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TWO α_2-ADRENERGIC RECEPTOR SUBTYPES, α_{2A} AND α_{2C}, INHIBIT TRANSMITTER RELEASE IN THE BRAIN OF GENE-TARGETED MICE

M. M. BÜCHELER, K. HADAMEK and L. HEIN*

Institut für Pharmakologie und Toxikologie, Universität Würzburg, Versbacher Strasse 9, 97078 Würzburg, Germany

Abstract—α_2-Adrenergic receptors play an essential role in regulating neurotransmitter release from sympathetic nerves and from adrenergic neurons in the CNS. However, the role of each of the three highly homologous α_2-adrenergic receptor subtypes (α_{2A}, α_{2B}, α_{2C}) in this process has not been determined unequivocally. To address this question, the regulation of norepinephrine and dopamine release was studied in mice carrying deletions in the genes encoding the three α_2-adrenergic receptor subtypes. Autoradiography and radioligand binding studies showed that α_2-receptor density in α_{2A}-deficient brains was decreased to $9 \pm 1\%$ of the respective wild-type value, whereas α_2-receptor levels were reduced to $83 \pm 4\%$ in α_{2C}-deficient mice. These results indicate that approximately 90% of mouse brain α_2-receptors belong to the α_{2A} subtype and 10% are α_{2C}-receptors. In isolated brain cortex slices from wild-type mice a non-subtype-selective α_2-receptor agonist inhibited release of [^{3}H]norepinephrine by maximally 96%. Similarly, release of [^{3}H]dopamine from isolated basal ganglion slices was inhibited by 76% by an α_2-receptor agonist. In α_{2A}-receptor-deficient mice, the inhibitory effect of the α_2-receptor agonist on norepinephrine and dopamine release was significantly reduced but not abolished. Only in tissues from mice lacking both α_{2A}- and α_{2C}-receptors was no α_2-receptor agonist effect on transmitter release observed. The time course of onset of presynaptic inhibition of norepinephrine release was much faster for the α_{2A}-receptor than for the α_{2C}-subtype. After prolonged stimulation with norepinephrine, presynaptic α_{2C}-adrenergic receptors were desensitized.

From these data we suggest that two functionally distinct α_2-adrenergic receptor subtypes, α_{2A} and α_{2C}, operate as presynaptic inhibitory receptors regulating neurotransmitter release in the mouse CNS. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: adrenergic receptors, presynaptic receptor, dopamine, norepinephrine, transgenic mouse.

Presynaptic receptors are essential regulators of signal transmission in the nervous system. These receptors are located in the presynaptic membrane in close proximity to the neurotransmitter release site and they are activated by transmitter which has been released into the synaptic cleft. Receptors which are located presynaptically and modulate the release of their own neurotransmitter are termed autoreceptors, those which regulate release of other transmitters are heteroreceptors (Starke et al., 1989). Within the adrenergic system, presynaptic β-receptors enhance norepinephrine release while α_2-receptors inhibit further transmitter exocytosis (Starke et al., 1989). Molecular cloning has led to the identification of three independent genes encoding α_2-adrenergic receptor subtypes, termed α_{2A}, α_{2B}, and α_{2C} (Ruffolo et al., 1993). As the available pharmacological ligands lack sufficient subtype selectivity, it has been difficult to assign unequiv-

ocally biological functions to individual α_2-adrenergic receptor subtypes. As an alternative to using pharmacological ligands, molecular genetic techniques have been used to inactivate or modify the genes of the α_2-receptor subtypes in embryonic stem cells from which mouse lines were established which are deficient in individual α_2-receptor subtypes (Link et al., 1995, 1996; MacMillan et al., 1996; Altman et al., 1999). These mice have recently been successfully used to assign specific functions of non-selective α_2-receptor agonists to individual α_2-receptor subtypes (for reviews, see MacDonald and Scheinin, 1995; Hein, 2001).

In mice lacking the α_{2A}-receptor, presynaptic autoinhibition by endogenous norepinephrine or α_2-receptor agonists was significantly blunted but not abolished (Altman et al., 1999). In sympathetic neurons, two α_2-receptors, α_{2A} and α_{2C}, were found to inhibit release of norepinephrine (Hein et al., 1999). These data leave open four major questions. First, whether α_{2C}-receptors are up-regulated in mice lacking α_{2A}-receptors or whether they are co-expressed with α_{2A}-receptors in wild-type mice. The second question is whether also in the CNS the same receptors serve as inhibitory α_2-autoreceptors and third, whether the release of other transmitters by α_2-heteroreceptors follows the same principles. Finally, we set out to determine whether presynaptic α_2-adrenergic receptor subtypes can be functionally distinguished.

EXPERIMENTAL PROCEDURES

Transgenic mouse lines lacking α_2-receptor subtypes

For this study mice carrying targeted deletions of the genes encoding α_{2A}-, α_{2B}-, or α_{2C}-adrenergic receptors (α_2-KO) were used. The generation of α_{2B}-KO and α_{2C}-KO mice has been described previously (Link et al., 1995, 1996). Mice lacking the α_{2A}-receptor subtype have been generated recently (Altman et al., 1999). Mice carrying deletions in α_{2A}- and α_{2C}-receptor genes were generated by crossing single-knockout strains (Hein et al., 1999). Independent lines of homozygous knockout mice and matching wild-type strains were maintained in a specified pathogen-free facility. Genotyping was performed by Southern blot analysis with gene probes which are external to the respective targeting vectors. For experiments, mice at 4–6 months of age were used. All experiments were conducted in accordance with the guidelines for the care and use of animals approved by the University of Würzburg and the responsible government authorities (Regierung von Unterfranken, State of Bavaria). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable data.

