Cocaine and Amphetamine Increase Extracellular Dopamine in the Nucleus Accumbens of Mice Lacking the Dopamine Transporter Gene

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Behavioral and biochemical studies suggest that dopamine (DA) plays a role in the reinforcing and addictive properties of drugs of abuse. Recently, this hypothesis has been challenged on the basis of the observation that, in mice genetically lacking the plasma membrane dopamine transporter [DAT-knock out (DAT-KO)], cocaine maintained its reinforcing properties of being self-administered and inducing place preference, despite the failure to increase extracellular dopamine in the dorsal striatum. Here we report that, in DAT-KO mice, cocaine and amphetamine increase dialysate dopamine in the medial part of the nucleus accumbens. Moreover, reboxetine, a specific blocker of the noradrenaline transporter, increased DA in the nucleus accumbens of DAT-KO but not of wild-type mice; in contrast, GBR 12909, a specific blocker of the dopamine transporter, increased dialysate dopamine in the nucleus accumbens of wild-type but not of DAT-KO mice. These observations provide an explanation for the persistence of cocaine reinforcement in DAT-KO mice and support the hypothesis of a primary role of nucleus accumbens dopamine in drug reinforcement.

Key words: dopamine; nucleus accumbens; DAT-knock-out mice; cocaine; amphetamine; reboxetine

Cocaine and amphetamine psychostimulants are abused by humans (Johanson and Schuster, 1995) and self-administered by primates (Bergman et al., 1989) and rats (Richardson and Roberts, 1996). Among brain monoamines, dopamine (DA) has been attributed an important role in the reinforcing properties of drugs of abuse and in particular of cocaine and amphetamine (Wise and Bozarth, 1987; Koob, 1992; Di Chiara et al., 1993; Di Chiara, 1995). These psychostimulants increase extracellular DA by blocking the DA transporter (DAT) on DA nerve terminals (cocaine) or by promoting the nonexocytotic release of DA (amphetamine). Recently, the DA hypothesis of the reinforcing properties of cocaine has been challenged on the basis of the report that, in mice genetically lacking DAT [DAT-knock-out (KO)] (Giros et al., 1996), cocaine was self-administered but failed to increase extracellular DA in the caudate putamen (CPu), (Rocha et al., 1998; Sora et al., 1998). Cocaine however, like most drugs of abuse, increases DA preferentially in the nucleus accumbens (NAc) compared with the dorsal CPu, and this property has been hypothesized to be related to the reinforcing properties of drugs of abuse (Di Chiara and Imperato, 1988; Carboni et al., 1989; Barrot et al., 2000). In view of this, failure of cocaine to increase extracellular DA in the caudate putamen of DAT-KO mice is not incompatible with the hypothesis of a role of DA in the reinforcing effects of cocaine. In fact, although ineffective in the CPu, cocaine might still increase extracellular DA in the NAc of DAT-KO mice. To test this possibility, we studied by brain microdialysis the effect of cocaine and amphetamine on extracellular DA in the NAc of DAT-KO compared with wild-type mice.

MATERIALS AND METHODS

Animals. Homozygous DAT $^{-/-}$ mice were obtained by homologous recombination as described previously (Giros et al., 1996). These mice were then backcrossed for more than 15 generations on a C57BL/6 background. DAT $^{-/-}$ and wild-type DAT $^{+/+}$ littermates were obtained from the mating of DAT $^{+/-}$ mice. The genotype of the mice was determined by PCR analysis as follows. Genomic DNA (50 ng) from tail biopsies was amplified with primers DAT-1 (CCCGTCTACCCATGAGTAAAA), DAT-2 (CTCCACCTTCCTAGCACTAAC), and NEO2 (TGACCGCTTCCTCGTGC), generating a 870 bp product (DAT-1/NEO2) for the recombined DAT gene and a 580 bp product (DAT-1/DAT-2) for the wild-type DAT gene. After weaning, mice were housed two to four per cage and maintained under standard housing conditions with food and water available *ad libitum*. All mice used were 8–12 weeks old, drug naïve, and were only used in one test. All animal experimentation was conducted in accordance with the guidelines for care and use of experimental animals of the European Economic Community (86/809; DL 27.01.92, Number 116).

