Cell Mol Neurobiol (2012) 32:859–869
DOI 10.1007/s10571-011-9781-3

ORIGINAL PAPER

Beta3 Adrenoceptors Substitute the Role of M_2 Muscarinic Receptor in Coping with Cold Stress in the Heart: Evidence from M_2KO Mice

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Received: 21 September 2011 / Accepted: 3 December 2011 / Published online: 6 January 2012

Abstract We investigated the role of beta3-adrenoceptors (AR) in cold stress (1 or 7 days in cold) in animals lacking main cardioinhibitive receptors—M2 muscarinic receptors (M_2KO). There was no change in receptor number in the right ventricles. In the left ventricles, there was decrease in binding to all cardiostimulative receptors (beta1-, and beta2-AR) and increase in cardiodepressive receptors (beta3-AR) in unstressed KO in comparison to WT. The cold stress in WT animals resulted in decrease in binding to beta1- and beta2-AR (to 37%/35% after 1 day in cold and to 27%/28% after 7 days in cold) while beta3-AR were increased (to 216% of control) when 7 days cold was applied. MR were reduced to 46% and 58%, respectively. Gene expression of M2 MR in WT was not changed due to stress, while M3 was changed. The reaction of beta1- and beta2-AR (binding) to cold was similar in KO and WT animals, and beta3-AR in stressed KO animals did not change. Adenylyl cyclase activity was affected by beta3-agonist CL316243 in cold stressed WT animals but CL316243 had almost no effects on adenylyl cyclase activity in stressed KO. Nitric oxide activity (NOS) was not affected by BRL37344 (beta3-agonist) both in WT and KO animals. Similarly, the stress had no effects on NOS activity in WT animals and in KO animals. We conclude that the function of M2 MR is substituted by beta3-AR and that these effects are mediated via adenylyl cyclase rather than NOS.

Keywords Cold stress · Muscarinic receptors · Beta-adrenoceptors · Heart · Adenylyl cyclase · Nitric oxide synthase

Introduction

Three β-adrenoceptor subtypes have been characterized to these days but only two of them have been previously assumed to exist in the heart (Lands et al. 1967): β_1-AR and β_2-AR. Both these subtypes produce positive inotropic and chronotropic effects. All β-adrenoceptors (β-AR) are members of the G-protein-coupled receptor family (GPCR). β_1-AR couples to G_s proteins, stimulates adenylyl cyclase (AC), which subsequently leads to increase in cAMP intracellular levels. The sufficiency of cAMP is the key point in activation of protein kinase A (PKA), which phosphorylates also the L-type Ca^{2+} channels and thus promotes calcium influx and enhances contraction. The G-protein coupling, signaling pathways, and functional effects specific for the β_2-AR resembles β_1 subtype. Accumulated evidence indicates that at least in the rat and murine heart, stimulation of β_1-AR causes not only positive inotropic and chronotropic effects, but can also promote apoptosis of cardiomyocytes (Xiao et al. 1999). Persistent β_1-AR stimulation changes the receptor signaling pathway from PKA to Ca^{2+}/calmodulin-dependent protein kinase II

Electronic supplementary material The online version of this article (doi:10.1007/s10571-011-9781-3) contains supplementary material, which is available to authorized users.

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(CaMKII) predominance, leading to myocyte maladaptive remodeling (Xiao et al. 2006). On the other hand, prolonged β_2-AR activation switches the receptor G-protein coupling from G_s to G_i and thereby possesses antiapoptotic effect resulting in cardioprotection (Communal et al. 1999; Chesley et al. 2000; Xiao et al. 2006). Challenge about the existence of another heart β-AR came at the end of the last century, when the 3rd β-adrenergic subtype, β_3-AR, was identified in the heart. The presence of β_3-AR mRNA has been confirmed repeatedly in the heart (Gauthier et al. 2000; Myslivecek et al. 2006) and the β_3-AR protein has been also identified using immunohistochemical detection (De Matteis et al. 2002). Furthermore, the presence of β_3-AR in human atrial tissue using a selective monoclonal antibody was demonstrated (Chamberlain et al. 1999). In full contrast with the other β-AR subtypes, stimulation of the β_3-AR produced a marked decrease in cardiac contractility in human heart (Gauthier et al. 2000). The negative inotropic effects were noted in many cases, when β_3-adrenoceptor agonists were used (Gauthier et al. 1998, 1999; Tavernier et al. 2003). 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 (Nantel et al. 1993). These features suggest that expression of the β_3-AR in the heart may have pathophysiological significance. In the heart, β_3-AR are not coupled to G_s, like β_1- and β_2-AR, but to the G_i protein. In human ventricles activation of G_i proteins causes an activation of the nitric oxide (NO) pathway, probably implicating the endothelial NO synthase (eNOS), which is expressed both in endothelial cells and cardiomyocytes (Gauthier et al. 1998; Xiao et al. 1999; Schulz et al. 2005; Moens et al. 2010). The NO production induces an activation of guanylyl cyclase leading to an increase in intracellular cyclic guanosine monophosphate (cGMP). Alternatively, NO could regulate cardiac function in a cGMP-independent way through modification of other proteins as cytochrome *c* oxidase or L-type calcium channel. It is supposed that β_3-AR/NO pathway could act as a negative feedback mechanism opposing the positive inotropic influences of catecholamines in the heart. When β_3 agonist was applied, changes in the heart activity or gene expression of β_3-AR in the heart were shown repeatedly (Gauthier et al. 1998, 1999, 2000; Moniotte et al. 2001, Tavernier et al. 2003), although no direct evidence of β_3-AR binding sites in the heart exist. Moreover, role of the β_3-AR in physiological and pathophysiological conditions has not been evaluated yet.