Autoradiography and radioligand binding

Mouse brain synaptosomes were prepared as described (Nagy et al., 1976). Synaptosomes were resuspended in binding buffer containing 25 mM glycylglycine, 40 mM HEPES (pH 8), 5 mM EGTA, 5 mM EDTA, 5 mM MgCl₂, 100 mM NaCl, 8 nM [³H](1,4-benzodioxan-2-methoxy-2-yl)2-imidazoline hydrochloride (RX821002) (MacMillan et al., 1996). Non-specific binding was determined in the presence of 1 μM atipamezole. Receptor autoradiography was performed in brains from 4-month-old mice. Brains were frozen in isopentane cooled to −40°C with liquid nitrogen, and stored at −80°C. Transverse sections (10 μm) were serially cut with a cryostat, thaw-mounted onto slides and incubated for 60 min in 50 mM Tris–HCl (pH 7.5), 1.5 mM EDTA, 8 nM [³H]RX821002. To detect non-specific binding, 1 μM atipamezole was included. Incubations were carried out in quadruplicate. Following incubations, the slides were washed 2×5 min in cold buffer, rinsed in distilled water, air-dried, and exposed to ³H-Hyperfilm (Amersham Pharmacia, Freiburg, Germany) for 18 weeks.

[³H]Norepinephrine release

[³H]Norepinephrine release of brain cortex was determined as described previously (Limberger et al., 1995; Altman et al., 1999; Trendelenburg et al., 2001). In brief, round brain cortex slices (2 mm diameter) were incubated with 0.1 μM [³H]norepinephrine (Amersham Pharmacia) in physiological buffer for 30 min. Tissue pieces were then superfused for 60 min with [³H]norepinephrine-free medium containing 1 μM desipramine at 1.8 ml/min. The superfusion medium contained the following components (mM): NaCl 118, KCl 4.7, CaCl₂ 1.3, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03. Transmitter release was elicited by rectangular electrical pulses of 2 ms duration and 20 V/cm voltage, giving a current strength of 60 mA. There were six periods of stimulation in each experiment with intervals of 20 min. For experiments with exogenous agonists (5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine [UK14,304] or norepinephrine; Fig. 1), tissues were stimulated with single trains of four pulses at 100 Hz. To mimic autoinhibition conditions (Fig. 2), each stimulation period consisted of either one, two or 64 trains (train interval 1 s) of four pulses at 100 Hz. In order to inhibit remaining presynaptic α_2-adrenergic receptors and to determine the degree of endogenous autoinhibition, the α_2-receptor antagonist rauwolscine (1 μM) was added to the superfusion medium 10 min before an electrical stimulation period. The results obtained with rauwolscine in wild-type specimens were identical to those obtained with the α_2-receptor antagonist atipamezole. For determination of the time course of onset of presynaptic inhibition, release was elicited with one

to 31 rectangular pulses at 1 Hz or 3 Hz (Fig. 5). Drugs were added to the superfusion media 15 min before electrical stimulation.

For desensitization experiments, mouse atria were incubated with [³H]norepinephrine in the presence of 1 μM rauwolscine (to avoid activation of presynaptic α_2-receptors during the loading period). Subsequently, atria were transferred to the superfusion chamber and superfused for 30 min with physiological buffer without or with 10 μM norepinephrine. Fifteen minutes after treatment with norepinephrine, 1 μM UK14,304 was added to the superfusion to inhibit electrically induced release of [³H]norepinephrine. At the end of the experiments, tissues were processed as described below.

[³H]Dopamine release

[³H]Dopamine release of basal ganglia was determined similar to the [³H]norepinephrine release. Basal ganglia were punched out from frontal sections of the mouse brain (Mayer et al., 1988). Tissue slices were preincubated for 60 min at 37°C in 1 ml medium containing 0.3 μM of [³H]dopamine (Amersham Pharmacia). Slices were then placed in a superfusion chamber ($t = 0$ min) and superfused for 60 min with tritium-free medium containing 1 μM nomifensine at a rate of 1 ml/min. There were eight periods of electrical stimulation. Stimulation consisted of rectangular pulses of 2 ms width and 30 V/cm voltage drop between the electrodes of each chamber. The first two stimulation periods at $t = 20$ min and $t = 40$ min with 20 pulses at 10 Hz were not used for determination of tritium overflow. The subsequent stimulation periods consisted of 20 pulses at 10 Hz for the UK14,304 experiment and of five, 10 or 50 pulses at 20 Hz for the determination of endogenous inhibition by released transmitter. Drugs were added 20 min before stimulation periods. At the end of [³H]norepinephrine and [³H]dopamine release experiments, tissues were solubilized and tritium was determined in superfusate samples and tissues. Electrically evoked overflow of total tritiated compounds was calculated as difference between total tritium outflow and basal outflow and was expressed as a percentage of tissue tritium at the time of stimulation.

Drugs

Rauwolscine HCl, desipramine HCl, nomifensine maleate and UK14,304 were purchased from RBI/Sigma (Deisenhofen, Germany). RX821002 was purchased from Amersham Pharmacia. Atipamezole was provided by Orion (Espoo, Finland).

