

Increased amphetamine-induced locomotor activity, sensitization, and accumbal dopamine release in M_5 muscarinic receptor knockout mice

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Abstract

Introduction Muscarinic M_5 receptors are the only muscarinic receptor subtype expressed by dopamine-containing neurons of the ventral tegmental area. These cells play an important role for the reinforcing properties of psychostimulants and M_5 receptors modulate their activity. Previous studies showed that M_5 receptor knockout ($M_5^{-/-}$) mice are less sensitive to the reinforcing properties of addictive drugs. **Materials and methods** Here, we investigate the role of M_5 receptors in the effects of amphetamine and cocaine on locomotor activity, locomotor sensitization, and dopamine release using $M_5^{-/-}$ mice backcrossed to the C57BL/6NTac strain.

Statistical analyses Sensitization of the locomotor response is considered a model for chronic adaptations to repeated substance exposure, which might be related to drug craving and relapse. The effects of amphetamine on locomotor activity and locomotor sensitization were enhanced in $M_5^{-/-}$ mice, while the effects of cocaine were similar in $M_5^{-/-}$ and wild-type mice.

Results Consistent with the behavioral results, amphetamine-, but not cocaine, -elicited dopamine release in nucleus accumbens was enhanced in $M_5^{-/-}$ mice.

Discussion The different effects of amphetamine and cocaine in $M_5^{-/-}$ mice may be due to the divergent pharmacological profile of the two drugs, where amphetamine, but not cocaine, is able to release intracellular stores of dopamine. In conclusion, we show here for the first time that amphetamine-induced hyperactivity and dopamine release as well as amphetamine sensitization are enhanced in mice lacking the M_5 receptor. These results support the concept that the M_5 receptor modulates effects of addictive drugs.

Keywords Muscarinic · M_5 · Cocaine · Amphetamine · Sensitization · Dopamine

Introduction

The reinforcing effects of psychostimulants are mediated by the mesocorticolimbic dopamine system (Koob 1992; Wise 1996; Koob et al. 1998). The cell bodies of this system are located in the ventral tegmental area (VTA) and project to the nucleus accumbens (NAc). These dopaminergic neurons receive cholinergic input from the laterodorsal tegmental (LDT) and pedunculo-pontine nuclei which, thus, modulate dopamine release in the nucleus accumbens (Bolam et al. 1991; Oakman et al. 1995; Blaha et al. 1996). The most recently cloned member of the muscarinic acetylcholine receptor family (M_1 – M_5 ; Bonner et al. 1988) and the only muscarinic acetylcholine receptor that can be detected in dopaminergic neurons in the VTA is the M_5 receptor subtype (Vilaro et al. 1990; Weiner et

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al. 1990). It has, therefore, been proposed that M_5 receptors play a critical role in cholinergic modulation of dopamine release from midbrain dopaminergic neurons (Vilaro et al. 1990; Weiner et al. 1990). While subtype-selective muscarinic receptor ligands are not available, M_5 receptor-deficient mice have been generated in order to investigate the physiological role of this receptor subtype. Studies using M_5 receptor knockout mice ($M_5^{-/-}$) have shown that morphine- (Basile et al. 2002) and muscarinic agonist-induced (Yamada et al. 2001) dopamine release in the striatum was, indeed, reduced in $M_5^{-/-}$ mice and that the prolonged accumbal dopamine release observed after electrical stimulation of the LDT was absent in $M_5^{-/-}$ mice (Forster et al. 2002).

Several studies have found $M_5^{-/-}$ mice to be less responsive to various drugs of abuse. Morphine-induced conditioned place preference and morphine withdrawal symptoms were reduced in $M_5^{-/-}$ mice (Basile et al. 2002); $M_5^{-/-}$ mice self-administered cocaine at a lower rate than wild-type controls in the acute self-administration model and showed less cocaine conditioned place preference (Fink-Jensen et al. 2003). Wang et al. (2004) found decreased hyperlocomotion in $M_5^{-/-}$ mice in response to the indirect catecholamine agonist amphetamine.

Using $M_5^{-/-}$ mice that have been extensively backcrossed to the C57BL/6Ntac strain, our group previously showed diminished acquisition of self-administration of a low dose of cocaine and decreased cocaine self-administration under a progressive ratio schedule compared to wild-type littermate controls (Thomsen et al. 2005).

In order to further investigate the role of the M_5 receptor in the addiction-related effects of amphetamine and cocaine, we examined baseline as well as amphetamine- and cocaine-induced locomotor activity of $M_5^{-/-}$ mice backcrossed to the C57BL/6Ntac strain. We also studied sensitization of the amphetamine- and cocaine-induced hyperlocomotor response, since it has been suggested that the sensitizing properties of addictive drugs play a role in relapse to drug-seeking behavior that can occur long after discontinuation of drug use (Robinson and Berridge 1993, 2008). Finally, we examined the effect of acute amphetamine and cocaine administration on extracellular dopamine levels in the nucleus accumbens measured using in vivo fixed potential amperometry.

Materials and methods

Animals

M_5 receptor knockout mice were generated by disrupting the muscarinic M_5 receptor gene in 129S6/SvEv embryonic stem cells as previously described (Yamada et al. 2001).

The founder mice of mixed genetic background were backcrossed to the C57BL/6Ntac strain for 13 generations resulting in mice with a high degree of congenicity. $M_5^{-/-}$, heterozygotes ($M_5^{+/-}$), and their wild-type ($M_5^{+/+}$) littermates were bred in the animal facilities at the Panum Institute, University of Copenhagen. Genotyping was performed on mouse-tail DNA using a PCR procedure. The mice were acclimatized to the animal facilities at Rigshospitalet University Hospital, where experiments were conducted for at least 1 week prior to any experiment. Animals were housed in standard cages (macrolon type III) on wood-chip bedding with food and water available ad libitum and kept on a 12-h light/dark cycle in a temperature- (22–24°C) and humidity- (55%) controlled room. Cardboard pipes and nesting material were provided for enrichment. All experiments were carried out with experimentally naïve adult mice, 8–17 weeks of age at the start of the experiment. All testing was conducted during the light phase of the circadian cycle (9.00 A.M.–5.00 P.M.). All procedures were conducted in accordance with guidelines from the Animal Experimentation Inspectorate, Denmark and the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Behavioral studies

Saline (0.9%), cocaine hydrochloride, and dexamphetamine sulfate were purchased from the pharmacy at Rigshospitalet University Hospital. All concentrations refer to the salt form of the drug.

Locomotor activity was assessed in an open field (40×40×80 cm, constructed of plywood and painted white), placed in a dimly lit room (16.6 lx at the bottom of the apparatus). A camera located on the ceiling above the apparatus recorded the experiments. The distance the animals moved was analyzed with the video-tracking program EthoVision (version 3.1, Noldus, Wageningen, The Netherlands).

