAMP (Choi et al., 1992). Moreover, various isoforms of adenylyl cyclases can be phosphorylated (and thus regulated) by protein kinase A (PKA), protein kinase C (PKC) and calmodulin-dependent protein kinases (CaMKs) (Hurley, 1999). Last but not least, MR can regulate tyrosine kinase pathway and mitogen-activated protein kinase (MAPK) pathway employing multiple mechanisms. Gq-coupled receptors can activate proline-rich tyrosine kinase 2 (PYK2) through calmodulin-dependent protein kinases and PKC-mediated mechanism. PYK2 can activate c-src to cause the activation of the ERK pathway (Gutkind, 1998, Gudermann et al., 2000).

Muscarinic receptors mediate a plenty of physiological and pathophysiological processes throughout the body. In the central nervous system, all subtypes of muscarinic receptors are involved. M_1-MR (Yamasaki et al., 2010), M_2–MR and M_4–MR (Ohno et al., 1994, Tzavara et al., 2003) have been implicated in attention, arousal and cognitive processes. M_2-MR mediate tremor, hypothermia and analgesia (Gomeza et al., 2001). M_3-MR are involved in certain types of learning (Poulin et al., 2010) and are required for the proper functioning of hypothalamic GHRH neurons (Gautam et al., 2009). M_4-MR are involved in the inhibitory control of striatal projecting neurons, where they control D_1-dopamine receptor-mediated locomotor stimulation (Gomeza et al., 2001). M_5-MR are likely involved in the acetylcholine-mediated vasodilatation of cerebral arteries and arterioles. Moreover, M_5-MR are expressed in the ventral tegmental area where they are involved in mesolimbic dopaminergic pathway for the regulation of morphine reward and withdrawal symptoms (Yamada et al., 2003). The role of muscarinic receptors in cardiovascular processes will be thoroughly discussed later. M_3-MR have been found to mediate a contraction of urinary bladder and selective M_3 antagonists are employed therapeutically to treat an overactive bladder syndrome (Chapple, 2004). The role of muscarinic receptors has been shown in mediating many pathophysiological processes including an involvement in cancer growth. M_2-MR have been shown to inhibit the growth of glioblastoma cell lines (Ferretti et al., 2012), M_1- and M_3-MR antagonist has been shown to inhibit a growth of non-small cell lung cancer cells (Hua et al., 2012). M_3-MR were also shown to play a role in proliferation and perineural invasion of cholangiocarcinoma cells (Feng et al., 2012).

1.5. G protein coupled receptors in the heart

1.5.1. α-adrenoceptors

The major subtype playing the role in the heart is α_1-subtype. In healthy human myocardium, α_1-adrenoceptors constitute about 10% of total adrenoceptor binding with α_{1A} and α_{1B} being the predominant subtypes (Jensen et al., 2009a) whereas α_{1D} is the predominant subtype in epicardial coronary arteries and smooth muscle cells (Jensen et al., 2009b).

The important feature concerning both α_{1A} and α_{1B} subtypes is that the amount of their binding sites is not diminished in heart failure both in left and right ventricles and their function in this conditions is maintained. This is in contrast with β-adrenoceptors that are down-regulated and also functionally uncoupled in heart failure (Jensen et al., 2009a) leading to diminished functional activation of potentially beneficial pathways including those mediating a positive inotropy.

1.5.2. β_1-adrenoceptors

β_1-AR constitute about 70-80% of all β-AR in the heart (Brodde, 1991). They are localized widely through cardiomyocytes without particular local inhomogenities. When stimulated, they elicit full contractile response. Upon stimulation, Gs protein is activated and further activates adenylyl cyclase (AC). AC converts adenosintriphosphate (ATP) into cyclic AMP (cAMP). cAMP further activates protein kinase A (PKA) resulting in phosphorylation of multiple proteins including those involved in coupling between excitation and contraction. This classical paradigm is true for only intermittent or short-term β_1-AR activation. Prolonged β_1-AR stimulation leads to G protein receptor kinase (GRK)-mediated receptor phosphorylation, which leads to the recruitment of β-arrestin to phosphorylated β_1-AR. β-arrestin plays two different roles in β_1-AR signaling: it works as a sterical inhibitor that blocks further interaction between the receptor and G protein and targets the receptor for internalization (Lefkowitz, 1998). Second, it serves as a scaffold protein that enables the interaction of other members of signaling cascade. Prolonged β_1-AR activation leads to the activation of calmodulin activated protein kinase II (CaMKII) that is responsible for clinically unfavorable effects of β_1-AR activation (i.e. increased apoptosis) that is observed in untreated heart failure (Yoo et al., 2009). The exact mechanism how β_1-AR is linked to CaMKII is still not completely resolved; however, the recent work has showed that β-arrestin (β-ARR) is required as a scaffold protein for an aggregation of CaMKII and Epac (exchange protein directly activated by cAMP) bringing them to the close proximity to cAMP generation site. Upon stimulation by cAMP, Epac exhibits guanine nucleotide exchange factor activity

toward Rap1. The exact mechanism how Epac activates CaMKII remains unknown but it seems to require phospholipase-ε (PLC-ε) and proteinkinase-ε (PKC-ε). As a result, CaMKII is activated and transmits the signal further in the cell (Mangmool et al., 2010). However, signaling through β_1-AR can have a protective role as well as it has been showed that β_1-AR can also signal through β_1-arrestin that further leads to matrix-metalloproteinase (MMP)-mediated cleavage and extracellular shedding of heparin-binding epidermal growth factor (HB-EGF) that ultimately leads to EGFR transactivation (Noma et al., 2007). This pathway requires specific G protein-coupled receptor kinases 5 and 6. It has been showed that this pathway confers cardioprotection.

1.5.3. β_2-adrenoceptors

Compared with β_1-AR, β_2-AR constitute only about 20-30% of total β-adrenoceptors in the heart (Brodde, 1991). There are multiple aspects of how β_2-signaling differs from β_1-signaling. First, β_2-ARs are not uniformly distributed throughout the cardiomyocyte, but they are enriched in caveolae with particular spatial distribution. Second, although β_2-AR couple to Gs protein (which is similar to β_1-AR signaling), they are also able to couple to Gi, which elicits the contrary effect. β_2-AR coupling to Gi is preceded by PKA-mediated phosphorylation of β_2-AR. The effect of Gi can possibly occur since prolonged β_2-AR stimulation induces the translocation of β_2-AR out of caveolae. Moreover, prolonged agonist stimulation activates PI3-kinase and functionally further inhibits Gs. It has been proposed that β_2-adrenoceptor signaling hinders apoptosis and functionally oppose β_1-signaling. Furthermore, only β_1-AR and not β_2-AR can activate CaMKII. The reason for this lies in the different amino-acid sequence of the C-terminal tail of the receptor, which has been showed in chimera studies (Mangmool et al., 2010). C-tail of β_1-AR mediates a unique conformation of β-arrestin that allows for scaffolding of Epac and CaMKII to form a stable complex, leading to subsequent cAMP/Epac-mediate activation of CaMKII. Both β_1-AR and β_2-AR work usually in a complementary manner; however, under certain circumstances their function might be opposing. It has been showed that the stimulation of β_1-adrenoceptors promotes apoptosis (Communal et al., 1999, Zaugg et al., 2000, Zhu et al., 2003) whereas the stimulation of β_2-adrenoceptors has anti-apoptotic effects both in vitro (Communal et al., 1999) and in vivo (Chesley et al., 2000). These findings might have a therapeutic potential in treatment of cardiac disorders (e.g. heart failure).

1.5.4. β_3- adrenoceptors

Challenge about the existence of another β-AR came at the end of the last century, when the 3rd subtype of β-adrenoceptor (β_3-AR) was cloned (Emorine et al., 1989). Messenger RNA (mRNA) of this receptor was identified in various tissues (adipose tissue, small and large intestine, gall and urinary bladder and myometrium) (Rouget et al., 2005) including the heart (Gauthier et al., 2000, Myslivecek et al., 2006). The β_3-AR protein has been also identified using immunohistochemical detection (De Matteis et al., 2002).

The activation of β_3-AR can produce different effects depending upon the heart chamber-atria vs. ventricles (Brixius et al., 2004, Spasojevic et al., 2011) and upon species (Gauthier et al., 2000, Skeberdis et al., 2008). In human ventricles, stimulation of the β_3-AR produces a marked decrease in cardiac contractility (Gauthier et al., 1996), which is in contrast to the other β-AR subtypes. It has been supposed that β_3-AR serves as a „brake" during excessively increased sympathetic overstimulation since it is activated at high catecholamine concentrations and counteracts the effects of β_1 and β_2 activation by producing a negative inotropic effect (Moens et al., 2010). On the other hand, it has been shown that in human atria β_3-AR stimulation increases contractility (Skeberdis et al., 2008). It has been observed as well that the expression of β_3-AR is increased in the left ventricles of patients with heart failure (Moniotte et al., 2001) suggesting the role of β_3-AR up-regulation in pathological conditions. β_3-AR is unique since it can couple both to Gs and Gi without the requirement for receptor phosphorylation (Liggett et al., 1993, Soeder et al., 1999). Compared to β_1- and β_2-AR, the β_3-AR presents a relative in vitro and in vivo lack of desensitization following activation with agonists, which is supposed to be due to the lack of the phosphorylation sites for protein kinase A and β-adrenoceptor kinase (β-ARK) that are critical players for mediating receptor desensitization (Nantel et al., 1993). However, most recent work suggests that in human embryonic kidney (HEK) cells, β_3-adrenoceptor desensitization occurs, but it does not involve reduced receptor numbers or significant reduction of Gs expression. Instead, its mechanism seems to be changes at the level of adenylyl cyclase function (Michel-Reher and Michel, 2013). In human ventricles, the activation of β_3-AR causes an activation of the nitric oxide (NO) pathway. It has been thought long that the NOS isoform involved is endothelial NO synthase (eNOS) (Brixius et al., 2004) that has a crucial role in regulating L-type calcium channel activity in cardiac tissue. Protein-kinase G (PKG) is a downstream effector of the eNOS/NO signaling cascade; it phosphorylates L-type Ca^{2+} channels, which deactivates them (van der Heyden et al., 2005). However, recent studies have showed that eNOS is not the only

NO isoform that mediates signaling through β_3-AR as NO production via the neuronal NOS (nNOS) has been demonstrated (Amour et al., 2007). The role of inducible NOS (iNOS) has not been fully clarified yet but it is likely that NO production through this isoform may also contribute to NO production followed by β_3-AR activation (Maffei et al., 2007).

1.5.5. β_4-adrenoceptors

At the end of 20th century, a fourth β-adrenoceptor was described (β_4-AR) (Kaumann and Molenaar, 1997) but later it was found out that this β_4-AR is only a low affinity state of β_1-AR (Granneman, 2001, Kaumann et al., 2001). However, some studies have shown a role of this receptor both in cardiac (Myslivecek et al., 2003) and vascular physiology (Mallem et al., 2004)- this receptor was found to mediate vasodilatation in normotensive but not hypertensive rats, which might have therapeutic consequences. The current concept of signaling through β-receptors is in Fig. 1.5.1

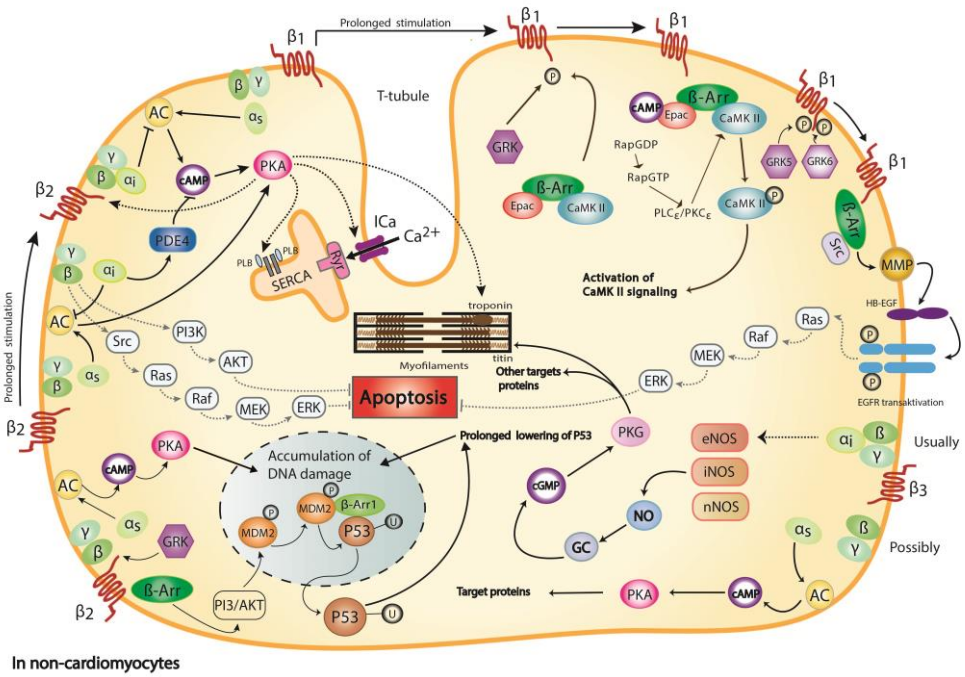
Prolonged stimulation
β1
β2
β3
T-tubule
AC
αs
αi
β
γ
cAMP
PKA
PDE4
ICa
Ca2+
PLB
SERCA
RyR
troponin
Myofilaments
titin
Activation of CaMK II signaling
Other targets proteins
Apoptosis
PI3K
AKT
Src
Ras
Raf
MEK
ERK
GRK
ß-Arr
Epac
CaMK II
RapGDP
RapGTP
PLCε/PKCε
GRK5
GRK6
MMP
HB-EGF
EGFR transaktivation
Accumulation of DNA damage
MDM2
ß-Arr1
P53
PI3/AKT
Prolonged lowering of P53
PKG
cGMP
GC
NO
eNOS
iNOS
nNOS
Usually
Possibly
Target proteins
In non-cardiomyocytes

Fig. 1.5.1. The current concept of signaling through β-receptors

The predominant signaling through β_1-AR occurs through activation of Gs protein and subsequent activation of adenylyl cyclase (AC) that activates protein kinase A. Protein kinase A then phosphorylates its target proteins including calcium channel, phospholamban (PLB) and troponins. Upon persistent stimulation, G protein receptor kinase (GRK) is recruited leading to β_1-AR receptor phosphorylation, which results in β-arrestin (β-ARR) recruitment. This allows a conformation change to occur in β-arrestin molecule enabling stable interaction with calmodulin-activated protein kinase II (CaMKII) and exchange protein directly activated by cAMP (Epac). β-arrestin-mediated translocation of CaMKII and Epac to the β_1-AR complex bring these molecules in close proximity to the location of cAMP generation by AC. cAMP directly binds to and stimulates Epac, which leads to CaMKII activation via mechanism that requires Rap (Ras-related GTP binding protein), PLCε and PKCε, ultimately leading to phosphorylation of downstream effectors. Activation of CaMKII plays a role in various cardiac pathologic states and is considered to be maladaptive. Under different circumstances, persistent stimulation of β_1-AR leads to the receptor phosphorylation by specific GRK: GRK5 and GRK6, which leads to β_1-AR phosphorylation and recruitment of β-arrestin. β-arrestin further recruits Src (non receptor protein tyrosin kinase) that leads to matrix metalloproteinase activation (MMP) activation and cleavage of heparin bound-epidermal growth factor (HB-EGF). This leads to its binding to the respective receptor (EGF-R) and its transactivation. Downstream signaling cascade is activated eliciting cardioprotective action (e.g. apoptosis inhibition).

Signaling through β_2-AR predominantly occurs via Gs protein as well, leading to cAMP formation and PKA activation. However, upon persistent stimulation, PKA phosphorylates β_2-receptor inducing its coupling with Gi protein. Signaling through Gi protein directly inhibits the activation of AC; moreover, signaling through βγ subunits leads to the activation of PI3-kinase pathway and Src pathway mediating cardioprotective action (e.g. apoptosis inhibition).

In non-cardiomyocyte cells (germline cells), chronic activation of β_2-AR leads to the recruitment of β-arrestin 1, which faciliates Akt-mediated activation of MDM_2 (mouse double minute 2 homolog, also known as E3 ubiquitin-protein ligase). MDM_2 targets p53, leading to its degradation. In this process, β-arrestin-1 acts as a molecular scaffold. Prolonged lowering of p53 then leads to DNA damage accumulation.

β_3-AR signaling was believed to occur through Gi protein activation subsequently leading to the NO synthase (NOS) activation. All isoforms (iNOS, eNOS, nNOS) are likely to be

involved. NOS activation leads to NO production with the subsequent activation of protein kinase G (PKG) that phosphorylates its target proteins (incl. titin). The data from this thesis support the hypothesis that β_3-AR signaling occurs also through Gs protein leading to PKA activation.

1.5.6. Muscarinic receptors

In the heart, the dominant muscarinic receptor (MR) subtype is M_2. When stimulated, M_2-receptors activate Gi protein that inhibits adenylyl cyclase and thus counteracts cAMP-PKA-dependent signaling pathways. Gi also directly increases the activity of potassium channel, which results in hyperpolarization of the sinoatrial and atrioventricular node (Szabo and Otero, 1989). Moreover, $\beta\gamma$ subunits directly activate the potassium channel operated by muscarinic receptor (Logothetis et al., 1987). Additionally, the stimulation of M_2-receptors can inhibit L-type calcium channel and thus directly reduce contractility of ventricular cardiomyocytes (Valenzuela et al., 1997). As a consequence, the stimulation of M_2-receptors markedly slows the heart, shortens atrial action potentials, increases smooth muscle contraction, inhibits the funny current (I_f, mixed sodium-potassium current responsible for starting the diastolic depolarization phase) and directly decreases contractility (Olshansky et al., 2008). The disruption of M_2-mediated signaling may have adverse consequences like heart failure (Zhang et al., 2002) or arrhythmias (Baba et al., 2004). The nature of non-M_2-subtypes in the myocardium is still a matter of debate and the results published so far are contradictory. Binding studies performed on rats suggested that the minor subtype comprise of M_1- and M_5-subtypes (Myslivecek et al., 2008). However, other studies suggested that the major non-M_2-subtypes were M_1, M_3 and M_4 (Wang et al., 2004, Li et al., 2010). M_3-receptors were showed to mediate cardioprotective role of acetylcholine on myocardium; they were showed to ameliorate ischaemia-induced arrhythmias (Wang et al., 2012) and decrease the infarct size and mortality rate employing the M_3/NF-κB/miR-376b-5p/BDNF axis (Pan et al., 2012).

1.6. Regulation of G protein-coupled receptors

The regulation of G protein-coupled receptors is very complex with many new findings emerging every year. Principally, receptor function can be regulated either on the level of their number present at the membrane or they can by regulated through post-translational modifications (e.g. phosphorylation). Signaling events induced by a certain receptor can lead to the regulation of the same receptor (i.e. homologous regulation) or a different receptor (i.e. heterologous regulation, cross-talk, cross-regulation).

1.6.1. Phosphorylation-dependent regulation of G protein-coupled receptors

G proteins can be phosphorylated either by so-called G protein receptor-coupled kinases (GRK) or second messenger-dependent kinases (most often proteinkinase A and proteinkinase C, PKA and PKC, respectively). Both kinase systems phosphorylate serine and/or threonine residues of G protein-coupled receptors (GPCR), but these phosphorylation sites are different for each of them. Even among GRK, different GRK phosphorylate GPCR on different sites, which ultimately leads to the employment of different downstream signaling pathways.

The family of GRK consists of 7 homologs. GRK 1 and 7 phosphorylate exclusively visual opsins; on the contrary, GRK 2-6 phosphorylate various members of GPCR as well as other surface receptors (Lefkowitz and Shenoy, 2005). The important feature of GRK phosphorylation is that GRK phosphorylate only receptors with bound agonists. When stimulated by an agonist, GPCR undergo conformational changes and expose their binding sites for heterotrimeric G proteins. Gα subunits of G proteins exchange GTP for GDP leading to G protein activation and downstream signaling. Moreover, receptors with bound agonists immediately become a substrate for GRK-mediated phosphorylation, which leads to the recruitment of the cytosolic adaptor proteins called β-arrestins. The family of arrestins comprises 4 members (Lefkowitz, 1998, Gurevich and Gurevich, 2006). Traditionally, the main function of β-arrestin was to mediate the receptor desensitization but the knowledge about the myriad of arrestin roles has expanded enormously in recent years (Ibrahim and Kurose, 2012, Hu et al., 2013, Kang et al., 2013). Generally, besides desensitization, β-arrestins mediate endocytosis and signaling. The endocytosis requires the involvement of clathrin and β-arrestin-clathrin interaction. Moreover, studies conducted in recent years revealed that additional binding partners are necessary for efficient receptor internalization; these include the clathrin adaptor protein 2 (AP2), N-ethylmaleinimid-sensitive fusion protein (NSF), the small G protein ARF6 and PI4P kinase (Nelson et al., 2008). β-arrestins also mediate scaffolding of the signaling kinases including MAP kinase, MAP kinase kinase and MAP kinase kinase kinase, but also Src and CaMKII (Engelhardt, 2007, Noma et al., 2007, Mangmool et al., 2010).

Prolonged desensitization of GPCR leads to receptor internalization, which is a process when phosphorylated arrestin-bound receptors are being removed from the plasma membrane to a membrane–associated intracellular compartment. The total amount of binding sites on the whole-cell level is unchanged, just the membrane fraction decreases (Tomankova and Myslivecek, 2011). Following internalization, GPCR can undergo different pathways. They

can be either lysosomally degraded (which leads to a diminished amount of binding sites; termed receptor down-regulation), can be recycled back to the plasma membrane (resensitization). β-arrestins can undergo a large variety of modifications that influence their subsequent signaling role. These modifications comprise phosphorylation, ubiquitination and S-nitrosylation. In terms of GPCR regulation, ubiquitination is of particular interest since an ubiquitinated β-arrestin can form a stable complex with GPCR that co-internalize into endocytotic vesicles. These complexes can be associated with activated protein kinase (e.g. MAPK) and form a so called „signalosomes“ spreading the signal further into the cell (Lefkowitz and Shenoy, 2005).

When a cell is facing a situation that a receptor is not adequately stimulated, the receptor can undergo a process of sensitization and up-regulation. Moreover, further class of proteins regulating the function of G-proteins was discovered- regulator of G protein signaling (RGS) proteins (Willars, 2006). These proteins work both as GTPase activating proteins (GAPs) (De Vries et al., 2000), but also regulate GPCR signaling independently of the GAP activity by modulation protein-protein interactions, protein translation or intracellular localization of signaling molecules (Abramow-Newerly et al., 2006). Some proteins of this family (RGS4, RGS6 etc.) play an important role in regulating cardiovascular function as well and dysfunction of these proteins may contribute to various pathological states including chronotropic incompetence or AV conduction block (Fu et al., 2007).

1.6.2. Phosphorylation-independent regulation of G protein-coupled receptors

It has been also proved that besides the phosphorylation-dependent regulation of G protein coupled receptors, certain GRK promote a signaling that does not require a GPCR phosphorylation. This process has been studied the most for the interaction of mGlu1 receptor and GRK2. GRK2 directly binds to the second intracellular loop and the C-terminal tail of mGlu1a receptor but only to the second intracellular loop in the case of mGlu1b (Dhami et al., 2005).

