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Genetic Dissection of α_2-Adrenoceptor Functions in Adrenergic versus Nonadrenergic Cells

Ralf Gilsbach, Christoph Röser, Nadine Beetz, Marc Brede, Kerstin Hadamek, Miriam Haubold, Jost Leemhuis, Melanie Philipp, Johanna Schneider, Michal Urbanski, Bela Szabo, David Weinshenker, and Lutz Hein

Institute of Experimental and Clinical Pharmacology and Toxicology (R.G., N.B., M.H., J.L., J.S., M.U., B.S., L.H.) and Centre for Biological Signaling Studies (N.B., L.H.), University of Freiburg, Freiburg, Germany; Institute of Pharmacology and Toxicology, University of Würzburg, Würzburg, Germany (C.R., M.B., K.H.); Institute of Cell Biology, Duke University, Durham, North Carolina (M.P.); and Department of Human Genetics, Emory University, Atlanta, Georgia (D.W.)

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ABSTRACT

α_2-Adrenoceptors mediate diverse functions of the sympathetic system and are targets for the treatment of cardiovascular disease, depression, pain, glaucoma, and sympathetic activation during opioid withdrawal. To determine whether α_2-adrenoceptors on adrenergic neurons or α_2-adrenoceptors on nonadrenergic neurons mediate the physiological and pharmacological responses of α_2-agonists, we used the dopamine β-hydroxylase (*Dbh*) promoter to drive expression of α_{2A}-adrenoceptors exclusively in noradrenergic and adrenergic cells of transgenic mice. *Dbh*-α_{2A} transgenic mice were crossed with double knockout mice lacking both α_{2A}- and α_{2C}-receptors to generate lines with selective expression of α_{2A}-autoreceptors in adrenergic cells. These mice were subjected to a comprehensive phenotype analysis and compared with wild-type mice, which express α_{2A}- and α_{2C}-receptors in both adrenergic and nonadrenergic cells, and α_{2A}/α_{2C} double-knockout mice, which do not express these receptors in any cell type. We were surprised to find that only a few functions previously ascribed to α_2-adrenoceptors were mediated by receptors on adrenergic neurons, including feedback inhibition of norepinephrine release from sympathetic nerves and spontaneous locomotor activity. Other agonist effects, including analgesia, hypothermia, sedation, and anesthetic-sparing, were mediated by α_2-receptors in nonadrenergic cells. In dopamine β-hydroxylase knockout mice lacking norepinephrine, the α_2-agonist medetomidine still induced a loss of the righting reflex, confirming that the sedative effect of α_2-adrenoceptor stimulation is not mediated via autoreceptor-mediated inhibition of norepinephrine release. The present study paves the way for a revision of the current view of the α_2-adrenergic receptors, and it provides important new considerations for future drug development.

Adrenergic receptors are important targets for the treatment of human diseases and conditions including hypertension and heart failure, psychiatric and neurological diseases, asthma, and pain (Westfall and Westfall, 2006). To date, nine different adrenergic receptor subtypes have been cloned and grouped into three receptor groups, including $\alpha_{1A,B,D}$, $\alpha_{2A,B,C}$, and $\beta_{1,2,3}$ (Bylund et al., 1994). However, the therapeutic potential of these subtypes has not been fully explored because of the lack of ligands with sufficient subtype-selectivity. At present, only four of the nine possible subtype distinctions (i.e., α_1, α_2, β_1, and β_2) have achieved clinical relevance (Westfall and Westfall, 2006). Especially within the α_1- and α_2-receptor subgroups, the physiological significance of individual receptor subtypes has remained unclear until recently. For the α_2-adrenoceptors, mouse models with targeted deletions of the individual subtypes have greatly advanced our understanding of the physiological role and the therapeutic potential of these receptors (Gilsbach and Hein, 2008). Activation of α_{2A}-receptors could be linked with bradycardia and hypotension (MacMillan et al., 1996), sedation (Lakhlani et al., 1997), and consolidation of working memory (Wang et al., 2007). In contrast, α_{2B}-receptors counteracted

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ABBREVIATIONS: A(+/+), wild-type murine α_{2A}-adrenoceptor gene; A(−/−), null allele of the murine α_{2A}-adrenoceptor gene; *Adra2a*, *Adra2b*, *Adra2c*, murine genes encoding α_{2A}-, α_{2B}-, or α_{2C}-adrenoceptors; Dbh, dopamine β-hydroxylase; SCG, superior cervical ganglia; Tg, transgenic line; CNS, central nervous system; LORR, loss of righting reflex; TH, tyrosine hydroxylase; PCR, polymerase chain reaction; qPCR, qualitative polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; DSP-4, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride; RX821002, 2-(2,3-dihydro-2-methoxy-1,4-benzodioxin-2-yl)-4,5-dihydro-1H-imidazole hydrochloride; UK14,304, brimonidine.

the hypotensive effect of α_{2A}-receptors (Link et al., 1996) and were essential for placenta vascular development (Philipp et al., 2002). α_{2C}-Receptors were identified as feedback regulators of adrenal catecholamine release (Brede et al., 2003), an essential pathway to limit the progression of cardiac hypertrophy and failure in experimental models (Lymperopoulos et al., 2007) and in humans with congestive heart failure (Small et al., 2002).

α_2-Receptors were initially identified as presynaptic receptors inhibiting the release of neurotransmitters in isolated tissues in vitro (Starke et al., 1975). The term "autoreceptors" has been introduced for those receptors that are "sensitive to the neuron's own transmitter." In contrast to autoreceptors, heteroreceptors are modulated by neurotransmitters derived from neighboring neurons (Bylund et al., 1994). Despite considerable progress in adrenergic biology, it is unknown whether the wide array of clinical actions of α_2-agonists is indeed mediated by the classic presynaptic α_2-autoreceptors or whether and to what degree α_2-adrenoceptors on nonadrenergic cells are involved. To address this question, we crossed transgenic mice with expression of α_{2A}-receptors under control of the dopamine β-hydroxylase promoter to mice with constitutive deletion of the α_{2A}- and α_{2C}-adrenoceptor genes, thus generating mice that exclusively express α_{2A}-receptors in adrenergic cells. These animals were subjected to a comprehensive phenotyping analysis to provide a comprehensive overview of adrenergic cell versus nonadrenergic cell functions for α_2-adrenoceptors. We were surprised to find that very few α_2-receptor functions were mediated by α_2-adrenoceptors in adrenergic cells; most effects of α_2-agonists were mediated by α_2-receptors on nonadrenergic neurons or cells. These results underline the importance of nonadrenergic cell α_2-adrenoceptors.

Materials and Methods

Generation of Transgenic Mice. A transgenic vector consisting of a 5.6-kilobase part of the human dopamine β-hydroxylase promoter as described previously (promoter plasmid kindly provided by Dr. R. Palmiter) (Mercer et al., 1991; Hoyle et al., 1994), the coding sequence of the murine α_{2A}-adrenoceptor with an aminoterminal epitope tag (flag epitope, DYKDDDD; Daunt et al., 1997; Hein et al., 1999), and the SV40 t intron and poly(A) signal was constructed as depicted in Fig. 1 a. The vector was linearized, separated from plasmid sequences, and microinjected into fertilized oocytes from superovulated FVB/N mice. Several independent transgenic *Dbh-Adra2a-Tg* founder lines were obtained, two of which, numbered A11 and A25, were investigated in detail. These transgenic mice were crossed with congenic C57BL6/J α_{2A}- and α_{2C}-deficient mice (Hein et al., 1999). Genotypes were confirmed by polymerase chain reactions (Fig. 1b) performed with genomic DNA isolated from tail biopsies (Hein et al., 1999). For detection of the *Dbh-Adra2a-Tg*, the following primers were used: forward primer, 5′-ATGTCGACGCCACCTTAGAT-3′; and reverse primer, 5′-AGGCAAACCAGCGTCAGTGT-3′. Dopamine β-hydroxylase knockout [Dbh(−/−)] mice, maintained on a mixed C57BL/SJ and 129SvEv background, were generated as described previously (Thomas et al., 1995; Szot et al., 2004). Littermate Dbh(+/−) mice, which have normal catecholamine levels and behavior, were used as controls (Thomas et al., 1998; Bourdélat-Parks et al., 2005). For experiments, age-matched adult (3–5 months) male litter mates that were maintained in specified pathogen-free facilities were used. All animal procedures were approved by the responsible animal care committees of the Universities of Freiburg and Würzburg, Germany. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

