Cocaine self-administration in dopamine-transporter knockout mice

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The plasma membrane dopamine transporter (DAT) is responsible for clearing dopamine from the synapse. Cocaine blockade of DAT leads to increased extracellular dopamine, an effect widely considered to be the primary cause of the reinforcing and addictive properties of cocaine. In this study we tested whether these properties are limited to the dopaminergic system in mice lacking DAT. In the absence of DAT, these mice exhibit high levels of extracellular dopamine, but paradoxically still self-administer cocaine. Mapping of the sites of cocaine binding and neuronal activation suggests an involvement of serotonergic brain regions in this response. These results demonstrate that the interaction of cocaine with targets other than DAT, possibly the serotonin transporter, can initiate and sustain cocaine self-administration in these mice.

The widespread abuse of cocaine, a highly addictive psychostimulant, places tremendous social, medical, and economic burdens on society. By improving our understanding of the underlying mechanisms of cocaine addiction, it may be possible to develop more effective therapeutic strategies and social policies aimed at reducing the abuse of cocaine. Cocaine inhibits the uptake of monoaminergic neurotransmitters from the extracellular space through its interaction with plasma membrane monoamine transporters[1]. This family of proteins, which includes the transporters for dopamine (dopamine transporter, DAT), norepinephrine (norepinephrine transporter, NET), and serotonin (serotonin transporter, SERT), acts to terminate monoaminergic transmission by rapid removal of the neurotransmitters from the synaptic cleft, back into the presynaptic terminals[2].

It is commonly believed that the reinforcing/addictive properties of cocaine depend on the ability of cocaine to block DAT, thereby increasing the extracellular concentration of the neurotransmitter dopamine within specific brain areas[3,4]. The interaction of cocaine with DAT and the resultant elevation of extracellular dopamine is correlated to its potency for inducing subjective[5] and reinforcing effects[6–9], thus providing a theoretical basis for its addictive properties. Therefore, disruption of the interaction between DAT and cocaine might be expected to attenuate the reinforcing effects of cocaine. Previous studies from our laboratory have shown that mice in which DAT has been genetically deleted undergo a series of profound neurochemical adaptations ($DAT^{-/-}$)[10,11]. For example, despite a marked decrease of dopamine in the tissue, these mice exhibit higher than normal levels of extracellular dopamine and spontaneous hyperlocomotion. However, they do not display the increase in locomotor activity typically observed upon administration of high doses of cocaine[10]. Based on the correlation between the strength of the psychomotor stimulant properties of a drug and the strength of its reinforcing or addictive effects[4], and the fact that the primary target for cocaine is absent in $DAT^{-/-}$ mice, cocaine would not be expected to serve as a positive reinforcer in these animals.

To test this hypothesis, $DAT^{-/-}$ and wild-type mice were trained in a cocaine reward paradigm (cocaine i.v. self-administration), in which animals perform an operant task (lever press) in exchange for a reward (food or cocaine). Contrary to our expectation, the $DAT^{-/-}$ mice still self-administer cocaine even in the absence of the presumed target. Interestingly, in these mice, specific binding of a cocaine analog and *c-fos* gene expression in response to cocaine were observed in serotonergic brain regions. These results suggest a potential interaction of cocaine with the SERT, which might participate in the reinforcing properties of cocaine.

Results

$DAT^{-/-}$ MICE SELF-ADMINISTER COCAINE

The i.v. cocaine self-administration method[12] was used to test the hypothesis that $DAT^{-/-}$ mice are insensitive to the reinforcing effects of cocaine. Food was used as a reinforcer to train the animals and to establish their ability to acquire an operant behavior. Wild-type and $DAT^{-/-}$ mice required comparable numbers

Table 1. Sessions required to meet food shaping or cocaine self-administration acquisition criteria in two-lever operant box

	Food	Cocaine
Wild type	5.7 ± 0.6	5.1 ± 0.4
$DAT^{-/-}$	5.0 ± 0.7	10.8 ± 0.6[1]

Criteria include ability to successfully press active lever a minimum number of times (15) and discriminate between active and inactive levers (≥3:1 active: inactive presses) for three successive sessions.
[1] t (4.4) = -2.66, $p < 0.05$.

