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Prejunctional actions of methylenedioxymethamphetamine in vas deferens from wild-type and $\alpha_{2A/D}$-adrenoceptor knockout mice

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Abstract

Methylenedioxymethamphetamine (MDMA, 'ecstasy') has major agonist actions at prejunctional $\alpha_{2A/D}$-adrenoceptors in the rat. We wished to establish whether MDMA has potency at more than one subtype of α_2-adrenoceptor, in line with affinity in ligand-binding studies. We have investigated the effects of MDMA in vas deferens from wild-type and from knockout mice lacking the $\alpha_{2A/D}$-adrenoceptor. The potency of the α_2-adrenoceptor agonist xylazine at inhibiting stimulation-evoked contractions to a single stimulus in the presence of cocaine was significantly reduced in knockout (pD_2 of 8.27 ± 0.07, $-\log$ M, $n = 4$) as compared with wild-type mice (8.69 ± 0.08, $n = 4$, $P < 0.05$), whereas potency of MDMA was unchanged (5.39 ± 0.06, $n = 4$ versus 5.38 ± 0.06, $n = 6$). Similar differences between xylazine and MDMA were seen for responses to stimulation at 10 Hz for 4 s. In studies of mouse atria pre-incubated with ^{3}H-noradrenaline, the stimulation-evoked release of tritium was inhibited to a similar extent by MDMA (10 μM) in tissues from wild-type and knockout mice. The prejunctional $\alpha_{2A/D}$-adrenoceptor is reported to be replaced by the α_{2C}-adrenoceptor in this knockout mouse, so that we have evidence that suggests that MDMA has similar potencies at both subtypes in functional studies.

Keywords: MDMA (methylenedioxymethamphetamine); α_2-Adrenoceptor; $\alpha_{2A/D}$-Adrenoceptor knockout; Vas deferens

1. Introduction

We have recently shown that MDMA (methylenedioxymethamphetamine, 'ecstasy') has major agonist actions at prejunctional α_2-adrenoceptors in rat vas deferens (Lavelle et al., 1999) and at central α_2-adrenoceptors-mediating depressor responses (McDaid and Docherty, 2001). MDMA inhibits isometric contractions of epididymal portions of rat vas deferens, and the potency of MDMA was shifted by the α_2-adrenoceptor antagonist yohimbine but not by the 5-HT_1-receptor antagonist cyanopindolol (Lavelle et al., 1999). In anaesthetised rats, blood pressure-lowering actions of MDMA were attenuated by the α_2-adrenoceptor antagonist methoxyidazoxan but not by 5-HT_1-receptor selective antagonists (McDaid and Docherty, 2001). In ligand-binding studies of α_2-adrenoceptors, MDMA showed similar potency at all three subtypes (Lavelle et al., 1999).

α_2-Adrenoceptors have been subdivided into three subtypes, α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors based on ligand-binding and molecular cloning studies (Bylund, 1992; Lorenz et al., 1990), and the rat α_{2D}-adrenoceptor is a species orthologue of the human α_{2A}-adrenoceptor (Lanier et al., 1991; Harrison et al., 1991). Functional prejunctional α_2-adrenoceptors in rat submandibular gland (Limberger et al., 1992; Smith et al., 1992; Smith and Docherty, 1992), rat vas deferens (Smith et al., 1992; Smith and Docherty, 1992), rat cerebral cortex (Trendelenburg et al., 1993), pithed rat heart (Smith et al., 1995) and mouse atria (Wahl et al., 1996) resemble the α_{2D}-adrenoceptor, whereas those in rabbit cerebral cortex (Trendelenburg et al., 1993) and human saphenous vein (Molderings and Gothert, 1995) resemble the α_{2A}-adrenoceptor.