Probe preparation. Concentric dialysis probes were prepared with a 7 mm piece of AN 69 (sodium methallyl sulfate copolymer) dialysis fiber (310 μm outer diameter, 220 μm inner diameter; Hospal, Dasco, Italy),

Received Nov. 29, 2000; revised Feb. 6, 2001; accepted Feb. 12, 2001.

This work was supported by funds from Consiglio Nazionale delle Ricerche and Ministero della Ricerca Scientifica e Tecnologica.

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sealed at one end with a drop of epoxy glue. Two 4-cm-long pieces of fused silica (Composite Metal Services, Worcester, UK) tubing were introduced in the dialysis fiber, taking care to have the inlet reaching the lower end and the outlet reaching the higher end of the dialyzing portion (1.0 mm) of the fiber. The inlet and the outlet were then sealed to the fiber and to a 18 mm piece of stainless steel (obtained from a 24 gauge needle) that were then inserted into a piece of 200 μl micropipette tip 6-mm-long and glued to it. The fiber was covered with a thin layer of epoxy glue, except for the dialyzing part. The probe was left to dry for 24 hr (Di Chiara, 1990; Di Chiara et al., 1996).

Surgery and experiments. Mice anesthetized with chloral hydrate (400 mg/kg, i.p.) were placed in a stereotaxic apparatus. The skull was exposed, and a small hole was drilled on the right side. The head position was adjusted so that bregma and lambda had the same height. The probe was implanted vertically in the medial accumbens (anterior, 1.2 mm; lateral, 0.6 mm; vertical, −5.2 mm from the bregma) or in the CPu (anterior, 0.0; lateral, 1.8 mm; vertical, 4 mm from the bregma), according to the atlas of Franklin and Paxinos (1997) and then fixed on the skull with dental cement. Mice were housed in a transparent plastic (Plexiglas) hemisphere, closed with a top hemisphere, with food and water available. Experiments were performed on freely moving mice 48 hr after probe implant. Ringer's solution (147 mM NaCl, 2.2 mM $CaCl_2$, and 4 mM KCl) was pumped through the dialysis probe at constant rate of 1 μl/min. Samples were taken every 20 min and analyzed.

Analytical procedure. Dialysate samples (20 μl) were injected without any purification into an HPLC apparatus equipped with reverse-phase column (LC-18 DB; Supelco, Bellefonte, PA) and a coulometric detector (Coulochem II; ESA Inc., Bedford, MA) to quantify DA. The first electrode was set at +130 mV and the second electrode at −125 mV. The composition of the mobile phase was 50 mM Na H_2 PO_4, 5 mM Na_2HPO_4, 0.1 mM Na_2EDTA, 0.5 mM octyl sodium sulfate, and 15% (vol/vol) methanol, pH 5.5. The mobile phase was pumped with an LKB 2150 pump at a flow rate of 1.0 ml/min. The sensitivity of the assay allowed for the detection of 5 fmol of DA.

Histology. At the end of the experiment, mice were anesthetized and transcardially perfused with 20 ml of saline (0.9% NaCl) and 20 ml of formaldehyde (10%). The probes were removed, and brains were cut on a vibratome in serial coronal slices oriented according the atlas of Franklin and Paxinos (1997). The position of the probe was ascertained by observation under stereomicroscope (10–20 magnification) and comparison with corresponding levels of the atlas of Franklin and Paxinos (1997). Results from mice implanted incorrectly were discarded.

Drugs. Cocaine HCl and amphetamine sulfate were obtained from SALARS (Como, Italy). GBR 12909 was a gift from Novo A/S (Bagsveerd, Denmark). Reboxetine was a gift from Pharmacia Upjohn (Milan, Italy).

Statistics. Statistical analysis was performed by Statistica (StatSoft Inc., Tulsa, OK). Three-way ANOVA for repeated measures (time points) was applied to the data expressed as percent of basal DA concentration obtained from the serial assays of DA after each treatment. Results from treatments showing significant overall changes were subjected to *post hoc* Tukey test with significance for $p < 0.05$. Basal values were the means of three consecutive samples differing <10%. Each implanted mouse was challenged with a single dose of the test drug only once.

