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# $\alpha_2$-Adrenoceptors in the enteric nervous system: a study in $\alpha_{2A}$-adrenoceptor-deficient mice

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**1** Mammals possess three types of $\alpha_2$-adrenoceptor, $\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$. Our aim was to determine the type of $\alpha_2$-adrenoceptor involved in the control of gastrointestinal motility.

**2** In transmitter overflow experiments, myenteric plexus longitudinal muscle (MPLM) preparations of the ileum were preincubated with [$^3$H]-choline and then superfused. The $\alpha_2$-adrenoceptor agonist medetomidine reduced the electrically evoked overflow of tritium from preparations taken from wild type but not $\alpha_{2A}$-adrenoceptor-knockout mice.

**3** In a second series of overflow experiments, MPLM preparations were preincubated with [$^3$H]-noradrenaline and then superfused. Again medetomidine reduced the electrically evoked overflow of tritium from wild type but not $\alpha_{2A}$-knockout preparations.

**4** In organ bath experiments, medetomidine reduced electrically evoked contractions of segments of the ileum from wild type but not $\alpha_{2A}$-knockout mice.

**5** In each of these three series, phentolamine antagonized the effect of medetomidine in wild-type preparations with greater potency than rauwolscine.

**6** In conscious mice, gastrointestinal transit was assessed by means of an intragastric charcoal bolus. In $\alpha_{2A}$-knockout mice, the speed of gastrointestinal transit was doubled compared to wild-type. Medetomidine, injected intraperitoneally, slowed gastrointestinal transit in wild type but not $\alpha_{2A}$-knockout mice.

**7** We conclude that the cholinergic motor neurons of the enteric nervous system of mice possess $\alpha_2$-heteroreceptors which mediate inhibition of acetylcholine release, of neurogenic contractions and of gastrointestinal transit. The noradrenergic axons innervating the intestine possess $\alpha_2$-autoreceptors. Both hetero- and autoreceptors are exclusively $\alpha_{2A}$. It is the $\alpha_{2A}$-adrenoceptor which *in vivo* mediates the inhibition of intestinal motility by the sympathetic nervous system.
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**Keywords:** Mouse ileum; knockout mice; enteric nervous system; acetylcholine release; noradrenaline release; intestinal motility; medetomidine; $\alpha_2$-autoreceptor; $\alpha_2$-heteroreceptor; $\alpha_{2A}$-adrenoceptor

**Abbreviations:** MPLM, myenteric plexus longitudinal muscle

## Introduction

The gastrointestinal tract is extensively innervated by postganglionic sympathetic noradrenergic fibres, and catecholamines modulate a variety of digestive functions (Furness & Costa, 1987; De Ponti *et al*., 1996). Intestinal $\alpha_2$-adrenoceptors play a prominent role in this modulation. Their activation inhibits motility, decreases mucosal fluid and electrolyte secretion (Burks, 1994; De Ponti *et al*., 1996), promotes peptide absorption (Berlioz *et al*., 2000), and promotes the proliferation of intestinal epithelial cells (Schaak *et al*., 2000).

$\alpha_2$-Adrenoceptors are located on intestinal smooth muscle and epithelial cells (Burks, 1994; Bülbring & Tomita, 1987; De Ponti *et al*., 1996). An especially important location, however, is on the cholinergic neurons of the intestinal plexuses where, when activated, the receptors produce hyperpolarization and a decrease in transmitter release (Burks, 1994; Vizi, 1979; Starke, 1981; Fuder & Muscholl, 1995; De Ponti *et al*., 1996; Stebbing *et al*., 2001). The noradrenergic axons themselves also possess $\alpha_2$-adrenoceptors: the ubiquitous presynaptic $\alpha_2$-autoreceptors which, when activated, reduce noradrenaline exocytosis (Starke, 1977; De Ponti *et al*., 1996; Starke *et al*., 1989).

Mammals are now known to possess three genetic $\alpha_2$-adrenoceptor subtypes, $\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$. The rodent orthologue of the $\alpha_{2A}$-adrenoceptor differs pharmacologically from the human orthologue and is sometimes called $\alpha_{2D}$-adrenoceptor (Bylund *et al*., 1994; Hieble *et al*., 1997). We use the designation $\alpha_{2A}$ in the present paper, irrespective of species.

Studies using $\alpha_2$-subtype-selective drugs have suggested that the $\alpha_2$-adrenoceptors inhibiting the release of [$^3$H]-acetylcholine in the guinea-pig ileum ($\alpha_2$-'heteroreceptors' to distinguish them from the autoreceptors) are $\alpha_{2A}$ (Shen *et al*., 1990; Blandizzi *et al*., 1991; 1993; Funk *et al*., 1995). The same diagnosis was suggested for the $\alpha_2$-adrenoceptors inhibiting neurogenic contractions of the guinea-pig ileum (Colucci *et al*.,

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1998), the $\alpha_2$-adrenoceptors inhibiting neurogenic contractions of the rat ileum (Liu & Coupar, 1997a), the $\alpha_2$-adrenoceptors inhibiting fluid secretion in the rat small intestine (Liu & Coupar, 1997b), the $\alpha_2$-adrenoceptors inhibiting faecal excretion in rats (Croci & Bianchetti, 1992), and the $\alpha_2$-adrenoceptors mediating contraction of the canine proximal colon (Zhang *et al*., 1992). The $\alpha_2$-autoreceptors of the guinea-pig ileum were classified as $\alpha_{2A}$ or $\alpha_{2B}$ (Shen *et al*., 1990; Blandizzi *et al*., 1993; Funk *et al*., 1995).

The limited selectivity of drugs often leaves some doubt in receptor classifications. The deletion of genes coding for a given receptor now offers new opportunities for the elucidation of receptor mechanisms. Experiments on mice lacking the $\beta_3$-adrenoceptor have clarified, for example, that this receptor mediates a slowing of gastrointestinal transit (Fletcher *et al*., 1998). As to $\alpha_2$-adrenoceptors, studies on mice lacking either the $\alpha_{2A}$-, the $\alpha_{2B}$-, the $\alpha_{2C}$-, or both the $\alpha_{2A}$- and the $\alpha_{2C}$-adrenoceptor have shown that the $\alpha_2$-autoreceptors in the heart, vas deferens and brain are predominantly $\alpha_{2A}$ with an admixture of $\alpha_{2C}$, and that deletion of these subtypes leads to anomalous sympathetic neurotransmission (Altman *et al*., 1999; Trendelenburg *et al*., 1999; 2001; Hein *et al*., 1999; Hein, 2001; Starke, 2001).