1.7. The role of G protein-coupled receptors in autonomic nervous system

The autonomic nervous system is the part of the peripheral nervous system that mediates an involuntary control over various mainly visceral organ and thus contributes significantly to the control of homeostasis. Among others, it exerts a control over the heart rate, blood pressure, the response of gastrointestinal tract to food, thermoregulation, contraction of the urinary bladder, focusing of the eyes and sexual arousal. Similarly to other parts of the

nervous system, autonomic nervous system has an afferent, central (integrative) and efferent part. It consists of two main branches: sympathetic and parasympathetic (McCorry, 2007). The enteric nervous system is also sometimes considered the part of the autonomic nervous system but sometimes it is considered to be independent. Sympathetic nervous system mediates a body response to a possibly dangerous event; it can be characterized as quick response mobilizing system. On the contrary, parasympathetic nervous system is activated more slowly and is active when the organism is relaxed. Usually, both systems work in opposition (e.g. in the cardiovascular system) but they may also work synergically (e.g. during sexual arousal and orgasm, wherein both play a role). Noradrenaline is the main mediator of sympathetic nervous system and acetylcholine is the main mediator of parasympathetic nervous system. The effect of both systems on the cardiovascular function occurs via G protein coupled receptors (β- adrenoceptors for sympathetic nervous system and muscarinic receptors for parasympathetic nervous system). Both systems exert a tonic influence and the resulting function (heart rate, blood pressure etc.) is the result of the net balance between the systems (McCorry, 2007). It should be mentioned that the proper function and balance between both systems is important in human physiology and disturbance in both sympathetic and parasympathetic function play a role in many disorders including high blood pressure and heart failure (Port and Bristow, 2001, Olshansky et al., 2008), which has important clinical implications.

1.8. The role of stress

Stress is a response of the body to inner or outer strain that is able to elicit a response characterized by stress response elements (i.e. by the pathways and their activation that is evoked by a stressor). The responses to stressors are considered as specific. Therefore, every response that consists of a part of the specific pathway could be understood as stress response element. The main stress response elements are: specific activation of prefrontal/orbitofrontal cortex and limbic system, activity of neurons in nucleus tractus solitarii (NTS), activation of catecholaminergic groups in brainstem/medulla) and can have both positive (eustress) or negative impact (distress) on the body. Since stress provokes a distinct response of the organism, it is frequently used in research. Stressors can be divided according to their nature into physical, chemical, psychological, social, stressors disturbing cardiovascular/metabolic homeostatis and those affecting multiple systems (Kvetnansky et al., 2009).

We have used cold stress in our experiments since it is natural event (compared with immobilization or myocardial infarction), can be very well dosed and measured and can be used over a longer period of time thus enabling to investigate the effect of prolonged stress.

1.9. The importance of gene-knockout organisms for studying physiologic functions

In the few past decades, the techniques of molecular genetics enabled to prepare mice where a particular gene from the genome was selectively suppressed - so called gene „knock-outs". The particular gene can be switched off either on the whole-body level or selectively in a particular organ (i.e. the heart or the central nervous system). Knock-out studies thus provide a valuable insight into the function of many genes. It has been found that organisms may be viable although they have a knock-out of a gene that has been thought to be indispensable for survival. For example, animals with whole-body (Hrabovska et al., 2010) as well as CNS specific (Farar et al., 2012) knock-out of acetylcholinesterase (key enzyme degrading acetylcholine in the synapse) are viable and even (as in the case of CNS specific knock-out) have only mild functional impairment. This observation is in the striking contrast with the clinical experience of organophosphate poisoning. If acetylcholinesterase is inhibited postnatally (through an application of an organophosphate), such treatment is not tolerated and is always lethal. Moreover, even genes encoding proteins with elementary signaling function have been proved not to be necessary for survival as mice with whole-body c-fos knock out have been prepared (Johnson et al., 1992). However, the absence of such an important gene leads to severe functional impairment (Paylor et al., 1994). The experiments with gene knock-outs are facing many challenges too, though. First, due to functional impairment, it is sometimes challenging to breed the genetic line and maintain the population (Fritz and Robertson, 1996). Second, a severe genetic hit can trigger multiple adaptation changes that do not have to be easily interpreted (Benes et al., 2012a, Benes et al., 2013). One have to be cautious when using gene knock-out as a disease model as the possible adaptation changes induced by the knock-out can distort the role of the simulated disease itself (Sharma et al., 2004). Despite these limitations, knock-out models can provide a valuable tool to study the gene function. Therefore, main knock-out models related to adrenoceptors and muscarinic receptors will be briefly discussed below.

1.9.1. β_1-knockout mice

Mice without functional β_1-adrenoceptors display a severe prenatal lethality (lethality rate about 90%) (Rohrer et al., 1996). Those surviving to adulthood have a grossly normal cardiac

morphology with similar heart weight/body weight ratios and similar myocardial catecholamine content. β_1KO mice have also slightly but significantly diminished binding of myocardial β_2-receptors. The activity of adenylyl cyclase is similar both under basal and forskolin-stimulated conditions. When measured for a short time, both heart rate and blood pressure were normal (Rohrer et al., 1996). However, the long-term heart rate measurement revealed that β_1KO mice have actually a decreased heart rate. A spectral analysis of heart rate variability measured as RR intervals directly from ECG tracing revealed that an absence of β_1-adrenoceptors increases a total autonomic variability through both time and frequency indexes (Ecker et al., 2006). When these mice were challenged by non-specific β-agonist isoprenaline, the increase in heart rate that is normally seen in WT mice was absent in KO. On the contrary, β_2-mediated hypotensive response was preserved both in WT and KO (Rohrer et al., 1996). β_1-receptors are also responsible for mediating a sympathetic nervous system mediated adaptive thermogenesis as β_1KO mice develop a hypothermia when challenged with a cold-stress. These mice are unable to perform a brown adipose tissue response upon the stimulation with dobutamine (selective β_1-agonist) or noradrenaline (Ueta et al., 2012).

1.9.2. β_2-knockout mice

Contrary to β_1KO mice, lacking of β_2-AR is not associated with prenatal lethality (Chruscinski et al., 1999). Adult β_2KO mice are normal and fully fertile. They have a normal resting heart rate and blood pressure and normal chronotropic response to the β-AR agonist isoprenaline (both β_1 and β_2 agonist). However, the hypotensive effect of isoprenaline is significantly attenuated in β_2KO mice. On the contrary, they have a more pronounced hypertensive response to adrenaline (Chruscinski et al., 1999). The spectral analysis of heart rate variability (HRV) revealed that β_2-receptors play (together with β_1-receptors) a role in mediating sympathetic regulation of HRV. The absence of β_2-receptors increases total autonomic variability through the frequency-domain indexes, total power and ultra-low frequency power (Ecker et al., 2006). When challenged with exercise test, β_2KO mice have higher total exercise capacity but their blood pressure during exercise is markedly higher. Finally, at comparable workload, β_2KO mice have a lower respiratory exchange ratio suggesting a difference in energy metabolism (Chruscinski et al., 1999).

1.9.3. β_3-knockout mice

β_3KO mice have a similar resting heart rate as WT mice but smaller left ventricle size and impaired LV relaxation (Varghese et al., 2000). When challenged with isoprenaline, β_3KO

mice are able to increase their heart rate to the similar extend as WT but they have much higher contractile response (Varghese et al., 2000). On the contrary, the cardioprotective properties are revealed during stress condition. In WT animals, nebivolol (clinically used β-blocker with β_3-agonistic effects) and CL316243 (specific β_3-agonist) were able to induce a cardioprotection during ischaemia/reperfusion, which was abolished in mice lacking β_3-adrenoceptor strongly suggesting a cardioprotective role of β_3-adrenoceptors (Aragon et al., 2011).

1.9.4. β_1/β_2-knockout mice

Similarly to β_1-knockouts, β_1/β_2-knockout mice suffer with severe prenatal lethality (90-95%). Surviving β_1/β_2-knockout mice are not different both in baseline heart rate or mean arterial blood pressure (Rohrer et al., 1999). However, similarly to β_1KO, when monitored over a longer time period, β_1/β_2KO mice show a lower baseline heart rate and heart rate variability changes both in time and frequency domain correlating with effect of both β_1 and β_2-receptors (Ecker et al., 2006). Upon a challenge with isoprenaline, β_1/β_2KO mice in comparison to WT have markedly attenuated both chronotropic and hypotensive effect of the drug. Moreover, β_1/β_2KO mice have significantly lower maximal heart rate during exercise and altered oxygen consumption and carbon dioxide production. β_1/β_2KO mice showed a mild but significant reduction in muscarinic receptors density (Rohrer et al., 1999).

1.9.5. $\beta_1/\beta_2/\beta_3$ -knockout mice

$\beta_1/\beta_2/\beta_3$-triple knockout (TKO) mice are fully viable without apparent cardiovascular disturbances. However, they have lower heart weight/body weight ratio with lower cardiomyocyte size indicating cardiac hypotrophia. Furthermore, a lower resting heart rate and lower ejection fraction was found. These changes are accompanied by diminished activity of sarcoplasmic Ca^{2+}-ATPase (SERCA 2a) despite the protein expressions of SERCA 2a, ryanodine receptor and Na^{+}/Ca^{2+}-exchanger were similar. The protein expression of phospholamban was increased but its PKA-dependent phosphorylation at serine 16 was absent and CaMKII-dependent phosphorylation at threonine 17 was decreased. Despite these profound changes, there was no significant functional impairment of $\beta_1/\beta_2/\beta_3$ -triple knockout mice in the running wheel tests. On the contrary, the mean velocity of running and the total trip distance were actually higher in TKO mice (Lee et al., 2010). Further experiments on these mice revealed normophagic obesity and cold-intolerance. Their brown fat has an impaired morphology and diminished expression of an uncoupling protein-1 (UCP-1)

following a challenge with cold. On the contrary, these mice have higher circulating levels of free fatty acids and glycerol both at basal and fasted states suggesting enhanced lipolysis (Jimenez et al., 2002).

1.9.6. M_2-knockout mice

Mice lacking the gene for M_2-receptor were generated in Wess laboratory (Gomeza et al., 1999) and further characterized in subsequent studies. The knock-out of M_2-receptor was found to have impact both on central nervous system as well as cardiovascular system. M_2-receptor subtype was proved to play a key role in mediating whole-body tremor, hypothermia and analgesia- three of the most striking central muscarinic effects (Gomeza et al., 2001). M_2-receptors were showed to be involved in learning and memory; M_2KO mice showed significant deficits in behavioral flexibility and working memory in the Barnes circular maze and the T-maze tests. These behavioral deficits were associated with profound changes in neuronal plasticity (measured at Schaffer collateral-CA1 synapse) and both short-term and long-term potentiation (STP and LTP, respectively) were severely impaired in hippocampi (Seeger et al., 2004). A study (LaCroix et al., 2008) examined the cardiovascular function of M_2KO mice and showed that in spite of fully comparable baseline characteristics, after a week of chronic isoprenaline infusion followed by the bolus injection of phenylephrine, M_2KO mice exhibited impaired ventricular function indicated by attenuated dp/dt and increased end-diastolic pressure. Furthermore, chronic isoprenaline infusion significantly increased matrix metalloproteinase (MMP) activity (LaCroix et al., 2008). Authors conclude that the deficiency of M_2-receptors increases susceptibility to chronic adrenergic stress. Despite M_2KO mice were so thoroughly investigated, virtually no attention has been paid to the role of other cardiac receptors.

2. Goals and Hypotheses

Despite M_2KO mice were already a matter of investigation (discussed in the previous section (LaCroix et al., 2008), (Seeger et al., 2004)), very little is known about the adaptation mechanisms that take place after the knock-out of M_2-gene. Similarly, the way they cope with stress is not well understood either.

The goal of the work was to perform in-depth analysis of M_2KO mice both at baseline and upon a challenge with a cold stress and to explore the role of opposing receptors i.e. adrenoceptors in adaptation to missing M_2-receptors. We have focused on the receptor level, subsequent changes in intracellular signaling as well as on the consequences in physiological functions on the whole-organism level.

We have hypothesized that:

1. There would be a down-regulation of cardiostimulatory β_1- and β_2-adrenoceptors based on the down-regulation of the β_1- and β_2-adrenoceptor gene expression. Since β_3-adrenoceptors have been proved to mediate cardioinhibitory function we have assumed that these receptors would be one of the receptors employed to overtake the missing function of M_2.

2. M_4-receptors would possibly take over the function of missing M_2. Other non-M_2-subtypes would not be significantly altered.

3. There would be no difference in the systolic and diastolic function of M_2KO hearts.

4. There would be no difference in the heart rate, activity and basal temperature.

5. There would be no difference in the level of circulating catecholamines and the activity of catecholamine synthesizing enzymes.

6. The condition of prolonged (one week) stress would lead to different adaptation responses in M_2KO mice with distinct adjustment in cardiac signaling.

Rationale

Ad 1) A number of previous studies showed that there was a need for the homeostatic balance between sympathetic and parasympathetic nerve tonus, which means there is also a need for the balance between the number of muscarinic receptors and β-adrenoceptors as well as their functional output (Myslivecek et al., 1996, Garofolo et al., 2002, Brodde and Leineweber, 2004, Stavrakis et al., 2011). Based upon these studies, we have hypothesized that cardiostimulatory β_1- and β_2-adrenoceptors would be down-regulated. Conversely, since β_3-adrenoceptors are supposed to mediate cardioinhibitory function we have assumed that these receptors would be either without change or up-regulated to compensate for the missing function of M_2-receptors.

Ad 2) Although muscarinic signaling is complex, even numbered subtypes (M_2- and M_4-receptors) inhibit adenylyl cyclase through pertussis toxin-sensitive G proteins of the Gi family. Therefore we have assumed that M_4-receptors would possibly take over the function of missing M_2-receptors.

Ad 3) Systolic and diastolic function was invasively assessed in the study of (LaCroix et al., 2008) and no difference between wild-type and M_2KO mice was found.

Ad 4) At any given time point, heart rate reflects the dynamic balance between sympathetic and parasympathetic branch of the autonomic nervous system. Previously published studies did not report a difference in heart rate between WT and M_2KO mice (LaCroix et al., 2008), so we have not expected to find a difference either. On the central level, muscarinic receptors mediate hypothermia. M_2-receptor deficient mice have attenuated hypothermic response to oxotremorine (Bymaster et al., 2001) but their basal temperature was reported not to be altered. Similarly, M_2KO mice were reported to have similar locomotor activity as WT littermates (Gomeza et al., 2001), so we have assumed that these parameters would be similar in our study as well.

Ad 5) The already published studies showed that M_2KO mice had similar basal heart rate and locomotor activity, which means they do not seem to be under chronic stress at baseline. Therefore we have supposed that the deletion of M_2-receptors would not result in the

difference in the overall sympathetic tonus and the level of circulating catecholamines with the respective activities of the enzymes would be similar.

Ad 6) Upon 1-week infusion of isoprenaline and subsequent increased afterload (increase in the arterial pressure by the infusion of phenylephrine), M_2KO mice did not show an increase in contractility, which means they are more susceptible to stress (LaCroix et al., 2008). These differences are likely to be accompanied by altered signaling.

At the beginning, we have performed binding study and gene expression study. Then we have moved into studying physiologic parameters on the whole organism level and performed echocardiographic analysis, long-term telemetric measurement of heart rate, activity and body temperature, analysis of heart rate variability and pharmacological study (with muscarinic and adrenergic agonist and antagonist: carbachol, atropine, isoprenaline, and propranolol). Finally, we have focused on signaling changes- we have made an analysis of NO-synthase and adenylyl cyclase activity, measurement of circulating catecholamines and analysis of gene expression of catecholamine-synthesizing enzymes.

In the following sections, the methods and results will be discussed in this order.

3. Material and Methods

3.1. Experimental animals

Mice lacking the gene for M_2–muscarinic receptor clone were generated in Wess laboratory (Gomeza et al. 1999) and then bred in our animal facility (Prague, Czech Republic).

M_2−/− (M_2KO) mice (i.e., mice with complete deletion of M_2-muscarinic receptor gene; genetic background: 129 J1× CF1) were generated as described previously (Gomeza et al. 1999). In all experiments, age-matched wild-type (WT) mice of the corresponding genetic background were used as controls (M_2+/+, 129 J1×CF1). Animals were treated in accordance with the legislature of the Czech Republic and the EU, and the experimental protocol was approved by the Committee for the Protection of Experimental Animals of the 1st Faculty of Medicine, Charles University, Prague. The animals were maintained under controlled environmental conditions (12/12 light/dark cycle, lights on at 6 AM). Food and water were available *ad libitum*. The average ambient light phase temperature±SEM was 24.45±0.4 °C; the average ambient dark phase temperature±SEM was 24.22±0.4 °C. The temperature in the experimental room was checked every 10 min using a thermometric chip (Maxim Integrated Products, Inc., Sunnyvale, CA, USA). Male M_2KO animals and their WT counterparts (weighing 24–38 g, 11–16 weeks old) were used in the study. The animals were housed at three individuals per cage. At the end of the experiments, the animals were sacrificed by decapitation; hearts were collected, connective tissue was carefully discarded, and the left and right ventricles were isolated, flash frozen, and kept at -80 °C until the experiments were performed.

3.2. Cold stress

The animals (WT and KO) were exposed to cold for 1 day (24 h) or 7 days in conditions simulating natural situation. In the biological night (from 8 p.m. to 8 a.m.) the animals were exposed to the temperature 5.91± 0.38°C, while in the biological day the temperature rose to 9.73± 0.59°C. The light/dark cycle was the same as in control animals.
The temperature in the experimental space was checked every 10 min using thermometric chip (Maxim Integrated Products, Inc., Sunnyvale, CA, USA). Animals were housed three per cage. At the end of experiment, animals were sacrificed by decapitation; hearts were collected, connective tissue was carefully discarded, the left and right ventricles were isolated, flash frozen and kept in -80°C until the experiments were performed.

3.3. Receptor binding

We performed receptor binding on membranes, which were prepared as follows: the tissue was weighed and homogenized in ice-cold Tris–EDTA buffer (Tris–HCl 50 mmol/l, EDTA 2 mmol/l, pH adjusted to 7.4) for determination of total β-adrenoceptors and muscarinic receptors (Myslivecek et al., 2007). For determination of β_3-adrenoceptors the following buffer was used: Tris-HCl 50 mmol/l, $MgCl_2$-100mmol/l, GTP-300μmol/l, Tiron-1μmol/l, ascorbic acid-1mmol/l as described previously (Muzzin et al., 1994). In all cases, the homogenate was centrifuged at 600g for 10 min at 4 °C, the supernatant was collected and the sediment was resuspended in the buffer and centrifuged again using the same conditions. The supernatants were mixed and centrifuged for 25 min at 32,000g at 4 °C. The supernatant was discarded; the sediment was re-suspended in the buffer and centrifuged again at the same velocity and time (25 min, 32,000g, 4°C). The amount of receptor binding sites was determined in duplicate using the following conditions: (a) total β_1-adrenoceptor + β_2-adrenoceptor binding sites (B_{max}): concentration rank 93.75–3,000 pmol/l [^{3}H]-CGP 12177, non-specific binding was determined with 50μmol/l propranolol; (b) β_3-adrenoceptor binding sites (B_{max}): concentration rank 4.68 - 150 nmol/l [^{3}H]-SB 206606, non-specific binding was determined with 1 mmol/l SR59230A; (c) muscarinic receptors binding sites: 32.5–2,000 pmol/l [^{3}H]-QNB, non-specific binding was determined with 5μmol/l atropine. The incubation was performed at 38 °C and lasted for 60 min with [^{3}H]-CGP 12177, 30 minutes for [^{3}H]-SB 206606 and 120 min with [^{3}H]-QNB. All incubation times were adjusted previously (Myslivecek et al., 2007). The amount of binding sites (B_{max}) per milligram of protein (determined using BCA, a method kit; Sigma, Czech Republic) and the affinity constant (K_D) were computed by non-linear regression using GraphPad Prism 5.01 program (GraphPad Software, San Diego, CA, USA). Affinity constants (K_D) were used for the "single-point" measurement in order to determine the number of receptors, saving the amount of tissue needed, using the following saturating concentrations of radioligands (2,000 pmol/l [^{3}H]-CGP 12177, 120 nmol/l [^{3}H]-SB 206606 and 1,500 pmol/l [^{3}H]-QNB). β_1- and β_2-adrenoceptors were determined using co-incubation of membranes with 400 pmol/l [^{3}H]-CGP 12177 and with subtype-selective antagonists in triplicates (10^{-7}mol/l CGP 20712A for β_1-adrenoceptors, or 10^{-8}mol/l ICI 118.552 for β_2-adrenoceptors).

3.4. Receptor gene expression

Total mRNA was isolated using the chloroform–isopropanol (RNA Bee, TelTest, TX, USA) method according to the manufacturer´s instructions. RNA yield and integrity was evaluated

spectrophotometrically (Tecan M200 Nanoquant; detection of 2µl of sample in 16 wells at the moment) at A_1=260 and A_1/ A_2=260/280 nm, respectively. Samples with A_1/A_2 between 1.6 and 1.9 were used for downstream procedures. Total RNA was purified to eliminate potentially contaminating genomic DNA using recombinant DNAse (Ambion). Purified mRNA was subsequently transcribed into cDNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare). qPCR was performed using TaqMan probes (Applied Biosystems, probe numbers: M_1: Mm01231010_m1, M_2: Mm01167087_m1, M_3: Mm01338409_m1, M_4: Mm01331561_s1, M_5: Mm01701883_s1, β_1-AR: Mm00431701_s1, β_2-AR: m02524224_s1) with Roche qPCRMastermix (Roche) using the following protocol: 2 min at 50 °C, 10 min at 95 °C followed by 50 cycles for 15 s at 95 °C and 1 min at 60 °C in a final volume of 10µl. In order to determine the relative gene expression, the δ-δ-C_t method was used. The levels of the analyzed transcripts were normalized to the level of the reference gene transcript (18SrRNA, the level of which was practically the same in WT and KO). Then, for each sample, the following formula was used: normalized ratio=$2^{CT_1 - CT_2}$, where the CT_1 is the CT of the reference transcript and the CT_2 is the CT of the target transcript; CT stands for the cycle threshold. No template controls and non-reverse transcription reactions were performed as negative controls.

3.5. Echocardiography

Animals were anesthetized with i.m. injection of Zoletil® 100, Rometar® 2 % 5:1, diluted ten times, 3.2 ml/kg. Chest hair was shaved and ventricular thicknesses and dimensions were measured by an experienced echocardiographer using a 14-MHz probe (Vivid 7 ultrasound system, GE Healthcare, Fairfield, CT, USA). Interventricular septum thickness, posterior wall thickness, left ventricle end-systolic, end-diastolic diameters and cycle lengths were measured from parasternal short axis projection. M-mode tracings were obtained close to the papillary muscle level. The probe position was adjusted in order to obtain a perfectly round shape of the ventricle. Subsequently, probe position was changed to obtain an apical four-chamber view (A4C), from which mitral valve E and A wave velocities, E/A ratio, mitral valve deceleration time and slope were measured using pulsed wave Doppler tracing mode. Probe position was slightly rotated then to obtain LV outflow tract measurements. Maximal velocity in the left ventricle outflow tract and left ventricle ejection time were measured. During the whole examination, care was taken to avoid excessive pressure, which could cause bradycardia. Finally, measured parameters were used to calculate the heart rate, left ventricle fractional

shortening, left ventricle mass, and left ventricular peak velocity of circumferential fiber shortening according to previously published equations (Tanaka et al., 1996).

