Mice Lacking Dopamine D4 Receptors Are Supersensitive to Ethanol, Cocaine, and Methamphetamine

Marcelo Rubinstein,[2] Tamara J. Phillips,[3,7]
James R. Bunzow,[4] Tomás L. Falzone,[2]
Gustavo Dziewczapolski,[1] Ge Zhang,[4]
Yuan Fang,[5] Jennifer L. Larson,[4]
John A. McDougall,[4] Julia A. Chester,[3]
Carmen Saez,[8] Thomas A. Pugsley,[9]
Oscar Gershanik,[5] Malcolm J. Low,[4]
and David K. Grandy[4,6,10]
[1] Instituto de Investigaciones Farmacológicas
[2] Instituto de Investigaciones en Ingeniería Genética y
Biología Molecular
CONICET
and Depto. Química Biológica
FCEyN
Universidad de Buenos Aires
Buenos Aires, Argentina
[3] Dept. of Behavioral Neuroscience
[4] Vollum Institute
[5] Dept. of Physiology and Pharmacology
[6] Dept. of Cell and Developmental Biology
Oregon Health Sciences University
Portland, Oregon 97201
[7] Dept. of Veterans Affairs Medical Center
Research Service
3710 S. W. U. S. Veterans Hospital Rd.
Portland, Oregon 97201
[8] Hospital Universitario "Virgen del Rocio"
Avenida Manuel Siurot
s/n 41013 Sevilla, Spain
[9] Parke-Davis Pharmaceutical Research
Division of Warner-Lambert Co.
Ann Arbor, Michigan 48105

Summary

The human dopamine D4 receptor (D4R) has received considerable attention because of its high affinity for the atypical antipsychotic clozapine and the unusually polymorphic nature of its gene. To clarify the in vivo role of the D4R, we produced and analyzed mutant mice ($D4R^{-/-}$) lacking this protein. Although less active in open field tests, $D4R^{-/-}$ mice outperformed wild-type mice on the rotarod and displayed locomotor supersensitivity to ethanol, cocaine, and methamphetamine. Biochemical analyses revealed that dopamine synthesis and its conversion to DOPAC were elevated in the dorsal striatum from $D4R^{-/-}$ mice. Based on these findings, we propose that the D4R modulates normal, coordinated and drug-stimulated motor behaviors as well as the activity of nigrostriatal dopamine neurons.

Introduction

Dopamine (DA) is a neurotransmitter in four well-characterized brain pathways (Lindvall and Bjorland, 1983) that

[10] To whom correspondence should be addressed.

modulate neuroendocrine, locomotor, cognitive, and emotional functions. The shortest of these tracts originates in the arcuate nucleus of the hypothalamus and projects to the intermediate lobe of the pituitary, as well as to the hypophyseal portal vessels of the median eminence where DA controls the synthesis and release of proopiomelanocortin-derived peptides and prolactin, respectively.

DA is also synthesized in the terminals of neurons that project from the substantia nigra pars compacta (SNc) to the striatum, a structure associated with the planning, initiation, and coordination of voluntary movement and complex behavioral repertoires (Graybiel, 1990). Some of these nigrostriatal DA neurons project to striosomes, or "patches", of the caudate/putamen (CPU) while others synapse in the striatal matrix (Graybiel, 1990). This heterogeneous topological organization of the striatum is thought to permit the complex integration of motivational, sensory, and motor inputs arriving via afferents from cortical and limbic areas (Graybiel et al., 1994). The importance of the nigrostriatal DA neurons is underscored by the fact that their loss results in Parkinson's disease (Hornykiewicz, 1966).

The two remaining central DA tracts of major importance originate in the ventral tegmental area (VTA) and constitute the mesocortical and mesolimbic DA pathways. The mesocortical neurons project primarily to the medial prefrontal, cingulate, and entorhinal corticies: limbic areas involved in emotional, motivational, and cognitive functions whose dysregulation may contribute to psychotic symptomology (Civelli et al., 1993). The mesolimbic DA neurons project to the nucleus accumbens, septum, olfactory tubercle, amygdaloid complex, and piriform cortex. Of these projections, the mesoaccumbens DA neurons continue to be the focus of much attention because they are thought to be involved in mediating some of the positive, reinforcing properties shared by drugs of abuse such as cocaine, methamphetamine, alcohol, and opiates (Di Chiara, 1995; White, 1996).

