The M2 muscarinic receptors are essential for signaling in the heart left ventricle during restraint stress in mice

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Abstract

We hypothesized that muscarinic receptors (MRs) in the heart have a role in stress responses and thus investigated changes in MR signaling (gene expression, number of receptors, adenylyl cyclase (AC), phospholipase C (PLC), protein kinase A and C (PKA and PKC) and nitric oxide synthase (NOS)) in the left ventricle, together with telemetric measurement of heart rate (HR) in mice (wild type [WT] and M2 knockout [KO]) during and after one (1R) or seven sessions (7R) of restraint stress (seven mice per group). Stress decreased M2 MR mRNA and cell surface MR in the left ventricle in WT mice. In KO mice, 1R, but not 7R, decreased surface MR. Similarly, AC activity was decreased in WT mice after 1R and 7R, whereas in KO mice, there was no change. PLC activity was also decreased after 1R in WT and KO mice. This is in accord with the concept that cAMP is a key player in HR regulation. No change was found with stress in NOS activity. Amount of AC and PKA protein was not changed, but was altered for PKC isoenzymes (PKCa, β, γ, η and ε) (increased) in KO mice, and PKCβ (increased) in WT mice. KO mice were more susceptible to stress as shown by inability to compensate HR during 120 min following repeated stress. The results imply that not only M2, but also M3 are involved in stress signaling and in allostasis. We conclude that for a normal stress response, the expression of M2 MR to mediate vagal responses is essential.

Introduction

It is well established that stimulation of cardiac muscarinic receptors (MRs) via vagal activation triggers bradycardic responses (decreases in heart rate, HR), negative chronotropy. There are five MR subtypes, denoted as M1, M2, M3, M4 and M5, encoded by five distinct genes (Caulfield & Birdsall, 1998). It is now firmly established that M2 MR represents the principal MR subtype found in the mammalian heart. M2 MR mRNA has been reported to constitute more than 90% of total muscarinic mRNAs in the rat heart (Krejci & Tucek, 2002) and over 99% in the mouse heart (Kitazawa et al., 2009). The principal signaling mechanism of M2 MR comprises G{i,0} activation, inhibition of adenylyl cyclase (AC) and thus decrease in protein kinase A (PKA) signaling (Alexander et al., 2011).

Cyclic AMP is a key player in HR regulation (Difrancesco, 2010) as it regulates funny channels (via hyperpolarization-activated cyclic nucleotide gated 4 channel, which is a major component of these channels). Funny channels are considered as sodium/potassium channels and are expressed in the pacemaker atrial cells, but not in the whole myocardium (Difrancesco, 2010). Thus, cAMP increased by β-adrenoceptors or decreased by M2 MR represents a basic physiological mechanism in HR regulation. In addition, G1 (Nagata et al., 2000) or G0 (Valenzuela et al., 1997) subunit (activated by M2 MR) can inhibit voltage-gated Ca2+ channels (L-type), which consequently reduces contractility of ventricular myocytes. Moreover, G protein βγ subunits can directly activate the muscarinic-gated potassium channel (Bunemann & Hosey, 2001) and have a role in parasympathetic HR control (Gehrmann et al., 2002). In addition to M2 MR, a wealth of data has also demonstrated the presence of non-M2 MR in the mammalian heart, albeit as minor populations (Dhein et al., 2001; Myslivecek et al., 2008a).

Consistent with previous functional approaches, studies on MR knockout (KO) mice lines have provided unambiguous evidence that MR-induced decreases in HR are mediated by M2 (Fisher et al., 2004; Stengel et al., 2000). While in M1 KO mice and M3 KO mice, the bradycardic responses to MR stimulation does not differ from responses in control mice, they are abolished in M2 KO mice in vitro as well as in vivo (Fisher et al., 2004; Kitazawa et al., 2009; Stengel et al., 2000, 2002). In addition, isolated spontaneously beating atria from M2 KO mice do not present MR-induced negative inotropic activity (decrease in heart contractility), clearly implicating M2 in mediating the inhibition of heart contractility (Kitazawa et al., 2009). Interestingly, atria from M2 KO mice respond to the MR agonist carbachol with positive inotropic activity (Kitazawa et al., 2009). Furthermore,
increase in HR is observed after carbachol in conscious M₂ KO mice monitored telemetrically (Benes et al., 2012b).

Despite the loss of MR agonist-induced or vagally mediated decreases in HR in M₂ KO mice, in vivo basal HR in anesthetized (Fisher et al., 2004) or conscious (Benes et al., 2012b) M₂ KO mice is not changed. Likewise, the spontaneous rate of beating in isolated atria from M₂ KO mice is not significantly different from wild type (WT) mice, indicating that M₂ do not play a significant role in modulating basal sinoatrial nodal function in vitro (Stengel et al., 2000). Absence of changes in HR in M₂ KO mice in vivo has been suggested to result from a lower resting vagal tone in mice than in large mammals (Fisher et al., 2004). However, it is possible that the impaired cholinergic signaling in M₂ KO mice is counterbalanced by an adaptation in the adrenergic system in the heart (Dhein et al., 2013), which exerts opposite effects to vagal stimulation on cardiac functions and elicits positive chronotropic, inotropic and bathmotropic responses (Myslivecek et al., 2008b). Indeed, the absence of M₂ in the left ventricle of M₂ KO mice is accompanied by decreases in mRNA and protein expression of β-adrenoceptors (β₁ and β₂) (Benes et al., 2012b). These changes in β-adrenoceptors in the heart of M₂ KO mice might represent possible mechanisms of almost normal cardiac function in M₂ KO mice.

