Dissociation of rewarding and dopamine transporter-mediated properties of amphetamine

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The interaction of amphetamine (AMPH) with the dopamine (DA) transporter (DAT) is thought to be critically important for the DA-elevating actions of this drug. It is commonly believed that DA elevations are involved in the rewarding/reinforcing properties of AMPH and other drugs of abuse. Here, we found that DAT deletion did not eliminate the rewarding effects of AMPH as measured by conditioned place preference (CPP). In fact, mice in which the DAT gene has been deleted (DAT-KO mice) exhibited AMPH-induced CPP for many weeks after the time when extinction occurred in WT mice. Moreover, systemic AMPH still increased extracellular DA in the nuclei accumbens (NAc) of mice lacking the DAT, although local infusion of AMPH into the NAc did not have this effect. By using voltammetry in NAc slices, we found that AMPH did not decrease the rate of DA clearance. The rate of ventral tegmental area DA neuron firing was dramatically inhibited by AMPH in brain slices from WT mice, but there was no inhibition of firing in DAT-KO mice. AMPH-induced CPP was abolished by pretreatment with WAY-100635, a serotonin 5-HT1A receptor antagonist, in DAT-KO mice, but the drug did not change AMPH place preference in WT mice. Therefore, despite the absence of the DAT, AMPH displays rewarding effects and causes an increase in extracellular DA in the NAc of DAT-KO mice, acting indirectly in this case. The 5-HT system may be involved in the rewarding effects of AMPH in these mice.

Materials and Methods

Animals. Mice with a deletion of the gene (Slc6a3) encoding the DAT were used in these studies. Homozygote DAT-KO and WT littermate mice were derived from the crossing of heterozygous DAT 129sV/C57Bl mice, as described in ref. 19. Mice were housed three to five per cage on a 12-h light/dark cycle with ad libitum access to water and food. All animal procedures were approved by the Wake Forest University Health Sciences Institutional Animal Care and Use Committee.

Cyclic Voltammetry in Brain Slices. Mice were killed by decapitation, and the brains were rapidly removed and cooled in ice-cold, preoxygenated (95% O2/5% CO2) modified Krebs buffer. The tissue was then sectioned into 400-μm-thick coronal slices containing the NAc with a vibrating tissue slicer (Leica VT1000S, Leica Instruments, Wetzlar, Germany). Slices were kept in a reservoir of oxygenated Krebs buffer at room temperature until required. Thirty minutes before each experiment, a brain slice was transferred to a submersion recording chamber, perfused at 1 ml/min with 34°C oxygenated Krebs, and allowed to equilibrate. DA release was evoked by a single, rectangular, electrical pulse (300 μA, 2 ms per phase, biphasic), applied every 15 min. DA was detected by using FSCV as described earlier (14, 15, 20). Measured time courses of DA before and after d-AMPH (Sigma–Aldrich) were analyzed with a Michaelis–Menten-based...
set of kinetic equations (21) to determine the maximal concentration of DA detected and the rate of DA clearance. Time courses in DAT-KO mice were evaluated as a pseudo first-order rate constant (k). To compare kinetics between genotypes, a rate constant k was calculated by dividing V_{max} by K_{m} values in WT mice (15, 20). Voltammetry experiments were performed in both core and shell regions because they have different innervations by DA, norepinephrine, and 5-HT terminals.

**Microdialysis.** Microdialysate samples were collected from the right NAc 24 h after surgery, separated, and quantified by HPLC as previously described for freely moving mice (14, 15, 17). The procedure and brain coordinates for the implantation of the probe were chosen according to previous investigation (17). Briefly, mice were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic instrument, and dialysis probes (1-mm membrane length, 0.24-mm o.d.; Cuprophane, 6-kDa cutoff; CMA-11, CMA/Microdialysis, Solna, Sweden) were implanted into the right NAc (antero-posterior, 1.2 mm; dorso-ventral, −5.2 mm; and lateral, 0.6 mm relative to bregma). Twenty-four hours after surgery, the dialysis probe was connected to a syringe pump and perfused at 1 µl/min with artificial cerebrospinal fluid (CMA/Microdialysis). After at least a 1-h equilibration period, perfusates were collected every 20 min.

**Electrophysiology.** Mice were killed by decapitation after halothane anesthesia. The full methodology for preparation of brain slices of the VTA from rats has been published (22, 23). Coronal sections (400 µm) containing the VTA were maintained at 35°C and submerged in artificial cerebrospinal fluid flowing at 2 ml/min. Electrodes were positioned into the VTA by visual guidance; mouse DA-ergic neurons have been shown to have distinctive electrophysiological characteristics (22), including broad (>2.5 msec) action potentials often with an inflection or “notch” on the rising phase, spontaneous and regular firing at 0.5–5 Hz, and are inhibited by DA. Only neurons meeting these electrophysiological criteria were studied.