The opposing effects on the heart rate and on the force of heart muscle contraction are mediated via muscarinic receptors and the mammal heart tissue is realized as M_2 predominant organ. Muscarinic receptors (MR), similarly to β-adrenoceptors, belong to G protein-coupled receptor family. To date, five muscarinic receptor subtypes have been described and cloned (Wess et al. 2003). The odd-numbered subtypes (M_1, M_3, M_5) stimulate phospholipase C (PLC; via pertussis toxin-insensitive Gq protein), which cleaves phosphatidilinositolbisphosphate (PIP_2) to inositoltrisphosphate (IP_3) and diacylglycerol (DAG). The even-numbered subtypes (M_2, M_4) inhibit adenylyl cyclase (AC; via pertussis toxin-sensitive Gi protein), i.e., they decrease the amount of cyclic adenosine monophosphate (Moscona-Amir et al. 1988) and decrease the activity of proteinkinase A (PKA). In the last decade, all muscarinic receptor subtypes became available (Wess et al. 2003, 2007) from which M_2 muscarinic receptor knockout (Gomeza et al. 2001) is of special interest in research of heart regulation as M_2 muscarinic receptors are predominant muscarinic receptor subtype in the heart (regulating heart rate, contractility, atrio-ventricular conduction). In atria of these mice there was no change in heart rat after carbachol while in wild type bradycardia was induced (Gomeza et al. 1999).

Also, the heart function can be also affected by another receptor type—i.e., α_1-adrenoceptors (Brodde et al. 2006). These receptors have cardiostimulative functions. However, the maximum positive inotropic effect following α_1-AR stimulation is by far less than that evoked by β-AR stimulation in the human heart (Brodde et al. 2006).

We have recently shown that heart stress reaction comprise also muscarinic receptors (Myslivecek et al. 2004, 2008). Therefore, exposure of M_2 MR KO mice to stress could reveal the role of both M_2 muscarinic receptors and also of cardioinhibitive β_3-adrenoceptors in the heart reaction to stress.

Moreover, we have repeatedly shown that activation of one heart (or lung) receptor system (i.e., muscarinic or adrenergic) affects not only activated system but also antagonistic system ((Myslivecek et al. 1996, 2004, 2007).

Therefore, we tested two hypotheses:

1. The number of cardiostimulative adrenoceptors would be decreased as a result of trend to maintain heart homeostasis in unstressed M_2KO mice.
2. The inhibition of cardiac adenylyl cyclase system would be provided by β_3-adrenoceptors in M_2KO mice. Moreover, the coping with stress would be also taken over by β_3-adrenoceptors when M_2 muscarinic receptors lack.

Methods

Experimental Animals

The M_2 muscarinic receptor mice lacking M_2 muscarinic receptor clone was generated in Wess laboratory (Gomeza et al. 1999) and then bred in our animal facility (Prague,

Czech Republic). Animals were treated in accordance with the legislature of the Czech Republic and the EU legislature, and the experimental protocol was approved by the Committee for the Protection of Experimental Animals of the 1st Medical Faculty, Charles University, Prague. The wild type line was mixed 129J1/CF1 line. Control animals were maintained under controlled environmental conditions (12/12 light/dark cycle, 22 ± 1°C, light on at 8 a.m.). Food and water were available ad libitum. Male M_2KO animals and their WT counterparts (weighting 20–25 g, 11–13 weeks old) were used in the study.

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 rise to 9.73 ± 0.59°C. The light/dark cycle was the same as in the control animal. 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.

Gene Expression of Muscarinic Receptor Subtypes (M_1–M_5) Using Real-Time PCR TaqMan Assays

Total mRNA was isolated using chloroform-isopropanol (RNA Bee, TelTest, TX, USA) method according to manufacturer's instructions. RNA yield and integrity was evaluated spectrophotometrically (Tecan M200 Nanoquant, detection of 2 µl of sample in 16 wells at the moment) at A1 = 260 nm and A1/A2 = 260/280 nm, respectively. Samples with A1/A2 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) with Roche qPCR Mastermix (Roche) using following protocol: 2 min at 50°C, 10 min at 95°C followed by 50 cycles 15 s at 95°C and 1 min at 60°C in a final volume 10 µl. In order to determine the relative gene expression, the Delta–Delta-Ct (ddCt) method was used. The levels of analyzed transcripts were normalized to the level of the reference gene transcript (18SrRNA, which level was practically the same in WT and KO). Then, for each sample the following formula was used: normalized ratio = 2CT1–CT2, where CT1 is CT of reference transcript and CT2 is CT of target transcript, where CT is the cycle threshold. After the qPCR reaction, melting curve analysis was performed to confirm the specificity of the analyzed transcript. No-template controls (NTC) and non-reverse transcription reactions (NRT) were performed as negative controls.