Quantitative Real-Time PCR. mRNA quantification by real-time polymerase chain reaction (qPCR) from murine tissues was performed as described previously (Gilsbach et al., 2007). For qPCR, 35 μl of the amplification mixture (Quantitect SYBR Green Kit; QIAGEN, Valencia, CA) was used containing 20 ng of reverse-transcribed RNA and 300 nM primers (MWG, Ebersberg, Germany) specific for α_{2A}-adrenoceptor (5′-CAAGATCAACGACCAGAAGT-3′ and 5′-GTCAAGGCTGATGGCGCACAG-3′) or ribosomal protein S29 (5′-ATGGGTCACCAGCAGCTCTA-3′ and 5′-AGCCTATGTCCTTCGCGTACT-3′) sequences. Reactions were run in triplicate on an MX3000P detector (Stratagene, Amsterdam, the Netherlands). The cycling conditions were the following: 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. Absolute copy numbers were determined using standard curves of corresponding linear DNA fragments (Gilsbach et al., 2007). Genomic DNA from tail biopsies (Hein et al., 1999) was used to determine transgene copy numbers. The α_{2B}-adrenoceptor gene was used as a reference control. Reactions were carried out as described above using primers for α_{2A}-adrenoceptor (see above) and α_{2B}-adrenoceptor (5′-GCAGAGGTCTCGGAGCTAA-3′ and 5′-GCCTCTCCGACAGAAGATA-3′) sequences.

Autoradiography. For receptor autoradiography, transverse brain sections (10 μm) were cut serially with a cryostat, thaw-mounted onto slides, and incubated for 60 min in 50 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, and 8 nM [^{3}H]RX821002 (GE Healthcare, Freiburg, Germany). To determine nonspecific binding, 1 μM atipamezole was included. After incubations, the slides were washed twice for 5 min in cold buffer, rinsed in distilled water, air-dried, and analyzed using a BAS5000 Fuji PhosphorImager (Fuji, Tokyo, Japan).

Immunohistochemistry. For immunodetection of Flag-tagged α_{2A}-receptors, cryostat sections (light microscopy) or vibrating microtome sections (electron microscopy) from perfusion-fixed mice (4% paraformaldehyde, 0.1% glutaraldehyde in phosphate-buffered saline) were used. Sections were blocked in 1% bovine serum albumin and 0.04% Triton X-100 in phosphate-buffered saline and incubated for 48 h at 4°C with a rabbit polyclonal antiserum against the epitope tag (antiserum "1809" in Hein et al., 1994), followed by overnight incubation with goat anti-rabbit biotinylated antibody and ABC Vectastain (Vector Laboratories, Burlingame, CA) incubation. Sections were incubated with 3,3′-diaminobenzidine and hydrogen peroxide, followed by 60-min treatment with OsO_4 and embedding in araldite (Durcupan, Fluka, Germany). Ultrathin sections that were contrast-stained with 2% uranyl acetate were inspected in a LEO AB912 electron microscope (Leo Electron Microscopy, Cambridge, UK).

Locus Ceruleus Microdissection. For microscopical microdissection of locus ceruleus specimens, tissues were frozen in liquid nitrogen. Cryostat sections (15 μm) were mounted on glass slides and dehydrated in ethanol and xylene followed by microdissection at a Leica AM6000 inverted microscope (Leica, Wetzlar, Germany). The area of the locus ceruleus (Paxinos and Franklin, 2001) was microdissected using MicroChisels (Eppendorf, Hamburg, Germany), aspirated via a micropipette, xylene was evaporated, and RNA was isolated using the RNeasy Micro-Kit (QIAGEN). The identity of the locus ceruleus was verified by qPCR determination of tyrosine hydroxylase and dopamine β-hydroxylase mRNA expression.

Isolation of Neurons from Superior Cervical Ganglia. Superior cervical ganglia (SCG) from mice were dissociated by treatment with trypsin, collagenase, and DNase in Neurobasal-A media (Invitrogen, Carlsbad, CA) supplemented with 0.5 mM glutamine, 1% penicillin/streptomycin, and 10 mM HEPES at 35°C and 850 rpm. Cells were plated onto poly(D-lysine)-coated coverslips in Neurobasal-A media with 0.5 mM glutamine, 2.5% fetal bovine serum, 1% B27 supplement, and 1% penicillin/streptomycin conditioned on astroglial culture for 24 h. For immunocytochemistry, neurons were fixed in methanol and incubated overnight with anti-tyrosine hy-