There are similarities in receptor gene expression, receptor presence, and thus signaling cascades between the right atrium and left ventricle that indicate the appropriateness of studying signaling in the left ventricle, given the limited size of the right atrium. Moreover, left ventricular contractility is a major component of increased cardiac output in the stress response.

In addition to the above-mentioned signaling pathways, carbachol also activates nitric oxide synthase (NOS) (Sterin-Borda et al., 1995) via activation of phospholipase C (PLC), calcium/calmodulin and protein kinase C (PKC). NOS and guanylate cyclase increase cAMP, hence modifying contractility.

In summary, signaling pathways connected to the MRs comprise: M₂ MR Gₛ-mediated inhibition of AC, changing cAMP level (involved in HR regulation) and mainly M₃ MR Gₛq-mediated activation of PLC, which in turn activates PKC and NOS (Figure 8).

Stress is usually connected with a response of the sympathetic nervous system (Pacak & Palkovits, 2001). Previously, we have found that stress changes not only heart adrenoceptors but also MRs (Benes et al., 2012a; Myslivecek et al., 2008c; Novakova et al., 2010). We have hypothesized that these receptor changes could be the mechanism for relatively unchanged heart function in mice that lack the main cardioinhibitory receptors (Benes et al., 2012b). In this study, we focused on changes in muscarinic signaling in response to stress. In order to elucidate the role of M₂ MR in response to stress, we have compared the changes elicited by restraint stress in WT mice and in M₂ KO mice in AC activity, PLC activity, NOS activity and AC, PKC and PKA protein amounts in the left ventricle. In addition, we measured changes in HR during and after stress in M₂ KO and WT mice. However, we acknowledge that there is no direct functional connection between the right atrium, where HR is regulated via the sinoatrial node, and the signaling changes we measured in the left ventricle. We have tested the hypothesis that M₂ MRs are indispensable for normal cardiac stress response.

**Methods**

**Animals**

The mouse clone lacking the M₂ MR gene was generated in the Wess Laboratory (Prague, Czech Republic; Gomeza et al., 1999) and then bred in our animal facility. Mice were treated in accordance with the legislation of the Czech Republic and EU legislation (European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe no. 123, Strasbourg, France, 1985)), and the experimental protocol was approved by the Committee for the Protection of Experimental Animals of the 1st Medical Faculty, Charles University, Prague. The WT mice were from the mixed 129J1/CF1 line. WT and KO genotypes were confirmed using polymerase chain reaction (PCR) analysis as previously described (Cea-del Rio et al., 2010).

The mice were maintained (three per cage) under controlled environmental conditions (12-h/12-h light/dark cycle, 22 ± 1 °C, light on at 06:00 h). Food and water were available ad libitum. Male M₂ KO mice and their WT counterparts (born the same week, weighting 20–25 g, 11–13 weeks old) were used in this study.

**Restraint stress**

WT and M₂ KO mice were exposed to acute (120 min, 1R) and repeated (120 min daily for seven days, 7R) restraint sessions: the mice were placed individually in a 50 ml tube (11.5 × 3 cm) with ventilation holes, which allowed only restricted movement. The stress session started at 08:00 h. The mice were killed (by conscious decapitation) 180 min after the end of restraint; the hearts were removed and dissected into right atrium, left atrium, left ventricle and right ventricle. As the amount of tissue in mice is small, the left ventricles were used for signaling analysis and other parts for gene expression studies. Control (not stressed) mice (in all cases) were killed at the same time as mice killed after one or seven sessions of restraint. No difference was found between the 1R and 7R control groups, i.e. between groups not influenced by stress, thus these groups were pooled into one cohort.

**MR gene expression**

Total mRNA was isolated using the chloroform–isopropanol (RNA Bee, TelTest, Friendswood, TX) method according to the manufacturer’s instructions. RNA yield and integrity were evaluated spectrophotometrically using a Tecan Infinite 200 Nanoquant (Tecan Group Ltd., Männedorf, Switzerland) at A₁/₂₅₄nm and A₁/₂₆₀nm and A₁/₂₆₀/₂₈₀nm, respectively. Samples with A₁/₂₆₀/₂₈₀nm values between 1.7 and 1.95 were used for downstream procedures. Total RNA was purified to eliminate potentially contaminating genomic DNA using recombinant DNase (Life Technologies Czech Republic Inc., Prague, Czech Republic), After purification, a final concentration of purified mRNA was assessed again using Tecan Infinite 200 Nanoquant (Tecan Group Ltd., Männedorf, Switzerland);
1000 ng of purified mRNA was subsequently transcribed into cDNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Pittsburgh, PA) and pd(N)6 primer (GE Healthcare). qPCR was performed using TaqMan probes (Life Technologies Czech Republic, Prague, Czech Republic), probe numbers: Hs 99999901_s1 for 18S rRNA, Mm01167087_m1 for M1, Mm01338409_m1 for M2, Mm0133561_s1 for M3 and Mm01701883_s1 for M5 with Roche qPCR Mastermix (Roche Czech Republic, Inc., Prague, Czech Republic) using the following protocol: 2 min at 50 °C, 10 min at 95 °C followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C in a final volume of 10 μL. All experiments were performed in duplicate. Then, for each sample, the following formula was used: normalized ratio = 2^(-ΔΔCT), where CT1 is cycle threshold (CT) of reference transcript and CT2 is CT of target transcript. No template controls and no reverse transcription reactions were performed as negative controls. MR subtype gene expression (M1 mRNA-M5 mRNA) normalized to housekeeper gene (18S rRNA) was evaluated using GraphPad Software (San Diego, CA). Variability of our results was less than 14%; sensitivity of assay was 10^-8 AU.