3.6. Telemetric assessment of heart rate

The telemetry system used was commercially available from Mini Mitter (Respironics, Andover, MA, USA). The transponders (E-Mitter, G_2-HR) were implanted in the peritoneal cavity under the same anesthesia as used in echocardiography (Zoletil® 100, Rometar® 2 % 5:1, diluted ten times, 3.2 ml/kg); mice were left for 1 week to recover from the surgery and then used in the experiment. The sensor leads were used for heart rate data acquisition; other parameters (temperature and activity) were acquired directly from the transponders. Receivers were connected in series and connected directly to the PC into a single computer port, allowing for the determination of all parameters. The heart rate (HR) data were collected every 10 s. VitalView, Chronos-fit, and GraphPad software were used for the evaluation of data.

3.7. Heart rate variability

Spectral analysis of heart rate variability obtained from telemetric recordings (heart rate collected every 10s) in intact animals was performed. Heart rate spectra were obtained using Fourier transformation. Subsequently, the area under each curve (AUC) was calculated and differences in AUC between WT and KO were compared.

3.8. Telemetric assessment of body temperature and animal activity

For picture completion of biorhythm changes and possible correlation of increased animal activity with heart rate, body temperature and animal activity were also assessed using the same telemetric system. The data were collected every 10 s in case of temperature and every 60 s in case of body activity.

3.9. Biorhythm analysis

The data collected by telemetry were grouped into half-hour sequences, the mean values were calculated and used for further analysis. The analysis was performed using Chronos-fit program (Arraj and Lemmer, 2006), employing Fourier analysis and stepwise regression technique. Then, the data were transferred into GraphPad Prism 5.04 program (San Diego, CA, USA) for further statistical analysis.

3.10. Drug application

Animals were held in separate cages; every single animal had its own cage with free access to food and water. We maintained the same regular light/dark regimen as stated before. The sensor had been implanted in general anesthesia (see "Telemetric assessment of heart rate" for details), and after 1 week of recovery period the experiment was started. The application route was intraperitoneal (i.p.) and approximately 100µl (according to the weight) was applied to each animal. All of the drugs were administered at the same daytime (9:30 AM to 9:45 AM) in order to minimize various influences of circadian heart rate variation. The telemetric system (discussed earlier) was used for heart rate monitoring. Carbachol (carbamoylcholine chloride, 0.5 mg/kg), isoprenaline (isoprenaline hydrochloride, 50 mg/kg), atropine (atropine monohydrate sulfate, 30 mg/kg), and propranolol (propranolol hydrochloride, 20 mg/kg) were dissolved in saline, and the same volume of saline was applied to control animals. The doses were derived from dose–response curves in WT animals from preliminary experiments.

3.11. Adenylyl cyclase activity (basal and forskolin-stimulated)

We have measured the basal level and forskolin stimulated activity of adenylyl cyclase using modified method of (Hoffert et al., 2005). Membranes were incubated in HBSS buffer (Hank's Stock Solutions, i.e. measurement of basal cAMP level) with or without presence of 40 µmol/l forskolin (stimulated adenylyl cyclase activity) for 10 min at 37°C. The reaction was stopped by adding 0.2 N HCl. Then, the mixture was incubated for 20 min at room temperature, which was followed by centrifugation at 10,000g for 10 min. Supernatants were saved for measurement of cAMP and pellets were used to measure protein content (BCA assay, Sigma). cAMP content was measured using an enzyme immunoassay kit (Cayman Chemical) at λ= 413 nm on a plate reader (Sunrise, Tecan). The final amount of cAMP was expressed as pmol/mg protein.

3.12. β_3-adrenoceptor effect on adenylyl cyclase activity

For the investigation of β_3-adrenoceptors (β_3-AR) mediated changes in adenylyl cyclase activity the tissue homogenates were pre-incubated with preferential β_3-AR agonist CL 316243 (100 nM) 10 min at room temperature. The activity of adenylyl cyclase was determined immediately using enzyme immunoassay kit as described above.

3.13. Determination of nitric oxide synthase activity

The determination of nitric oxide synthase activity (NOS) in tissue homogenates was performed by the Ultrasensitive Colorimetric NOS Assay Kit (Oxford Biomedical Research, Inc., USA). The first phase of the experiment (in microcentrifuge tube) was proceeded with tissue homogenates (30µl) mixed with 50 mM HEPES with 0.5 mM EDTA (200µl), NADPH Part A containing $NADP^+$, Glucose 6-Phosphate and L-Arginine (10µl), and NADPH Part B containing Glucose 6-Phosphate dehydrogenase (10µl). After mixing and incubating for 1 h at 37°C (water bath) the reaction mixtures were chilled on ice for 5 min and subsequently nitrate reductase (10µl) was added to each sample. After vortexing and incubating for 20 min at room temperature the reaction mixtures were centrifuged at 12,500 rpm for 5 min at 4°C. The amount of protein (determined using the Pierce BCA protein Assay Kit) in the reaction mixtures (total volume of 260µl) was within the range of 138–339µg. The second phase of the experiment (in flat-bottom microtiter plate) was performed with nitrite standard solutions (100µl) diluted from 100 to 0.5µM in duplicates and samples (100µl) also in duplicates followed by addition of the Griess reagents, SA in 3 N HCl (50µl), and NED (50µl). After shaking for 5 min at room temperature the absorbance was measured at 540 nm in multi-detection microplate reader (Synergy HT).

3.14. β_3-adrenoceptor effect on NOS activity

For the investigation of β_3-adrenoceptor (β_3-AR) mediated changes in NO-synthase (NOS) activity the tissue homogenates were pre-incubated with preferential β_3-AR agonist BRL 37344 (1 mM) 10 min at room temperature. The time dependence (0, 5,10, 15, 30 min) of NOS activation by BRL 37344 was determined in preliminary experiments showing 10 min as the start of peak level of activation. The activity of NOS was determined using the Ultrasensitive Colorimetric NOS Assay Kit (Oxford Biomedical Research, Inc.).

3.15. Catecholamine blood concentration

Blood samples were collected in general anesthesia (see “Telemetric assessment of heart rate” for details), which was administered 25 min prior to sampling. Blood was drawn from the tail vein and the amount was more than 100µl (the plasma amount used for subsequent analysis was 50µl). Catecholamines were determined by enzyme immunoassay for the quantitative determination of Adrenaline (A) and Noradrenaline (NA) using 2-CAT Research ELISA kit (DEE5400, Demeditec Diagnostics GmbH, Germany). Briefly, A and NA were extracted using a cis-diol specific-affinity gel, acylated and then converted enzymatically. The solid

phase-bound antigen and A or NA in the samples/standards/controls competed for a fixed number of antiserum (rabbit) binding sites. After the system reached equilibrium, free antigen and free antigen–antiserum were washed out. Antirabbit IgG and peroxidase conjugate detects solid-phase bound antiserum. After converting TMB (substrate), the reaction was stopped and absorbance was read at 450 nm.

3.16. Gene expression of catecholamine-synthesizing enzymes

In order to assess if there is no chronic adrenergic stress in KO animals, the gene expression of catecholamine-synthesizing enzymes was measured. Total RNA was prepared from frozen mice adrenal medullae using the RNAzolTM reagent (Tel-Test, Friendswood, TX, USA) according to the manufacturer's instructions. Reverse transcription was performed from 1.5μg of total RNA using Ready-To-Go You-Prime First-Strand Beads (Amersham Bioscience, Buckinghamshire, UK) and pd(N)6 primer according to the manufacturer's protocol. To determine mRNA levels in the adrenal medullae of mice, the following primers were used:

tyrosinhydroxylase (TH):

For: 5´-GAAGGGCCTCTATGCTACCCA-3´, Rev: 5´-TGGGCGCTGGATACGAGA-3´;

dopamine-β-hydroxylase (DBH): For: 5´-GACTCAACTACTGCCGGCACGT-3´,

Rev: 5´-CTGGGTGCACTTGTCTGTGCAGT-3´,

phenylethanolamine N-methyltransferase (PNMT):

For: 5´-TACCTCCGCAACAACTACGC-3´, Rev: 5´-AAGGCTCCTGGTTCCTCTCG-3´,

glyceraldehyde-3-phosphate dehydrogenase (GAPDH):

For: 5´-AGATCCACAACGGATACATT-3´, Rev: 5´-TCCCTCAAGATTGTCAGCAA-3´.

After the initial denaturation at 94 °C for 5 min, each cycle of amplification consisted of 60 s at 94 °C, 30 or 60 s at the respective annealing temperature and with a specific number of cycles (Benes et al., 2012b), and 60s at 72°C. Final polymerization was performed for 7 min at 72 °C. All reactions were performed in the linear phase of amplification. PCR products were separated on 2 % agarose/ethidium bromide gels. The density of individual bands was evaluated by STS 6220I system (Ultra-Lum, Inc., BLD Science, Garner, NC, USA), and relative expression values were calculated by the analysis of band intensities using PCBAS 2.08e software (Raytest, Inc., Dusseldorf, Germany). Semiquantitative values were expressed relative to the housekeeping gene (GAPDH).

3.17. Statistical analysis

Results are presented as mean± S.E.M. and each group represents an average of six to eight animals. In some cases we had to pool the tissue; in these cases, the number of experimental objects in the group is lower. Statistical differences among groups were determined by 1-way analysis of variance (ANOVA). For multiple comparisons, modified Newman–Keuls correction was used. The data from drug application experiments were evaluated using 2-way ANOVA (repeated measures; factors: treatment, phenotype). Heart rate variability data were analyzed using SPSS 19 software (IBM, Inc.) and GraphPad Prism 5.01 (GrapPad software, Inc.). The comparison of two groups was performed using Student t-test or Mann–Whitney test (if data did not reveal Gaussian distribution as assessed by F test). Values of $p<0.05$ were considered to be significant.

4. Results

We have initially performed binding experiments and subsequently gene expression experiments both in left and right ventricles. In right ventricles, no difference in binding and gene expression in all analyzed muscarinic and β-receptor subtypes were found between WT and KO animals except for binding of muscarinic receptors that was diminished to the similar extent as in left-ventricle (6% of WT). Therefore, other signaling parameters (adenylyl cyclase, NO-synthase) were analyzed in left ventricles only. All data presented below thus pertain to the situation in left ventricles.

4.1. Binding characteristics, basal state

Gene disruption of M_2-receptor gene changed the amount of all β_1-, β_2-, and β_3- adrenoceptor binding sites (Fig. 4.1.1). The cardiostimulatory β_1- and β_2-AR were decreased in comparison to WT. On the contrary, the amount of β_3-AR binding sites was increased in M_2KO mice. The binding of muscarinic receptor specific radioligand in M_2KO mice was not completely abolished with 13 % of remaining binding sites.

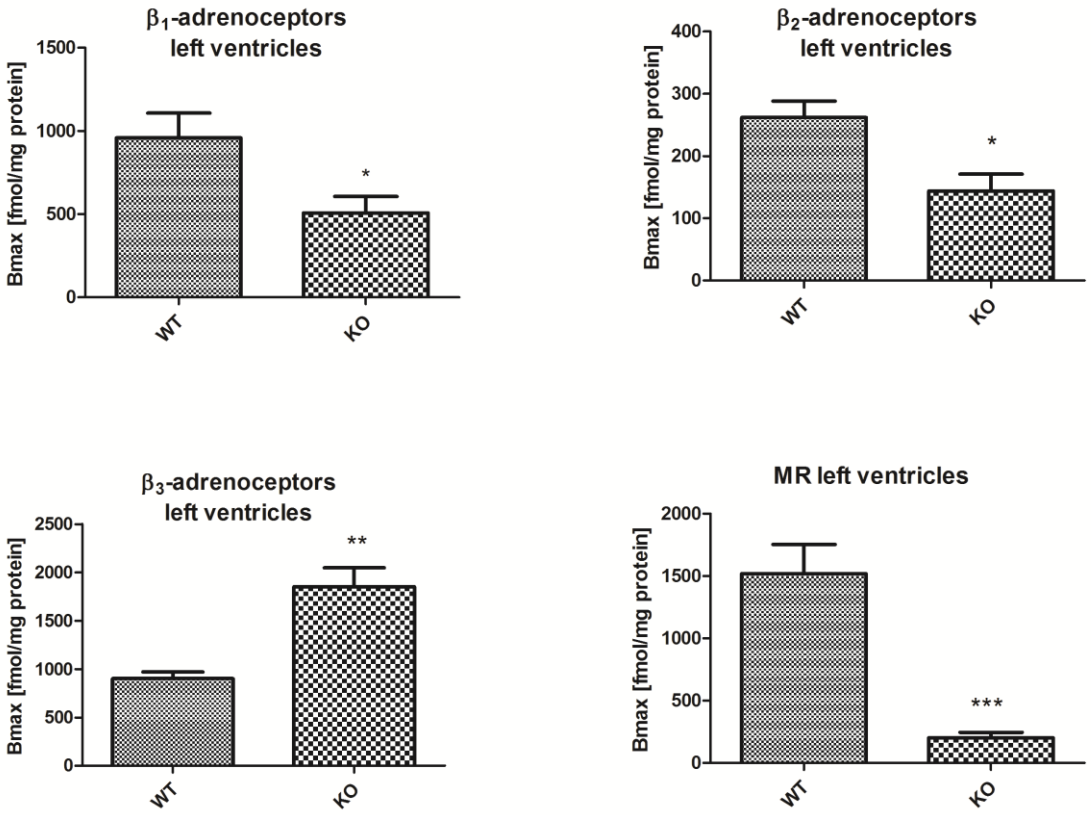

Fig. 4.1.1. The changes in the number of β_1-, β_2-, β_3-adrenoceptor binding sites and muscarinic receptor binding sites in M_2KO mice.

β_1- adrenoceptor binding sites (top left), β_2- adrenoceptor binding sites (top right), β_3-adrenoceptor binding sites (bottom left) and muscarinic receptor binding sites (bottom right). *p<0.05 different from WT, **p<0.01 different from WT, ***p<0.001 different from WT, n= 3–4 (β_1- and β_2-AR) and n= 6 (MR and β_3-AR).

The experiments with saturation binding and competition with CGP20712A and ICI118.151 proved that the affinity of receptors (K_D) was not affected by M_2-receptor knockout (155±25 pmol/l vs. 208±48 pmol/l for β-AR WT and KO, respectively; 50.1±2.5 pmol/l vs. 52.2± 4.5 pmol/l for MR in WT and KO, respectively, Fig. 4.1.2). Similarly, saturation experiments showed that K_D was similar for β_3-AR WT and KO mice (1030±169.6 pmol/l vs. 760.3±134.9 pmol/l, p= n.s.). These data suggest that observed changes occur in the amount of binding sites rather than in the affinities of receptors to ligands.

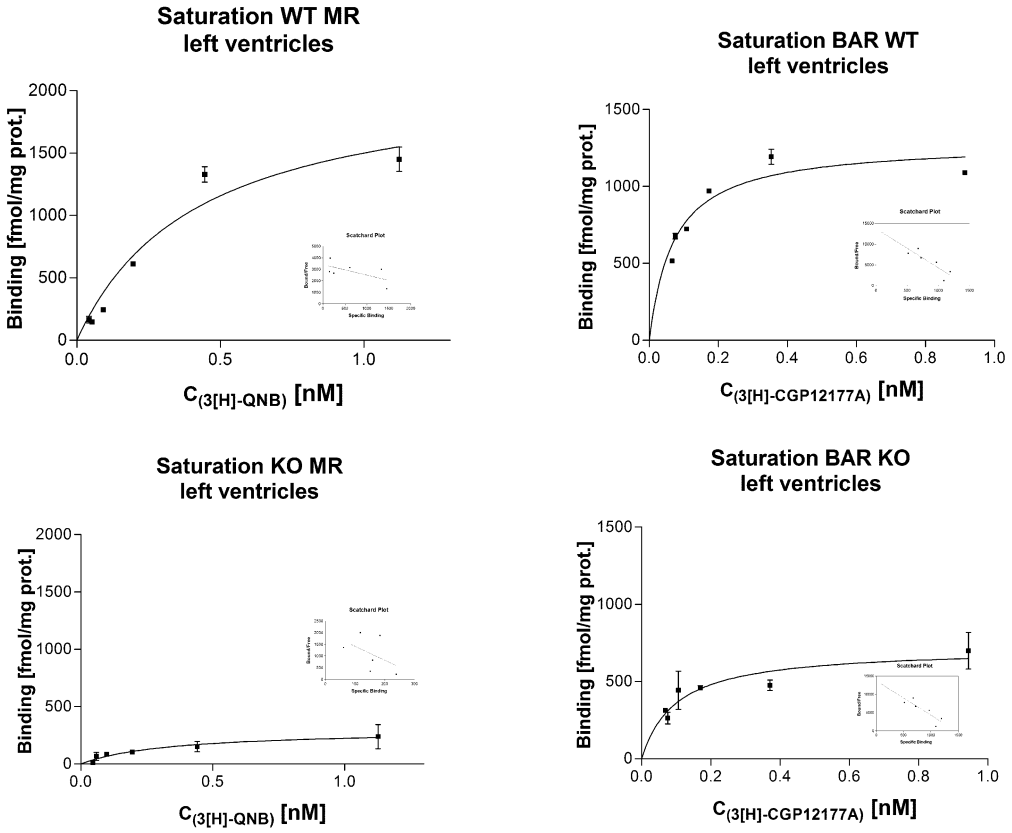

Fig. 4.1.2. Saturation binding to muscarinic (left) and β-adrenoceptors (right) in WT (top) and KO (bottom) animals.

Abscissa: concentration of free radioligand [nmol.l^{-1}].Ordinate: B_{max} [fmol.mg.prot^{-1}]. Insert: Scatchard plot of binding. MR, Muscarinic receptors; BAR, β-adrenoceptors.

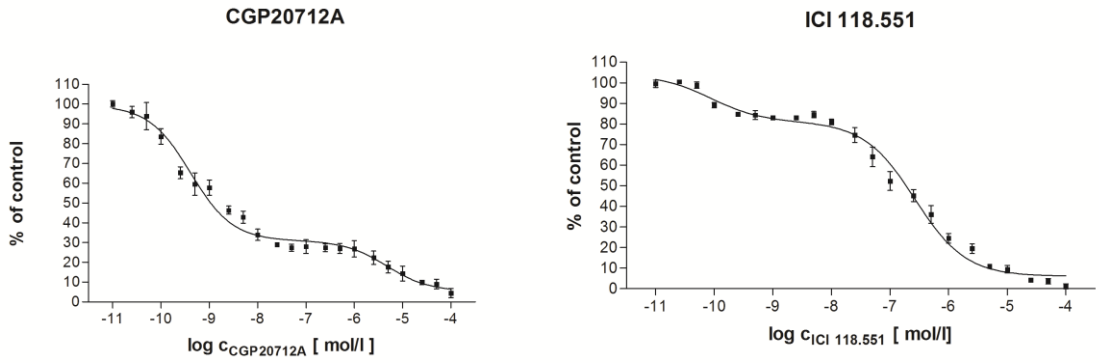

Fig. 4.1.3. Competition binding curves for CGP 20712A (β_1- AR antagonist) and ICI 118.551 (β_2-AR antagonist) with ^{3}H-CGP12177.

Average data from 4 independent experiments. Abscissa: log of antagonist concentration. Ordinate: Bound [% of binding in the absence of antagonist].

4.2. Receptor binding upon stress

In WT animals, the cold stress resulted in a decrease in β_1- and β_2-AR (both after 1 day stay in the cold and after 7 days in cold) and also in a decrease in MR (see Fig. 4.2.1a, b). β_1-AR decreased to 37% of control values after 1 day of cold and further decreased to 27% of control values after 7 days of cold (1-way ANOVA, P= 0.0007, F= 20.94, df= 10). β_2-AR were diminished to 35% after 1 day of cold and to 28% after 7 days in cold, respectively (1-way ANOVA, P= 0.0003, F= 27.33, df= 10). On the contrary, β_3-AR increased (to 216% of control values) after 7 days of cold (1-way ANOVA, P=0.0001, F= 31.80, df= 17), Fig. 4.2.1. Muscarinic receptors decreased to 46% of control values after 1 day of cold and the changes prevailed till the day 7 (when 58% of control values were measured; 1-way ANOVA, p= 0.0182, F= 5.298, df= 17). The magnitude of changes expressed as percent of WT unstressed animals is shown in Tab. 4.2.3.

The reaction of cardiostimulatory β_1- and β_2-AR to cold was similar in KO animals. In KO animals, there was a decrease in β_1-AR number after 1 day of cold (to 45% of KO control values) and further persisted- 32% of KO control values were measured after 7 days of cold. The changes in β_2-AR were almost the same as those in WT: β_2-AR decreased to 44% and to 29% in KO animals (after day 1 and day 7, respectively), Fig. 4.2.2.

In contrast to cardioinhibitive receptors in wild types, β_3-AR in KO animals (which were already increased at baseline compared to WT) did not further change in reaction to cold (Fig. 4.2.2.). The magnitude of changes expressed as percent of WT unstressed animals is shown in Table 4.2.3. Muscarinic receptors (that were greatly attenuated at baseline in KO) did not further change upon cold stress (Tab. 4.2.3.).

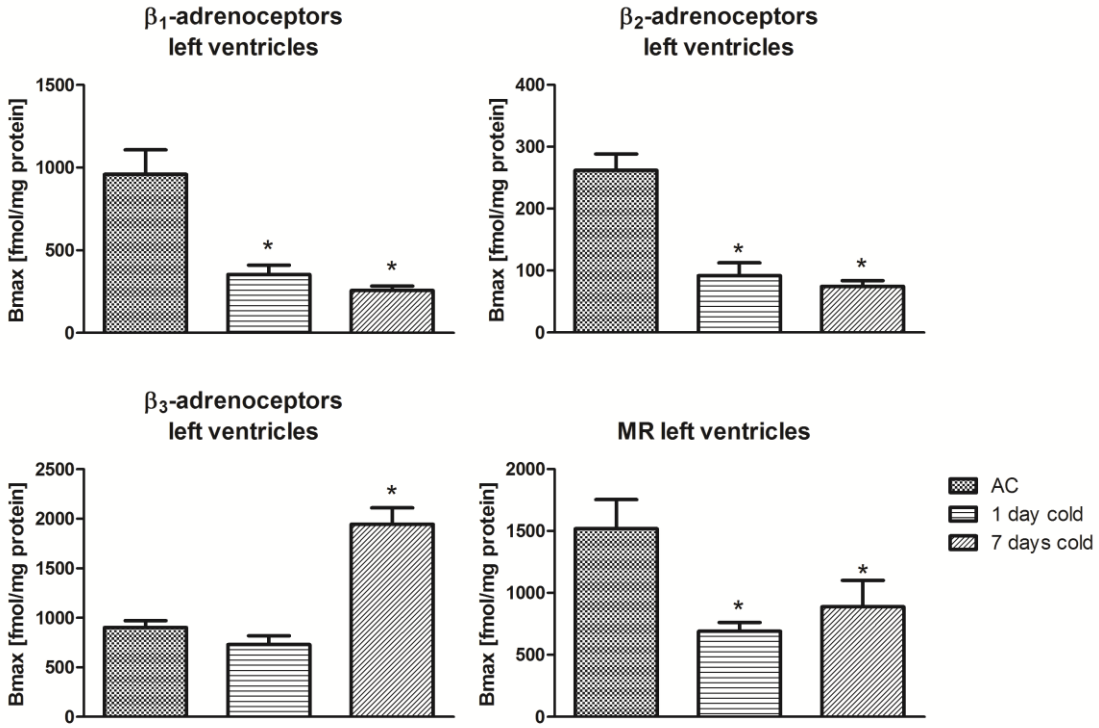

Fig. 4.2.1. Changes of receptor binding during exposure to cold in wild type animals in left ventricles.