However, some studies have produced results suggesting that prejunctional α_2-adrenoceptors may not always be of the $\alpha_{2A/D}$-subtype, or that more than one subtype may be present prejunctionally (see Docherty, 1998). The functional prejunctional α_2-adrenoceptors in rat isolated atrium do not closely resemble the α_{2D}-adrenoceptor (Smith et al., 1992; see also Limberger et al., 1992). Due to this

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discrepancy, prejunctional α_2-adrenoceptors in rat atrium and rat cerebral cortex were directly compared employing eight adrenoceptor antagonists (Ho et al., 1998). The results suggested that, in addition to the α_{2D}-adrenoceptor, a second subtype of adrenoceptor resembling the α_{2C}-adrenoceptor subtype is found in the rat atrium but not the cerebral cortex. Studies in α_2-adrenoceptor knockout mice confirm that two subtypes of α_2-adrenoceptor can be present prejunctionally (Hein and Kobilka, 1998; Altman et al., 1999). Therefore, in $\alpha_{2A/D}$-adrenoceptor knockout mice, the prejunctional $\alpha_{2A/D}$-adrenoceptor is replaced by another α_2-adrenoceptor (Trendelenburg et al., 1999), the α_{2c}-adrenoceptor (Hein et al., 1999).

We now investigate the effects of MDMA in comparison with α_2-adrenoceptor agonists in vas deferens and atrium from wild-type and from knockout mice lacking the $\alpha_{2A/D}$-adrenoceptor. We wished to establish whether MDMA has potency at more than one subtype of α_2-adrenoceptor, in line with affinity in ligand-binding studies. Some of these results have been published in abstract form (Rajamani et al., in press).

2. Methods

C57BL/6 mice (18–28 g, male and female, wild-type and homozygous $\alpha_{2A/D}$-knockout: Jackson Laboratories, Bar Harbor, ME, USA) were killed by CO_2 overdose.

2.1. Isometric contractions of mouse vas deferens

Tissues were placed between platinum electrodes and attached to myograph transducers under 0.5 g tension in organ baths at 37 °C in Krebs–Henseleit solution of the following composition: (mM): NaCl, 119; $NaHCO_3$, 25; D-glucose, 11.1; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.0. Tissues were stimulated every 5 min with a single stimulus or trains of 40 pulses at 10 Hz (0.5 ms pulses, supramaximal voltage) using a Grass S88 Stimulator. Isometric contractile responses were recorded on a MacLab system. Peak contractions were measured, even for responses to 10 Hz stimulation, which had a biphasic form. The early peak was almost always larger than the later sustained contraction (see Fig. 3). Once consistent control responses to electrical stimulation at 5-min intervals had been obtained, tissues were given vehicle, nifedipine (10 μM) or cocaine (3 μM). Once consistent responses had again been obtained (usually by 15 min), MDMA, adrenoceptor agonists or vehicle were added cumulatively in 0.5 or 1 log unit increments at 5-min intervals, immediately after an electrical stimulation. Hence, effects of agonists on stimulation-evoked contractions were examined 5 min after administration. Effects of agonists on stimulation-evoked responses were not corrected for the relatively small changes occurring in experiments in which multiple additions of vehicle replaced test drug.

2.2. Radioactive overflow experiments

Mouse whole right and left atria from both males and females were used. All results were combined as there were no obvious differences. The Krebs–Henseleit solution had the composition stated above with the addition of corticosterone (30 μM) propranolol (1 μM), EDTA (30 μM) and ascorbic acid (280 μM). Isolated tissues were pre-incubated for 30 min in 1 ml of Krebs–Henseleit medium containing [^{3}H]noradrenaline (0.5 μM, specific activity 39 Ci/mmol), before being placed in a Brandel Superfusor and superfused at 37 °C with [^{3}H]noradrenaline-free Krebs–Henseleit solution at a rate of 0.33 ml/min. Desipramine (1 μM) was present after pre-incubation with [^{3}H]noradrenaline to block re-uptake of noradrenaline (and uptake of MDMA).

In all experiments, tissues were stimulated (supramaximal voltage, 0.5 ms pulses) with short bursts of 20 pulses at 100 Hz every 5 s for 3 min, three times (S_0–S_2) at intervals of 27 min. After 99 min of superfusion, the tissues were stimulated to remove loosely bound tritium (S_0). Effluent samples were collected in 1-ml aliquots, beginning after 120 min of superfusion, so that sampling began 6 min before the control stimulation period at 126 min (S_1). MDMA or an equivalent volume (1%) of distilled water (vehicle) were superfused in the Krebs–Henseleit solution beginning 12 min before the test stimulation period at 153 min (S_2). At the end of the experiment, tissues were dissolved in 0.5 ml of tissue solubiliser (Soluene, Packard). A volume of 1 ml superfusate or dissolved tissue was added to 3 ml of liquid scintillant (Ecoscint A) and the radioactivity was measured by liquid scintillation spectroscopy in a LKB 1214 RackBeta counter with, on average, 48% counting efficiency for tritium and automatic quench correction.