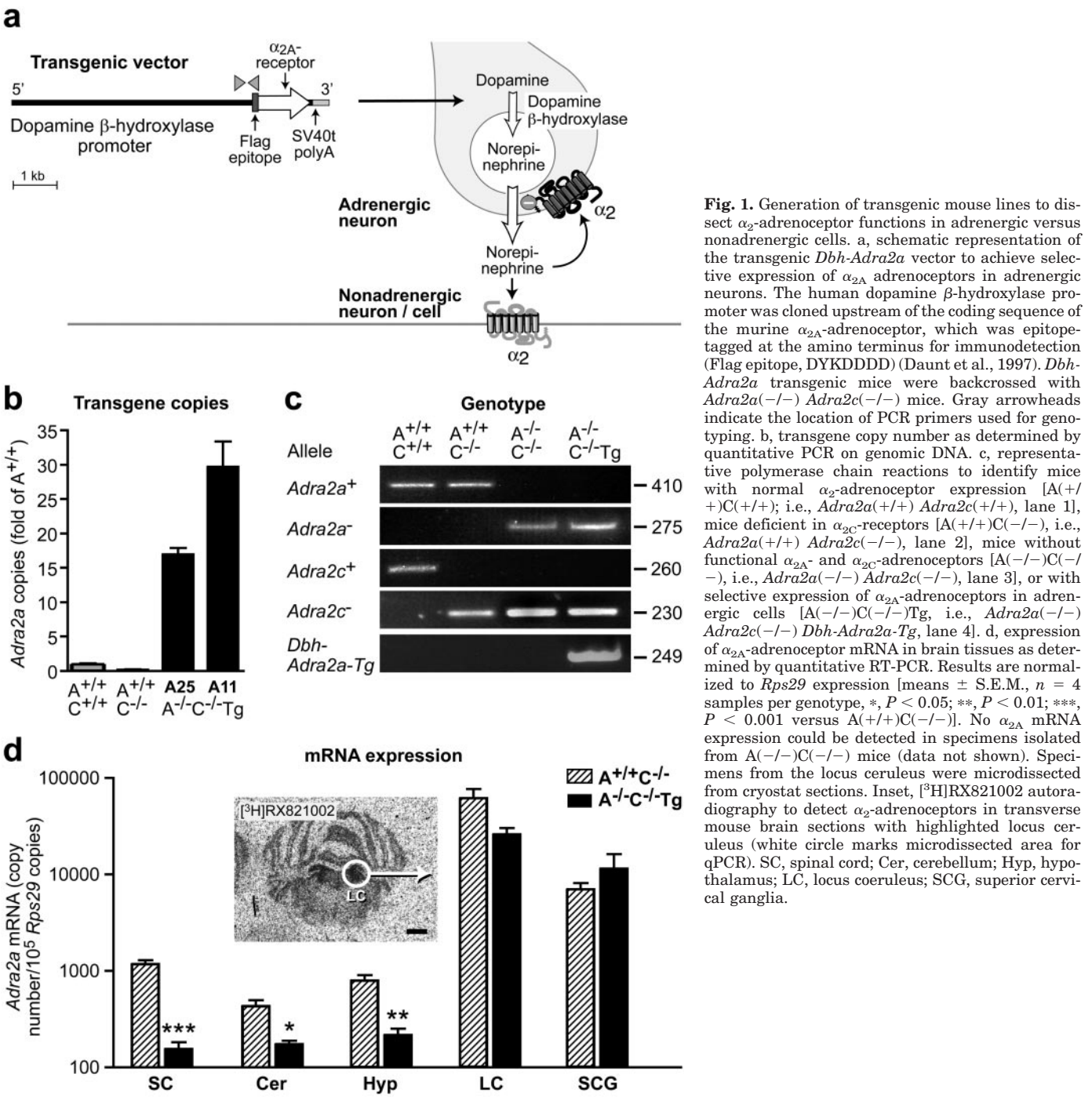

Fig. 1. Generation of transgenic mouse lines to dissect α_2-adrenoceptor functions in adrenergic versus nonadrenergic cells. a, schematic representation of the transgenic *Dbh-Adra2a* vector to achieve selective expression of α_{2A} adrenoceptors in adrenergic neurons. The human dopamine β-hydroxylase promoter was cloned upstream of the coding sequence of the murine α_{2A}-adrenoceptor, which was epitope-tagged at the amino terminus for immunodetection (Flag epitope, DYKDDDD) (Daunt et al., 1997). *Dbh-Adra2a* transgenic mice were backcrossed with *Adra2a*(−/−) *Adra2c*(−/−) mice. Gray arrowheads indicate the location of PCR primers used for genotyping. b, transgene copy number as determined by quantitative PCR on genomic DNA. c, representative polymerase chain reactions to identify mice with normal α_2-adrenoceptor expression [A(+/+)C(+/+); i.e., *Adra2a*(+/+) *Adra2c*(+/+), lane 1], mice deficient in α_{2C}-receptors [A(+/+)C(−/−), i.e., *Adra2a*(+/+) *Adra2c*(−/−), lane 2], mice without functional α_{2A}- and α_{2C}-adrenoceptors [A(−/−)C(−/−), i.e., *Adra2a*(−/−) *Adra2c*(−/−), lane 3], or with selective expression of α_{2A}-adrenoceptors in adrenergic cells [A(−/−)C(−/−)Tg, i.e., *Adra2a*(−/−) *Adra2c*(−/−) *Dbh-Adra2a-Tg*, lane 4]. d, expression of α_{2A}-adrenoceptor mRNA in brain tissues as determined by quantitative RT-PCR. Results are normalized to *Rps29* expression [means ± S.E.M., $n = 4$ samples per genotype, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus A(+/+)C(−/−)]. No α_{2A} mRNA expression could be detected in specimens isolated from A(−/−)C(−/−) mice (data not shown). Specimens from the locus ceruleus were microdissected from cryostat sections. Inset, [^{3}H]RX821002 autoradiography to detect α_2-adrenoceptors in transverse mouse brain sections with highlighted locus ceruleus (white circle marks microdissected area for qPCR). SC, spinal cord; Cer, cerebellum; Hyp, hypothalamus; LC, locus coeruleus; SCG, superior cervical ganglia.

droxylase and anti-Flag M2 antiserum followed by Alexa Fluor 488- and Alexa Fluor 568-coupled secondary antibodies.

Electrophysiology. For patch-clamp recording, slides with cultured SCG neurons were fixed at the glass bottom of a superfusion chamber and superfused with buffer at room temperature at a flow rate of 1.5 ml/min. The buffer had the following composition: 126 mM NaCl, 1.2 mM NaH_2PO_4, 3 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose, pH 7.35 (after the solution was gassed with 95% O_2/5% CO_2). The superfusion buffer contained tetrodotoxin (0.3 μM) to block voltage-gated sodium channels. Neurons were visualized with infrared videomicroscopy. Recordings were obtained with an EPC-9 amplifier under the control of TIDA software (HEKA Elektronik, Lambrecht, Germany). Series resistance compensation of 50% was usually applied. Series resistance was measured before and after recordings, and experiments with major changes in series resistance (>20%) were discarded. Cell were patch-clamped with pipettes (2–3 MΩ resistance) containing the following buffer: 100 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, 0.5 mM $CaCl_2$, 20 mM tetraethylammonium chloride, 10 mM EGTA, 3 mM magnesium-ATP, and 0.3 mM sodium-GTP, pH 7.35 adjusted with CsOH. Calcium currents were evoked every 15 s by voltage ramps (from −70 to 50 mV; ramp speed, 0.5 mV/ms). Leak currents were determined by running hyperpolarizing ramps (from −70 to −94 mV; ramp speed, 0.1 mV/ms) 5 s after the depolarizing ramps. The maximum leak subtracted current measured during the ramp was used for statistical evaluation.

[^{3}H]Norepinephrine Release. Electrically evoked [^{3}H]norepinephrine (GE Healthcare) release from isolated mouse atria was

determined as described previously (Hein et al., 1999; Gilsbach et al., 2007). In brief, freshly isolated mouse atria were incubated in 2 ml of medium containing 0.1 μM [^{3}H]norepinephrine (GE Healthcare) for 45 min at 37°C. Atria were transferred to superfusion chambers. After 45 min of superfusion, successive 2-min superfusate samples were collected. The preincubation medium consisted of 118 mM NaCl, 4.8 mM KCl, 0.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 11 mM glucose, 0.57 mM ascorbic acid, and 0.03 mM Na_2-EDTA, saturated with 5% CO_2 in O_2. The superfusion medium was the same but contained 2.5 mM $CaCl_2$ and 1 μM desipramine. Six periods of electrical stimulation (20 pulses/50 Hz, 1-ms pulse width, 80 mA) were applied at 16-min intervals. At the end of the experiments, tissues were solubilized, and tritium was determined in superfusate samples and tissues. The electrically evoked overflow of total tritium reflects exocytotic release of [^{3}H]norepinephrine.

Locomotor Activity. Two weeks after implantation of a telemetry device (DSI; Transoma Medical, St. Paul, MN), locomotor activity was recorded in conscious unrestrained mice both during the day (7:00 A.M. to 7:00 P.M.) and night (7:00 PM to 7:00 AM) (Gilsbach et al., 2007).

Nociception. Thermal antinociception was determined using an automated tail-flick system (Ugo Basile, Comerio, Italy). The time to withdrawal of the tail from an infrared light source shining onto the base of the tail was automatically recorded.

Body Temperature. Body temperature (measured in degrees Celsius) was recorded with a rectal thermometer probe (FMI, Seeheim-Ober Beerbach, Germany). Saline or drug were injected (intraperitoneally) 60 min before the test.

Sedation/Hypnosis. Mice were injected with different doses of medetomidine (intraperitoneally) and the time after injection, at which the righting reflex of mice was lost and the recovery time of this reflex were monitored (Lakhlani et al., 1997). The observer was blinded with respect to the genotype of the mice. For determination of the anesthetic sparing effect mice pretreated with subanesthetic drug concentrations were placed in an air-tight Plexiglas chamber, and isoflurane was continuously introduced at increasing concentrations (0–1.2 vol% in O_2) (Lakhlani et al., 1997). Mice were equilibrated to each concentration of isoflurane for 5 min before the righting reflex.

Statistical Analysis. Data are presented as means ± S.E.M. of individual data points. Data were analyzed using one- or two-way analysis of variance followed by Bonferroni post hoc tests or Student's *t* test, respectively. Results from electrophysiological measurements were analyzed using the Mann-Whitney test. A *P* value of less than 0.05 was considered statistically significant. Graphs and statistical analyses were generated by Prism (version 4.0c; GraphPad Software, San Diego, CA).

Results

Transgenic Mouse Models to Dissect α_2-Adrenoceptor Functions in Adrenergic versus Nonadrenergic Cells. To distinguish between functions that are mediated by α_2-autoreceptors in adrenergic cells and α_2-receptors expressed in nonadrenergic neurons or cells, a transgenic mouse strain with specific expression of α_{2A}-adrenoceptors in adrenergic cells was generated. The transgenic construct (Fig. 1a) consisted of the coding sequence of an epitope-tagged murine α_{2A}-receptor (Daunt et al., 1997) downstream of the dopamine β-hydroxylase (*Dbh*) promoter sequence followed by an SV40 *t*-intron and polyadenylation signal. The *Dbh* promoter has been successfully used to drive the expression of target genes in adrenergic neurons in vivo (Mercer et al., 1991; Hoyle et al., 1994). After pronuclear injection of the linearized transgenic vector, several transgenic founder mice were obtained and identified by PCR genotyping (Fig. 1, b and c). Two of five transgenic founder lines, A11 and A25, which contained 30 ± 4 and 17 ± 1 transgene copies (Fig. 1b), respectively, were used for further studies. Transgenic offspring were born at the expected Mendelian ratio and did not show any signs of developmental or structural defects (data not shown). To generate mouse strains with selective expression of α_{2A}-adrenoceptors in adrenergic cells, transgenic mice were crossed with mice lacking α_{2A}- and α_{2C}-adrenoceptors (*Adra2a*(−/−) *Adra2c*(−/−); Hein et al., 1999). Previous experiments have demonstrated that α_{2A}- and α_{2C}-adrenoceptors are the major presynaptic feedback regulators in the adrenergic system (Hein et al., 1999). Thus, mice with selective expression of α_{2A}-autoreceptors in adrenergic neurons [termed A(−/−)C(−/−)Tg; genotype *Adra2a*(−/−) *Adra2c*(−/−) *Dbh-Adra2a-Tg*] were compared with wild-type mice [termed A(+/+)C(+/+), genotype *Adra2a*(+/+) *Adra2c*(+/+)], mice lacking α_{2C}-adrenoceptors [termed A(+/+)C(−/

