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α_2-Adrenoceptors modulating neuronal serotonin release: a study in α_2-adrenoceptor subtype-deficient mice

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1 The release-inhibiting α_2-adrenoceptors of cerebral serotoninergic axons were studied in mice. Slices of the hippocampus or the occipito-parietal cortex from NMRI mice, from mice lacking the $\alpha_{2A/D}$-, the α_{2B}-, the α_{2C}- or both the $\alpha_{2A/D}$- and the α_{2C}-adrenoceptor, and from mice sharing the genetic background of the receptor-deficient animals (WT) were preincubated with [^{3}H]-serotonin and then superfused and stimulated electrically, in most experiments by trains of 8 pulses at 100 Hz.

2 The concentration-response curves of the α_2-adrenoceptor agonist medetomidine were virtually identical in hippocampal slices from NMRI and WT mice, with maximally 70% inhibition and an EC_{50} of about 2 nM. In hippocampal slices from NMRI mice, phentolamine and rauwolscine were equipotent antagonists against medetomidine.

3 The effect of medetomidine was greatly reduced, with maximally 20% inhibition, in hippocampal slices from $\alpha_{2A/D}$-adrenoceptor-deficient mice; was slightly reduced, with maximally 59% inhibition, in hippocampal slices from α_{2C}-adrenoceptor-deficient mice; was not changed in hippocampal slices from α_{2B}-adrenoceptor-deficient mice; and was abolished in hippocampal slices from mice lacking both the $\alpha_{2A/D}$- and the α_{2C}-adrenoceptor.

4 Similar results were obtained in: (i) occipito-parietal slices from NMRI and $\alpha_{2A/D}$-adrenoceptor-deficient mice and (ii) hippocampal slices that were preincubated with [^{3}H]-serotonin in the presence of oxaprotiline to rule out cross-labelling of noradrenergic axons.

5 The serotoninergic axons of the mouse brain possess both $\alpha_{2A/D}$-heteroreceptors, which predominate, and α_{2C}-heteroreceptors but lack α_{2B}-adrenoceptors. The situation resembles the coexistence of $\alpha_{2A/D}$- and α_{2C}-autoreceptors but lack of α_{2B}-autoreceptors at the noradrenergic axons of mice.

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Abbreviations: KO, knockout; MK 912, (2*S*, 12b*S*) 1′,3′-dimethylspiro (1, 3, 4, 5′, 6, 6′, 7, 12b-octahydro-2H-benzo[b]furo [2, 3-a] quinolizine)-2,4′-pyrimidin-2′-one HCl; WT, wildtype

Introduction

The release of serotonin in the brain is reduced when α_2-adrenoceptors are activated, which are presumably located on the serotoninergic terminal axons (Starke & Montel, 1973; Frankhuyzen & Mulder, 1980; Göthert & Huth, 1980; Göthert *et al*., 1981). These presynaptic α_2-adrenoceptors, often called α_2-heteroreceptors to distinguish them from the α_2-autoreceptors of noradrenergic neurones, have been found in several brain areas and several species including man (reviewed by Mongeau *et al*., 1997; humans: Raiteri *et al*., 1990; Feuerstein *et al*., 1993).

When the three genetically distinct α_2-adrenoceptor subtypes $\alpha_{2A/D}$, α_{2B} and α_{2C} became known, the question arose to which subtype the α_2-heteroreceptors at the serotoninergic axons belonged; the $\alpha_{2A/D}$-adrenoceptors are orthologous receptors, the pharmacological properties of which differ between species, the pharmacological α_{2A} version occurring for example in humans and rabbits and the α_{2D} version in rats and mice (see Bylund *et al*., 1994). Experiments using series of antagonists initially showed that the receptors were not α_{2B} (Gobbi *et al*., 1990; Maura *et al*., 1992), and later that they were at least predominantly α_{2A} in the rabbit and α_{2D} in the rat (Trendelenburg *et al*., 1994). They thus obeyed the rule that all mammalian presynaptic α_2-adrenoceptors, autoreceptors as well as heteroreceptors, belonged predominantly to the $\alpha_{2A/D}$ group of orthologous receptors (see Starke *et al*., 1995).

Recently, $\alpha_{2A/D}$-, α_{2B}- and α_{2C}-adrenoceptor-deficient mice were generated by disruption of the respective genes. Experiments with these mice confirmed that the presynaptic α_2-autoreceptors of noradrenergic neurones were mainly $\alpha_{2A/D}$, but showed that non-$\alpha_{2A/D}$-autoreceptors existed as well (Altman *et al*., 1999; Trendelenburg *et al*., 1999). In a further step, $\alpha_{2A/D}$-adrenoceptor- and α_{2C}-adrenoceptor-deficient mice were crossed to produce animals lacking both subtypes. Studies on these animals demonstrated that the non-$\alpha_{2A/D}$-autoreceptors were α_{2C} (Hein *et al*., 1999).

We have now used $\alpha_{2A/D}$-adrenoceptor-deficient mice (α_{2A}KO), α_{2B}-deficient mice (α_{2B}KO), α_{2C}-deficient mice (α_{2C}KO) and the both $\alpha_{2A/D}$- and α_{2C}-adrenoceptor-deficient

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animals of Hein *et al*. (1999) (α_{2AC}KO) to re-examine the α_2-heteroreceptors at the serotoninergic axons of the brain. Slices of the hippocampus or the occipito-parietal cortex were preincubated with [^{3}H]-serotonin, and release of [^{3}H]-serotonin was then elicited by electrical stimulation. Medetomidine was used as a selective α_2-adrenoceptor agonist.