Baseline, amphetamine-, and cocaine-induced locomotor activity

All animals were allowed at least 1 h of habituation to the experimental room. Male $M_5^{+/+}$, $M_5^{+/-}$, and $M_5^{-/-}$ mice were used. Before the baseline activity measurement, mice were weighed and then placed in the open field apparatus without any prior treatment, and activity was recorded. For the amphetamine- and cocaine-induced activity assessment, mice were weighed and injected with saline, amphetamine (2.0 or 4.0 mg/kg, i.p.), or cocaine (2.5, 5.0 or 10.0 mg/kg, i.p.) immediately before testing. The recording sessions lasted 20 min. Each mouse was tested only once. The order

of testing was randomized with respect to both dose and genotype.

Sensitization to the locomotor activating effect of amphetamine and cocaine

Male $M_5^{+/+}$ and $M_5^{-/-}$ mice were transported to the experimental room 3 days before the experiment began and housed there in a ventilated housing cabinet (Scanntainer, Scanbur BK A/S, Karlslunde, Denmark) throughout the experiment. To induce behavioral sensitization, we paired amphetamine or cocaine injections with exposure of the mice to the open field apparatus for 45 min/day. After an initial day (day 0) with saline injections, $M_5^{+/+}$ and $M_5^{-/-}$ mice were divided into groups of similar activity levels that received injections of amphetamine (2.0 mg/kg, i.p.), cocaine (5.0 mg/kg, i.p.), or saline for 6 days (days 1–7). After a 6-day injection- and test-free pause, all mice received a single injection of their respective drug (or saline), and locomotor activity was assessed (day 12). After another eight injection- and test-free days, all mice were given amphetamine or cocaine and tested in the open field (day 20). On the next day (day 21), all mice were injected with saline and again exposed to the open field to assess how much of the hyperlocomotion observed could be ascribed to context conditioning (Post et al. 1981; Ohmori et al. 2000; Robinson and Berridge 2001).

Electrochemical studies

Thirty-five drug-naïve $M_5^{-/-}$ mice and their $M_5^{+/+}$ littermates were transported to the University of Memphis where electrochemical studies were conducted. Animals were housed five per cage in a temperature-controlled environment ($21 \pm 1^\circ\text{C}$) with a 12-h light–12-h dark cycle (lights on at 6:00 A.M.). Food and water were available ad libitum. Mice were acclimatized to housing facilities for at least 1 week prior to any experiments. These experiments were approved by the Institutional Animal Care and Use Committee at the University of Memphis and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Efforts were made to reduce the number of animals used and to minimize animal pain and discomfort.

Surgery

Mice were anesthetized with urethane (1.5 g/kg, i.p.; Sigma–Aldrich Chemical Co., St Louis, MO, USA) and mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) within a mouse head holder adaptor (Stoelting, Wood Dale, IL, USA), ensuring the skull was flat. Body temperature was maintained with a temperature-

regulated heating pad (TC-1000, CWE Inc., New York, NY, USA). In each mouse, a concentric bipolar stimulating electrode (SNE-100; Rhodes Medical Co., CA, USA) was implanted into the left medial forebrain bundle (MFB) (coordinates: AP -2.1 mm from bregma, ML $+1.1$ mm from midline, and DV -4.8 mm from dura, Paxinos and Franklin 2001). An Ag/AgCl reference and stainless-steel auxiliary electrode combination was placed in surface contact with contralateral cortical tissue approximately 3 mm posterior from bregma. A carbon fiber microelectrode with an active surface of 250 μm (length) by 10 μm (o.d.) (Thornel Type P, Union Carbide, Pittsburgh, PA, USA) was implanted into the left nucleus accumbens (coordinates: AP $+1.5$ mm from bregma, ML $+1.0$ mm from midline, and -4.0 mm from dura; Paxinos and Franklin 2001).

Fixed potential amperometry recordings and MFB stimulation

Fixed potential amperometry in combination with carbon fiber microelectrodes is a technique used for the continuous and selective neurochemical monitoring of rapid changes in dopamine oxidation current (efflux) recorded in the NAc, evoked by electrical stimulation (Gonon 1988; Chergui et al. 1994; Dugast et al. 1994; Michael and Wightman 1999; Forster and Blaha 2003). Amperometric recordings were made within a Faraday cage to increase the signal-to-noise ratio. A fixed positive potential (0.8 V) was applied to the recording electrode and oxidation current was monitored continuously (10,000 samples/second) with an electrometer (ED410 e-corder 401 and EA162 Picostat, eDAQ Inc., Colorado Springs, CO, USA), filtered at 50 Hz. Following implantation of the recording electrode, a series of cathodal monophasic constant current pulses (800 A) was delivered to the stimulating electrode via an optical isolator and programmable pulse generator (Iso-Flex/Master-8; AMPI, Jerusalem, Israel). The stimulation protocol consisted of a 50-Hz train of 15 pulses (0.5 ms duration) applied every 30 s. After a 5-min baseline recording of MFB stimulation-evoked dopamine efflux, all mice received a systemic injection of saline (0.9%), and changes in dopamine efflux were monitored for 40 min. Amphetamine (2 mg/kg i.p.; University Hospital Rigshospitalet, Copenhagen, Denmark) or cocaine (5 mg/kg i.p.; Sigma–Aldrich Chemical Co., St Louis, MO, USA) was then administered and changes in MFB stimulation-evoked dopamine efflux were monitored over the course of each drug's effects.

At the conclusion of each electrochemical recording session, an iron deposit was made in the MFB stimulation site by passing direct anodic current (100 μA for 10 s) through the stimulating electrode. Mice were then euthanized with a 0.25-ml intracardial injection of urethane

(0.345 g/ml). Brains were removed and immersed overnight in 10% buffered formalin containing 0.1% potassium ferricyanide and then stored in 30% sucrose/10% formalin solution until sectioning. A Prussian blue spot resulting from a redox reaction of ferricyanide marked the stimulating site. Placements of the stimulating and electrochemical recording electrodes were verified under a light microscope.

Autoradiography

For receptor autoradiography, $M_5^{-/-}$ and $M_5^{+/+}$ mice were sedated with CO_2 and decapitated, the brains were dissected and immersed in $-40^\circ C$ isopentane for 1 min and stored at $-80^\circ C$ until sectioning. Using a cryostat, coronal sections (15 μm) were obtained from caudate-putamen (CPu) and nucleus accumbens (NAc) (1.54 to 0.74 mm relative to bregma; Franklin and Paxinos 1997), ventral tegmental area/substantia nigra (VTA/SN) (-3.08 to -3.64 mm), and the prefrontal cortex (PFC) (2.80 to 2.10 mm) for D1- and D2-family receptor autoradiography, while only CPu/NAc and VTA/SN were processed for dopamine transporter (DAT) autoradiography. The sections were thaw-mounted onto Superfrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany), and stored at $-80^\circ C$ until use.