RESULTS

Basal dialysate DA from the NAc of wild-type and DAT-KO mice were 46.33 ± 6.74 and 192 ± 28 fmol/20 μl, respectively ($t = -5.31$; df = 46; $p < 0.0001$). Basal dialysate DA from the CPu of wild-type and DAT-KO mice were 38.8 ± 5.4 and 161 ± 31 fmol/20 μl, respectively ($t = -3.88$; df = 16; $p < 0.005$).

Cocaine (20 mg/kg, i.p.) and amphetamine (5 and 2 mg/kg, i.p.) increase dialysate DA in the NAc of both DAT-KO and wild-type mice (Figs. 1*A*, 2*A*,*B* respectively). The maximal increase of DA elicited by cocaine or by amphetamine in the NAc of DAT-KO mice did not differ significantly from that of wild-type mice. Three-way ANOVA of the results shown in Figure 1*A* revealed a significant effect of treatment ($F_{(1,15)} = 15.89$; $p < 0.005$) and no effect of gene patrimony ($F_{(1,15)} = 1.32$; $p = 0.26$). The results in Figure 2, *A* and *B*, revealed a significant effect of treatment ($F_{(1,14)} = 29.44$; $p < 0.0005$; and $F_{(1,13)} = 15.85$; $p < 0.005$, respectively) and no effect of gene patrimony ($F_{(1,14)} = 0.3$; $p = 0.59$; and $F_{(1,13)} = 1.18$; $p = 0.29$, respectively).

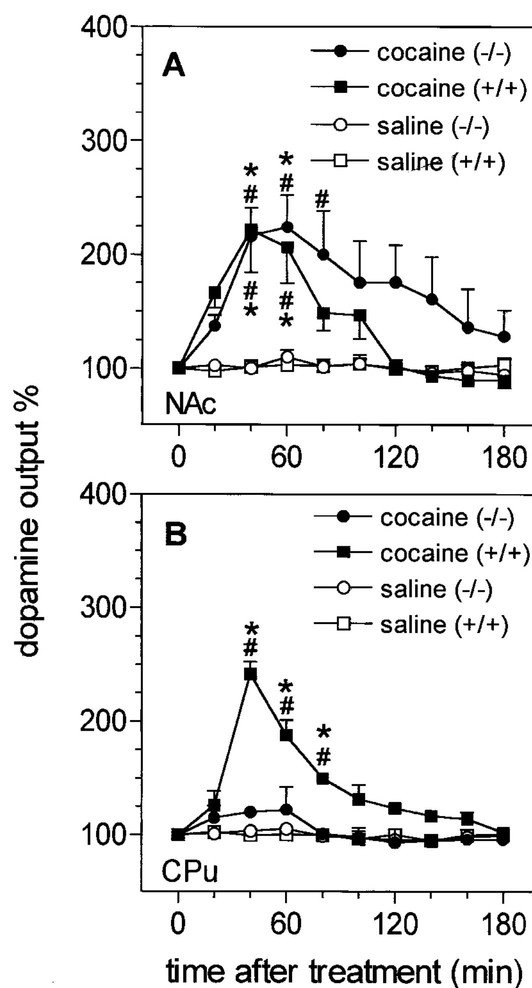

Figure 1. Effect of cocaine (20 mg/kg, i.p.) on dopamine concentration in dialysate obtained by *in vivo* microdialysis from the NAc (*A*) and the CPu (*B*) in both DAT-KO (−/−) and wild-type (+/+) mice. Each *point* is the mean ± SEM of at least three to six determinations. #$p < 0.05$ from basal values concentration; *$p < 0.05$ from the corresponding time point of vehicle group.

Figure 1*B* shows that cocaine increased dialysate DA in the CPu of wild-type but not DAT-KO mice. Three-way ANOVA revealed a significant effect of treatment ($F_{(1,8)} = 40.8$; $p < 0.001$), gene patrimony ($F_{(1,8)} = 26.35$; $p < 0.001$), and interaction ($F_{(9,72)} = 10.46$; $p < 0.0001$). Figure 3*A* shows that GBR 12909 (10 mg/kg, i.p.; *A*) increased significantly dialysate DA in the NAc of wild-type mice but not DAT-KO mice, whereas reboxetine (20 mg/kg, i.p.; *B*) increased significantly dialysate DA in DAT-KO but not wild-type mice. Three-way ANOVA of the results shown in *A* revealed a significant effect of treatment ($F_{(1,10)} = 86.29$; $p < 0.0001$), gene patrimony ($F_{(1,10)} = 129.8$; $p < 0.0001$), and interaction ($F_{(9,90)} = 18.58$; $p < 0.0001$). The results in *B* revealed a significant effect of treatment ($F_{(1,17)} = 7.61$; $p < 0.05$) and gene patrimony ($F_{(1,17)} = 8.97$; $p < 0.01$). Fluoxetine (10 mg/kg, i.p.) did not modify dialysate DA in the NAc of either wild-type and KO-DAT mice ($F_{(1,13)} = 0.75$; $p = 0.40$).