Studies on intestinal functions in $\alpha_2$-adrenoceptor subtype-deficient mice are lacking. The aim of the present work was to characterize the $\alpha_2$-heteroreceptors of the ileal cholinergic neurons and the ileal $\alpha_2$-autoreceptors by means of mice lacking the $\alpha_{2A}$-adrenoceptor ($\alpha_{2A}$-knockout). Four functions were studied: release of [$^3$H]-acetylcholine from myenteric plexus longitudinal muscle (MPLM) preparations of the ileum; release of [$^3$H]-noradrenaline from MPLM preparations; neurogenic contractions of the ileum; and gastrointestinal transit *in vivo*.

## Methods

### *Animals*

The $\alpha_{2A}$-knockout mice have been described previously (Altman *et al*., 1999). Naval Medical Research Institute mice (NMRI; Charles River, Sulzfeld, Germany) were used as wild type animals. The mice were kept with free access to food and water under an alternate 12-h light/dark cycle. All experiments were carried out on male mice aged at least 9 weeks. The study was approved by the Animal Experiments Committee of the regional council of Freiburg.

### *Drugs*

[Methyl-$^3$H]-choline chloride, specific activity 75–80 Ci mmol$^{-1}$, (−)-[ring-2,5,6-$^3$H]-noradrenaline, specific activity 70.7 Ci mmol$^{-1}$ (DuPont, Dreieich, Germany); phentolamine HCl (Ciba-Geigy, Basel, Switzerland); morphine HCl (Merck, Darmstadt, Germany); hemicholinium-3 bromide, tetrodotoxin citrate, *trans*-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide HCl (U-50488) (Biotrend, Köln, Germany); medetomidine HCl (Orion, Espoo, Finland); atropine sulphate (Serva, Heidelberg, Germany); desipramine HCl, rauwolscine HCl (Sigma, Deisenhofen, Germany). Drugs were dissolved in distilled water except for *in vivo* experiments (0.9% saline).

### *[$^3$H]-Acetylcholine and [$^3$H]-noradrenaline release*

Mice were killed by cervical dislocation. The 5–7 distal centimetres of the ileum were discarded. The lumen of the ~16 cm proximal to this portion was flushed. The myenteric plexus longitudinal muscle or MPLM preparation (the longitudinal muscle with the myenteric plexus attached) was dissected as described by Paton & Vizi (1969; see also Kilbinger, 1982). The strip was cut into 14–20 segments of about 5×8 mm size. The segments were preincubated for 45 min at 37°C in 4 ml medium containing either 0.1 $\mu$M [$^3$H]-choline or 0.1 $\mu$M [$^3$H]-noradrenaline. They were then washed with five 4-ml volumes of [$^3$H]-choline- or [$^3$H]-noradrenaline-free medium and transferred to 12 superfusion chambers equipped with platinum electrodes, one segment per chamber, where they were superfused at 37°C at a rate of 1.2 ml min$^{-1}$. Successive 2-min superfusate samples were collected from $t=60$ 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 segments.

The preincubation medium consisted of (mM): NaCl 118, KCl 4.8, $CaCl_2$ 0.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $KH_2PO_4$ 1.2, glucose 11, ascorbic acid 0.57, $Na_2EDTA$ 0.03. The superfusion medium was the same but contained 2.5 mM $CaCl_2$ and either 3 $\mu$M hemicholinium-3 ([$^3$H]-acetylcholine release) or 1 $\mu$M desipramine ([$^3$H]-noradrenaline release). The medium was saturated with 5% $CO_2$ in $O_2$.

Seven periods of electrical stimulation were applied. Each consisted of rectangular pulses of 1 ms width and a current strength of 80 mA, which yielded a voltage of 40–50 V between the electrodes of each chamber. The first stimulation period, at $t=30$ min, consisted of a train of 180 pulses/3 Hz and was not used for determination of tritium overflow. The subsequent six stimulation periods were applied at $t=64$ ($S_1$), 82 ($S_2$), 100 ($S_3$), 118 ($S_4$), 136 ($S_5$) and 154 min ($S_6$) and consisted either of a train of 60 pulses/1 Hz ([$^3$H]-acetylcholine) or a train of 30 pulses/50 Hz ([$^3$H]-noradrenaline). Medetomidine was added to the superfusion medium at cumulatively 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.

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 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, $S_n/S_1$ overflow ratios were calculated. Overflow ratios were also calculated as a percentage of the average corresponding ratio from controls in which no medetomidine was applied. The basal efflux of tritium was evaluated similarly.

### *Neurogenic contractions*

A length of ~16 cm of the ileum was prepared as described above. The lumen was flushed and the ileum cut into

segments of 2–2.5 cm length. The segments were placed vertically in 5-ml organ baths between platinum electrodes at the top and the bottom of the bath, and were connected to isometric transducers (Hugo Sachs, March, Germany). The medium was identical with the superfusion medium above but was hemicholinium-3- and desipramine-free. The temperature was 37°C and the bath medium was bubbled with 5% $CO_2$ in $O_2$. A resting tension of 0.5 g was applied and kept constant during a 30-min equilibration period, with washing every 10 min. After the 30 min, electrical stimulation with pairs of pulses was started (1 ms pulse width, 50 ms interval, 40–60 V). The pulse-pair frequency was adjusted to match the frequency of spontaneous contractions (usually between 0.2–0.4 Hz; Smith *et al*., 1988). Mechanical activity was recorded on a Rikadenki recorder (Hugo Sachs). When twitch responses had stabilized, medetomidine, U-50488 or morphine was added to the organ bath cumulatively at 3-min intervals. Phentolamine and rauwolscine, when used as antagonists against medetomidine, were added at least 30 min prior to the first dose of medetomidine.

About the last 40 twitch contractions before drug addition were averaged to yield $t_0$. At least the last 14 twitch contractions in the presence of the *n*th drug concentration were averaged to yield $t_n$. Twitch ratios $t_n/t_0$ were then formed. Twitch ratios were also calculated as a percentage of the average corresponding ratio from controls in which no drug was applied.

### *Gastrointestinal transit*

Mice were fasted 6 h prior to the experiments, with free access to water. At the time of the experiment, a charcoal suspension (10% charcoal in 5% gummi arabicum, 0.25–0.3 ml per mouse) was administered intragastrically (Pol *et al*., 1996). Thirty minutes later mice were killed by cervical dislocation. The stomach and small intestine were removed. The distance travelled by the charcoal suspension was expressed as a percentage of total small intestine length. Medetomidine or 0.9% saline was injected intraperitoneally 20 min before administration of the charcoal suspension.

### *Statistics*

Where possible, concentration-response curves of medetomidine were evaluated by logistic curve fitting (equation no. 25 of Waud, 1976) using either the data for 'agonist given alone' or, in a simultaneous fit, the data for 'agonist given alone' *and* 'agonist in the presence of antagonist', essentially as described previously (Trendelenburg *et al*., 1997). The calculation yielded the maximal effect ($E_{max}$) and $EC_{50}$ of agonist given alone as well as its $EC_{50}$ in the presence of 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.mean as defined by Waud, 1976). Groups were compared by the Mann–Whitney test with Bonferroni correction if Kruskal-Wallis analysis indicated a significant difference. *n* represents the number of tissue segments or, for *in vivo* experiments, the number of animals. $P<0.05$ was taken as the limit of statistical difference.