MR density
Radioligand binding studies were performed on a cell membrane fraction and compared with binding to whole tissue homogenate, reflecting total number of receptors. The tissue was weighed and homogenized in ice-cold Tris-ethylenediaminetetraacetic acid (EDTA) buffer (Tris-HCl 50 mmol.l⁻¹, EDTA 2 mmol.l⁻¹, pH 7.4). The homogenates were divided into two aliquots. The first was centrifuged at 32,000 x g for 20 min at 4 °C; the sediment was re-suspended in 700 μl of Tris-EDTA buffer; this homogenate portion was used to indicate the total number of receptors in the sample. The second aliquot was centrifuged at 600 x g for 10 min at 4 °C; supernatant was collected and centrifuged again for 20 min at 32,000 x g at 4 °C. The sediment was re-suspended as the membrane fraction. The amount of MR-binding sites was determined in duplicate in homogenates and in membrane fractions. The incubation medium contained 2 nmol.l⁻¹ [3H]-(±)-Quinuclidinyl α-hydroxydiphenylacetate, L-[benzilic-4,4'-3H]-([3H]QNB; As = 1.35 TBq.mmol⁻¹⁻¹; Perkin Elmer, Boston, MA) in Tris-EDTA buffer; non-specific binding was determined in the presence of 5 μmol.l⁻¹ atropine. The incubation was performed at room temperature for 120 min and terminated by rapid filtration through Whatman GF/B glass fiber filters in a Brandel cell harvester (Brandel Inc., Gaithersburg, MD). The filters were washed with ice-cold distilled water, and the retained radioactivity was measured by liquid scintillation spectrometry. The amount of binding sites (Bmax) per mg protein (determined using a BCA kit, Sigma Czech Republic, Prague, Czech Republic) and the affinity constant (KD) were computed by non-linear regression from preliminary saturation experiments (concentration range of [3H]QNB was 66.5–2000 pmol.l⁻¹) using the GraphPad Prism 5.01 program (GraphPad Software, San Diego, CA). Radioligand-binding data were evaluated as described before (Benes et al., 2012b). Sensitivity of this assay is 1 fmol/mg protein, variability of results was less than 13%.

AC activity
The determination of AC activity in tissue homogenates was performed using the Screen Quest™ cAMP Colorimetric ELISA Assay Kit (AAT Bioquest, Inc., Sunnyvale, CA) according to the manufacturer’s instructions. Briefly, frozen tissues were weighed and homogenized in 0.05 mmol.l⁻¹ Tris-HCl buffer (pH 7.4) with 0.002 M EDTA (50 mg of tissue.ml⁻¹). Tissue homogenates were centrifuged (32,000 g) for 10 min at 4 °C, and the supernatants were immediately assayed for AC activity. cAMP standards, control and test samples were added (75 μl per well) to the anti-cAMP Ab-coated 96-well plate. After incubation (10 min at room temperature), horse radish peroxidase–cAMP conjugate working solution was added (25 μl per well), and the plate was incubated at room temperature for 3 h by placing the plate on a shaker. Plate contents were aspirated and washed four times with wash solution (200 μl per well). Subsequently, Amplite™ Green (100 μl per well) was added, and the plate was incubated at room temperature for 3 h, protected from light. Absorbance increase was measured at 405 nm using a multi-detection microplate reader (BioTek, Winooski, VT). To investigate actions of muscarinic acetylcholine (ACH) receptor agonist, the effect of carbachol on AC activity was studied; supernatants (prepared as described above) were pre-incubated with carbachol (50 nmol.l⁻¹) for 20 min at room temperature. The activity of AC was determined immediately using the Screen Quest™ cAMP Colorimetric ELISA Assay Kit (AAT Bioquest, Inc.). The optimal concentration of carbachol and incubation time were determined in preliminary experiments. Variability of our results was 11%, and the sensitivity of assay was 10 pmol/mg protein.