Abscissa: number of days in cold. Ordinate: receptor binding expressed as B_{max} [fmol/mg. prot.$^{-1}$]. a, b, c, d: appropriate type of receptor is marked in the heading.

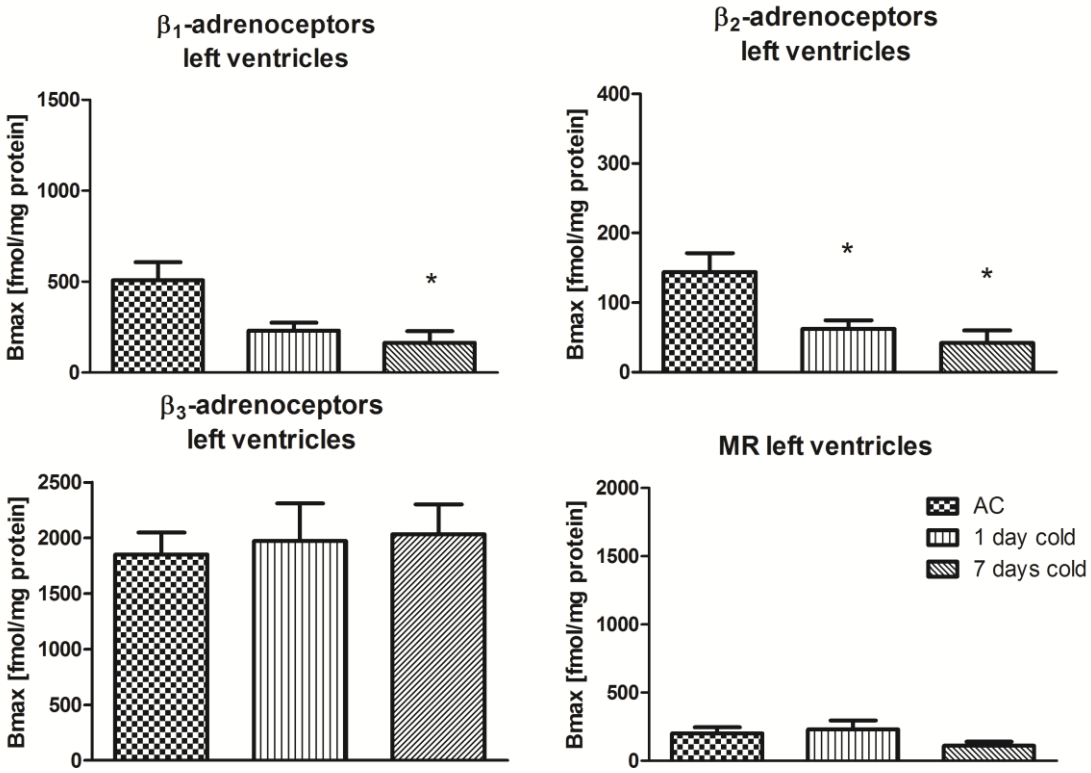

Fig 4.2.2: Changes of receptor binding during exposure to cold in knockout animals. Abscissa: number of days in cold. Ordinate: receptor binding expressed as B_{max} [fmol/mg. prot.$^{-1}$]. a, b, c, d: appropriate type of receptor is marked in the heading.

Genotype	Receptor subtype	Exposure to stress		
		Unstressed	1-day-stressed	7-day-stressd
WT	β_1-AR	100 ± 15%	37 ± 6%*	27 ± 3%*
M_2KO		53 ± 10%*	24 ± 5%*	17 ± 7%*
WT	β_2-AR	100 ± 10%	35 ± 8%*	28 ± 4%*
M_2KO		54 ± 10%	24 ± 5%*	16 ± 7%*
WT	β_3-AR	100 ± 8%	82 ± 10%	219 ± 19%*
M_2KO		208 ± 22%*	222 ± 38%	228 ± 30%
WT	MR	100 ± 15%	46 ± 5%*	58 ± 14%*
M_2KO		13 ± 3%*	15 ± 4%	7 ± 2%

WT unstressed animals are considered as standard (100%), *$P < 0.05$ significant difference from unstressed WT

Table 4.2.3. The proportional changes in binding sites in the left ventricles summarizing the magnitude of changes between groups

4.3. Receptor gene expression, basal state

In the left ventricles of WT animals, M_1- and M_5-receptors were not expressed. The gene knockout of M_2-receptors naturally abolished their gene expression. Moreover, M_3-receptor gene expression was diminished in M_2KO animals (Fig. 4.3.1.). M_4-receptor gene expression remained unchanged in M_2KO animals. The gene expression of both β_1- and β_2-adrenoceptors was decreased to approximately 60 % in KO animals (Fig. 4.3.1.).

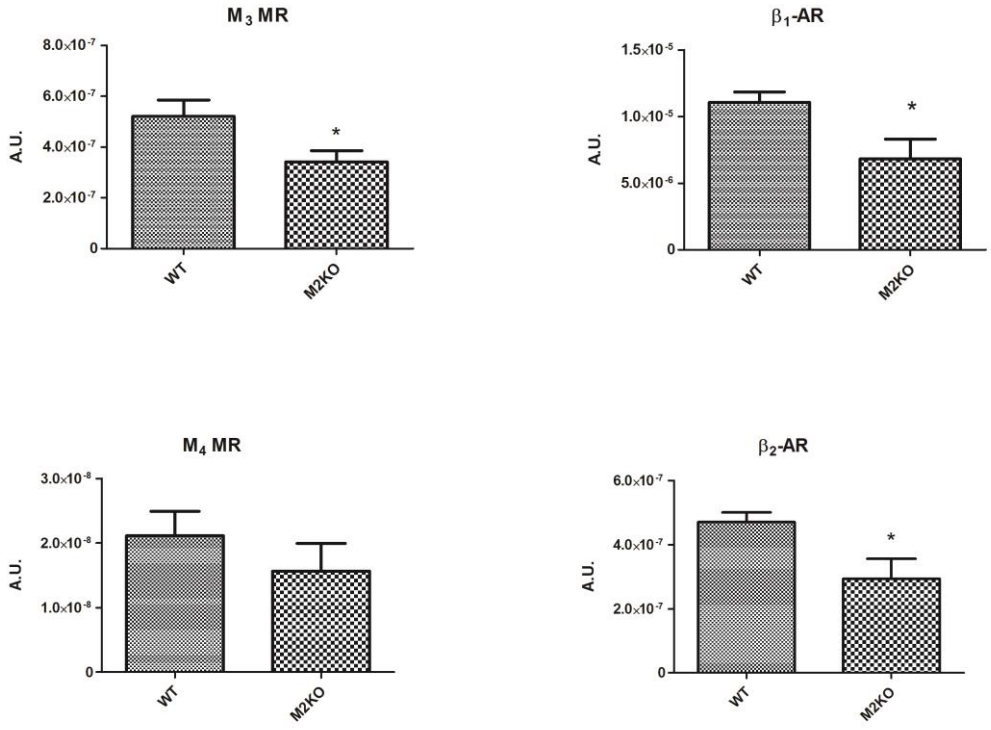

Fig. 4.3.1. The changes in M_3, M_4, and β_1- and β_2-adrenoceptor gene expression in left ventricles in M_2KO animals.

*p<0.05 different from WT, n=8 (WT and KO). M_2-receptor gene expression was undetectable in KO animals, in addition to that, M_1- and M_5-receptor gene expressions were undetectable both in WT and KO mice.

4.4. Receptor gene expression upon stress

Further, we have performed a gene expression assay determining the amount of mRNA for all MR subtypes (M_1-M_5) after the application of cold stress. As expected, M_2-receptors were not expressed in KO animals. In addition to that, the gene expression of M_1- and M_5-receptors was undetectable both in the unstressed and stressed wild types as well as in unstressed and stressed knockout animals. The expression of M_4-receptors were not changed in WT and KO upon stress (Fig. 4.4.1.d,e). On the contrary, the expression of M_3-receptors was decreased upon cold stress in WT but not in KO animals (Fig. 4.4.1.b,c). The magnitude of changes expressed as percent of WT unstressed animals is shown in Table 4.4.2.

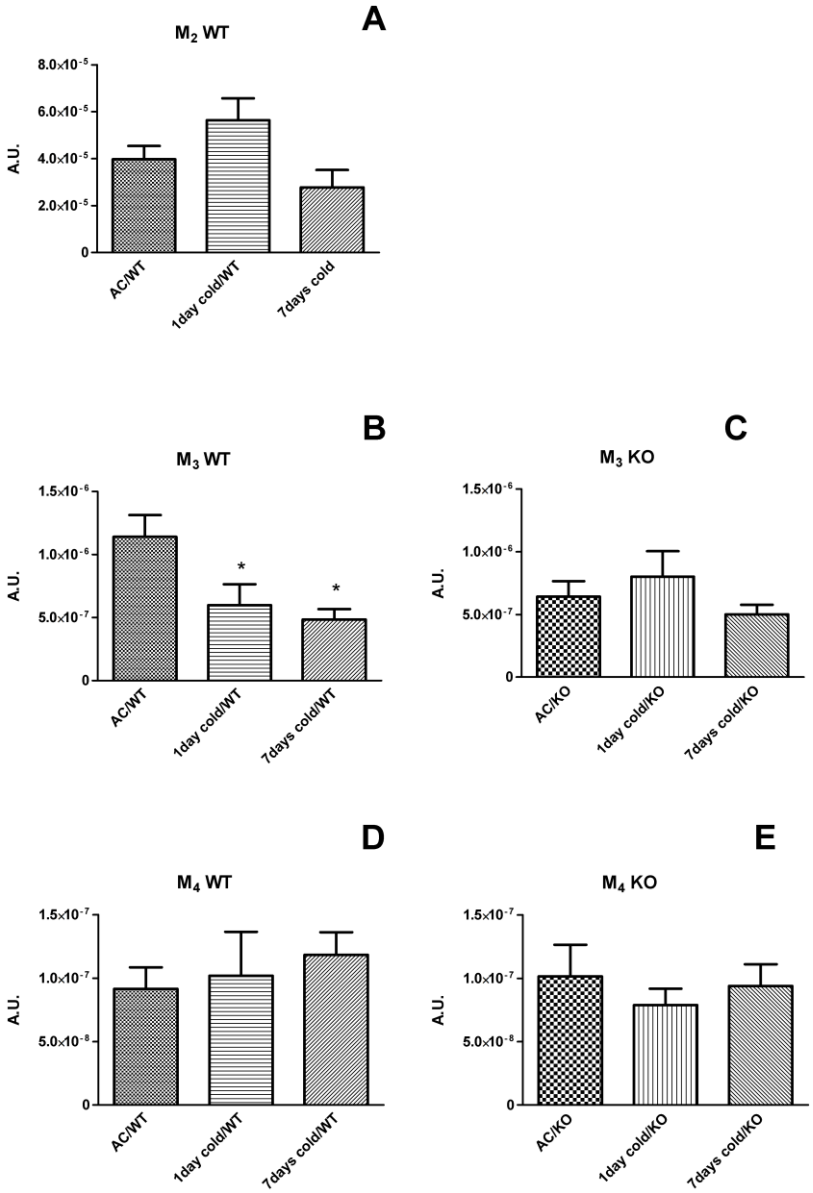

Fig 4.4.1: Changes of gene expression of muscarinic receptor subtypes (M_1–M_5) during exposure to cold in wild type and M_2 knockout animals.

Abscissa: number of days in cold. Ordinate: the relative amount of mRNA expressed in A.U. [arbitrary units]. M_2 WT: M_2-receptor gene expression, KO animals are not shown as M_2-MR were deleted. M_1- and M_5-MR were not detectable. A: M_2 in WT, B: M_3 in WT, C: M_3 in KO, D: M_4 in WT, E: M_4 in KO.

Genotype	Receptor subtype	Exposure to stress		
		Unstressed	1-day-stressed	7-day-stressed
WT	M_1MR	Non detectable		
M_2KO				
WT	M_2MR	100 ± 14%	142 ± 24%	70 ± 19%
M_2KO		Non detectable		
WT	M_3MR	100 ± 15%	52 ± 13%*	42 ± 7%*
M_2KO		56 ± 11%*	70 ± 18%	44 ± 7%
WT	M_4MR	100 ± 19%	111 ± 38%	129 ± 20%
M_2KO		111 ± 27%	86 ± 14%	103 ± 19%
WT	M_5MR	Non detectable		
M_2KO				

WT unstressed animals are considered as standard (100%), *$P < 0.05$ significant difference from unstressed WT

Table 4.4.2. The proportional changes in gene expression of M_1-M_5 muscarinic receptor subtypes in the left ventricles summarizing the magnitude of changes between groups

4.5. Echocardiographic parameters

The echocardiographic parameters describing both LV systolic and diastolic function were similar both in WT and KO animals. The data are summarized in the Table 4.5.1 below. Representative echocardiograms of WT and KO animals are shown in Fig. 4.5.2.

Parameter	**WT** (n=8)	**KO** (n=8)	**Units**
BW	32.12±1.30	34.41±0.90	[g]
M-Mode			
IVSd-PSAX	0.98±0.08	0.87±0.05	[mm]
LVIDd-PSAX	3.24±0.17	3.62±0.16	[mm]
LVIDs-PSAX	1.95±0.19	2.09±0.18	[mm]
LVPWd-PSAX	1.12±0.14	0.83±0.05	[mm]
RR-PSAX	201.25±22.77	205.50±25.55	[ms]
Doppler			
MV E Vel	0.58±0.03	0.61±0.02	[m/s]
MV DecT	41.00±3.37	40.50±2.38	[ms]
MV Dec Slope	15.23±1.32	15.88±1.18	[m/s^2]
MV A Vel	0.26±0.02	0.30±0.04	[m/s]
MV E/A Ratio	2.29±0.15	2.40±0.35	
LVOT Vmax	0.66±0.03	0.60±0.04	[m/s]
LVOT maxPG	1.80±0.17	1.51±0.18	[mmHg]
LVET	86.13±5.26	86.63±5.73	[ms]
Calculated parameters			
HR	331.11±36.74	322.49±31.30	[min^{-1}]
LV FS	41.01±3.90	43.18±2.81	[%]
LV mass, echo	135.41±14.45	107.74±4.02	[mg]
Vcf	5.04±0.70	5.31±0.68	[circ/s]

Table 4.5.1: Echocardiographic parameters in WT and KO animals

The data are shown as mean±SEM. *IVSd-PSAX* interventricular septum thickness in diastole (measured in parasternal short axis, PSAX), *LVIDd-PSAX* left ventricle end-diastolic diameter measured in PSAX, *LVIDs-PSAX* left ventricle end-systolic diameter measured in PSAX, *LVPWd-PSAX* left ventricle posterior wall thickness measured in PSAX, *RR-PSAX* cycle

length measured in PSAX, *MV E Vel* mitral valve E wave velocity, *MV DecT* mitral valve deceleration time, *MV Dec slope* mitral valve deceleration time slope, *MV A Vel* mitral valve A wave velocity, *MV E/A Ratio* mitral valve E/A ratio, *LVOT Vmax* maximal velocity in the left ventricle outflow tract, *LVOT maxPG* maximal pressure gradient in the left ventricle outflow tract, *LVET* left ventricle ejection time, *HR* heart rate, *LV FS%* left ventricle fractional shortening, *LV mass*, echocardiographic calculation of LV mass, *Vcf* left ventricular peak velocity of circumferential fiber shortening

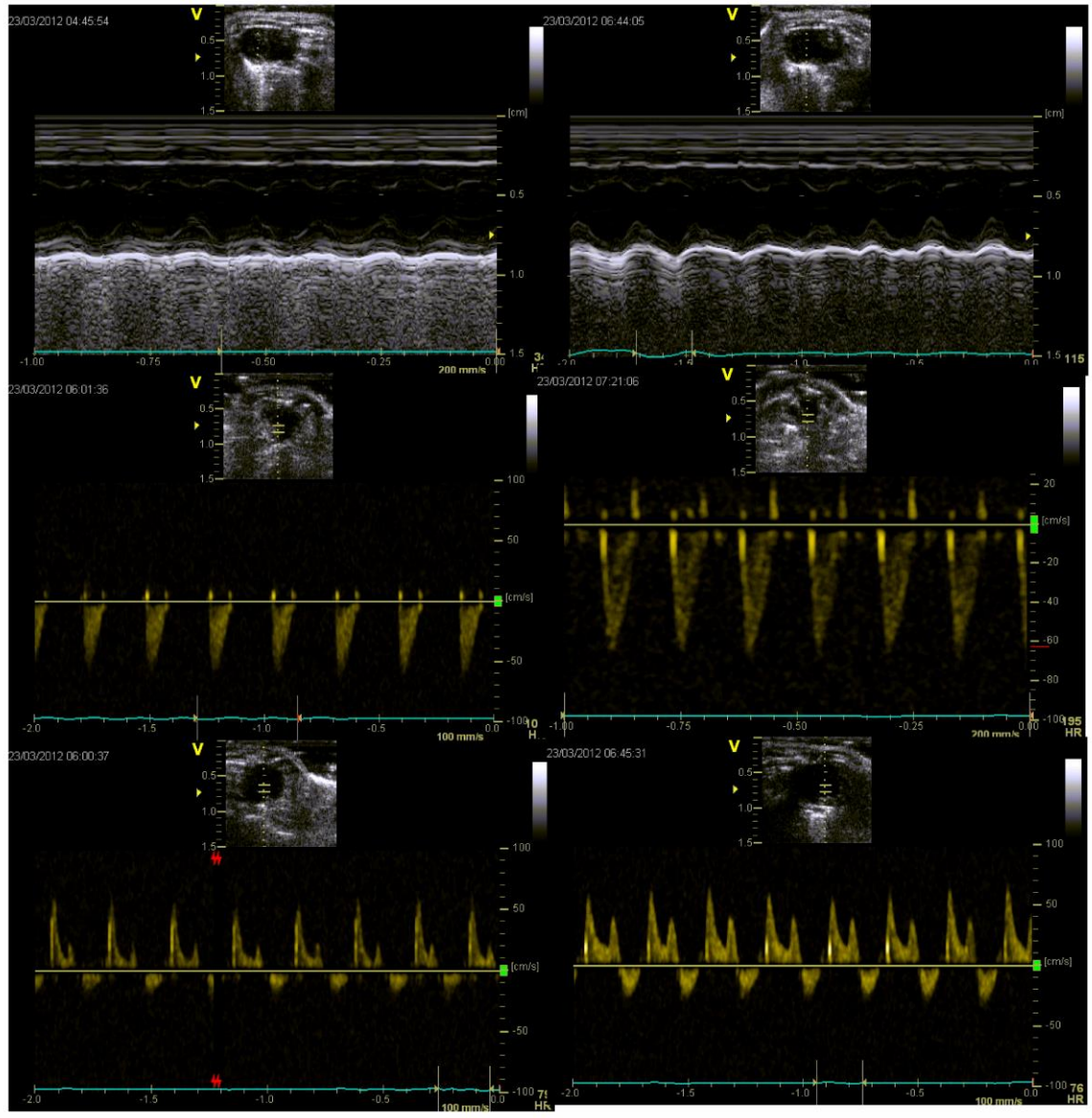

Fig. 4.5.2. Representative echocardiograms of WT (left) and KO (right) animals. Left: WT mice, right: KO mice. Top: Two-dimensional echocardiogram: end diastole, M-mode echocardiogram from the same mouse in the parasternal short-axis view. Middle: left

ventricle outflow tract (two-dimensional echocardiogram, pulsed wave Doppler tracing). Bottom: mitral view (two-dimensional echocardiogram, pulsed wave Doppler tracing)

4.6. Heart rate

When comparing means in WT and KO animals as averaged in 30-min intervals, we have found an increase in basal heart rate in M_2KO animals (513.1±5.01 beats per minute, n=8 vs. 469.6±12.24, n=8, p=0.0054, KO vs. WT, i.e. an increase of 6 % in KO animals, Fig. 4.6.1. left). The difference between animals was also seen when comparing light phase and dark phase means; the increase was slightly higher in the nighttime (8% vs. 5% in the daytime; Fig. 4.6.1, middle). In order to better characterize the differences between wild-type and M_2KO animals we have employed biorhythm analysis. Both WT and KO animals displayed a typical circadian pattern in HR with peak values in the dark span. However, WT and KO animals differed in biorhythm parameters (day mean 492.7±7.05 beats per minute, vs. 449.5±16.73, p=0.0320, KO vs. WT animals; night mean 530.6±10.61 vs. 488.5±9.03, p=0.0091; mesor 512.1±4.87, vs. 468.5±12.55, p=0.006; peak (i.e., maximal values) 585.1±19.28 vs. 531.6±7.51, p=0.0410; and minimal values 458.0±10.62 vs. 398.8±22.58, p=0.0240, n=6-8 for all parameters). In summary, the differences were between 8 and 15 %. The comparison of biorhythms in KO and WT animals is showed in Fig. 4.6.1. (right).

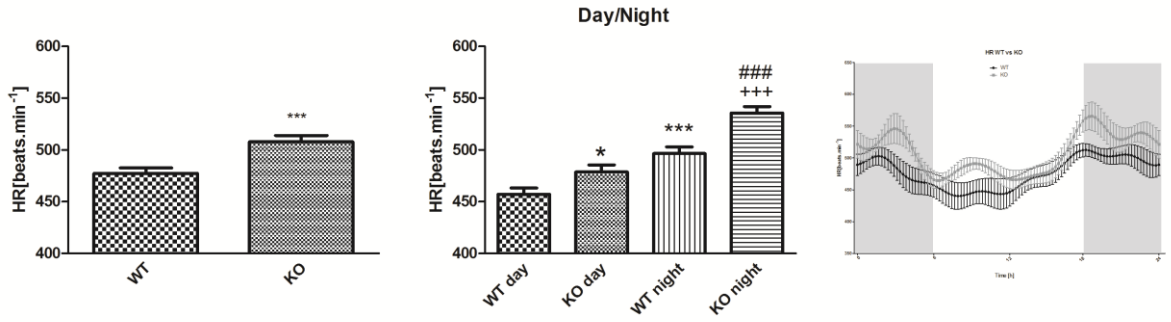

Fig. 4.6.1. Heart rate in WT and M_2KO mice.