DISCUSSION

This study shows that the psychostimulants cocaine and amphetamine increase dialysate dopamine in the NAc of both DAT-KO

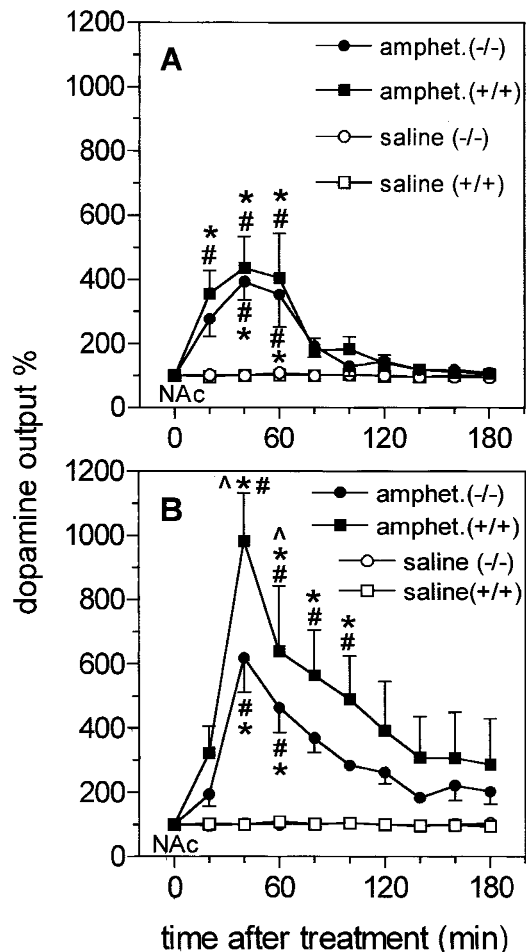

Figure 2. Effect of amphetamine (2 and 5 mg/kg, i.p.; *A* and *B*, respectively) on dopamine concentration in dialysate obtained by *in vivo* microdialysis from the NAc in both DAT-KO (−/−) and wild-type (+/+) mice. Each *point* is the mean ± SEM of at least three to six determinations. #$p < 0.05$ from basal values concentration; *$p < 0.05$ from the corresponding time point of vehicle group.