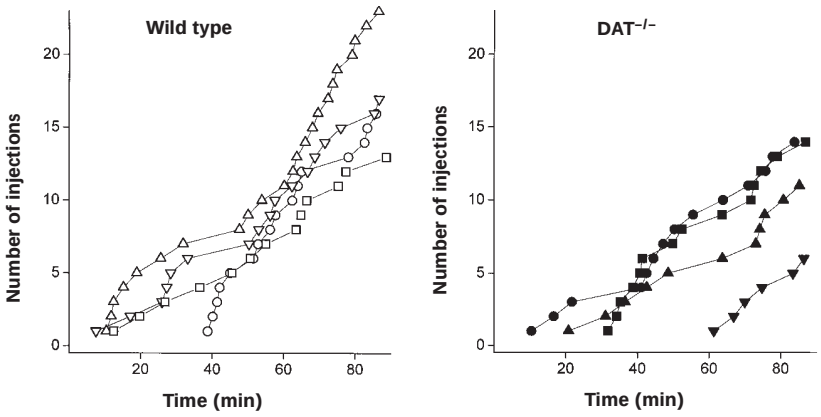

Fig. 1. Rate of cocaine self-administration. Wild type (n=4; l, g, h, p) and DAT$^{-/-}$ mice (n=4; L, G, H, P) were tested under an FR2 schedule, with unlimited number of injections (0.5 mg/kg/injection) within 90 min. The y-axis shows the number of injections taken during the session; the x-axis shows the duration of the session. Data are presented as individual responses for each animal, showing the time when the injections were taken during the 90-min session.

of sessions to initiate a response for food in a two-lever operant box (Table 1). However, when cocaine was used as the reinforcer, the DAT$^{-/-}$ mice required significantly more sessions to meet self-administration acquisition criteria than their counterparts (Table 1). This observation supports the idea that DAT blockade facilitates cocaine-taking behavior. Nevertheless, once cocaine self-administration was acquired, DAT$^{-/-}$ mice consistently and dose-dependently self-administered cocaine (Fig. 1 and 2). A global multivariate repeated measures analysis of variance (ANOVA) revealed a significant effect of dose of cocaine within subjects (F(4,48) = 11.86; p<0.0005), but not between the genotypes (F(1,12) = 1.26; p>0.05) or between genotypes and doses (F(4,48) = 1.9; p>0.05). Although there seems to be a trend toward a leftward shift in the curve for DAT$^{-/-}$, the possibility that there are differences in either the efficacy of cocaine, or the propensity to self-administer, will require testing at lower doses and under a progressive ratio schedule. Following dose–response testing, saline was substituted for cocaine to confirm extinction of the self-administration behavior. Both DAT$^{-/-}$ and wild-type mice exhibited behavior indicative of an 'extinction-burst' phenomenon, i.e. increased number of lever presses in both active and inactive levers in the first day under saline (data not shown), but both genotypes extinguished the self-administration behavior by the third day under saline (Fig. 2). Following extinction, DAT$^{-/-}$ and wild-type mice restarted cocaine self-administration when cocaine was available upon presses of the opposite lever (data not shown). Thus, reversal and reinstatement of cocaine self-administration were observed in both genotypes.

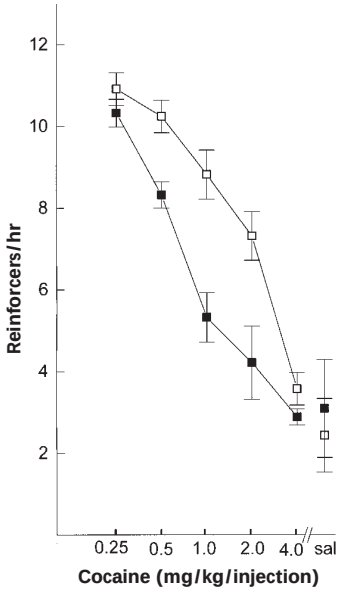

Fig. 2. Dose–response curve for cocaine self-administration. Wild-type (n=8; l) and DAT$^{-/-}$ mice (n=6; L) were tested with various doses of cocaine (0.25, 0.5, 1.0, 2.0 and 4.0 mg per kg per injection plotted on a logarithmic scale on x-axis). The y-axis shows the number of injections taken per hour during a 90-min period. Each dose was tested independently on three consecutive days; subsequently, saline was substituted for cocaine. When saline was substituted for cocaine, both genotypes showed extinction behavior. Values represent the mean and standard error of injections taken per hour during the third day of each dose for the wild-type and DAT$^{-/-}$ mice.