Average heart rate measured in 30-min intervals over a 24-hour period (left). Heart rate in the light and dark phase (middle). Comparison of biorhythm in WT and KO animals (right). *p<0.05 different from WT day, ***p<0.001 different from WT day, +++p<0.001 different from WT night, ###p<0.001 different from KO day.

4.7. Heart rate variability

Both animal groups (WT and KO) showed a similar distribution of power spectral densities (PSDs), Fig. 4.7.1. When areas under the curves (AUCs) were compared, Mann–Whitney test showed no difference between groups ($6.03\times10^5\pm1.76\times10^5$ vs. 5.84×10^5 $\pm1.71\times10^5$, WT vs. KO, respectively, p=0.916).

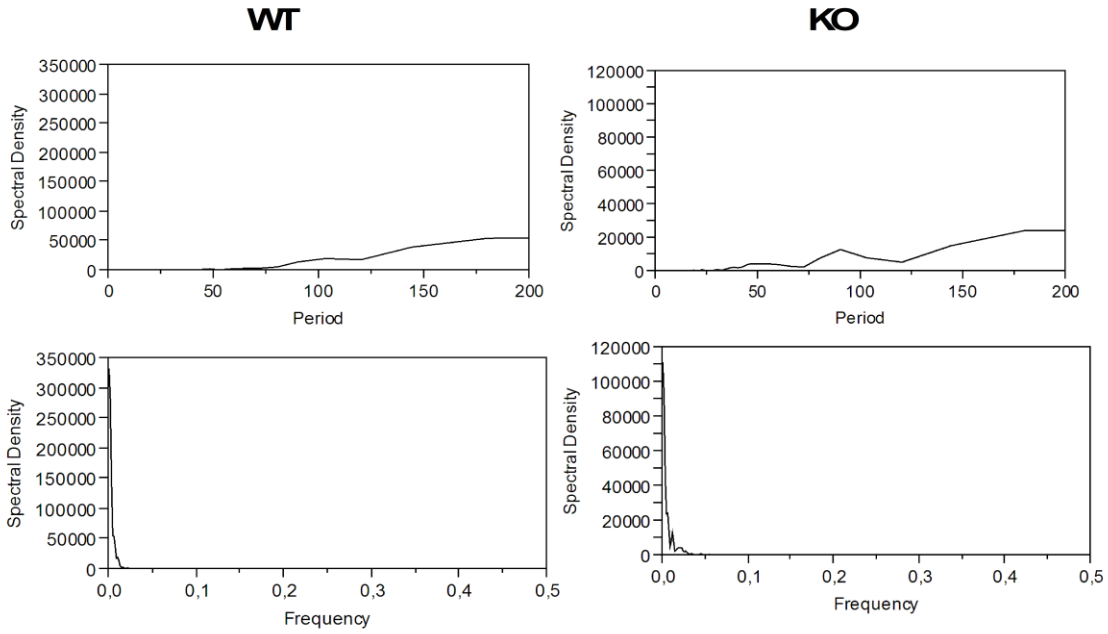

Fig. 4.7.1. Representative figures of heart rate variability

The representative figure of spectral density vs. period and spectral density vs. frequency in WT and KO animals. No difference was observed between KO and WT mice

4.8. Activity

In order to determine whether the heart rate differences could be caused by an increase in activity (and therefore by higher demands for blood perfusion in KO animals), we have analyzed activity of WT and KO mice. Both WT and KO animals displayed a typical circadian pattern in their activity with peak values in the dark span. No difference in activity was observed both when the data were averaged in 30-min intervals over the whole 24-hours period as well as when the data from the light phase and the dark phase were treated separately. No difference in biological rhythm analysis was seen as well, Fig. 4.8.1.

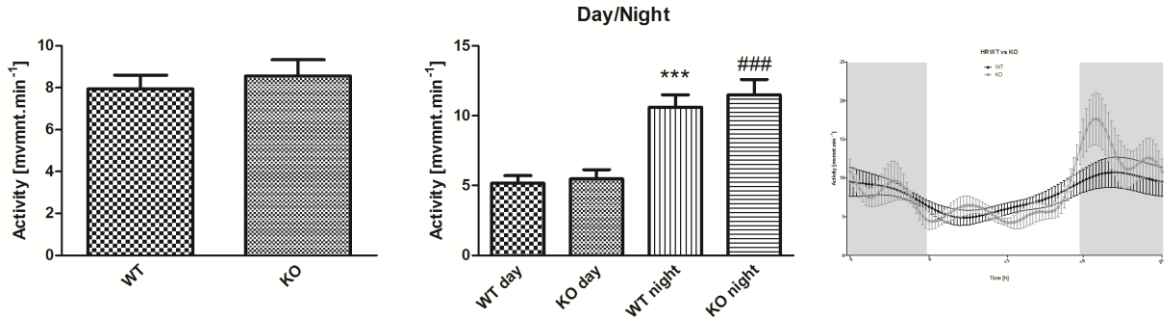

Fig. 4.8.1. Activity

Activity in WT and M_2KO mice. Left: average activity. Middle: the activity in the light and dark phase (day or night). Right: comparison of biorhythm in WT and KO animals. ***p<0.001 different from WT day. ###p<0.001 different from KO day, 1-way ANOVA (p< 0.0001, F=15.83, df=93)

4.9. Body temperature

The body temperature in M_2KO animals was lower than in WT animals. When comparing the light and the dark phase, the same picture was obtained. The temperature of KO animals was lower both in the light phase and in the dark phase. In both groups (WT and KO) there was an increase in body temperature in the dark phase. Both WT and KO animals displayed a typical circadian pattern in temperature with peak values in the dark span. There was no difference in biological rhythm analysis (Fig. 4.9.1.).

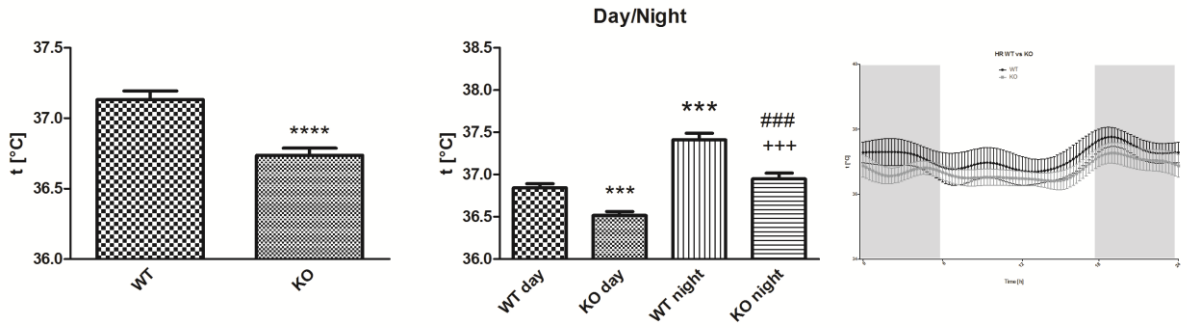

Fig. 4.9.1. Body temperature

Body temperature in WT and M_2 KO mice. Left: average temperature. Middle: temperature in the light and dark phase (day or night). Right: comparison of biorhythm in WT and KO animals. ****p<0.0001 different from WT, ***p<0.001 different from WT day, ###p<0.001

different from KO day, +++p<0.001 different from WT night, 1-way ANOVA (p< 0.0001, F=36.18, df=93).

4.10. Actograms

The pattern of increased values of HR, activity, and temperature in WT and KO is showed in Fig. 4.10.1. As can be observed, the periods of increased values are similar in all parameters in WT and KO mice.

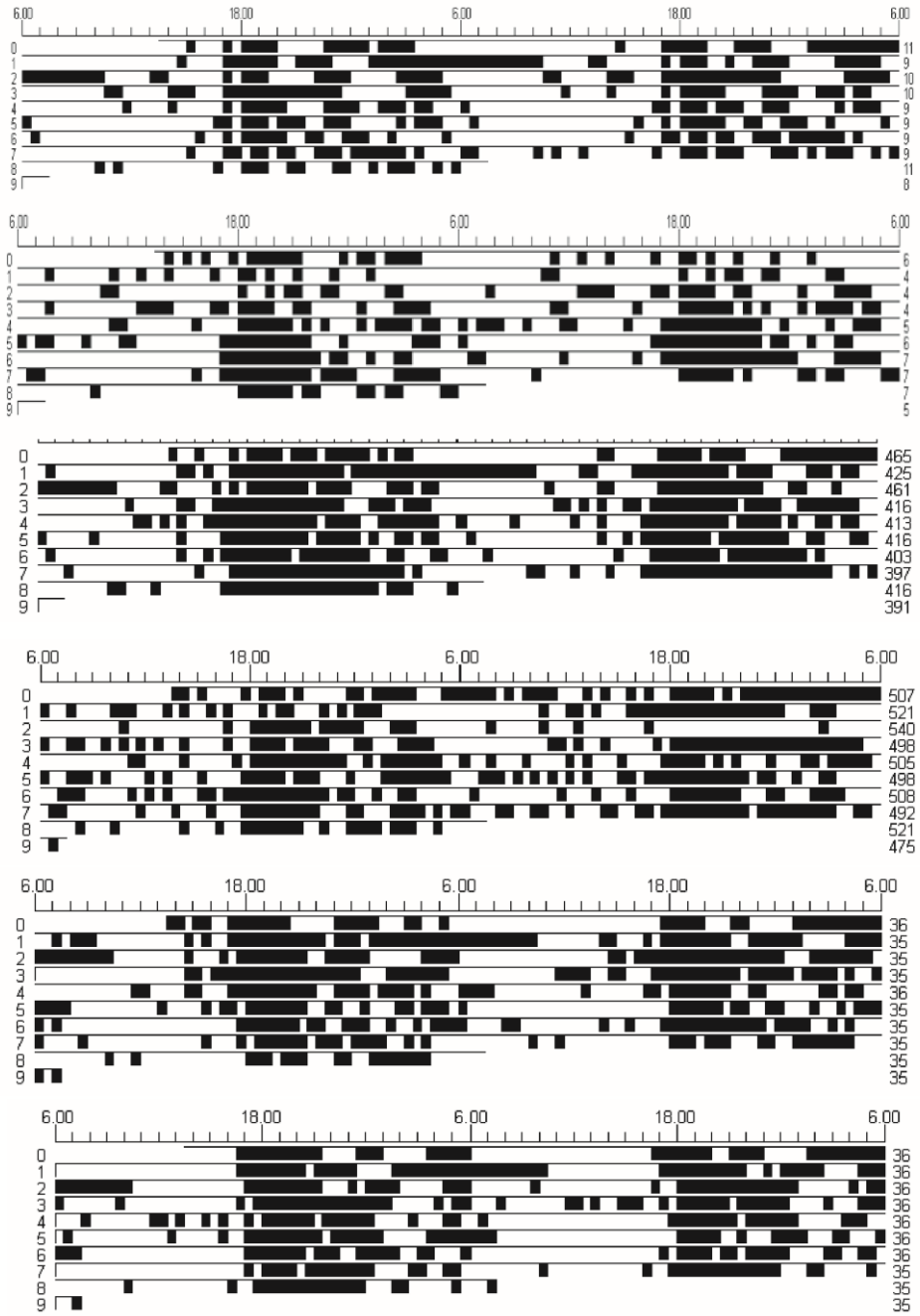

Fig. 4.10.1. The actograms of representative WT and KO mice showing increased values of respective parameters (black rectangles). **Abscissa**: time starting at 6:00 AM and showing two consecutive days in one row. Ordinate: The number of row representing two days. From top to bellow: WT activity, KO activity, WT HR, KO HR, WT temperature, KO temperature

4.11. Drug application

Carbachol (see Fig. 4.11.1.a) caused expected effects of WT animals (bradycardia, 257.4±18.03 vs. 464.5±8.645 in WT animals treated with saline, i.e., decrease to 55 %). (The response of KO animals to saline treatment did not differ from the response in WT animals except for the first 3 min when the heart rate was slightly higher). In M_2KO animals, carbachol induced tachycardia (651.4±24.56 vs. 546.9±10.91, i.e., increased by 19 %) when compared to saline-treated KO mice.

Isoprenaline (see Fig. 4.11.1.b) also caused expected effects in WT animals (tachycardia, 687.1±6.593 vs. 390±3.29, i.e., 76 % increase compared to WT animals treated with saline). The same effects were observed in KO animals, where isoprenaline also evoked tachycardia although the effects were not as high as in WT animals (659.9±7.2 vs. 453.2± 1.5, i.e., 46 % increase compared to saline-treated KO animals). There was also no difference in the response of KO animals to saline application when compared to WT counterparts.

Atropine (see Fig. 4.11.1.c) was able to increase the heart rate in WT animals (512.6±11.26 vs. 401.9±1.85) but failed to affect the heart rate in KO mice (480.4±4.314 vs. 479.2±3.14). Propranolol (see Fig. 4.11.1.d) was able to significantly decrease the heart rate in WT animals (243.1±16.88 vs. 510.4 ±8.662). Similarly, in KO animals, the heart rate under propranolol treatment fell from 586.6±10.27 to 408.2±5.56. Therefore, a decrease to 48 % of saline-treated animals was found in WT mice but only up to 70 % in KO counterparts.

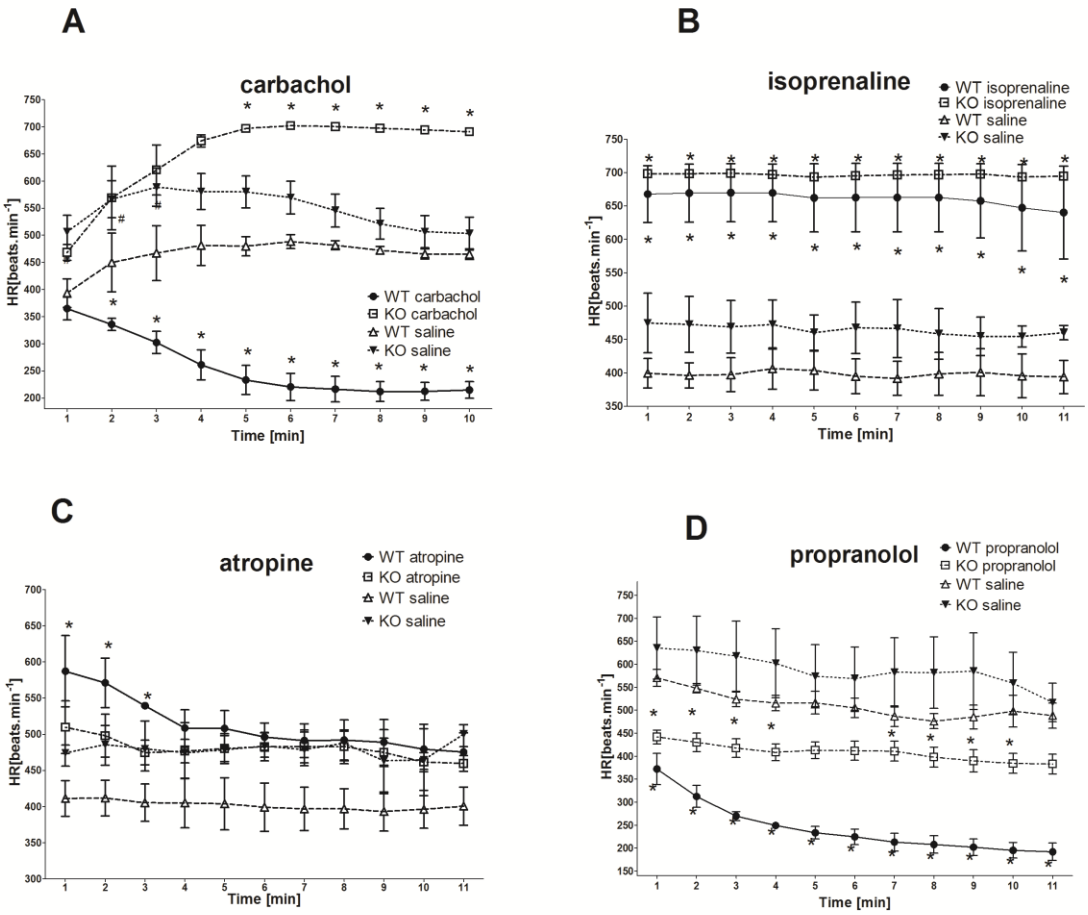

Fig. 4.11.1. The effects of carbachol (0.5 mg/kg, a), isoprenaline (50 mg/kg, b), atropine (30 mg/kg, c), and propranolol (20 mg/kg, d) on heart rate as assessed by telemetric measurement.

The drugs were applied i.p. and saline application served as control. Abscissa, time from the onset of drug effects; ordinate, heart rate [beats per min]; n=91–111. The onset of drug effects was: carbachol in the 1st minute, isoprenaline in the 21st minute, atropine in the 25th minute, propranolol in the 6th minute, respectively. The data were evaluated using 2-way ANOVA (repeated measures, factors: treatment, phenotype). The doses were derived from dose–response curves in WT animals from the preliminary experiments. *$p<0.05$ from saline-treated animals, #$p<0.05$ from WT saline-treated animals. Higher significance ($p<0.01$, $p<0.001$) is not shown.

4.12. Adenylyl cyclase activity

Adenylyl cyclase activity increased in unstressed WT animals when it was stimulated by forskolin, which was abolished by CL 316243 (Fig. 4.12.1.a). CL 316243 alone had no effect on adenylyl cyclase activity in unstressed animals (Fig. 4.12.1.a, 1-way ANOVA, p= 0.0072, F=5.347, df= 23). In contrast, CL 316243 was able to increase adenylyl cyclase activity after one (Fig. 4.12.1.c, 1-way ANOVA, p=0.0001, F= 13.37, df= 23) and 7 days of cold (Fig. 4.12.1.e, 1-way ANOVA, P= 0.0087, F= 5.63, df= 18). In stressed animals, forskolin also increased the amount of cAMP produced by adenylyl cyclase, but CL 316243 did not abolish these effects (Figs. 4.12.1.c, e).

In KO animals, forskolin was not able to increase the adenylyl cyclase activity in unstressed animals (Fig. 4.12.1.b), there was only difference between combined treatment with CL 316243 with forskolin and other groups (1-way ANOVA, P= 0.0005, F= 9.26, df= 23). When stress was applied (after 1 or 7 days) there was marked increase after forskolin treatment (Fig. 4.12.1.d,f, 1 day: 1-way ANOVA, p= 0.0183, F= 4.05, df= 27; 7 days: 1-way ANOVA, p= 0.0019, F= 6.91, df= 25). In contrast to WT animals, CL 316243 had almost no effects on adenylyl cyclase activity in KO animals itself (Fig. 4.12.1.b, d, f); it only abolished the effects of forskolin after 7 days of cold.

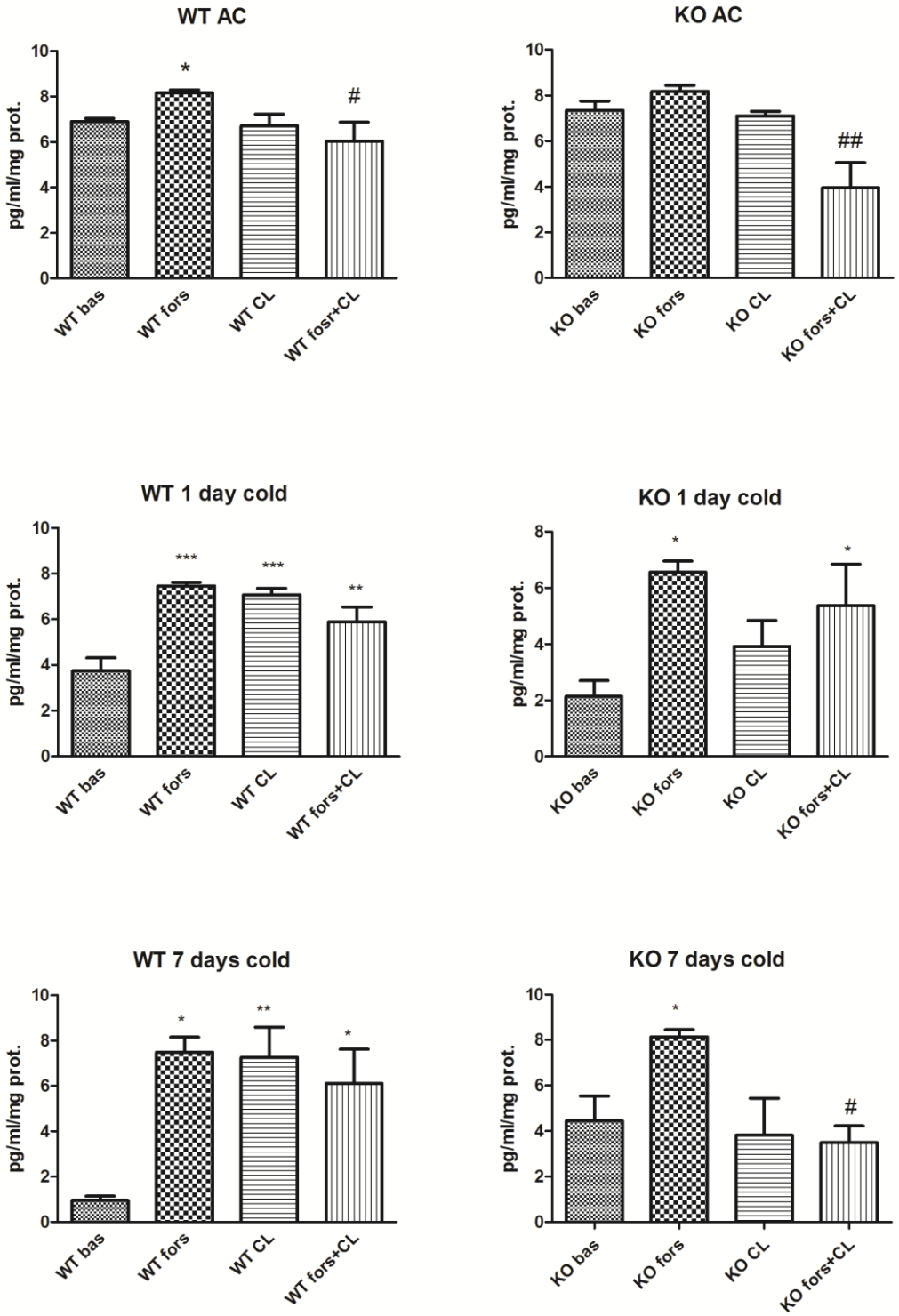

Fig 4.12.1 Changes of adenylyl cyclase activity during exposure to cold in wild type and knockout animals.

Abscissa: type of the drug added to the mixture (bas: basal level, i.e., no drug was added, fors: forskolin, CL: CL 316243, fors+CL: forskolin and CL 316243 were applied). Ordinate: the

amount of measured cAMP expressed as pg.ml^{-1}.mg of protein A: WT AC: unstressed WT animals, B: KO AC: unstressed KO animals, C: WT 1 day cold, D: KO 1 day cold, E: WT 7 days cold, F: KO 7 days cold.