Methods

Animals, tissues and superfusion

Male Naval Medical Research Institute (NMRI) mice, male α_{2A}KO, α_{2B}KO and α_{2C}KO mice (Altman *et al*., 1999), male or female α_{2AC}KO mice (Hein *et al*., 1999), and male mice of the wildtype strain from which the KO animals were generated (C57BL/6 × 129Sv; WT) were killed at the age of at least 2 months. Six to seven round slices of the occipito-parietal cortex (Limberger *et al*., 1995b) and 20–30 frontal slices, 0.3 mm thick, of the hippocampus were obtained from each animal. The occipito-parietal and hippocampal slices were preincubated for 45 min at 37°C in 4-ml volumes of medium containing 0.1 μM [^{3}H]-serotonin. They were then washed with five 3-ml volumes of [^{3}H]-serotonin-free medium and transferred to 12 superfusion chambers equipped with platinum electrodes, one slice (occipito-parietal cortex) or 2–3 slices (hippocampus) per chamber, where they were superfused with [^{3}H]-serotonin-free medium at a rate of 1.2 ml min^{-1}. Successive 2-min superfusate samples were collected from $t = 50$ min onwards ($t = 0$ being the start of superfusion). At the end of experiments tissues were dissolved and tritium was determined in superfusate samples and slices.

The superfusion medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03 and nitroquipazine 0.001. Unless stated otherwise, the preincubation medium was the same but nitroquipazine-free.

Stimulation and drug application

Seven periods of electrical stimulation were applied. Each consisted of rectangular pulses of 1 ms width and a current strength of 60 mA, which yielded a voltage of 20–30 V between the electrodes of each chamber. The first stimulation (18 pulses at 3 Hz) was delivered at $t = 30$ min and was not used for the determination of tritium overflow. The subsequent six stimulation periods differed, depending on the type of experiment.

In the first type of experiment (hippocampus only), various pulse patterns were applied as follows: a single pulse at $t = 54$ and 126 min (S_1 and S_4); eight pulses at 100 Hz at $t = 72$ and 144 min (S_2 and S_5); and 360 pulses at 3 Hz at $t = 90$ and 162 min (S_3 and S_6). Drug effects were determined by addition of the drug at $t = 96$ min, i.e. 30 min before S_4, at a fixed concentration.

In the second type of experiment (hippocampus and occipito-parietal cortex), which was used predominantly, each stimulation period consisted of a train of 8 pulses/100 Hz. Stimulation periods were applied at $t = 54$ (S_1), 72 (S_2), 90 (S_3), 108 (S_4), 126 (S_5) and 144 min (S_6). Concentration-response curves for medetomidine were determined by addition of the agonist at increasing concentrations 12 min before S_2, S_3, S_4, S_5 and S_6. Phentolamine and rauwolscine, when tested as antagonists against medetomidine, were present throughout superfusion.

Evaluation

The outflow of tritium was calculated as a fraction of the tritium content of the tissue at the onset of the respective collection period (fractional rate; min^{-1}). The overflow elicited by electrical stimulation was calculated as the difference 'total tritium outflow during the 4 min (one pulse, eight pulses at 100 Hz) or 34 min (360 pulses at 3 Hz) after onset of electrical stimulation' minus 'basal outflow'; basal tritium outflow was assumed to decline linearly from the 2-min period before to the 2-min period after these stimulation peaks. The evoked overflow was then expressed as a percentage of the tritium content of the tissue at the onset of stimulation. For further evaluation, overflow ratios were calculated: S_4/S_1, S_5/S_2 and S_6/S_3 (pairs with identical stimulation conditions) for the first type of experiment and S_2/S_1, S_3/S_1, S_4/S_1, S_5/S_1 and S_6/S_1 for the second. Overflow ratios were also calculated as a percentage of the average corresponding ratio from controls in which no drug was applied after S_1. The basal efflux of tritium was evaluated similarly, with calculation of ratios of fractional outflow rates such as b_2/b_1 (b_2 being efflux immediately before S_2 and b_1 efflux immediately before S_1).

Where possible, concentration-response curves of medetomidine, were evaluated by logistic curve fitting (equation no. 25 of Waud, 1976), using either the data for 'medetomodine given alone' or, in a simultaneous fit, the data for 'medetomidine given alone' and 'medetomidine in the presence of phentolamine or rauwolscine', essentially as described previously (Trendelenburg *et al*., 1997). The calculation yielded the maximal effect (E_{max}) and EC_{50} of medetomidine given alone as well as its EC_{50} in the presence of an antagonist. Apparent antagonist pK_d values were calculated from the antagonist-induced EC_{50} increase. The pK_d values are *apparent* because the competitive character of the interaction was not verified.

Results are expressed as arithmetic means ± s.e.mean except in the case of E_{max} and EC_{50} (means ± s.e. as defined by Waud, 1976). Groups were compared by the Mann-Whitney test with Bonferroni correction if Kruskal-Wallis analysis indicated a significant difference. $P < 0.05$ was taken as the limit of statistical difference. n Represents the number of superfusion chambers. n Superfusion chambers contained slices from approximately $n/6$ to n animals.

Materials

5-[1,2-^{3}H(N)]-Hydroxytryptamine creatinine sulphate, specific activity 21.4–24.0 Ci mmol^{-1} (NEN, Köln, Germany); tetrodotoxin citrate (Biotrend, Köln, Germany); (+)-oxaprotiline HCl, phentolamine HCl (Ciba-Geigy, Basel, Switzerland); (2S, 12bS) 1′,3′-dimethylspiro (1, 3, 4, 5′, 6, 6′, 7, 12b-octahydro-2H-benzo[b]furo[2,3-a]quinolizine)-2,4′- pyrimidin-2′-one HCl (MK 912; MSD Sharp & Dohme, München, Germany); medetomidine HCl (Orion, Espoo, Finnland); (±)-idazoxan HCl (Reckitt & Colman, Kingston-upon-Hull, U.K.); methiothepin mesylate, 6-nitroquipazine maleate, rauwolscine HCl (Sigma, Deisenhofen, Germany). Drugs

were dissolved in distilled water except MK 912 (10 mM HCl).