Dopamine is unaffected by cocaine in DAT KO mice

The above results clearly show that cocaine can serve as an effective positive reinforcer in the DAT$^{-/-}$ mice, inducing a regular pattern of self-administered injections characteristic of a fixed-ratio schedule of reinforcement (Fig. 1). Thus, in apparent contradiction to a wide variety of reports [3,4,8,9], these findings would seem to suggest that cocaine binding to DAT is not required for the reinforcing effects of cocaine. However, it should be noted that previous studies have shown that the removal of DAT (DAT$^{-/-}$) switches the organism to a hyperactive mode of dopamine neurotransmission, with the very prolonged lifetime of extracellular dopamine inducing marked adaptive changes in the dopamine system[10,11]. For example, levels of D1 and D2 receptors are reduced approximately 50%[10], tyrosine hydroxylase activity is increased even though protein levels are down nearly 90%, and total tissue dopamine levels are only 5% of normal, whereas extracellular dopamine is increased at least fivefold in the striatum[11]. Importantly, administration of a dose of cocaine (40 mg per kg, i.p.) that led to an approximate three-fold increase in extracellular dopamine levels in wild-type mice did not alter extracellular dopamine in the DAT$^{-/-}$ mice as measured by microdialysis (Fig. 3). Thus, in the DAT$^{-/-}$ mice, the extracellular levels of dopamine in the dorso/ventral striatal region are persistently higher than those found transiently after cocaine administration in wild-type mice (Fig. 3). Therefore, it is remarkable that the DAT$^{-/-}$ mice, which are already under the influence of the primary pharmacological action of cocaine, elevated dopamine, still self-administer the drug. These results indicate that the molecular target(s) for cocaine and the pathways that underlie the acquisition and maintenance of the drug-taking behavior in DAT$^{-/-}$ mice must involve neuronal pathways other than the dopamine system.

Cocaine binds to serotonergic sites in DAT$^{-/-}$ mice

To identify the putative site of cocaine action in these mice, cocaine-binding sites were mapped by autoradiography with

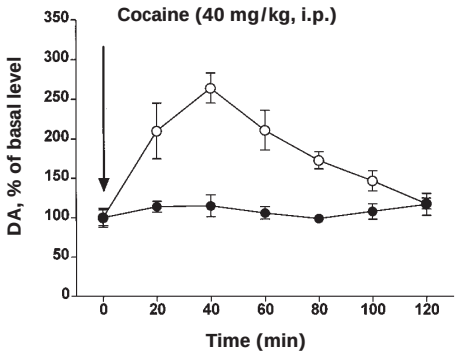

Fig. 3. Effect of cocaine on extracellular dopamine as measured by microdialysis. *In vivo* microdialysis was performed in the dorso/ventral striatal region of freely moving mice. Open circles are data from wild-type mice and closed circles from homozygous DAT$^{-/-}$ mice. Note that the steady-state striatal extracellular dopamine concentrations are at least fivefold higher in DAT$^{-/-}$ than in wild-type mice[11]. The data are expressed as percentages of average values of four basal values before cocaine administration (means ± SEM). Basal levels of dopamine in striatal dialysates were 3.25 ± 0.4 nM (n=5) for wild-type and 19.4 ± 5.0 nM (n=4) for DAT$^{-/-}$ mice. All data points representing this effect of cocaine on striatal dopamine levels in wild-type mice, except for the effect of cocaine 120 min after administration, were significantly different (p<0.05) from saline-treated controls (data not shown). There was no significant effect of cocaine in DAT$^{-/-}$ mice**.** (p), DAT +/+. (P), DAT$^{-/-}$.