## Results

### *[$^3$H]-Acetylcholine release*

Stimulation by 60 pulses/1 Hz ($S_1$ to $S_6$) produced clear peaks of tritium overflow from MPLM segments taken from wild-type (NMRI) or $\alpha_{2A}$-knockout mice and preincubated with [$^3$H]-choline (Figure 1A,B). The overflow of tritium evoked by 60 pulses/1 Hz at $S_1$ was similar in wild type preparations (0.46 ± 0.04% of tissue tritium, corresponding to 0.63 nCi on average; $n=25$) and $\alpha_{2A}$-knockout preparations (0.51 ± 0.02% of tissue tritium; 0.51 nCi; $n=45$). In experiments without medetomidine, the evoked overflow remained similar from $S_1$ to $S_6$, irrespective of the mouse strain, giving $S_n/S_1$ values close to unity (filled circles in Figure 1A,B). The evoked overflow was abolished by tetrodotoxin (0.3 $\mu$M) or omission of calcium (data not shown).

In wild type mice the $\alpha_2$-adrenoceptor agonist medetomidine, when added at increasing concentrations after $S_1$, caused a concentration-dependent decrease of the evoked overflow of tritium (Figure 1A, empty circles; concentration-response curve in Figure 2, filled circles). The $EC_{50}$ of medetomidine was 0.99 ± 0.53 nM and its $E_{max}$ 39.9 ± 3.2% inhibition ($n=14$, from Figure 2). In $\alpha_{2A}$-knockout mice, the effect of medetomidine no longer occurred (Figure 1B, empty circles; concentration-response curve in Figure 2A, empty circles).

The receptors mediating the inhibition by medetomidine in wild type mice were also characterized pharmacologically by means of the α-adrenoceptor antagonists phentolamine and rauwolscine (0.3 $\mu$M each). Neither antagonist, when present throughout superfusion, changed the overflow of tritium at $S_1$ (phentolamine present 0.51 ± 0.05% of tissue tritium; $n=11$; rauwolscine present 0.47 ± 0.05% of tissue tritium; $n=11$). Control $S_n/S_1$ ratios (no medetomidine) were close to unity also in the presence of the antagonists (data not shown). Rauwolscine tended to shift the concentration-response curve of medetomidine to the right, and phentolamine shifted it to the right significantly. Phentolamine was more potent than rauwolscine (Figure 2B). The flat concentration-response curves of medetomidine in the presence of phentolamine and rauwolscine did not permit logistic curve fitting, so $EC_{50}$ values for medetomidine in the presence of the antagonists were interpolated, assuming that the $E_{max}$ had not changed. The apparent $pK_d$ values were 9.2 and 7.7 for phentolamine and rauwolscine, respectively.

The basal efflux of tritium before $S_1$ was similar in wild-type and $\alpha_{2A}$-knockout MPLM pieces (Figure 1A,B). Medetomidine reduced basal tritium outflow from wild type strips by maximally 17% (Figure 1A) but caused no change in $\alpha_{2A}$-knockout strips (Figure 1B).

### *[$^3$H]-Noradrenaline release*

Trains of 60 pulses at 1 Hz gave too small and variable tritium overflow peaks in experiments on the release of [$^3$H]-noradrenaline. For this reason, MPLM segments preincubated with [$^3$H]-noradrenaline were stimulated by trains of 30 pulses/50 Hz (Trendelenburg *et al*., 2000). Under these conditions, the evoked overflow of tritium was similar to the [$^3$H]-acetylcholine experiments. It was higher in wild type preparations (0.43 ± 0.04% of tissue tritium, corresponding to

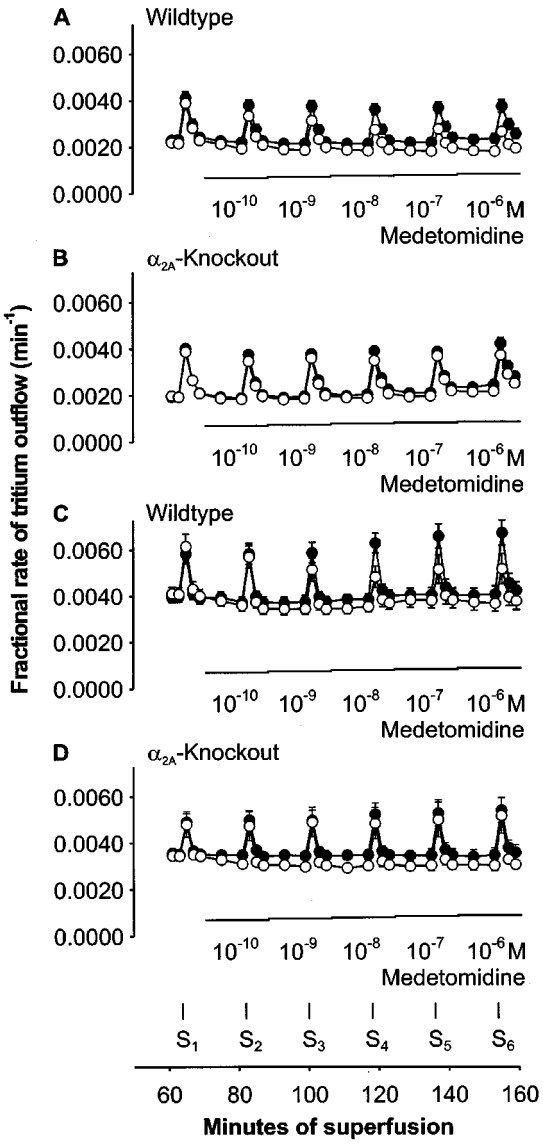


**Figure 1** Tritium efflux from MPLM segments preincubated with [$^3$H]-choline (A,B) or [$^3$H]-noradrenaline (C,D). Segments were taken from either wild type (NMRI) or $\alpha_{2A}$-knockout mice. After preincubation, segments were superfused and stimulated six times by either 60 pulses/1 Hz (A,B) or 30 pulses/50 Hz (C,D) ($S_1-S_6$). Filled circles represent experiments without medetomidine. Empty circles represent experiments in which medetomidine was added as indicated. Each value is the mean$\pm$s.e.mean from 7–24 preparations.