Results

Evoked tritium overflow: stimulation conditions

In an initial series of experiments, hippocampal slices preincubated with [^{3}H]-serotonin were exposed to various electrical pulse patterns in order to find suitable stimulation conditions. Single pulses elicited only very small increases in tritium outflow, averaging 0.20% of tissue tritium for NMRI and 0.19% of tissue tritium for α_{2A}KO mice (see legend to Table 1). Trains of eight pulses at 100 Hz, in contrast, produced acceptable outflow peaks, 0.84 and 0.85% of tissue tritium on average for NMRI and α_{2A}KO mice, respectively, and for trains of 360 pulses at 3 Hz the evoked overflow was even larger (see legend to Table 1). The evoked overflow, in particular that evoked by eight pulses at 100 Hz or 360 pulses at 3 Hz, was greatly reduced by tetrodotoxin (0.3 μM; Table 1) and at most slightly increased by the serotonin receptor antagonist methiothepin (1 μM; Table 1), indicating only a minor degree of 5-HT-receptor-mediated autoinhibition. Pulse trains consisting of eight pulses at 100 Hz were chosen for most of the following experiments.

Evoked tritium overflow: NMRI versus WT hippocampus

Repeated stimulation by eight pulses at 100 Hz, without drug administration after S_1, produced tritium overflow from hippocampal slices that was very similar from S_1-S_6, irrespective of the mouse strain and irrespective of the absence or presence of antagonists (filled circles in Figure 1). In one series of experiments, the effect of medetomidine in hippocampal slices from NMRI mice was compared with its effect in hippocampal slices taken from the strain from which the knockout animals were generated (WT); experiments on NMRI and WT animals were carried out strictly in parallel. The overflow of tritium evoked by eight pulses at 100 Hz at S_1, before addition of medetomidine, was identical in the two groups (Table 2; compare NMRI 1 and WT). Medetomidine caused concentration-dependent inhibition, and its effects in the two groups were indistinguishable, whether viewed in efflux-versus-time curves (Figure 1, upper two panels) or concentration-response curves (NMRI and WT curves were superimposed; not shown). The EC_{50} of medetomidine in NMRI hippocampal slices was 1.77 ± 0.05 nM and its E_{max} $69.1\pm0.3\%$ inhibition; the EC_{50} in WT hippocampal slices was 1.64 ± 0.04 nM and the E_{max} $70.4\pm0.3\%$ inhibition. NMRI mice were used for comparison with the transgenic animals in all subsequent experiments.

Evoked tritium overflow: NMRI versus α_{2A}KO, α_{2B}KO, α_{2C}KO and α_{2AC}KO hippocampus

Results obtained in the NMRI mice of this series agreed well with the preceding series, although the overflow evoked by S_1 was slightly lower (Table 2; compare NMRI 2, no drug, with NMRI 1). The EC_{50} of medetomidine was 1.78 ± 0.15 nM and its E_{max} $70.5\pm0.9\%$ inhibition (from concentration-response curve in Figure 2).

Disruption of the any of the α_2-adrenoceptor genes did not affect the overflow response to S_1 (Table 2; compare α_{2A}KO, α_{2B}KO and α_{2C}KO, no drug, with NMRI 2, no drug). In hippocampal slices from α_{2B}KO animals, the effect of medetomidine likewise was as in NMRI slices; the EC_{50} was 1.67 ± 0.17 nM and the E_{max} $69.8\pm1.2\%$ inhibition (Figure 2). In slices from α_{2A}KO and α_{2C}KO animals, however, the effect of medetomidine was reduced. The reduction was slight in α_{2C}KO slices, in which the EC_{50} was 2.70 ± 0.33 nM and the E_{max} $58.7\pm0.7\%$ inhibition (Figure 3). The reduction was marked in slices from α_{2A}KO animals: the concentration-response curve, although apparently not shifted to the right or left, was so flat that fitting of a sigmoid was not possible (Figures 1 and 2). Nevertheless,

Table 1 Evoked tritium overflow from hippocampal slices taken from NMRI and α_{2A}KO mice and preincubated with [^{3}H]-serotonin: drug effects

Drug added before S_4	*Tritium overflow* (% of control) *elicted by* *One pulse*	*Eight pulses at 100 Hz*	*360 pulses at 3 Hz*
−(Control)	100±20	100±5	100±6
	100±18	*100±1*	*100±3*
Tetrodotoxin (0.3 μM)	36±10	17±3*	14±2*
	*16±4**	*12±3**	*12±1**
Methiothepin (1 μM)	160±26	137±6*	144±6*
	97±11	*109±2*	*115±3**
Phentolamine (1 μM)	93±14	111±5	121±1*
	79±10	*102±4*	*92±6*
Idazoxan (1 μM)	96±21	112±4	109±1
	95±19	*93±4*	*80±3**
MK 912 (0.1 μM)	109±16	111±9	109±6
	86±8	*100±3*	*91±4*

Slices were stimulated successively by single pulses (S_1, S_4), trains of eight pulses at 100 Hz (S_2, S_5) and trains of 360 pulses at 3 Hz (S_3, S_6). In slices from NMRI mice, evoked tritium overflow was $0.20\pm0.01\%$ (single pulse; S_1; corresponding to 0.07 nCi on average), $0.84\pm0.02\%$ (eight pulses at 100 Hz; S_2; 0.30 nCi) and $9.4\pm0.3\%$ (360 pulses at 3 Hz; S_3; 3.1 nCi) of tissue tritium ($n=36$ each). In slices from α_{2A}KO mice, evoked tritium overflow was $0.19\pm0.01\%$ (single pulse; S_1; 0.07 nCi), $0.85\pm0.02\%$ (eight pulses at 100 Hz; S_2; 0.31 nCi) and $10.5\pm0.2\%$ (360 pulses at 3 Hz; S_3; 3.6 nCi) of tissue tritium ($n=36$ each). Drugs were added 30 min before S_4. Values represent the evoked overflow, based on S_4/S_1, S_5/S_2 and S_6/S_3 ratios and expressed as a percentage of the corresponding average control ratio without drug application. Each value is the mean±s.e.mean from six superfusion chambers. First lines: NMRI mice; *second lines, figures in italics*: α_{2A}KO mice. Significant differences from control: $^*P<0.05$.

two concentrations of medetomidine still caused significant inhibition in the α_{2A}KO preparations (Figure 2). The greatest inhibition, at a concentration of 0.01 μM, was by 19.8%.