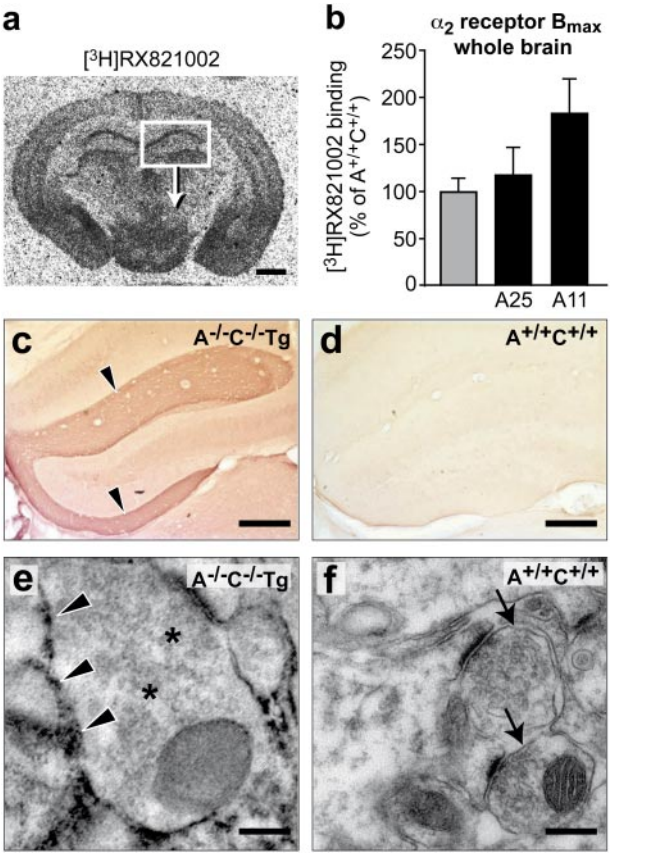

Fig. 2. Expression and localization of transgenic α_{2A}-adrenoceptors. a, representative [^{3}H]RX821002 autoradiography to detect α_2-adrenoceptors in transverse mouse brain sections in the hippocampus (white box highlights area enlarged in immunohistochemistry, c and d). b, maximal density (B_{max}) of α_2-adrenoceptor protein was measured by [^{3}H]RX821002 binding in synaptosomes of whole-brain samples from wild-type mice and two independent transgenic lines, A25 and A11 (means ± S.E.M., n = 3 independent experiments performed in triplicate). c–f, immunohistochemical detection of epitope-tagged, transgenic α_{2A}-adrenoceptors in A(−/−)C(−/−)Tg (c and e) versus A(+/+)C(+/+) hippocampus (d and f) by anti-Flag antiserum followed by peroxidase-coupled secondary antibody. e and f, expression of Flag-tagged α_{2A}-adrenoceptors can be detected by immunoelectron peroxidase labeling in presynaptic plasma membranes of A(−/−)C(−/−)Tg hippocampus (e, arrowheads), but not in A(+/+)C(+/+) samples (f, arrows). Presynaptic transmitter vesicles were not labeled by the Flag antiserum (e, asterisks). Scale bars: a, 1 mm; c and d, 0.5 mm; e and f, 200 nm.

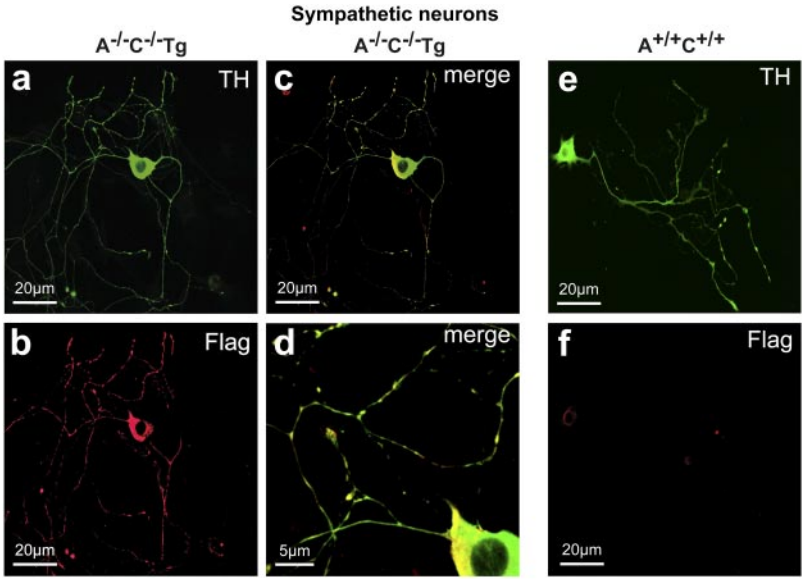

Fig. 3. Expression of α_{2A}-adrenoceptors in sympathetic neurons. a to f, neurons were isolated from SCG, cultivated in vitro for 24 h, and immunostained to detect TH (a, c, d, and e) or Flag-tagged α_{2A}-adrenoceptors (Flag; b, c, d, and f). To demonstrate overlapping expression of TH and Flag, images were merged (c and d). Flag-tagged α_{2A}-adrenoceptors were localized in the plasma membrane of the soma and neuronal processes (b–d).

−), genotype *Adra2a*(+/+) *Adra2c*(−/−)], and mice lacking both α_{2A}- and α_{2C}-adrenoceptors [A(−/−)C(−/−), genotype *Adra2a*(−/−) *Adra2c*(−/−)] (Fig. 1c).

Validation of α_2-Adrenoceptor Transgene Expression. Expression of the *Dbh-Adra2a* transgene was validated by different methods, including quantitative RT-PCR, radioligand binding, autoradiography, and immunohistochemical detection methods. mRNA for the *Dbh-Adra2a* transgene could be detected in peripheral and central nervous tissues, including superior cervical ganglia and locus ceruleus, which was microdissected from mouse brains (Fig. 1d), as well as adrenal medulla (data not shown). Using primers that recognize endogenous and transgenic α_{2A}-adrenoceptor mRNA, expression of the transgenic α_{2A}-receptor [in A(−/−)C(−/−)Tg] was similar to the expression level of the endogenous α_{2A}-receptor (Fig. 1d). In nonadrenergic regions of the central nervous system, including spinal cord, cerebellum, and hypothalamus, transgenic α_{2A}-receptor mRNA expression was 26- to 169-fold lower than in locus ceruleus and sympathetic ganglia (Fig. 1d). No specific mRNA could be detected with these RT-PCR primers in A(−/−)C(−/−) brain or sympathetic tissue (data not shown). To assess α_{2A}-adrenoceptor protein expression, radioligand binding experiments with the α_2-adrenoceptor antagonist [^{3}H]RX821002 (Fagerholm et al., 2004) were performed with synaptosomes prepared from whole brains. α_2-Adrenoceptor density (B_{max}) in the transgenic line A25 was similar to the receptor level in wild-type control brain (Fig. 2b), whereas the amount of α_{2A}-adrenoceptors in the A11 strain was 80% higher than the respective wild-type value (Fid. 2b). Both transgenic lines were phenotyped independently and yielded identical results with respect to the assignment of α_2-adrenoceptor functions to adrenergic versus nonadrenergic cells (data not shown).

