Cardiovascular Research (2010) **86**, 432–442
doi:10.1093/cvr/cvq014

Sympathetic α_2-adrenoceptors prevent cardiac hypertrophy and fibrosis in mice at baseline but not after chronic pressure overload

Ralf Gilsbach[1], Johanna Schneider[1], Achim Lother[1], Stefanie Schickinger[2], Jost Leemhuis[1], and Lutz Hein[1]*

[1]Institute of Experimental and Clinical Pharmacology and Toxicology, University of Freiburg, Albertstrasse 25, Freiburg 79104, Germany; and [2]Institute of Pharmacology and Toxicology, University of Würzburg, Würzburg, Germany

Received 26 October 2009; revised 11 January 2010; accepted 13 January 2010; online publish-ahead-of-print 18 January 2010

Time for primary review: 19 days

Aims α_2-Adrenoceptors modulate cardiovascular function by vasoconstriction or dilatation, by central inhibition of sympathetic activity, or by feedback inhibition of norepinephrine release from sympathetic neurons. Despite detailed knowledge about subtype-specific functions of α_2-receptors, the relative contributions of sympathetic vs. non-sympathetic receptors involved in these cardiovascular effects have not been identified. The aim of this study was to define the physiological and pharmacological role of α_{2A}-adrenoceptors in adrenergic vs. non-adrenergic cells at baseline and during sympathetic stress.

Methods and results Transgenic mice expressing α_{2A}-adrenoceptors under control of the dopamine β-hydroxylase (Dbh) promoter were generated and crossed with mice carrying a constitutive deletion in the α_{2A}- and α_{2C}-adrenoceptor genes. α_{2AC}-deficient mice showed increased norepinephrine plasma levels, cardiac hypertrophy, and fibrosis at baseline. Expression of the Dbh-α_{2A} transgene in sympathetic neurons prevented these effects. In contrast, Dbh-α_{2A} receptors mediated only a minor part of the bradycardic and hypotensive effects of the α_2-agonist medetomidine. After chronic pressure overload as induced by transverse aortic constriction in mice, the Dbh-α_{2A} transgene did not reduce norepinephrine spillover, cardiac dysfunction, hypertrophy, or fibrosis. In isolated wild-type atria, α_2-agonist-induced inhibition of [^{3}H]norepinephrine release was significantly desensitized after pressure overload. In primary sympathetic neurons from Dbh-α_{2A} transgenic mice, norepinephrine and medetomidine induced endocytosis of α_{2A}-adrenoceptors into neurite processes.

Conclusion α_{2A}-Adrenoceptors expressed in adrenergic cells are essential feedback inhibitors of sympathetic norepinephrine release to prevent cardiac hypertrophy and fibrosis at baseline. However, these receptors are desensitized by chronic pressure overload which in turn may contribute to the pathogenesis of this condition.

Keywords Adrenoceptors • Norepinephrine • Transgenic mouse model • Pressure overload • Cardiac hypertrophy

1. Introduction

α_2-Adrenoceptors are members of the family of G protein-coupled receptors which mediate the biological functions of the endogenous catecholamines, epinephrine, and norepinephrine.[1] These receptors were initially identified as feedback inhibitors of neurotransmitter release in adrenergic and other neurons (for review see Starke[2]). In addition to the endogenous ligands epinephrine and norepinephrine, they may be activated by several agonist drugs, including clonidine, brimonidine, and moxonidine.[1] Three different α_2-adrenoceptor subtypes have been cloned, termed α_{2A}, α_{2B}, and α_{2C}.[1] Mouse models with targeted deletions in the α_2-adrenoceptor genes have helped to identify subtype-specific functions for each of these receptors.[3] The α_{2B}-subtype plays an important role in the control of placenta development and vascular tone,[4,5] whereas α_{2C}-receptors are considered as feedback regulators of adrenal catecholamine release.[6,7] Activation of α_{2A}-receptors induces bradycardia and hypotension,[8] sedation[9] and facilitates working memory.[10] Furthermore, the

* Corresponding author. Tel: +49 761 203 5314; fax: +49 761 203 5318, Email: lutz.hein@pharmakol.uni-freiburg.de

α_{2A}-subtype serves as the major feedback inhibitor of norepinephrine release.[11–13]

Previous studies have suggested that inhibitory α_2-feedback receptors in sympathetic neurons (α_2-autoreceptors) may be protective during the development of cardiac failure by limiting the amount of norepinephrine stimulating cardiac myocyte adrenergic receptors.[14,15] However, α_2-receptors in other cell types or organs may contribute to cardiovascular regulation.[16] Thus, it remains unclear, whether α_2-autoreceptors are essential for the cardiovascular effects of α_2-agonist drugs and whether they limit maximal sympathetic activation during the development of cardiac hypertrophy and failure.

In order to address these questions for the α_{2A}-subtype, a mouse model with selective expression of α_{2A}-adrenoceptors in adrenergic cells including post-ganglionic sympathetic neurons[17] was generated. Recently, this model was applied to distinguish whether α_2-agonists mediate their effects on the central nervous system by the classic feedback α_{2A}-autoreceptors in adrenergic neurons or by α_2-adrenoceptors expressed in non-adrenergic neurons or other cell types.[17] Surprisingly, most α_2-agonist effects including analgesia, hypothermia, sedation, and anaesthetic-sparing were mediated by α_{2A}-adrenoceptors in non-adrenergic neurons.[17] Thus, the aim of this study was to define the role of α_{2A}-autoreceptors in adrenergic neurons for cardiovascular effects of α_2-agonist drugs and to investigate their potential protective role in cardiac hypertrophy and fibrosis by limiting sympathetic norepinephrine release. Our data suggest that α_{2A}-adrenoceptors in adrenergic cells are primarily inhibiting sympathetic activity at baseline, whereas these receptors were desensitized during chronic sympathetic stimulation as induced by left ventricular (LV) pressure overload. In contrast, sympathetic α_{2A} receptors mediated only a minor part of the bradycardic and hypotensive effects of the α_2-agonist medetomidine.

2. Methods

2.1 Generation of transgenic mice

A transgenic vector consisting of the human dopamine β-hydroxylase (Dbh) promoter, the murine α_{2A}-adrenoceptor with an aminoterminal epitope tag ('flag' epitope, DYKDDDD[18]) and the SV40t intron and poly A signal was constructed to generate transgenic mice (*Figure 1A*).[17] Dbh-α_{2A} transgenic mice were crossed with congenic C57BL/6J α_{2A}- and α_{2C}-deficient mice.[19] All animal procedures were approved by the responsible animal care committee of the University of Freiburg, Germany. The study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Haemodynamic measurements and echocardiography

For LV catheterization with a 1.4 F pressure–volume catheter (Millar Instruments, Houston, TX, USA), mice were anaesthetized with isoflurane (2 vol% in O_2) and their body temperature was kept at 37°C.[20] The microtip catheter was inserted into the right carotid artery and the pressure tip was advanced into the left ventricle. Data were recorded and analysed with Chart v5.4 (AD Instruments, Castle Hill, Australia). Medetomidine was applied via an infusion pump (Harvard Instruments) connected to a polyethylene tubing (0.61 mm outer diameter) which was inserted into the left jugular vein. Echocardiography was performed using a Vivid 7 Dimension (GE Healthcare, Munich, Germany) echocardiograph equipped with a 14 MHz transducer. Fractional shortening (FS) and ejection fraction (EF) were calculated as described.[21]