the potent cocaine congener [^{125}I]RTI-55 (also called β-CIT). Both [^{125}I]RTI-55[13–15] and the closely related [^{3}H]WIN 35,428[16] have been extensively used to map cocaine binding sites in brain. [^{125}I]RTI-55 has a binding profile similar to cocaine[1] in that it binds to DAT, SERT, and to a lesser degree NET[13,17]. In agreement with studies in rat[18,19], significant [^{125}I]RTI-55 binding was observed in several brain regions, including striatum, nucleus accumbens, olfactory tubercle, septum, and to a lesser degree parietal and cingulate cortex, in wild-type brains (Fig. 4, upper panels). Binding was also observed in anterior olfactory nuclei, cerebral cortex, amygdala, hippocampus (CA1, CA3), mediodorsal thalamic nuclei, laterodorsal thalamic nuclei, medial habenular nucleus, dorsomedial hypothalamic and lateral geniculate nucleus (data not shown). In DAT$^{-/-}$ mice, however, [^{125}I]RTI-55 binding was markedly reduced in dorsal striatum (Fig. 4, lower panels), though still present in septum, olfactory tubercle, nucleus accumbens, parietal and cingulate cortex (Fig. 4, lower panels), anterior olfactory nuclei, cerebral cortex, amygdala, mediodorsal thalamic nuclei, laterodorsal thalamic nucleus, lateral geniculate nucleus, and hippocampus (data not shown). The addition of the SERT inhibitor alaproclate[20] drastically reduced [^{125}I]RTI-55 binding in non-dopaminergic regions of wild-type brains (Fig. 4, upper panels). There was some faint binding in septum, lateral dorsal thalamic nuclei, and paraventricular thalamic nuclei (data not shown), which may represent binding to NET[21]. In mice lacking DAT binding sites, the signal was drastically reduced by the SERT inhibitor (Fig. 4, lower panels), with only faint binding in septum, lateral dorsal and paraventricular thalamic nuclei. Addition of the NET inhibitor nisoxetine did not significantly reduce the [^{125}I]RTI-55 binding signal in any of the regions examined in either wild-type or DAT$^{-/-}$ mice (data not shown), although NET binding may be difficult to discern in the presence of DAT and/or SERT binding. Although several studies have suggested that norepinephrine may be involved in the reinforcing effect of cocaine[22,23], NET does not appear to be a significant target of [^{125}I]RTI-55 in DAT$^{-/-}$ mice. As [^{125}I]RTI-55 and cocaine are similar in their binding profiles[1], these results suggest that SERT represents the primary target of cocaine in the DAT$^{-/-}$ mice.

C-FOS INDUCTION IN NON-DOPAMINE AREAS IN DAT$^{-/-}$ MICE

In order to identify brain regions that which are activated by cocaine in the DAT$^{-/-}$ mice, we examined the expression of the immediate early gene *c-fos*. The Fos protein is rapidly induced in neurons of striatum and nucleus accumbens in animals given various cocaine dosing regimens, suggesting its involvement in the cascade of events initiated by cocaine[24–26]. *In situ* hybridization revealed that one hour after administration of a single dose