The distribution pattern of transgenic α_{2A}-adrenoceptors in the autoradiography experiments was identical with results obtained by immunohistochemical detection (Fig. 2, a and c). Flag-tagged transgenic α_{2A}-receptors were detected in brain regions with high levels of adrenergic target innervation, including cortex, amygdala, and hippocampus (Fig. 2, a, c, and e). Flag-tagged α_{2A}-receptors were readily detectable in the stratum lacunosum moleculare of the hippocampus (Fig. 2, c and e), resembling expression of endogenous α_{2A}-receptors (Fagerholm et al., 2004). No anti-Flag staining was observed in tissue sections from nontransgenic A(+/+)C(+/+) mice (Fig. 2d). Immunoelectron microscopy was used to determine the subcellular localization of Flag-tagged α_{2A}-receptors in the hippocampus (Fig. 2e). High levels of peroxidase reaction product indicating the presence of Flag-tagged α_{2A}-receptors was observed in the hippocampus in the presynaptic plasma membrane of axon terminals (Fig. 2e, arrowheads) but not in neurotransmitter vesicles of axon terminals (Fig. 2e, asterisks). No specific immunostaining was observed in hippocampus sections from A(+/+)C(+/+) mice (Fig. 2f). Taken together, these findings indicate that the *Dbh-Adra2a* transgene was expressed in a tissue-specific and subcellular pattern that resembles the localization of endogenous α_{2A}-autoreceptors.

Validation of Transgenic α_{2A}-Adrenoceptor Function. To examine whether *Dbh*-transgenic α_{2A}-receptors expressed in adrenergic cells were functional, sympathetic neurons were isolated from mouse SCG and cultivated in vitro for further studies (Fig. 3). Neurons isolated from transgenic mouse strains [A(−/−)C(−/−)Tg] exhibited overlapping staining for tyrosine hydroxylase (TH) with the anti-Flag immune serum (Fig. 3, a–d), whereas no anti-Flag staining could be observed in TH-positive neurons from nontransgenic mice (Fig. 3, e and f). The function of transgenic α_{2A}-adrenoceptors in sympathetic neurons was assessed by determining the inhibition of voltage-gated Ca^{2+} channel currents (Fig. 4, a and b). SCG neurons were held at −70 mV, and voltage-gated calcium channels were activated by ramp depolarization (Fig. 4a). The mean amplitude of calcium currents during the initial reference period was 0.27 ± 0.04 nA (n = 19) (Fig. 4b). The α_2-adrenoceptor agonist medetomidine (100 nM) inhibited calcium currents in wild-type neurons [A(+/+)C(+/+)] to 26 ± 21% of the prestimulation value. In neurons prepared from α_{2C}-adrenoceptor-deficient mice [A(+/+)C(−/−)], medetomidine inhibited calcium currents to 63 ± 8% of control. In neurons prepared from $\alpha_{2A/C}$-adrenoceptor-deficient mice [A(−/−)C(−/−)], medetomidine failed to inhibit calcium currents. However, in neurons derived from $\alpha_{2A/C}$-adrenoceptor-deficient-Dbh α_{2A}-adrenoceptor mice

[A(−/−)C(−/−)Tg], medetomidine inhibited calcium currents to 28 ± 13% of the prestimulation value. To test whether transgenic α_{2A}-adrenoceptors operated as presynaptic autoreceptors in sympathetic neurons, the inhibition of electrically evoked release of [^{3}H]norepinephrine by α_2-agonists was tested in tissue samples with dense adrenergic innervation. For this purpose, tissue specimens from mouse cardiac atria were incubated in [^{3}H]norepinephrine-containing physiological buffer in vitro, and neurotransmitter release was elicited by short pulse trains of electrical field stimulation (Hein et al., 1999) (Fig. 4c). To inhibit [^{3}H]norepinephrine release, the α_{2A},α_{2C}-preferring agonist UK14,304 (brimonidine) was used. UK14,304 does not efficiently activate α_{2B}-receptors that may be present at low levels in sympathetic postganglionic neurons (Trendelenburg et al., 2003). In A(+/+)C(+/+) and A(+/+)C(−/−) control atria, UK14,304 inhibited the electrically evoked release of [^{3}H]norepinephrine in a concentration-dependent manner. This inhibitory effect was completely absent in specimens from A(−/−)C(−/−) mice (Hein et al., 1999). It is noteworthy that transgenic expression of α_{2A}-autoreceptors [A(−/−)C(−/−)Tg] completely rescued the defect in α_2-mediated inhibition in α_{2A}/α_{2C}-deficient mice [A(−/−)C(−/−), Fig. 4c]. The EC_{50} value and the degree of maximal inhibition did not differ significantly between atria from A(−/−)C(−/−)Tg and A(+/+)C(−/−) mice [logEC_{50} A(+/+)C(−/−) − 7.96 ± 0.07 versus A(−/−)C(−/−)Tg −7.83 ± 0.11, n = 10]. However, the ability of medetomidine to inhibit norepinephrine release from isolated wild-type sympathetic nerves was slightly but significantly more potent [logEC_{50}, A(+/+)C(+/+) −8.37 ± 0.07, $P < 0.05$ versus A(+/+)C(−/−)].

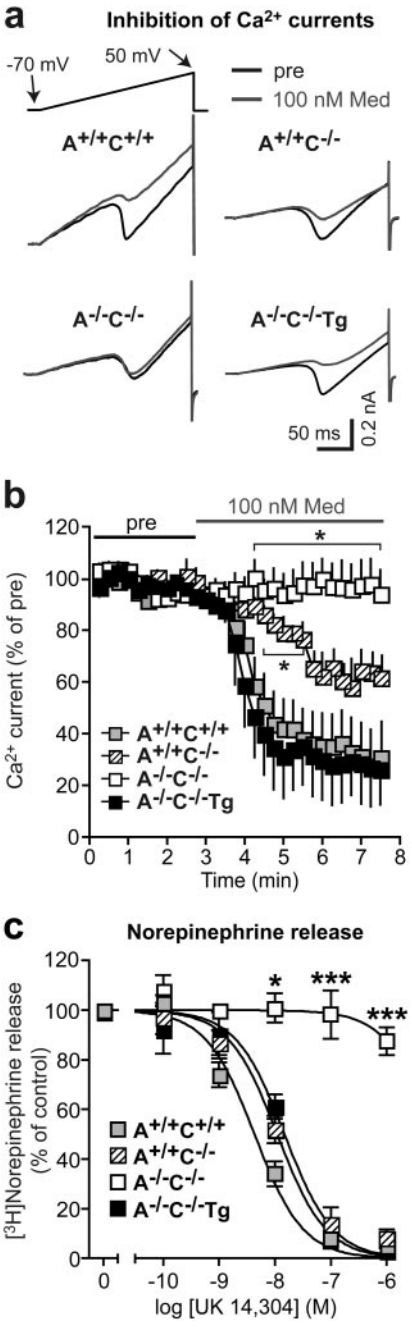

Fig. 4. Function of α_{2A}-adrenoceptors in sympathetic neurons. a and b, effect of medetomidine on voltage-gated calcium channels in cultured SCG sympathetic neurons. Calcium currents were evoked every 15 s by depolarizing voltage ramps (−70–50 mV). After an initial reference period (pre), medetomidine (100 nM) superfusion started. Calcium current amplitudes were expressed as a percentage of the initial reference value (pre). a, original tracings. b, statistical evaluation: mean ± S.E.M. of three [A(+/+) C(+/+)], six [A(+/+) C(−/−)], five [A(−/−) C(−/−)], and five [A(−/−) C(−/−) Tg) experiments. *, $P < 0.05$ versus A(+/+)C(+/+). c, feedback control of norepinephrine release from sympathetic nerves. The α_{2A},α_{2C}-agonist UK14,304 inhibited overflow of [^{3}H]norepinephrine from isolated A(+/+)C(+/+), A(+/+)C(−/−), and A(−/−)C(−/−)Tg atria but not from A(−/−)C(−/−) atria. Atria were stimulated by field stimulation with 20 rectangular electrical pulses at 50 Hz (1-ms pulse width, 80 mA) applied at 16-min intervals [*, $P < 0.05$ versus A(+/+)C(+/+) control, n = 6–10 samples per genotype].