4.13. Nitric oxide synthase (NOS) activity

As it can be deduced from Fig. 4.13.1., BRL 37344 was not able to stimulate NOS activity both in WT animals (Fig. 4.13.1.a, 1-way ANOVA, p= 0.2352, F= 1.451, df= 35) and KO animals (Fig. 4.13.1.b, 1-way ANOVA, p= 0.5565, F= 0.8030, df= 35). Similarly, the stress had no effect on NOS activity in WT animals (after 1 or 7 days in cold) as well as in KO animals.

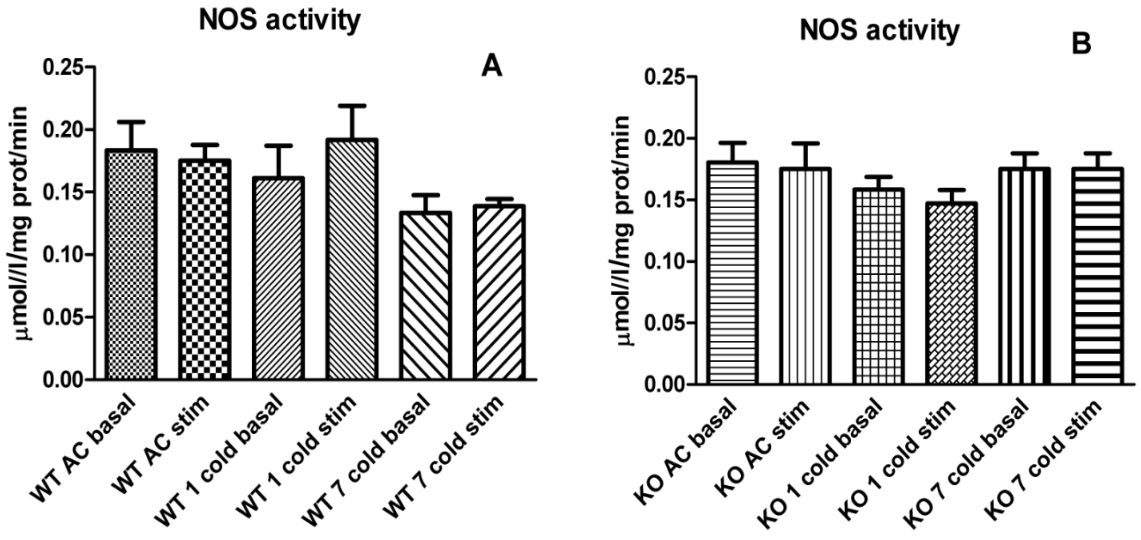

Fig. 4.13.1. Changes of nitric oxide synthase activity during exposure to cold in wild type (a) and knockout (b) animals.

Abscissa: type of stimulation used for NOS determination (basal basal level, i.e., no drug was added, stim: BRL 37344 treatment). Number of days in cold are shown as 1 or 7 cold (i.e., 1 day in the cold, 7 days in the cold). Ordinate: the amount of measured NO expressed as µmol.l^{-1}.mg.prot^{-1}.min^{-1}

4.14. Catecholamine blood concentration

Both adrenaline (A) and noradrenaline (NA) concentrations did not differ between KO and WT animals. Noradrenaline levels were 2.26±0.15 ng.ml^{-1} in WT animals and 3.03±0.46

ng.ml^{-1} in KO animals and adrenaline levels 0.45±0.04 ng.ml^{-1} in WT animals and 0.37±0.08 ng.ml^{-1} in KO animals (Fig. 4.14.1.).

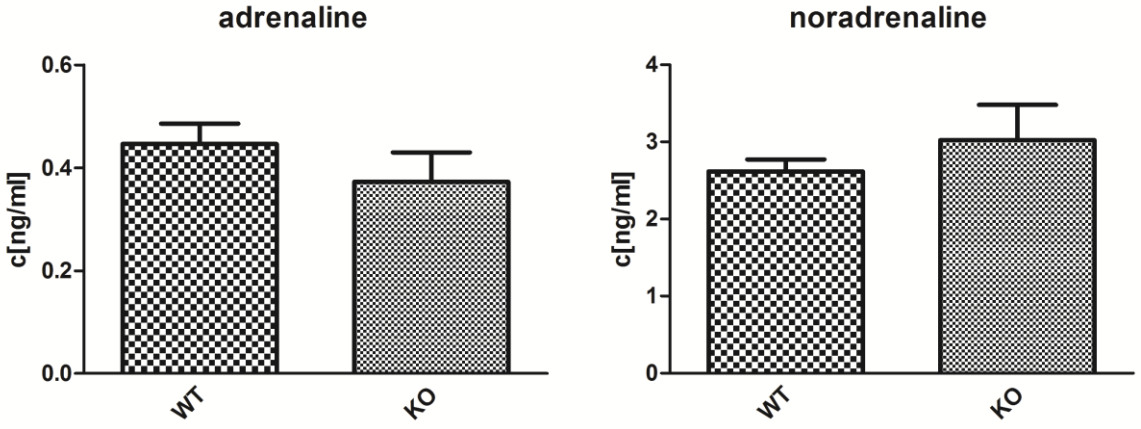

Fig. 4.14.1. Concentrations of adrenaline and noradrenaline in the blood in WT and KO animals.

Ordinate, catecholamine concentration [ng.ml^{-1}], n= 3–10. No difference between WT and KO animals were observed.

4.15. Gene expression of catecholamine-synthesizing enzymes

We have determined the gene expression of catecholamine-synthesizing enzymes (tyrosine-hydroxylase (TH), dopamine-β-hydroxylase (DBH) and phenylethanolamine-N-methyltransferase (PNMT)). No change in the gene expression of these enzymes between WT and KO animals was found (Fig. 4.15.1.).

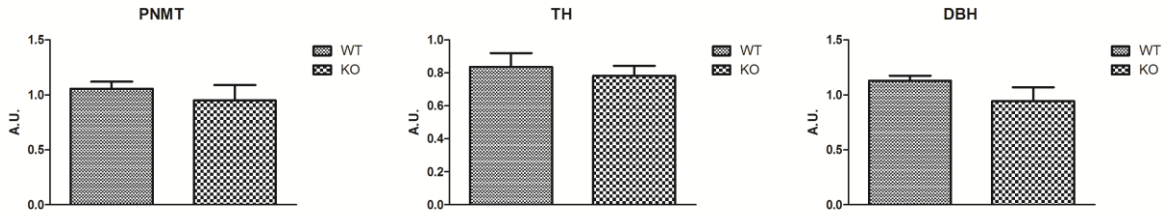

Fig. 4.15.1: The gene expression of TH, DBH and PNMT in WT and KO animals.

No change in the gene expression was observed. Ordinate: the relative amount of mRNA expressed in A.U. [arbitrary units].

4.16. Changes in gene expression of catecholamine synthesizing enzymes

There was no difference in the gene expression of tyrosine hydroxylase and dopamine-β-hydroxylase in both WT and KO and in basal state and upon cold stress. There was a higher level of gene expression of phenylethanolamine-N-methyl-transferase in WT animals after 1 and 7 days of cold (Fig. 4.16.1.).

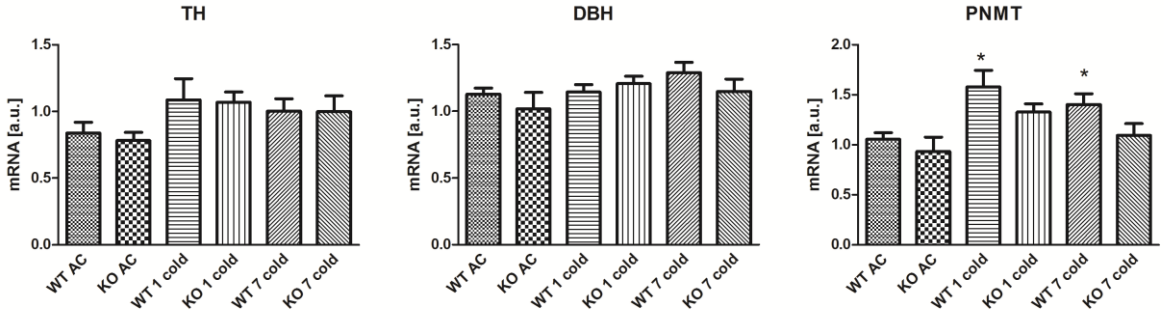

Fig. 4.16.1. The expression of catecholamine synthesizing enzymes

Abscissa: animal type and number of days in cold. Ordinate: the relative amount of mRNA expressed in A.U. [arbitrary units]. * $p<0.05$ from WT AC animals.

5. Discussion

In the presented work we show that the disruption of M_2-receptor gene caused a decrease in muscarinic receptor binding as well as β_1- and β_2-adrenoceptor binding sites (to 52 and 55 %, respectively). In contrast to that, β_3-adrenoceptor binding was increased at the baseline. When challenged by cold-stress, both WT and KO animals showed a decrease in β_1-and β_2-adrenoceptor binding. WT animals showed a decrease in muscarinic binding as well; on the contrary, the remaining non-M_2-binding preserved in M_2KO mice was not further altered. β_3-AR binding (already increased at baseline in M_2KO mice) continued to be increased to a similar extent upon stress stimulus (it was not further altered). WT mice showed eventually an increase in β_3-AR binding, but a prolonged stimulus was necessary to be applied (7-day cold stress), no changes were observed upon 1-day cold stress. M_2KO mice had lower baseline gene expression of M_3-receptors, β_1-adrenoceptors and β_2-adrenoceptors. Cold stress lead to the decrease of gene expression of M_3-receptors in WT but not KO mice. The expression of other receptor subtypes was not further altered. All these changes were chamber-specific (found in the left ventricle only). The overall cardiac function was not altered; no changes were found both in LV systolic and diastolic function as assessed by echocardiography (e.g. similar LV end-systolic and end-diastolic diameter, fractional shortening, mitral flow characteristics and maximal velocity in LV outflow tract). Only marginal changes have been found in physiological parameters; slightly higher basal heart rate during both dark and light phase in KO animals and slightly lower basal body temperature of M_2KO animals have been found. Heart rate variability was similar in both groups either. The effects of isoprenaline and propranolol on HR were similar in WT and KO (in KO we have recorded the changes of lesser extent). Atropine was not able to increase HR in KO animals and carbachol decreased the HR in WT but increased the HR in KO. Our study suggests that the downstream signaling through β_3-AR occurs via adenylyl cyclase rather than NO synthase. There was a higher level of gene expression of phenylethanolamine-N-methyl-transferase in WT animals after 1 and 7 days of cold (and no difference in KO animals). The gene expression of tyrosine hydroxylase and dopamine-β-hydroxylase was not altered both in WT and KO.

5.1. Gene expression and binding

Our results show that the disruption of M_2-MR gene caused changes in the gene expression of M_3-receptors, β_1-adrenoceptors and β_2-adrenoceptors together with the presumed decrease in muscarinic receptor binding sites as well as β_1- and β_2-adrenoceptor binding sites (to 52 and 55 %, respectively). The decrease of β_1-adrenoceptor and β_2-adrenoceptor binding sites was

caused by changes in the gene expression of the respective gene, as can be deduced from the gene expression study. In contrast to that, β_3-adrenoceptor binding was increased at the baseline. This phenomenon, i.e. that when one subtype receptor down-regulation occurs and it is followed by down-regulation of an antagonistic receptor as well was demonstrated multiple times (Paraschos and Karliner, 1994, Werry et al., 2003, Myslivecek et al., 2007) and it is fully in accordance with our hypothesis. Since the regulation is region specific (present in the left but not the right ventricle) heterologous regulation (i.e. local mechanism) is the most likely to be the responsible mechanism (Werry et al., 2003). This is an important fact suggesting that each ventricle has distinct mechanisms for receptor regulation.

We would like to point out that the binding of muscarinic-specific radioligand in M_2KO animals was not completely abolished, confirming the previous finding about another (minor) muscarinic population in the heart (Ito et al., 2009). On the level of gene expression, we were able to detect M_2-, M_3-, and M_4-receptor gene expression in WT animals; M_1- and M_5-receptors were not expressed. This is in a good agreement with some previously published data (Yang et al., 2005, Li et al., 2010, Pan et al., 2012). On the other hand, according to our previously published binding studies, the minor muscarinic population seemed to be M_1 (Myslivecek et al., 2008). However, this previous study was performed on rats; on the contrary, this present study has been done with mice, so difference between species may play a role. Further, we have found a progressive decrease in M_3-gene expression upon cold stress in WT mice but not in M_2KO mice. The expression of M_4-receptors was not altered. M_3-receptors were showed to mediate cardioprotective role of acetylcholine on myocardium; they were showed to ameliorate ischaemia-induced arrhythmias (Wang et al., 2012) and decrease of infarct size and mortality rate employing the M_3/NF-κB/miR-376b-5p/BDNF axis (Pan et al., 2012). This supports the important role of non-M_2-receptors for cardiac function. In our study, we have not found a compensatory up-regulation of M_4-receptors (that together with M_2-receptors inhibit adenylyl cyclase), so this hypothesis was not confirmed.

The receptor binding study on β_3-adrenoceptors showed a baseline increase in M_2KO that was sustained over the period of 7-day stress compared to WT mice, where an increase in β_3-AR appeared only after a rather prolonged stressful stimulus (7-day cold stress). This finding is in a good agreement with previously published studies and with the general paradigm on the function of β_3-adrenoceptors - they are perceived to be a „brake“ that is activated upon

excessively increased sympathetic overstimulation causing a negative inotropic effect counteracting the effects of β_1 and β_2 activation (Moens et al., 2010).

An application of 7-day cold stress was necessary to provoke an adrenergic response great enough to induce an up-regulation of β_3-AR. Our results show that CL 316243 (10,000 times more selective to β_3-adrenoceptors than to β_1- and β_2-adrenoceptors) was able to abolish the forskolin effects in WT unstressed animals. In contrast to that, CL316243 increases the amount of produced cAMP in cold, which suggests that β_3-adrenoceptors can switch from Gi coupling to Gs coupling. This is the property of β_3-adrenoceptors in the adipose tissue (Hamilton and Doods, 2008). Another possibility is that it can reveal the secondary state of β_3-adrenoceptors as it has been demonstrated earlier (Baker, 2005). Nevertheless, the increase of cAMP production by the β_3-AR activation is unobvious and can be seen in cold stressed WT animals only. Conversely, CL 316243 was not able to increase cAMP levels in KO animals, where β_3-AR are constantly increased. This fact gives evidence about the compensatory role of β_3-AR in the heart when M_2-MR are missing.

The possible interaction between increased level of cAMP and heart homeostasis should be evaluated further. As it has been demonstrated recently, there can be a connection of β_3-AR to Ca^{2+} signaling (Li et al., 2010) which could be also the possibility how to explain an increase in cAMP levels in WT only. In these experiments (Li et al., 2010), β_3-AR inhibits the Ca^{2+} currents induced by activation of β_1- and β_2-AR, which could explain the differences in CL 316243 effects in WT and KO animals (different amounts of β_3-AR binding sites). Concerning the downstream signaling pathway activated by β_3-AR, we have showed that the activity of adenylyl cyclase is importantly affected by β_3-adrenoceptor agonist, which suggests that adenylyl cyclase can be regulated during cold stress reaction by β_3-AR. This finding does not correlate well with the previous work suggesting that β_3-AR signaling occurs via the activation of NO synthase and subsequent production of NO. It has been thought long that the NOS isoform involved is endothelial NO synthase (eNOS) (Brixius et al., 2004) that has a crucial role in regulating L-type calcium channel activity in cardiac tissue. Protein kinase G (PKG) is a downstream effector of the eNOS/NO signaling cascade; it phosphorylates L-type Ca^{2+} channels which deactivates them (van der Heyden et al., 2005). However, recent studies show that eNOS is not the only NO isoform that mediates signaling through β_3-AR as NO production via the neuronal NOS (nNOS) has been demonstrated (Amour et al., 2007). The role of inducible NOS (iNOS) has not been fully clarified yet either, but it is likely that NO production through this isoform may also contribute to the NO production followed by β_3-AR activation (Maffei et al., 2007). Our work suggests that the

signaling through β_3-adrenoceptors can occur through the activation of cAMP as well. This illustrates the complexity of β-AR signaling and well supports the concept of „signalome“ where an individual adrenoceptor associates with multiple signaling pathways in time and spatially specific manner (Park and Palczewski, 2005).

Overall, since the main difference found was on the level of receptors, we propose that they serve as a tool to compensate M_2-knockout to preserve cardiac function.

5.2. Biorhythms (heart rate, temperature, activity)

We should emphasize that although analyzed and discussed separately, there is a mutual connection between heart rate, activity and temperature (both the temperature of animals and ambient temperature). We have found that M_2KO mice have slightly but significantly increased heart rate (507.8±6.15 vs. 477.3± 5.19 beats.min^{-1}, i.e., 6 %) both during light phase and dark phase with similar spectra of heart rate variability. Heart rate reflects the dynamic balance between sympathetic and parasympathetic branch of the autonomic nervous system, but the ratio between both of them might vary between species and other factors especially experimental conditions (ambient temperature) needed to be taken into account. In humans, the sinoatrial node is under the dominant influence of parasympathetic nervous system as the resting heart rate is much slower than the intrinsic rate of the sinoatrial node (Brubaker and Kitzman, 2011). On the other hand, it was long believed that in mice the intrinsic rate of the sinoatrial node is significantly lower than the actual heart rate (Gehrmann et al., 2000), which was interpreted that mice were under a dominant tonus on the sympathetic nervous system. It has been found recently that the ratio between sympathetic and parasympathetic tone depends greatly upon the ambient temperature. At 30°C, wild-type mice have the heart rate about 350 bpm whereas M_2KO mice 416 bpm (during the light phase) demonstrating that under these conditions the heart rate in mice is (similarly to humans) under dominant tone of parasympathetic nervous system (Swoap et al., 2008). However, when the ambient temperature drops to 21-23°C (normally used in animal facilities) the heart rate rises to cca 600 bpm (light phase) both in WT and M_2KO mice and the difference between groups disappears. Therefore, we have measured ambient temperature very carefully and have showed that in such well controlled environment (24.45 °C light phase/24.21 °C dark phase) it was possible to reveal a slight but significant increase in the heart rate in M_2KO animals, which is fully in accordance with previously published studies (Swoap et al., 2004, Swoap et al., 2008). On the other hand, some previously published work (Bymaster et al., 2001, LaCroix et al., 2008) reported no difference in the heart rate between WT and M_2KO mice.

However, certain factors have biased these observations. Both of these studies were performed on anesthetized animals, the experiments were performed during the day and the ambient temperature was not provided. Our analysis revealed that the difference in heart rate was greater during the night when mice are naturally more active. During the day the differences were more subtle and there were even intervals where there was virtually no difference at all. We believe we were able to record a difference between WT and M_2KO mice because we used telemetric monitoring on wake animals and monitored the ambient temperature very carefully.

The long term telemetric analysis of activity (averaged values and biorhythm analysis) of the animals did not find any difference between WT and M_2KO mice. This finding is in accordance with previously published data (Gomeza et al., 2001). In this case, data obtained from long term telemetric monitoring are in accordance with the data obtained during the experiment. No difference in activity (KO vs. WT) together with lower body temperature in KO animals in relation to unchanged left ventricular function as assessed by echocardiography makes the possibility that the HR increase is caused by higher heart performance rather improbable. This data suggest that M_2-receptors do not affect activity behavior in mice.

On the other hand, the long term measurement of the basal temperature of M_2KO mice revealed that is was decreased (without change in biorhythm). This can explain the differences in oxotremorine-induced tremor and hypothermia in M_2KO mice as published previously (Gomeza et al., 1999).

5.3. Cardiac function

No changes were found in any echocardiographic parameters (related to left ventricular systolic and diastolic function): there were no differences in left ventricular end-diastolic and end-systolic diameters, as well as in left ventricle hemodynamic indicators (maximal velocity in the left ventricle outflow tract, mitral flow characteristics—E velocity, A velocity, E/A ratio, left ventricle ejection time). Similarly, left ventricular peak velocity of circumferential fiber shortening showed no changes in KO animals. This observation is in agreement with previously published work (LaCroix et al., 2008) but in contradiction with study when M_2-MR were knocked-out postnatally (a study using M_2-MR coding plasmid DNA-immunized mice), where the impairment of heart function and changes in left ventricular echocardiographic parameters were found (Gimenez et al., 2005). These results indicate the

important differences between pre-natal and postnatal adaptation processes. Preserved systolic and diastolic function is fully in accordance with our hypotheses.

5.4. Drug application

The effects of carbachol in WT animals were expectable. On the other hand, the tachycardia in M_2KO animals was not regularly observed. (Gomeza et al., 1999) referred that incubation of atria derived from M_2KO mice with the carbachol (1μM) had no significant effect on atrial beating frequencies. Similarly, in vivo study with carbachol in M_2KO mice (Bymaster et al., 2001) showed no influence on the heart rate. On the other hand, (Kitazawa et al., 2009) described an increase in inotropy in the atria of M_2KO animals. There are at least two explanations of this phenomenon that originate from our results: (a) the first possibility is that the decrease of β-adrenoceptors (to approximately one half of control values) when heart muscarinic receptors are almost absent in the heart is able to cope with basal conditions of the heart while it is not sufficient to cope with the sustained activation of muscarinic receptors; (b) the second possibility is that the effects of minor muscarinic receptor subtype (as described by (Willmy-Matthes et al., 2003, Wang et al., 2004, Wang et al., 2007, Wang et al., 2012) and considered as cardiostimulatory) can be revealed whilst it is hidden (in WT animals) by action mediated via major M_2-receptors. According to our knowledge, this is the first observation of cardiostimulatory effects of carbachol *in vivo* in mice lacking the main cardioinhibitory muscarinic receptor subtype. This phenomenon further confirms the role of minor muscarinic receptor subtype in the heart. The third possibility is increased noradrenaline release from sympathetic nerve endings as described by (Trendelenburg et al., 2005) in M_2/M_3KO, but this rather would not be the case as we have not found a difference in blood catecholamines between WT and KO animals.

Upon the application of isoprenaline, we have observed an increase in HR both in WT and KO mice, but the effect on M_2KO animals was attenuated. This observation is in the contradiction with the previously published data (LaCroix et al., 2008) that reported a significantly increased heart rate upon isoprenaline application in M_2KO mice. The reason why we have observed opposite results is not clear; however, in our study all the measurements were performed by telemetry without using sedation, which we believe helped to obtain more reliable and unbiased results. The lesser extent of tachycardia in KO animals treated with isoprenaline can have the nature in having a decreased number of β-adrenoceptors (52% for β_1-adrenoceptors and 55 % for β_2-adrenoceptors). On the other

hand, the effects of β-adrenoceptor agonist persisted in KO animals, suggesting the compensatory role of receptor changes in heart physiology.

Atropine application increased the heart rate in WT animals but did not have this effect in M_2KO, confirming the absence of main cardioinhibitory receptors (M_2) in the heart. It was reported previously that atropine had no effect in WT animals (LaCroix et al., 2008), which is in contradiction with our results. Again, we believe that the measurements performed by telemetry without using sedation helped us to obtain more reliable and unbiased results.

Propranolol decreased the heart rate both in WT and M_2KO animals; however, the drop in M_2KO animals was attenuated (a decrease to 48% in WT but only to 70% in M_2KO). This supports the hypothesis mentioned earlier that the decrease in β-adrenoceptors could be responsible for the diminished effects of β-adrenoceptor drugs in M_2KO animals. Once again, these data also support the hypothesis about the compensatory role of receptors in maintaining stable and physiological cardiac output.