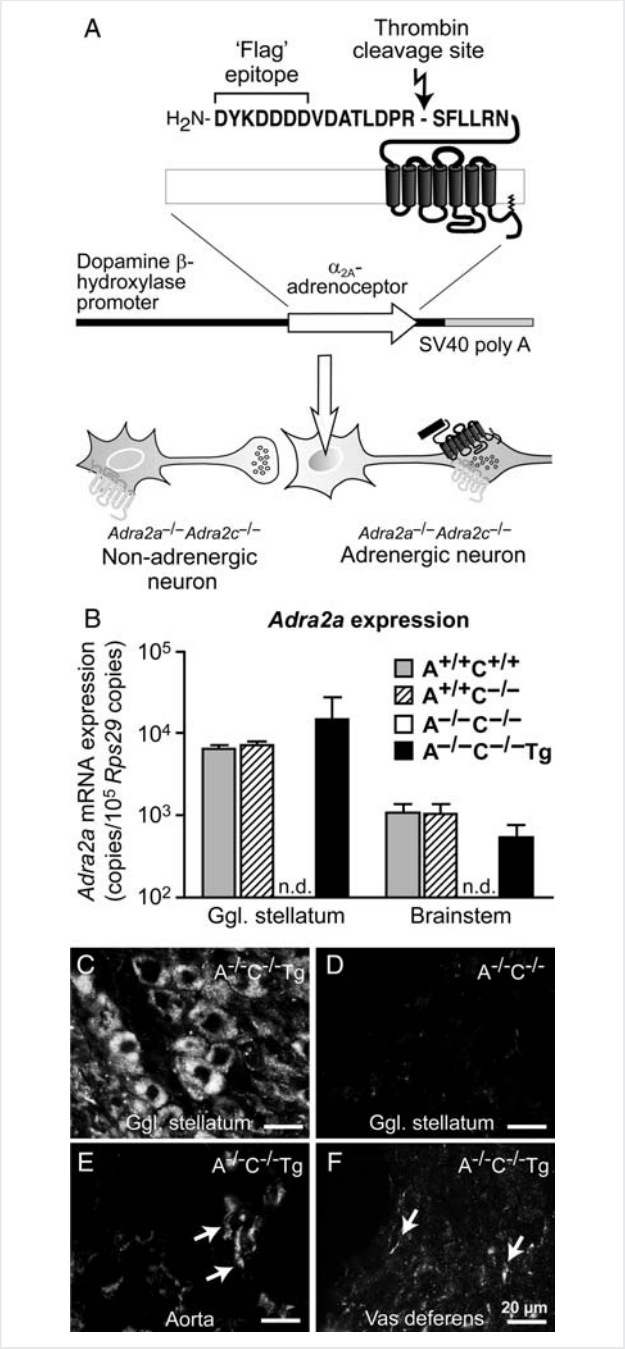

Figure 1 Transgenic model to dissect α_2-adrenoceptor functions in adrenergic vs. non-adrenergic cells. (*A*) Transgenic vector to achieve selective expression of epitope-tagged ('flag' epitope, DYKDDDD[18]) α_{2A}-receptors in adrenergic neurons. Dbh-α_{2A} transgenic mice were backcrossed with α_{2AC}-deficient (*Adra2a*$^{-/-}$ *Adra2c*$^{-/-}$) mice.[19] (*B*) Expression of α_{2A}-adrenoceptor mRNA in stellate sympathetic ganglia and brainstem was determined by quantitative real-time PCR and normalized to ribosomal S29 protein (*Rps29*) expression ($n = 5–7$ samples per genotype). (*C–F*) Detection of flag-tagged α_{2A}-adrenoceptors by immunofluorescence in cryostat sections from A$^{-/-}$C$^{-/-}$Tg stellate ganglia (*C*), aorta (*E*), or vas deferens (*F*). In stellate ganglia from non-transgenic A$^{-/-}$C$^{-/-}$ mice (*D*), no specific flag staining could be identified (bars, 20 µm). Arrows indicate sympathetic terminals in aorta (*E*) or vas deferens (*F*).

2.3 Histology

Hearts were fixed with 4% paraformaldehyde in phosphate-buffered saline, embedded in paraffin, cut into 3 μm slices, and stained with haematoxylin–eosin, Sirius-red or fluorescent wheat germ agglutinin (Alexa Fluor 488 conjugate, Invitrogen, Karlsruhe, Germany) and nuclei were counterstained with propidium iodide.[20] For immunodetection of epitope-tagged α_{2A}-adrenoceptors in stellate ganglia, aorta, and vas deferens, cryostat sections from perfusion-fixed mice (4% paraformaldehyde and 0.1% glutaraldehyde in phosphate-buffered saline) were permeabilized with 0.1% Triton X-100, blocked with 3% bovine serum albumin and 3% normal goat serum in PBS, and incubated overnight with DYDDDDK tag antibodies, followed by Cy3-coupled secondary antibodies (see below).

2.4 Gene expression analysis

Total RNA was prepared from cardiac ventricles and stellate ganglia with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and RNA quality was assessed on RNA LabChips (Agilent, Böblingen, Germany). Microarray experiments were carried out using GeneChip Mouse Genome 430A 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Results were analysed with ArrayAssist 5.0 software (Stratagene, Amsterdam, The Netherlands) using the GCRMA algorithm. Microarray data have been deposited in NCBI's Gene Expression Omnibus (accession number GSE18004).

For quantitative real-time polymerase chain reactions (qPCR), 35 μL of the amplification mixture (Qiagen, Hilden, Germany, Quantitect SYBR Green Kit) was used containing 20 ng of reverse transcribed RNA and 300 nmol/L primers (*Table 1*, Eurofins MWG Operon, Ebersberg, Germany). Reactions were run in triplicate on a MX3000P detector (Stratagene, Amsterdam, The Netherlands). The cycling conditions were: 15 s polymerase activation at 95°C and 40 cycles at 95°C for 15 s, at 58°C for 30 s, and at 72°C for 30 s.[22]

2.5 Catecholamine high-performance liquid chromatography

Using reversed phase high-performance liquid chromatography with electrochemical detection (3 μm Prontosil C18 AQ column, Bischoff, Leonberg, Germany), catecholamines were determined in plasma samples from anaesthetized mice.[22]

Table 1 Sequences of primers used for quantitative real-time PCR

Gene	Primer [5′→3′]	Product size (bp)
Adra2a	s: CAAGATCAACGACCAGAAGT as: GTCAAGGCTGATGGCGCACAG	214
Cartpt	s: CCCGAGCCCTGGACATCTA as: GCTTCGATCTGCAACATAGCG	102
Ctgf	s: TGACCCCTGCGACCCACA as: TACACCGACCCACCGAAGACACAG	117
Myh7	s: ACTGTCAACACTAAGAGGGTCA as: TTGGATGATTTGATCTTCCAGGG	114
Nppa	s: GCTTCCAGGCCATATTGGAG as: GGGGGCATGACCTCATCTT	126
Rps29	s: ATGGGTCACCAGCAGCTCTA as: AGCCTATGTCCTTCGCGTACT	152

s, Sense primer; as, antisense primer.

2.6 [^{3}H]norepinephrine release

Mouse atria were incubated in a medium containing 0.1 μmol/L [^{3}H]norepinephrine (GE Healthcare, Munich, Germany) for 45 min at 37°C.[19,22] The superfusion medium consisted of (in mmol/L): NaCl 118, KCl 4.8, $CaCl_2$ 0.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, Na_2EDTA 0.03, $CaCl_2$ 2.5, and desipramine 0.001 saturated with 5% CO_2 in O_2. Six periods of electrical stimulation (20 pulses/50 Hz, 1 ms pulse width, 80 mA) were applied at 16 min intervals. Atria were solubilized and tritium was determined in superfusate samples and atria.[19,22]

2.7 Isolation of neurons from sympathetic ganglia

Superior cervical and stellate ganglia from mice were dissociated by treatment with trypsin, collagenase, and DNase in DMEM (Invitrogen, Karlsruhe, Germany). Cells were plated on poly-D-lysine coated coverslips in DMEM media with 0.5 mmol/L glutamine, 10% foetal calf serum, and 1% penicillin/streptomycin. After agonist stimulation (10 μmol/L norepinephrine or 0.1 μmol/L medetomidine for 30 min at 37°C), neurons were fixed with 4% paraformaldehyde, permeabilized with Triton X-100 (0.1%), and incubated overnight with antibodies to detect epitope-tagged α_{2A}-adrenoceptors (DYDDDDK tag antibody, Cell Signaling, New England Biolabs, Frankfurt/Main, Germany) or tyrosine hydroxylase (Sigma-Aldrich, Munich, Germany) followed by secondary antibodies labelled with Cy3, Alexa Fluor 488 or Fluor 568.[17] To detect internalized α_{2A}-adrenoceptors, the epitope tag was removed from cell surface receptors by brief thrombin treatment (6.5 U/mL thrombin, 10 min, 15°C).[18,23] For quantification of immunostaining, cells were visualized with a Zeiss Axiovert 200 microscope system and images were processed using Metamorph (Molecular Devices, Downingtown, PA, USA) 7.1 software.

2.8 Statistical analysis

Data are presented as means ± standard error of the mean of individual data points. Data were analysed using one-way or two-way ANOVA followed by Bonferroni *post hoc* tests. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Transgenic model with the expression of α_{2A}-adrenoceptors in adrenergic cells

A mouse model with the expression of epitope-tagged α_{2A}-adrenoceptors in neurons which synthesize (nor)epinephrine was generated as described recently[17] (*Figure 1A*). These transgenic mice that expressed α_{2A}-adrenoceptors under control of the dopamine β-hydroxylase promoter (Dbh-α_{2A}) were crossed with mice lacking α_{2A}- and α_{2C}-adrenoceptors[19] (*Figure 1A*). Mice with an additional deletion of the α_{2B}-adrenoceptor gene could not be generated due to lethality of the triple knockout mice on a C57BL/6J background.[5,24] Thus for the present study, mice with the expression of α_{2A}-adrenoceptors in adrenergic neurons ($A^{-/-}C^{-/-}$Tg) were compared with wild-type mice ($A^{+/+}C^{+/+}$), mice lacking α_{2C}-adrenoceptors ($A^{+/+}C^{-/-}$), or mice deficient in both α_{2A}- and α_{2C}-adrenoceptors ($A^{-/-}C^{-/-}$) (*Figure 1*).