Statistical analysis

Data displayed show means ± S.E.M. For all experiments, one-way or two-way analysis of variance tests followed by appropriate post-hoc tests or t-tests were used to determine statistical significance ($P < 0.05$) using Prism 2.0 software (GraphPad, San Diego, CA, USA).

RESULTS

Expression of α_2-adrenergic receptor subtypes

In order to determine the localization and density of brain α_2-adrenergic receptors, autoradiography and radioligand binding experiments were performed (Fig. 1). In frontal sections from wild-type brains, α_2-adrenergic receptors were primarily detected in the brain cortex and in the hippocampus (Fig. 1A). Signal intensity was slightly reduced in sections from mice lacking α_{2C}-receptors and was greatly decreased in α_{2A}-KO brains. Only in sections from mice lacking both α_{2A}- and
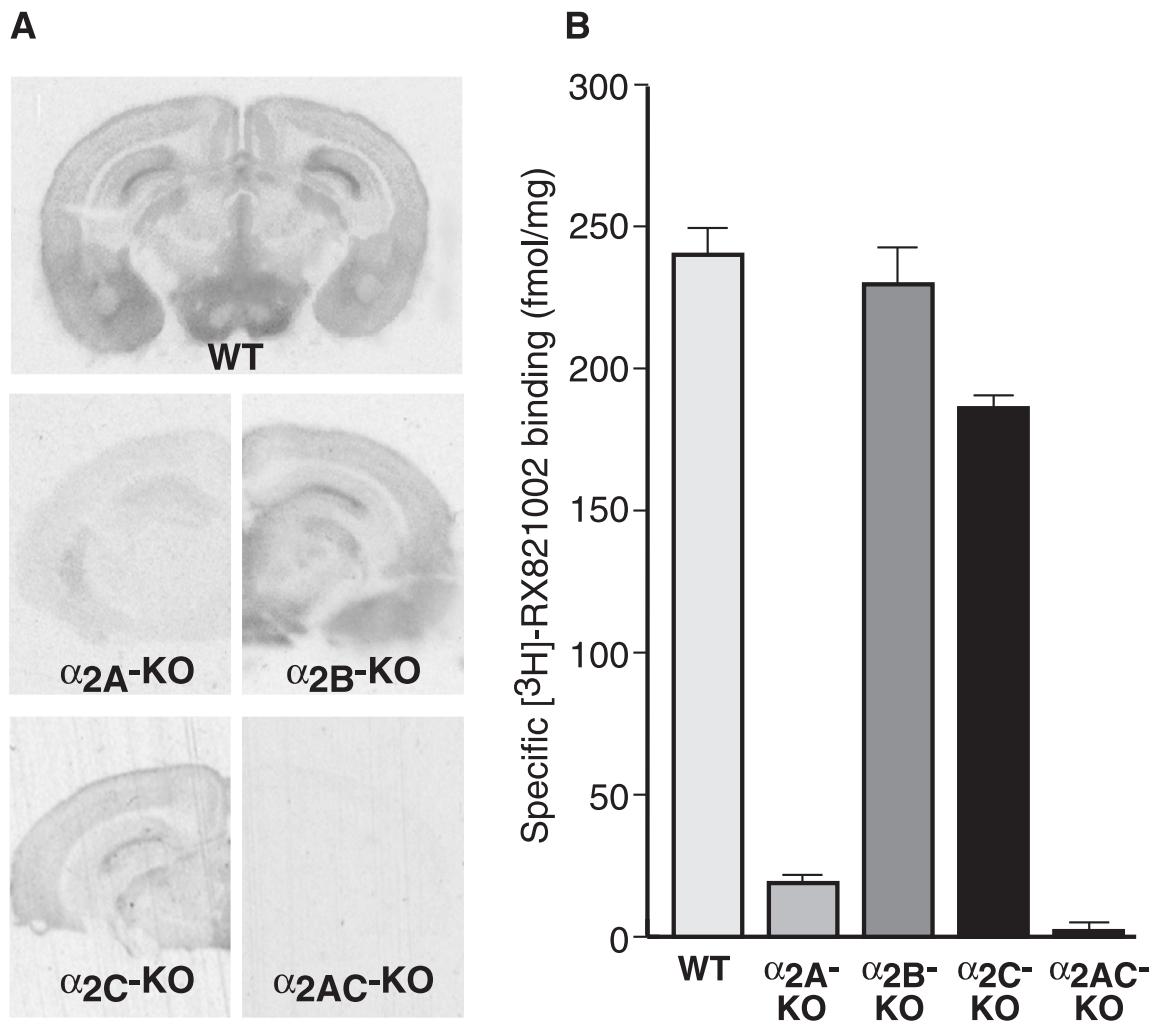
Fig. 1. Expression of α_2-adrenergic receptor subtypes in the brain of α_2-KO mice. (A) Autoradiograms of [^{3}H]RX821002 specific binding in the brain of wild-type (WT) and α_2-receptor knockout mice. In wild-type mouse brain, highest levels of α_2-receptors were expressed in brain cortex and hippocampus. In the presence of the α_2-receptor antagonist atipamezole, the signal was reduced to the background level (data not shown). (B) α_2-Adrenergic receptor density in mouse brain synaptosomes. Total α_2-receptor binding was determined in synaptosomes in the presence of 8 nM RX821002 (fmol/mg membrane protein). For non-specific binding, 1 μM atipamezole was used. In synaptosomes from α_{2A}-KO mice, α_2-receptor density was decreased by 90%, whereas in mice lacking α_{2C}-receptors, a 17% reduction in receptor levels was found. Only in specimens from α_{2AC}-KO mice, no specific α_2-receptor binding could be detected. Data shown are representative autoradiograms from three experiments with slices slices per genotype and radioligand binding data are means ± S.E.M. ($n = 9$).