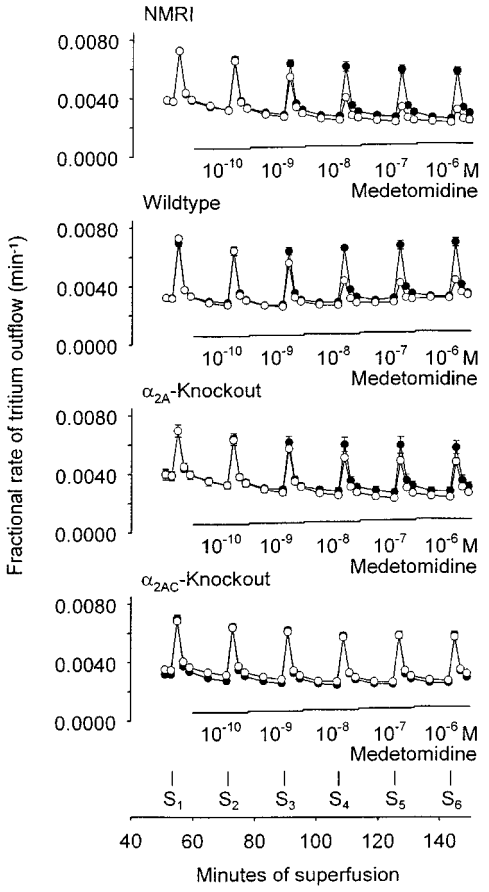

Figure 1 Tritium efflux from hippocampal slices preincubated with [^{3}H]-serotonin and effect of medetomidine. Slices were from NMRI mice, from the strain from which the knockout animals had been generated (wildtype), from α_{2A}-knockout mice or from α_{2AC}-knockout mice. After preincubation, slices were superfused and stimulated six times by eight pulses at 100 Hz (S_1-S_6). Filled circles represent slices that were superfused with medetomidine-free medium throughout. Empty circles represent experiments in which medetomidine (0.1 nM – 1 μM) was added as indicated. Each value is the mean ± s.e.mean from 5 – 10 superfusion chambers.

In hippocampal slices from animals in which both the α_{2A} gene and the α_{2C} gene had been disrupted, the overflow of tritium evoked by S_1 again did not differ from NMRI values (Table 2; compare α_{2AC}KO with NMRI 2, no drug). In these tissues, any inhibitory effect of medetomidine was abolished (Figures 1 and 2).

Effects of phentolamine (0.3 μM) and rauwolscine (0.3 μM) against medetomidine were studied in hippocampal slices from NMRI and α_{2A}KO mice. Phentolamine and rauwolscine shifted the concentration-response curve of medetomidine in slices from NMRI mice to the right with similar potency, the pK_d values being 8.6 and 8.4, respectively (Figure 3a). The flat concentration-response curve of medetomidine in α_{2A}KO

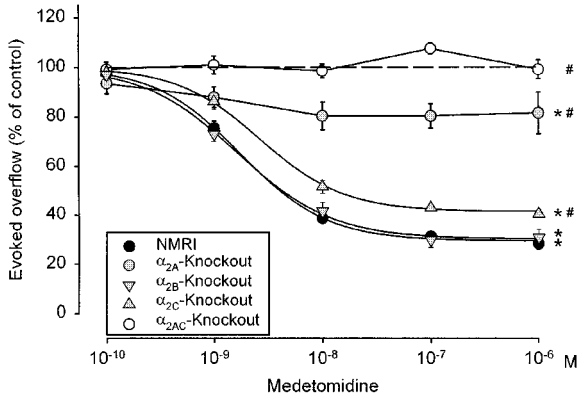

Figure 2 Effect of medetomidine on electrically evoked tritium overflow from hippocampal slices taken from NMRI, α_{2A}-knockout, α_{2B}-knockout, α_{2C}-knockout and α_{2AC}-knockout mice. S_1-S_6 consisted of eight pulses at 100 Hz. Medetomidine was added at increasing concentrations before S_2-S_6. Ordinates, evoked tritium overflow, calculated from S_n/S_1 ratios and expressed as a percentage of the corresponding average control ratio (no medetomidine). Each value is the mean ± s.e.mean from 7 – 11 superfusion chambers. *Indicates significant ($P<0.05$) inhibition in the respective group by 1 nM – 1 μM medetomidine (NMRI, α_{2B}KO, α_{2C}KO) or 10 and 100 nM medetomidine (α_{2A}KO). # Indicates significantly ($P<0.05$) less inhibition, by 1 nM – 1 μM medetomidine, in the respective group than in NMRI mice.

Table 2 Overflow of tritium evoked by eight pulses at 100 Hz

Mouse strain	*Drug present throughout superfusion*	*Evoked tritium overflow (S_1, per cent of tissue tritium)* *Hippocampus*	*Occipito-parietal cortex*
NMRI 1[a]	–	0.87 ± 0.03 (12)	
WT	–	0.87 ± 0.03 (12)	
NMRI 2[b]	–	0.78 ± 0.03 (20)	0.50 ± 0.03 (14)
NMRI	Phentolamine (0.3 μM)	0.94 ± 0.04 (12)*	0.64 ± 0.06 (12)
NMRI	Rauwolscine (0.3 μM)	0.85 ± 0.03 (12)*	0.58 ± 0.05 (12)
α_{2A}KO	–	0.70 ± 0.05 (14)	0.47 ± 0.03 (15)
α_{2A}KO	Phentolamine (0.3 μM)	0.87 ± 0.06 (12)	0.51 ± 0.04 (12)
α_{2A}KO	Rauwolscine (0.3 μM)	0.67 ± 0.03 (12)	0.45 ± 0.04 (20)
α_{2B}KO	–	0.71 ± 0.02 (12)	
α_{2C}KO	–	0.81 ± 0.03 (12)	
α_{2AC}KO	–	0.78 ± 0.03 (19)	