Previous experiments have indicated that the Dbh-α_{2A} transgene was expressed in a tissue-specific and subcellular pattern that resembled the localization of endogenous α_{2A}-autoreceptors.[17] In stellate ganglia and in the brain stem, transgenic α_{2A}-receptor mRNA was detected at levels which were similar to the expression of the endogenous α_{2A}-subtype (*Figure 1B*). No Dbh-α_{2A} mRNA was found in tissue samples from heart, blood vessels, or kidney

(data not shown). Flag-tagged α_{2A}-adrenoceptors were identified in sympathetic neurons of stellate ganglia from $A^{-/-}C^{-/-}$Tg but not from $A^{-/-}C^{-/-}$ mice (*Figure 1C* and *D*). These receptors could also be detected in sympathetic nerves innervating peripheral tissues, including aorta and vas deferens (*Figure 1E* and *F*).

3.2 Pharmacological effects of the α_2-agonist medetomidine

Medetomidine dose dependently reduced heart rate, systolic and diastolic blood pressure as well as LV FS and EF in wild-type and in $A^{+/+}C^{-/-}$ mice (*Figure 2*). The bradycardic effect of medetomidine in $A^{-/-}C^{-/-}$ mice was reduced to 16 $\pm$ 4% of the respective wild-type control (*Figure 2C* and *E*). At low doses (25–50 μg/kg), medetomidine did not affect systolic blood pressure, LV FS or EF in $A^{-/-}C^{-/-}$ mice (*Figure 2D–H*). Surprisingly, all of these agonist effects were only partially rescued by the expression of the Dbh-α_{2A} transgene (*Figure 2C–H*). In order to test whether bradycardia and hypotension were mediated by sympathetic inhibition or by withdrawal of parasympathetic tone, atropine was applied to inhibit muscarinic cholinergic receptors (*Figure 2E* and *F*). In the presence of atropine, maximal medetomidine-induced bradycardia and hypotension in $A^{+/+}C^{+/+}$ mice were significantly reduced to the levels observed in $A^{-/-}C^{-/-}$Tg mice (*Figure 2E* and *F*). Similarly, the inhibitory effect of medetomidine on LV function was only partially rescued by the Dbh-α_{2A}-transgene. Medetomidine (125 μg/kg) lowered LV EF by 21.2 $\pm$ 0.8% in wild-type mice but only by 8.0 $\pm$ 0.4% in $A^{-/-}C^{-/-}$Tg mice (*Figure 2H*). Taken together, these results indicate that α_{2A}-adrenoceptors in adrenergic neurons contribute only in part to the acute cardiovascular effects of α_2-agonists, like medetomidine.

3.3 Physiological significance of α_{2A}-adrenoceptors in adrenergic cells

The role of α_{2A}-adrenoceptors expressed in adrenergic cells was assessed in non-operated control mice and after chronic cardiac pressure overload as induced by transverse aortic constriction (TAC). After TAC, perioperative or 8-week mortality did not differ significantly between genotypes (data not shown). Moreover, the degree of aortic stenosis was similar in all mouse strains (data not shown).

At baseline, circulating norepinephrine levels were significantly elevated in $A^{-/-}C^{-/-}$ mice when compared with $A^{+/+}C^{+/+}$ or $A^{+/+}C^{-/-}$ mice (*Figure 3A*) which is consistent with previous reports.[14,19] Transgenic expression of α_{2A}-receptors completely normalized plasma norepinephrine levels in $A^{-/-}C^{-/-}$Tg mice (*Figure 3A*). After TAC, plasma norepinephrine levels were significantly higher when compared with baseline values but they did not differ between genotypes (*Figure 3A*). In control mice plasma epinephrine levels were increased in mice deficient in α_{2C}-adrenoceptors as previously reported[14,19] but were not normalized in $A^{-/-}C^{-/-}$Tg mice (data not shown).

In order to assess whether increased circulating norepinephrine affected cardiovascular function, haemodynamic parameters were determined by direct aortic and LV catheterization during isoflurane anaesthesia at baseline and after TAC (*Figure 3B* and *C*; *Table 2*). At baseline, systolic blood pressure was significantly higher in $A^{-/-}C^{-/-}$ than in wild-type mice ($A^{-/-}C^{-/-}$ 113.9 $\pm$ 2.4 mmHg vs. $A^{+/+}C^{+/+}$ 90.9 $\pm$ 2.9 mmHg, $P < 0.01$). However, in $A^{-/-}C^{-/-}$Tg mice, systolic blood pressure did not differ significantly from wild-type mice, indicating that re-expression of α_{2A}-adrenoceptors in adrenergic cells was sufficient to reduce systolic blood pressure. Chronic pressure overload increased LV systolic pressure in all genotypes to similar levels (*Figure 3B*). LV end-diastolic pressure after TAC was significantly elevated in $A^{-/-}C^{-/-}$ and in $A^{-/-}C^{-/-}$Tg mice (*Figure 3C*). Importantly, transgenic α_{2A}-adrenoceptors did not affect any of the haemodynamic parameters after TAC (*Figure 3B* and *C*; *Table 2*).

At baseline, hearts from $A^{-/-}C^{-/-}$ mice showed significant cardiac hypertrophy, increased myocyte cross-sectional areas and interstitial fibrosis when compared with $A^{+/+}C^{+/+}$ mice (*Figure 4A–G*; *Table 2*). At baseline, the expression of transgenic α_{2A}-adrenoceptors in $A^{-/-}C^{-/-}$Tg mice prevented cardiac hypertrophy and LV interstitial fibrosis when compared with $A^{-/-}C^{-/-}$ mice (*Figure 4B*, *E*, *G*). Chronic pressure overload induced cardiac hypertrophy and LV interstitial fibrosis in all genotypes (*Figure 4*). Ventricular weight and myocyte cross-sectional areas increased to similar levels in $A^{-/-}C^{-/-}$ and in $A^{-/-}C^{-/-}$Tg mice (*Figure 4B* and *E*). Furthermore, the Dbh-α_{2A} transgene did not attenuate interstitial cardiac fibrosis after TAC (*Figure 4G*). Analysis of cardiac gene expression further supported the histological results. Expression of α_{2A}-adrenoceptors in $A^{-/-}C^{-/-}$Tg mice reduced elevated natriuretic peptide (*Nppa*) mRNA expression when compared with $A^{-/-}C^{-/-}$ mice at baseline (*Figure 3D*). TAC led to increased expression of *Nppa*, α-myosin heavy chain (*Myh7*), and connective tissue growth factor (*Ctgf*) in all genotypes when compared with control mice (*Figure 3D–F*). The increase in *Nppa* and *Ctgf* mRNA levels after TAC were more pronounced in $A^{-/-}C^{-/-}$ and $A^{-/-}C^{-/-}$Tg hearts when compared with wild-type mice (*Figure 3D–F*). Taken together, these results demonstrate that adrenergic cell α_{2A}-adrenoceptors did not affect elevated circulating norepinephrine levels and resulting cardiac dysfunction and remodelling induced by TAC.

3.4 Transcriptome analysis of stellate ganglia after chronic pressure overload

As transgenic α_{2A}-receptors did not affect cardiac hypertrophy or dysfunction after TAC, we hypothesized that alterations in sympathetic ganglia attenuated pre-synaptic α_2-adrenoceptor function during chronic sympathetic stress. In order to identify changes in sympathetic gene expression in response to TAC, mRNA was isolated from sympathetic stellate ganglia of control and TAC-operated mice (*Figure 5*). After TAC, 69 probe sets representing 57 genes were significantly regulated in their expression in stellate ganglia (*Figure 5A* and *B*). Interestingly, none of the adrenergic target genes including receptors, transporters, enzymes (*Figure 5C*) as well as G protein-coupled receptor kinases (GRK) or arrestins (data not shown) differed in their expression in stellate ganglia between TAC and control conditions (*Figure 5C*). However, peptide cotransmitters of the sympathetic system, including CART (cocaine amphetamine regulated transcript, *Cartpt*), somatostatin, galanin, and neuropeptide Y were significantly increased in their expression after TAC in all genotypes (*Figure 5B* and *D*).