α_{2C}-receptors, no signal above background could be identified (Fig. 1A). Similar results were obtained with [^{3}H]RX821002 binding in synaptosomes prepared from mouse brains (Fig. 1B). α_2-Receptor density in α_{2A}-deficient brains was decreased to $9 \pm 1\%$ of the respective wild-type value, whereas α_2-receptor levels were reduced to $83 \pm 4\%$ in α_{2C}-deficient mice. These results indicate that approximately 90% of mouse brain α_2-receptors belong to the α_{2A}-receptor subtype and 10% are α_{2C}-receptors. Most importantly, α_{2C}-receptors are expressed in the wild-type brain and are not only up-regulated in knockout mice lacking α_{2A}-receptors.

Norepinephrine release from brain cortex

Small pieces of the parieto-occipital cortex were used to examine the presynaptic regulation of norepinephrine release in wild-type and α_2-receptor-deficient mice. Elec-trical stimulation by single trains of four pulses at 100 Hz elicited release of [^{3}H]norepinephrine (Fig. 2) which was similar in wild-type and α_2-receptor-deficient tissues. Under these conditions, the α_2-receptor antagonist rauwolscine (1 μM) did not increase [^{3}H]norepinephrine release, because the single-pulse trains are too short for significant autoinhibition to develop (Marshall, 1983; Singer, 1988). In wild-type brain cortex, the α_2-agonist UK14,304 caused concentration-dependent inhibition of the norepinephrine release by maximally 96% (Fig. 2A, B). In mice lacking α_{2B}- or α_{2C}-receptors, the effect of the α_2-receptor agonist was not different from wild-type mice. However, in α_{2A}-deficient cortex UK14,304 inhibited transmitter release by 24% (Fig. 2B). Thus the α_{2A}-receptor is the principal autoreceptor regulating norepinephrine release in the brain cortex as has been suggested previously from pharmacological experiments (Limberger et al., 1995). In order to determine which
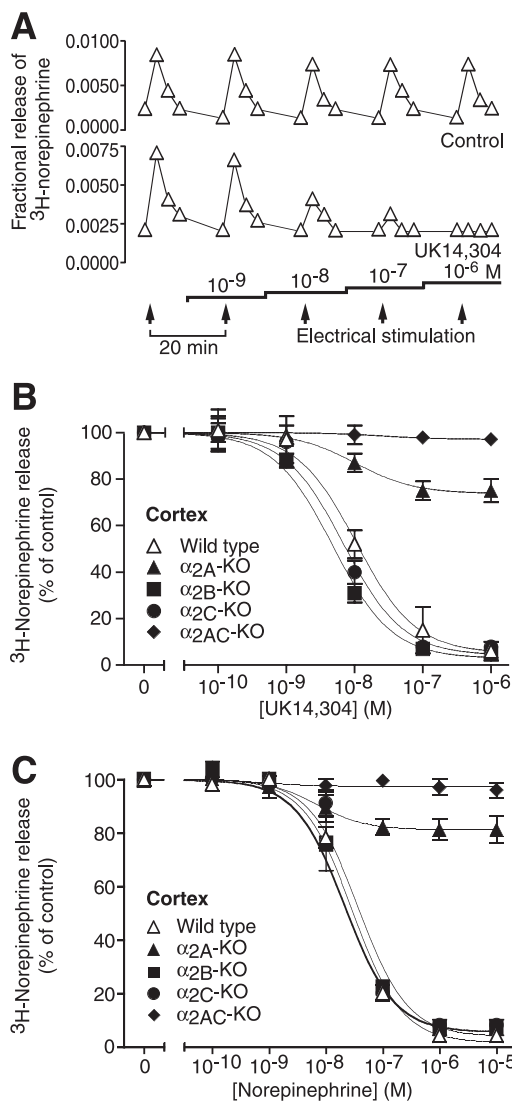
A

B

C

Fig. 2. Inhibition of [³H]norepinephrine release in mouse brain cortex slices from wild-type or α₂-KO mice by the α₂-receptor agonist UK14,304. (A) Tritium efflux-versus-time curves from brain cortex slices of wild-type mice. Cortex slices were stimulated with single trains of four pulses at 100 Hz (arrow) at 20-min intervals. In the absence of agonist, release of [³H]norepinephrine was constant for five stimulation periods (upper trace), but was completely inhibited by addition of the α₂-receptor agonist UK14,304 (lower trace). (B) Concentration–response curves for UK14,304. Release of [³H]norepinephrine could be inhibited by UK14,304 in cortex slices from wild-type mice or animals lacking single α₂-receptor subtypes. No agonist effect was observed in tissues from mice deficient in α₂A- and α₂C-receptor subtypes (α₂AC-KO). (C) Inhibition of norepinephrine release from mouse brain cortex by exogenous norepinephrine. Inhibition of transmitter release did not differ between wild-type, α₂B-KO and α₂C-KO tissues. However, the inhibitory effect of norepinephrine was greatly reduced in the α₂A-deficient mice and was completely absent in mice lacking α₂A- and α₂C-adrenergic receptors. Data are means ± S.E.M. from 8–16 tissue pieces.

subtype is involved in mediating the remaining α₂-receptor agonist effect in α₂A-KO cortex, mouse strains lacking α₂A-, α₂B-, or α₂C-receptor subtypes were crossed to generate mice lacking two receptor subtypes. At present, mice lacking α₂A- and α₂C-receptors (α₂AC-KO) are the only viable animals lacking two α₂-receptor subtypes

that could be generated (Hein et al., 1999). In these mice, the inhibitory effect of UK14,304 was abolished (Fig. 2B) indicating that α₂A- and α₂C-receptors are required for the presynaptic inhibitory feedback loop. The α₂B-subtype does not appear to be involved in this process.