Table summarizes tritium overflow evoked by S_1 from all experiments in which S_1-S_6 consisted of eight pulses at 100 Hz, except experiments in which the influence of the presence of uptake inhibitors during preinculation with [^{3}H]-serotinin was examined. Each value is the mean ± s.e.mean from (n) superfusion chambers. [a]NMRI mice used for comparison with the wildtype (WT). [b]NMRI mice used for comparison with receptor-deficient mice and for study of the interaction of phentolamine and rauwolscine with medetomidine. Significant differences from NMRI 2: *$P<0.05$. All occipito-parietal values are significantly ($P<0.05$) smaller than corresponding hippocampal values.

slices did not permit quantification of antagonist effects. However, phentolamine and rauwolscine obviously antagonized the small effect of medetomidine that remained in the α_{2A}KO preparations ($n=3-11$; data not shown). Phentolamine and rauwolscine, when present throughout superfusion of NMRI hippocampal slices, significantly increased the overflow of tritium elicited by S_1 (Table 2).

Evoked tritium overflow: NMRI versus α_{2A}KO occipito-parietal cortex

As in the hippocampus, S_1-S_6, all eight pulses per 100 Hz, produced overflow peaks of similar magnitude when no drug was added after S_1, irrespective of the mouse strain and irrespective of the absence or presence of antagonists (data not shown). In all groups, the overflow of tritium evoked by S_1, before addition of medetomidine, was lower than in hippocampal slices (Table 2). Medetomidine reduced the evoked overflow of tritium from NMRI occipito-parietal cortex with an EC_{50} of 2.57 ± 0.91 nM and an E_{max} of $58.4\pm3.6\%$ inhibition (Figure 4), similar to NMRI hippocampus.

Disruption of the α_{2A} gene did not change the overflow evoked by S_1 (Table 2; compare α_{2A}KO, no drug, with NMRI 2, no drug). As in the hippocampus, however, the effect of medetomidine was greatly changed: no obvious shift of the concentration-response curve to the right or left but marked flattening, so that a sigmoid could not be fitted to the data. One concentration, 1 μM, still caused significant inhibition by 17.4% (Figure 4).

As in NMRI hippocampal slices, phentolamine (0.3 μM) and rauwolscine (0.3 μM) shifted the concentration-response curve of medetomidine in NMRI occipito-parietal cortex slices to the right to a similar extent, the apparent pK_d values being 7.8 for phentolamine and 8.0 for rauwolscine (Figure 3b). Phentolamine (0.3 μM) and rauwolscine (0.3 μM) also antagonized the small effect of medetomidine that remained in the α_{2A}KO preparations. However, the flatness of the agonist concentration-response curves prevented quantification ($n=3-8$; data not shown). The antagonists, when present throughout superfusion, did not change the overflow of tritium elicited by S_1 (Table 2).

Evoked tritium overflow: effect of α-adrenoceptor antagonists given alone

In NMRI hippocampal slices, phentolamine and rauwolscine, when present throughout superfusion, had increased the overflow of tritium elicited by S_1, as mentioned above (Table 2). Drug effects on the evoked overflow can be better assessed when the drugs are administered after S_n, so that S_n is the internal control for each single preparation. For this reason, phentolamine (1 μM) and the α_2-selective antagonists idazoxan (1 μM) and MK 912 (0.1 μM) were given after S_3 in experiments on NMRI and α_{2A}KO hippocampal slices in which single pulses, eight pulses per 100 Hz and 360 pulses per 3 Hz were applied successively. None of the three drugs changed the overflow elicited by single pulses or eight pulses per 100 Hz. Phentolamine increased the response to 360 pulses per 3 Hz in NMRI hippocampus and idazoxan reduced the response to 360 pulses per 3 Hz in α_{2A}KO hippocampus. Both changes were small (Table 1).

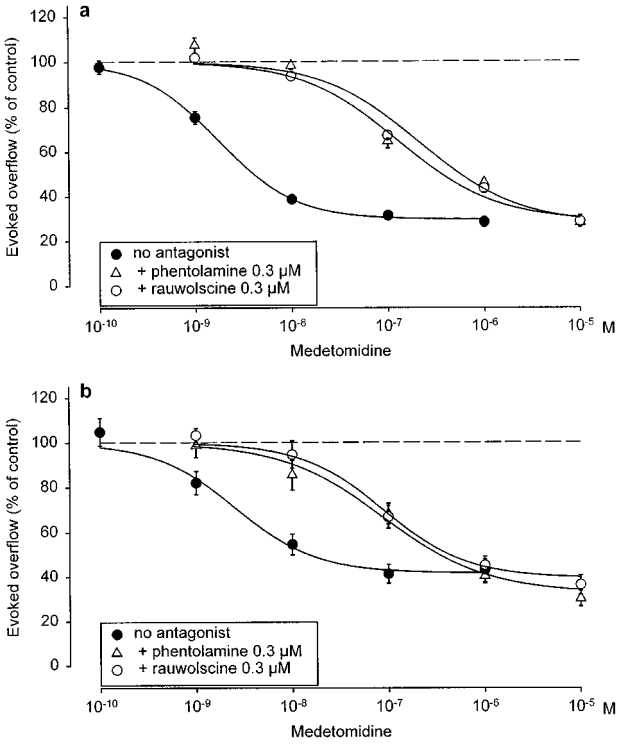

Figure 3 Interaction of medetomidine with phentolamine and rauwolscine on electrically evoked tritium overflow from hippocampal (a) or occipito-parietal cortex slices (b) taken from NMRI mice. S_1-S_6 consisted of eight pulses at 100 Hz. Medetomidine was added at increasing concentrations before S_2-S_6. Phentolamine and rauwolscine were present throughout superfusion. Ordinates, evoked tritium overflow, calculated from S_n/S_1 ratios and expressed as a percentage of the corresponding average control ratio (no medetomidine). The 'no antagonist' curves are (a) from Figure 2 and (b) from Figure 4, respectively. Each value is the mean$\pm$s.e.mean from 3–11 superfusion chambers.