3.5 Desensitization of sympathetic α_{2A}-adrenoceptors after chronic sympathetic stimulation

Transgenic α_{2A}-receptors did not affect the expression of adrenergic target genes in sympathetic ganglia. Thus, we reasoned that

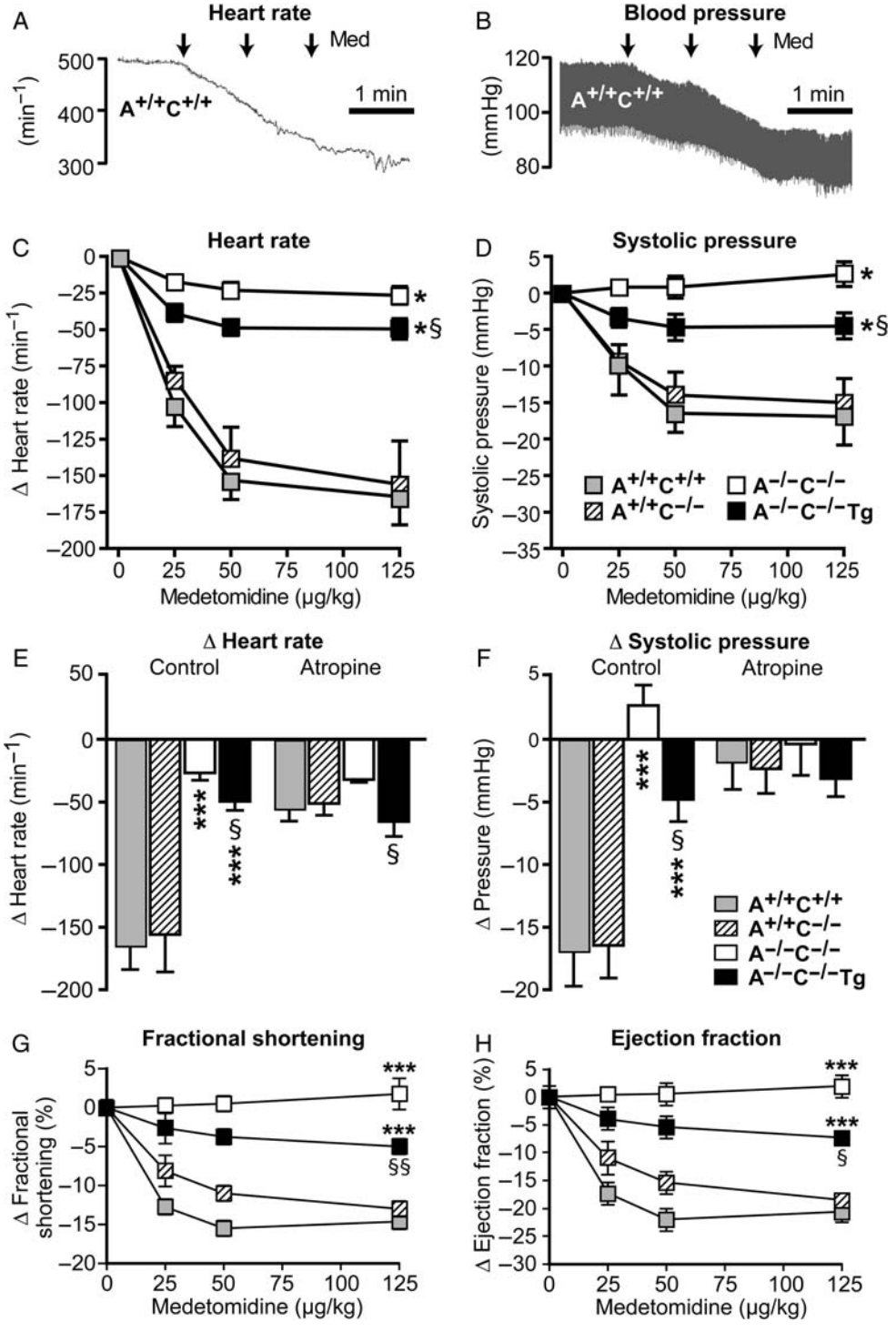

Figure 2 Haemodynamic effects of the α_2-adrenoceptor agonist medetomidine. Changes in heart rate (*A*, *C*, *E*) and systolic pressure (*B*, *D*, *F*) in response to intravenous infusion of medetomidine were determined by arterial microtip catheterization during isoflurane anaesthesia. (*A*–*D*) Original trace recordings of heart rate and arterial pressure in $A^{+/+}C^{+/+}$ mice before and after intravenous injection of medetomidine (Med) at increasing doses (arrowheads, 25, 50, 125 μg/kg). Bradycardic (*C*) and hypotensive (*D*) effects of medetomidine were significantly attenuated or absent in $A^{-/-}C^{-/-}$ or $A^{-/-}C^{-/-}$Tg mice, respectively, when compared with $A^{+/+}C^{+/+}$ or $A^{+/+}C^{-/-}$ control mice. (*E* and *F*) Maximal bradycardia (*E*) and hypotension (*F*) as observed after intravenous injection of medetomidine (125 μg/kg) in the absence ('control') or presence of atropine (1 mg/kg i.p.). (*G* and *H*) Left ventricular function was assessed by transthoracic echocardiography during isoflurane anaesthesia. Left ventricular fractional shortening (*G*) and ejection fraction (*H*) were determined after i.p. injection of medetomidine at increasing doses. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. $A^{+/+}C^{+/+}$; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ vs. $A^{-/-}C^{-/-}$; $n = 5$–6 per genotype group.

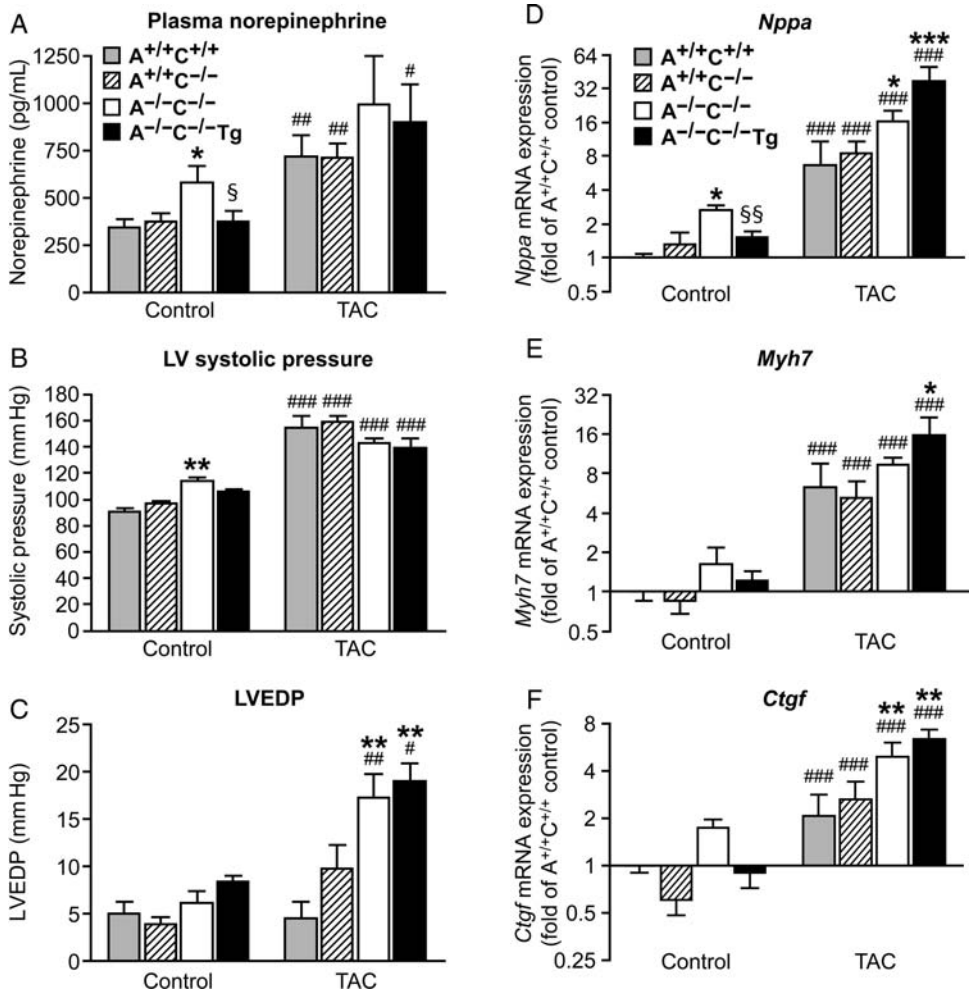

Figure 3 Plasma norepinephrine, cardiac function, and cardiac gene expression at baseline or after chronic LV pressure overload. (*A*) Plasma norepinephrine levels were determined in control mice vs. 8 weeks after transverse aortic constriction (TAC) during isoflurane anaesthesia. (*B* and *C*) LV systolic pressure (*B*) and LV end-diastolic pressure (LVEDP, *C*) were determined by microtip catheterization during isoflurane anaesthesia ($n = 5–18$ per genotype group). (*D–F*) Cardiac mRNA expression of atrial natriuretic peptide (*Nppa*), β-myosin heavy chain (*Myh7*), or connective tissue growth factor (*Ctgf*, $n = 6–10$ per genotype). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. $A^{+/+}C^{+/+}$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. control; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ vs. $A^{-/-}C^{-/-}$.

α_{2A}-adrenoceptors might be desensitized during chronic sympathetic activation. Atria from control or TAC-operated mice were incubated *in vitro* in physiological buffer containing [^{3}H]norepinephrine.[19,25] Activation of pre-synaptic α_2-adrenoceptors by an exogenous α_2-agonist inhibited electrically evoked transmitter release by 95.1 ± 3.9% in atria from control mice (*Figure 5E*). After TAC, the maximal inhibitory effect of the α_2-agonist on norepinephrine release was significantly reduced to 58.7 ± 11.0% (*Figure 5E*). Thus cardiac sympathetic α_2-adrenoceptor function was desensitized after chronic pressure overload.