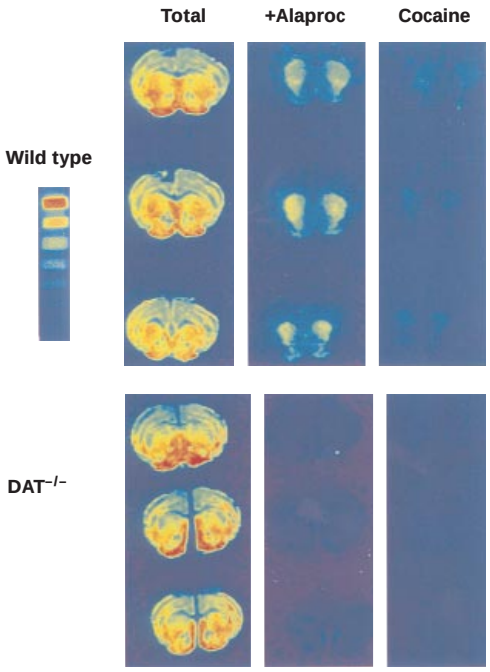

Fig. 4. *In vitro* autoradiographic binding of cocaine analogue [^{125}I]RTI-55. *In vitro* autoradiography was performed in wild-type and DAT$^{-/-}$ brain. Upper panels, [^{125}I]RTI-55 labeling in wild-type brain. Labeling was most pronounced in striatum, nucleus accumbens, olfactory tubercle, septum, and to a lesser degree in cortical regions. Addition of alaproclate (Alaproc; 1 μM), a SERT inhibitor, significantly reduced binding to serotonergic fields, whereas cocaine (50 μM) eliminated nearly all binding. Lower panels, [^{125}I]RTI-55 labeling in DAT$^{-/-}$ brain. Robust labeling was observed in several brain regions, including olfactory tubercle, cortical regions, and septum, but greatly reduced in striatum. The addition of either alaproclate (1 μM) or cocaine (50 μM) eliminated [^{125}I]RTI-55 binding in the DAT$^{-/-}$ brain. Each microscale bar represents a twofold dilution of preceding bar, with top band equal to 677 nCi/mg. For further analysis, tissue sections were analyzed by gamma scintillation spectroscopy. Values for wild-type and DAT$^{-/-}$ mice were as follows (dpm/slide ± SEM; three sections (20 μm)/slide; four separate experiments): total 48844 ± 5151 versus 39912 ± 7157, + alaproclate 17897 ± 2220 versus 5390 ± 3044*; + cocaine 2990 ± 1915 versus 3010 ± 515. * significantly different from wild type (p < 0.05).

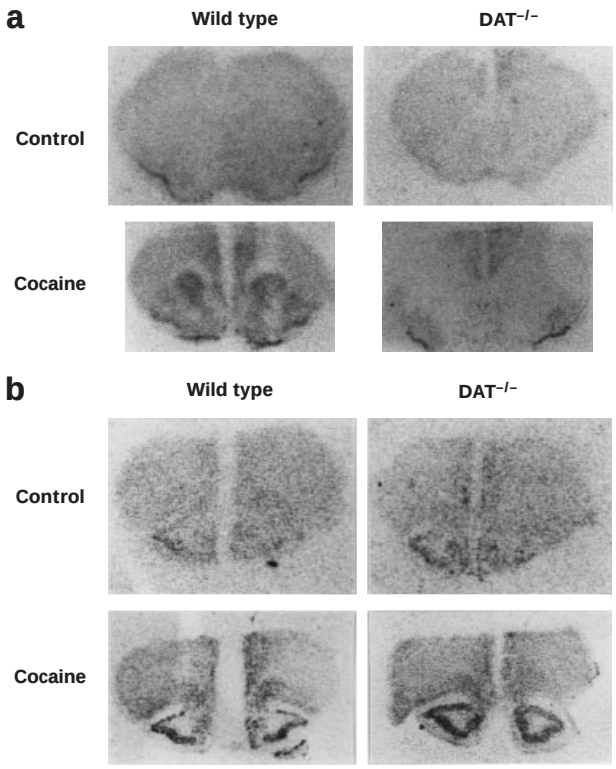

Fig. 5. *In situ* hybridization of brain mRNA expression of *c-fos* in mice treated with cocaine. Representative data are shown in each panel (four animals/group). **(a)** Cocaine (40 mg/kg, i.p. for 1 hr) induced a marked elevation of *c-fos* mRNA in the striatum and nucleus accumbens of wild-type with no apparent increase detected in DAT$^{-/-}$ mice. An increase in *c-fos* gene expression was also observed in the piriform cortex. **(b)** Cocaine also induced a marked elevation in the anterior olfactory nuclei both in wild-type and DAT$^{-/-}$ mice. Densitometric analysis of autoradiograms was performed to obtain relative intensities for sections of wild-type and DAT$^{-/-}$ mice. Quantitation of *c-fos* mRNA expression revealed a twofold (versus saline-treated) increase in the striatum of cocaine-treated, wild-type mice with no significant change in DAT$^{-/-}$ mice (wild type: 227 ± 4%; DAT$^{-/-}$ 88 ± 24%). In the anterior olfactory nucleus, *c-fos* expression increased by twofold in both genotypes (wild type 207 ± 4 %; DAT$^{-/-}$ 215 ± 2 %).