α_2-Adrenoceptor Effects on Sedation/Hypnosis. Stimulation of central α_2-adrenoceptors by α_2-agonists is well documented to cause sedation and hypnosis (Lakhlani et al., 1997; Maze et al., 2001). Sedation induced by the α_2-agonist medetomidine was assessed using the righting reflex (Fig. 5a). At a dose of 1000 μg/kg, medetomidine induced a strong sedative effect in A(+/+)C(+/+) and A(+/+)C(−/−) mice, because all of the drug-treated mice lost their righting reflex (LORR) (Fig. 5a). None of the A(−/−)C(−/−) or A(−/−)C(−/−)Tg mice lost the righting reflex, indicating that α_{2A}-receptors in adrenergic cells were not required for the sedative effects of the α_2-agonist medetomidine. To confirm that α_2-mediated LORR is independent of modulating norepinephrine release, we tested dopamine β-hydroxylase-deficient mice [Dbh(−/−)], which are unable to synthesize norepinephrine (Thomas et al., 1995; Weinshenker et al., 2008). Medetomidine induced LORR in all control [Dbh(+/−)] and Dbh(−/−) mice (Fig. 5b). To exclude that α_2-receptor deletion and/or transgenic expression affected sensitivity to sedative stimuli nonspecifically, mice received the $GABA_A$ receptor agonist pentobarbital, and the times until loss (Fig. 5c) or recovery (Fig. 5d) of the righting reflex were recorded. Induction and duration of the hypnotic effect of pentobarbital did not differ between the four α_2-genotypes.

Because α_2-agonists have been documented to lower the dose of inhalation anesthetics required for anesthesia (Lakhlani et al., 1997), we evaluated the anesthetic-sparing effect of nonsedative doses of medetomidine during isoflurane anesthesia (Fig. 6). In A(+/+)C(+/+) and A(+/+)C(−/−) mice, medetomidine shifted the isoflurane dose-response curve to the left (Fig. 6, a and b). This anesthetic-sparing effect of medetomidine was ablated by deletion of α_{2A}-/α_{2C}-receptors [A(−/−)C(−/−)] and was not rescued by transgenic expression of α_{2A}-autoreceptors [A(−/−)C(−/−)Tg] (Fig. 6, c and d).

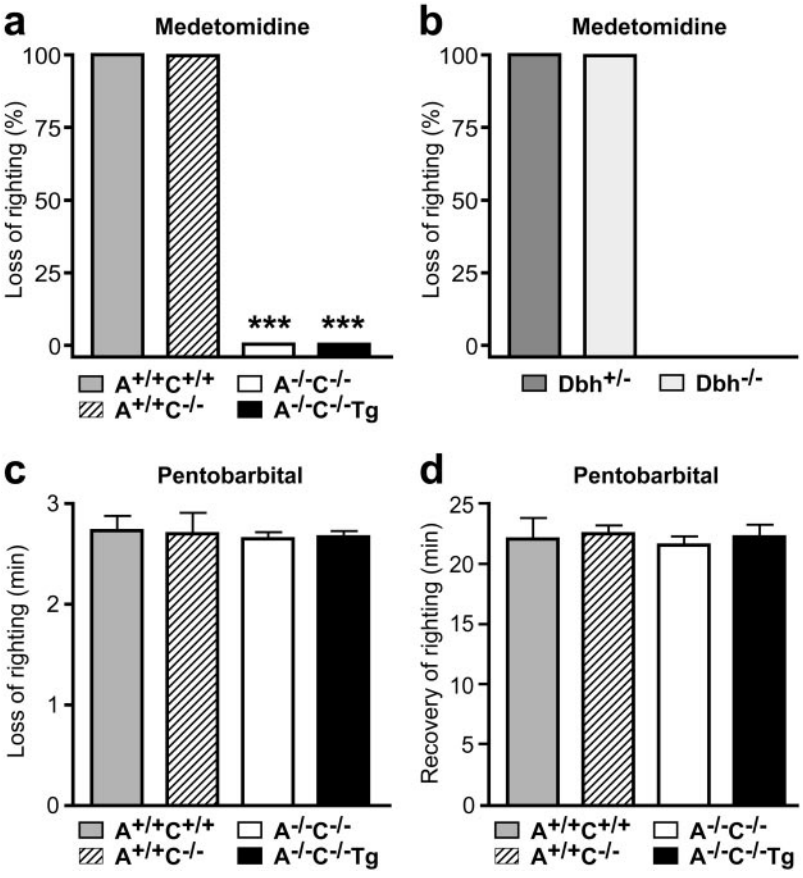

Fig. 5. α_2-Agonist-mediated loss of righting reflex. a, intraperitoneal medetomidine (1000 μg/kg) induced a loss of the righting reflex in A(+/+)C(+/+) and A(+/+)C(−/−) mice but not in A(−/−)C(−/−) or A(−/−)C(−/−)Tg mice (∗, $P < 0.05$; $n = 6$–9 per genotype). b, medetomidine (1000 μg/kg, i.p.) induced a loss of the righting reflex in dopamine β-hydroxylase-deficient mice [Dbh(+/−), Dbh(−/−)] ($n = 6$ per genotype). c and d, onset (c) and recovery time (d) of pentobarbital-induced loss of the righting reflex did not differ between genotype groups (50 mg/kg pentobarbital i.p., $n = 6$).

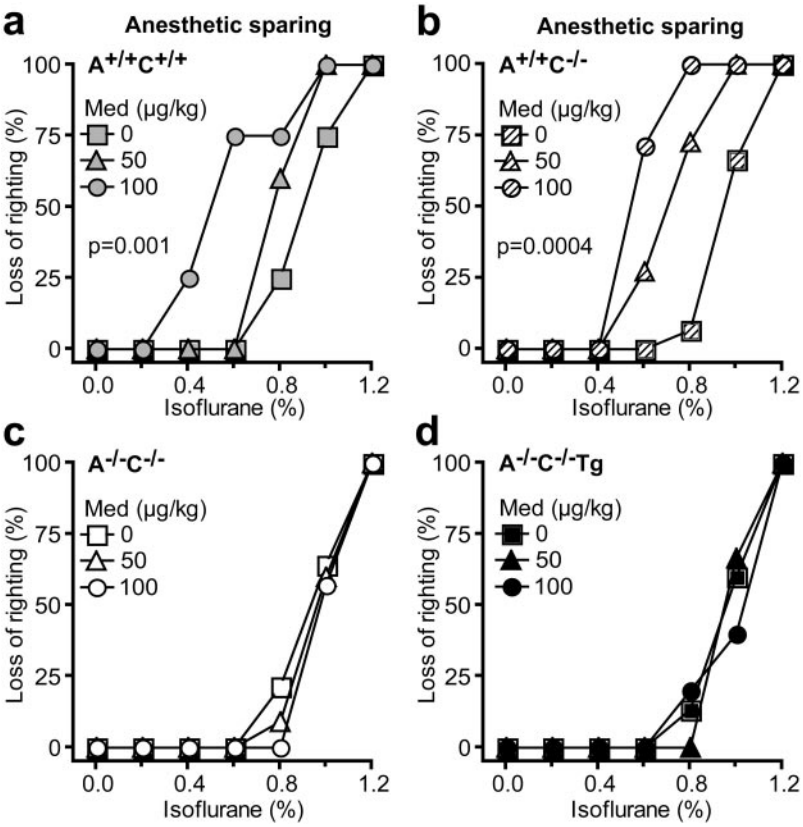

Fig. 6. Anesthetic-sparing effect of the α_2-adrenoceptor agonist medetomidine. Medetomidine was applied intraperitoneally at doses that did not cause a loss of the righting reflex (50 and 100 μg/kg) 15 min before exposure to increasing concentrations of isoflurane (in 1 L/min O_2 flow). Medetomidine induced a leftward shift of the isoflurane-mediated loss of righting reflex curves in A(+/+)C(+/+) (a) and A(+/+)C(−/−) mice (b) but not in A(−/−)C(−/−) (c) or A(−/−)C(−/−) Tg (d) mice ($n = 10$–15 per genotype).