The basal efflux of tritium was expressed as a percentage rate, i.e. the efflux of total tritiated compounds per minute was expressed as a percentage of the tritium content of the tissue at the time of collection. To quantify the effects of a drug on the basal efflux, the rate of efflux in the 3 min before stimulation in the presence of the drug (S_2) was divided by the rate of efflux in the 3 min before the control stimulation period (S_1), and compared with the equivalent ratio obtained in vehicle experiments without the test drug (B_2/B_1 ratios).

The stimulation-evoked overflow of total tritium was calculated by subtraction of the basal overflow and was expressed as a percentage of the tritium content of the tissue at the onset of the respective stimulation periods (see Smith et al., 1992). To quantify the effects of a drug on stimulation-evoked overflow of tritium, the evoked overflow in the presence of the drug (S_2) was divided by

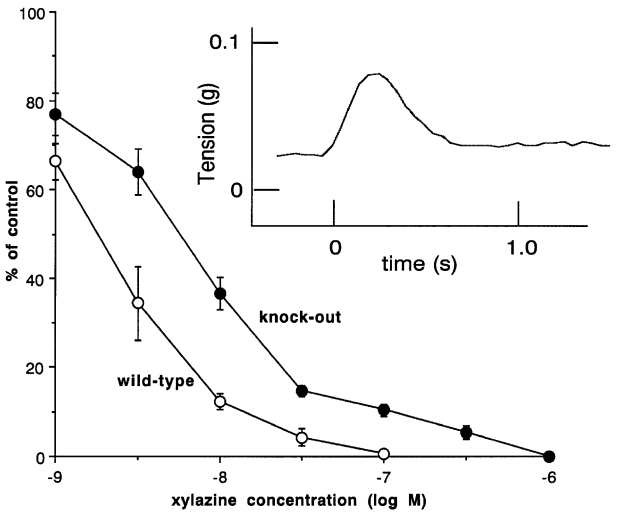

Fig. 1. Effects of the α_2-adrenoceptor agonist xylazine on isometric contractions of mouse vas deferens evoked by single pulse stimulation in the presence of cocaine (3 μM). Open symbols: responses in wild-type mice; filled symbols: responses in knockout mice. Responses are expressed as a percentage of the control response, just prior to the addition of xylazine. Vertical bars represent S.E.M. from four experiments. The inset shows a typical response to a single stimulus in vas deferens from a knockout mouse (responses in wild-type were qualitatively similar). Time and tension scales have arbitrary zero points.

the overflow evoked in the control stimulation period (S_1), and compared with the equivalent ratio obtained in vehicle experiments without the test drug (S_2/S_1 ratios).

2.3. Drugs

Cocaine hydrochloride (Sigma, Ireland); corticosterone (Sigma, UK); (±)-MDMA hydrochloride (methylenedioxymethamphetamine: Sigma); nifedipine (Sigma); oxymetazoline hydrochloride (Sigma); (−)-propranolol hydrocholoride (Sigma); xylazine hydrochloride (Gift: Bayer, Ireland).

Drugs were dissolved in distilled water, except for corticosterone and nifedipine (100% ethanol).

2.4. Statistics

Values are mean ± S.E.M. from n experiments. Agonist pD_2 ($-\log IC_{50}$: concentration producing 50% of maximum inhibition of stimulation-evoked contractions) values were obtained using the GraphPad Prism programme. In tissues from wild-type animals, the IC_{50} was approximately 50% inhibition of the evoked contraction, so that in experiments employing tissues from knockout animals in which a full concentration–response curve could not be obtained, IC_{50} was estimated as 50% inhibition. Differences in pD_2 values or maximum inhibition between groups were compared by Student's t-test for paired or unpaired data when appropriate, and by Analysis of Variance. Statistical and graphical analysis was carried out using Instat for Macintosh and GraphPad Prism for PC.