The many physiological effects of DA are mediated by a family of receptors transcribed from five distinct genes (Civelli et al., 1993). Of the three D2-like subtypes, the D4 receptor (D4R) continues to receive considerable attention because it displays the highest affinity for the atypical antipsychotic clozapine (Seeman and Van Tol, 1994), and it is expressed in brain regions that are associated with organizational planning, affect, psychotic behavior, motivation, and reward (Meador-Woodruff et al., 1994; Mrzljak et al., 1996; Ariano et al., 1997). In addition, the number of D4Rs has been reported to be elevated in postmortem brains of schizophrenics (Seeman et al., 1993), although this remains controversial (Reynolds and Mason, 1994).

The discovery that the human gene *Drd4* is highly polymorphic, especially in the number of 48 nucleotide repeats in the exon encoding the D4R's putative third cytoplasmic loop (Van Tol et al., 1992), led many laboratories to search for associations between these alleles and various psychological conditions. Although no study

has demonstrated that a given *Drd4* allele is associated with a particular mental disorder, there have been reports showing that certain alleles may predispose an individual to alcoholism (George et al., 1993; Muramatsu et al., 1996; Geijer et al., 1997), novelty-seeking behavior (Benjamin et al., 1996; Ebstein et al., 1996), and opiate abuse (Kotler et al., 1997). In addition, a genetic mapping study of ethanol drinking in mice has provisionally localized a quantitative trait locus (QTL) within 13 cM of murine *Drd4* (Phillips et al., 1994).

Owing to its low abundance and the lack of specific ligands, the actual contribution of D4Rs to complex behaviors has proven to be elusive. Consequently, in an effort to determine a role for D4Rs in vivo, we have produced mice lacking this receptor ($D4R^{-/-}$) by means of homologous recombination in embryonic stem cells. Here we report that, although $D4R^{-/-}$ mice are physically indistinguishable from their wild-type littermates, they displayed reduced spontaneous locomotor activity and rearing, they outperformed $D4R^{+/+}$ animals on the rotarod, they were supersensitive to the stimulation of locomotor activity elicited by ethanol, cocaine, and methamphetamine, and they consistently showed elevated DA synthesis and turnover in their dorsal striata. Based on these findings, we have concluded that the D4R modulates normal and drug-stimulated locomotor behaviors as well as the activity of nigrostriatal DA neurons.

Results

Targeted Disruption of the Dopamine D4 Receptor Gene in the Mouse Results in a Mutant Transcript

A 129/SvEv mouse genomic phage library was screened with a human *D4R* cDNA, and a 12.3 kb mouse genomic clone was isolated. The targeting vector, constructed from this phage minus exon II (Figure 1A), was linearized with NotI and electroporated into embryonic stem (ES) cells. Pools of neo^r-gancyclovir^r ES cells were screened for homologous recombination by PCR. Several recombinant clones were confirmed by Southern blotting analysis, and three were eventually expanded and injected into 3.5-day–old C57BL/6J blastocysts.

Four male chimeras carrying the mutated *D4R* gene were bred with C57BL/6J females to generate F1 heterozygotes. Breeding of these F1 offspring produced F2 hybrids, which segregated the mutated *D4* receptor allele (Figure 1B) in a Mendelian fashion. Homozygous F2 male and female mice ($D4R^{-/-}$) grew and reproduced normally.