Similar to the α₂-receptor agonist UK14,304, exogenous norepinephrine was also able to inhibit transmitter release in a concentration-dependent manner (Fig. 2C). Compared with wild-type or α₂C-KO cortex, inhibition by norepinephrine was reduced in α₂A-KO cortex and absent in α₂AC-KO tissues (Fig. 2C). In brain cortex from α₂B-KO mice, the inhibitory effect of norepinephrine on transmitter release did not differ from wild-type mice (Fig. 2C).

To test whether endogenous norepinephrine regulates its own release via the same receptor subtypes, tissue specimens were repeatedly stimulated with one to 64 electrical pulses to mimic autoinhibition conditions (Fig. 3A). With increasing number of pulses, the amount of transmitter released from α₂A-KO tissues was significantly higher than from wild-type, α₂B-KO or α₂C-KO
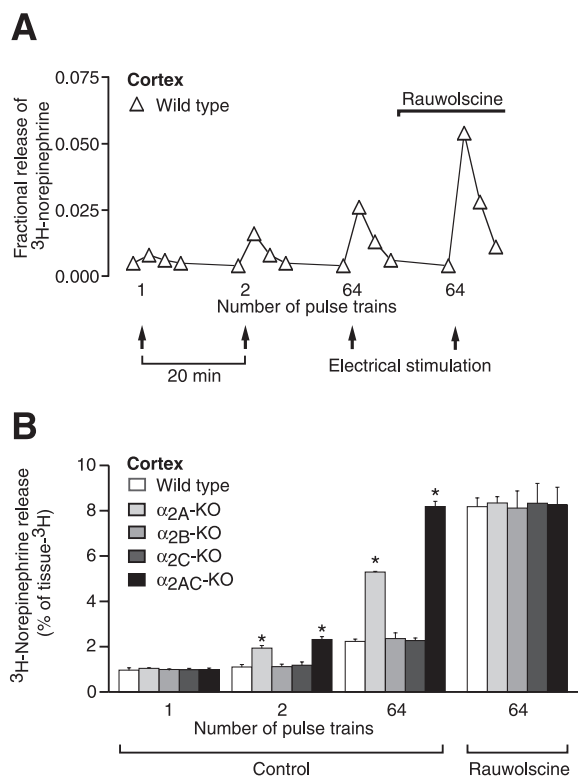
A

B

Fig. 3. Autoinhibition of [³H]norepinephrine release by endogenously released transmitter in brain cortex slices. (A) Representative tritium efflux-versus-time curve from brain cortex of wild-type mice. After loading with [³H]norepinephrine, cortex slices were stimulated with one, two, or 64 pulse trains at 1 Hz (four pulses at 100 Hz per train). Blockade of α₂-receptors with the non-subtype-selective antagonist rauwolscine (1 μM) increased release elicited by 64 pulse trains significantly. (B) [³H]Norepinephrine release in brain cortex from wild-type, α₂A-KO, α₂B-KO, α₂C-KO, and α₂AC-KO mice (n = 8–16 tissue slices). Transmitter release was significantly higher in specimens from α₂A-KO or α₂AC-KO compared with wild-type mice (P < 0.05), indicating lack of presynaptic α₂-autoinhibition in these mice.
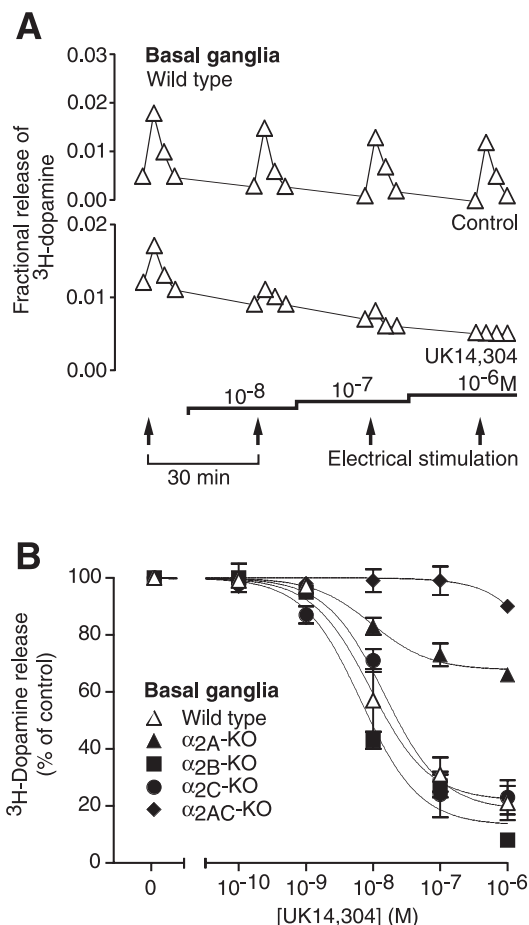
Fig. 4. Inhibition of dopamine release from basal ganglia of wild-type and α2-KO mice. Basal ganglion specimens were incubated with [3H]dopamine and release of dopamine was elicited by single electrical pulse trains. (A) Representative tritium efflux curves from basal ganglia of wild-type mice. In the absence of α2-receptor agonist, the amount of released transmitter remained constant over four stimulation periods (upper trace), but it was completely inhibited by addition of the α2-receptor agonist UK14,304 (lower trace). (B) The α2-receptor agonist UK14,304 inhibited dopamine release in basal ganglia from wild-type mice and mice lacking single α2-receptor subtypes. The maximal inhibitory effect of UK14,304 was greatly reduced in α2A-KO mice, and there was no agonist effect in α2AC-KO mice. Data are means ± S.E.M. from nine to 12 tissue slices per genotype.