Receptor Binding

We performed receptor binding on membranes prepared as follows: the tissue was weighted 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-adrenergic receptors and muscarinic receptors (Myslivecek et al. 2007) or in another buffer (Tris–HCl 50 mmol/l, $MgCl_2$ 100 mmol/l, GTP 300 µmol/l, Tiron 1 µmol/l, ascorbic acid 1 mmol/l) for determination of β_3-adrenoceptors (Muzzin et al. 1994). The homogenate was centrifuged at 600×g for 10 min at 4°C, the supernatant was collected and the sediment was re-suspended in the buffer and centrifuged again using the same conditions. Supernatants were mixed and centrifuged for 25 min at 32,000×g at 4°C. The supernatant was discarded; the sediment was re-suspended in the buffer and centrifuged again in the same velocity and time (25 min, 32000×g, 4°C). The experiments were performed similarly as has been reported before (Myslivecek et al. 2007). The amount of receptor binding sites was determined in duplicates using following conditions: (a) total β_1-adrenoceptor + β_2-adrenoceptor binding sites (B_{max}): concentration rank 93.75—3000 pmol/l [^{3}H]CGP 12177, non-specific binding was determined with 50 µmol/l propranolol; (b) β_3-adrenoceptor binding sites: 4.68–150 nmol/l [^{3}H]SB 206606, non-specific binding was determined with 1 mmol/l SR59230A; (c) muscarinic receptors binding sites: 65–2000 pmol/l [^{3}H]QNB, non-specific binding was determined with 5 µmol/l atropine. The incubation was performed at 38°C and lasted 60 min with [^{3}H]CGP 12177, 30 min with [^{3}H]SB 206606, and 120 min with [^{3}H]QNB. All incubation times were adjusted previously (Muzzin et al. 1994; Myslivecek et al. 2007). The amount of binding sites (B_{max}) per mg protein (determined using BCA a method kit; Sigma) and the affinity constant (K_D) was computed by non-linear regression using GraphPad Prism 5.01 program (GraphPad Software). Affinity constants (K_D) were used for the "single-point" measurement to determine the number of receptors saving the amount of tissue needed, using following saturating concentrations of radioligands (2000 pmol/l [^{3}H]CGP 12177, 1500 pmol/l

[^{3}H]QNB, and 120 nmol/l [^{3}H]SB 206606 using the equation as previously. β_1- and β_2-adrenoceptors were determined using co-incubation of membranes with 400 pmol/l [^{3}H]CGP, and with subtype-selective antagonists in triplicates (10^{-7} mol/l CGP 20712A, β_1-adrenoceptors or 10^{-8} mol/l ICI 118.552, β_2-adrenoceptors).

Adenylyl Cyclase Activity

We measured the basal level of cAMP and forskolin-stimulated activity of adenylyl cyclase using modified method of Hoffer et al. (2005) in left ventricles. 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 RT, what was followed by centrifugation at 10,000×g 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 $\lambda = 413$ nm on a plate reader (Sunrise, Tecan). The final amount of cAMP was expressed as pmol/mg protein.

β_3-adrenoceptor Effect on Adenylyl Cyclase Activity

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

Determination of Nitric Oxide Synthase Activity

The determination of nitric oxide synthase activity (NOS) in tissue homogenates (murine right ventricles) 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 (30 μl) mixed with 50 mM HEPES with 0.5 mM EDTA (200 μl), NADPH Part A containing NADP+, Glucose 6-Phosphate and L-Arginine (10 μl), and NADPH Part B containing Glucose 6-Phosphate dehydrogenase (10 μl). 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 (10 μl) 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 0.26 ml) was within the range of 138–339 μg. The second phase of the experiment (in flat-bottom microtiter plate) was performed with nitrite standard solutions (100 μl) diluted from 100 to 0.5 μM in duplicates and samples (100 μl) also in duplicates followed by addition of the Griess reagents, SA in 3 N HCl (50 μl), and NED (50 μl). After shaking for 5 min at room temperature the absorbance was measured at 540 nm in multi-detection microplate reader (Synergy HT).

β_3-adrenoceptor Effect NOS Activity

For the investigation of β_3-adrenergic receptors (β_3-AR) mediated changes in NO-synthase (NOS) activity the tissue homogenates (murine left ventricles) 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 start of peak level of activation. Immediately the activity of NOS was determined using the Ultrasensitive Colorimetric NOS Assay Kit (Oxford Biomedical Research, Inc.).

Statistical Analysis

Results are presented as mean ± S.E.M. and each group represents an average of 6 animals. Statistical differences among groups were determined by one-way or two-way analysis of variance (ANOVA), and for multiple comparisons an adjusted t-test modified by SNK (Student–Newman–Keuls) correction was used. Values of $P < 0.05$ were considered to be significant. When appropriate, Student's t-test was used.

Results

Basic Binding Characteristics

In unstressed animals, we have found changes in the amount of β_1-, β_2-, and β_3- adrenoceptors binding sites in the left ventricles (compare Figs. 1, 2) and no changes in right ventricles (data not shown). β_1-, β_2-AR were decreased (Fig. 1a, b vs. Fig. 2a, b to approximately one half, t-test, $P = 0.0039$, degrees of freedom, $df = 10$) in comparison to WT as a result of M_2 muscarinic receptors knockout, β_3-AR were doubled (please compare Figs. 1c, 2c) in comparison to control (t-test, $P = 0.001$, $df = 10$). The affinity of receptors (K_D) was not affected by M_2