Autoradiographic detection of D1-family receptors

The brain sections were preincubated in a buffer containing 50 mM TRIS base, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 nM MDL 100.907 (H. Lundbeck, Valby, Denmark) at $0^\circ C$ for 15 min before being incubated with $^3[H]SCH23390$ (1.0 nM, PerkinElmer Danmark A/S, Hvidovre, Denmark, 85 Ci/mmol) for 1 h at $4^\circ C$ in the same buffer, followed by two 5-min washes at $0^\circ C$ in buffer. Nonspecific binding was determined in the presence of the D1-, D2-, and 5-HT₂ receptor antagonist cis(Z)-flupentixol (10 μM , H. Lundbeck, Valby, Denmark).

After being dipped in demineralized water, slides were dried and exposed to Kodak Biomax MR-films (Amersham Biosciences, Hillerød, Denmark). The films were exposed for 40 days (CPu/NAc and VTA/SN) or 60 days (PFC) in autoradiography cassettes at $-20^\circ C$ before being developed.

Autoradiographic detection of D2-family receptors

The brain sections were preincubated in a buffer containing 50 mM TRIS base, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ at $0^\circ C$ for 15 min before being incubated with $^3[H]raclopride$ (4 nM, PerkinElmer Danmark A/S, Hvidovre, Denmark, 60.1 Ci/mmol) for 1 h at $4^\circ C$ in the same buffer, followed by two 30-min washes at $0^\circ C$ in buffer. Nonspecific binding was determined in the presence of 10 μM sertindole (H. Lundbeck, Valby, Denmark), a D2-, 5-HT₂-

and alpha1 receptor antagonist. Slides were further processed as described above. Exposure time was 40 days for both CPu/NAc and VTA/SN.

DAT autoradiography

The brain sections were preincubated in a buffer containing 50 mM Tris-HCl, 120 mM NaCl, and 5 mM KCl at $4^\circ C$ for 15 min before being incubated with $^3[H]mazindol$ (8 nM, PerkinElmer Danmark A/S, Hvidovre, Denmark, 24.5 Ci/mmol) and desipramine (300 nM, Sigma-Aldrich Danmark A/S, Brøndby, Denmark) for 1 h at $4^\circ C$ in the same buffer, followed by two 5-min washes at $4^\circ C$ in buffer. Nonspecific binding was determined in the presence of the high-affinity DAT inhibitor GBR12909 (0.5 μM , Sigma-Aldrich Danmark A/S, Brøndby, Denmark). After being dipped in demineralized water the slides were dried and exposed to 3H -Hyperfilms (Amersham Biosciences, Hillerød, Denmark) at $-20^\circ C$ for 10 weeks.

Developed films were analyzed and quantitated in a computer-assisted video densitometer (Scion-Image; Scion Corporation, Frederick, MD) using the standard curve generated from 3H standards (Amersham Biosciences, Hillerød, Denmark).

Statistical analyses

Behavioral studies

Baseline activity was analyzed by one-way analysis of variance (ANOVA). For amphetamine- and cocaine-induced activity, distance moved during the 20-min open-field session was analyzed by two-way ANOVA with genotype and drug treatment as between-subjects factors. Significant main effects or interactions were further analyzed by one-way ANOVA, followed by Tukey's honestly significant difference post hoc test.

The development of sensitization to the locomotor activating effect of amphetamine and cocaine over 10 days of the experiment was analyzed using mixed-model ANOVA with day as within-subject factor and genotype and drug treatment as between-subjects factors. Significant main effects or interactions were followed by appropriate planned post hoc tests.

Electrochemical studies

The dopamine oxidation current evoked by MFB stimulation after saline injection in $M_5^{-/-}$ and $M_5^{+/+}$ mice was compared by two-tailed independent samples Student's *t* test. Saline currents recorded prior to drug injection were then normalized to zero current values, with data points

occurring within the range of 0.25 s before and 1.0 s after the onset of the stimulation train extracted from the continuous record at 5-min intervals. In each mouse, the increase in dopamine oxidation current evoked by MFB stimulation after drug administration was expressed as percent change from pre-drug saline responses. For each condition, the resulting percentage changes were subsequently averaged across animals. For each drug, mean changes in oxidation current were compared between $M5^{-/-}$ and $M5^{+/+}$ mice over the first 45 min after drug injection using mixed-model ANOVA with time as within-subject factor and genotype as between-subjects factor, followed by Bonferroni corrected post hoc t test when appropriate. In order to compare reuptake of dopamine (DAT efficiency), peak increase in dopamine oxidation currents after drug administration was normalized within each treatment group; curves were fitted with nonlinear regression using GraphPad Prism (GraphPad Software, Inc., La Jolla, USA) using the equation $Y = \text{SPAN} \times \exp(-K \times X) + \text{PLATEAU}$ and half-lives determined for each mouse by the software. Half-lives were compared with two-tailed independent samples Student's t test.

Autoradiography

D1- and D2-family receptor and DAT density was determined for each animal with a total of six measurements for each brain area (each cerebral hemisphere in three sections). The mean of these six measurements was used for further statistical analysis. Two-tailed independent samples Student's t tests were used to compare $M5^{-/-}$ and $M5^{+/+}$ mice.

The alpha level for all analyses was set at 0.05.

Results

Behavioral studies

Baseline locomotor activity

Basal open field activity did not differ between genotypes in mice backcrossed to the C57BL/6Ntac strain. All genotypes displayed similar levels of activity ($M5^{+/+} = 65.39 \pm 2.35$ cm; $M5^{+/-} = 58.60 \pm 3.19$ cm; $M5^{-/-} = 60.32 \pm 1.74$ cm; means \pm SEM; $n = 14-21$).

Amphetamine- and cocaine-induced locomotor activity

Distance moved during the 20-min session is presented in Fig. 1 for amphetamine- and in Fig. 2 for cocaine-induced locomotor activity. For amphetamine, the two-way ANOVA revealed a significant increase in locomotor activity with

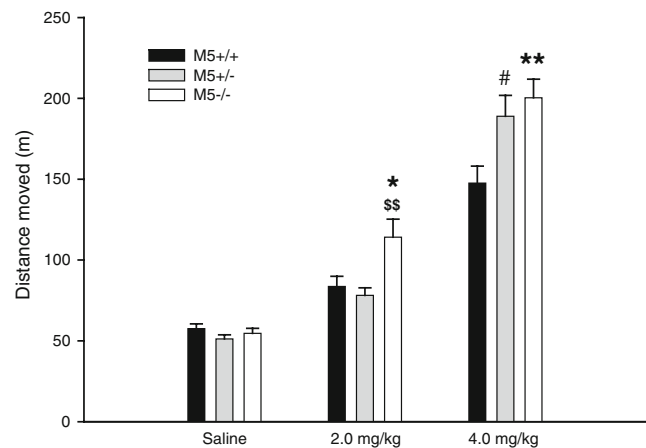


Fig. 1 Amphetamine-induced hyperlocomotor activity during a 20-min session in the open field. $M5^{-/-}$ animals moved significantly longer distances than $M5^{+/+}$ animals after injection with 2.0 and 4.0 mg/kg of amphetamine. After injection with 4.0 mg/kg amphetamine, also $M5^{+/-}$ mice moved more than $M5^{+/+}$ mice. * $p < 0.05$; ** $p < 0.01$ vs. $M5^{+/+}$; \$\$ $p < 0.01$ vs. $M5^{+/-}$; # $p < 0.05$ vs. $M5^{+/+}$. Abscissa amphetamine dose. Ordinate locomotor activity measured as distance moved. Data are group means \pm SEM. Group sizes: $n = 10-14$