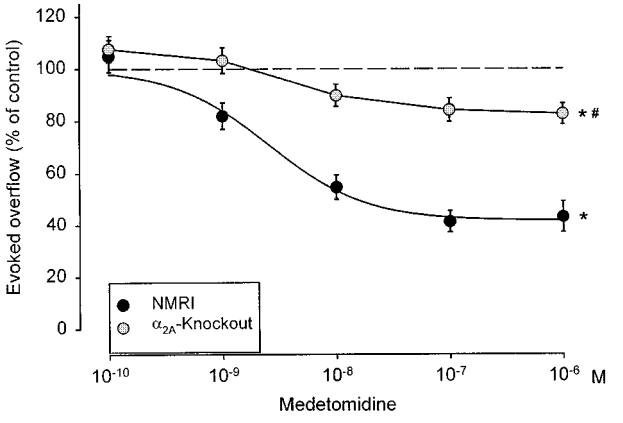

Figure 4 Effect of medetomidine on electrically evoked tritium overflow from occipito-parietal cortex slices taken from NMRI and α_{2A}-knockout mice. S_1-S_6 consisted of eight pulses at 100 Hz. Medetomidine was added at increasing concentrations before S_2-S_6. Ordinates, evoked tritium overflow, calculated from S_n/S_1 ratios and expressed as a percentage of the corresponding average control ratio (no medetomidine). Each value is the mean$\pm$s.e.mean from 5–8 superfusion chambers. *Indicates significant ($P<0.05$) inhibition in the respective group by 1 nM–1 μM medetomidine (NMRI) or 1 μM medetomidine (α_{2A}KO). # Indicates significantly ($P<0.05$) less inhibition, by 1 nM–1 μM medetomidine, in α_{2A}KO than in NMRI mice.

Preincubation with [^{3}H]-serotonin in the presence of uptake inhibitors

A final series of experiments was performed in order to avoid erroneous conclusions due to uptake of [^{3}H]-serotonin into noradrenergic axon terminals during preincubation. For this purpose, hippocampal slices from NMRI or α_{2A}KO mice were preincubated with [^{3}H]-serotonin in the presence of the selective inhibitor of the high affinity noradrenaline transporter, oxaprotiline (to prevent uptake into noradrenergic axons), or the selective inhibitor of the high affinity serotonin transporter, nitroquipazine (to prevent entry into serotoninergic axons). Experiments without uptake inhibitors were carried out in parallel.

NMRI and α_{2A}KO hippocampal preparations that had been preincubated with [^{3}H]-serotonin in inhibitor-free medium contained 66.0 ± 5.6 and 51.4 ± 3.0 nCi of tritiated compounds at the beginning of sample collection, i.e. at $t = 50$ min of superfusion, respectively ($n = 8$ each, $P > 0.05$). Presence of 10 μM oxaprotiline during preincubation with [^{3}H]-serotonin did not significantly reduce these values (NMRI -22.2% and α_{2A}KO -7.8% on average, $n = 8$ each, $P > 0.05$). Presence of 10 μM nitroquipazine during preincubation with [^{3}H]-serotonin, however, caused a significant decrease (NMRI -65.3% and α_{2A}KO -62.3% on average, $n = 8$ each, $P < 0.05$). Tritium overflow elicited by eight pulses per 100 Hz from slices that had been preincubated with [^{3}H]-serotonin in inhibitor-free medium was similar to the preceding series of experiments (see Table 2). Tritium overflow from slices that had been preincubated with [^{3}H]-serotonin in the presence of oxaprotiline or nitroquipazine was slightly but significantly lower ($P < 0.05$; data not shown).

Concentration-response curves of medetomidine in NMRI hippocampal slices are shown in Figure 5a. Experiments on slices preincubated with [^{3}H]-serotonin in inhibitor-free medium yielded results almost identical with those shown in Figure 2; the EC_{50} of medetomidine was 2.25 ± 0.45 nM and its E_{max} $65.9 \pm 2.0\%$ inhibition (filled circles in Figure 6a). When oxaprotiline had been present during preincubation with [^{3}H] serotonin, the concentration-response curve of medetomidine remained unchanged (Figure 5a). When nitroquipazine had been present during preincubation, the curve was shifted slightly to the left and downward, with an EC_{50} of 0.52 ± 0.13 nM and an E_{max} of $79.5 \pm 1.5\%$ inhibition (Figure 6a).

Concentration-response curves of medetomidine in α_{2A}KO hippocampal slices are shown in Figure 5b. Experiments on slices preincubated with [^{3}H]-serotonin in inhibitor-free medium gave results virtually identical with the series shown in Figure 2: a flat concentration-response curve of medetomidine, but significant ($P < 0.05$) inhibition by 0.1 (22.7% inhibition, the largest effect) and 1 μM medetomidine (filled circles in Figure 5b). Presence of oxaprotiline or nitroquipazine during labelling did not change the concentration-response curve of medetomidine (Figure 5b).