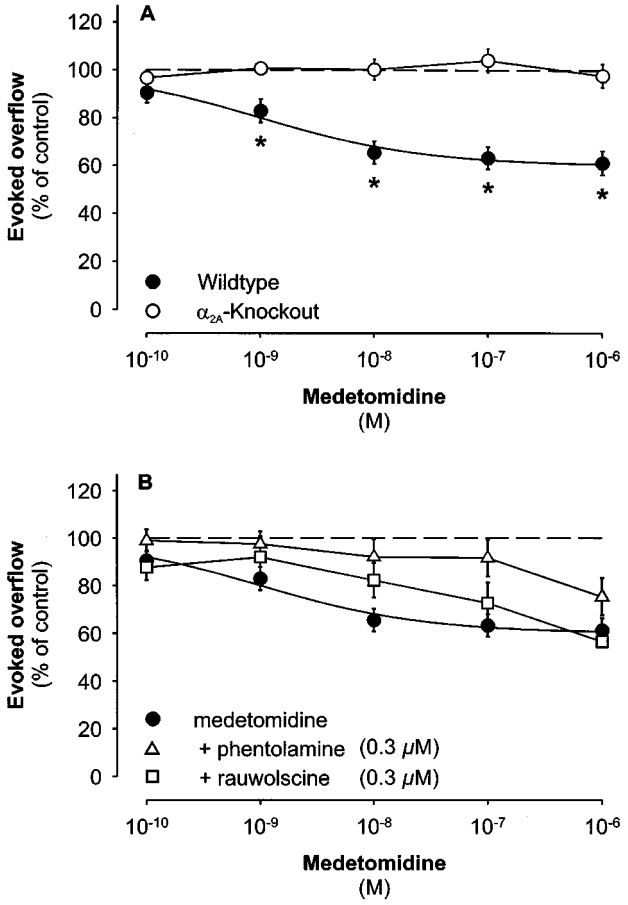


**Figure 2** Effect of medetomidine on electrically evoked tritium overflow from MPLM segments preincubated with [$^3$H]-choline. After preincubation, segments were superfused and stimulated six times by 60 pulses/1 Hz ($S_1-S_6$). Medetomidine was added at increasing concentrations before $S_2-S_6$. (A) shows effect of medetomidine in segments taken from wild type or $\alpha_{2A}$-knockout mice as indicated. (B) shows interaction of medetomidine with phentolamine and rauwolscine in preparations taken from wild type mice. Phentolamine and rauwolscine were present from the start of 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). Each value is the mean$\pm$s.e.mean from 5–24 preparations. *Indicates significantly ($P<0.05$) less inhibition by medetomidine in $\alpha_{2A}$-knockout than wild type mice. In $\alpha_{2A}$-knockout preparations, medetomidine caused no significant change.

0.41 nCi on average; $n=22$) than in $\alpha_{2A}$-knockout preparations ($0.30\pm0.04\%$ of tissue tritium; 0.22 nCi; $n=15$; $P<0.05$). In experiments without medetomidine, the evoked overflow increased slightly from $S_1$ to $S_6$, giving $S_n/S_1$ values slightly above unity (filled circles in Figure 1C,D). The evoked overflow was abolished by tetrodotoxin (0.3 $\mu$M) or omission of calcium (data not shown).

In wild-type mice medetomidine produced a concentration-dependent decrease of evoked tritium outflow (Figure 1C, empty circles; concentration-response curve in Figure 3, filled circles). The $EC_{50}$ of medetomidine was $0.57\pm0.24$ nM and its $E_{max}$ $57.6\pm3.6\%$ inhibition ($n=10$, from Figure 3). As in [$^3$H]-acetylcholine experiments, no inhibition remained in the $\alpha_{2A}$-knockout preparations (Figure 1D, empty circles, concentration-response curve in Figure 3A, empty circles).

The receptors mediating the inhibition by medetomidine in wild type mice were characterized also by means of phentolamine and rauwolscine (0.3 $\mu$M each). The stimulation-evoked overflow at $S_1$ tended to be higher in the presence of phentolamine ($0.65\pm0.10\%$; $n=12$) and rauwolscine ($0.55\pm0.06\%$; $n=16$) than in their absence; however, the tendencies did not reach statistical significance. Control $S_n/S_1$ values (no medetomidine) were slightly higher than unity also in the presence of the antagonists (data not shown). Both antagonists shifted the concentration-response curve of medetomidine to the right, phentolamine being more potent than rauwolscine (Figure 3B). The apparent p$K_d$ values were 8.9 and 8.4 for phentolamine and rauwolscine, respectively.

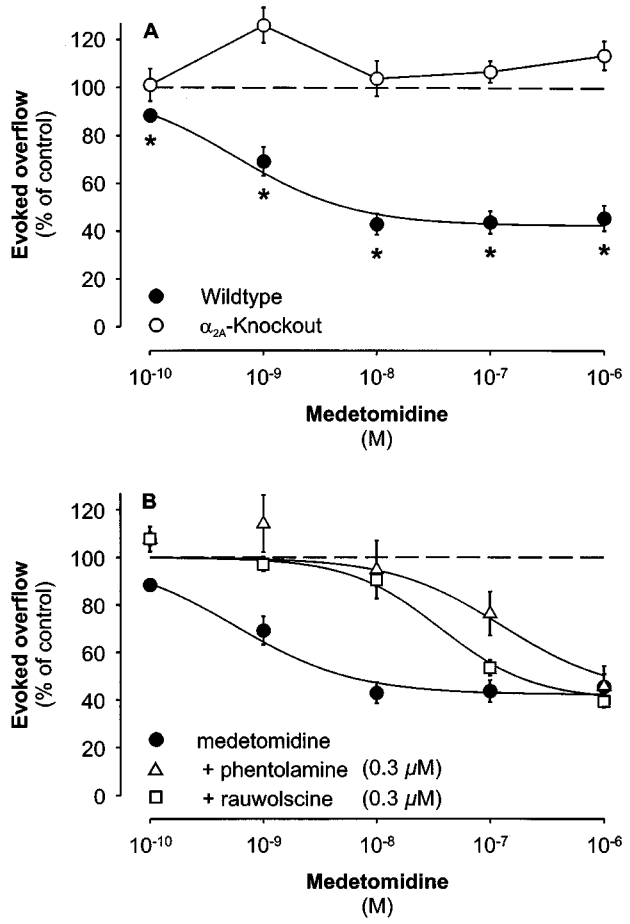


**Figure 3** Effect of medetomidine on electrically evoked tritium overflow from MPLM segments preincubated with [$^3$H]-noradrenaline. After preincubation, segments were superfused and stimulated six times by 30 pulses/50 Hz ($S_1-S_6$). Medetomidine was added at increasing concentrations before $S_2-S_6$. (A) Shows effect of medetomidine in segments taken from wild type or $\alpha_{2A}$-knockout mice as indicated. (B) Shows interaction of medetomidine with phentolamine and rauwolscine in preparations taken from wild type mice. Phentolamine and rauwolscine were present from the start of 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). Each value is the mean ± s.e.mean from 7–10 preparations. *Indicates significantly ($P<0.05$) less inhibition by medetomidine in $\alpha_{2A}$-knockout than wild type mice. In $\alpha_{2A}$-knockout preparations, medetomidine caused no significant change.