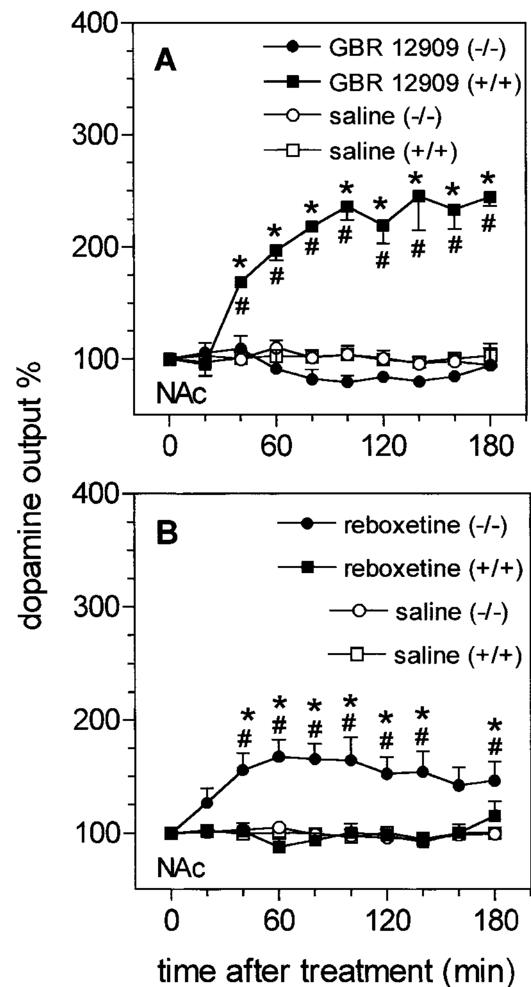

Figure 3. Effect of GBR 12909 (10 mg/kg i.p.; *A*) and reboxetine (20 mg/kg, i.p.; *B*) on dopamine concentration in dialysate obtained by *in vivo* microdialysis from the NAc in both DAT-KO (−/−) and wild-type (+/+) mice. Each *point* is the mean ± SEM of at least three determinations. #$p < 0.05$ from basal values concentration; *$p < 0.05$ from the corresponding time point of vehicle group.

and wild-type mice. In agreement with previous studies (Rocha et al., 1998), no significant change in dialysate DA was observed in the CPu of DAT-KO mice after cocaine, whereas basal dialysate DA in DAT-KO mice was approximately fourfold higher than in wild-type mice. In contrast to cocaine and amphetamine, GBR 12909, a specific blocker of DAT (Andersen, 1989), failed to increase dialysate DA in the NAc of DAT-KO at doses that are fully effective in wild-type mice and in rats (Carboni et al. 2000). Cocaine and amphetamine, unlike GBR 12909, also block the norepinephrine transporter (NET), as well as the serotonin transporter (SERT). However, a role of SERT blockade alone in the psychostimulant-induced increase of DA in NAc of DAT-KO mice is made unlikely by the observation that fluoxetine, a potent SERT inhibitor, failed to increase DA in the NAc of DAT-KO mice (see Results). This in turn is consistent with the fact that DA is not a good substrate for SERT (Raiteri et al., 1977). A better candidate as a substrate for psychostimulant-induced increase of DA in the NAc of DAT-KO mice is NET, reportedly even more efficient than DAT in taking up DA (Raiteri et al., 1977; Giros and Caron, 1993). Indeed, in the rat prefrontal cortex, in which norepinephrine (NE) innervation prevails over DA innervation, DA has been reported to be cleared from the extracellular space by NET rather than by DAT (Carboni et al., 1990; Tanda et al., 1997). Although NET blockade in the medial NAc does not seem to contribute to a significant extent to the clearance of DA from the extracellular space (Tanda et al., 1997), the NAc shell to which the medial NAc corresponds receives in the rat a consistent NE projection (Berridge et al., 1997). We speculated that, in the DAT-KO mice, the NET expressed by NE terminals of the NAc could, because of the absence of DAT, act as an alternative site for DA clearance from the extracellular compartment.

To test this hypothesis, we investigated the effect of the specific NET blocker reboxetine (Wong et al. 2000) on extracellular DA in the medial NAc of DAT-KO mice. Results show that reboxetine increased DA in the NAc of DAT-KO but not of wild-type mice. It is notable that the maximal increase of dialysate DA after reboxetine in the NAc of DAT-KO mice was not different from that obtained after cocaine in the same area ($F_{(1,10)} = 0.678$; $p = 0.429$). Like cocaine and amphetamine, reboxetine failed to increase DA in the CPu of DAT-KO mice (data not shown). These observations suggest that cocaine and amphetamine increase DA in the medial NAc of DA-KO mice by blocking NET. This mechanism appears to take place in the DAT-KO and not in

wild-type mice as a result of diversion of DA reuptake to NET in the absence of DAT. In turn, the ability of reboxetine and psychostimulants to increase DA in the medial NAc but not in the CPu of DAT-KO mice is consistent with the presence of NET-containing terminals in the caudal half of the accumbens shell but not in the caudate putamen (Berridge et al., 1997).

The present observations, although offering an explanation for the persistence of cocaine reinforcement in DAT-KO mice, predict that NET blockade would be reinforcing specifically in DAT-KO mice. If this prediction will hold true, not only the DA hypothesis of drug reinforcement will be confirmed but also that of a specific role of NAc DA (Wise and Bozarth, 1987; Di Chiara and Imperato, 1988; Koob, 1992; Di Chiara, 1995) will receive a strong support. From a more general viewpoint, the present study provides a remarkable example of compensation for the influence of a complete genetic deletion of the substrate of a central drug effect (DAT).

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