3.6 Endocytosis of α_{2A}-adrenoceptors in primary sympathetic neurons

As expression of GRKs and arrestins in sympathetic ganglia was unchanged after TAC, we hypothesized that receptor endocytosis might contribute to α_2-adrenoceptor desensitization. Sympathetic neurons were isolated from superior cervical and stellate ganglia from $A^{-/-}C^{-/-}$Tg mice and were maintained *in vitro*. In unstimulated, tyrosine hydroxylase-positive neurons, flag-tagged α_{2A}-adrenoceptors were readily detected in somata and neuronal processes (*Figure 6*). In order to distinguish between cell surface and intracellular receptors, the flag epitope was cleaved off from cell surface receptors by brief thrombin treatment and the amount of intracellular epitope-tagged α_{2A}-adrenoceptors was visualized and quantified by immunostaining (*Figure 6E–K*). Unstimulated cells showed little intracellular flag-α_{2A} staining (*Figure 6E*, *F*, *K*). However, stimulation with norepinephrine or medetomidine for 30 min resulted in a significant increase in the number of flag-containing intracellular vesicles in neurites and somata (*Figure 6G*, *H*, *K*). When the flag epitope was first removed from cell surface receptors by brief thrombin cleavage followed by incubation with norepinephrine, no increase in intracellular flag staining could be detected (*Figure 6I*, *J*, *K*). Thus, agonist stimulation of epitope-tagged α_{2A}-adrenoceptors resulted in receptor endocytosis in processes of sympathetic neurons *in vitro*.

Table 2 Ventricle weight, body weight, and haemodynamic parameters

Parameter	$A^{+/+}C^{+/+}$		$A^{+/+}C^{-/-}$		$A^{-/-}C^{-/-}$		$A^{-/-}C^{-/-}Tg$	
	Control	TAC	Control	TAC	Control	TAC	Control	TAC
Ventricle weight (mg)	118.5 ± 3.1 ($n = 10$)	164.6 ± 7.4$^{\#\#\#}$ ($n = 12$)	125.7 ± 4.45 ($n = 13$)	181.6 ± 9.4$^{\#\#\#}$ ($n = 14$)	142.3 ± 4.6 ($n = 14$)	213.7 ± 7.4$^{***\#\#\#}$ ($n = 15$)	138.0 ± 3.6 ($n = 12$)	227.8 ± 15.0$^{***\#\#\#}$ ($n = 11$)
Body weight (g)	30.3 ± 1.5	28.1 ± 0.8	28.3 ± 0.8	28.8 ± 1.1	29.2 ± 0.9	28.1 ± 1.0	30.5 ± 0.8	27.4 ± 1.0
Lung weight (mg)	145.9 ± 4.2	178.5 ± 17.3	139.6 ± 13.1	171.8 ± 18.1	161 ± 4.4	262.7 ± 41.4$^{*\#\#}$	164.7 ± 6.3	297.1 ± 46.8$^{**\#\#}$
Ventricle/body weight (mg/g)	4.0 ± 0.2	5.9 ± 0.3$^{\#\#\#}$	4.4 ± 0.1	6.3 ± 0.2$^{\#\#\#}$	4.9 ± 0.1*	7.7 ± 0.3$^{***\#\#\#}$	4.5 ± 0.1	8.4 ± 0.6$^{***\#\#\#}$
Heart rate (min^{-1})	516.5 ± 26.9	551.1 ± 20.6	509.4 ± 13.9	547.0 ± 16.9	522.3 ± 7.8	558.3 ± 10.8	531.2 ± 9.6	538.2 ± 11
LV systolic pressure (mmHg)	90.9 ± 2.2	154.8 ± 9.0$^{\#\#\#}$	96.5 ± 1.8	158.8 ± 5.0$^{\#\#\#}$	113.9 ± 2.4**	142.8 ± 3.8$^{\#\#\#}$	106.2 ± 1.1	139.3 ± 7.1$^{\#\#}$
Aortic diastolic pressure (mmHg)	66.6 ± 0.6	69.4 ± 5.2	65.3 ± 1.2	75.7 ± 3.1	75.5 ± 3.2	74.7 ± 2.4	71.9 ± 3.8	75.0 ± 2.2
LV end-diastolic pressure (mmHg)	4.9 ± 1.3	4.5 ± 1.7	3.8 ± 0.8	9.7 ± 2.5	6.1 ± 1.2	17.2 ± 2.5$^{**\#\#}$	8.3 ± 0.6	19.0 ± 1.8$^{**\#}$
dp/dt_{max} (mmHg/s)	6646 ± 512	5626 ± 610	6280 ± 567	6380 ± 250	6493 ± 378	5644 ± 235	7152 ± 265	5125 ± 311$^{\#\#}$
dp/dt_{min} (mmHg/s)	−8508 ± 808	−6204 ± 473$^{\#}$	−6747 ± 513*	−6840 ± 354	−6294 ± 529**	−5749 ± 210	−7555 ± 595	−5374 ± 328$^{\#}$

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. $A^{+/+}C^{+/+}$.
$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. control.

4. Discussion

The present study describes the physiological and pharmacological role of α_{2A}-adrenoceptors in adrenergic vs. non-adrenergic cells at baseline and during sympathetic stress in a transgenic mouse model. At baseline, adrenergic cell α_{2A}-adrenoceptors were essential as pre-synaptic feedback receptors to inhibit the release of norepinephrine and thus to prevent hypertension, cardiac hypertrophy, and fibrosis. After chronic sympathetic activation as induced by LV pressure overload, these receptors were desensitized. In control mice, the pharmacological effects of α_2-agonists were mostly mediated by α_{2A}-receptors in non-adrenergic cells, as transgenic expression of α_{2A}-adrenoceptors in sympathetic neurons of α_{2AC}-deficient mice only partially restored the bradycardic or hypotensive effect of the α_2-agonist.

4.1 Transgenic model with the expression of α_{2A}-adrenoceptors in adrenergic cells

Data presented in this study and results from a recent report[17] indicate that the Dbh promoter was sufficient to drive the expression of flag-tagged α_{2A}-receptors in adrenergic neurons in the central nervous system as well as in the periphery, including sympathetic neurons in superior cervical ganglia[17] and stellate ganglia. Dbh-α_{2A}-adrenoceptors were detected at the mRNA and protein levels, they were targeted to the plasmamembrane and they inhibited neuronal voltage-gated Ca^{2+} channels and electrically evoked [^{3}H]norepinephrine release from isolated mouse atria.[17] Thus transgenic Dbh-α_{2A}-adrenoceptors fulfilled all criteria for pre-synaptic inhibitory feedback receptors.

4.2 Physiological function of α_2-adrenoceptors as feedback inhibitors

The results of the present study indicate that the main role of adrenergic cell α_{2A}-receptors is to control norepinephrine release under control conditions. This conclusion is based on the fact that the effects of constitutive deletion of the α_{2A}-adrenoceptor gene on plasma norepinephrine levels, resting systolic blood pressure, LV hypertrophy, and fibrosis could be rescued by transgenic expression of Dbh-α_{2A}-adrenoceptors. These findings may be of clinical relevance as genetic variation of human α_{2A}-adrenoceptors may contribute to increased cardiovascular risk.[26,27] Single nucleotide polymorphisms in the human *ADRA2A* gene were associated with increased resting blood pressure in cohorts of European origin[28] and with elevated norepinephrine plasma levels in healthy African-American volunteers.[29]

4.3 Desensitization of post-ganglionic sympathetic α_{2A}-adrenoceptors after TAC

Sympathetic α_{2A}-adrenoceptors were desensitized during chronic sympathetic activation as induced by chronic LV pressure overload. Desensitization was apparent in cardiac sympathetic nerves investigated *ex vivo*. As one possible mechanism which may contribute to desensitization, norepinephrine induced endocytosis of α_{2A}-adrenoceptors in primary sympathetic neurons isolated from Dbh-α_{2A} transgenic mice. However, in addition to endocytosis,[30] other processes may contribute to desensitization, including phosphorylation of receptors by GRKs or protein kinase C,[31] arrestin