5.5. Catecholamine blood concentration and gene expression of catecholamine-synthesizing enzymes

In order to test if down-regulation of cardiac β-adrenoceptors (observed in our study) could be caused by the increased catecholamine release (carbachol decreases noradrenaline release from sympathetic nerves and this decrease is diminished in M_2/M_3KO animals (Trendelenburg et al., 2005)), we have measured the blood catecholamines. We have showed that the levels of circulating catecholamines were similar in WT and KO animals, which supports the hypothesis that heterologous regulation of β-adrenoceptors via intracellular signaling pathways is likely the responsible mechanism. The levels of circulating catecholamines were similar as referred by (Grouzmann et al., 2003). Similarly, the expression of the catecholamine-synthesizing enzymes (TH, DBH, and PNMT) showed that there was no increase in M_2KO animals indicating that M_2KO mice are not under chronic adrenergic stress. This is fully in accordance with our hypotheses.

5.6. Specific changes in cardiac autonomic receptor expression and function in M_2KO mice

The ability to manipulate the mice genes (knock-out and knock-in mice) provides the possibility for researchers to investigate the specific functions of the particular protein and to analyze its physiological roles more specifically than in the past. It was believed that this method was superior in ability to see the effect of a specific gene, but the initial hopes put into

the method were gradually corrected and now it is believed that it is rather an illustration of organism adaptation.

On the other hand, the knockout of M_2-receptors is quite specific as the proportion of minor muscarinic heart receptors is minute, and therefore the general deletion of M_2-receptor gene means that specifically in the heart main cardioinhibitory receptors are absent (minor subpopulation of cardioinhibitory M_4-receptors are present). The presence of M_2- receptors in peripheral tissue like in the intestine, lung, salivary glands and smooth muscles could not rather play a role in the regulation of heart function. On the other hand, the presence of M_2-receptors on cardiac nerves (as autoinhibitory receptors), on autonomic ganglia (in a similar function, i.e., as autoreceptors), or in the hypothalamus (as receptors that can affect basal autonomic tonus) could change the picture of general knockout. Although there are some data about the presence of M_2-MR in parasympathetic ganglia (or postsynaptic nerves) (Racke et al., 2006) and about the presence of MR on sympathetic ganglia (Fasano and Niel, 2009), which are rather M_1-MR, their role is not strong enough to justify their importance in heart regulation. Moreover, our data suggest that they are not present in the heart (left and right ventricles) at all (missing M_1-receptor gene expression in both WT and KO mice). The release-inhibiting heart receptors are more likely heterogenous (Zhou et al., 2002) with a defined role of M_4-MR rather than M_2-MR. On the other hand, MR are present in the medial preoptic area (Imeri et al., 1996), affecting the sleep–wake cycle and thermoregulation, and M_2-MR appear to regulate adrenocorticotropin hormone responses to cholinergic stimulation (in males, not in females) (Rhodes et al., 2005). Moreover, little attention was paid to the counter-regulatory changes. Usually, the investigation concentrates on specific knockout changes - i.e., M_2-receptors (Stengel et al., 2000), β_1-adrenoceptors (Rohrer et al., 1996, Ecker et al., 2006), β_2-adrenoceptors (Chruscinski et al., 1999, Ecker et al., 2006), β_3-adrenoceptors (Varghese et al., 2000), β_1/β_2-adrenoceptors (Kiriazis et al., 2008) or alternatively on total β-adrenoceptor knockout, i.e $\beta_1/\beta_2/\beta_3$-adrenoceptor knockout (Lee et al., 2010). Only a few papers have described the alteration of counter-acting receptor responses in knockout animals. For example, (LaCroix et al., 2008) have noticed a changed response to isoprenaline in M_2KO animals. Some authors have investigated the effects of downstream signaling molecules knockout (adenylyl cyclase, G proteins) on heart functions (Boknik et al., 2009, Gottle et al., 2009). According to our knowledge, our investigation is the first to emphasize the role of counter-regulatory receptors in the maintenance of heart functions.

6. Conclusion

In cardiac left ventricles, the disruption of M_2-receptor gene caused a compensatory decrease of cardiostimulatory β_1-adrenoceptors and β_2-adrenoceptors with corresponding down-regulation of the gene expression, M_3-receptors were down-regulated as well. Missing M_2-receptors were functionally replaced by the main cardioinhibitory β_3-adrenoceptors that were up-regulated, not by cardioinhibitory M_4-receptors. β_3-adrenoceptors were found to signal through adenylyl cyclase instead of NO synthase. All these changes were found in the left ventricle only, so heterologous regulation is likely to be the responsible mechanism. Slightly higher basal heart rate and lower basal body temperature of M_2KO animals underscore the importance of telemetric measurement to avoid biased recordings. Despite all the changes found the overall cardiac function was unaltered.

7. References

Abramow-Newerly M, Roy AA, Nunn C, Chidiac P (2006) RGS proteins have a signalling complex: interactions between RGS proteins and GPCRs, effectors, and auxiliary proteins. Cellular signalling 18:579-591.

Ahlquist RP (1948) A study of the adrenotropic receptors. Am J Physiol 153:586-600.

Alexander SP, Mathie A, Peters JA (2011) Guide to Receptors and Channels (GRAC), 5th edition. British journal of pharmacology 164 Suppl 1:S1-324.

Amour J, Loyer X, Le Guen M, Mabrouk N, David JS, Camors E, Carusio N, Vivien B, Andriantsitohaina R, Heymes C, Riou B (2007) Altered contractile response due to increased beta3-adrenoceptor stimulation in diabetic cardiomyopathy: the role of nitric oxide synthase 1-derived nitric oxide. Anesthesiology 107:452-460.

Andre C, Couton D, Gaston J, Erraji L, Renia L, Varlet P, Briand P, Guillet JG (1999) beta2-adrenergic receptor-selective agonist clenbuterol prevents Fas-induced liver apoptosis and death in mice. The American journal of physiology 276:G647-654.

Aragon JP, Condit ME, Bhushan S, Predmore BL, Patel SS, Grinsfelder DB, Gundewar S, Jha S, Calvert JW, Barouch LA, Lavu M, Wright HM, Lefer DJ (2011) Beta3-adrenoreceptor stimulation ameliorates myocardial ischemia-reperfusion injury via endothelial nitric oxide synthase and neuronal nitric oxide synthase activation. Journal of the American College of Cardiology 58:2683-2691.

Arraj M, Lemmer B (2006) Circadian rhythms in heart rate, motility, and body temperature of wild-type C57 and eNOS knock-out mice under light-dark, free-run, and after time zone transition. Chronobiology international 23:795-812.

Baba A, Yoshikawa T, Fukuda Y, Sugiyama T, Shimada M, Akaishi M, Tsuchimoto K, Ogawa S, Fu M (2004) Autoantibodies against M2-muscarinic acetylcholine receptors: new upstream targets in atrial fibrillation in patients with dilated cardiomyopathy. European heart journal 25:1108-1115.

Baker JG (2005) Evidence for a secondary state of the human beta3-adrenoceptor. Molecular pharmacology 68:1645-1655.

Benes J, Mravec B, Krizanova O, Kvetnansky R, Myslivecek J (2012a) The restructuring of dopamine receptor subtype gene transcripts in c-fos KO mice. Brain research bulletin 87:511-520.

Benes J, Mravec B, Kvetnansky R, Myslivecek J (2013) The restructuring of muscarinic receptor subtype gene transcripts in c-fos knock-out mice. Brain research bulletin.

Benes J, Novakova M, Rotkova J, Farar V, Kvetnansky R, Riljak V, Myslivecek J (2012b) Beta3 adrenoceptors substitute the role of M(2) muscarinic receptor in coping with cold stress in the heart: evidence from M(2)KO mice. Cellular and molecular neurobiology 32:859-869.

Bockaert J, Pin JP (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. The EMBO journal 18:1723-1729.

Boknik P, Grote-Wessels S, Barteska G, Jiang M, Muller FU, Schmitz W, Neumann J, Birnbaumer L (2009) Genetic disruption of G proteins, G(i2)alpha or G(o)alpha, does not abolish inotropic and chronotropic effects of stimulating muscarinic cholinoceptors in atrium. British journal of pharmacology 158:1557-1564.

Briones AM, Daly CJ, Jimenez-Altayo F, Martinez-Revelles S, Gonzalez JM, McGrath JC, Vila E (2005) Direct demonstration of beta1- and evidence against beta2- and beta3-adrenoceptors, in smooth muscle cells of rat small mesenteric arteries. British journal of pharmacology 146:679-691.

Brivanlou AH, Darnell JE, Jr. (2002) Signal transduction and the control of gene expression. Science (New York, NY) 295:813-818.

Brixius K, Bloch W, Pott C, Napp A, Krahwinkel A, Ziskoven C, Koriller M, Mehlhorn U, Hescheler J, Fleischmann B, Schwinger RH (2004) Mechanisms of beta 3-adrenoceptor-induced eNOS activation in right atrial and left ventricular human myocardium. British journal of pharmacology 143:1014-1022.

Brodde OE (1991) Beta 1- and beta 2-adrenoceptors in the human heart: properties, function, and alterations in chronic heart failure. Pharmacological reviews 43:203-242.

Brodde OE, Leineweber K (2004) Autonomic receptor systems in the failing and aging human heart: similarities and differences. European journal of pharmacology 500:167-176.

Brubaker PH, Kitzman DW (2011) Chronotropic incompetence: causes, consequences, and management. Circulation 123:1010-1020.

Bymaster FP, Carter PA, Zhang L, Falcone JF, Stengel PW, Cohen ML, Shannon HE, Gomeza J, Wess J, Felder CC (2001) Investigations into the physiological role of muscarinic M2 and M4 muscarinic and M4 receptor subtypes using receptor knockout mice. Life sciences 68:2473-2479.

Calzada B, de Artinano A (2001) Alpha-adrenoceptor subtypes. Pharmacological research : the official journal of the Italian Pharmacological Society 44:195-208.

Communal C, Singh K, Sawyer DB, Colucci WS (1999) Opposing effects of beta(1)- and beta(2)-adrenergic receptors on cardiac myocyte apoptosis : role of a pertussis toxin-sensitive G protein. Circulation 100:2210-2212.

Daly CJ, McGrath JC (2011) Previously unsuspected widespread cellular and tissue distribution of beta-adrenoceptors and its relevance to drug action. Trends in pharmacological sciences 32:219-226.

Davies DE (2009) The role of the epithelium in airway remodeling in asthma. Proceedings of the American Thoracic Society 6:678-682.

De Matteis R, Arch JR, Petroni ML, Ferrari D, Cinti S, Stock MJ (2002) Immunohistochemical identification of the beta(3)-adrenoceptor in intact human adipocytes and ventricular myocardium: effect of obesity and treatment with ephedrine and caffeine. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity 26:1442-1450.

De Vries L, Zheng B, Fischer T, Elenko E, Farquhar MG (2000) The regulator of G protein signaling family. Annual review of pharmacology and toxicology 40:235-271.

Dhami GK, Babwah AV, Sterne-Marr R, Ferguson SS (2005) Phosphorylation-independent regulation of metabotropic glutamate receptor 1 signaling requires g protein-coupled receptor kinase 2 binding to the second intracellular loop. The Journal of biological chemistry 280:24420-24427.

Downes GB, Gautam N (1999) The G protein subunit gene families. Genomics 62:544-552.

Ecker PM, Lin CC, Powers J, Kobilka BK, Dubin AM, Bernstein D (2006) Effect of targeted deletions of beta1- and beta2-adrenergic-receptor subtypes on heart rate variability. American journal of physiology Heart and circulatory physiology 290:H192-199.

Emorine LJ, Marullo S, Briend-Sutren MM, Patey G, Tate K, Delavier-Klutchko C, Strosberg AD (1989) Molecular characterization of the human beta 3-adrenergic receptor. Science 245:1118-1121.

Engelhardt S (2007) Alternative signaling: cardiomyocyte beta1-adrenergic receptors signal through EGFRs. The Journal of clinical investigation 117:2396-2398.

Farar V, Mohr F, Legrand M, Lamotte d'Incamps B, Cendelin J, Leroy J, Abitbol M, Bernard V, Baud F, Fournet V, Houze P, Klein J, Plaud B, Tuma J, Zimmermann M, Ascher P, Hrabovska A, Myslivecek J, Krejci E (2012) Near-complete adaptation of the PRiMA knockout to the lack of central acetylcholinesterase. Journal of neurochemistry 122:1065-1080.

Fasano C, Niel JP (2009) The mammalian sympathetic prevertebral ganglia: models for the study of neuronal networks and basic neuronal properties. Autonomic neuroscience : basic & clinical 150:8-20.

Feng YJ, Zhang BY, Yao RY, Lu Y (2012) Muscarinic acetylcholine receptor M3 in proliferation and perineural invasion of cholangiocarcinoma cells. Hepatobiliary & pancreatic diseases international : HBPD INT 11:418-423.

Ferretti M, Fabbiano C, Di Bari M, Ponti D, Calogero A, Tata AM (2012) M2 muscarinic receptors inhibit cell proliferation in human glioblastoma cell lines. Life sciences 91:1134-1137.

Fritsche A, Stumvoll M, Haring HU, Gerich JE (2000) Reversal of hypoglycemia unawareness in a long-term type 1 diabetic patient by improvement of beta-adrenergic sensitivity after prevention of hypoglycemia. The Journal of clinical endocrinology and metabolism 85:523-525.

Fritz JD, Robertson D (1996) Gene targeting approaches to the autonomic nervous system. Journal of the autonomic nervous system 61:1-5.

Fu Y, Huang X, Piao L, Lopatin AN, Neubig RR (2007) Endogenous RGS proteins modulate SA and AV nodal functions in isolated heart: implications for sick sinus syndrome and AV block. American journal of physiology Heart and circulatory physiology 292:H2532-2539.

Garofolo MC, Seidler FJ, Auman JT, Slotkin TA (2002) beta-Adrenergic modulation of muscarinic cholinergic receptor expression and function in developing heart. American journal of physiology Regulatory, integrative and comparative physiology 282:R1356-1363.

Gautam D, Jeon J, Starost MF, Han SJ, Hamdan FF, Cui Y, Parlow AF, Gavrilova O, Szalayova I, Mezey E, Wess J (2009) Neuronal M3 muscarinic acetylcholine receptors are essential for somatotroph proliferation and normal somatic growth. Proc Natl Acad Sci U S A 106:6398-6403.

Gauthier C, Langin D, Balligand JL (2000) Beta3-adrenoceptors in the cardiovascular system. Trends in pharmacological sciences 21:426-431.

Gauthier C, Tavernier G, Charpentier F, Langin D, Le Marec H (1996) Functional beta3-adrenoceptor in the human heart. The Journal of clinical investigation 98:556-562.

Gehrmann J, Hammer PE, Maguire CT, Wakimoto H, Triedman JK, Berul CI (2000) Phenotypic screening for heart rate variability in the mouse. American journal of physiology Heart and circulatory physiology 279:H733-740.

Gibson SK, Gilman AG (2006) Gialpha and Gbeta subunits both define selectivity of G protein activation by alpha2-adrenergic receptors. Proceedings of the National Academy of Sciences of the United States of America 103:212-217.

Gimenez LE, Hernandez CC, Mattos EC, Brandao IT, Olivieri B, Campelo RP, Araujo-Jorge T, Silva CL, Campos de Carvalho AC, Kurtenbach E (2005) DNA immunizations with M2 muscarinic and beta1 adrenergic receptor coding plasmids impair cardiac function in mice. Journal of molecular and cellular cardiology 38:703-714.

Gomeza J, Shannon H, Kostenis E, Felder C, Zhang L, Brodkin J, Grinberg A, Sheng H, Wess J (1999) Pronounced pharmacologic deficits in M2 muscarinic acetylcholine receptor knockout mice. Proceedings of the National Academy of Sciences of the United States of America 96:1692-1697.

Gomeza J, Zhang L, Kostenis E, Felder CC, Bymaster FP, Brodkin J, Shannon H, Xia B, Duttaroy A, Deng CX, Wess J (2001) Generation and pharmacological analysis of M2 and M4 muscarinic receptor knockout mice. Life Sci 68:2457-2466.

Gottle M, Geduhn J, Konig B, Gille A, Hocherl K, Seifert R (2009) Characterization of mouse heart adenylyl cyclase. The Journal of pharmacology and experimental therapeutics 329:1156-1165.

Granneman JG (2001) The putative beta4-adrenergic receptor is a novel state of the beta1-adrenergic receptor. American journal of physiology Endocrinology and metabolism 280:E199-202.

Grouzmann E, Cavadas C, Grand D, Moratel M, Aubert JF, Brunner HR, Mazzolai L (2003) Blood sampling methodology is crucial for precise measurement of plasma catecholamines concentrations in mice. Pflugers Archiv : European journal of physiology 447:254-258.

Gudermann T, Grosse R, Schultz G (2000) Contribution of receptor/G protein signaling to cell growth and transformation. Naunyn-Schmiedeberg's archives of pharmacology 361:345-362.

Gurevich EV, Gurevich VV (2006) Arrestins: ubiquitous regulators of cellular signaling pathways. Genome biology 7:236.

Gutkind JS (1998) The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. The Journal of biological chemistry 273:1839-1842.

Gyires K, Zadori ZS, Shujaa N, Minorics R, Falkay G, Matyus P (2007) Analysis of the role of central and peripheral alpha2-adrenoceptor subtypes in gastric mucosal defense in the rat. Neurochemistry international 51:289-296.

Hague C, Uberti MA, Chen Z, Hall RA, Minneman KP (2004) Cell surface expression of alpha1D-adrenergic receptors is controlled by heterodimerization with alpha1B-adrenergic receptors. The Journal of biological chemistry 279:15541-15549.

Hamilton BS, Doods HN (2008) Identification of potent agonists acting at an endogenous atypical beta3-adrenoceptor state that modulate lipolysis in rodent fat cells. European journal of pharmacology 580:55-62.

Hanyu R, Wehbi VL, Hayata T, Moriya S, Feinstein TN, Ezura Y, Nagao M, Saita Y, Hemmi H, Notomi T, Nakamoto T, Schipani E, Takeda S, Kaneko K, Kurosawa H, Karsenty G, Kronenberg HM, Vilardaga JP, Noda M (2012) Anabolic action of parathyroid hormone regulated by the beta2-adrenergic receptor. Proceedings of the National Academy of Sciences of the United States of America 109:7433-7438.

Hara MR, Kovacs JJ, Whalen EJ, Rajagopal S, Strachan RT, Grant W, Towers AJ, Williams B, Lam CM, Xiao K, Shenoy SK, Gregory SG, Ahn S, Duckett DR, Lefkowitz RJ (2011) A stress response pathway regulates DNA damage through beta2-adrenoreceptors and beta-arrestin-1. Nature 477:349-353.

Hein L, Altman JD, Kobilka BK (1999) Two functionally distinct alpha2-adrenergic receptors regulate sympathetic neurotransmission. Nature 402:181-184.

Hiraizumi-Hiraoka Y, Tanaka T, Yamamoto H, Suzuki F, Muramatsu I (2004) Identification of alpha-1L adrenoceptor in rabbit ear artery. The Journal of pharmacology and experimental therapeutics 310:995-1002.

Hoffert JD, Chou CL, Fenton RA, Knepper MA (2005) Calmodulin is required for vasopressin-stimulated increase in cyclic AMP production in inner medullary collecting duct. The Journal of biological chemistry 280:13624-13630.

Hrabovska A, Farar V, Bernard V, Duysen EG, Brabec J, Lockridge O, Myslivecek J (2010) Drastic decrease in dopamine receptor levels in the striatum of acetylcholinesterase knock-out mouse. Chemico-biological interactions 183:194-201.

Hu S, Wang D, Wu J, Jin J, Wei W, Sun W (2013) Involvement of beta-arrestins in cancer progression. Molecular biology reports 40:1065-1071.

Hua N, Wei X, Liu X, Ma X, He X, Zhuo R, Zhao Z, Wang L, Yan H, Zhong B, Zheng J (2012) A novel muscarinic antagonist R2HBJJ inhibits non-small cell lung cancer cell growth and arrests the cell cycle in G0/G1. PloS one 7:e53170.

Hurley JH (1999) Structure, mechanism, and regulation of mammalian adenylyl cyclase. The Journal of biological chemistry 274:7599-7602.

Hurowitz EH, Melnyk JM, Chen YJ, Kouros-Mehr H, Simon MI, Shizuya H (2000) Genomic characterization of the human heterotrimeric G protein alpha, beta, and gamma subunit genes. DNA research : an international journal for rapid publication of reports on genes and genomes 7:111-120.

Chapple CR (2004) Darifenacin: a novel M3 muscarinic selective receptor antagonist for the treatment of overactive bladder. Expert opinion on investigational drugs 13:1493-1500.

Chesley A, Lundberg MS, Asai T, Xiao RP, Ohtani S, Lakatta EG, Crow MT (2000) The beta(2)-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G(i)-dependent coupling to phosphatidylinositol 3'-kinase. Circulation research 87:1172-1179.

Childress AC, Sallee FR (2012) Revisiting clonidine: an innovative add-on option for attention-deficit/hyperactivity disorder. Drugs of today (Barcelona, Spain : 1998) 48:207-217.

Choi EJ, Wong ST, Hinds TR, Storm DR (1992) Calcium and muscarinic agonist stimulation of type I adenylylcyclase in whole cells. The Journal of biological chemistry 267:12440-12442.

Chruscinski A, Brede ME, Meinel L, Lohse MJ, Kobilka BK, Hein L (2001) Differential distribution of beta-adrenergic receptor subtypes in blood vessels of knockout mice lacking beta(1)- or beta(2)-adrenergic receptors. Molecular pharmacology 60:955-962.

Chruscinski AJ, Rohrer DK, Schauble E, Desai KH, Bernstein D, Kobilka BK (1999) Targeted disruption of the beta2 adrenergic receptor gene. The Journal of biological chemistry 274:16694-16700.

Ibrahim IA, Kurose H (2012) beta-arrestin-mediated signaling improves the efficacy of therapeutics. Journal of pharmacological sciences 118:408-412.

Imeri L, Bianchi S, Angeli P, Mancia M (1996) Muscarinic receptor subtypes in the medial preoptic area and sleep-wake cycles. Neuroreport 7:417-420.

Ito Y, Oyunzul L, Seki M, Fujino Oki T, Matsui M, Yamada S (2009) Quantitative analysis of the loss of muscarinic receptors in various peripheral tissues in M1-M5 receptor single knockout mice. British journal of pharmacology 156:1147-1153.

Jensen BC, Swigart PM, De Marco T, Hoopes C, Simpson PC (2009a) {alpha}1-Adrenergic receptor subtypes in nonfailing and failing human myocardium. Circulation Heart failure 2:654-663.

Jensen BC, Swigart PM, Laden ME, DeMarco T, Hoopes C, Simpson PC (2009b) The alpha-1D Is the predominant alpha-1-adrenergic receptor subtype in human epicardial coronary arteries. Journal of the American College of Cardiology 54:1137-1145.