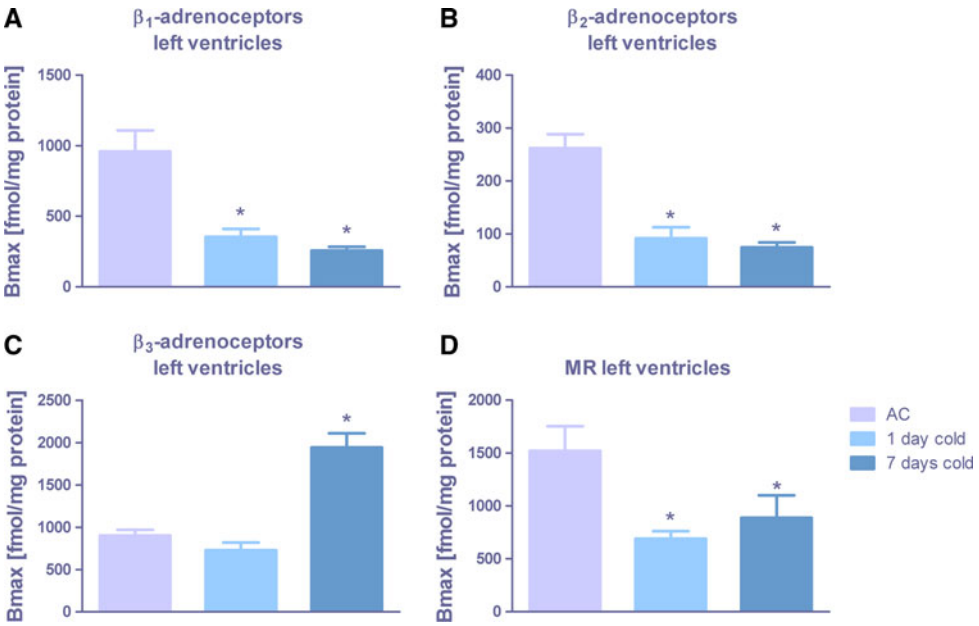

Fig. 1 Changes of receptor binding during exposure to cold in wild type animals in left ventricles. *Abscissa*: number of days in cold. *Ordinate*: receptor binding expressed as B_{max} [fmol/mg prot.]. **a**, **b**, **c**, **d**: appropriate type of receptor is marked in the heading

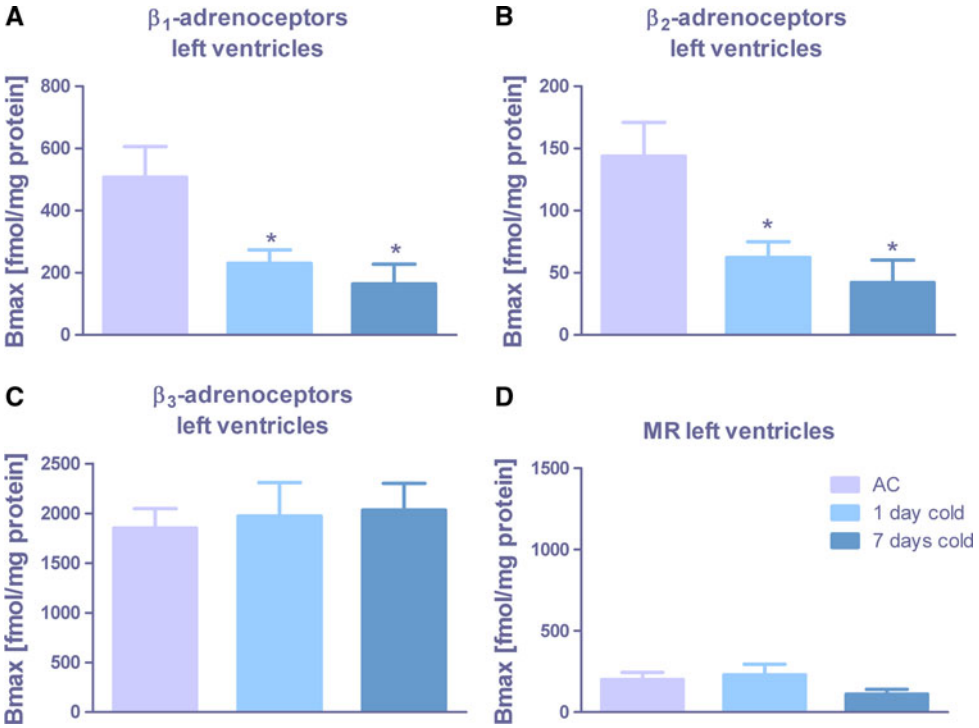

Fig. 2 Changes of receptor binding during exposure to cold in knockout animals. *Abscissa*: number of days in cold. *Ordinate*: receptor binding expressed as B_{max} [fmol/mg prot.]. **a**, **b**, **c**, **d**: appropriate type of receptor is marked in the heading

muscarinic receptor knockout (245.2 ± 43.94 pmol/l vs. 207.8 ± 19.25 pmol/l for β_1/β_2-AR WT and KO, respectively; 36.84 ± 5.7 pmol/l vs. 38.1 ± 10.5 pmol/l for MR WT and KO, respectively) suggesting changes in receptor levels but not in their affinities to ligands without change in the receptor affinity. In addition to changes in β-adrenoceptors, we also found remaining binding to muscarinic receptors (see Figs. 1d, 2d, *t*-test, $P = 0.0003$, $df = 10$).

The Cold Stress Effects on Receptor Binding

The reaction to cold stress in WT animals was chamber specific. While there was important receptor reaction to cold in left ventricles, we did not found any change in receptor numbers in right ventricles. Similarly to WT animals, there was no change in receptor number in right ventricles of M_2KO mice.