Basal tritium outflow

The basal efflux of tritium from hippocampal slices before S_1 (b_1) was similar in the various animal strains (see Figure 1). The same was true for occipito-parietal cortex slices. Two

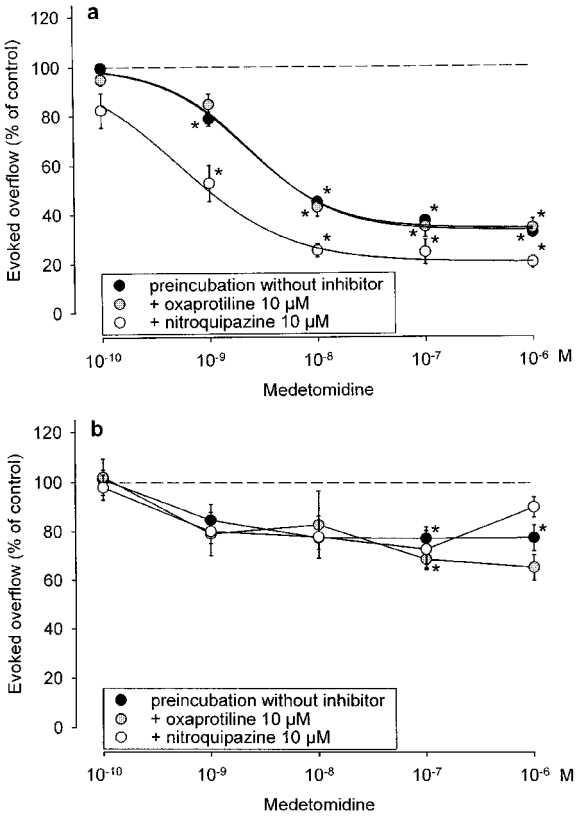

Figure 5 Effect of medetomidine on electrically evoked tritium overflow from hippocampal slices taken from NMRI (a) or α_{2A}KO (b) mice: tritium labelling in the presence of oxaprotiline and nitroquipazine. Slices were first incubated for 30 min with 10 μM oxaprotiline or nitroquipazine and then, in the continued presence of oxaprotiline or nitroquipazine, with [^{3}H]-serotonin. Subsequently they were superfused with the usual medium containing nitroquipazine (1 μM). Otherwise identical experiments on slices preincubated with [^{3}H]-serotonin in the absence of oxaprotiline and nitroquipazine were carried out in parallel. S_1-S_6 consisted of eight pulses at 100 Hz. Medetomidine was added at increasing concentrations before S_2-S_6. Ordinates, evoked tritium overflow, calculated from S_n/S_1 ratios and expressed as a percentage of the corresponding average control ratio (no medetomidine). Each value is the mean $\pm$ s.e.mean from four superfusion chambers. *Indicates significant ($P < 0.05$) inhibition by medetonidine.

drugs tended to cause changes. Tetrodotoxin (0.3 μM) tended to reduce basal tritium outflow from NMRI and α_{2A}KO hippocampal slices (experiments of Table 1). Medetomidine (0.1 nM – 1 μM) also tended to reduce basal efflux, except in slices from α_{2AC}KO mice; the effect seemed to be attenuated by α-adrenoceptor antagonists. However, only some of these tendencies reached statistical significance.

Discussion

Action potential-evoked release of [^{3}H]-serotonin from mouse brain preparations has rarely been studied (e.g. Figueroa *et al*., 1985; Nakazi *et al*., 2000). Therefore, in initial experiments we established parameters of electrical stimulation which (i) gave well measurable tritium overflow peaks and (ii) triggered release that was largely sensitive to tetrodotoxin and (iii) not subject to major 5-HT-receptor-mediated autoinhibition: the latter because ongoing presynaptic autoinhibition

might minimize any α_2-adrenoceptor-mediated inhibition due to the well-known interaction between presynaptic inhibitory receptors (see p. 164 of Mongeau *et al*., 1997; Schlicker & Göthert, 1998). Trains of eight pulses at 100 Hz fulfilled these three conditions (Figure 1, Table 1). The only minor effect of methiothepin on release of [^{3}H]-serotonin elicited by one pulse or eight pulses at 100 Hz was expected (Fischer *et al*., 1990). Upon stimulation with longer pulse trains, autoinhibition and, hence the effect of methiothepin usually is greater (Fischer *et al*., 1990), but small increases similar to the present ones have also been reported (a 31% increase for 360 pulses at 3 Hz in rat brain cortex: Göthert, 1980).

α_2-Adrenergic inhibition of serotonin release in the brain of mice does not seem to have been reported previously. Our results show that it occurs both in the hippocampus and the occipito-parietal cortex: the α_2-adrenoceptor agonist medetomidine inhibited the release of [^{3}H]-serotonin, and its effect was antagonized by phentolamine and rauwolscine in a manner compatible with competitive kinetics. Mice strains may differ in serotoninergic neurone function (Daszuta *et al*., 1984). We made sure that both the magnitude of electrically evoked release and the inhibition by medetomidine were virtually identical in NMRI mice and the WT strain that shared the genetic background of the transgenic animals (Table 2, Figure 1). NMRI mice therefore could be used for comparison with the latter.

Our experiments show that serotoninergic axons innervating the mouse *hippocampus* (i) possess prominent $\alpha_{2A/D}$-heteroreceptors – or more specifically α_{2D}-heteroreceptors if the rodent orthologue of this receptor is called by its traditional designation; (ii) possess less prominent α_{2C}-heteroreceptors; and (iii) lack α_{2B}-heteroreceptors. The evidence is as follows. First, in the hippocampus of NMRI mice, phentolamine and rauwolscine were approximately equipotent antagonists against medetomidine (Figure 3a); a potency ratio phentolamine $\geqslant$ rauwolscine is unique to the α_{2D}-adrenoceptor (see Simonneaux *et al*., 1991; Limberger *et al*., 1995a), and the equipotency hence indicates a major population of α_{2D}-heteroreceptors. Second, disruption of the $\alpha_{2A/D}$-adrenoceptor gene to generate α_{2A}KO mice reduced the maximal inhibitory effect of medetomidine by about 70% (Figure 2), in accord with an α_{2D} predominance but disclosing an additional non-$\alpha_{2A/D}$ pathway. Third, disruption of the α_{2C}-adrenoceptor gene to produce α_{2C}KO mice also reduced the maximal inhibitory effect of medetomidine, although only by 17%, whereas disruption of the α_{2B} gene to produce α_{2B}KO mice did not change the medetomidine concentration-response curve (Figure 2); the 2 fold observation indicates that the non-$\alpha_{2A/D}$ pathway is an α_{2C} pathway. Fourth and finally, the small inhibition by medetomidine that remained in α_{2A}KO hippocampal slices was lost in α_{2AC}KO slices, i.e. when both the $\alpha_{2A/D}$- and the α_{2C}-adrenoceptor had been deleted (Figure 2), thus confirming the α_{2C} and ruling out an α_{2B} component.

