Sympathetic $\alpha_2$-adrenoceptors prevent cardiac hypertrophy and fibrosis in mice at baseline but not after chronic pressure overload

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Aims

$\alpha_2$-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 $\alpha_2$-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 $\alpha_2A$-adrenoceptors in adrenergic vs. non-adrenergic cells at baseline and during sympathetic stress.

Methods and results

Transgenic mice expressing $\alpha_2A$-adrenoceptors under control of the dopamine $\beta$-hydroxylase (Dbh) promoter were generated and crossed with mice carrying a constitutive deletion in the $\alpha_2A$- and $\alpha_2C$-adrenoceptor genes. $\alpha_2AC$-deficient mice showed increased norepinephrine plasma levels, cardiac hypertrophy, and fibrosis at baseline. Expression of the Dbh-$\alpha_2A$ transgene in sympathetic neurons prevented these effects. In contrast, Dbh-$\alpha_2A$ receptors mediated only a minor part of the bradycardic and hypotensive effects of the $\alpha_2$-agonist medetomidine. After chronic pressure overload as induced by transverse aortic constriction in mice, the Dbh-$\alpha_2A$ transgene did not reduce norepinephrine spillover, cardiac dysfunction, hypertrophy, or fibrosis. In isolated wild-type atria, $\alpha_2$-agonist-induced inhibition of $[^3H]$norepinephrine release was significantly desensitized after pressure overload. In primary sympathetic neurons from Dbh-$\alpha_2A$ transgenic mice, norepinephrine and medetomidine induced endocytosis of $\alpha_2A$-adrenoceptors into neurite processes.

Conclusion

$\alpha_2A$-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

$\alpha_2$-Adrenoceptors are members of the family of G protein-coupled receptors which mediate the biological functions of the endogenous catecholamines, epinephrine, and norepinephrine. These receptors were initially identified as feedback inhibitors of neurotransmitter release in adrenergic and other neurons (for review see Starke$^6$). 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 $\alpha_2$-adrenoceptor subtypes have been cloned, termed $\alpha_2A$, $\alpha_2B$, and $\alpha_2C$.$^1$ Mouse models with targeted deletions in the $\alpha_2$-adrenoceptor genes have helped to identify subtype-specific functions for each of these receptors.$^3$ The $\alpha_2B$-subtype plays an important role in the control of placenta development and vascular tone,$^4,5$ whereas $\alpha_2C$-receptors are considered as feedback regulators of adrenal catecholamine release.$^6,7$ Activation of $\alpha_2A$-receptors induces bradycardia and hypotension,$^8$ sedation$^9$ and facilitates working memory.$^{10}$ Furthermore, the

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α2A-subtype serves as the major feedback inhibitor of norepinephrine release.11–13

Previous studies have suggested that inhibitory α2-feedback receptors in sympathetic neurons (α2-A2-autoreceptors) may be protective during the development of cardiac failure by limiting the amount of norepinephrine stimulating cardiac myocyte adrenergic receptors.14,15 However, α2-receptors in other cell types or organs may contribute to cardiovascular regulation.16 Thus, it remains unclear, whether α2A-autoreceptors are essential for the cardiovascular effects of α2-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 α2A-subtype, a mouse model with selective expression of α2A-adrenoceptors in adrenergic cells including post-ganglionic sympathetic neurons17 was generated. Recently, this model was applied to distinguish whether α2 agonists mediate their effects on the central nervous system by the classic feedback α2A-autoreceptors in adrenergic neurons or by α2A-adrenoceptors expressed in non-adrenergic neurons or other cell types.18,19 Surprisingly, most α2-agonist effects including analgesia, hypothermia, sedation, and anesthetic-sparing were mediated by α2A-adrenoceptors in non-adrenergic neurons.20 Thus, the aim of this study was to define the role of α2A-autoreceptors in adrenergic neurons for cardiovascular effects of α2-agonist drugs and to investigate their potential protective role in cardiac hypertrophy and fibrosis by limiting sympathetic norepinephrine release. Our data suggest that α2A-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 α2A receptors mediated only a minor part of the bradycardic and hypotensive effects of the α2-agonist medetomidine.