Table 1 Sequences of used primers and conditions of PCR

Gene	Primer sequence	Annealing temperature (°C)/time (s)	Number of cycles
TH	For: 5′-GAAGGGCCTCTATGCTACCCA-3′	63/30	35
	Rev: 5′-TGGGCGCTGGATACGAGA-3′		
DBH	For: 5′-GACTCAACTACTGCCGGCACGT-3′	60/30	35
	Rev:5′-CTGGGTGCACTTGTCTGTGCAGT-3′		
PNMT	For: 5′-TACCTCCGCAACAACTACGC-3′	60/30	35
	Rev: 5′-AAGGCTCCTGGTTCCTCTCG-3′		
GAPDH	For: 5′-AGATCCACAACGGATACATT-3′	60/60	30
	Rev: 5′-TCCCTCAAGATTGTCAGCAA-3′		

The cold stress in WT animals resulted in decrease in β_1- and β_2-AR (both after 1 day stay in the cold and after 7 days in cold) and also to dramatic decrease in MR in the heart left ventricles (see Fig. 1a, b). Exactly, there was decrease in β_1-AR to 37% of control in animals living in cold for 1 day and decrease to 27% of control after 7 days in cold (one-way ANOVA, $P = 0.0007$, $F = 20.94$, $df = 10$). β_2-AR were diminished to 35% after 1 day and to 28% after 7 days in cold, respectively (one-way ANOVA, $P = 0.0003$, $F = 27.33$, $df = 10$). On the other hand, β_3-AR (Fig. 1c) were increased (to 216% of control) when 7 days cold was applied (one-way ANOVA, $P < 0.0001$, $F = 31.80$, $df = 17$). MR were reduced (Fig. 1d) to 46% and to 58%, respectively (one-way ANOVA, $P = 0.0182$, $F = 5.298$, $df = 17$). The magnitude of changes expressed as percent of WT unstressed animals is shown in Tables 1, 2.

The reaction of cardiostimulative β_1- and β_2-adrenoceptors to cold was similar in KO animals to that in WT animals (see Fig. 2a, b). There was decrease in β_1-AR number after 1 day cold (to 45% of KO control) and after 7 days cold (to 32% of KO control). The changes in β_2-AR were almost same as those in WT: β_2-AR decreased to 44% and to 29% in KO animals (while there was decrease to 35% and to 28% in WT), respectively (see Fig. 1b). In contrast to cardioinhibitive receptors in wild types, β_3-AR in KO animals did not change in reaction to cold (see Fig. 2c). The magnitude of changes expressed as percent of WT unstressed animals is shown in Table 2. As a result of M_2 gene disruption, there was no change in MR number in the left ventricles (see Fig. 2d). The magnitude of changes expressed as percent of WT unstressed animals is shown in Table 2.

Table 2 The proportional changes in binding sites in the left ventricles summarizing the magnitude of changes between groups

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

The Changes in Muscarinic Gene Expression in Unstressed and Stressed Animals

In order to better characterize the changes in muscarinic receptors we have investigated the changes in muscarinic receptor gene expression (M_1–M_5 subtypes) in WT and KO animals. As expected, M_2 muscarinic receptors were not expressed in KO animals (therefore not depicted on Fig. 3). In addition to that, the gene expression of M_1 and M_5 muscarinic receptors was undetectable in the unstressed and stressed wild types as well as in unstressed and stressed knockouts. M_4 muscarinic receptors were not changed (see Fig. 3d, e) in WT and KO by stress. On the other hand, M_3 muscarinic receptors gene expression was changed (see Fig. 3b, c) in cold stress (one-way ANOVA, $P = 0.0293$, $F = 4.11$, $df = 26$) in WT but not in KO animals. The magnitude of changes expressed as percent of WT unstressed animals is shown in Table 3.

Adenylyl Cyclase Activity Changes During Cold Stress

Adenylyl cyclase activity increased in unstressed WT animals when it was stimulated by forskolin which was abolished by CL 316243 (see Fig. 4a). CL 316243 alone had no effect on adenylyl cyclase activity in unstressed animals (Fig. 4a, one-way ANOVA, $P = 0.0072$, $F = 5.347$, $df = 23$). In contrast, CL 316243 was able to increase adenylyl cyclase activity after one (Fig. 4c,