of cocaine, the expression of *c-fos* mRNA was increased in the striatum, nucleus accumbens, and olfactory tubercle of wild-type mice, but not of the DAT$^{-/-}$ mice (Fig. 5a). In conjunction with the microdialysis experiments demonstrating that cocaine does not alter extracellular dopamine in the dorso/ventral striatal region, the *c-fos* studies suggest that two of the events thought to participate in the early steps of cocaine action, transient increases in extracellular dopamine and *c-fos* induction in the striatum and nucleus accumbens, are not obligatory for self-administration in DAT$^{-/-}$ mice. As it is likely that the DAT$^{-/-}$ mice have already undergone the regulatory changes that occur from the high levels of extracellular dopamine, increases in the immediate-early-gene response may not be observed.

Although c-fos activation was absent in the striatum and nucleus accumbens of the DAT$^{-/-}$ mice, other regions appeared to be activated. Interestingly, cocaine treatment increased *c-fos* mRNA in the anterior olfactory nuclei and piriform cortex, and to a lesser extent the orbital cortex in both DAT$^{-/-}$ mice and wild-type littermates (Fig. 5b). Several studies suggest that these brain regions, which are predominantly serotonergic but also contain dopamine and norepinephrine, may be important in drug addiction. In intracranial self-stimulation, in which electrical stimulation of a particular brain region is used as the reinforcer, the anterior olfactory nuclei support this response, and the rate of stimulation is increased by amphetamine and cocaine[27]. Neuronal activation in the piriform cortex has been observed following treatment with nicotine, methamphetamine, or cocaine[28–30]. In addition, several other drugs of abuse, such as lysergic acid diethylamide (LSD), the phenethylamine hallucinogen 1-(2,5-dimethoxy-4-iodophenyl-2-aminopropane (DOI), and phencyclidine (PCP), markedly activate serotonergic transmission in the piriform cortex[31,32]. Recent *in vivo* studies in human cocaine addicts suggest that the reinforcing effects of cocaine are mediated, at least in part, by striato-thalamic-orbitofrontal circuits[33]. The induction of *c-fos* in the anterior olfactory nuclei, piriform cortex, and orbital cortex demonstrated in the present study, in conjunction with previous studies showing activation of related areas by other drugs of abuse, indicates that these circuits may support the reinforcing effects of cocaine.

Discussion

It is well established that, in addition to efficiently inhibiting dopamine uptake, cocaine also inhibits the uptake of serotonin[6]. In wild-type mice, drugs that selectively inhibit DAT are readily self-administered[34,35], but drugs that block only SERT are not[36,37]. Lesions of the dopamine system attenuate cocaine self-administration, whereas lesions of either the serotonergic or noradrenergic systems do not[38–41]. However, serotonin-uptake inhibitors and receptor ligands have been shown to modulate the reinforcing and subjective effects of cocaine, although with conflicting results[42–45]. Manipulations of the 5HT1B receptor highlight this controversy. For example, pharmacological stimulation of the 5HT1B receptor enhances the reinforcing effects of the selective DAT inhibitor GBR-12909[44]. Genetic deletion of the 5HT1B receptor, which represents the opposite of pharmacological stimulation, also enhances the reinforcing effects of cocaine[46,47]. Thus, although there are numerous discrepancies within the literature with respect to the direction of the effect, overall, these studies support the concept that dopamine is primarily involved in the effect of cocaine and that other neurotransmitters, in particular serotonin, modulate these properties of cocaine. Our data extend this concept by demonstrating that the putative effects of serotonin are mediated independently of alterations in extracellular dopamine levels, as cocaine had no effect on these parameters in the striatum of DAT$^{-/-}$ mice.