PLC activity
PLC activity in tissue homogenates was determined using an EnzChek® Direct PLC Assay Kit (Invitrogen Corporation, Carlsbad, CA). To detect PLC activity, this assay uses a proprietary substrate (glycero-phosphoethanolamine with a dye-labeled sn-2 acyl chain), which releases the dye-labeled diacylglycerol when cleaved by phosphatidylcholine-specific PLC. The amount of protein (determined using a Pierce BCA Protein Assay Kit, Life Technologies Czech Republic, Prague, Czech Republic) in the reaction mixtures (total volume of 0.2 ml) was 150 μg. The concentration of carbachol in the incubation mixture was 50 nmol.l⁻¹. Fluorescence (485 nm excitation and 535 nm emission) of the dye-labeled product was measured after 100 min with a Synergy™ HT Multi-Mode Microplate Reader (BioTek, Winooski, VT). The sensitivity of our assay was 100 mU/ml/mg protein, the variability of results was less than 6%.

NOS activity
NOS activity in tissue homogenates was determined with an Ultrasensitive Colorimetric NOS Assay Kit (Oxford Biomedical Research, Inc., Rochester Hills, MI) as described previously (Benes et al., 2012a). The amount of protein (determined using the Pierce BCA Protein Assay Kit, Life Technologies Czech Republic, Prague, Czech Republic) in the reaction mixtures (total volume of 0.26 ml) was 100 μg. To investigate effects of carbachol on NOS activity, tissue
homogenates were pre-incubated with carbachol (10 μmol.l⁻¹) for 20 min at 37 °C (water bath), as described previously (Borda et al., 1998). NOS activity was determined immediately using an Ultraviolet Sensitive Colorimetric NOS Assay Kit (Oxford Biomedical Research, Inc.). Variability of our results was less than 16%, and the sensitivity of the assay was 0.01 μmol/l/mg protein/min.

**AC protein expression**

Tissue lysates were first denaturated with Laemmli Sample Buffer at 95 °C for 5 min. For the subsequent SDS-PAGE analysis in 10% resolving gel, 75 μg of total protein was loaded per lane. On completion of protein migration, gels were immediately transferred onto nitrocellulose membranes (TE 77X Large Semi-Dry Transfer Unit) and the blots then probed with antibodies (anti-AC antibody (A cyclase (R-32): sc-1701, Santa Cruz Biotechnology Inc., Santa Cruz, CA, dilution 1:200) using a One-Hour Western Basic Kit by GenScript (Piscataway, NJ). Immunoreactivity was visualized by chemiluminescence with the Odyssey Fc system (LI-COR Biosciences, Lincoln, NE) and finally quantified by densitometry with MCID Core imaging software. β-actin (detected with anti-beta actin antibody (ab75186), concentration of 1 μg.ml⁻¹, Abcam, Cambridge, UK) served as a loading control for each blot as a housekeeper gene product. Variability of results was less than 17%.

**PKA protein expression**

PKA expression was performed on cell lysates using a procedure similar to that described above for AC protein expression. As an antibody, PKAα/β/γ cat (H-95): sc-28892 (dilution 1:200, Santa Cruz Biotechnology Inc.) was used. β-actin (anti-beta Actin antibody – Loading Control (ab75186), Abcam) protein expression served as control housekeeper protein expression. Variability of results was 21%.

**PKC protein expression**

PKC expression was measured using a procedure similar to that described above for AC protein expression. PKC (H-300): sc-10800 (dilution 1:200, Santa Cruz Biotechnology Inc.) was used as the detection antibody. Three isoforms of PKC were detected with MW = 94.6 kDa, 87.8 kDa, 79.6 kDa, respectively, corresponding to PKCe (94.6 kDa), the group of PKCs: α,β,γ,η (87.8 kDa) and PKCδ (79.6 kDa). β-actin was used as housekeeper comparison protein. Variability of results was 21%.

**Changes in HR**

To assess overall functional changes in stressed mice, we used telemetry to measure HR in intact and stressed mice. The Mini Mitter telemetry system was used (Respironics, Andover, MA). The transponders (E-Mitter, G2-HR, dimensions 15.5 × 6.5 mm) were implanted in the peritoneal cavity under anesthesia (Zoletil® 100, Rometar® 2% 5:1, diluted 10 times, 3.2 ml kg⁻¹, i.p.); mice were left one week for recovery from the surgery and then used in this experiment. The sensor leads were used for HR data acquisition; other parameters (temperature and activity) were acquired directly from the transponders. Receivers were connected in series and connected directly to a PC via a single computer port allowing for the determination of all parameters. The HR data were collected every 10 s. VitalView (Respironics, Andover, MA) and GraphPad Software were used for the evaluation of data. For analysis purposes, we compared 120-min periods during stress (Respironics, Andover, MA) and the next 120-min recovery period. Detailed analysis of sequential 15-min periods was also performed. Variability of results was less than 1%.

**Statistical analysis**

Statistical significance between groups was determined using one-way ANOVA with Student–Newman–Keuls post hoc analysis. When comparing two groups, only (typically WT vs. KO) Student’s t-test was used.

**Results**

**MR subtypes gene expression**

As expected, M₂ MR gene expression was detected in WT mice only (Figures 1 and 2 and Supplementary Figure 1).