tissues (Fig. 3B). In α2AC-KO cortex, norepinephrine release was even higher than in α2A-deficient specimens and reached the same magnitude as pharmacological inhibition of α2-receptors with the antagonist rauwolscine (Fig. 3B). Thus, α2A- and α2C-receptors can both inhibit release of endogenous norepinephrine under autoinhibition conditions.

α2-Heteroreceptors regulating dopamine release

Physiological and pharmacological experiments suggest that α2-adrenergic receptors inhibit presynaptic release of a number of other neurotransmitters, including dopamine, serotonin and acetylcholine (Starke et al., 1989). To determine whether α2-heteroreceptor subtypes differ from the subtypes operating as autoreceptors, release of [3H]dopamine was measured in small speci-

mens of basal ganglia from wild-type and α2-receptor-deficient mice. When basal ganglia were preincubated with [3H]dopamine, electrical stimulation with short pulse trains elicited release of dopamine (Fig. 4A). The amount of transmitter released by short pulse trains did not differ between wild-type and α2-receptor-deficient tissues. Uptake or release of [3H]dopamine was not changed in the presence of the norepinephrine transport inhibitor desipramine (data not shown). In non-transgenic basal ganglia, the α2-receptor agonist UK14,304 inhibited dopamine release in a concentration-dependent manner by maximally 79% (Fig. 4A, B). The inhibitory effect of the α2-receptor agonist was not changed in α2B- or α2C-receptor-deficient tissues when compared with wild-type basal ganglia. However, α2-receptor-mediated inhibition was greatly reduced in α2A-KO basal ganglia and it was completely absent in specimens from α2AC-KO mice (Fig. 4B). Thus, similar to the α2-autoreceptors regulating norepinephrine release in the brain cortex, dopamine release from basal ganglia is modulated by the two receptor subtypes α2A and α2C.

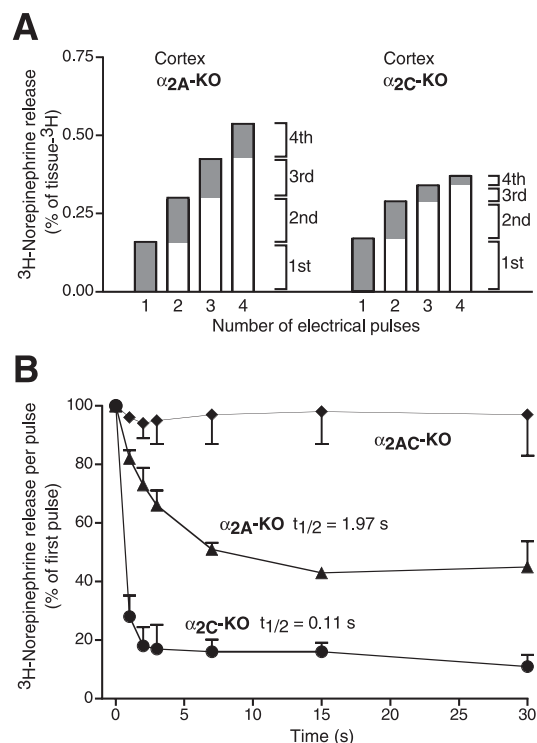Fig. 5. Time course of the onset of presynaptic inhibition of [3H]norepinephrine release in mouse brain cortex by α2A- and α2C-receptors. (A) Transmitter release was stimulated by one, two, three, or four sequential electrical pulses (interval 1 s) in α2A-KO and α2C-KO tissues. The magnitude of transmitter release during the first, second, third and fourth pulse is indicated by the shaded area. Data shown are from a representative experiment. (B) Time course of the transmitter release per pulse from α2A-KO, α2C-KO and α2AC-KO cortex. The time course of presynaptic inhibition was determined at 1 Hz stimulation frequency. In tissues from α2AC-KO mice, the amount of transmitter released during up to 31 sequential electrical pulse trains remained constant, indicating the absence of presynaptic inhibition. In α2C-KO cortex, presynaptic inhibition was much faster than in α2A-KO tissue. Data are means ± S.E.M. from four to eight experiments.