2. Methods

2.1 Generation of transgenic mice

A transgenic vector consisting of the human dopamine β-hydroxylase (Dbh) promoter, the murine α2A-adrenoceptor with an aminoterminal epitope tag (‘flag’ epitope, DYKDDDDK)18 and the SV40T intron and poly A signal was constructed to generate transgenic mice (Figure 1A).21 Dbh-α2A transgenic mice were crossed with congenic C57BL/6J α2A- and α2C-deficient mice.22 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 O2) 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 α2-adrenoceptor functions in adrenergic vs. non-adrenergic cells. (A) Transgenic vector to achieve selective expression of epitope-tagged (‘flag’ epitope, DYKDDDDK) α2A-receptors in adrenergic neurons. Dbh-α2A transgenic mice were backcrossed with α2AC-deficient (Adra2a−/− Adra2c−/−) mice.21 Detection of flag-tagged α2A-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−/− Tg 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 α2A-adrenoceptors in stellate ganglia, aorta, and vas deferens, cryostat sections from perfusion-fixed mice (4% paraformaldehyde 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) using the GCRMA algorithm. Microarray data have been deposited in NCBI’s Gene Expression Omnibus (accession number GSE18004).

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).

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

2.6 [3H]norepinephrine release

Mouse atria were incubated in a medium containing 0.1 μmol/L [3H]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, CaCl2 0.2, MgSO4 1.2, NaHCO3 25, KH2PO4 1.2, glucose 11, ascorbic acid 0.57, Na2EDTA 0.03, CaCl2 2.5, and desipramine 0.001 saturated with 5% CO2 in O2. 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 DNsase in DMEM (Invitrogen, Karlsruhe, Germany). Cells were plated on poly-D-lysine coated coverslips in DMEM media with 0.5 mmol/L glucose, 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 α2A-adrenoceptors (DYDDD DK 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 α2A-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

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 α2A-adrenoceptors in adrenergic cells

A mouse model with the expression of epitope-tagged α2A-adrenoceptors in neurons which synthesize (nor)epinephrine was generated as described recently22 (Figure 1A). These transgenic mice that expressed α2A-adrenoceptors under control of the dopamine β-hydroxylase promoter (Dbh-α2A) were crossed with mice lacking α2A- and α2C-adrenoceptors19 (Figure 1A). Mice with an additional deletion of the α2B-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 α2A-adrenoceptors in adrenergic neurons (A−/−C−/−Tg) were compared with wild-type mice (A+/−C+/+), mice lacking α2C-adrenoceptors (A−/−C−/−), or mice deficient in both α2A- and α2C-adrenoceptors (A−/−C−/−) (Figure 1).

Previous experiments have indicated that the Dbh-α2A transgene was expressed in a tissue-specific and subcellular pattern that resembled the localization of endogenous α2A-autoreceptors.17 In stellate ganglia and in the brain stem, transgenic α2A+ receptor mRNA was detected at levels which were similar to the expression of the endogenous α2A-subtype (Figure 1B). No Dbh-α2A mRNA was found in tissue samples from heart, blood vessels, or kidney.
(data not shown). Flag-tagged α2A-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 α2A-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 ± 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-α2A 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-α2A-transgene. Medetomidine (125 μg/kg) lowered LV EF by 21.2 ± 0.8% in wild-type mice but only by 8.0 ± 0.4% in A−/−C−/−Tg mice (Figure 2H). Taken together, these results indicate that α2A-adrenoceptors in adrenergic neurons contribute only in part to the acute cardiovascular effects of α2A-agonists, like medetomidine.