Based on the design of our targeting vector, we predicted that the loss of exon II would result in a *D4R* mRNA 113 nucleotides shorter than the wild-type transcript (Fishburn et al., 1995). This was confirmed by Southern blotting of RT-PCR products (Figure 1C). Furthermore, juxtaposition of exons 1 and 3 should produce a 131 amino acid mutant polypeptide of which the first 91 residues correspond to D4R (including putative transmembrane [TM] domain 1, the first cytoplasmic loop, and most of TM2). Automated DNA sequencing of RT-PCR products synthesized from $D4R^{+/+}$ and $D4R^{-/-}$
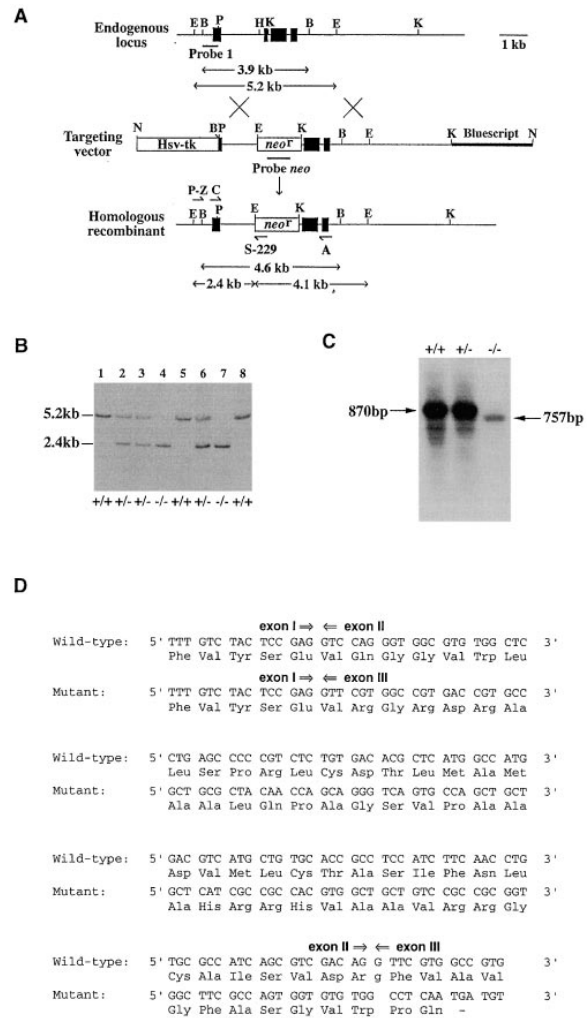
Figure 1. Targeted Disruption of the Murine *D4* Receptor Gene

(A) Alignment of the D4 receptor locus, targeting vector, and mutant locus. Solid boxes are coding exons, and potential crossovers are indicated by Xs. Probe 1, fragment used to detect wild-type and mutant alleles on Southern blots; A, P-Z, C, and S-229 are PCR primers; *neo^r*, neomycin resistance; Hsv-tk, herpes simplex virus-thymidine kinase; B, BamHI; E, EcoRI; H, HindIII; K, KpnI; N, NotI; P, PstI.

(B) Southern analysis of genomic DNA digested with EcoRI. +/+, wild-type; +/−, heterozygotes; −/−, homozygotes.

(C) RT-PCR of RNA from +/+, +/−, and −/− mice using primers A and C. Products were Southern blotted and probed with a human D4 cDNA.

(D) Comparison of wild-type and mutant *D4R* cDNA sequences.

mice provided direct confirmation that, in the mutant transcript, exons 1 and 3 were spliced together, causing a shift in the reading frame and the premature appearance of a termination codon (Figure 1D).

D4 Receptor-Deficient Mice Are Less Sensitive to Clozapine

The atypical antipsychotic clozapine has a high affinity for the D4R in vitro (Seeman and Van Tol, 1994) and has been shown to block apomorphine-induced locomotion
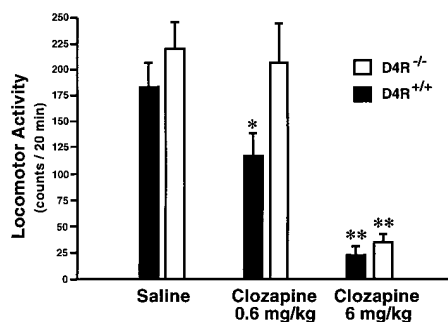
Figure 2. Effects of Clozapine on Apomorphine-Induced Reversal of Akinesia in Catecholamine-Depleted Mice

Depleted $D4R^{+/+}$ and $D4R^{-/-}$ mice received an injection of saline, 0.6 mg/kg, i.p., or 6 mg/kg, i.p. clozapine. All mice received 0.25 mg/kg apomorphine, i.p., 40 min later, and locomotor activity was immediately monitored for 1 hr. Bars correspond to mean ± SEM of at least seven mice. An ANOVA was performed followed by the Dunnett's t test. (asterisk) $p < 0.05$ versus the corresponding genotype receiving saline; (double asterisk) $p < 0.01$ versus the corresponding genotype receiving saline.