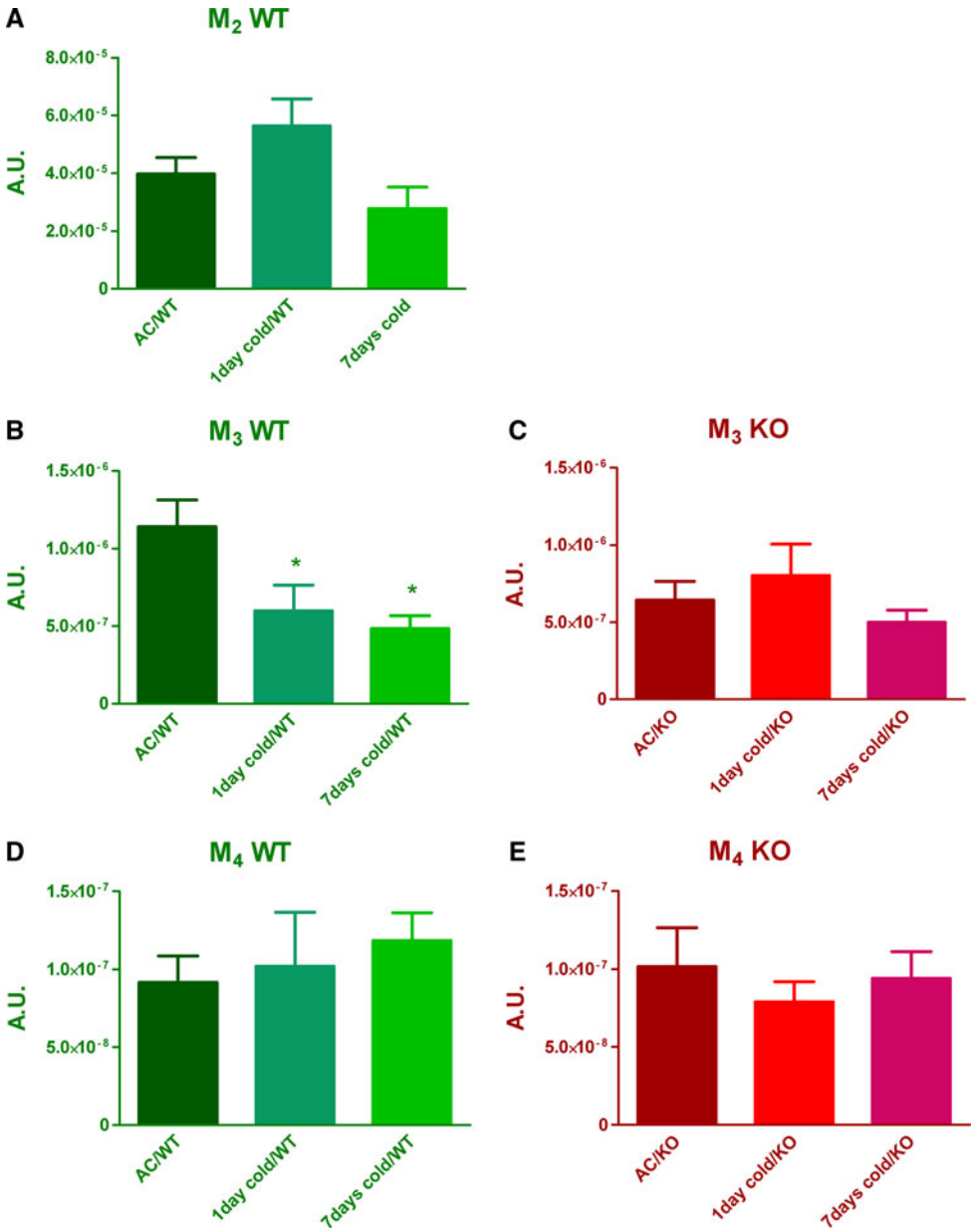

Fig. 3 Changes of gene expression of muscarinic receptor subtypes (M_1–M_5) during exposure to cold in wild type and knockout animals. *Abscissa*: number of days in cold. *Ordinate*: the relative amount of mRNA expressed in A.U. [arbitrary units]. *M2 WT*: M_2 muscarinic receptor gene expression, KO animals are not shown as M2 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

one-way ANOVA, $P < 0.0001$, $F = 13.37$, $df = 23$) and 7 days cold (Fig. 4e, one-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. 4c, e). In KO animals, forskolin was not able to increase the adenylyl cyclase activity in unstressed animals (Fig. 4b), there was only difference between combined treatment with CL 316243 with forskolin and other groups (one-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. 4d, 1 day: one-way ANOVA, $P = 0.0183$, $F = 4.05$, $df = 27$; 7 days: (Fig. 4f) one-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. 4b, d, f); it only abolished the forskolin effects after 7 days cold.

Nitric Oxide Synthase Activity During Cold Stress

As it can be deduced from Fig. 5, BRL 37344 was not able to stimulate NOS activity both in WT animals (Fig. 5a (one-way ANOVA, $P = 0.2352$, $F = 1.451$, $df = 35$)) and KO animals (Fig. 5b (one-way ANOVA, $P = 0.5565$, $F = 0.8030$, $df = 35$). Similarly, the stress had no effects on NOS activity in WT animals (after 1 or 7 days in cold) as well as on KO animals.

Table 3 The proportional changes in gene expression of M1–M5 muscarinic receptor subtypes in the left ventricles summarizing the magnitude of changes between groups

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

Changes in Gene Expression of Catecholamine Synthesizing Enzymes

The changes of gene expression of catecholamine synthesizing enzymes are shown in supplementary material.

Discussion

Our results show that gene disruption of M_2 gene itself changed receptor levels on cardiac tissue membranes and that this effects was limited to the left ventricles. This is an important fact, as the trend for maintaining homeostasis between antagonistic receptors is not present in the right ventricles. That means only physiologically important heart regions are able to change the other (antagonistic) receptor properties. Concerning the mechanism, it is the most probable that the antagonistic receptor regulation is caused by heterologous regulation (i.e., local mechanism) as it is region specific. In other case, all regions should be affected. The fact that in case of one subtype receptor down-regulation there is also down-regulation of antagonistic receptor was demonstrated multiple times (Lee and Fraser 1993; Paraschos and Karliner 1994; Myslivecek et al. 1998).

Another finding of this article is that cold stress is able to decrease the number of β_1-, β_2-adrenoceptor, and muscarinic receptor binding sites and to increase the number of β_3-adrenoceptor in left murine ventricles. In mice, lacking main M_2 muscarinic receptors was the loss of main cardioihibitive receptors compensated by increase in β_3-adrenoceptors. This finding, according to our knowledge, is new. Concerning the mechanism, the decrease of β_1- and β_2-AR could be caused by the decrease of protein translation and/or decrease of their membrane surface presentation. The data on β_3-AR [based on our previous findings (Tillinger et al. 2008)] suggest increase in β_3-AR gene expression. Also, it is necessary to point out that the previous study was performed on rat and the present results are from mice. Usually, strain differences exist.

We demonstrate here that knocking out of M_2 muscarinic receptors changes also the gene expression changes in left ventricles during stress reaction which is, according to our knowledge, also new finding. There is decrease in M_3 muscarinic receptor gene expression in WT, while the gene expression is not changed KO animals during cold stress. On the other hand, M_2 muscarinic receptor mRNA is not changed in WT animals suggesting that changes in muscarinic receptor binding are consequence of changes in protein properties (decrease in protein translation and/or decrease of their membrane surface presentation and/or increase of their removal from the membrane and/or degradation) rather than changes in gene expression. M_3 muscarinic receptors are coupled to PLC (Wang et al. 2007) and could have cardiostimulative effects. Therefore, their changes can be understood as a part of compensatory mechanisms with mutual interplay between cardiostimulating and cardioinhibitive receptors. But, in M_3 knockout animals, the changes in heart rate in comparison to WT animals after muscarinic agonist treatment are not substantial (Fisher et al. 2004) which question the role of M_3 muscarinic receptors in regulation of heart rate. Another possibility is that changes in M_3 gene expression are related to the ability to induce delayed cardioprotection which have been shown recently (Zhao et al. 2010).