**Right atrium**

M₂ MR gene expression was decreased in the WT right atrium (Figure 1) to 19% after one restraint session and to 36% after seven restraint sessions (one-way ANOVA, p = 0.017, F = 5.318, degrees of freedom (df) = 2 (treatment), 16 (residual), 18 (total)). In addition, there was also a decrease in M₃ MR gene expression (in WT and KO mice) after one session of restraint (to 35% and 30% in WT and KO, respectively, one-way ANOVA, p = 0.029, F = 2.795, df = 5 (treatment), 41 (residual) and 46 (total)).

**Left ventricle**

M₂ MR gene expression was decreased to 28% of control values after one session of restraint stress (one-way ANOVA, p < 0.015, F = 5.237, df = 2 (treatment), 19 (residual), 21 (total)) and the same decrease was found after seven restraint sessions (to 29%) (Figure 2). Similarly, as in the right atrium, there was a decrease in M₃ MR gene expression (one-way ANOVA, p = 0.016, F = 5.042, df = 2 (treatment), 22 (residual), 24 (total)) after one session of restraint (to 44%). Gene expression of other MR subtypes (M₄; Figure 2) was not changed by stress in WT and KO mice.

**Left atrium and right ventricle**

There was a decrease in M₂ MR gene expression after one stress session in the right ventricle and an increase in both chambers in M₄ MR after seven sessions of restraint (Supplementary Figure 1).

**MR density**

**Membrane receptors**

The number of membrane receptors was decreased in WT mice after one and seven restraint sessions (one-way ANOVA, p < 0.016, Fₜ = 6.831, df = 2 (treatment), 9 (residual), 11
In homogenates, receptor levels were not changed by stress in either WT (one-way ANOVA, \( p = 0.49, F = 0.785, df = 2 \) (treatment), 8 (residual), 10 (total)) or in KO mice (one-way ANOVA, \( p = 0.21, F = 1.90, df = 2 \) (treatment), 8 (residual), 10 (total); Figure 3).

**Total receptors**

In homogenates, receptor levels were not changed by stress in either WT (one-way ANOVA, \( p = 0.49, F = 0.785, df = 2 \) (treatment), 8 (residual), 10 (total)) or in KO mice (one-way ANOVA, \( p = 0.21, F = 1.90, df = 2 \) (treatment), 8 (residual), 10 (total); Figure 3).

**AC activity**

Carbachol (50 nmol l\(^{-1}\)) did not inhibit basal activity of AC in WT or KO mice (Figure 4). Activity of AC in WT mice but not in M\(_2\) MR KO mice was decreased by one or seven sessions of restraint stress (one-way ANOVA, \( p < 0.0001, F = 8.382, df = 11 \) (treatment), 32 (residual) and 43 (total)).

**PLC activity**

Carbachol (50 nmol l\(^{-1}\)) stimulated PLC activity in WT controls (Figure 5; one-way ANOVA, \( p < 0.0001, F = 18.25, df = 11 \) (treatment), 28 (residual) and 39 (total)). One session, but not seven sessions, of restraint stress decreased PLC activity in WT mice. Moreover, one session, but not seven sessions, of restraint prevented stimulation of PLC activity by carbachol. A similar decrease in PLC activity after one restraint session, but not seven sessions, was seen in KO mice. However, carbachol did not affect PLC activity in control KO mice or after one restraint session but it stimulated PLC after seven stress sessions.

**NOS activity**

There were no effects of carbachol or stress on left ventricle NOS activity in either WT or KO mice (Table 1; one-way ANOVA, \( p = 0.72, F = 0.7037, df = 11 \) (treatment), 28 (residual) and 39 (total)).

**AC protein expression**

AC protein expression did not differ between WT and KO mice and was not altered by stress (Table 2; one-way ANOVA, \( p = 0.24, F = 1.531, df = 5 \) (treatment), 14 (residual) and 19 (total)). Western blotts are shown in Supplementary Figure 2.
Figure 2. The effect of stress on gene expression for M2, M3, and M4 MR in the left ventricle. AU, arbitrary units (green); WT, wild type (red); KO, M2 muscarinic receptor knockout; MR, muscarinic receptors. Relative amount of gene expression is lower for M3 and M4 than for M2 MR. See key for explanation of specific columns. *p < 0.05 vs. WT control, #p < 0.05 between WT and KO. One-way ANOVA with SNK post-hoc analysis (n = 6–12 in respective group). Data are mean ± SEM.

Figure 3. Comparison of total and surface MR number changes with stress in left ventricle. MR, muscarinic receptors. WT, wild type (green); KO, M2 muscarinic receptor knockout (red). Note different scales for WT and KO binding (Bmax) indicating predominant M2 receptor subtype expression in WT and decrease in M2 receptor number with stress. Decrease in surface receptors with stress in M2 KO mice indicates also a minor decrease in non-M2 receptors. *p < 0.05 vs. control. One-way ANOVA with SNK post-hoc analysis (n = 4 in each group). Data are mean ± SEM.
PKA protein expression

PKA protein expression was not different between groups (Table 3; one-way ANOVA, \( p = 0.2771, F = 1.432, df = 5 \) (treatment), 13 (residual) and 18 (total)). Western blots are shown in Supplementary Figure 2.