The results in the *occipito-parietal cortex* are similar but less complete: the approximate antagonist equipotency of phentolamine and rauwolscine in NMRI occipito-parietal cortex slices (Figure 3b) and the 70% loss of inhibitory efficacy of medetomidine in α_{2A}KO slices (Figure 4) indicate prevailing α_{2D}-heteroreceptors and an additional non-$\alpha_{2A/D}$ mechanism. The latter, however, was not studied further.

The existence of prominent $\alpha_{2A/D}$-heteroreceptors is in accord with previous work on rats and rabbits (Trendelenburg *et al*., 1994). The coexistence of α_{2C}- (but not α_{2B}-) heteroreceptors with $\alpha_{2A/D}$-heteroreceptors at the serotoninergic axons is reminiscent of the coexistence of α_{2C}- (but not α_{2B}-) autoreceptors with $\alpha_{2A/D}$-autoreceptors at the noradrenergic axons of mice (Altman *et al*., 1999; Hein *et al*., 1999; Trendelenburg *et al*., 1999): the hetero- and autoreceptors constitute the same, dual, types (*cf*. Trendelenburg *et al*., 1994).

Previous work has shown that the α_2-heteroreceptors at cerebral serotoninergic axons are not activated by endogenous noradrenaline to any major extent under *in vitro* conditions such as the present ones (see p. 163 of Mongeau *et al*., 1997). We have seen here the same. Phentolamine and rauwolscine enhanced the release of [^{3}H]-serotonin elicited by eight pulses at 100 Hz (S_1) in NMRI hippocampus when they were present throughout superfusion (Table 2), and phentolamine also enhanced the release elicited by 360 pulses at 3 Hz (S_6) in NMRI hippocampus when it was added after S_3 (Table 1). The increases were small, however, and idazoxan and MK 912 failed to cause any increase (Table 1). Still, it seems of interest that no increase occurred in α_{2A}KO hippocampus (Tables 1 and 2), indicating perhaps that the α_{2D}-heteroreceptor rather than the α_{2C}-heteroreceptor was involved. *In vivo*, a functionally important α_2-adrenergic inhibition of [^{3}H]-serotonin release by endogenous noradrenaline is likely (see Mongeau *et al*., 1997).

[^{3}H]-Serotonin may be taken up into, and subsequently released from, noradrenergic terminal axons. An α_2-autoreceptor-mediated modulation of [^{3}H]-serotonin release from noradrenergic axons may then simulate an α_2-heteroreceptor-mediated modulation of [^{3}H]-serotonin release from serotoninergic axons. Several considerations indicate, however, that cross-labelling of noradrenergic axons played a minimal role in our study. First, the affinity of serotonin for the noradrenaline transporter is much lower ($K_i > 10$ μM) than its affinity for the serotonin transporter (K_m 0.5 μM; Hoffman *et al*., 1991; Pacholczyk *et al*., 1991). Second, α_2-adrenoceptor antagonists increased the release of [^{3}H]-serotonin elicited by 360 pulses at 3 Hz by maximally 21% (Table 1). They increase the release of noradrenaline from mouse brain noradrenergic axons elicited by pulse trains of this kind, which lead to major α_2-autoinhibition, by more than 300% (Trendelenburg *et al*., 1999). Third, only nitroquipazine but not oxaprotiline, when present during preincubation with [^{3}H]-serotonin, significantly reduced the uptake and subsequent retention of [^{3}H]-serotonin. About one-third of the accumulation and retention was resistant to nitroquipazine. The same has been observed in rabbit brain cortex where the resistant fraction was *not* due to uptake into noradrenergic axons (Limberger *et al*., 1986). Fourth, the concentration-response curves of medetomidine in NMRI as well as α_{2A}KO hippocampal slices were unchanged when uptake of [^{3}H]-serotonin into noradrenergic axons had been prevented by oxaprotiline (Figure 5).

Some uptake of [^{3}H]-serotonin into, and subsequent release from, noradrenergic axons did occur, however, at least in slices labelled in the presence of nitroquipazine: these slices responded to electrical stimulation with medetomidine-sensitive tritium overflow (Figure 5). In NMRI hippocampus, medetomidine acted on the noradrenergic axons (preincuba-

tion with nitroquipazine) even with lower EC_{50} and higher E_{max} than on the serotoninergic axons (preincubation with oxaprotiline) (Figure 5a). This may reflect the high potency and large maximal effect of medetomidine at the noradrenergic axons of the mouse brain (in single pulse experiments: EC_{50} about 0.25 nM and maximal inhibition by about 98%; Trendelenburg *et al*., 1999).

In conclusion, the serotoninergic axons innervating the hippocampus and the occipito-parietal cortex of mice possess release-inhibiting α_2-heteroreceptors. Characterization by phentolamine and rauwolscine suggests that these receptors are predominantly α_{2D}. Experiments on animals lacking $\alpha_{2A/D}$-, α_{2B}-, α_{2C}- or both $\alpha_{2A/D}$- and α_{2C}-adrenoceptors confirm this predominance but show that the axons possess in addition a minor population of α_{2C}-heteroreceptors. The coexistence of $\alpha_{2A/D}$- and α_{2C}-heteroreceptors at the serotoninergic axons agrees with the coexistence of $\alpha_{2A/D}$- and α_{2C}-autoreceptors at the noradrenergic axons of the mouse brain.

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