drug dose ($F_{2,87} = 198.76$; $p < 0.001$); this response differed significantly between genotypes ($F_{2,87} = 8.66$; $p < 0.001$), and there was a significant interaction between genotype and drug treatment ($F_{4,87} = 4.81$; $p < 0.01$). Post hoc analysis showed that $M5^{-/-}$ mice moved significantly longer distances than $M5^{+/+}$ mice at both 2.0 and 4.0 mg/kg amphetamine ($p < 0.05$). Furthermore, $M5^{-/-}$ mice displayed more hyperactivity than $M5^{+/-}$ animals ($p < 0.01$) at the 2.0-mg/kg dose. Both, $M5^{+/-}$ and $M5^{-/-}$ mice showed increased hyperactivity at the 4.0-mg/kg dose compared to $M5^{+/+}$ mice, revealing a gene-dose effect. In all three genotypes, both the 2.0- and the 4.0-mg/kg doses induced significant hyperactivity relative to saline ($p < 0.05$).

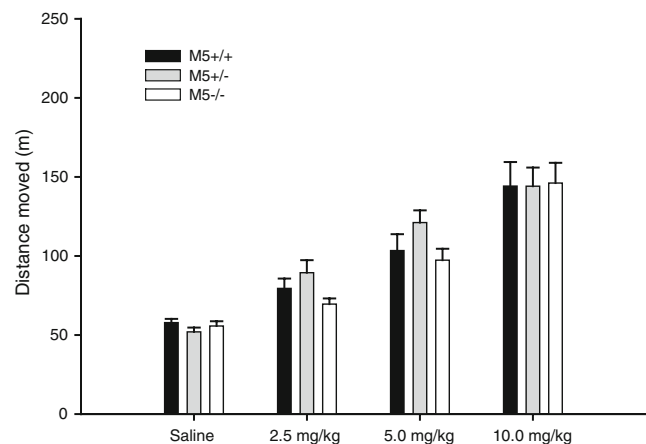


Fig. 2 Cocaine-induced hyperlocomotor activity during a 20-min session in the open field. Cocaine doses of 2.5, 5.0, and 10 mg/kg induced similar levels of hyperactivity in all three genotypes. Abscissa cocaine dose. Ordinate locomotor activity measured as distance moved. Data are group means \pm SEM. Group sizes: $n = 10-25$

Cocaine also increased locomotor activity dose-dependently ($F_{3,142}=71.09$; $p<0.001$) but with no effect of genotype and no interaction between genotype and drug treatment. Doses of 5.0 and 10.0 mg/kg cocaine induced significant hyperactivity compared to saline ($p<0.001$).

Sensitization to the locomotor-activating effect of amphetamine and cocaine

Sensitization to the locomotor-activating effect of amphetamine is shown in Fig. 3. Mixed-model ANOVA showed a significant main effect of day ($F_{9,450}=35.35$; $p<0.001$), amphetamine treatment ($F_{1,50}=71.35$; $p<0.001$), and genotype ($F_{1,50}=8.6$; $p<0.005$) as well as significant interactions between all factors entered into the model ($p<0.05$). Post hoc analysis showed that the saline treated $M_5^{+/+}$ and $M_5^{-/-}$ mice never differed throughout the experiment. Likewise, the genotypes did not differ on the first day of the experiment (day 0), when all animals received a saline injection. In contrast, when injected with amphetamine, $M_5^{-/-}$ animals displayed significantly more hyperactivity than $M_5^{+/+}$ animals on the remaining days of the experiment ($p<0.05$, Fig. 3).

$M_5^{-/-}$ mice displayed a significantly higher level of sensitization compared to $M_5^{+/+}$ mice as revealed by an increased effect of amphetamine in previously amphetamine-injected $M_5^{-/-}$ mice, expressed as percentage increase over the respective control group of previously saline injected animals on day 20 of the experiment ($M_5^{+/+}=207.65\pm$

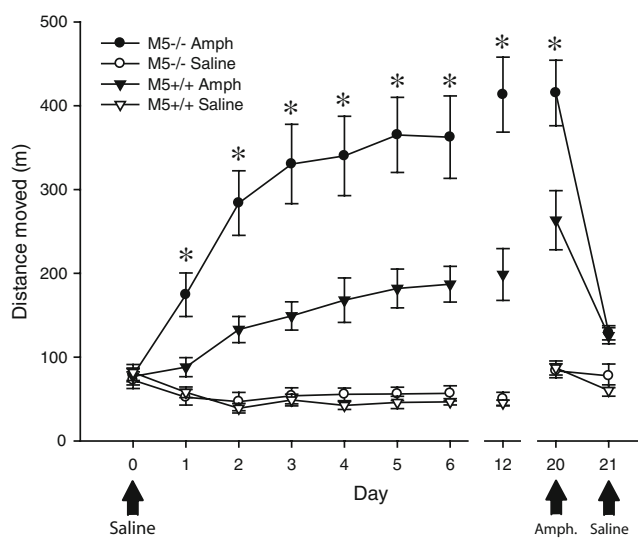


Fig. 3 Locomotor activity during sensitization to amphetamine (2.0 mg/kg, i.p.). Repeated administration of amphetamine significantly increased the hyperlocomotor response in both genotypes; however, this was dramatically enhanced in $M_5^{-/-}$ mice. Black arrows indicate when treatment differed from that implied by the legend. * $p<0.05$ vs. amphetamine treated $M_5^{+/+}$ mice. Abscissa number of days in the treatment regimen. Ordinate locomotor activity measured as distance moved. Data are group means \pm SEM. Groups sizes: $n=11-13$

46.91%; $M_5^{-/-}=395.58\pm 46.68\%$; $p<0.01$). The activity of both amphetamine-treated groups also differed significantly from their controls on this day of the experiment ($p<0.001$).

On the last day of the experiment (day 21), when all animals were again injected with saline to assess the contribution of context conditioning to the observed levels of hyperactivity, the two genotypes did not differ significantly. However, both groups of previously amphetamine-treated animals displayed higher levels of activity after saline injection than the corresponding saline-treated animals, showing significant and similar levels of context conditioning (see Fig. 3).