Aqueous solutions of AMPH were added to the flowing artificial cerebrospinal fluid with a calibrated infusion pump. AMPH was administered for 6–7 min to ensure that measurements were made after the full AMPH concentration was reached in the tissue and the peak drug effect was attained. Extracellular recording electrodes were filled with 0.9% NaCl, and tip resistances ranged from 4 to 8 MΩ. An IBM personal computer-based data acquisition system was used to calculate, display, and store the frequency of firing over 5-sec and 1-min intervals. Firing rate was determined before, during, and after drug application. Firing rate was calculated over a 1-min interval immediately before drug administration and over a 1-min interval during the peak drug effect. Drug-induced changes in firing rate were expressed as the percentage change from the control firing rate according to the formula \((FR_{D} - FR_{C})/FR_{C} \times 100\), where \(FR_{D}\) is the firing rate during the peak drug effect and \(FR_{C}\) is the control firing rate. Averaged numerical values were expressed as the mean ± SEM.

**CPP.** The CPP apparatus consisted of white and black chambers (21×28×25 cm) connected by an anteroom (21×12×25 cm) with guillotine doors. During the preconditioning phase, mice were allowed access for 15 min to both chambers. During the conditioning phase, mice received an i.p. dose of AMPH (2.5 or 5.0 mg/kg) or saline and were confined to one chamber of the apparatus for 15 min. Mice were returned to their home cage for 8 h and then given an injection of either drug or saline, whichever they had not yet received, and were placed in the opposite chamber. This pairing was repeated in the first group of mice for 4 days and in the second group for 2 days. The third experimental group (4 days of pairings) received WAY-100635 (2 mg/kg) 20 min before AMPH (5 mg/kg) or saline before saline injection.

On the next and subsequent days, up to 55 days (first group only) after the last pairing (postconditioning phase), mice were placed in the anteroom and the doors opened. Time spent in each compartment was measured electronically by infrared photo-beams during a 15-min observation period. CPP was assessed by the amount of time difference between the preconditioning and postconditioning phase. Data were analyzed with Student’s t test.

**Statistics.** Statistical analyses by using repeated measures ANOVA and paired or unpaired Student’s t tests were carried out with PRISM (GraphPad, San Diego) and SIGMSTAT (SPSS, Chicago). The data are presented as mean ± SEM. Differences of P < 0.05 are reported.

**Results**

**Effect of AMPH on Extracellular DA Levels.** Baseline dialysate DA levels measured by microdialysis in the NAc of DAT-KO mice were higher than in the WT controls (2.9 ± 0.6 vs. 0.7 ± 0.1 nM, \(P < 0.001; n = 10\)). DA levels were significantly elevated by systemic administration of AMPH (5 mg/kg, i.p.) in both WT and DAT-KO mice (\(P < 0.001\) vs. respective saline-treated controls) (Fig. 1A). The AMPH-induced percentage increase in DA levels in WT mice was greater than in DAT-KO animals (\(P < 0.05\)). However, the absolute level of DA at the peak time after AMPH was greater in DAT-KO mice (7.1 ± 0.8 vs. 3.7 ± 0.9 nM, \(P < 0.05; n = 10\)). In contrast to systemic administration, infusion of AMPH (50 and 100 µM) directly into the NAc by means of the microdialysis probe did not produce an increase in DA levels in DAT-KO mice (\(P > 0.05; n = 4\), although there were robust drug-induced increases in WT mice (Fig. 1B; \(P < 0.01; n = 4\))