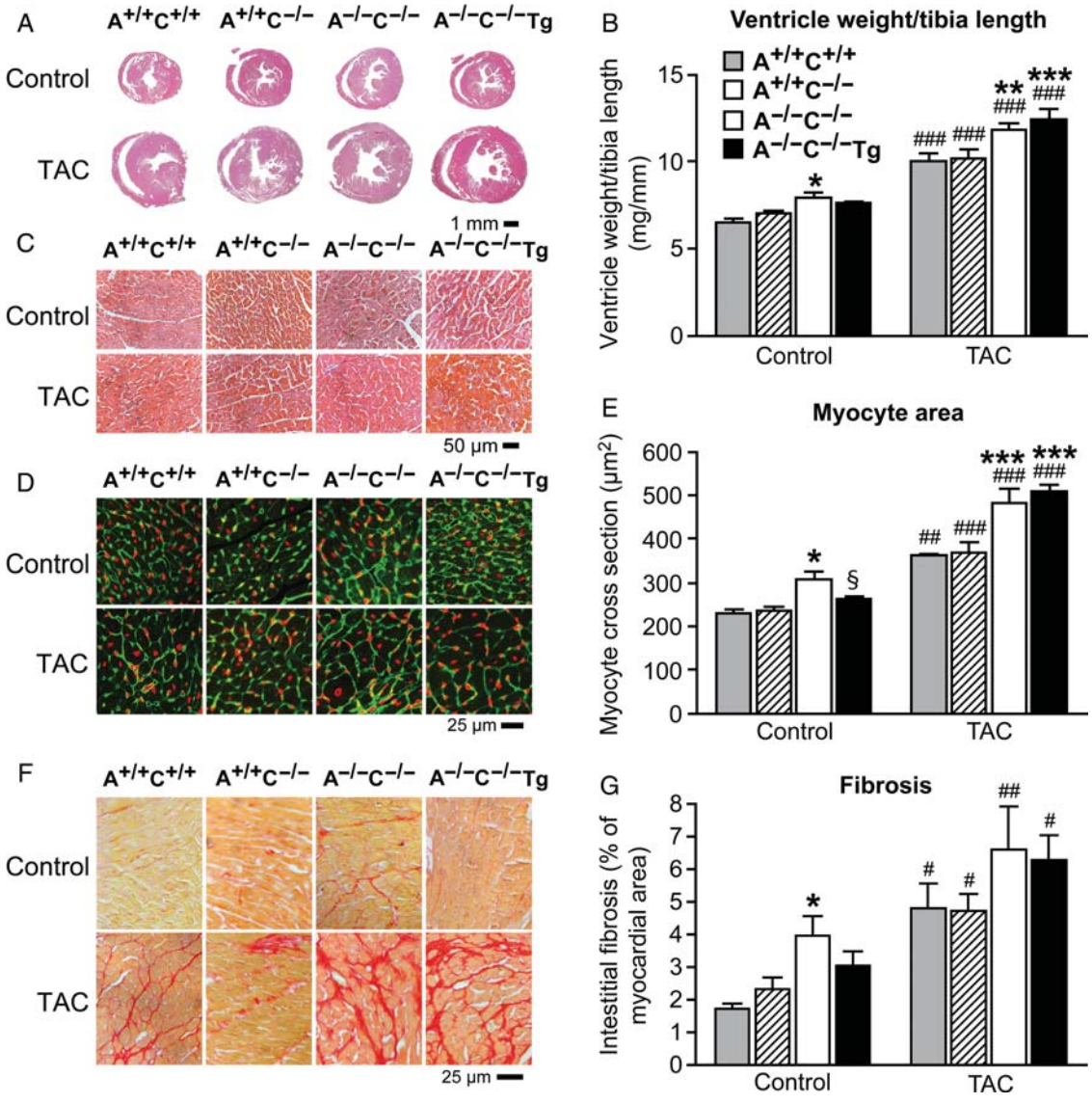

Figure 4 Cardiac hypertrophy and fibrosis after chronic LV pressure overload. (*A* and *C*) Mid-equatorial cross sections through ventricles stained with haematoxylin–eosin revealed cardiac hypertrophy after TAC. (*B*) Ventricle weight/tibia length ratios in control and TAC-operated mice ($n = 10$–14). (*D* and *E*) LV myocyte cross-sectional areas as determined by wheat germ agglutinin staining ($n = 5$–9). (*F*) Sirius red staining to detect interstitial fibrosis in mid-ventricular cardiac sections of control hearts or 8 weeks after TAC. (*G*) Morphometric analysis of Sirius red stained sections to determine LV interstitial collagen deposition ($n = 6$–8 per genotype). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. $A^{+/+}C^{+/+}$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. control; $^{\S}P < 0.05$ vs. $A^{-/-}C^{-/-}$.

binding.[32] Further studies are required to determine the precise molecular mechanism of desensitization of α_{2A}-adrenoceptors *in vivo*.

4.4 α_2-Adrenoceptor function in human hypertension and heart failure

The present study in transgenic mice may also help to interprete the findings of clinical trials applying α_2-agonists in humans. The well recognized sympathoinhibitory actions of intravenously administered clonidine have been explained in the context of two possible sites of action, including α_2-adrenoceptors in peripheral sympathetic nerves or in brain stem nuclei modulating sympathetic and parasympathetic outflow.[33] Commonly, α_2-adrenoceptors are described as feedback inhibitors in sympathetic nerves to attenuate further detrimental stimulation of cardiac myocyte β-adrenoceptors by high levels of norepinephrine in heart failure.[34] Several experimental and clinical studies have thus tested the concept of sympathetic inhibition by α_2-receptor agonists. Activation of central α_2-adrenoceptors by clonidine or moxonidine suppressed the sympathetic nervous system in congestive heart failure.[35] However, moxonidine had serious adverse effects and was even associated with increased mortality in chronic heart failure.[35]

Thus, α_2-agonist treatment during chronic heart failure may differ at least in two important aspects from inhibition of β-adrenoceptors by beta-blockers: first, in addition to lowering sympathetic tone in the central nervous system, α_2-agonists induce a strong activation of parasympathetic activity as has also been suggested in human and