The basal efflux of tritium before $S_1$ was comparable in wild-type and $\alpha_{2A}$-knockout MPLM pieces (Figure 1C,D). Medetomidine reduced basal tritium outflow from wild type as well as $\alpha_{2A}$-knockout strips by maximally 11% (Figure 1C,D).

### *Neurogenic contractions*

In most experiments, electrical field stimulation of ileal segments with pairs of pulses produced stable twitch contractions; preparations in which stability was not reached were discarded. Twitches before drug addition ($t_0$) were similar in ileal segments from wild-type mice (0.50 ± 0.05 g; $n=17$) and $\alpha_{2A}$-knockout mice (0.52 ± 0.07 g; $n=10$). The twitches were abolished by tetrodotoxin (0.3 μM) and greatly reduced but in most cases not abolished by atropine (1 μM).

In wild type ileal segments medetomidine, when added at increasing concentrations, reduced the twitch contractions in a concentration-dependent manner (Figure 4, filled circles). The $EC_{50}$ of medetomidine was 0.69 ± 0.58 nM and its $E_{max}$ 49.9 ± 6.8% inhibition ($n=17$, from Figure 4). In segments from $\alpha_{2A}$-knockout mice, no significant inhibition by medetomidine remained (Figure 4A, empty circles).

The receptors mediating the twitch inhibition by medetomidine in wild type mice were again characterized also by means of phentolamine (0.3 μM) and rauwolscine (0.3 μM). Neither antagonist changed the twitches *per se*, and twitches were also stable in the presence of the antagonists. Both antagonists shifted the concentration-response curve of medetomidine to the right, phentolamine being more potent than rauwolscine (Figure 4B). The apparent $pK_d$ values were 8.7 for phentolamine and 8.2 for rauwolscine.

The effects of the selective κ-opioid-receptor agonist U-50488 and the selective μ-opioid-receptor agonist morphine on electrically evoked contractions were investigated for comparison. U-50488 reduced the twitch responses with almost identical concentration-response curves in ileal segments from wild type and $\alpha_{2A}$-knockout mice (Figure 5A). The same was true for morphine (Figure 5B).

### *Gastrointestinal transit*

Finally, the effect of medetomidine on gastrointestinal transit was examined *in vivo* (Figure 6). In saline-treated wild type mice, the distance travelled by the charcoal marker within 30 min was 36.2 ± 3.3% of the total length of the small intestine ($n=11$). Intraperitoneal administration of medetomidine to wild type mice caused a dose-dependent reduction of the transit speed. After injection of the highest dose, 0.2 mg kg$^{-1}$, gastrointestinal transit was reduced to 8.5 ± 2.8% of total small intestine length ($n=8$). Deletion of the $\alpha_{2A}$-adrenoceptor resulted in two changes. First, gastrointestinal transit in saline-treated $\alpha_{2A}$-knockout mice was increased to 70.1 ± 3.6% of total small intestine length ($n=8$). Second, medetomidine 0.2 mg kg$^{-1}$ failed to produce any inhibition (Figure 6).

## Discussion

Neuronal $\alpha_2$-adrenoceptors in the intestine have not been studied in mice previously. So the demonstrations of $\alpha_2$-adrenoceptors inhibiting [$^3$H]-acetylcholine release, [$^3$H]-noradrenaline release and neurogenic contractions in mice are new findings. An inhibition of gastrointestinal transit through $\alpha_2$-adrenoceptors in mice has been already shown (Pol *et al.*, 1996).

Our main result is that all these $\alpha_2$-adrenoceptors are exclusively, or at least far predominantly, $\alpha_{2A}$. The evidence is as follows.

Any inhibitory effect of the $\alpha_2$-adrenoceptor agonist medetomidine on the release of [$^3$H]-acetylcholine was abolished when the $\alpha_{2A}$-adrenoceptor had been eliminated by disruption of its gene. Moreover, in wild type mice, phentolamine was more potent than rauwolscine in antagonizing the inhibitory effect of medetomidine, a potency order found for the guinea-pig, rat and mouse $\alpha_{2A}$-adrenoceptor and not compatible with an $\alpha_{2B}$- or $\alpha_{2C}$-

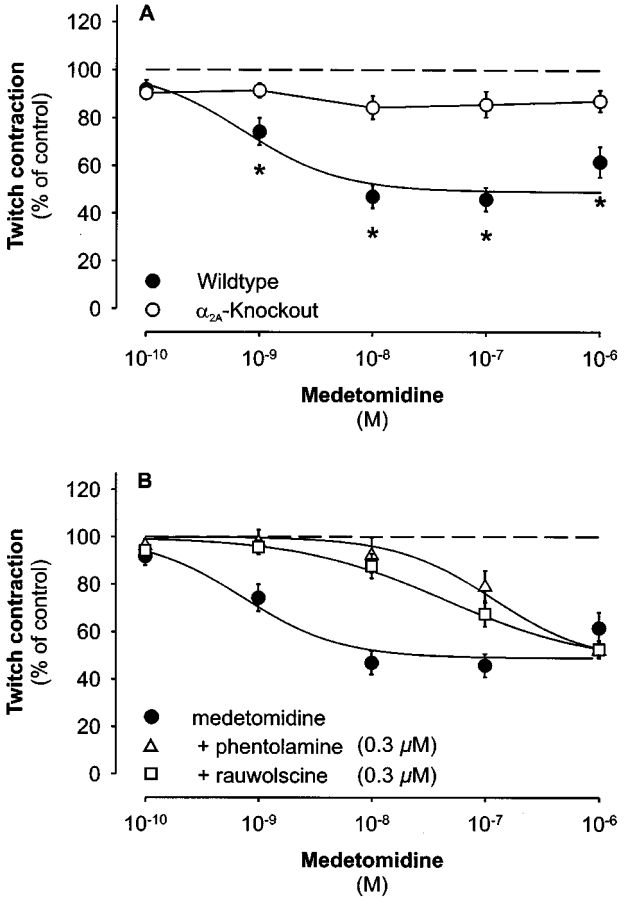


**Figure 4** Effect of medetomidine on electrically evoked contractions. Ileum segments were continuously stimulated with pairs of pulses at a frequency of 0.2–0.4 Hz. Medetomidine was added at increasing concentrations every 3 min. (A) shows effect of medetomidine in segments taken from wild type or $\alpha_{2A}$-knockout mice as indicated. (B) shows interaction of medetomidine with phentolamine and rauwolscine in preparations taken from wild type mice. Phentolamine and rauwolscine were present in the medium from 30 min prior to the first dose of medetomidine. Ordinates, twitch responses, calculated from ratios of twitch amplitudes after the *n*th dose of medetomidine and twitch amplitudes before addition of medetomidine ($t_n/t_0$) and expressed as a percentage of the corresponding average control ratio (no medetomidine). Each value is the mean ± s.e.mean from 9–17 preparations. *Indicates significantly ($P<0.05$) less inhibition by medetomidine in $\alpha_{2A}$-knockout than wild type mice. In $\alpha_{2A}$-knockout preparations, medetomidine caused no significant change.