3. Results

3.1. Isometric contractions of mouse vas deferens

In the absence of cocaine, responses to single pulse electrical stimulation were generally small and inconsistent, so that cocaine was employed. Single pulse stimulation produced a contraction of 0.120 ± 0.040 (mean ± S.E.M., $n = 16$) and 0.090 ± 0.016 g ($n = 12$) in wild-type and knockout mice, respectively, in the presence of cocaine (3 μM) (no significant difference). A typical trace is shown in Fig. 1. The potency of the α_2-adrenoceptor agonist xylazine was significantly reduced in knockout (pD_2 of 8.27 ± 0.07, $-\log$ M, $n = 4$) as compared with wild-type mice (8.69 ± 0.08, $n = 4$; Student's t-test, $P < 0.05$) (Fig. 1), whereas potency of MDMA was unchanged (5.39 ± 0.06, $n = 4$ versus 5.38 ± 0.06, $n = 6$) (Fig. 2).

Stimulation with 40 pulses at 10 Hz in the absence of cocaine produced a contraction of 0.40 ± 0.05 ($n = 32$) and 0.75 ± 0.08 g ($n = 23$) in wild-type and knockout mice, respectively ($P < 0.001$). A typical trace is shown in Fig. 3. These responses were reduced to 0.12 ± 0.03 g ($n = 9$) and 0.20 ± 0.05 g ($n = 8$), respectively, following exposure to nifedipine (10 μM) to reduce postjunctional α_1-adrenoceptor-mediated actions of agonists ($P < 0.05$). Responses were significantly larger in knockout mice than

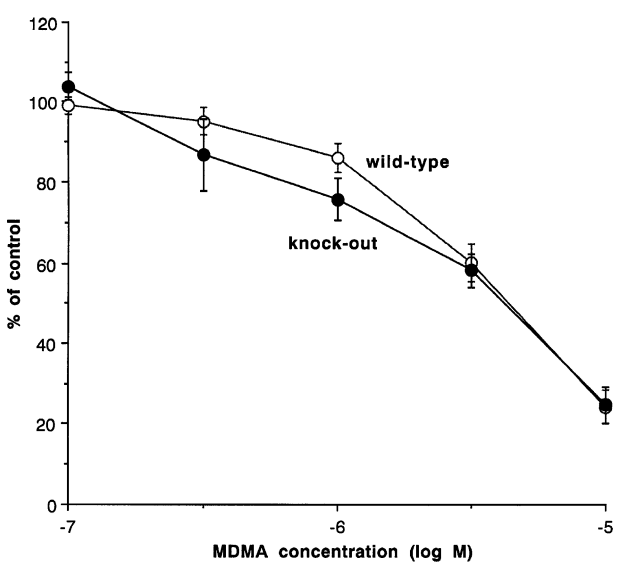

Fig. 2. Effects of MDMA on isometric contractions of mouse vas deferens evoked by single pulse stimulation in the presence of cocaine (3 μM). Open symbols: responses in wild-type mice; filled symbols: responses in knockout mice. Responses are expressed as a percentage of the control response, just prior to the addition of MDMA. Vertical bars represent S.E.M. from four experiments.

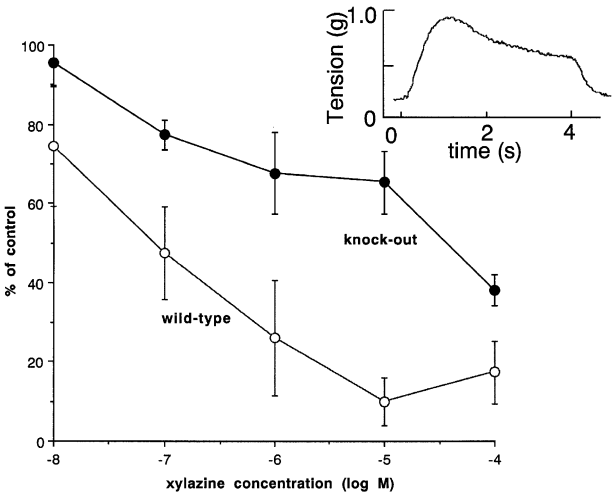

Fig. 3. Effects of the α_2-adrenoceptor agonist xylazine on isometric contractions of mouse vas deferens evoked by 10 Hz 4 s stimulation in the presence of nifedipine (10 μM). Open symbols: responses in wild-type mice; filled symbols: responses in knockout mice. Responses are expressed as a percentage of the control response, just prior to the addition of xylazine. Vertical bars represent S.E.M. from at least five experiments. The inset shows a typical response to 10 Hz stimulation for 4 s, prior to nifedipine in vas deferens from a knockout mouse (responses in wild-type were qualitatively similar). Time and tension scales have arbitrary zero points.