3.3 Physiological significance of α2A-adrenoceptors in adrenergic cells

The role of α2A-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.α19 Transgenic expression of α2A-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 α2C-adrenoceptors as previously reportedα19 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). At baseline, systolic blood pressure was significantly higher in A−/−C−/− than in wild-type mice (A−/−C−/− 113.9 ± 2.4 mmHg vs. A+/+C+/+ 90.9 ± 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 α2A-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 α2A-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 α2A-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-α2A transgene did not attenuate interstitial cardiac fibrosis after TAC (Figure 4G). Analysis of cardiac gene expression further supported the histological results. Expression of α2A-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 α2A-adrenoceptors did not affect elevated circulating norepinephrine levels and resulting cardiac dysfunction and remodeling induced by TAC.

3.4 Transcriptome analysis of stellate ganglia after chronic pressure overload

As transgenic α2A-receptors did not affect cardiac hypertrophy or dysfunction after TAC, we hypothesized that alterations in sympathetic ganglia attenuated pre-synaptic α2-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, Carpt), 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 α2A-adrenoceptors after chronic sympathetic stimulation

Transgenic α2A-receptors did not affect the expression of adrenergic target genes in sympathetic ganglia. Thus, we reasoned that...
Figure 2 Haemodynamic effects of the α₂-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^+/+; †P < 0.05, ‡P < 0.01 vs. A^−/− C^−/−; n = 5–6 per genotype group.
a2A-adrenoceptors might be desensitized during chronic sympathetic activation. Atria from control or TAC-operated mice were incubated in vitro in physiological buffer containing [3H]norepinephrine. Activation of pre-synaptic a2-adrenoceptors by an exogenous a2-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 a2-agonist on norepinephrine release was significantly reduced to 58.7 ± 11.0% (Figure 5E). Thus cardiac sympathetic a2-adrenoceptor function was desensitized after chronic pressure overload.

3.6 Endocytosis of a2A-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 a2-adrenoceptor desensitization. Sympathetic neurons were isolated from superior cervical and stellate ganglia from A2/C-/-Tg mice and were maintained in vitro. In unstimulated, tyrosine hydroxylase-positive neurons, flag-tagged a2A-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 a2A-adrenoceptors was visualized and quantified by immunostaining (Figure 6E–K). Unstimulated cells showed little intracellular flag-a2A 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 treatment followed by incubation with norepinephrine, no increase in intracellular flag staining could be detected (Figure 6I, J, K). Thus, agonist stimulation of epitope-tagged a2A-adrenoceptors resulted in receptor endocytosis in processes of sympathetic neurons in vitro.

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), b-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. A+/−C+/+. §p < 0.05, §§p < 0.01 vs. A−/−C−/−.
4. Discussion

The present study describes the physiological and pharmacological role of α2A-adrenoceptors in adrenergic vs. non-adrenergic cells at baseline and during sympathetic stress in a transgenic mouse model. At baseline, adrenergic cell α2A-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 α2-agonists were mostly mediated by α2A-receptors in non-adrenergic cells, as transgenic expression of α2A-adrenoceptors in sympathetic neurons of α2AC-deficient mice only partially restored the bradycardic or hypotensive effect of the α2-agonist.

4.1 Transgenic model with the expression of α2A-adrenoceptors in adrenergic cells

Data presented in this study and results from a recent report indicate that the Dbh promoter was sufficient to drive the expression of flag-tagged α2A-receptors in adrenergic neurons in the central nervous system as well as in the periphery, including sympathetic neurons in superior cervical ganglia and stellate ganglia. Dbh-α2A-adrenoceptors were detected at the mRNA and protein levels, they were targeted to the plasmamembrane and they inhibited neuronal voltage-gated Ca2+ channels and electrically evoked [3H]norepinephrine release from isolated mouse atria. Thus transgenic Dbh-α2A-adrenoceptors fulfilled all criteria for pre-synaptic inhibitory feedback receptors.