**Effect of AMPH on DA Clearance.** The rate of DA clearance, monitored by FSCV, reported as a rate constant k (calculated as a first-order rate constant with the formula \(V_{max}/K_{m}\)), was dramatically slower in NAc core (0.024 sec⁻¹ vs. 8.65 sec⁻¹; \(P < 0.0001; n = 4\)) and in NAc shell (0.017 sec⁻¹ vs. 3.34 sec⁻¹; \(P < 0.0001; n = 4\)) of DAT-KO mice as compared with WT mice (Figs. 2 and 3). No significant difference was observed in the clearance rate of DA between the NAc core and shell in slices from DAT-KO mice (0.024 sec⁻¹ vs. 0.017 sec⁻¹; \(P > 0.05; n = 4\)), although the clearance rate constant in the NAc shell slices of WT mice was significantly slower than that of the NAc core (3.34 sec⁻¹ vs. 8.65 sec⁻¹; \(P < 0.05; n = 5\)) (Fig. 3). Application of 1 and 10 µM AMPH for 20 min prolonged the clearance of DA in both the core and shell NAc in slices from WT mice (Figs. 2 and 3). However, these parameters were unaltered by AMPH in NAc slices from DAT-KO mice (Figs. 2 and 3). There were significant changes in single pulse-evoked DA release after AMPH (1 and 10 µM) in both NAc core (0.76 ± 0.11, 0.48 ± 0.04, respectively, vs. predrug, 0.89 ± 0.11 µM, \(P < 0.05; n = 4\)) and NAc shell (0.35 ± 0.07, 0.27 ± 0.06, respectively, vs. predrug, 0.56 ± 0.02 µM, \(P < 0.05; n = 4\)) of WT animals. AMPH had no effect on single pulse-evoked DA release in either core or shell NAc of DAT-KO mice (data not shown).

**Effect of AMPH on Firing Rate of VTA DA Neurons.** Extracellular single unit recordings were made from 10 VTA neurons from WT mice and 7 VTA neurons from DAT-KO mice. All neurons were identified as dopaminergic according to electrophysiological criteria (see Materials and Methods). All neurons fired spontaneous action potentials with regular interspike intervals at rates ranging from 0.87 to 3.44 Hz for WT mice and from 0.68 to 2.68 Hz for DAT-KO mice. The mean firing rate for VTA neurons from WT mice was 1.9 ± 0.3 Hz (±SEM, \(n = 10\)) and
the mean firing rate for VTA neurons from DAT-KO mice was 1.8 ± 0.3 Hz \((n = 7)\). A \(t\) test comparing the baseline firing rates of VTA neurons from mice subjected to the two treatment conditions indicated no significant difference in firing rates \((P > 0.05)\). AMPH inhibited the firing rate of VTA neurons from WT mice \((n = 10\) cells from six animals) in a concentration-dependent manner over the concentration range of 1–10 \(\mu M\) (Fig. 4). In contrast, AMPH did not significantly inhibit the firing rate of dopaminergic VTA neurons from DAT-KO mice \((n = 7\) cells from five animals) over this concentration range; the apparent increase in firing rate produced by AMPH was not statistically significant. The difference between the AMPH concentration–response curves over the range of 2–10 \(\mu M\) for WT and DAT-KO mice was statistically significant (Fig. 4, two-way ANOVA, \(F(1, 34) = 24.74; P < 0.001\); Student–Newman–Keuls test, \(P < 0.05\)). There were no significant changes in the mean spontaneous firing rate of VTA DA neurons in either WT or DAT-KO mice in response to 5-HT \((1–50 \mu M, F = 1.03, df = 6,63; P > 0.05, n = 6)\) or S-CT \((1–10 \mu M, F = 0.214, df = 3,30; P > 0.05, n = 6)\), a 5-HT \(_{1}\) receptor agonist (data not shown).

**Rewarding Effect of AMPH.** We found that WT and DAT-KO mice exhibited similar results within the parameters of a traditional place conditioning paradigm (Fig. 5A). However, when the postconditioning test was repeated on day 6 and 13, DAT-KO mice continued to display a significant place preference, in contrast to WT control. Because WT mice did not exhibit a preference for AMPH anymore, these animals were not tested further. An extended testing of DAT-KO mice has shown significant AMPH CPP on day 40 and tendency \((P = 0.08)\) on day 30. No significant drug preference was observed on day 55 \((P > 0.05)\). Pairing of AMPH \((5 \, mg/kg)\) for 2 days significantly increased the time spent in the drug-paired side in DAT-KO mice but not in WT mice (Fig. 5B). Under the same conditions...
We tried to attenuate AMPH CPP by blockade of 5-HT receptors. Pretreatment with WAY-100635 (2 mg/kg), the 5-HT1A receptor antagonist, completely abolished AMPH-induced CPP (4 days of pairing) in DAT-KO mice (Fig. 6). However, AMPH-induced CPP was insensitive to WAY-100635 in WT mice.