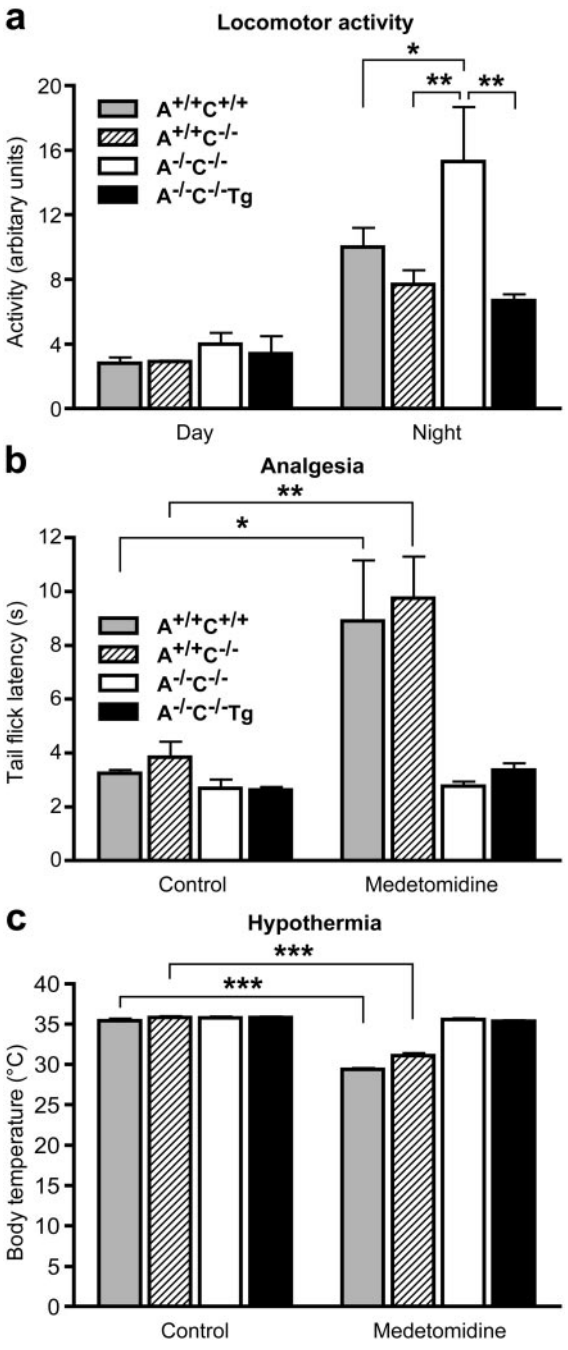

Fig. 7. Locomotor, analgesic, and hypothermic effects of the α_2-agonist medetomidine in α_{2A}-transgenic mice. a, locomotor activity was monitored 2 weeks after implantation of a telemetric activity monitor. At nighttime, spontaneous locomotor activity was significantly greater in A(−/−)C(−/−) mice compared with A(+/+)C(+/+), A(+/+)C(−/−), or A(−/−)C(−/−)Tg mice (n = 6 per genotype). b, medetomidine (250 μg/kg i.p.) prolonged the tail-flick latency in A(+/+)C(+/+) and A(+/+)C(−/−) but not in A(−/−)C(−/−) or A(−/−)C(−/−)Tg mice (n = 6–9 per genotype). c, medetomidine (250 μg/kg i.p.) significantly lowered body temperature in A(+/+)C(+/+) and A(+/+)C(−/−) but not in A(−/−)C(−/−) or A(−/−)C(−/−)Tg mice (n = 8–12 per genotype, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Spontaneous locomotor activity was determined in mice 2 weeks after subcutaneous implantation of a telemetric activity monitor, and activity was measured during a controlled light/dark cycle in 2-min intervals (Fig. 7a). During the light cycle, activity levels did not differ between genotypes (Fig. 7a). During the dark cycle, however, locomotor activity of A(−/−)C(−/−) mice was significantly enhanced compared with A(+/+)C(+/+) and A(+/+)C(−/−) mice (Fig. 7a). Transgenic expression of adrenergic cell α_{2A}-receptors reduced locomotor activity to the level of wild-type control mice.

Antinociceptive Effects Mediated by α_2-Adrenoceptors. α_2-agonists mediate strong analgesic effects at spinal and supraspinal levels (Pertovaara, 2006; Sanders and Maze, 2007). Thus, we determined the antinociceptive effect of medetomidine in the tail-flick assay (Fig. 7b). At baseline, the time to withdrawal of the tail from the infrared light source did not differ significantly between genotypes. Medetomidine (250 μg/kg i.p.) significantly prolonged the latency time of tail withdrawal in A(+/+)C(+/+) or A(+/+)C(−/−) mice but not in A(−/−)C(−/−) or in A(−/−)C(−/−)Tg mice (Fig. 7b). Thus, α_{2A}-autoreceptors are not essential for the antinociceptive effect of medetomidine in the tail-flick assay.

α_2-Agonist-Mediated Hypothermia. At baseline, body temperature did not differ between genotypes (Fig. 7c). When applied at a nonsedative dose of 250 μg/kg, medetomidine lowered body core temperature significantly by 5.9 ± 0.4°C in A(+/+)C(+/+) and A(+/+)C(−/−) mice (Fig. 7 c). However, the hypothermic effect was completely absent in A(−/−)C(−/−) or in A(−/−)C(−/−)Tg mice, indicating that adrenergic cell α_2-adrenoceptors are not required for this function (Fig. 7c).

Discussion

α_2-Adrenoceptors and receptors for acetylcholine and GABA were among the first receptors to be identified as inhibitory feedback receptors, which are located on their own transmitter's nerve terminals to inhibit the release of neurotransmitter. The discovery and investigation of these prototypic presynaptic or "autoreceptors" has greatly advanced our understanding of the neurobiology of transmitter release (Sudhoff and Starke, 2008). For most transmitter systems, several receptor subtypes were identified as candidate autoreceptors by molecular cloning. In isolated tissues from gene-targeted mice, all three cloned α_2-adrenoceptors, α_{2A}, α_{2B}, and α_{2C}, were shown to operate as inhibitory feedback receptors to control norepinephrine release in vitro (Hein et al., 1999; Trendelenburg et al., 2003). However, it remained unknown which receptor subtype operated as an inhibitory autoreceptor in vivo and which effects of pharmacological α_2-receptor ligands are mediated via these autoreceptors on adrenergic neurons.

To distinguish between α_2-adrenoceptor functions in adrenergic versus nonadrenergic cells, we have generated a transgenic model with specific expression of α_2-receptors in adrenergic neurons and subjected these mice to a comprehensive phenotype program. The primary finding of this study is that the majority of α_2-receptor agonist effects were mediated via α_2-receptors in nonadrenergic cells and not via adrenergic cell α_2-receptors (Fig. 8 and Table 1). The results of this study emphasize the importance of nonadrenergic α_2-adrenoceptors, but they do not completely exclude contribution of α_2-adrenoceptor functions in adrenergic cells.

Transgenic Model to Distinguish Receptor Functions in Adrenergic Cells from Nonadrenergic Cells. Several lines of evidence indicate that the transgenic model generated to functionally separate α_2-receptors in adrenergic

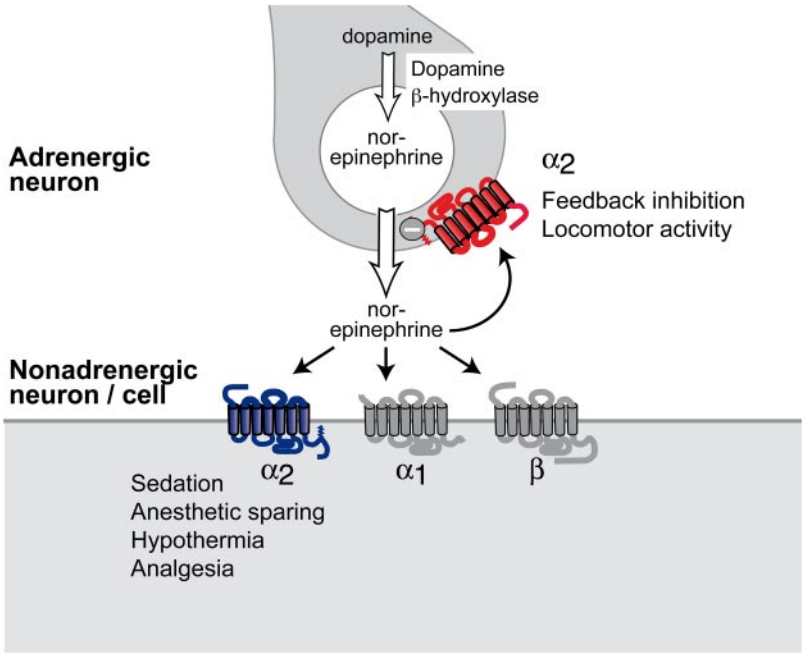

Fig. 8. Schematic representation of α_2-adrenoceptor functions mediated by receptors in adrenergic cells versus nonadrenergic cells.

cells from nonadrenergic cells was successful. First, the expression of α_{2A}-receptors under control of the dopamine β-hydroxylase promoter could be identified in adrenergic cells and tissues (e.g., locus ceruleus and sympathetic ganglia). On the protein level, transgenic α_{2A}-receptors were detected by radioligand autoradiography and by immunohistochemistry. Using immunoperoxidase labeling, transgenic α_{2A}-receptors were identified in the presynaptic plasma membrane but not in postsynaptic or intracellular transmitter vesicle membranes of hippocampal neurons (Fig. 2e). Furthermore, transgenic α_{2A}-receptors completely restored the α_2-autoreceptor function in peripheral tissue innervated by sympathetic nerve fibers. Thus, taken together, expression of transgenic α_{2A}-adrenoceptors recapitulated the subcellular localization and in vitro function of α_2-adrenoceptors in adrenergic cells.