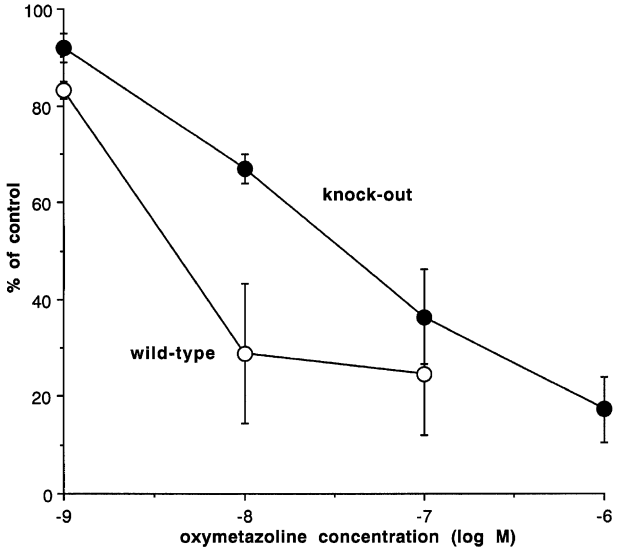

Fig. 5. Effects of the α_2-adrenoceptor agonist oxymetazoline on isometric contractions of mouse vas deferens evoked by 10 Hz 4 s stimulation in the presence of nifedipine (10 μM). Open symbols: responses in wild-type mice; filled symbols: responses in knockout mice. Responses are expressed as a percentage of the control response, just prior to the addition of oxymetazoline. Vertical bars represent S.E.M. from at least five experiments.

wild-type in the presence or absence of nifedipine. In experiments carried out in the presence of nifedipine, the potency of the α_2-adrenoceptor agonist xylazine was significantly reduced in vas deferens from knockout (4.54 ± 0.17, $n = 4$) as compared with wild-type mice (7.01 ± 0.48, $n = 4$) (Fig. 3), whereas potency of MDMA was unchanged (4.26 ± 0.24, $n = 4$ versus 4.32 ± 0.14, $n = 4$) (Fig. 4). Potency of the α_2-adrenoceptor agonist oxymetazoline was significantly reduced in knockout (6.72 ± 0.49, $n = 5$; $P < 0.05$) as compared with wild-type (8.56 ± 0.48, $n = 40$) (Fig. 5).

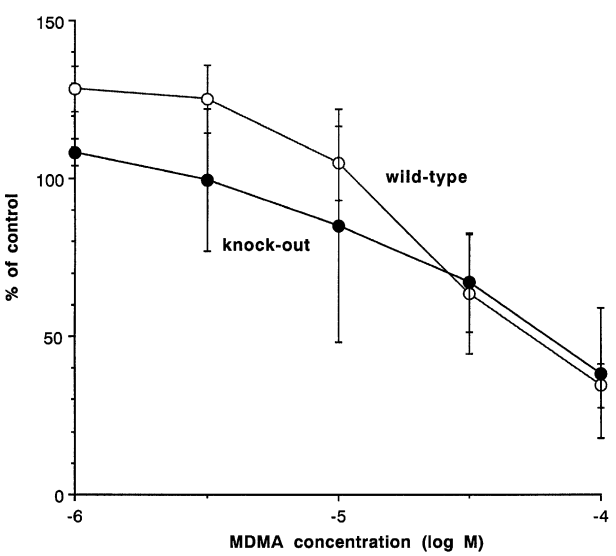

Fig. 4. Effects of MDMA on isometric contractions of mouse vas deferens evoked by 10 Hz 4 s stimulation in the presence of nifedipine (10 μM). Open symbols: responses in wild-type mice; filled symbols: responses in knockout mice. Responses are expressed as a percentage of the control response, just prior to the addition of MDMA. Vertical bars represent S.E.M. from four experiments.