In DAT$^{-/-}$ mice, the cocaine congener [^{125}I]RTI-55 seems to bind to SERT, and cocaine induces immediate-early-gene activation in brain regions rich in serotonergic innervation. Together with the demonstration that the DAT$^{-/-}$ mice self-administer cocaine, these data point to a positive contribution of the serotonergic system in the maintenance of the reinforcing effects of cocaine, although contributions of the norepinephrine or other neurotransmitter systems cannot be totally ruled out. Nevertheless, the finding the DAT$^{-/-}$ mice self-administer cocaine does not preclude a role for dopamine. Indeed, in agreement with current dogma, it seems that elevated extracellular dopamine, whether transient as with cocaine administration in wild-type mice, or persistent as in the DAT$^{-/-}$ mice, is obligatory for cocaine reinforcing effects. Most important, however, is that DAT$^{-/-}$ mice retain the ability to acquire and maintain self-administration of

cocaine, which argues against the interaction of cocaine with DAT being the sole mediator of cocaine addiction. The serotonin system may provide an additional component of reinforcement, which in the case of the DAT$^{-/-}$ mice, seems to be sufficient to initiate the self-administration behavior. This raises the possibility that therapeutic management of cocaine addiction that targets both the dopamine and serotonin systems could be advantageous. The DAT$^{-/-}$ mice provide an exciting opportunity to elucidate the contribution of other neurotransmitter systems to cocaine's reinforcing properties.

Methods

ANIMALS. Male homozygotes and wild type littermates derived from the crossing (over 30 generations) of heterozygous DAT 129/SvJ/C57Bl6 mice were used for this study. Animals were housed individually on a 12-h light/dark cycle with free access to water and food. All animal procedures were approved by the institutional animal care and use committee.

SELF-ADMINISTRATION. The apparatus and procedures used for self-administration were as described.[46,47]. Food-shaping and cocaine self-administration experiments took place in mouse operant chambers (model ENV-300; Med Associates, Inc., Georgia, VT). The front panel contained a liquid dipper (model ENV-202A) situated between two ultra-sensitive mouse levers (model ENV-310A) with a single stimulus light (model ENV-221, 3 W) on the chamber ceiling. The chambers were placed in sound- and light-attenuating enclosures. Data were recorded using OPN software.

For food shaping, mice were trained to press a lever under a fixed ratio schedule (FR1, one lever press required for delivery of reinforcer), and subsequently under FR2 (two lever presses required) schedule, using food as a reinforcer (sweetened condensed milk solution: 2 parts water/1 part milk). Sessions lasted for either one hour or for up to 20 reinforcers. Mice were required to meet acquisition criteria for food-shaping (3:1 active:inactive lever response ratio) for one lever, and subsequently for the other lever. The number of sessions required for total acquisition of food shaping took into account acquisition of food-shaping in both levers and was used to measure latency for acquisition of operant behavior.

For cocaine self-administration, mice were implanted with a right external jugular catheter (0.22 mm ID, 0.67 mm OD, Dow Corning, Midland, MI) as previously described[46,47], following acquisition of food-shaping. The acquisition phase of cocaine self-administration started two days following catheter implantation. An active lever was randomly assigned for each of the animals, which were then allowed to self-administer cocaine at 2.0 mg/kg/injection (0.02ml/5 s), under FR1 schedule, with a maximum of 20 cocaine injections within a 180 min session. At the start of each session, a priming injection of cocaine (same dose used during the session) was given through the catheter. Each injection was accompanied by a flashing of the stimulus light followed by a 30-s time-out in the dark when lever pressing had no consequences. The timeout period was included to prevent overdose. Acquisition criteria were defined as intake of at least 15 injections within the session (approximately 5 reinforcers/h) and a 3:1 ratio of active to inactive lever presses (75% of active lever presses) occurring over three consecutive days. Once cocaine self-administration was acquired, the mice went to a training phase under FR2 schedule until presenting a stable baseline. Each FR2 training session lasted for 90 min and consisted of unlimited number of reinforcers; a stable baseline was defined as a rate of cocaine intake (measured as number of reinforcers per hour) that did not vary by more than 20% across three consecutive training sessions. Each subject presenting a stable baseline was submitted to dose–response tests in which each dose of cocaine (0.25, 0.5, 1.0, 2.0 and 4.0 mg/kg/injection) was randomly tested over three consecutive days. Testing sessions were identical to training sessions. Following dose–response tests, saline was substituted for cocaine over three consecutive days to test for behavior extinction. Subsequently, cocaine was reintroduced, but the response producing cocaine injection (active lever) was switched to the other lever to test reinstatement and reversal of cocaine self-administration.