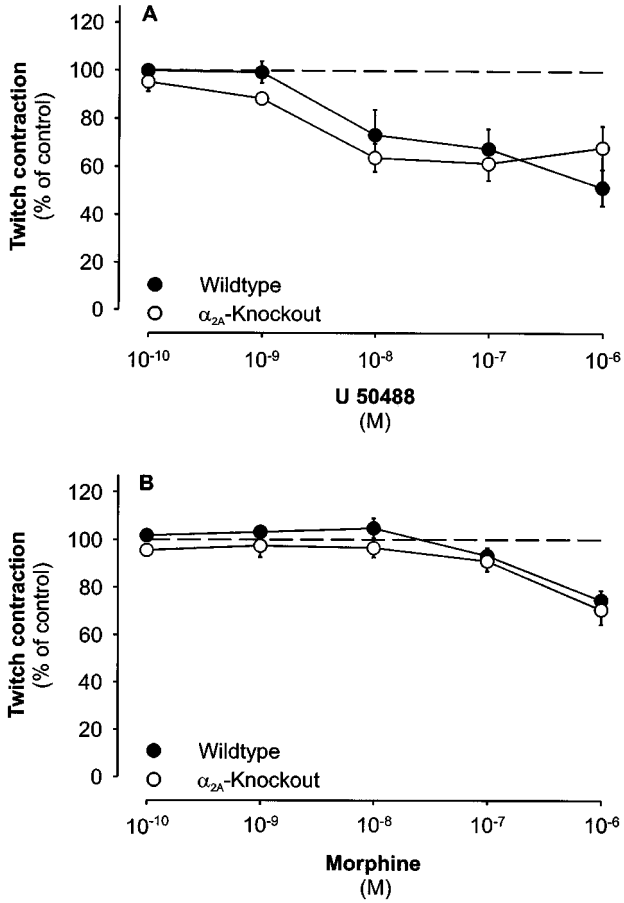


**Figure 5** Effect of (A) U-50488 and (B) morphine on electrically evoked contractions. Ileum segments were continuously stimulated with pairs of pulses at a frequency of 0.2–0.4 Hz. U-50488 or morphine was added at increasing concentrations every 3 min. Ordinates, twitch responses, calculated from ratios of twitch amplitudes after the *n*th dose of opioid agonist and twitch amplitudes before addition of agonist ($t_n/t_0$) and expressed as a percentage of the corresponding average control ratio (no agonist). Each value is the mean ± s.e.mean from 4–10 preparations.

adrenoceptor (Simonneaux *et al*., 1991; Trendelenburg *et al*., 1997). The $\alpha_2$-heteroreceptors at the murine ileal cholinergic neurons hence are $\alpha_{2A}$. They thus agree with the corresponding $\alpha_{2A}$-heteroreceptors in the guinea-pig ileum (Shen *et al*., 1990; Blandizzi *et al*., 1991; 1993; Funk *et al*., 1995).

The same pair of observations – loss of the effect of medetomidine after $\alpha_{2A}$ gene deletion and an antagonist potency ratio phentolamine > rauwolscine in wild type mice – was made concerning the modulation of the release of [$^3$H]-noradrenaline. The ileal $\alpha_2$-autoreceptors of the mouse, hence, are equally $\alpha_{2A}$. This diagnosis is in accord with that of Shen *et al*. (1990) and Funk *et al*. (1995) for the guinea-pig ileum. Blandizzi *et al*. (1993) suggested that the $\alpha_2$-autoreceptors in the guinea-pig ileum were $\alpha_{2B}$, but their experimental basis was narrower (Funk *et al*., 1995).

Again precisely the same pair of observations proves the $\alpha_{2A}$ nature of the $\alpha_2$-adrenoceptors mediating the inhibition of neurogenic contractions. The contractions were greatly reduced by atropine, indicating that acetylcholine was the major, although not the only, motor transmitter (Kunze & Furness, 1999). The [$^3$H]-acetylcholine experiments and the contraction experiments hence examined the same physiological process, acetylcholine release, and the conclusions – $\alpha_{2A}$-heteroreceptors at the cholinergic neurons – support each other. As a control, an opioid $\kappa$-receptor agonist and the $\mu$-receptor agonist morphine were also tested against neurogenic twitches. As expected, the effects in wild type and $\alpha_{2A}$-knockout ilea were practically identical, indicating that the gene knockout specifically prevented the effect of the $\alpha_2$-adrenoceptor agonist.

In conscious mice, finally, medetomidine failed to cause any reduction of the speed of gastrointestinal transit when the $\alpha_{2A}$-adrenoceptor had been deleted. If we assume that the slowing of gastrointestinal transit by $\alpha_2$-adrenoceptor activation is largely due to an inibition of the cholinergic motor neurons (Furness & Costa, 1987; Burks, 1994; Fuder &

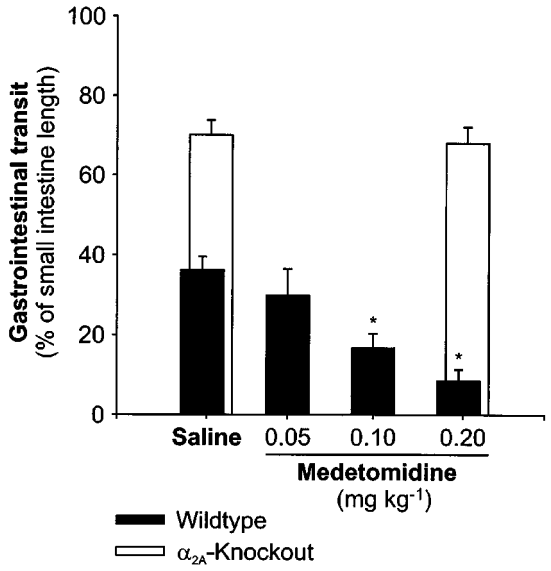


**Figure 6** Effect of medetomidine on gastrointestinal transit in conscious wild type and $\alpha_{2A}$-knockout mice. Mice received an intragastric bolus of charcoal suspension. Thirty minutes later, they were killed and the distance travelled by the charcoal was expressed as a percentage of total small intestine length. Medetomidine or saline was injected i.p. 20 min before the charcoal application. Each value is the mean ± s.e.mean from 8–11 mice. *Indicates significant ($P<0.05$) inhibition by medetomidine as compared to saline.