The development of sensitization to the locomotor-activating effect of cocaine is shown in Fig. 4. Mixed-model ANOVA showed a significant main effect of day ($F_{9,459}=40.98$; $p<0.0001$), cocaine treatment ($F_{3,459}=21.64$; $p<0.0001$) as well as significant interaction between these two factors ($F_{27,459}=5.82$; $p<0.0001$), while the effect of genotype was not significant and did not enter into any significant interaction. Post hoc analysis showed no differences between any of the groups on the first day of the experiment (day 0), when all animals received a saline injection and baseline locomotor activity was assessed. Saline treated $M_5^{+/+}$ and $M_5^{-/-}$ mice displayed similar locomotor activity throughout the experiment and so did the cocaine-treated $M_5^{-/-}$ and $M_5^{+/+}$ animals. Cocaine sensitization was also calculated as percent increase in locomotor activity on day 20 over the respective control group of previously saline-injected animals. The cocaine-treated $M_5^{-/-}$ and $M_5^{+/+}$ mice showed a significantly higher

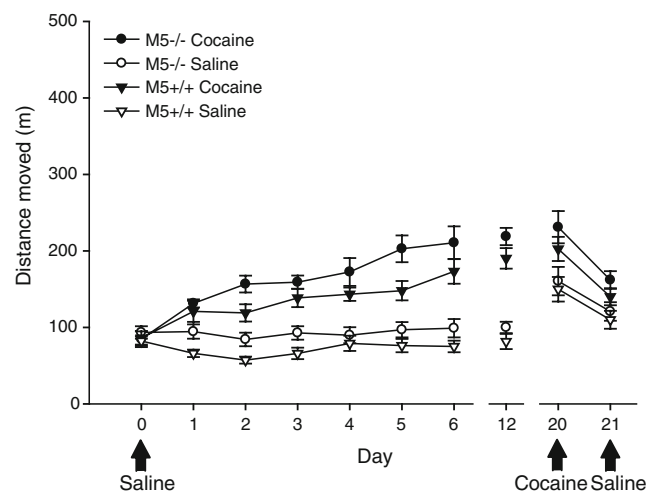


Fig. 4 Locomotor activity during sensitization to cocaine (5.0 mg/kg, i.p.). Cocaine induced similar levels of sensitization of the hyperlocomotor response in $M_5^{+/+}$ and $M_5^{-/-}$ mice. Black arrows indicate when treatment differed from that implied by the legend. Abscissa number of day in the treatment regimen. Ordinate locomotor activity measured as distance moved. Data are group means \pm SEM. Groups sizes: $n=12-15$

locomotor response compared to controls that received their first cocaine injection on that day ($p < 0.05$). However, the level of sensitization did not differ between $M5^{-/-}$ and $M5^{+/+}$ mice. On day 21, all mice were injected with saline to examine if the sensitization protocol induced similar levels of context conditioning in the two genotypes. No significant differences were found between the previously cocaine- and saline-treated groups or between the genotypes, indicating no contribution of context to the sensitized response.

Electrochemical studies

The mean change in dopamine oxidation current evoked by MFB stimulation after saline injection did not differ significantly between $M5^{-/-}$ and $M5^{+/+}$ mice ($M5^{-/-} = 0.43 \pm 0.05$ nA, $M5^{+/+} = 0.34 \pm 0.04$ nA). The effect of amphetamine on MFB stimulation-evoked dopamine efflux in the NAc is shown in Fig. 5, expressed as the mean change in oxidation current from saline levels. Amphetamine induced an increase in dopamine release in both genotypes, but the effect was much more pronounced in the $M5^{-/-}$ mice than in the $M5^{+/+}$ mice (Fig. 5). Mixed-model ANOVA showed a significant effect of time ($F_{9,144} = 11.58$; $p < 0.0001$) and genotype ($F_{1,144} = 12.05$; $p < 0.01$) and a significant interaction ($F_{9,144} = 6.16$; $p < 0.0001$). Post hoc tests showed that genotypes differed significantly from 20 through 40 min after amphetamine administration ($p < 0.05$). Cocaine also induced a marked increase in dopamine release in both genotypes (Fig. 6). For cocaine, mixed-model ANOVA showed a significant effect of time ($F_{9,135} = 16.27$; $p < 0.0001$) but no effect of genotype and no interaction.

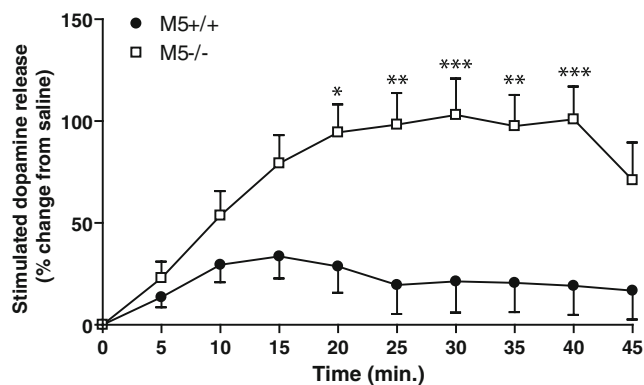


Fig. 5 The effect of amphetamine on MFB stimulated dopamine release in NAc. Amphetamine increased dopamine efflux in both genotypes. This effect was significantly enhanced in $M5^{-/-}$ compared to $M5^{+/+}$ mice during the entire period of measurement. * $p < 0.05$. Abscissa time in minutes after amphetamine injection. Ordinate stimulated dopamine release in percent change from saline values. Data are group means \pm SEM. Group sizes: $n = 8-10$

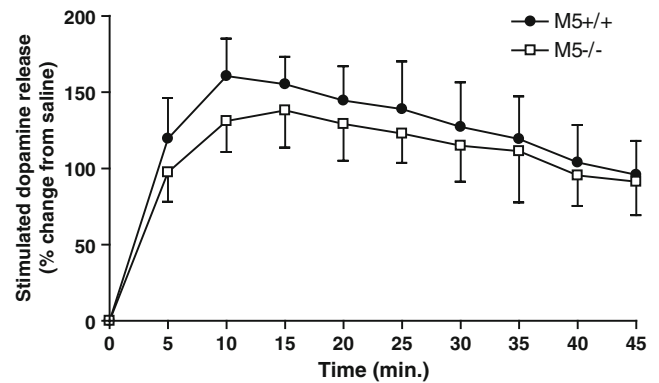


Fig. 6 The effect of cocaine on MFB stimulated dopamine release in NAc. Cocaine induced similar changes in dopamine release in both genotypes. Abscissa time in minutes after cocaine injection. Ordinate stimulated dopamine release in percent change from saline values. Data are group means \pm SEM. Group sizes: $n = 8-9$

Half-life of the decay of the peak dopamine oxidation current in the $M5^{-/-}$ and $M5^{+/+}$ mice was also compared for each drug (Fig. 7). No significant differences between the genotypes were found for either cocaine or amphetamine, indicating that DAT was equally effective in both genotypes under influence of both amphetamine and cocaine.

Autoradiography

Receptor autoradiography on D1- and D2-family receptors and the DAT was performed to investigate potential compensatory changes in density after disruption of the M5 gene. Brain areas with dopaminergic projections of importance for locomotion and reinforcement were investigated (i.e., PFC, CPu, NAc, VTA/SN). In addition, D1- and D2-family receptor density was measured in the olfactory tubercle because of the large concentration of dopamine receptors in this area.