PKC isoform protein expression

PKC\( \alpha, \beta, \gamma, \delta \) protein expression was increased in KO mice stressed once compared to control KO mice (Figure 6; one-way ANOVA, \( p = 0.0053, F = 6.745, df = 5 \) (treatment), 10 (residual) and 15 (total)). No change was observed with stress in WT mice. However, expression in KO mice (after one and seven sessions of restraint) was significantly higher than in WT mice.

Expression of PKC\( \alpha, \beta, \gamma, \delta \) was not changed by one or seven restraint sessions in WT mice, but increased by one restraint session in KO mice (one-way ANOVA, \( p = 0.0002, F = 14.06, df = 5 \) (treatment), 11 (residual) and 16 (total)).

PKC\( \epsilon \) expression was increased (one-way ANOVA, \( p = 0.0057, F = 7.965, df = 5 \) (treatment), 8 (residual) and 13 (total)) after one session of restraint in WT, but not KO mice. Western blots are shown in Supplementary Figure 2.
HR changes

As expected, HR increased in restrained mice (repeated measures one-way ANOVA, \( p < 0.0001 \), \( F = 133.5 \), \( df = 31 \) (treatment), 11 (individual) and 341 (residual)) both in WT and KO. During the first restraint session, HR was similar in WT and KO; thereafter (from sessions 2–5) the increase in HR was greater in KO than in WT mice (Figure 7). During stress session 6, the increases in HR were not significantly different between control and KO mice. During the recovery period (120 min after restraint), HR decreased. Repeated restraint led gradually to normalization of HR during the 120-min recovery period in WT mice (no difference in HR compared to unstrained control WT mice from day 5–7).

Table 2. Protein expression of adenylyl cyclase in mouse heart left ventricle (expressed as arbitrary units, relative to expression of \( \beta \)-actin).

|            | Control  | 1 x restraint | 7 x restraint |
|------------|----------|---------------|--------------|
| WT Mean ± SEM | 0.40 ± 0.04 | 0.51 ± 0.15 | 0.49 ± 0.06 |
| KO Mean ± SEM | 0.63 ± 0.036 | 0.78 ± 0.17 | 0.59 ± 0.10 |

No differences were found between groups. WT: wild type mice. KO: \( M_2 \) MR knockout mice. Control, intact control; 1 x restraint, mice restrained for 120 min once; 7 x restraint, mice restrained for 120 min seven times.

Table 3. Protein expression of protein kinase A in mouse heart left ventricle (expressed as arbitrary units, relative to expression of \( \beta \)-actin).

|            | Control  | 1 x restraint | 7 x restraint |
|------------|----------|---------------|--------------|
| WT Mean ± SEM | 1.57 ± 0.29 | 1.19 ± 0.16 | 2.17 ± 0.73 |
| KO Mean ± SEM | 2.22 ± 0.29 | 1.23 ± 0.11 | 1.04 ± 0.18 |

No differences were found between groups. WT: wild type mice. KO: \( M_2 \) MR knockout mice. Control, intact control; 1 x restraint, mice restrained for 120 min once; 7 x restraint, mice restrained for 120 min seven times. No significant differences between groups, one-way ANOVA.

Discussion

In this study, we tested the hypothesis that \( M_2 \) MRs are indispensable for normal heart responses to stress. Our data show that restraint decreased \( M_2 \) MR gene expression in the left ventricle in WT mice (which obviously cannot be seen in \( M_2 \) MR KO mice) accompanied by decrease in WT surface but not total number of MRs. In \( M_2 \) MR KO mice, only a single restraint session decreased surface MRs in the left ventricle, and, repeated (seven restraint sessions) did not affect the surface receptor number. Similarly, AC activity was decreased in the left ventricle in WT mice after one and seven restraint sessions, whereas in the KO mice, there was no difference in AC activity in stressed mice. PLC activity in the left ventricle was also decreased after one restraint session in WT but not KO mice. No change was found in NOS activity. The amount of targeted enzyme protein was either not changed by stress or by \( M_2 \) MR KO (AC, PKA), or changes were selective for specific isoenzymes (PKCa,\( \beta, \gamma, \eta \) and PKC\( \epsilon \) in KO mice, and PKC\( \iota \) in WT mice). A schematic summary of signaling changes is shown in Figure 9. Thus, stress responses in signaling mechanisms in the left ventricle in \( M_2 \) KO mice are significantly altered.

We recognize that changes in the left ventricle do not directly indicate signaling changes in the SA node. However, as can be inferred from Figure 1 and Supplementary Figure 1,
the changes in gene expression are similar in the left ventricle and right atrium, but differ from those in the left atrium and right ventricle where no changes in M2 MR were identified, but M4 MR expression was increased. Direct study of the mouse right atrium for tissue signaling analysis is difficult as the amount of tissue is a limiting factor. However, the changes in HR can be interpreted as consistent with changes in left ventricle signaling; in particular, the decrease in cAMP levels with stress or after carbachol in the left ventricle in WT, but not the KO mice shown in this study, implies that this decrease could underlie stress-induced adaptation.