in rodents (Krisch et al., 1994). Therefore, it was of interest to assess clozapine's actions on locomotion in both wild-type and mutant mice. In these studies, the mutated *Drd4* gene was maintained on a mixed F2 background (C57BL/6J and 129/Ola), resulting in heterogeneity among individuals with regard to baseline locomotion. To eliminate both individual variations in locomotion and the confound of drug effects on presynaptic DARs, wild-type and mutant mice were rendered akinetic by injecting them with a combination of α-methyl p-tyrosine (an inhibitor of tyrosine hydroxylase, [TH], activity) and reserpine (a monoamine vesicle-depleting agent). The mice were injected 20 min later with saline or clozapine (0.6 or 6 mg/kg, intraperitoneally [i.p.]). All mice were immediately placed 40 min later in activity monitors after receiving 0.25 mg/kg apomorphine, i.p. Under these conditions, 0.6 mg/kg clozapine attenuated the locomotor activity of the wild-type mice by almost 40% but had no effect on the $D4R^{-/-}$ mice whereas 6 mg/kg clozapine completely blocked the apomorphine-induced locomotion in both genotypes of mice (Figure 2).

Locomotion and Rearing Are Reduced in the Mutant Mice

Coordinated locomotor behavior depends on DA transmission in the striatum that is mediated by D1- and D2-like receptor stimulation (Clark and White, 1987; Waddington and Daly, 1993). Recently, low levels of *D4R* mRNA (Surmeier et al., 1996) and immunoreactivity (Ariano et al., 1997) were detected in rat striatum. Therefore, to explore whether the loss of D4Rs had an impact on gross locomotor behaviors, several basal activity measures were quantified in an open field environment.

Locomotor activity of wild-type and mutant mice of both genders was recorded after saline injection (i.p.) in three separate studies. The Omnitech digiscan automated activity monitor constituted a novel environment on the first test day and a familiar environment 24 hr later. In all studies, both genotypes showed habituation from day 1 to day 2 as evidenced by a decrease in

overall activity on the second day. However, under these testing conditions, there were still significant differences between the two genotypes on both days. Data from a representative study are shown in Table 1. On the first day, the $D4R^{-/-}$ mice covered less horizontal distance, had fewer rearing episodes, and moved more slowly than wild-type mice. On the second day of testing, in addition to scoring low on the previously noted measures, the $D4R^{-/-}$ mice initiated fewer movements and spent less time in motion than the wild-type mice. Interestingly, even though mutant female mice displayed slightly greater activity levels than the males, both genders of D4R-deficient mice were significantly less active than wild-type animals.

Anatomical and Pharmacological Evaluation of the Mutant Mice

Given the behavioral differences between the mutant and wild-type mice, we performed a general anatomical survey of Nissl-stained coronal brain sections prepared from both genotypes of mice. This analysis failed to reveal any gross structural abnormalities in the mutant mice (data not shown). Next, we evaluated dopamine binding sites in serial coronal brain sections that included striata from $D4R^{-/-}$ and $D4R^{+/+}$ mice. These sections were labeled with either [^{3}H]-SCH23390, a D1-like receptor-selective antagonist or [^{3}H]-spiperone, a D2-like receptor-selective. By this qualitative method of analysis, no differences in D1 or D2 binding were detected between the wild-type and mutant genotypes (data not shown). Quantitative saturation binding analyses of D1-like (using [^{3}H]SCH23390) and D2-like (using [^{3}H]nemonapride) receptors in striatal membranes prepared from wild-type and mutant mice also failed to reveal any significant differences in B_{max} (in fmol/mg protein: D1R WT 418 ± 3, KO 401 ± 5; D2R WT 231 ± 6, KO 228 ± 16) or K_d (in nM: D1R WT 0.18 ± 0.01, KO 0.17 ± 0.01; D2R WT 0.06 ± 0.01, KO 0.06 ± 0.01).