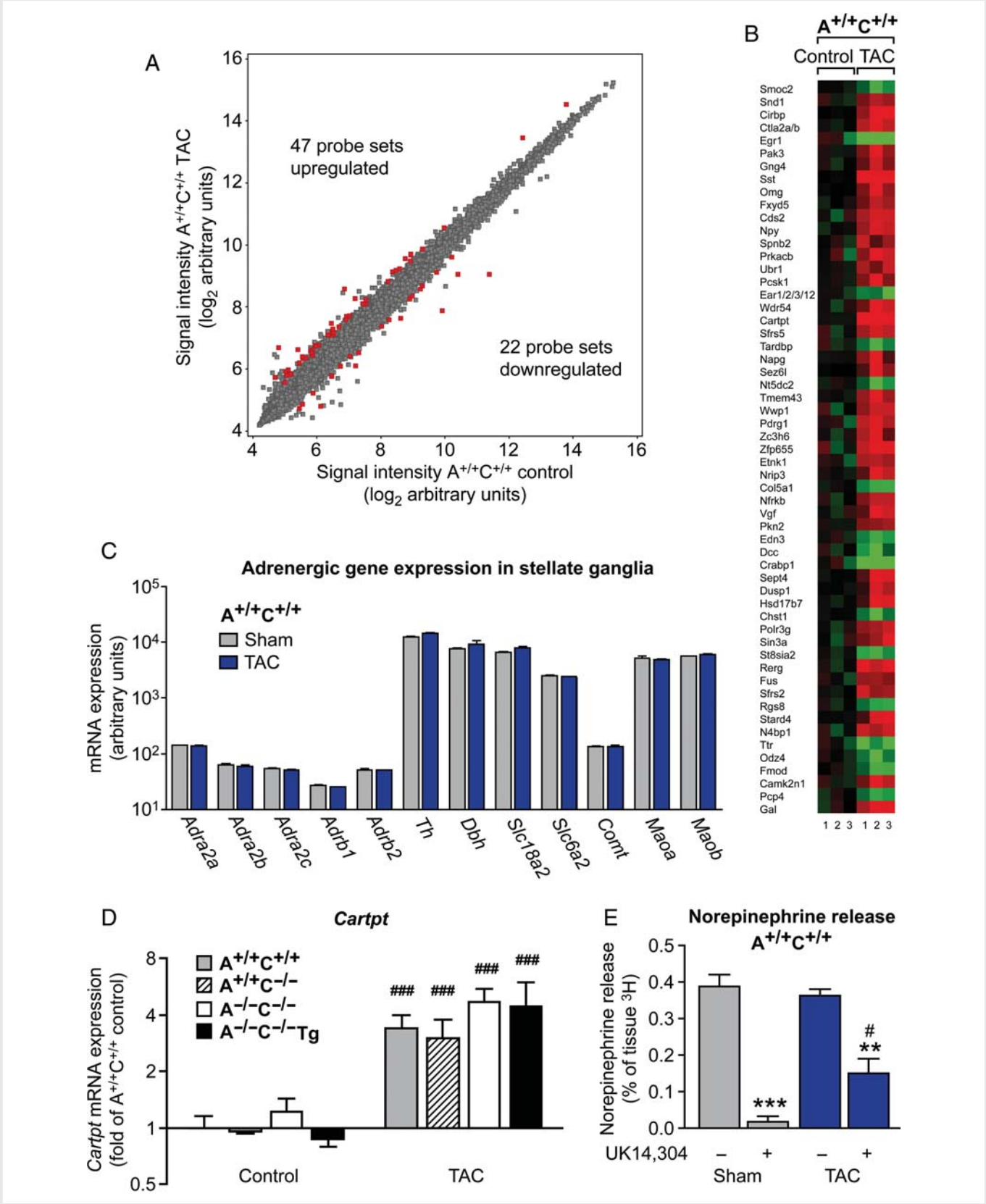

Figure 5 Microarray analysis of gene expression in stellate sympathetic ganglia after cardiac pressure overload. (*A*) Microarray analysis of total ventricular mRNA from control vs. TAC-operated $A^{+/+}C^{+/+}$ mice revealed 69 probe sets which were differentially expressed (>1.5-fold, $P < 0.05$) in sympathetic ganglia. (*B*) Genes which were differentially expressed in sympathetic ganglia after TAC (red colour depicts higher expression than mean of control; green indicates lower expression). (*C*) Expression of adrenergic receptors, enzymes, and transporters in sympathetic ganglia ($n = 3$ control, 3 TAC samples). (*D*) Expression of cocaine and amphetamine-regulated transcript (*Cartpt*) in sympathetic ganglia ($^{###}P < 0.001$ vs. control; $n = 7–10$ per genotype group). (*E*) α_2-Adrenoceptor-mediated inhibition of [^{3}H]norepinephrine release in isolated $A^{+/+}C^{+/+}$ mouse atria by the α_2-agonist UK14 304, 1 μmol/L. $^{**}P < 0.01$, $^{***}P < 0.001$ UK14 304 vs. control, $^{\#}P < 0.05$ TAC UK14 304 vs. control UK14 304; $n = 5–6$ per group.

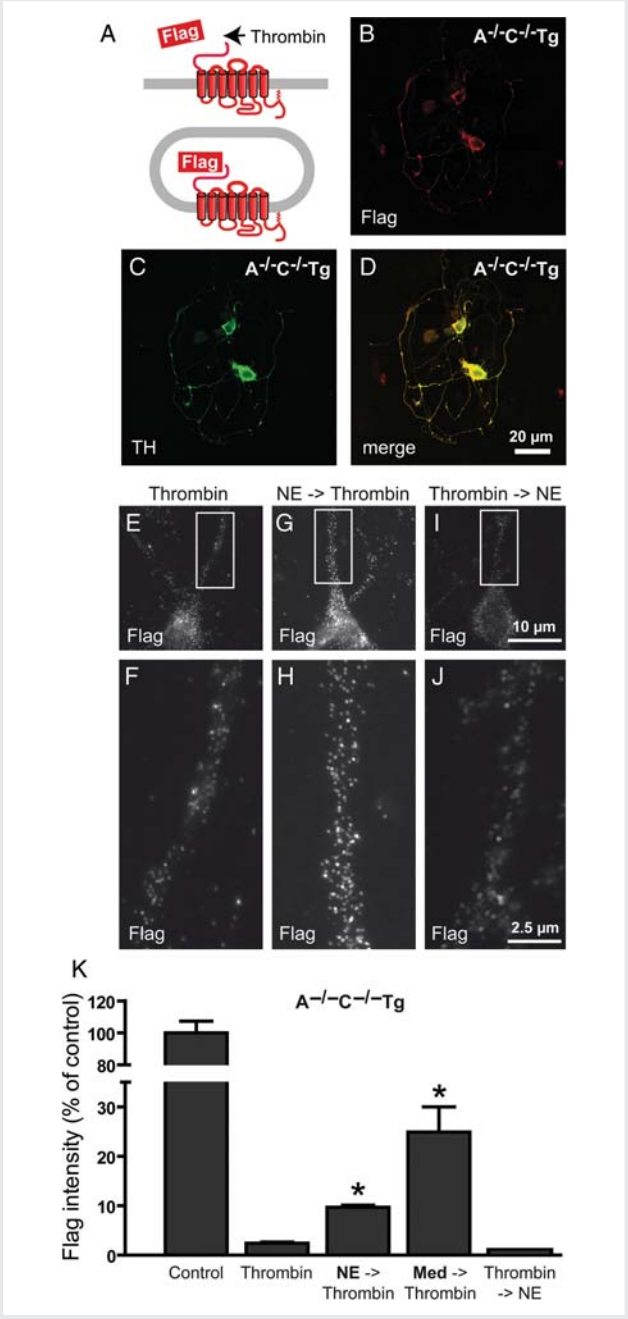

Figure 6 α_2-Adrenoceptor endocytosis in primary sympathetic neurons from $A^{-/-}C^{-/-}$Tg mice. (*A*) Thrombin cleaves off the flag epitope from cell surface α_{2A}-adrenoceptors but not from intracellular receptors. (*B–D*) Neurons were isolated from superior cervical ganglia and stellate ganglia and immunostained to detect flag-tagged α_{2A}-adrenoceptors (Flag, *B*, *D*) or tyrosine hydroxylase (TH, *C*, *D*). (*E–K*) Detection of internalized α_{2A}-adrenoceptors after thrombin cleavage of cell surface receptors. When compared with unstimulated neurons (*E* and *F*), stimulation with norepinephrine (10 µmol/L) or medetomidine (0.1 µmol/L) for 30 min resulted in a significant increase in internalized flag-tagged α_{2A}-adrenoceptors in sympathetic neurites and somata (*G*, *H*, *K*). Cleavage of cell surface receptors by thrombin followed by norepinephrine treatment did not result in increased intracellular flag staining (*I*, *J*). (*K*) Quantification of intracellular receptor staining from neurite regions >5 µm from the perikaryon. *$P < 0.05$ vs. thrombin treated; $n = 3$ experiments.

experimental studies.[36,37] Parasympathetic activation does not require the presence of α_2-adrenoceptors in adrenergic cells, but rather relies on the expression of α_2-heteroreceptors in non-adrenergic cells, presumably brain stem neurons.[33] Secondly, cardiac pre-synaptic α_2--adrenoceptors may be desensitized, thus preventing α_2-agonists from lowering sympathetic outflow directly in sympathetic terminals innervating cardiac myocytes. Data from human studies also support these findings. In patients with chronic heart failure, the effects of clonidine on norepinephrine spillover or bradycardia were significantly diminished.[38,39]

4.5 Limitations

The present study has several limitations. First, the Dbh promoter may not be restricted in its expression to neurons synthesizing norepinephrine but may also be expressed in other neurons (for further discussion, refer Gilsbach *et al.*[17]). However, incorrect expression of α_{2A}-receptors under control of the Dbh promoter may overestimate rather than underestimate the significance of α_2-autoreceptors in adrenergic neurons. In our initial study describing this transgenic model, the expression pattern, subcellular targeting, and intracellular signalling of flag-tagged adrenoceptors in neurons were characterized in detail to rule out dysfunction or incorrect expression of the transgenic receptors.[17] In addition, the present study does not allow us to distinguish whether α_2-receptor subtypes differ in their function between adrenergic and non-adrenergic cells. To this end, Dbh-α_{2A} transgenic mice were crossed with mice lacking α_{2A}- and α_{2C}-subtypes, because these receptors were previously identified as the major pre-synaptic feedback inhibitors of catecholamine release *in vivo*.[14,19] α_{2B}-Deficient mice could not be included in this study as deletion of this gene on a C57BL/6J background—which was used in the present study—was lethal during development.[5,24]

Future studies are required to determine whether sympathetic α_2-adrenoceptors also desensitize upon chronic sympathetic stimulation which is induced by other means than TAC. In addition to elevated sympathetic activity after TAC,[14] other transmitter and hormone systems can be activated by TAC and may thus affect the response to α_2-receptor stimulation. Thus, it would be important to determine the response of the transgenic model to myocardial infarction, chronic stress, or exercise.

4.6 Conclusions

The present study assigns specific functions to α_{2A}-adrenoceptors in adrenergic vs. non-adrenergic cells. In adrenergic neurons, α_{2A}-adrenoceptors primarily operate as inhibitory feedback receptors to limit basal norepinephrine release from sympathetic nerves at baseline. Dysfunction of these feedback receptors may result in typical cardiovascular consequences of increased norepinephrine levels, including increased blood pressure, cardiac hypertrophy, and fibrosis. Thus, genetic variation of α_2-adrenoceptors may contribute to the cardiovascular risk profile. However, chronic agonist activation of pre-synaptic sympathetic α_{2A}-adrenoceptors may not represent an effective strategy to inhibit sympathetic activation due to desensitization of these receptors. Further studies are now required to determine the precise molecular mechanism(s) of desensitization of sympathetic α_2-adrenoceptors during the development of cardiac hypertrophy and failure.

Acknowledgements

We thank the EMBL GeneCore (Heidelberg, Germany) staff, especially Vladimir Benes and Tomi Ivacevic, for performing the Affymetrix microarray experiments.

Conflict of interest: none declared.

Funding

This study was supported by the Deutsche Forschungsgemeinschaft (He 2073/2-2).