D2-family receptors were not measured in PFC because this receptor family is not detectable in this structure in mouse brain (Camps et al. 1990). After 3 months of film exposure, D2-family receptors were still not measurable in the VTA/SN region, and this area had to be excluded from the investigation.

Student's *t* tests showed that D1-family receptor density in the PFC, CPu, NAc, olfactory tubercle, and VTA/SN did not differ between $M5^{-/-}$ mice and their $M5^{+/+}$ controls. Likewise, D2-family receptor expression in the CPu, NAc, and olfactory tubercle did not differ between $M5^{-/-}$ mice and their $M5^{+/+}$ controls (see Table 1 and Fig. 8).

DAT expression was investigated in the NAc, CPu, SNc, and VTA. No significant differences were found between $M5^{-/-}$ mice and their $M5^{+/+}$ controls in any brain region (see Table 2 and Fig. 9).

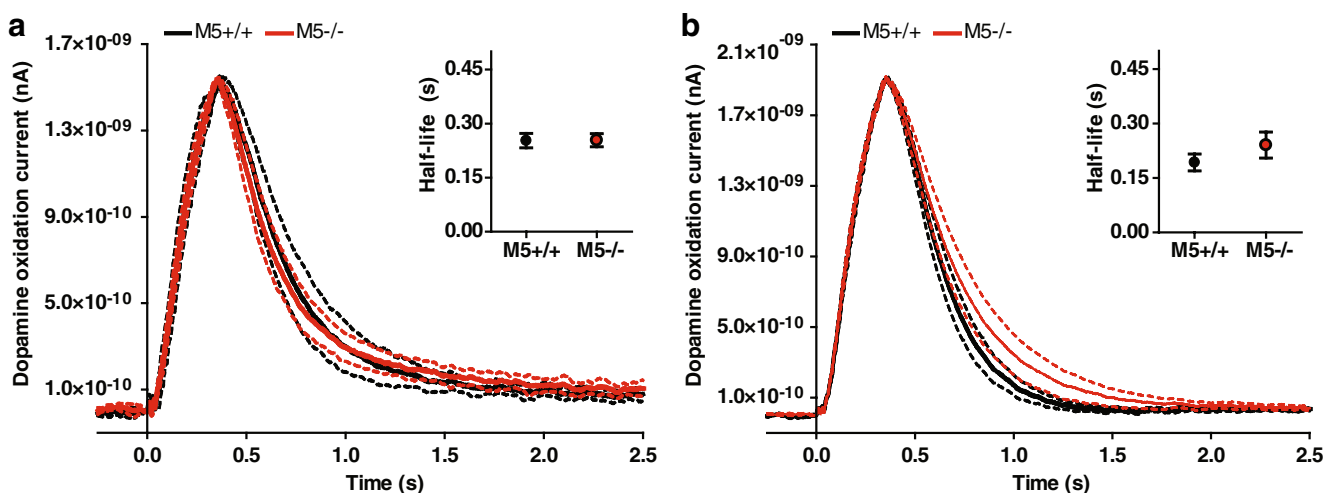


Fig. 7 Peak dopamine oxidation current of $M_5^{+/+}$ (black line) and $M_5^{-/-}$ (red line) mice after cocaine (a) and amphetamine (b) administration. The insert shows the half-life of the decay of the current. No significant differences between the genotypes were found for either cocaine or

amphetamine. *Abscissas* time in seconds post MFB stimulation. *Ordinates* dopamine oxidation current measured in nano-amperes. Data are group means \pm SEM. Group sizes: $n=8-10$

Discussion

The mesocorticolimbic dopamine system plays an important role in mediating the behavioral and reinforcing actions of psychostimulants (Koob et al. 1998). It receives input from the VTA, where M_5 receptors modulate dopamine release (Vilaro et al. 1990; Weiner et al. 1990). In the present study, we show increased amphetamine-induced locomotor activity, increased sensitization to the locomotor-activating effect of amphetamine and increased evoked accumbal dopamine efflux after amphetamine administration in M_5 receptor-deficient mice extensively backcrossed to the C57BL/6Ntac strain. Cocaine induced comparable levels of hyperlocomotion, sensitization, and evoked dopamine release in $M_5^{-/-}$ and $M_5^{+/+}$ mice. We found no compensatory changes in the expression of D1- and D2-family receptors and DAT.

All three genotypes displayed similar levels of baseline locomotor activity in the open field. This is in agreement with previous studies, using $M_5^{-/-}$ and $M_5^{+/+}$ mice of mixed genetic background (Yamada et al. 2001; Chintoh et al. 2003) as well as backcrossed mice (Thomsen et al. 2007). Thus, the M_5 receptor does not appear to modulate basal locomotor activity, as has been concluded previously (Yamada et al. 2001; Chintoh et al. 2003).

Acute amphetamine administration produced more locomotor activation in $M_5^{-/-}$ mice compared to wild-type mice at doses of 2.0 and 4.0 mg/kg. Previous studies found no difference in amphetamine-induced hyperactivity between $M_5^{+/+}$ and $M_5^{-/-}$ mice (Yamada et al. 2001; Yeomans et al. 2001), while one study observed reduced amphetamine-induced hyperlocomotion in $M_5^{-/-}$ mice (Wang et al. 2004). Several minor methodological differences may account for these divergent results. However, it is most likely that the

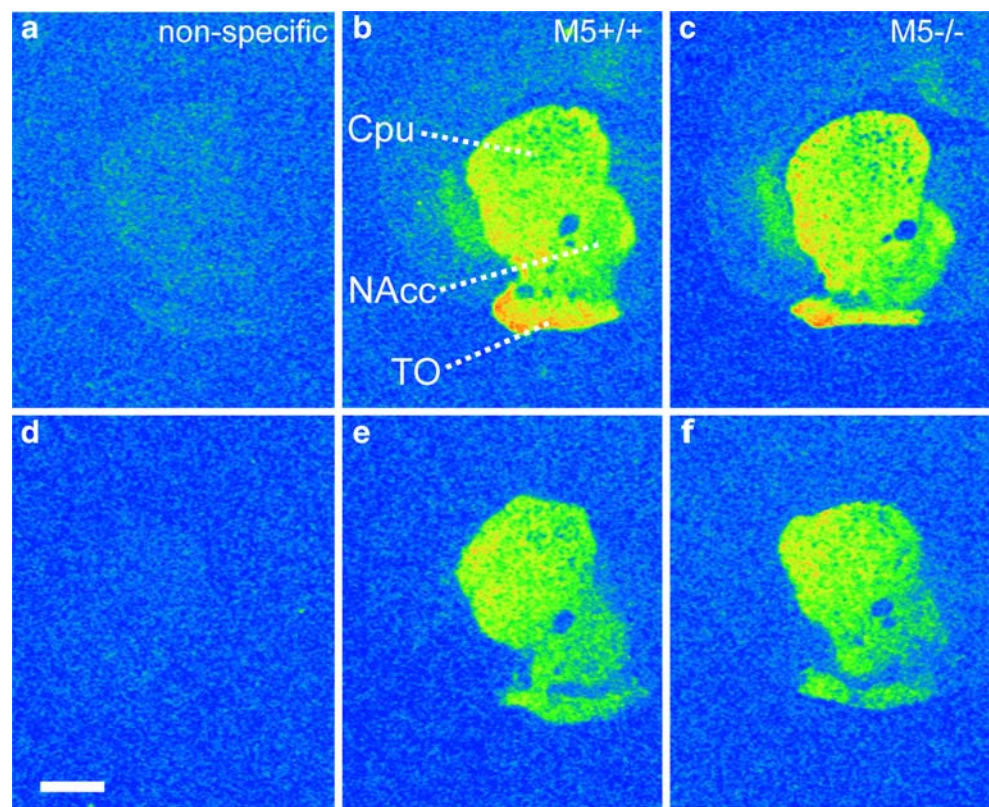
Table 1 D1- and D2-family receptor levels in $M_5^{+/+}$ and $M_5^{-/-}$ mice