DOPAC Levels and L-DOPA Accumulation Are Elevated in the Striata of D4 Receptor-Deficient Mice

The majority of the brain's supply of DA is synthesized by TH-positive neurons in the SNc and the VTA. The rate at which TH synthesizes DA is thought to be modulated, in part, through feedback inhibition via stimulation of presynaptic D2-like autoreceptors. Aware of reports that D4Rs are expressed in rat and monkey midbrain structures (Mrzljak et al., 1996; Surmeier et al., 1996; Ariano et al., 1997), we investigated whether DA synthesis was affected in the $D4R^{-/-}$ mice. Initially we evaluated TH immunoreactivity in the VTA and SNc of both wild-type and mutant mice but failed to detect any qualitative difference between the two genotypes (data not shown).

We next turned to biochemical measurements of DA synthesis and turnover in the dorsal striatum-caudate putamen (CPU) and nucleus accumbens (NAc) dissected from wild-type and mutant mice. HPLC-coupled electrochemical detection was used to quantitate DA, HVA, DOPAC, 5-HT, and 5-HIAA. Although both genotypes displayed similar amounts of DA, HVA, 5-HT, and

Table 1. Locomotor Behavior of D4R$^{+/+}$ (Wild-Type) and D4R$^{-/-}$ (Mutant) Mice

	Day 1[a]		Day 2[b]	
	D4R$^{+/+}$	D4R$^{-/-}$	D4R$^{+/+}$	D4R$^{-/-}$
Horizontal distance (cm)	5157 ± 239	3910 ± 167^{c}**	3894 ± 277	2576 ± 171**
Number of movements initiated	360 ± 9	339 ± 9	299 ± 13	225 ± 14**
Time in motion (s)	493 ± 17	454 ± 15	329 ± 18	267 ± 16*
Number of rearing events	95 ± 9	30 ± 4**	72 ± 8	19 ± 3**
Speed (cm/s)	10.3 ± 0.24	8.5 ± 0.13^{c}**	11.5 ± 0.31	9.6 ± 0.18^{c}**

* $p < 0.05$; ** $p < 0.001$; n = 25–30 mice/genotype.
[a] Day 1 constitutes a novel environment.
[b] Day 2 is considered a familiar environment.
[c] Gender differences.

5-HIAA in their CPU and NAc, DOPAC content was found to be increased 93% in CPU dissected from the mutant mice (Table 2), resulting in a DOPAC/DA ratio 1.9 times higher than in wild-type mice.

When clozapine (6 mg/kg, i.p.) was administered 2 hr before sacrifice, both the striatal DOPAC content and the DOPAC/DA ratio were elevated in the CPU of wild-type mice to the same extent as mutant mice receiving either saline or clozapine (Figure 3A). This same dose of clozapine had no effect on DOPAC/DA ratio in the NAc of either genotype (Figure 3B). In contrast, haloperidol (0.6 mg/kg, i.p.) increased the DOPAC/DA ratio in both CPU and NAc to the same extent in both genotypes (Figures 3A and 3B, respectively).

To explore whether the increased DOPAC/DA ratio detected in CPU might be due, in part, to increased DA synthesis, we determined the accumulation of L-DOPA in mice pretreated with the DOPA-decarboxylase inhibitor NSD-1015, which blocks the conversion of L-DOPA to DA (Carlsson et al., 1975). HPLC-coupled electrochemical detection consistently revealed that CPU from $D4R^{-/-}$ mice accumulated more L-DOPA than CPU taken from wild-type mice (Figure 3C).

D4 Receptor-Deficient Mice Outperform Their Wild-Type Littermates on the Rotarod

Balanced dopamine neurotransmission within the basal ganglia is critical for maintaining coordinated motor activity. Our observation that DA synthesis and turnover were elevated in the CPU of mutant mice suggested that, by comparing the performances of both genotypes in a test of coordination, additional phenotypic differences might be revealed. The rotarod is one widely employed measure of a rodent's ability to sustain complex

Table 2. Dopamine, Serotonin, and Their Metabolites in Mutant (KO) and Wild-Type (WT) Mice