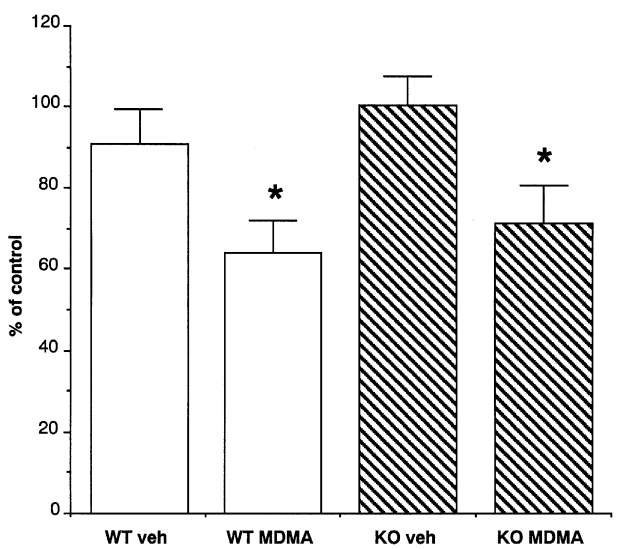

Fig. 6. Effects of MDMA (10 μM) or vehicle on release of tritium evoked by 20 pulses at 100 Hz every 5 s for 3 min in atria from wild-type (open columns) or alpha$_{2A/D}$-knockout mice (hatched columns). Abbreviations: WT, wild-type mice; KO, knockout mice; veh, vehicle. Responses are expressed as a percentage of the control response, just prior to the addition of MDMA or vehicle. Vertical bars represent S.E.M. from at least six experiments.

3.2. Stimulation-evoked overflow in mouse atrium

Experiments were carried out using short trains of 20 pulses at 100 Hz every 5 s for 3 min in the presence of the noradrenaline uptake blocker desipramine (1 μM). Basal outflow of tritium was 0.079 ± 0.020% ($n = 9$) and 0.073 ± 0.018% of tissue tritium/min ($n = 11$), and stimulation produced an evoked release of tritium of 0.664 ± 0.133% and 0.688 ± 0.117% of tissue tritium content, in tissues from wild-type and knockout mice, respectively. MDMA (10 μM) did not increase the basal release of tritium but significantly reduced the stimulation-evoked release of tritium in wild-type and knockout mice, as compared to the effects of vehicle (Fig. 6).

4. Discussion

In this study, we have looked at the prejunctional effects of MDMA in vas deferens from wild-type and $\alpha_{2A/D}$-adrenoceptor knockout mice in comparison to the effects of α_2-adrenoceptor agonists. Two stimulus parameters were employed in mouse vas deferens: single stimulus and 10 Hz 4 s. Responses to a single stimulus did not differ significantly between wild-type and knockout, but responses to 10 Hz 4 s were significantly greater in tissues from knockout mice (see below). Single pulse stimulation allowed investigation of prejunctional inhibitory effects of exogenous agonists in the absence of endogenous feedback. However, contractile responses to a single stimulus were small, but were increased by cocaine, presumably by an action to block re-uptake of noradrenaline. Although agonist potency against trains of pulses at 10 Hz were reduced (xylazine and MDMA were 30 and 10 times less potent than at inhibiting the response to a single stimulus), the Ca^{2+} entry blocker nifedipine could be used to reduce the postjunctional α_1-adrenoceptor actions of agonists.

The α_2-adrenoceptor agonist xylazine was significantly more potent in wild-type mice than knockout mice at inhibiting contractions to both single pulse and 10 Hz 4 s stimulation. The shift in xylazine potency between wild-type and knockout was approximately three-fold with a single stimulus but 30-fold at 10 Hz. This might be consistent with the suggestion that α_{2C}-adrenoceptors are mainly involved in inhibiting release to low frequency stimulation (Hein et al., 1999) (but see below).