The transgenic strategy applied in the current study may have several limitations. Expression of the Dbh-transgene may not be restricted to adrenergic cells (Mercer et al., 1991; Hoyle et al., 1994). However, mRNA expression of the Dbh-α_{2A}-transgene was 26- to 169-fold lower in nonadrenergic regions of the CNS than in adrenergic nuclei, including locus ceruleus or sympathetic ganglia (Fig. 1d). Misexpression of α_{2A}-receptors under the control of the Dbh promoter used for the present study may lead to false-positive assignments of α_2-functions as autoreceptor (i.e., receptors in adrenergic cells). Furthermore, higher-than-physiological levels of α_{2A}-receptor expression may result in a gain of function that is not achieved by endogenously expressed receptors. Indeed, we observed that transgenic α_{2A}-receptors compensated for the loss of both α_{2A} and α_{2C} in sympathetic ganglia (Fig. 4b). Finally, α_{2A}-adrenoceptors expressed under control of the *Dbh* promoter may alter their expression pattern during embryonic development. Although we cannot rule out alterations in neuronal development, thorough macroscopical or microscopical investigation did not reveal differences between transgenic and wild-type brains.

TABLE 1
Summary of α_2-adrenoceptor functions in adrenergic versus nonadrenergic cells

Function	Adrenergic Cell	Nonadrenergic Cell
Agonist-mediated inhibition of Ca^{2+} currents in sympathetic ganglia in vitro	+	
Agonist-mediated inhibition of electrically-evoked norepinephrine release from atria in vitro	+	
Spontaneous locomotor activity at night	+	
Agonist-induced loss of righting reflex		+
Agonist-induced anesthetic-sparing		+
Agonist-induced analgesia (tail flick)		+
Agonist-induced hypothermia		+

Transgenic lines were crossed with mice lacking α_{2A}- and α_{2C}-adrenoceptors because these two subtypes represent the major presynaptic feedback inhibitors of norepinephrine release in vivo (Hein et al., 1999; Brede et al., 2003). α_{2B}-Adrenoceptor-deficient mice were not included mainly for two reasons. First, the contribution of α_{2B} in presynaptic feedback inhibition has been demonstrated in isolated tissue preparations but not in vivo (Trendelenburg et al., 2003). Second, homozygous deletion of the α_{2B}-adrenoceptor gene was lethal during embryonic development and perinatally (Philipp et al., 2002). However, future experiments will address whether α_2-adrenoceptor subtypes mediate specific functions in adrenergic versus nonadrenergic cells.

α_2-Adrenoceptor Functions in Adrenergic versus Nonadrenergic Cells. Using this model, only a few α_2-adrenoceptor functions could be ascribed to receptors expressed in adrenergic cells: inhibition of Ca^{2+} currents in SCG neurons, inhibition of electrically evoked norepinephrine from sympathetic nerves, and modulation of spontaneous locomotor activity. All other tested α_2-receptors functions, including analgesia, sedation, anesthetic sparing, and hypothermia, required the presence of α_2-adrenoceptors on nonadrenergic cells (Fig. 8). In addition to revising the view of the physiology of the adrenergic system, the present results offer new insight into the mechanisms of α_2-agonist

drugs. Most importantly, all CNS effects of α_2-agonists on pain, sedation, and anesthetic sparing, as well as body temperature, were mediated by α_2-heteroreceptors. Several mechanisms for the antinociceptive effects of α_2-agonists have been identified on supraspinal and spinal levels (Pertovaara, 2006; Sanders and Maze, 2007). Antinociceptive α_2-receptors were suggested to reside in spinal terminals of primary nociceptor neurons, in spinal pain relay neurons, and on dorsal horn excitatory interneurons. Because none of these neuron groups synthesizes (nor)epinephrine, they should be classified as nonadrenergic cell α_2-adrenoceptors. The present study demonstrates that intraperitoneal medetomidine indeed mediates its antinociceptive effect via nonadrenergic cell α_2-receptors in the tail-flick assay (Fig. 7b). This result is consistent with previous reports from G protein-coupled inwardly rectifying potassium channel-2 mutant mice, linking the analgesic effect also to postsynaptic α_2-receptors (Blednov et al., 2003).

In clinical anesthesia, α_2-agonists may be used during the induction of anesthesia for their anesthetic-sparing effect or in the perioperative period as potent sedative and analgesic drugs (Kamibayashi and Maze, 2000). However, it has been difficult to identify whether α_2-receptors mediating sedation and hypnosis are pre- or postsynaptic receptors. According to one concept, the locus ceruleus plays an important role in the sedative effect of α_2-agonists (Mizobe et al., 1996). In mice lacking functional α_{2A}-adrenoceptors (α_{2A}-D79N), the sedative and anesthetic-sparing effects of the α_2-agonist dexmedetomidine were ablated (Lakhlani et al., 1997). In brain slices from α_{2A}-D79N mice, clonidine failed to inhibit spontaneous firing of locus ceruleus neurons (Lakhlani et al., 1997). Based on these and other experiments, it was hypothesized that α_2-agonists lower locus ceruleus neuron activity via presynaptic inhibitory autoreceptors (Jones, 2005). Although the present results confirm the role of the α_{2A}-subtype for the sedative effect of α_2-agonists, they also demonstrate that α_2-autoreceptors are not essential for this effect. Neither the anesthetic-sparing nor the sedative effects of the α_2-agonist medetomidine could be rescued by transgenic expression of α_2-adrenoceptors in adrenergic cells (Fig. 5, 6). Furthermore, the α_2-agonist medetomidine still resulted in a loss of the righting reflex in mice with genetic deficiency in dopamine β-hydroxylase, the key enzyme required for synthesis of norepinephrine from dopamine (Thomas et al., 1995; Weinshenker et al., 2008). In accordance with these data, earlier studies have shown that depletion of norepinephrine from central adrenergic neurons by the neurotoxin DSP-4 or by direct injection of 6-hydroxydopamine into the locus ceruleus of rats did not affect the sedative effects of clonidine (Spyraki and Fibiger, 1982). Thus, the sedative effects of α_2-agonists may essentially require α_2-adrenoceptors in nonadrenergic neurons in the CNS.

Norepinephrine has anticonvulsant properties in most seizure models, but the effects of α_2-agonists has been ambiguous. A previous study using dopamine β-hydroxylase knockout mice showed that the proconvulsant effects of α_2-agonists were mediated by the α_{2A}-autoreceptor, whereas the anticonvulsant effects of α_2-agonists were mediated by α_{2A}-receptors on nonadrenergic neurons (Weinshenker and Szot, 2002; Szot et al., 2004). These results suggest that the development of selective α_{2A}-agonists for nonadrenergic cell receptors may also be effective antiseizure medications. Furthermore, a recent study has identified a neuronal pathway linking α_2-adrenoceptors in nonadrenergic brain cortex neurons via cAMP and HCN channel signaling with working memory (Wang et al., 2007).

The present study paves the way to a search for new α_2-adrenoceptor-based therapeutic strategies. The majority of pharmacological effects of α_2-agonists are mediated by receptors in nonadrenergic cells, which may greatly differ in their localization and intracellular signal transduction and effector coupling. In conclusion, following up these pathways will result in a better understanding of receptor subtype and functional diversity within the adrenergic system and may provide important new considerations for future drug development.

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Address correspondence to: Dr. Lutz Hein, Institute of Experimental and Clinical Pharmacology and Toxicology, University of Freiburg, Albertstrasse 25, 79104 Freiburg, Germany. E-mail: lutz.hein@pharmakol.uni-freiburg.de