Jimenez M, Leger B, Canola K, Lehr L, Arboit P, Seydoux J, Russell AP, Giacobino JP, Muzzin P, Preitner F (2002) Beta(1)/beta(2)/beta(3)-adrenoceptor knockout mice are obese and cold-sensitive but have normal lipolytic responses to fasting. FEBS letters 530:37-40.

Johnson RS, Spiegelman BM, Papaioannou V (1992) Pleiotropic effects of a null mutation in the c-fos proto-oncogene. Cell 71:577-586.

Kang DS, Tian X, Benovic JL (2013) beta-Arrestins and G protein-coupled receptor trafficking. Methods in enzymology 521:91-108.

Katz A, Wu D, Simon MI (1992) Subunits beta gamma of heterotrimeric G protein activate beta 2 isoform of phospholipase C. Nature 360:686-689.

Kaumann AJ, Engelhardt S, Hein L, Molenaar P, Lohse M (2001) Abolition of (-)-CGP 12177-evoked cardiostimulation in double beta1/beta2-adrenoceptor knockout mice. Obligatory role of beta1-adrenoceptors for putative beta4-adrenoceptor pharmacology. Naunyn-Schmiedeberg's archives of pharmacology 363:87-93.

Kaumann AJ, Molenaar P (1997) Modulation of human cardiac function through 4 beta-adrenoceptor populations. Naunyn-Schmiedeberg's archives of pharmacology 355:667-681.

Kiriazis H, Wang K, Xu Q, Gao XM, Ming Z, Su Y, Moore XL, Lambert G, Gibbs ME, Dart AM, Du XJ (2008) Knockout of beta(1)- and beta(2)-adrenoceptors attenuates pressure overload-induced cardiac hypertrophy and fibrosis. British journal of pharmacology 153:684-692.

Kitazawa T, Asakawa K, Nakamura T, Teraoka H, Unno T, Komori S, Yamada M, Wess J (2009) M3 muscarinic receptors mediate positive inotropic responses in mouse atria: a study with muscarinic receptor knockout mice. The Journal of pharmacology and experimental therapeutics 330:487-493.

Kjelsberg MA, Cotecchia S, Ostrowski J, Caron MG, Lefkowitz RJ (1992) Constitutive activation of the alpha 1B-adrenergic receptor by all amino acid substitutions at a

single site. Evidence for a region which constrains receptor activation. The Journal of biological chemistry 267:1430-1433.

Kvetnansky R, Sabban EL, Palkovits M (2009) Catecholaminergic systems in stress: structural and molecular genetic approaches. Physiological reviews 89:535-606.

Lacey RJ, Berrow NS, London NJ, Lake SP, James RF, Scarpello JH, Morgan NG (1990) Differential effects of beta-adrenergic agonists on insulin secretion from pancreatic islets isolated from rat and man. Journal of molecular endocrinology 5:49-54.

LaCroix C, Freeling J, Giles A, Wess J, Li YF (2008) Deficiency of M2 muscarinic acetylcholine receptors increases susceptibility of ventricular function to chronic adrenergic stress. American journal of physiology Heart and circulatory physiology 294:H810-820.

Lands AM, Arnold A, McAuliff JP, Luduena FP, Brown TG, Jr. (1967) Differentiation of receptor systems activated by sympathomimetic amines. Nature 214:597-598.

Langer SZ, Hicks PE (1984) Alpha-adrenoreceptor subtypes in blood vessels: physiology and pharmacology. Journal of cardiovascular pharmacology 6 Suppl 4:S547-558.

Lee S, Grafweg S, Schneider T, Jimenez M, Giacobino JP, Ghanem A, Tiemann K, Bloch W, Muller-Ehmsen J, Schwinger RH, Brixius K (2010) Total beta-adrenoceptor deficiency results in cardiac hypotrophy and negative inotropy. Physiological research / Academia Scientiarum Bohemoslovaca 59:679-689.

Lefkowitz RJ (1998) G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. The Journal of biological chemistry 273:18677-18680.

Lefkowitz RJ, Shenoy SK (2005) Transduction of receptor signals by beta-arrestins. Science (New York, NY) 308:512-517.

Li DL, Liu BH, Sun L, Zhao M, He X, Yu XJ, Zang WJ (2010) Alterations of muscarinic acetylcholine receptors-2, 4 and alpha7-nicotinic acetylcholine receptor expression after ischaemia / reperfusion in the rat isolated heart. Clinical and experimental pharmacology & physiology 37:1114-1119.

Liggett SB, Freedman NJ, Schwinn DA, Lefkowitz RJ (1993) Structural basis for receptor subtype-specific regulation revealed by a chimeric beta 3/beta 2-adrenergic receptor. Proceedings of the National Academy of Sciences of the United States of America 90:3665-3669.

Lipworth BJ (1996) Clinical pharmacology of beta 3-adrenoceptors. British journal of clinical pharmacology 42:291-300.

Lodish H BA, Zipursky SL, et al. (2000) Molecular Cell Biology. 4th edition. New York: W. H. Freeman.

Logothetis DE, Kurachi Y, Galper J, Neer EJ, Clapham DE (1987) The beta gamma subunits of GTP-binding proteins activate the muscarinic K+ channel in heart. Nature 325:321-326.

MacDonald E, Scheinin M (1995) Distribution and pharmacology of alpha 2-adrenoceptors in the central nervous system. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society 46:241-258.

Maffei A, Di Pardo A, Carangi R, Carullo P, Poulet R, Gentile MT, Vecchione C, Lembo G (2007) Nebivolol induces nitric oxide release in the heart through inducible nitric oxide synthase activation. Hypertension 50:652-656.

Mallem MY, Toumaniantz G, Serpillon S, Gautier F, Gogny M, Desfontis JC, Gauthier C (2004) Impairment of the low-affinity state beta1-adrenoceptor-induced relaxation in spontaneously hypertensive rats. British journal of pharmacology 143:599-605.

Mangmool S, Shukla AK, Rockman HA (2010) beta-Arrestin-dependent activation of Ca(2+)/calmodulin kinase II after beta(1)-adrenergic receptor stimulation. The Journal of cell biology 189:573-587.

McCorry LK (2007) Physiology of the autonomic nervous system. American journal of pharmaceutical education 71:78.

Mendez-Ferrer S, Battista M, Frenette PS (2010) Cooperation of beta(2)- and beta(3)-adrenergic receptors in hematopoietic progenitor cell mobilization. Annals of the New York Academy of Sciences 1192:139-144.

Migeon JC, Nathanson NM (1994) Differential regulation of cAMP-mediated gene transcription by m1 and m4 muscarinic acetylcholine receptors. Preferential coupling of m4 receptors to Gi alpha-2. J Biol Chem 269:9767-9773.

Michel-Reher MB, Michel MC (2013) Agonist-induced desensitization of human beta3-adrenoceptors expressed in human embryonic kidney cells. Naunyn-Schmiedeberg's archives of pharmacology 386:843-851.

Moens AL, Yang R, Watts VL, Barouch LA (2010) Beta 3-adrenoreceptor regulation of nitric oxide in the cardiovascular system. Journal of molecular and cellular cardiology 48:1088-1095.

Moniotte S, Kobzik L, Feron O, Trochu JN, Gauthier C, Balligand JL (2001) Upregulation of beta(3)-adrenoceptors and altered contractile response to inotropic amines in human failing myocardium. Circulation 103:1649-1655.

Muramatsu I, Tanaka T, Suzuki F, Li Z, Hiraizumi-Hiraoka Y, Anisuzzaman AS, Yamamoto H, Horinouchi T, Morishima S (2005) Quantifying receptor properties: the tissue segment binding method - a powerful tool for the pharmacome analysis of native receptors. Journal of pharmacological sciences 98:331-339.

Muzzin P, Boss O, Mathis N, Revelli JP, Giacobino JP, Willcocks K, Badman GT, Cantello BC, Hindley RM, Cawthorne MA (1994) Characterization of a new, highly specific, beta 3-adrenergic receptor radioligand, [3H]SB 206606. Molecular pharmacology 46:357-363.

Myslivecek J, Duysen EG, Lockridge O (2007) Adaptation to excess acetylcholine by downregulation of adrenoceptors and muscarinic receptors in lungs of acetylcholinesterase knockout mice. Naunyn-Schmiedeberg's archives of pharmacology 376:83-92.

Myslivecek J, Klein M, Novakova M, Ricny J (2008) The detection of the non-M2 muscarinic receptor subtype in the rat heart atria and ventricles. Naunyn-Schmiedeberg's archives of pharmacology 378:103-116.

Myslivecek J, Novakova M, Palkovits M, Krizanova O, Kvetnansky R (2006) Distribution of mRNA and binding sites of adrenoceptors and muscarinic receptors in the rat heart. Life sciences 79:112-120.

Myslivecek J, Ricny J, Kolar F, Tucek S (2003) The effects of hydrocortisone on rat heart muscarinic and adrenergic alpha 1, beta 1 and beta 2 receptors, propranolol-resistant binding sites and on some subsequent steps in intracellular signalling. Naunyn-Schmiedeberg's archives of pharmacology 368:366-376.

Myslivecek J, Trojan S, Tucek S (1996) Biphasic changes in the density of muscarinic and beta-adrenergic receptors in cardiac atria of rats treated with diisopropylfluorophosphate. Life sciences 58:2423-2430.

Nantel F, Bonin H, Emorine LJ, Zilberfarb V, Strosberg AD, Bouvier M, Marullo S (1993) The human beta 3-adrenergic receptor is resistant to short term agonist-promoted desensitization. Molecular pharmacology 43:548-555.

Nathanson NM (2000) A multiplicity of muscarinic mechanisms: enough signaling pathways to take your breath away. Proceedings of the National Academy of Sciences of the United States of America 97:6245-6247.

Nelson CD, Kovacs JJ, Nobles KN, Whalen EJ, Lefkowitz RJ (2008) Beta-arrestin scaffolding of phosphatidylinositol 4-phosphate 5-kinase Ialpha promotes agonist-

stimulated sequestration of the beta2-adrenergic receptor. The Journal of biological chemistry 283:21093-21101.

Noma T, Lemaire A, Naga Prasad SV, Barki-Harrington L, Tilley DG, Chen J, Le Corvoisier P, Violin JD, Wei H, Lefkowitz RJ, Rockman HA (2007) Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. The Journal of clinical investigation 117:2445-2458.

Nonogaki K (2000) New insights into sympathetic regulation of glucose and fat metabolism. Diabetologia 43:533-549.

Ohno M, Yamamoto T, Watanabe S (1994) Blockade of hippocampal M1 muscarinic receptors impairs working memory performance of rats. Brain Res 650:260-266.

Oka Y, Saraiva LR, Kwan YY, Korsching SI (2009) The fifth class of Galpha proteins. Proceedings of the National Academy of Sciences of the United States of America 106:1484-1489.

Olshansky B, Sabbah HN, Hauptman PJ, Colucci WS (2008) Parasympathetic nervous system and heart failure: pathophysiology and potential implications for therapy. Circulation 118:863-871.

Pan SJ, Hancock J, Ding Z, Fogt D, Lee M, Ivy JL (2001) Effects of clenbuterol on insulin resistance in conscious obese Zucker rats. American journal of physiology Endocrinology and metabolism 280:E554-561.

Pan Z, Guo Y, Qi H, Fan K, Wang S, Zhao H, Fan Y, Xie J, Guo F, Hou Y, Wang N, Huo R, Zhang Y, Liu Y, Du Z (2012) M3 subtype of muscarinic acetylcholine receptor promotes cardioprotection via the suppression of miR-376b-5p. PloS one 7:e32571.

Paraschos A, Karliner JS (1994) Receptor crosstalk: effects of prolonged carbachol exposure on beta 1-adrenoceptors and adenylyl cyclase activity in neonatal rat ventricular myocytes. Naunyn-Schmiedeberg's archives of pharmacology 350:267-276.

Park PS, Palczewski K (2005) Diversifying the repertoire of G protein-coupled receptors through oligomerization. Proceedings of the National Academy of Sciences of the United States of America 102:8793-8794.

Paylor R, Johnson RS, Papaioannou V, Spiegelman BM, Wehner JM (1994) Behavioral assessment of c-fos mutant mice. Brain research 651:275-282.

Port JD, Bristow MR (2001) Altered beta-adrenergic receptor gene regulation and signaling in chronic heart failure. Journal of molecular and cellular cardiology 33:887-905.

Poulin B, Butcher A, McWilliams P, Bourgognon JM, Pawlak R, Kong KC, Bottrill A, Mistry S, Wess J, Rosethorne EM, Charlton SJ, Tobin AB (2010) The M3-muscarinic

receptor regulates learning and memory in a receptor phosphorylation/arrestin-dependent manner. Proc 107:9440-9445.

Racke K, Juergens UR, Matthiesen S (2006) Control by cholinergic mechanisms. Eur J Pharmacol 533:57-68.

Rhodes ME, Billings TE, Czambel RK, Rubin RT (2005) Pituitary-adrenal responses to cholinergic stimulation and acute mild stress are differentially elevated in male and female M(2) muscarinic receptor knockout mice. Journal of neuroendocrinology 17:817-826.

Roehrborn CG, Schwinn DA (2004) Alpha1-adrenergic receptors and their inhibitors in lower urinary tract symptoms and benign prostatic hyperplasia. The Journal of urology 171:1029-1035.

Rohrer DK, Desai KH, Jasper JR, Stevens ME, Regula DP, Jr., Barsh GS, Bernstein D, Kobilka BK (1996) Targeted disruption of the mouse beta1-adrenergic receptor gene: developmental and cardiovascular effects. Proceedings of the National Academy of Sciences of the United States of America 93:7375-7380.

Rohrer DK, Chruscinski A, Schauble EH, Bernstein D, Kobilka BK (1999) Cardiovascular and metabolic alterations in mice lacking both beta1- and beta2-adrenergic receptors. The Journal of biological chemistry 274:16701-16708.

Rouget C, Bardou M, Breuiller-Fouche M, Loustalot C, Qi H, Naline E, Croci T, Cabrol D, Advenier C, Leroy MJ (2005) Beta3-adrenoceptor is the predominant beta-adrenoceptor subtype in human myometrium and its expression is up-regulated in pregnancy. The Journal of clinical endocrinology and metabolism 90:1644-1650.

Seeger T, Fedorova I, Zheng F, Miyakawa T, Koustova E, Gomeza J, Basile AS, Alzheimer C, Wess J (2004) M2 muscarinic acetylcholine receptor knock-out mice show deficits in behavioral flexibility, working memory, and hippocampal plasticity. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:10117-10127.

Sharma S, Adrogue JV, Golfman L, Uray I, Lemm J, Youker K, Noon GP, Frazier OH, Taegtmeyer H (2004) Intramyocardial lipid accumulation in the failing human heart resembles the lipotoxic rat heart. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 18:1692-1700.

Shekim WO, Bylund DB, Hodges K, Glaser R, Ray-Prenger C, Oetting G (1994) Platelet alpha 2-adrenergic receptor binding and the effects of d-amphetamine in boys with attention deficit hyperactivity disorder. Neuropsychobiology 29:120-124.

Shulman GI (1999) Cellular mechanisms of insulin resistance in humans. The American journal of cardiology 84:3J-10J.

Skeberdis VA, Gendviliene V, Zablockaite D, Treinys R, Macianskiene R, Bogdelis A, Jurevicius J, Fischmeister R (2008) β3-adrenergic receptor activation increases human atrial tissue contractility and stimulates the L-type Ca2+ current. The Journal of Clinical Investigation 118:3219-3227.

Soeder KJ, Snedden SK, Cao W, Della Rocca GJ, Daniel KW, Luttrell LM, Collins S (1999) The beta3-adrenergic receptor activates mitogen-activated protein kinase in adipocytes through a Gi-dependent mechanism. The Journal of biological chemistry 274:12017-12022.

Spasojevic N, Gavrilovic L, Dronjak S (2011) Regulation of atrial catecholamine enzymes and adrenoceptor gene expression after chronic stress. Acta Physiologica Hungarica 98:433-441.

Stavrakis S, Kem DC, Patterson E, Lozano P, Huang S, Szabo B, Cunningham MW, Lazzara R, Yu X (2011) Opposing cardiac effects of autoantibody activation of beta-adrenergic and M2 muscarinic receptors in cardiac-related diseases. International journal of cardiology 148:331-336.

Stengel PW, Gomeza J, Wess J, Cohen ML (2000) M(2) and M(4) receptor knockout mice: muscarinic receptor function in cardiac and smooth muscle in vitro. The Journal of pharmacology and experimental therapeutics 292:877-885.

Swoap SJ, Li C, Wess J, Parsons AD, Williams TD, Overton JM (2008) Vagal tone dominates autonomic control of mouse heart rate at thermoneutrality. American journal of physiology Heart and circulatory physiology 294:H1581-1588.

Swoap SJ, Overton JM, Garber G (2004) Effect of ambient temperature on cardiovascular parameters in rats and mice: a comparative approach. American journal of physiology Regulatory, integrative and comparative physiology 287:R391-396.

Szabo G, Otero AS (1989) Muscarinic activation of potassium channels in cardiac myocytes: kinetic aspects of G protein function in vivo. Trends in pharmacological sciences Suppl:46-49.

Tanaka N, Dalton N, Mao L, Rockman HA, Peterson KL, Gottshall KR, Hunter JJ, Chien KR, Ross J, Jr. (1996) Transthoracic echocardiography in models of cardiac disease in the mouse. Circulation 94:1109-1117.

Tomankova H, Myslivecek J (2011) Mechanisms of G protein-coupled receptors regulation. Neuro endocrinology letters 32:607-615.

Trendelenburg AU, Meyer A, Wess J, Starke K (2005) Distinct mixtures of muscarinic receptor subtypes mediate inhibition of noradrenaline release in different mouse peripheral tissues, as studied with receptor knockout mice. British journal of pharmacology 145:1153-1159.

Tzavara ET, Bymaster FP, Felder CC, Wade M, Gomeza J, Wess J, McKinzie DL, Nomikos GG (2003) Dysregulated hippocampal acetylcholine neurotransmission and impaired cognition in M2, M4 and M2/M4 muscarinic receptor knockout mice. Mol Psychiatry 8:673-679.

Uberti MA, Hague C, Oller H, Minneman KP, Hall RA (2005) Heterodimerization with beta2-adrenergic receptors promotes surface expression and functional activity of alpha1D-adrenergic receptors. The Journal of pharmacology and experimental therapeutics 313:16-23.

Ueta CB, Fernandes GW, Capelo LP, Fonseca TL, Maculan FD, Gouveia CH, Brum PC, Christoffolete MA, Aoki MS, Lancellotti CL, Kim B, Bianco AC, Ribeiro MO (2012) beta(1) Adrenergic receptor is key to cold- and diet-induced thermogenesis in mice. The Journal of endocrinology 214:359-365.

Valenzuela D, Han X, Mende U, Fankhauser C, Mashimo H, Huang P, Pfeffer J, Neer EJ, Fishman MC (1997) G alpha(o) is necessary for muscarinic regulation of Ca2+ channels in mouse heart. Proceedings of the National Academy of Sciences of the United States of America 94:1727-1732.

van der Heyden MA, Wijnhoven TJ, Opthof T (2005) Molecular aspects of adrenergic modulation of cardiac L-type Ca2+ channels. Cardiovascular research 65:28-39.

Varghese P, Harrison RW, Lofthouse RA, Georgakopoulos D, Berkowitz DE, Hare JM (2000) beta(3)-adrenoceptor deficiency blocks nitric oxide-dependent inhibition of myocardial contractility. The Journal of clinical investigation 106:697-703.

Vetter IR, Wittinghofer A (2001) The guanine nucleotide-binding switch in three dimensions. Science (New York, NY) 294:1299-1304.

Wang H, Lu Y, Wang Z (2007) Function of cardiac M3 receptors. Autonomic & autacoid pharmacology 27:1-11.

Wang S, Han HM, Jiang YN, Wang C, Song HX, Pan ZY, Fan K, Du J, Fan YH, Du ZM, Liu Y (2012) Activation of cardiac M3 muscarinic acetylcholine receptors has cardioprotective effects against ischaemia-induced arrhythmias. Clinical and experimental pharmacology & physiology 39:343-349.

Wang Z, Shi H, Wang H (2004) Functional M3 muscarinic acetylcholine receptors in mammalian hearts. British journal of pharmacology 142:395-408.

Werry TD, Wilkinson GF, Willars GB (2003) Mechanisms of cross-talk between G-protein-coupled receptors resulting in enhanced release of intracellular Ca2+. The Biochemical journal 374:281-296.

Willars GB (2006) Mammalian RGS proteins: multifunctional regulators of cellular signalling. Seminars in cell & developmental biology 17:363-376.

Willmy-Matthes P, Leineweber K, Wangemann T, Silber RE, Brodde OE (2003) Existence of functional M3-muscarinic receptors in the human heart. Naunyn-Schmiedeberg's archives of pharmacology 368:316-319.

Xiao RP, Zhu W, Zheng M, Cao C, Zhang Y, Lakatta EG, Han Q (2006) Subtype-specific alpha1- and beta-adrenoceptor signaling in the heart. Trends in pharmacological sciences 27:330-337.

Yamada M, Basile AS, Fedorova I, Zhang W, Duttaroy A, Cui Y, Lamping KG, Faraci FM, Deng C-X, Wess J (2003) Novel insights into M5 muscarinic acetylcholine receptor function by the use of gene targeting technology. Life sciences 74:345-353.

Yamasaki M, Matsui M, Watanabe M (2010) Preferential localization of muscarinic M1 receptor on dendritic shaft and spine of cortical pyramidal cells and its anatomical evidence for volume transmission. J 30:4408-4418.

Yang B, Lin H, Xu C, Liu Y, Wang H, Han H, Wang Z (2005) Choline produces cytoprotective effects against ischemic myocardial injuries: evidence for the role of cardiac m3 subtype muscarinic acetylcholine receptors. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology 16:163-174.

Yoo B, Lemaire A, Mangmool S, Wolf MJ, Curcio A, Mao L, Rockman HA (2009) Beta1-adrenergic receptors stimulate cardiac contractility and CaMKII activation in vivo and enhance cardiac dysfunction following myocardial infarction. American journal of physiology Heart and circulatory physiology 297:H1377-1386.

Zaugg M, Xu W, Lucchinetti E, Shafiq SA, Jamali NZ, Siddiqui MA (2000) Beta-adrenergic receptor subtypes differentially affect apoptosis in adult rat ventricular myocytes. Circulation 102:344-350.

Zhang L, Hu D, Li J, Wu Y, Liu X, Yang X (2002) Autoantibodies against the myocardial beta1-adrenergic and M2-muscarinic receptors in patients with congestive heart failure. Chinese medical journal 115:1127-1131.

Zhou H, Meyer A, Starke K, Gomeza J, Wess J, Trendelenburg AU (2002) Heterogeneity of release-inhibiting muscarinic autoreceptors in heart atria and urinary bladder: a study with M(2)- and M(4)-receptor-deficient mice. Naunyn-Schmiedeberg's archives of pharmacology 365:112-122.

Zhu WZ, Wang SQ, Chakir K, Yang D, Zhang T, Brown JH, Devic E, Kobilka BK, Cheng H, Xiao RP (2003) Linkage of beta1-adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca2+/calmodulin kinase II. The Journal of clinical investigation 111:617-625.