Muscholl, 1995; De Ponti *et al*., 1996, Stebbing *et al*., 2001), the *in vivo* finding once again reflects the $\alpha_{2A}$-adrenergic inhibition of these neurons.

The $\alpha_2$-autoreceptors studied here are presumably located on the postganglionic sympathetic axons (Starke *et al*., 1989). The location of the $\alpha_2$-heteroreceptors is less certain: preganglionic parasympathetic axons, cholinergic nerve cell bodies in the enteric plexuses, and axons arising from such cell bodies are all possible. The third location probably is a major one (Fuder & Muscholl, 1995).

Under appropriate conditions such as an adequate action potential frequency, the $\alpha_2$-heteroreceptors as well as the $\alpha_2$-autoreceptors in the intestine mediate an inhibition by endogenous, previously released noradrenaline (Starke, 1977; Fuder & Muscholl, 1995). Under the present *in vitro* conditions, no endogenous inhibition developed: phentolamine and rauwolscine neither increased the release of [$^3$H]-acetylcholine nor the release of [$^3$H]-noradrenaline nor the neurogenic contractions. A physiological role of the $\alpha_{2A}$-heteroreceptor is borne out, however, by the *in vivo* experiments: deletion of the receptor doubled the speed of gastrointestinal transit. Experiments with $\alpha_2$-adrenoceptor antagonists have not clarified the question of whether the sympathetic nervous system tonically inhibits gastrointestinal motility (De Ponti *et al*., 1996). The gene knockout experiments support the operation of a tonic inhibition and show that the inhibition is mediated at least partly through $\alpha_{2A}$-adrenoceptors.

Studies on $\alpha_2$-adrenoceptor subtype-deficient animals have clarified the nature of the $\alpha_2$-autoreceptors in the heart, the vas deferens and the brain (see Introduction). They have also clarified the nature of the $\alpha_2$-heteroreceptors at cerebral dopaminergic and serotoninergic axons (Bücheler *et al*., 1999; Scheibner *et al*., 2001). All these receptors agreed in that they constituted a mixture of predominant $\alpha_{2A}$- and minor $\alpha_{2C}$-adrenoceptors. The present investigation adds the $\alpha_2$-autoreceptors of the sympathetic axons and the $\alpha_2$-heteroreceptors of the cholinergic neurons of the intestine. Interestingly, both seem to differ from the previously classified transmitter release-inhibiting receptors in that they are purely $\alpha_{2A}$, no $\alpha_{2C}$-adrenoceptor component being detectable.

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## References

ALTMAN, J.D., TRENDELENBURG, A.U., MACMILLAN, L., BERNSTEIN, D., LIMBIRD, L., STARKE, K., KOBILKA, B.K. & HEIN, L. (1999). Abnormal regulation of the sympathetic nervous system in $\alpha_{2A}$-adrenergic receptor knockout mice. *Mol. Pharmacol.,* **56,** 154–161.

BERLIOZ, F., MAORET, J.J., PARIS, H., LABURTHE, M., FARINOTTI, R. & ROZÉ, C. (2000). $\alpha_2$-Adrenergic receptors stimulate oligopeptide transport in a human intestinal cell line. *J. Pharmacol. Exp. Ther.,* **294,** 466–472.

BLANDIZZI, C., DODA, M., TARKOVÁCS, G., DEL TACCA, M. & VIZI, E.S. (1991). Functional evidence that acetylcholine release from Auerbach's plexus of guinea-pig ileum is modulated by $\alpha_{2A}$-adrenoceptor subtype. *Eur. J. Pharmacol.,* **205,** 311–313.

BLANDIZZI, C., TARKOVÁCS, G., NATALE, G., DEL TACCA, M. & VIZI, E.S. (1993). Functional evidence that [$^3$H] acetylcholine and [$^3$H] noradrenaline release from guinea pig ileal myenteric plexus and noradrenergic terminals is modulated by different presynaptic $\alpha_2$-adrenoceptor subtypes. *J. Pharmacol. Exp. Ther.,* **267,** 1054–1060.

BÜCHELER, M., LOHSE, M.J. & HEIN, L. (1999). Two presynaptic $\alpha_2$-adrenergic receptor subtypes are required for regulation of dopamine release in mouse basal ganglia. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **359,** R26.

BÜLBRING, E. & TOMITA, T. (1987). Catecholamine action on smooth muscle. *Pharmacol. Rev.,* **39,** 49–96.

BURKS, T.F. (1994). Neurotransmission and Neurotransmitters. In *Physiology of the Gastrointestinal Tract*. ed. Johnson, L.R., 3rd ed. Vol. 1. pp. 211–242. New York: Raven.

BYLUND, D.B., EIKENBERG, D.C., HIEBLE, J.P., LANGER, S.Z., LEFKOWITZ, R.J., MINNEMAN, K.P., MOLINOFF, P.B., RUFFOLO, JR. R.R. & TRENDELENBURG, U. (1994). International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol. Rev.,* **46,** 121–136.

COLUCCI, R., BLANDIZZI, C., CARIGNANI, D., PLACANICA, G., LAZZERI, G. & DEL TACCA, M. (1998). Effects of imidazoline derivatives on cholinergic motility in guinea-pig ileum: involvement of presynaptic $\alpha_2$-adrenoceptors or imidazoline receptors? *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **357,** 682–691.

CROCI, T. & BIANCHETTI, A. (1992). Stimulation of faecal excretion in rats by $\alpha_2$-adrenergic antagonists. *J. Pharm. Pharmacol.,* **44,** 358–360.

DE PONTI, F., GIARONI, C., COSENTINO, M., LECCHINI, S. & FRIGO, G. (1996). Adrenergic mechanisms in the control of gastrointestinal motility: from basic science to clinical applications. *Pharmacol. Ther.,* **69,** 59–78.

FLETCHER, D.S., CANDELORE, M.R., GRUJIC, D., LOWELL, B.B., LUELL, S., SUSULIC, V.S. & MACINTYRE, D.E. (1998). *Beta*-3 adrenergic receptor agonists cause an increase in gastrointestinal transit time in wild-type mice, but not in mice lacking the *beta*-3 adrenergic receptor. *J. Pharmacol. Exp. Ther.,* **287,** 720–724.

FUDER, H. & MUSCHOLL, E. (1995). Heteroreceptor-mediated modulation of noradrenaline and acetylcholine release from peripheral nerves. *Rev. Physiol. Biochem. Pharmacol.,* **126,** 265–412.

FUNK, L., TRENDELENBURG, A.U., LIMBERGER, N. & STARKE, K. (1995). Subclassification of presynaptic $\alpha_2$-adrenoceptors: $\alpha_{2D}$-autoreceptors and $\alpha_{2D}$-adrenoceptors modulating release of acetylcholine in guinea-pig ileum. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **352,** 58–66.