	D1-like		D2-like	
	$M_5^{+/+}$ (Bq/mg)	$M_5^{-/-}$ (Bq/mg)	$M_5^{+/+}$ (Bq/mg)	$M_5^{-/-}$ (Bq/mg)
NAc	752.5 \pm 39.8	753.9 \pm 32.2	436.5 \pm 26.0	489.4 \pm 33.1
CPu	796.6 \pm 28.5	770.5 \pm 36.4	435.41 \pm 39.3	515.7 \pm 14.5
TO	1,100.5 \pm 40.5	1,079.5 \pm 30.8	494.46 \pm 17.0	502.7 \pm 33.8
VTA/SN	919.6 \pm 54.5	995.7 \pm 39.8	ND	ND
PFC	107.9 \pm 28.1	111.1 \pm 27.0	ND	ND

Density of D1- and D2-family receptors was assessed by autoradiography with 3 [H]SCH23390 and 3 [H]raclopride, respectively. Receptor density did not differ between genotypes in any brain area examined. Data are group means \pm SEM. Groups sizes: $n=6-7$

CPu caudate putamen, NAc nucleus accumbens, ND not determined, PFC prefrontal cortex, TO olfactory tubercle, VTA/SN ventral tegmental area/substantia nigra

Fig. 8 Pseudocolored autoradiograms showing ^3H SCH23390 binding to D1-family receptors (**a–c**) and ^3H raclopride binding to D2-family receptors (**d–f**) at the level of caudate putamen (*CPu*), nucleus accumbens (*NAc*), and olfactory tubercle (*TO*). **a** Nonspecific binding in $M_5^{+/+}$ mouse brain. **b** Total D1 binding in $M_5^{+/+}$ mouse brain. **c** Total D1 binding in $M_5^{-/-}$ mouse brain. **d** Nonspecific binding in $M_5^{+/+}$ mouse brain. **e** Total D2 binding in $M_5^{+/+}$ mouse brain. **f** Total D2 binding in $M_5^{-/-}$ mouse brain. The density of D1- and D2-family receptors did not differ between $M_5^{-/-}$ and $M_5^{+/+}$ mice. Scale bar=1 mm



main reason for these discrepancies might be found in the genetic background of the animals. In previous studies, non-backcrossed mice maintained on mixed genetic backgrounds were used. Here, we used animals that had been backcrossed extensively to the C57BL/6Ntac strain. It is well known that gene knockouts and mutations may display different phenotypes on different genetic backgrounds (Gerlai 1996; Banbury Conference 1997). The mouse lines may also have different compensatory changes in adaptation to the M_5 receptor deletion that could account for the discrepancies. Wang et al. (2004) reported increased levels of dopamine D2 receptor mRNA in several brain regions of their $M_5^{-/-}$ mice. D2 agonists decrease locomotor activity in mice (Geter-

Douglass et al. 1997; Halberda et al. 1997; Ralph and Caine 2005), and a higher expression of D2 receptors may, therefore, contribute to the attenuated amphetamine-response in $M_5^{-/-}$ mice reported by Wang et al. (2004). In the present study, D1- and D2-family receptor and DAT binding was examined in relevant brain regions, and no changes were found which is consistent with results obtained before extensive backcrossing of this mouse line (Basile et al. 2002).

Cocaine also increased locomotor activity in a dose-dependent manner but in a similar fashion for $M_5^{-/-}$, $M_5^{-/+}$, and $M_5^{+/+}$ mice. From previous studies examining cocaine-conditioned place preference, as well as acute and chronic cocaine self-administration in $M_5^{-/-}$ mice (Fink-Jensen et al. 2003; Thomsen et al. 2005), reduced effects of cocaine in $M_5^{-/-}$ mice could be expected. However, the present findings are in agreement with a previous report on locomotor activity: Thomsen et al. (2007) also found similar levels of cocaine-induced hyperactivity in $M_5^{-/-}$, $M_5^{-/+}$, and $M_5^{+/+}$ mice at doses up to 56.0 mg/kg. These results underline the importance of using multiple behavioral models when investigating the neural basis of the stimulus properties of addictive drugs.

Similar effects of cocaine and amphetamine on locomotion might be expected as both drugs inhibit the reuptake of monoamines. However, amphetamine also redistributes vesicular dopamine to the cytosol from where it can be released into the synapse by reversing the transporter

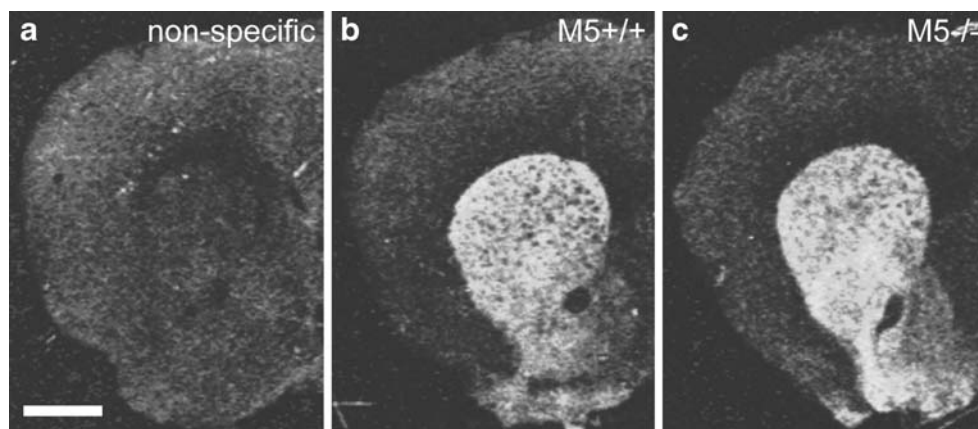
Table 2 DAT levels in $M_5^{+/+}$ and $M_5^{-/-}$ mice