**Involvement of M3 MR in stress signaling**

Mice lacking M2 MR were more susceptible to stress as shown by inability to compensate HR during 120 min in repeated stress sessions (6–7 restraints). Our results imply that not only M2 MR but also M3 MR are involved in stress signaling and in maintaining allostasis. This can be deduced from the finding that we identified M2, M3 and M4 MR mRNA in the heart. Stress changed both AC activity (inhibited by M2) and PLC activity (activated by M3). In mice lacking M2 MR, the number of surface MRs was decreased after one session of restraint, thus these receptors cannot be M2 MR. In KO mice, the M2 MR preferential pathway (AC activity) was not changed during stress, while PLC activity and PKC expression (preferential M3 MR pathway) was changed. There was also switch in PKC protein expression with stress: while in WT mice, only PKCα was increased, in the KO mice PKCα, β, γ, η and PKCζ were increased. Hence, M1 MR is involved in stress signaling. This conclusion can be made from finding that signaling changes in the M2 KO mice were in the preferential M1 MR pathway.

**MR subtypes in different species**

Concerning this issue, MR subtype heterogeneity is found in many species. For example, in the human heart, all five MR subtypes have been detected by RT-PCR; M1, M3 and M4 have been found in addition to M2, as indicated by competition binding assays and immunoblotting with subtype-specific antibodies (Wang et al., 2001). In the canine heart, functional and molecular evidence indicate the co-existence of M2, M3 and M4 (Shi et al., 1999). Heterogeneity of the MR population has also been reported in other species (Gadbut et al., 1994; Gallo et al., 1993; Mckinnon & Nathanson, 1995). Detailed functional studies with subtype-prefering antagonists combined with the use of M2, M3 KO mice and double M2/3 KO mice have demonstrated that MR-induced positive inotropy in atria from M2 KO and WT mice is mediated by M3, giving functional support for the presence of native M3 in the mouse heart (Kitazawa et al., 2009).

**The effects of stress on cholinergic signaling**

It is difficult to compare our data with previous findings as data on the effect of stress on cholinergic receptor signaling are sparse, although this system opposes the sympathetic nervous system that is activated in the stress response. There are few reports on the effect of different stressors on HR and blood pressure (considered as indicating adrenergic/cholinergic balance), such as after hemorrhage (Madwed & Cohen, 1991). Some cholinergic parameters after immobilization stress (1, 5 and 15 d) have been measured, but in a study focused on arrhythmias, and changes in signaling were not measured (Pshennikova et al., 1995). However, regarding immobilization stress, a decrease in MR number was observed in previous studies (Myslivecek et al., 2004, 2008c; Laukova et al., 2014). Furthermore, the balance between adrenoceptors (AR) and MR was reported to be disrupted in M2 KO mice (Lacroix et al., 2008), hence these mice are more susceptible to stress. In that report, mice were given bolus injection or were subjected to chronic infusion of isoproterenol, which induced a greater increase in HR in M2 KO than in WT mice; chronic infusion also impaired ventricular function. These authors also showed that M2 MR may mediate an inhibitory action of matrix metalloproteinase. Likewise, MR have been shown to play a role in acute adaptation of the rat heart to ischemia/reperfusion stress (Pisarenko et al., 1999).

![Changes in HR during and after restraint](image)

Figure 7. Changes in heart rate (HR) during and after restraint. Intact, after (A) (no stress), 1 d–7 d: HR during 120 min period of restraint on first to seventh day of stress, respectively; 1 d–7 d: HR during 120-min recovery period immediately after restraint on the first to seventh day. *p < 0.05, **p < 0.01, ***p < 0.001 vs. respective intact (after) group (or between indicated groups), ns, no significant difference, ###p < 0.01, ####p < 0.001 between wild type (WT) and knockout (KO). Repeated measures one-way ANOVA with SNK post-hoc analysis (n = 8 mice per group). Data are mean ± SEM.
60 min of reperfusion decreased HR and increased M2 MR mRNA and M2 MR protein expression in the left and right ventricles (Li et al., 2010). In another study, coronary occlusion increased production of reactive oxygen species (Kong et al., 2012) via the adenosine monophosphate activated protein kinase – PKC – NADPH oxidase pathway, which was prevented by vagal stimulation. Concerning cold stress, specific alternation of rhythm in temperature
Figure 9. Schematic representation of signal-changing in left ventricle in WT and KO mice in response to restraint stress. WT: wild type mice, KO: M2 muscarinic receptor (MR) knockout mice. Solid arrows indicate change, and dashed arrows indicate signaling sequence. In WT mice, the decrease in M2 MR mRNA with stress results in decrease of surface MR, with consequent decrease in adenyl cyclase (AC) and phospholipase C (PLC) activity, which in turn specifically increases expression of protein kinase C (PKC) isoenzyme (PKCγ1 protein was increased). No change was seen in nitric oxide synthase activity (NOS; not shown). In contrast, KO mice without M2 MR, showed only changes in PKC isoenzyme protein expression with stress (PKCζ and PKCζ,β,γ,η were increased). No change was seen in NOS (not shown). In the absence of M2, two possible routes are indicated by which M1 MR can increase PKC: either discrete and time dependent change in PLC that leads to PKC change, or another, undefined pathway is involved.