References

1. Bylund DB, Eikenberg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP *et al*. International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol Rev* 1994;**46**:121–136.
2. Starke K. Presynaptic autoreceptors in the third decade: focus on alpha2- adrenoceptors. *J Neurochem* 2001;**78**:685–693.
3. Gilsbach R, Hein L. Presynaptic metabotropic receptors for acetylcholine and adrenaline/noradrenaline. *Handb Exp Pharmacol* 2008;**184**:261–288.
4. Link RE, Desai K, Hein L, Stevens ME, Chruscinski A, Bernstein D *et al*. Cardiovascular regulation in mice lacking alpha2-adrenergic receptor subtypes b and c. *Science* 1996; **273**:803–805.
5. Philipp M, Brede ME, Hadamek K, Gessler M, Lohse MJ, Hein L. Placental alpha(2)-adrenoceptors control vascular development at the interface between mother and embryo. *Nat Genet* 2002;**31**:311–315.
6. Brede M, Nagy G, Philipp M, Sorensen JB, Lohse MJ, Hein L. Differential control of adrenal and sympathetic catecholamine release by alpha 2-adrenoceptor subtypes. *Mol Endocrinol* 2003;**17**:1640–1646.
7. Lymperopoulos A, Rengo G, Funakoshi H, Eckhart AD, Koch WJ. Adrenal GRK2 upregulation mediates sympathetic overdrive in heart failure. *Nat Med* 2007;**13**: 315–323.
8. MacMillan LB, Hein L, Smith MS, Piascik MT, Limbird LE. Central hypotensive effects of the alpha2a-adrenergic receptor subtype. *Science* 1996;**273**:801–803.
9. Lakhlani PP, MacMillan LB, Guo TZ, McCool BA, Lovinger DM, Maze M *et al*. Substitution of a mutant α_{2a}-adrenergic receptor via 'hit and run' gene targeting reveals the role of this subtype in sedative, analgesic, and anesthetic-sparing responses in vivo. *Proc Natl Acad Sci USA* 1997;**94**:9950–9955.
10. Wang M, Ramos BP, Paspalas CD, Shu Y, Simen A, Duque A *et al*. Alpha2A-adrenoceptors strengthen working memory networks by inhibiting cAMP-HCN channel signaling in prefrontal cortex. *Cell* 2007;**129**:397–410.
11. Altman JD, Trendelenburg AU, MacMillan L, Bernstein D, Limbird L, Starke K *et al*. Abnormal regulation of the sympathetic nervous system in alpha2A-adrenergic receptor knockout mice. *Mol Pharmacol* 1999;**56**:154–161.
12. Bucheler M, Hadamek K, Hein L. Two alpha2-adrenergic receptor subtypes, alpha2A and alpha2C, inhibit transmitter release in the brain of gene-targeted mice. *Neuroscience* 2002;**109**:819–826.
13. Olli-Lahdesmaki T, Kallio J, Scheinin M. Receptor subtype-induced targeting and subtype-specific internalization of human alpha(2)-adrenoceptors in PC12 cells. *J Neurosci* 1999;**19**:9281–9288.
14. Brede M, Wiesmann F, Jahns R, Hadamek K, Arnolt C, Neubauer S *et al*. Feedback inhibition of catecholamine release by two different alpha2-adrenoceptor subtypes prevents progression of heart failure. *Circulation* 2002;**106**:2491–2496.
15. Perrino C, Rockman HA. Reversal of cardiac remodeling by modulation of adrenergic receptors: a new frontier in heart failure. *Curr Opin Cardiol* 2007;**22**:443–449.
16. Cohn J. Sympathetic nervous system in heart failure. *Circulation* 2002;**106**:2417–2418.
17. Gilsbach R, Roser C, Beetz N, Brede M, Hadamek K, Haubold M *et al*. Genetic dissection of alpha2-adrenoceptor functions in adrenergic versus nonadrenergic cells. *Mol Pharmacol* 2009;**75**:1160–1170.
18. Daunt DA, Hurt C, Hein L, Kallio J, Feng F, Kobilka BK. Subtype-specific intracellular trafficking of alpha2-adrenergic receptors. *Mol Pharmacol* 1997;**51**:711–720.
19. Hein L, Altman JD, Kobilka BK. Two functionally distinct alpha2-adrenergic receptors regulate sympathetic neurotransmission. *Nature* 1999;**402**:181–184.
20. Beetz N, Hein L, Meszaros J, Gilsbach R, Barreto F, Meissner M *et al*. Transgenic simulation of human heart failure-like L-type Ca2+-channels: implications for fibrosis and heart rate in mice. *Cardiovasc Res* 2009;**84**:396–406.
21. Kawahara Y, Tanonaka K, Daicho T, Nawa M, Oikawa R, Nasa Y *et al*. Preferable anesthetic conditions for echocardiographic determination of murine cardiac function. *J Pharmacol Sci* 2005;**99**:95–104.
22. Gilsbach R, Brede M, Beetz N, Moura E, Muthig V, Gerstner C *et al*. Heterozygous alpha 2C-adrenoceptor-deficient mice develop heart failure after transverse aortic constriction. *Cardiovasc Res* 2007;**75**:728–737.
23. Hein L, Ishii K, Coughlin SR, Kobilka BK. Intracellular targeting and trafficking of thrombin receptors. A novel mechanism for resensitization of a G protein-coupled receptor. *J Biol Chem* 1994;**269**:27719–27726.
24. Muthig V, Gilsbach R, Haubold M, Philipp M, Ivacevic T, Gessler M *et al*. Upregulation of soluble vascular endothelial growth factor receptor 1 contributes to angiogenesis defects in the placenta of alpha 2B-adrenoceptor deficient mice. *Circ Res* 2007;**101**: 682–691.
25. Trendelenburg AU, Philipp M, Meyer A, Klebroff W, Hein L, Starke K. All three alpha2-adrenoceptor types serve as autoreceptors in postganglionic sympathetic neurons. *Naunyn Schmiedebergs Arch Pharmacol* 2003;**368**:504–512.
26. Small KM, Brown KM, Seman CA, Theiss CT, Liggett SB. Complex haplotypes derived from noncoding polymorphisms of the intronless {alpha}2A-adrenergic gene diversify receptor expression. *Proc Natl Acad Sci USA* 2006;**103**:5472–5477.
27. Small KM, Forbes SL, Brown KM, Liggett SB. An asn to lys polymorphism in the third intracellular loop of the human alpha 2A-adrenergic receptor imparts enhanced agonist-promoted Gi coupling. *J Biol Chem* 2000;**275**:38518–38523.
28. Sober S, Org E, Kepp K, Juhanson P, Eyheramendy S, Gieger C *et al*. Targeting 160 candidate genes for blood pressure regulation with a genome-wide genotyping array. *PLoS One* 2009;**4**:e6034.
29. Kurnik D, Muszkat M, Li C, Sofowora GG, Solus J, Xie HG *et al*. Variations in the alpha2A-adrenergic receptor gene and their functional effects. *Clin Pharmacol Ther* 2006;**79**:173–185.
30. Lu R, Li Y, Zhang Y, Chen Y, Shields AD, Winder DG *et al*. Epitope-tagged receptor knock-in mice reveal that differential desensitization of alpha2-adrenergic responses is because of ligand-selective internalization. *J Biol Chem* 2009;**284**:13233–13243.
31. Eason MG, Liggett SB. Subtype-selective desensitization of alpha 2-adrenergic receptors. Different mechanisms control short and long term agonist-promoted desensitization of alpha 2C10, alpha 2C4, and alpha 2C2. *J Biol Chem* 1992;**267**:25473–25479.
32. Wang Q, Limbird LE. Regulation of alpha2AR trafficking and signaling by interacting proteins. *Biochem Pharmacol* 2007;**73**:1135–1145.
33. Guyenet PG. The sympathetic control of blood pressure. *Nat Rev Neurosci* 2006;**7**: 335–346.
34. Salazar NC, Chen J, Rockman HA. Cardiac GPCRs: GPCR signaling in healthy and failing hearts. *Biochim Biophys Acta* 2007;**1768**:1006–1018.
35. Cohn JN, Pfeffer MA, Rouleau J, Sharpe N, Swedberg K, Straub M *et al*. Adverse mortality effect of central sympathetic inhibition with sustained-release moxonidine in patients with heart failure (MOXCON). *Eur J Heart Fail* 2003;**5**:659–667.
36. Tank J, Diedrich A, Szczech E, Luft FC, Jordan J. Alpha-2 adrenergic transmission and human baroreflex regulation. *Hypertension* 2004;**43**:1035–1041.
37. Tank J, Jordan J, Diedrich A, Obst M, Plehm R, Luft FC *et al*. Clonidine improves spontaneous baroreflex sensitivity in conscious mice through parasympathetic activation. *Hypertension* 2004;**43**:1042–1047.
38. Aggarwal A, Esler MD, Socratous F, Kaye DM. Evidence for functional presynaptic alpha-2 adrenoceptors and their down-regulation in human heart failure. *J Am Coll Cardiol* 2001;**37**:1246–1251.
39. Lang CC, Stein CM, Nelson RA, He HB, Belas FJ, Blair IA *et al*. Sympathoinhibitory response to clonidine is blunted in patients with heart failure. *Hypertension* 1997; **30**:392–397.