Discussion

Systemic Administration of AMPH Increases Extracellular DA Levels in the NAc of DAT-KO Mice Without Altering Clearance. The present in vivo microdialysis study confirms the observation that systemic injection of AMPH increases extracellular DA in the NAc of DAT-KO mice (17). This observation is in contrast to previous findings in the CPu, where no increase in dialysate DA was measured with AMPH (14, 17). It was postulated that intrastriatal differences in the density of monoamine uptake sites could play a role in the divergence. Because the NAc has a greater norepinephrine innervation than the CPu (24, 25), it was initially suggested that the norepinephrine transporter may contribute to DA clearance in the absence of the DAT in this brain region (17, 20). In fact, in the prefrontal cortex, such promiscuous uptake does take place in WT and DAT-KO mice (26). However, AMPH failed to change the DA clearance rate in mutant mice in either core or shell NAc. Identical results were obtained previously with desipramine, a norepinephrine transporter inhibitor, and fluoxetine, a 5-HT transporter inhibitor (20). Thus, despite the fact that serotonin transporter and norepinephrine transporter levels are higher in the NAc than in the CPu, these transporters do not contribute significantly to DA clearance in the NAc of mice with a genetic deletion of the DAT. In fact, in the NAc of DAT-KO mice, DA clearance, measured by using in vivo amperometry, was found to be similar to that observed in the CPu (27). This finding is in agreement with data that baseline DA dialysate levels from the NAc were not different from dorsal striatal levels in DAT-KO mice (17). If alternative clearance mechanisms had been operating, faster clearance and subsequently lower basal levels of DA would be expected in the NAc. Therefore, with no DAT, diffusion is most likely the primary mechanism of DA clearance.
mechanism of DA clearance, and the rate of this process is the same in the CPu and NAc.

In agreement with the lack of effect of AMPH on DA clearance in vitro, infusion of this drug into the NAc of freely moving DAT-KO mice did not alter extracellular DA levels. This observation rules out other local effects of AMPH, such as presynaptic facilitation of DA release, through activation of heteroreceptors (e.g., glutamate or serotonin) on DA terminals. Therefore, in DAT-KO mice, NAc DA terminals are not the primary site of acute AMPH actions.

An Enhanced Firing Rate of DA Neurons May Be Involved in the AMPH-Induced DA Increase in the NAc of DAT-KO Mice. In vivo, acutely injected AMPH has a non-DA-mediated excitatory effect on DA neurons in the VTA in the presence of DA autoreceptor inhibition (28), although the exact mechanisms are unclear. Therefore, AMPH may enhance phasic or spike-dependent release of DA, which is important in the neural processing of reward (29). However, under normal conditions, this effect is likely concealed by the inhibitory influence of AMPH-induced DA increases. Locally in the VTA, there is activation of inhibitory autoreceptors on DA cell bodies. From the NAc, there are direct and indirect feedback-inhibitory circuits to the VTA (28, 30). In fact, AMPH dose-dependently decreased the firing rate of VTA DA neurons in brain slices from WT mice, which is consistent with in vivo findings (28) when autoreceptors are not blocked.

In contrast to WT mice, DA autoreceptor function is impaired in DAT-KO mice (31). Therefore, the autoinhibitory AMPH actions on impulse-dependent DA release are markedly decreased. These consequences of DAT deletion may lead to a condition in which non-DA-mediated excitation becomes the dominant effect of AMPH on VTA DA neurons. Hence, the elevation of extracellular DA observed in the NAc of DAT-KO mice could be a consequence of increased DA release, resulting from activation of VTA DA neuron firing. The process of autoreceptor desensitization is not unique for the mutant mice. In fact, the depression of firing rate in DA neurons induced by acute AMPH administration switches to excitation after multiple injections of AMPH (5.0 mg/kg) (32, 33). Therefore, the increase in DA levels because of repeated DAT inhibition and genetic DAT deletion can modify the effect of AMPH on the firing rate of DA neurons. This finding suggests that in WT animals, critical neurochemical adaptations may occur when DA levels are repeatedly increased by AMPH. The interaction of AMPH with the DAT is the first link in these events. However, AMPH-induced increases in the firing rate of DA neurons may be more important for the development of addiction, and these appear only after repeated drug administration and are not related to effects of AMPH on the DAT.

How Might AMPH Increase the Firing Rate of VTA DA Neurons in DAT-KO Mice? There are several possibilities as to how AMPH might increase the firing rate of VTA DA neurons, none of which are exclusive. These possibilities include, but are not limited to, the activation of opioid (34), glutamate (35), norepinephrine (28), and 5-HT (36) systems, which modulate dopaminergic activity.