(Hata et al., 2001) caused changes in blood pressure that were affected by AF-DX 116 (M2 muscarinic agonist) indicating the role of the cholinergic system in stress responses. In contrast, heat acclimation decreases HR (Horowitz, 2002) and is also connected with changes in sympathetic activity (increased activation during short-term acclimation and decreased during long-term acclimation (Horowitz, 2002)). When studying heat acclimation of rats (Shmeeda et al., 2002), changes in the phospholipid milieu were found, but this can be an effect of membrane restructuring rather than change in signaling. It is not surprising that acetylcholinesterase inhibitors (sometimes considered as chemical stressors) are able to affect HR (Allon et al., 2005). Oxidative stress in an in vitro isolated heart preparation (Sand et al., 2003), enhances the negative inotropic response to ACh. Ischemia (oxidative stress)-induced myocardial injuries are diminished (hemodynamic improvement, decreasing ventricular arrhythmias and protection of cardiomyocytes from apoptotic death) by choline (ACH precursor), and this is reversed by M1 MR-selective antagonists but not by M2 MR selective antagonist, hence indicating the role of M1 MR in cardiac function (Yang et al., 2005). Conditioned fear also increases HR and mean blood pressure in mice (Carrieve, 2006). Hypoxia is connected with bradycardia and increase in β1-AR, β2-AR and M2 MR mRNA in zebrafish larvae, and the expression of β1-AR, β2-AR and M2 MR mRNA is altered in larvae experiencing M2 MR receptor knockdown (Steele et al., 2009). In adult male rats treated with glucocorticoid neonatally, AC activity in the heart is increased, together with increased β-AR and MR binding (Adigun et al., 2010). To the best of our knowledge, MR signaling in stressed mice has not been investigated previously.

cAMP levels as key player in HR regulation

Our results also imply that changes in cAMP levels in the heart during stress affect recovery, as when there was no change in AC activity the HR remained elevated during two hours after restraint (Figure 7). This can be seen when comparing WT and the KO mice. In WT mice, cAMP in the left ventricle was decreased, while in KO mice, there was no change in cAMP levels after one or seven sessions of restraint (Figure 4). This association is in good agreement with the concept that cAMP is a key player in HR regulation (Difrancesco, 2010). However, we measured the biochemical changes in the left ventricle, while HR is regulated from the SA node, and it remains to be tested whether there are similar changes in cAMP production in the right atrium. Furthermore, stress-induced decreases in cardiac β-adrenoceptors (Myslivecek et al., 2004; Torda et al., 1985) might underlie any such decreased AC activity and cAMP levels.

In contrast to AC activity, PLC activity returned to initial values after seven sessions of restraint (Figures 4 and 5), which indicates a minor role in allostasis. Similarly to M2 KO mice, the frequency of spontaneous beating of isolated atria from M1 KO and M4 KO mice and basal in vivo HR in M1 KO mice are not different from their WT littermates (Hardouin et al., 2002; Stengel et al., 2000, 2002).

Detailed analysis of HR also showed some similarities with a previously published study on stress in rats (Ngampramuan et al., 2008), although we used a different stress protocol. In this study, we show an initial HR increase lasting 15 min that was followed by a steady state level for the next 30 min, then a decrease in HR (though still elevated) that remained stable until the end of restraint. This pattern of HR changes was only seen in the first restraint session. Only M2 KO mice showed maintained fluctuation in HR during stress sessions (Figure 8). As in rats, the recovery period began with a steep decrease in HR and then an increase followed by a decrease (first session). Other sessions differed from that pattern (Figure 8).

Central MR activation

In order to evaluate possible central effects of MR activation, we compared the stress effects on overall animal activity and temperature. As can be seen in Supplementary Figure 3, no differences in activity were seen between WT and KO mice. Thus, HR differences cannot be explained by differences in
movements. Central MR activation of movement probably does not play a role in HR differences between M2 KO and WT mice. The pattern of temperature changes (Supplementary Figure 4) is more complex. KO mice show greater increase in temperature during stress and also in the recovery period. However, there was a clear difference between the pattern of HR changes and the pattern of temperature changes in the recovery period, while HR decreased from the fourth to seventh day, temperature remained stable both in WT and KO mice. As shown previously (Bymaster et al., 2001), oxotremorine (muscarinic agonist) produces hypothermia in WT but significantly less in M2 KO (6–8 °C in WT vs. 4.4 °C in KO); this shows that M2 MR has a role in central temperature control. The M2 MR probably does not affect HR stress-induced changes directly via central thermoregulatory mechanisms (we cannot exclude an additive effect) as the temperature change patterns were similar in WT and KO mice, although with greater increases in temperature in the KO mice.

In conclusion, restraint stress decreases M2 mRNA expression and MR number, AC activity (probably a M2 signaling pathway) and PLC activities (probably a M1 signaling pathway), but has no or little effect on proteins such as PKA, PKC and AC itself. Furthermore, the applied stress conditions do not change NOS activity (probable M3 signaling pathway); NOS is unlikely to have a role in stress responses in the heart. Our studies show that for a normal heart stress response, the presence of M2 MR is essential. This indicates that not only the classical stress pathway (adrenaline-adrenoreceptors) in the heart but also MRs should be taken into account in understanding stress reactions.

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Declaration of interest
The authors declare that no competing interests exist. All authors have approved the final article.

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Supplementary material available online

Supplementary Figures 1–4.