	DAT	
	$M_5^{+/+}$ (Bq/g)	$M_5^{-/-}$ (Bq/g)
NAC	84.6±6.6	88.2±6.6
CPu	197.9±8.1	197.3±10.3
SNc	250.6±38.8	210.8±15.9
VTA	269.8±33.6	215.9±15.8

Density of DAT was assessed by autoradiography with ^3H mazindol. DAT density did not differ between genotypes in any brain area examined. Data are group means ± SEM. Groups sizes: $n=10-11$

CPu caudate putamen, *NAc* nucleus accumbens, *SNc* substantia nigra, pars compacta, *VTA* ventral tegmental area

Fig. 9 Autoradiogram showing ^3H mazindol binding to DAT at the level of striatum. **a** Nonspecific binding in $M_5^{+/+}$ mouse brain. **b** Total DAT binding in $M_5^{+/+}$ mouse brain. **c** Total DAT binding in $M_5^{-/-}$ mouse brain. The density of DAT did not differ between $M_5^{-/-}$ and $M_5^{+/+}$ mice. Scale bar=1 mm



(Sulzer et al. 2005). This difference in pharmacological properties might be responsible for the observed differences in the two drugs ability to stimulate locomotor activity in $M_5^{+/+}$ and $M_5^{-/-}$ mice. Reduction in dopamine cell activity, induced by pharmacological denervation of dopamine axonal transmission, dramatically enhances synthesis and storage of dopamine in terminal vesicles (Brown et al. 1991). Similarly, M_5 receptor deletion could alter the firing properties of midbrain dopaminergic neurons, leading to an accumulation of dopamine in the terminals, which can be released by amphetamine, but not cocaine, thus unveiling an otherwise hidden phenotype. This hypothesis is supported by the results of our amperometry study, showing that amphetamine-, but not cocaine-, elicited dopamine release in nucleus accumbens is enhanced in $M_5^{-/-}$ mice compared to $M_5^{+/+}$ mice.

We also investigated the role of M_5 receptors in the more chronic effects of amphetamine and cocaine treatment. We used a regimen of repeated administration of psychostimulants that leads to progressive enhancement of behavioral responses, sensitization, which is accompanied by persistent neurochemical changes and can be observed even after long periods of abstinence (Robinson and Berridge 1993, 2008). Sensitization of the appetitive effects of addictive drugs has been suggested to underlie the transition from drug use to drug abuse (Robinson and Berridge 1993, 2008). Sensitization to psychostimulants can be measured as increased hyperlocomotion seen after repeated administrations of the drug. Behavioral sensitization of the locomotor response to amphetamine and cocaine was found in both $M_5^{+/+}$ and $M_5^{-/-}$ mice. From the first day of the treatment regimen, $M_5^{-/-}$ mice showed significantly enhanced hyperlocomotion compared to wild-type mice when amphetamine was administered. This is consistent with our observations after acute amphetamine administration. Moreover, increased sensitization in $M_5^{-/-}$ mice was attested by a greatly enhanced response on the test day (day 20) compared to the control group and to the sensitized wild-type mice. Like acute cocaine-induced

hyperlocomotion, sensitization to cocaine was alike in $M_5^{+/+}$ and $M_5^{-/-}$ mice.

Environmental factors may modify the expression of sensitized behavior, and in some cases, sensitization is context-dependent (Ohmori et al. 2000; Badiani and Robinson 2004). We, therefore, tested the sensitized mice with saline to evaluate the contribution of context conditioning to the hyperlocomotor response. Both genotypes expressed low and similar levels of context conditioning in the amphetamine sensitization experiment and no significant context conditioning in the cocaine sensitization experiment. Consequently, we ascribe the increased hyperlocomotor response observed in the $M_5^{-/-}$ mice to augmented amphetamine sensitization.

The activity of midbrain dopaminergic neurons projecting to the NAc is implicated in mediating the reinforcing effects of psychostimulants (Wise 1996; Koob et al. 1998). Thus, we used fixed potential amperometry recordings of MFB-stimulated dopamine release in the NAc to investigate the effect of amphetamine and cocaine administration in $M_5^{+/+}$ and $M_5^{-/-}$ mice. Evoked dopamine efflux measured after saline injection was similar in $M_5^{+/+}$ and $M_5^{-/-}$ mice. Consistent with the locomotor activity data, amphetamine had a significantly larger effect on dopamine release in $M_5^{-/-}$ compared to $M_5^{+/+}$ mice, while cocaine induced comparable increases in evoked dopamine release in both genotypes. These results indicate that the augmented amphetamine-induced hyperlocomotor response observed in $M_5^{-/-}$ mice is correlated with an increase in dopamine released into the extracellular space. Previous studies have provided evidence that M_5 receptors play a facilitating role in striatal dopamine release (Yamada et al. 2001; Basile et al. 2002; Forster et al. 2002). As mentioned earlier, genetic background and compensatory changes may account for some of the discrepancies between the present and previous studies. In backcrossed mice, Thomsen et al. (2005) found decreased reinforcing efficacy of cocaine in $M_5^{-/-}$ mice at low and moderate cocaine doses, and this effect could be overcome by higher cocaine doses. It is, therefore, possible

that a subtle effect of M_5 receptor deletion on cocaine-enhanced evoked dopamine release is not detectable in the present experiments. Although not significant, $M_5^{-/-}$ mice showed a tendency to lower increase in dopamine efflux after cocaine compared to wild-type mice (see Fig. 6). In contrast to cocaine, amphetamine is both a dopamine releaser and a dopamine reuptake inhibitor (Sulzer et al. 2005). Changes in the regulation of dopamine homeostasis by M_5 receptor deletion may be expressed differently in response to amphetamine as compared to cocaine due to amphetamine's multiple modes of action. As mentioned previously, we speculate that M_5 receptor deletion may affect intracellular dopamine levels by altering the firing properties of midbrain dopaminergic neurons and lead to an accumulation of dopamine in the terminals, which can be released by amphetamine, but not cocaine (Brown et al. 1991).

In conclusion, our data show that M_5 receptor disruption dramatically influences the acute and chronic effects of amphetamine. In contrast, cocaine-induced hyperlocomotion, sensitization, and evoked dopamine release in the NAc were unaffected by the M_5 receptor deletion. Differences in the mechanisms of action of the two drugs may account for the differential responses to the two psychostimulants observed in $M_5^{+/+}$ and $M_5^{-/-}$ mice. Sensitization to the appetitive effects of addictive drugs has been suggested as a possible mechanism underlying the transition from drug use to drug abuse by enhancing drug craving. Therefore, our observation of increased amphetamine sensitization in $M_5^{-/-}$ mice suggests that the M_5 receptor may be a valid target for pharmacological treatment of abuse of amphetamine and its derivatives and the prevention of relapse to drug-seeking behavior.

Ethical standards

The “Principles of Laboratory Animal Care” were followed, and all procedures were conducted in accordance with guidelines from the Animal Experimentation Inspectorate, Denmark and the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

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