AMPH is a potent inhibitor of the serotonin transporter (37), and 5-HT is well known to be a strong modulator of DA neurotransmission, with various receptors causing different and often opposite effects (36, 38–45). Therefore, a consideration of 5-HT receptors as candidates for the AMPH-induced increase in dopaminergic activity of DAT-KO mice may be promising. As one intriguing example, several studies in vivo have shown that stimulation of 5-HT1A receptors can enhance the activity of VTA but not substantia nigra DA neurons, whereas application of 5-HT1A antagonists induces the opposite effect (38, 39, 44). For example 8-OH-DPAT, a 5-HT1A receptor agonist, increased the firing rate, regularity of firing, and burst firing of VTA DA neurons in rats (38, 39). In contrast, the same drug did not alter any of the firing patterns of DA neurons in the substantia nigra (38). This result raises the possibility that serotonergic afferents into the midbrain may mediate, in part, the DA-elevating actions of AMPH in the NAc of DAT-KO mice. However, neither AMPH, 5-HT, nor 5-CT, a 5-HT1A agonist, had any significant effects on the firing rate of DA neurons in VTA slices from DAT-KO mice, in which afferent connections that are functional in vivo have been severed. The lack of effects of 5-HT drugs in VTA slices does not rule out involvement of this brain region in the effects of AMPH; rather, it indicates that a complex situation exists with regard to modulation of VTA DA neuron excitability. Given that the microdialysis data clearly demonstrated that AMPH elevated DA in the NAc without direct terminal effects, activation of DA neurons by AMPH remains the most likely explanation of the results.

AMPH Is Rewarding in DAT-KO Mice. An increase in DA neurotransmission in the NAc, especially in the shell compartment, is induced by natural rewards and drugs of abuse (46), as well as stressors (47–49). To examine the possibility of AMPH-induced rewarding properties in DAT-KO mice, we used a CPP paradigm, a valuable method for examining genetic influences involved in reward-related behaviors (50, 51). By using a traditional CPP paradigm, we found that AMPH was similarly rewarding in WT and DAT-KO mice. However, DAT-KO mice demonstrated CPP for AMPH over a long period (40 days), whereas WT mice showed preference only on the first day of testing. One interpretation of the data is that AMPH provokes a stronger response in the DAT-KO than in WT mice, because the drug is more rewarding in the absence of the DAT. However, a long-lasting AMPH-induced CPP may also be attributed to a learning deficit, which is consistent with an earlier finding of impaired learning in DAT-KO mice (16). These mice may be unable to extinguish inappropriate responses, therefore exhibiting extreme perseveration. However, it should be noted that the mutant mice associated rewarding properties of AMPH with cues in their environment. In addition, AMPH-induced CPP was reached faster (with two pairings) in the DAT-KO than in WT mice, demonstrating that the DAT-KO mice are readily able to learn this task. Thus, it is possible that the mutant mice are more sensitive to the rewarding effects of AMPH than WT mice. Additional experiments will be required to fully clarify this issue, but it is clear that AMPH can induce rewarding effects in the absence of the DAT.

These findings are consistent with reports that another powerful drug of abuse and DAT inhibitor, cocaine, can induce rewarding effects (50–53) and increase extracellular DA levels in the NAc in DAT-KO mice (17). Therefore, the increase in accumbal DA, which was induced even in the absence of DAT, may still be involved in AMPH and cocaine reward but not in the stimulating properties of these drugs. These data indirectly support the hypothesis for a segregation of AMPH reward and locomotor stimulation.

Serotonergic Mechanisms May Be Involved in the Rewarding/Reinforcing Properties of AMPH in DAT-KO Mice. The suggestion that AMPH can increase mesolimbic DA release through activation of 5-HT receptors is in good agreement with the hypothesis that the 5-HT system is essential for drug reinforcement in mice with reduced DAT function (52, 53). In fact, fluoxetine is itself reinforcing in DAT-KO mice (51, 53) and increases NAc DA levels in these mice (53). That the elimination of cocaine induced CPP in DAT-serotonin transporter double-knockout mice but not in DAT-KO mice (51, 54) is also consistent with this hypothesis. In the present study, AMPH-induced CPP was
removal of pretreatment with WAY-100635, a 5-HT₁A receptor antagonist, in DAT-KO mice, whereas the drug did not change AMPH place preference in WT mice. These data do not exclude the possibility that other types of 5-HT receptors may also be involved in this AMPH effect. Therefore, we suggest that serotonergic mechanisms may mediate, at least in part, the rewarding/reinforcing properties of AMPH in DAT-KO mice.

In conclusion, despite the absence of the DAT, AMPH displays rewarding effects and causes an increase in extracellular DA in the NAc of DAT-KO mice, acting indirectly in this case.

One possible circuit mediating the effect of AMPH on mesolimbic DA responses may involve 5-HT receptors. However, similar roles for other neurotransmitter systems cannot be excluded at this time.

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