FURNESS, J.B. & COSTA, M. (1987). *The Enteric Nervous System*. Edinburgh, London, Melbourne, New York: Churchill Livingstone.

HEIN, L. (2001). Transgenic models of $\alpha_2$-adrenergic receptor subtype function. *Rev. Physiol. Biochem. Pharmacol.,* **142,** 161–185.

HEIN, L., ALTMAN, J.D. & KOBILKA, B.K. (1999). Two functionally distinct $\alpha_2$-adrenergic receptors regulate sympathetic neurotransmission. *Nature,* **402,** 181–184.

HIEBLE, J.P., RUFFOLO, JR. R.R. & STARKE, K. (1997). Identification, characterization and subclassification of $\alpha_2$-adrenoceptors: an overview. In *$\alpha_2$-Adrenergic Receptors: Structure, Function and Therapeutic Implications*. eds. Lanier, S.M. & Limbird, L.E. pp. 1–18. Reading: Harwood Academic Publishers.

KILBINGER, H. (1982). The Myenteric Plexus-Longitudinal Muscle Preparation. In *Progress in Cholinergic Biology: Model Cholinergic Synapses*. eds. Hanin, I. & Goldberg, A.M. pp. 137–167. New York: Raven.

KUNZE, W.A.A. & FURNESS, J.B. (1999). The enteric nervous system and regulation of intestinal motility. *Annu. Rev. Physiol.,* **61,** 117–142.

LIU, L. & COUPAR, I.M. (1997a). Characterisation of pre- and postsynaptic α-adrenoceptors in modulation of the rat ileum longitudinal and circular muscle activities. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **356,** 248–256.

LIU, L. & COUPAR, I.M. (1997b). Role of $\alpha_2$-adrenoceptors in the regulation of intestinal water transport. *Br. J. Pharmacol.,* **120,** 892–898.

PATON, W.D. & VIZI, E.S. (1969). The inhibitory action of noradrenaline and adrenaline on acetylcholine output by guinea-pig ileum longitudinal muscle strip. *Br. J. Pharmacol.,* **35,** 10–28.

POL, O., VALLE, L., FERRER, I. & PUIG, M.M. (1996). The inhibitory effects of $\alpha_2$-adrenoceptor agonists on gastrointestinal transit during croton oil-induced intestinal inflammation. *Br. J. Pharmacol.,* **119,** 1649–1655.

SCHAAK, S., CUSSAC, D., CAYLA, C., DEVEDJIAN, J.C., GUYOT, R., PARIS, H. & DENIS, C. (2000). Alpha$_2$ adrenoceptors regulate proliferation of human intestinal epithelial cells. *Gut,* **47,** 242–250.

SCHEIBNER, J., TRENDELENBURG, A.U., HEIN, L. & STARKE, K. (2001). $\alpha_2$-Adrenoceptors modulating neuronal serotonin release: a study in $\alpha_2$-adrenoceptor subtype-deficient mice. *Br. J. Pharmacol.,* **132,** 925–933.

SHEN, K.Z., BARAJAS-LOPEZ, C. & SURPRENANT, A. (1990). Functional characterization of neuronal pre and postsynaptic $\alpha_2$-adrenoceptor subtypes in guinea-pig submucosal plexus. *Br. J. Pharmacol.,* **101,** 925–931.

SIMONNEAUX, V., EBADI, M. & BYLUND, D.B. (1991). Identification and characterization of $\alpha_{2D}$-adrenergic receptors in bovine pineal gland. *Mol. Pharmacol.,* **40,** 235–241.

SMITH, C.F.C., WALDRON, C. & BROOK, N.A. (1988). Opioid receptors in the mouse ileum. *Arch. Int. Pharmacodyn. Thér.,* **291,** 122–131.

STARKE, K. (1977). Regulation of noradrenaline release by presynaptic receptor systems. *Rev. Physiol. Biochem. Pharmacol.,* **77,** 1–124.

STARKE, K. (1981). α-adrenoceptor subclassification. *Rev. Physiol. Biochem. Pharmacol.,* **88,** 199–236.

STARKE, K. (2001). Presynaptic autoreceptors in the third decade: focus on $\alpha_2$-adrenoceptors. *J. Neurochem.,* **78,** 685–693.

STARKE, K., GÖTHERT, M. & KILBINGER, H. (1989). Modulation of neurotransmitter release by presynaptic autoreceptors. *Physiol. Rev.,* **69,** 864–989.

STEBBING, M.J., JOHNSON, P.J., VREMEC, M.A. & BORNSTEIN, J.C. (2001). Role of $\alpha_2$-adrenoceptors in the sympathetic inhibition of motility reflexes of guinea-pig ileum. *J. Physiol.,* **534,** 465–478.

TRENDELENBURG, A.U., COX, S.L., SCHELB, V., KLEBROFF, W., KHAIRALLAH, L. & STARKE, K. (2000). Modulation of [$^3$H]-noradrenaline release by presynaptic opioid, cannabinoid and bradykinin receptors and $\beta$-adrenoceptors in mouse tissues. *Br. J. Pharmacol.,* **130,** 321–330.

TRENDELENBURG, A.U., HEIN, L., GAISER, E.G. & STARKE, K. (1999). Occurrence, pharmacology and function of presynaptic $\alpha_2$-autoreceptors in $\alpha_{2A/D}$-adrenoceptor-deficient mice. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **360,** 540–551.

TRENDELENBURG, A.U., KLEBROFF, W., HEIN, L. & STARKE, K. (2001). A study of presynaptic $\alpha_2$-autoreceptors in $\alpha_{2A/D}$-, $\alpha_{2B}$- and $\alpha_{2C}$-adrenoceptor deficient mice. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **364,** 117–130.

TRENDELENBURG, A.U., SUTEJ, I., WAHL, C.A., MOLDERINGS, G.J., RUMP, L.C. & STARKE, K. (1997). A re-investigation of questionable subclassifications of presynaptic $\alpha_2$-autoreceptors: rat vena cava, rat atria, human kidney and guinea-pig urethra. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **356,** 721–737.

VIZI, E.S. (1979). Presynaptic modulation of neurochemical transmission. *Prog. Neurobiol.,* **12,** 181–290.

WAUD, D.R. (1976). Analysis of Dose-Response Relationships. In *Advances in General and Cellular Pharmacology*. eds. Narahashi, T. & Bianchi, C.P. Vol. 1. pp. 145–178. New York, London: Plenum.

ZHANG, L., KEEF, K.D., BRADLEY, M.E. & BUXTON, I.L.O. (1992). Action of $\alpha_{2A}$-adrenergic receptors in circular smooth muscle of canine proximal colon. *Am. J. Physiol.,* **262,** G517–G524.

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