The activity of adenylyl cyclase is importantly affected by β_3-adrenoceptor agonist which suggest that adenylyl cyclase can be regulated during cold stress reaction by β_3-ARs. As it has been demonstrated earlier (Gauthier et al. 2000), β_3-adrenoceptor can activate G_i proteins which causes an activation of the nitric oxide (NO) pathway, probably implicating the endothelial NO synthase (eNOS). 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, in cold stress CL 316243 increases the amount of produced cAMP which suggests that β_3-adrenoceptor can switch from G_i coupling to G_s coupling. This is the properties 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-adrenoceptor as it have been demonstrated by (Baker 2005). In summary, the increase of cAMP production by the β_3-AR activation is unobvious and (see Fig. 4) can be seen in cold stressed WT animals only. With deep contrast,

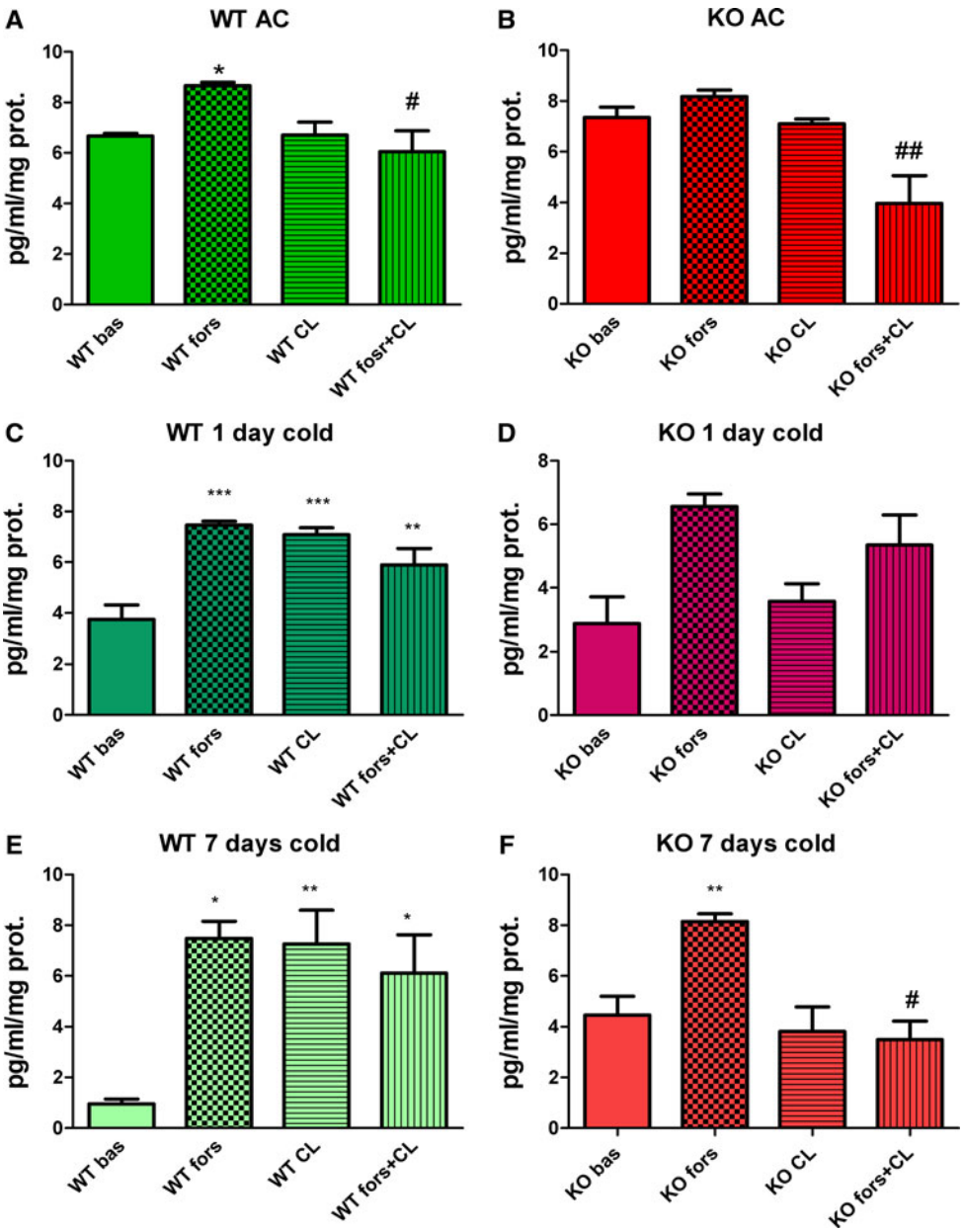

Fig. 4 Changes of adenylyl cyclase activity during exposure to cold in wild type and knockout animals. *Abscissa*: type of the drug added to the mixture (*bas* basal level, i.e., no drug was added, *fors* forskolin, *CL* CL 316243, *fors* + *CL* forskolin and CL 316243 was applied). *Ordinate*: the amount of measured cAMP expressed as pg/ml/mg prot. **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: appropriate type of animals and stress

CL 316243 was not able to increase cAMP levels in KO animals (where β_3-AR (see Fig. 2) are constantly increased). This fact gives evidence about the compensatory role of β_3-AR in the heart when M_2 MR are lacking. The possible interaction between increased level of cAMP and heart homeostasis should be evaluated further. As it has been demonstrated recently, there can be connection of β_3-AR to Ca^{++} signaling (Li et al. 2010) which could be also the possibility how to explain increase cAMP levels in WT only. In these experiments (Li et al. 2010), β_3-AR inhibits the Ca^{++} 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 numbers of β_3-AR binding sites).