Time course of feedback inhibition by α$_{2A}$- and α$_{2C}$-receptors

To test whether α$_{2A}$- and α$_{2C}$-receptors are functionally different, the time course of the onset of presynaptic inhibition of norepinephrine release in brain cortex was determined. For this purpose, tissue slices were stimulated with one to 31 single electrical pulses at 1 Hz and release of transmitter during each pulse was calculated as illustrated in Fig. 5A. To determine transmitter release elicited by the *n*-th pulse, the amount of released transmitter by (*n*−1) pulses was subtracted from the amount released by *n* pulses. With one or two electrical pulses, norepinephrine release was similar for α$_{2A}$-KO and α$_{2C}$-KO cortex (Fig. 5A). However, the amount of norepinephrine released during the following pulses declined much faster in α$_{2C}$-KO tissues than in α$_{2A}$-KO tissues. Half-maximal inhibition occurred within 0.11 s in the α$_{2C}$-KO but required 1.97 s in α$_{2A}$-KO (Fig. 5B). The time course of inhibition did not depend on the stimulation frequency as the half-times for inhibition did not differ significantly between 3 Hz and 1 Hz (data not shown). Transmitter release from α$_{2AC}$-KO cortex did not change between one and 31 pulses, indicating the absence of endogenous autoinhibition. These results demonstrate that the α$_{2A}$-receptor system operates at higher speed to inhibit transmitter release than the α$_{2C}$-subtype.

Desensitization of presynaptic α$_2$-adrenergic receptors

In order to address whether presynaptic α$_2$-receptors are subject to desensitization upon prolonged stimulation, tissues were incubated with 10 μM norepinephrine for 30 min before the inhibitory effect of the α$_2$-receptor agonist UK14,304 on transmitter release was determined (Fig. 6). In brain cortex slices, no effect of norepinephrine pretreatment on the inhibitory effect of UK14,304 could be detected (data not shown). However, in isolated mouse atria 30 min incubation with norepinephrine attenuated the effect of UK14,304 in specimens from α$_{2A}$-KO mice but not in tissues from wild-type mice or other genotypes (Fig. 6).

DISCUSSION

These studies demonstrate that two α$_2$-adrenergic receptor subtypes operate as presynaptic inhibitory receptors regulating transmitter release. Both α$_{2A}$- and α$_{2C}$-receptor subtypes were found to inhibit norepinephrine release in the brain cortex and dopamine release from basal ganglia. Presynaptic inhibition by the α$_{2A}$-receptor subtype occurred significantly faster than signalling by the α$_{2C}$-receptor system. Upon prolonged exposure to agonist, α$_{2C}$-receptors were subject to desensitization.

Previous pharmacological experiments have identified the α$_{2A}$-adrenergic receptor as the principal inhibitory autoreceptor in adrenergic neurons (Trendelenburg et al., 1993). In gene-targeted mice lacking single α$_2$-receptor subtypes, presynaptic feedback inhibition was
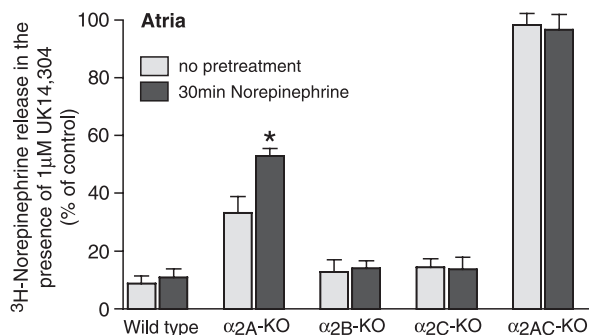

Fig. 6. Desensitization of presynaptic α$_2$-adrenergic receptors in sympathetic nerves after incubation with norepinephrine. Mouse atria were incubated with [³H]norepinephrine followed by 30 min incubation in the presence or absence of 10 μM norepinephrine. Electrically induced release of [³H]norepinephrine was determined in the presence of 1 μM UK14,304. After norepinephrine stimulation, the inhibitory effect of the α$_2$-receptor agonist UK14,304 was significantly attenuated in atria from α$_{2A}$-KO mice, but not in tissues from wild-type mice or animals lacking α$_{2B}$- or α$_{2C}$-receptors. Data are means ± S.E.M. from six to seven experiments.

reduced in tissues from α$_{2A}$-KO mice (Altman et al., 1999; Hein et al., 1999; Trendelenburg et al., 1999). The presence of additional or other α$_2$-receptor subtypes was previously suggested based on experiments with somewhat subtype-selective ligands (Ho et al., 1998). However, due to the lack of sufficiently selective antagonists, it was impossible to identify unequivocally the nature of the second subtype. By selective deletion of the three α$_2$-receptor subtypes in mice, the α$_{2C}$-receptor subtype could be identified as a second inhibitory presynaptic receptor in sympathetic neurons (Hein et al., 1999). However, previous experiments did not distinguish whether α$_{2A}$- and α$_{2C}$-receptors are present in wild-type neurons, or whether up-regulation of one receptor subtype compensates for loss of the other subtype in knockout mice.

Several lines of evidence suggest that α$_{2A}$- and α$_{2C}$-receptors operate also in wild-type mice as integral parts of the presynaptic feedback loop. Autoradiography and radioligand binding experiments (Fig. 1) indicate that both α$_{2A}$- and α$_{2C}$-receptors are present in normal mouse brain, although α$_{2C}$-receptors seem to be expressed at 10-fold lower levels than α$_{2A}$-receptors. Experiments on peripheral tissues, e.g. heart atria, demonstrate that the α$_{2C}$-receptor subtype mediates autoinhibition by low concentrations of norepinephrine in wild-type mice, whereas the potency of norepinephrine at the α$_{2A}$-receptor subtype was lower (Hein et al., 1999). This potency difference of norepinephrine for the α$_2$-receptors correlates with the affinity difference of norepinephrine for the α$_{2A}$- and α$_{2C}$-receptor subtypes, respectively. Furthermore, there is no evidence so far that the expression of the remaining α$_2$-receptor subtypes is altered in mice carrying deletions in α$_{2A}$- or α$_{2C}$-receptor genes (Link et al., 1995; Altman et al., 1999). Our findings confirm and extend the studies of Trendelenburg et al., 2001, who have recently shown in several tissues that inhibitory presynaptic regulation of norepinephrine release is mediated by α$_{2A}$- and α$_{2C}$-receptors.