4.2 Physiological function of α2-adrenoceptors as feedback inhibitors

The results of the present study indicate that the main role of adrenergic cell α2A-receptors is to control norepinephrine release under control conditions. This conclusion is based on the fact that the effects of constitutive deletion of the α2A-adrenoceptor gene on plasma norepinephrine levels, resting systolic blood pressure, LV hypertrophy, and fibrosis could be rescued by transgenic expression of Dbh-α2A-adrenoceptors. These findings may be of clinical relevance as genetic variation of human α2A-adrenoceptors may contribute to increased cardiovascular risk. Single nucleotide polymorphisms in the human ADRA2A gene were associated with increased resting blood pressure in cohorts of European origin and with elevated norepinephrine plasma levels in healthy African-American volunteers.

4.3 Desensitization of post-ganglionic sympathetic α2A-adrenoceptors after TAC

Sympathetic α2A-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 α2A-adrenoceptors in primary sympathetic neurons isolated from Dbh-α2A transgenic mice. However, in addition to endocytosis, other processes may contribute to desensitization, including phosphorylation of receptors by GRKs or protein kinase C, arrestin...
Further studies are required to determine the precise molecular mechanism of desensitization of $\alpha_2$-adrenoceptors in vivo.

4.4 $\alpha_2$-Adrenoceptor function in human hypertension and heart failure

The present study in transgenic mice may also help to interpret the findings of clinical trials applying $\alpha_2$-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 $\alpha_2$-adrenoceptors in peripheral sympathetic nerves or in brain stem nuclei modulating sympathetic and parasympathetic outflow. Commonly, $\alpha_2$-adrenoceptors are described as feedback inhibitors in sympathetic nerves to attenuate further detrimental stimulation of cardiac myocyte $\beta$-adrenoceptors by high levels of norepinephrine in heart failure. Several experimental and clinical studies have thus tested the concept of sympathetic inhibition by $\alpha_2$-receptor agonists. Activation of central $\alpha_2$-adrenoceptors by clonidine or moxonidine suppressed the sympathetic nervous system in congestive heart failure. However, moxonidine had serious adverse effects and was even associated with increased mortality in chronic heart failure.

Thus, $\alpha_2$-agonist treatment during chronic heart failure may differ at least in two important aspects from inhibition of $\beta$-adrenoceptors by beta-blockers: first, in addition to lowering sympathetic tone in the central nervous system, $\alpha_2$-agonists induce a strong activation of parasympathetic activity as has also been suggested in human and

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**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; §$p < 0.05$ vs. A$^{-/-}$C$^{-/-}$.
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) $\alpha_2$-Adrenoceptor-mediated inhibition of $[^3H]$norepinephrine release in isolated A^{+/+}C^{+/+} mouse atria by the $\alpha_2$-agonist UK14 304, 1 $\mu$mol/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.
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4.6 Conclusions

The present study assigns specific functions to α2A-adrenoceptors in adrenergic vs. non-adrenergic cells. In adrenergic neurons, α2A-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 α2A-adrenoceptors may contribute to the cardiovascular risk profile. However, chronic agonist activation of presynaptic sympathetic α2A-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 α2A-adrenoceptors during the development of cardiac hypertrophy and failure.

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 Gilbsbach et al.17). However, incorrect expression of α2A-receptors under control of the Dbh promoter may overestimate rather than underestimate the significance of α2-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 α2-receptor subtypes differ in their function between adrenergic and non-adrenergic cells. To this end, Dbh-α2A transgenic mice were crossed with mice lacking α2A- and α2C-subtypes, because these receptors were previously identified as the major pre-synaptic feedback inhibitors of catecholamine release in vivo.16,19 α2B-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 α2A-adrenoceptors also desensitize upon chronic sympathetic stimulation which is induced by other means than TAC. In addition to elevated sympathetic activity after TAC, other transmitter and hormone systems can be activated by TAC and may thus affect the response to α2-receptor stimulation. Thus, it would be important to determine the response of the transgenic model to myocardial infarction, chronic stress, or exercise.
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Conflict of interest: none declared.

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