It was demonstrated that modulation of NOS (rather eNOS) activity and increase in NO formation after the application of BRL 37344 is specifically coupled to a stimulation of the cardiac β-AR (Moens et al. 2010). We have investigated whether the preferential β_3-AR agonist BRL 37344 induces an activation of the NOS in the murine right ventricles. The activities of NOS expressed as μmoles of NO produced/μg protein/unit time were compared at tissue homogenates non-stimulated and stimulated with BRL (1 mM). No NOS activation was observed after application of BRL 37344 in tested samples of tissue homogenates. These data make the possibility that NOS is activated by β_3-AR in cold stress rather improbable.

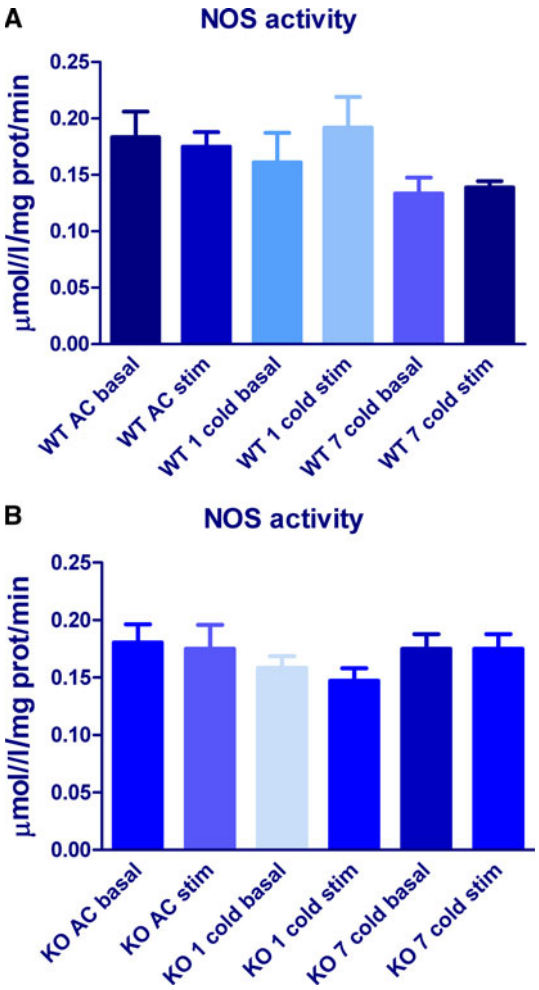

Fig. 5 Changes of nitric oxide synthase activity during exposure to cold in wild type (**a**) and knockout (**b**) animals. *Abscissa*: type stimulation used for NOS determination (*basal* basal level, i.e., no drug was added, *stim* BRL 37344 treatment). Number of days in cold are shown as 1 or 7 cold (i.e., 1 day in the cold, 7 days in the cold). *Ordinate*: the amount of measured NO expressed as µmol/l/mg prot/min

In conclusion, we demonstrate here that the cold stress decreases the number of β_1-, β_2-adrenoceptors and muscarinic receptors and increases the number of β_3-adrenoceptors in left murine ventricles (in WT animals). The stress is connected with increased levels of catecholamines that are able to decrease β_1-, β_2-adrenoceptors. These receptors are cardiostimulative (activate adenylyl cyclase, increase Ca^{++} release from sarcoplasmic reticulum (Krizanova et al. 2007) increasing the force and rate of heart contraction). Therefore, when β_1-, β_2-adrenoceptors are decreased, opposite receptors should change accordingly (i.e., there should be decrease in MR that inhibit adenylyl cyclase, decrease Ca^{++} release from reticulum and decrease the heart rate and contraction). In addition to that M_3 muscarinic receptor gene expression decreases were observed during cold stress in WT but not in KO animals. As no attitude in muscarinic receptor subtype detection is available, we can only speculate that minor (M_3) muscarinic receptor subtype, which is considered as cardiostimulating, should changed in the same manner as other receptors to maintain the balance between antagonistic receptors. β_3-adrenoceptors were assumed to be cardioinhibitive. We demonstrate here, that increase in β_3-AR is connected with increase of adenylyl cyclase activity that is probably due to imbalance between β_1-AR (β_2-AR) and β_3-AR as deduced from the β_3-AR changes in KO animals (where β_3-AR did not increased the adenylyl cyclase activity). Please also note, that the basal adenylyl cyclase activity decreases when comparing control, 1 day and 7 days cold. β_3-AR are not coupled to NOS.

When M_2 muscarinic receptors are lacking then there is persistent increase in β_3-adrenoceptors which leads to abolishing of β_3-AR induced adenylyl cyclase activity increase. Similarly as in WT, β_3-AR are not coupled to NOS in KO animals. This give evidence that β_3-AR substitute the role of lacking MR in the cold stressed animals in which they are not able to increase the adenylyl cyclase activity.

Acknowledgments This study was supported by grant GACR309/09/0406 and by Grant GAUK 111409. Technical assistance of Petra Svatosova is greatly acknowledged.

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