Two α_2-adrenergic receptor subtypes are also operating as heteroreceptors regulating dopamine release. Similar to the situation in the brain cortex, α_{2A}- and α_{2C}-receptors inhibited the release of dopamine from mouse basal ganglia by exogenous α_2-receptor agonists. Previous studies using pharmacological ligands had shown that the α_{2A}-receptor subtype is the principal α_2-receptor subtype involved in the control of dopamine exocytosis (Trendelenburg et al., 1994). Whereas the α_{2A}-receptor subtype shows a wide distribution in the brain, the α_{2C}-receptor is expressed at much lower densities and is concentrated primarily in basal ganglia, olfactory cells and hypothalamus (Nicholas et al., 1993, 1996; Scheinin et al., 1994; MacDonald and Scheinin, 1995; Talley et al., 1996; Tavares et al., 1996). Radioligand binding to the α_{2C}-receptor occurs in highest density in the striatum, relative to other brain regions (Link et al., 1995). However, the precise cellular localization of the α_{2C}-receptor subtype in basal ganglia is unknown at present. Basal ganglia receive only a sparse innervation by noradrenergic terminals and the total amount of norepinephrine in basal ganglia is much lower than the concentration of stored dopamine (Moore and Card, 1984). It has been demonstrated that activation of α_2-adrenergic receptors by dopamine inhibits adenylyl cyclase in basal ganglia (Zhang et al., 1999), indicating that α_2-receptor stimulation by dopamine may contribute to presynaptic regulation of dopamine exocytosis. Using gene targeting in mice, the role of dopamine D2 and D3 receptors in presynaptic regulation of dopamine release has been recently investigated (Koeltzow et al., 1998; L'hirondel et al., 1998). These mouse models are essential tools to further evaluate the role of dopamine and α_2-adrenergic receptors in regulating endogenous dopamine release. These studies extend the observation that also serotonin release is controlled by these two α_2-adrenergic receptors (Scheibner et al., 2001).

The present studies do not show evidence for a specific role of the α_{2B}-receptor subtype to regulate norepinephrine or dopamine release in the brain cortex or basal ganglia, respectively. In total brain membrane fractions, the abundance of α_{2B}-receptors was too low for detection by radioligand binding (Fig. 1B). However, several groups have reported that mRNA for the α_{2B}-adrenergic receptor was detectable in thalamic nuclei of the rat brain (Nicholas et al., 1993, 1996; Scheinin et al., 1994; MacDonald and Scheinin, 1995). Further studies using knockout mice will have to show whether α_{2B}-receptors are involved in the regulation of neurotransmitter release in the thalamus.

It is interesting to speculate about the physiological relevance of the two presynaptic α_2-receptor subtypes. Inhibition by both subtypes may be physiologically relevant as the time course of the onset of presynaptic inhibition was different for the two subtypes. Inhibition of norepinephrine release by the α_{2A}-receptor was much faster than inhibition by the α_{2C}-receptor subtype. In addition, experimental evidence was obtained that presynaptic α_{2C}-receptors are subject to agonist-dependent desensitization (Fig. 6). In atria from mice lacking the α_{2A}-receptor subtype, stimulation with norepinephrine attenuated the inhibitory effect of an α_2-receptor agonist on transmitter release. No desensitization could be observed in wild-type tissues or specimens from mice lacking α_{2B}- or α_{2C}-receptors. Thus only under conditions of decreased presynaptic receptor reserve (deletion of the α_{2A}-receptor subtype) could desensitization of presynaptic receptors be detected. Interestingly, *in vitro* studies have previously suggested that α_2-receptor subtypes are subject to differential desensitization while some reports have indicated that α_{2C}-receptors cannot be turned off by homologous desensitization (Eason et al., 1992; Eason and Liggett, 1992). However, the present studies clearly indicate that signaling via neuronal α_{2C}-receptors is attenuated after prolonged agonist exposure.

Thus, the presence of two inhibitory presynaptic receptors may improve regulation of neurotransmission. Several mechanisms may contribute to the observed functional difference between α_{2A}- and α_{2C}-receptors. Different intracellular signalling pathways have been identified by which G protein-coupled receptors can inhibit transmitter release (Hille, 1994; Jeong and Ikeda, 2000). Thus, these two α_2-receptor subtypes may be differentially coupled to these pathways. Alternatively, presynaptic α_2-receptors may be targeted to specific domains of the presynaptic membrane. Indeed, all three α_2-adrenergic receptor subtypes show distinct intracellular distribution and agonist-induced trafficking when transfected cells *in vitro* (Daunt et al., 1997; Olli-Lahdesmaki et al., 1999). These transgenic mice lacking individual α_2-receptor subtypes will be instrumental to define further the role of the α_{2A}- and α_{2C}-adrenergic receptors in presynaptic regulation of adrenergic and dopaminergic neurotransmission.

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