Oxymetazoline was only studied in studies of contractions to 10 Hz 4 s stimulation, but again, its potency was reduced in vas deferens from knockout mice. Reduction in potency of an agonist can be caused by reduction in number of receptors, or by change in receptor subtype if the agonist has lower affinity for the new subtype, or both. We know that one receptor subtype is lost and replaced in these receptor knockout mice, but compensation may not be complete, so that the number of the new receptors may be less. Compounds like xylazine and oxymetazoline are likely to be weak agonists, so that either of these possibilities could explain the present findings. Oxymetazoline is known to be selective for $\alpha_{2A/D}$-adrenoceptors (Bylund, 1992) but the selectivity of xylazine has not been fully established. However, the lack of change in potency of MDMA between wild-type and knockout might suggest that any difference for agonists between wild-type and knockout is due to differences in agonist affinity between α_2-adrenoceptor subtypes. Certainly, the effects of MDMA would suggest that sufficient receptors remain to allow full inhibition of neurotransmission, or alternatively, that MDMA has higher affinity for α_{2C}- than α_{2D}-adrenoceptors. However, ligand-binding studies suggest that MDMA affinity is similar for α_{2C} and α_{2D} (Lavelle et al., 1999). These results with MDMA tend to rule out the suggestion that α_{2C}-adrenoceptors are mainly involved in inhibiting release to low frequency stimulation (Hein et al., 1999)

In radioactive overflow experiments employing mouse atrium, desipramine (1 μM) was chosen as noradrenaline re-uptake blocker, as we have previously found it to be more effective than cocaine at preventing displacement of noradrenaline from nerve terminals by MDMA in rat (Lavelle et al., 1999). The present study confirmed this in mouse. When tissues were stimulated at 5 Hz for 3 min, MDMA (10 μM) did not inhibit stimulation-evoked overflow of tritium in atrium from wild-type or knockout animals (results not shown). However, when the stimulus parameters were changed to 20 pulses every 5 s at 100 Hz for 3 min, MDMA significantly inhibited stimulation-evoked release. This difference can be explained by the fact that the former stimulation parameters allow endogenous feedback by noradrenaline to become marked, whereas the latter parameters reduced the degree of endogenous feedback (see also Limberger et al., 1992). The major finding from this component of the study was that MDMA inhibited release to a similar extent in both wild-type and $\alpha_{2A/D}$-knockout mice, again demonstrating the adaptive changes occurring in this knockout (see also Trendelenburg et al., 1999).

In ligand-binding studies of α_2-adrenoceptor sites, MDMA showed approximately equal affinity for all subtypes (Lavelle et al., 1999). Hence, it might be expected that MDMA has agonist actions at all subtypes of α_2-adrenoceptor. This potency at all three subtypes could be examined at functional models of α_2-adrenoceptor, but subtypes other than the $\alpha_{2A/D}$ are relatively difficult to investigate. This study of knockout mice allows us to investigate α_{2C}-adrenoceptors in mouse vas deferens/atrium and the results obtained confirm that MDMA has potency at these receptors.

In studies of mouse vas deferens employing single pulse stimulation in the presence of cocaine, contractile responses obtained in vas deferens from knockout mice tended to be smaller, but this did not reach statistical significance. However, contractile responses to 10 Hz 4 s were significantly greater in vas deferens from knockout

mice than in vas deferens from wild-type mice. This suggests that, although an α_{2c}-adrenoceptor replaces the α_{2D}-adrenoceptor in the knockout mice, endogenous feedback by noradrenaline released by nerve stimulation still declines, resulting in increased release of noradrenaline and increased contractile responses. This effect would not be seen with a single stimulus (absence of negative feedback) but would become increasingly important with higher frequencies and longer trains of pulses. Results obtained in overflow studies of mouse atrium did not confirm an increased release of noradrenaline in tissues from wild-type. Admittedly, these parameters with multiple short trains of pulses at 100 Hz were designed to limit endogenous feedback. Other studies of $\alpha_{2A/D}$-knockout mice reported an increased evoked release in vas deferens (Altman et al., 1999) and decreased release in ileum (Blandizzi et al., in press) as compared to wild-type.

It is concluded that MDMA is a potent agonist at more than one subtype of α_2-adrenoceptor. Its potency at inhibiting nerve-stimulation-evoked contractions is similar in wild-type and $\alpha_{2A/D}$-adrenoceptor knockout mice. The prejunctional $\alpha_{2A/D}$-adrenoceptor is reported to be replaced by the α_{2C}-adrenoceptor in this knockout mouse (see Altman et al., 1999), so that we now have evidence which suggests that MDMA has similar potencies at both subtypes in functional studies.

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