MICRODIALYSIS. Intracerebral microdialysis was performed using concentric microdialysis probes (2 mm membrane length; cutoff 6000 Da; CMA-11, CMA/Microdialysis, Solna, Sweden). Stereotaxic coordinates were slightly different to correct for the marked size difference in the wild-type and DAT$^{-/-}$ mice: anterioposterior (AP) 0.0, dorsoventral (DV) -4.4, lateral (L) 2.5 for wild-type mice, and AP 0.0, DV -3.2, L 1.8 for DAT$^{-/-}$ relative to bregma[48]. At coordinates used in mice, the dialysis probe samples both dorsal and ventral striatal regions. The dialysis probes were perfused during implantation into the brain and for 1 h afterward with artificial cerebrospinal fluid (in mM): Na^+ 150, K^+ 3.0, Ca^{2+} 1.4, Mg^{2+} 0.8, PO_4^{3-} 31.0, Cl^- 155 (ESA Inc., Bedford, MA), pH 7.3. After operation, animals were returned to their home cages; 24 h after surgery the dialysis probe was perfused at 1.0 µl/min for 60 min before the experiment. Perfusate samples were collected every 20 min. At least four predrug samples were collected before cocaine was administered. Measurements of dopamine in microdialysis samples were performed by HPLC-EC as described[11].

AUTORADIOGRAPHY. *In vitro* autoradiography was performed as described[15]. Briefly, slide-mounted tissue sections (20 µm) were thawed and equilibrated in sucrose buffer (320 mM sucrose, 10 mM sodium phosphate, 10 mM sodium iodide, pH 7.4) for 10 min at room temperature. Sections were then incubated in the absence or presence of cocaine (50 µM), alaproclate (1µM), or nisoxetine (700 nM) for 20 min prior to the addition of [^{125}I]RTI-55 (NEN; 2200 Ci/mmol; 50 pM) for 60 min at room temperature. Free and nonspecifically bound [^{125}I]RTI-55 was removed by washing the sections in 2×20 min in ice-cold sucrose buffer, followed by 2×5 s water and 1×10 s 20% ethanol. Sections were then dried under a cool stream of air and desiccated at 4°C. Autoradiograms were prepared by exposing the slide-mounted tissue sections and [^{125}I] Microscale standards to [^{3}H] Hyperfilm (Amersham, Arlington Heights, IL) for 8–16 h at -20°C. Autoradiograms were digitized using an AlphaImager 2000 image analysis system (Alpha Innotech, Inc., San Leandro, CA) and color scale assigned using NIH Image. Tissue sections were then transferred to test tubes for gamma counting (Model 1274, LKB Instruments, Inc., Piscataway, NJ). Non-specific binding was determined in the presence of 50 µM cocaine with specific activity ranging between 90% and 95% of total activity.

IN SITU HYBRIDIZATION. Animals (four per genotype) were handled daily prior to rapid decapitation in order to minimize stress-induced increase in *c-fos* mRNA levels. *In situ* hybridization was performed.[49], using a cRNA probe for *c-fos* mRNA levels. The riboprobes were labeled using [α-^{35}S]UTP (Amersham, Arlington Heights, IL), slides were incubated overnight at 55°C and then washed under high stringency conditions (1x SSC containing 10 mM 2-mercaptoethanol for 30 min at room temperature, RNase buffer containing 20 mg/ml RNase A for 30 min at 37°C, RNase buffer at 37°C for 30 min, 1x SSC at room temperature for 30 min, 0.5x SSC for 30 min at 65° C and 0.5x SSC for 30 min at room temperature) and dried. The slides were apposed to$^{-/-}$dak X-Omat film for autoradiography for 48 h. Control sense RNA probes were used to hybridize adjacent sections as a negative control and revealed no specific hybridization.

DATA ANALYSIS. For acquisition data, the number of sessions required to meet acquisition criteria for food or cocaine as well as microdialysis data were compared between wild-type and DAT$^{-/-}$ mice by Student's *t* test. For dose–response data, results were scored as the number of reinforcers obtained per hour in the last day of each dose of cocaine or saline, and was used as the dependent variable analyzed for dose–response testing by repeated measures analysis of variance (ANOVA). Differences between genotypes for autoradiographic binding were analyzed by ANOVA.

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