			Saline[a]	Haloperidol (0.6 mg/kg)	Clozapine (6 mg/kg)
DA	Str	WT	88.17 ± 5.90[b]	80.68 ± 3.61	89.20 ± 6.88
		KO	90.37 ± 4.63[c]	74.95 ± 3.87	88.53 ± 4.22
	NAc	WT	95.06 ± 4.50	82.59 ± 5.68	94.98 ± 7.56
		KO	91.61 ± 4.08	85.54 ± 6.64	99.42 ± 7.49
DOPAC	Str	WT	7.02 ± 0.43	24.79 ± 1.07	12.26 ± 0.89
		KO	13.54 ± 1.53**	25.56 ± 1.99	11.85 ± 1.50
	NAc	WT	16.04 ± 1.93	32.48 ± 4.55	22.95 ± 5.03
		KO	17.10 ± 2.43	35.52 ± 3.22	20.47 ± 1.51
HVA	Str	WT	6.13 ± 0.27	17.06 ± 1.00	5.63 ± 0.37
		KO	5.73 ± 0.34	13.96 ± 1.16	5.61 ± 0.42
	NAc	WT	8.71 ± 0.67	19.61 ± 2.30	10.12 ± 1.40
		KO	7.04 ± 0.78	17.89 ± 1.44	7.72 ± 0.67
5-HT	Str	WT	3.43 ± 0.35	3.34 ± 0.42	3.61 ± 0.33
		KO	3.73 ± 0.32	3.35 ± 0.27	4.25 ± 0.26
	NAc	WT	3.06 ± 0.17	3.16 ± 0.21	3.48 ± 0.21
		KO	3.48 ± 0.12	3.29 ± 0.28	3.78 ± 0.32
5-HIAA	Str	WT	2.39 ± 0.30	2.80 ± 0.24	2.31 ± 0.15
		KO	2.17 ± 0.10	2.61 ± 0.16	2.23 ± 0.27
	NAc	WT	2.68 ± 0.16	3.35 ± 0.45	3.15 ± 0.35
		KO	2.32 ± 0.17	2.98 ± 0.21	2.78 ± 0.18

** $p < 0.01$; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindole acetic acid; Str, striatum; NAc, nucleus accumbens.
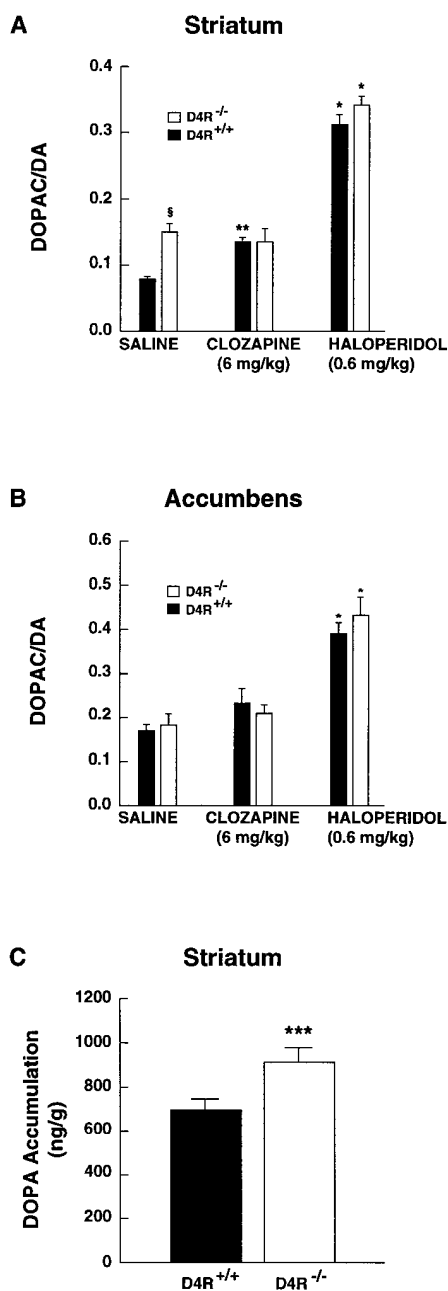[a] Mean values are pmol/mg tissue ± SEM.
[b] n = 7.
[c] n = 6.

A **Striatum**

B **Accumbens**

C **Striatum**

Figure 3. Dysregulation of Dopamine Biosynthesis and Turnover in $D4R^{-/-}$ Mice

DOPAC/DA ratio in striata (A) and nucleus accumbens (B) of $D4R^{+/+}$ or $D4R^{-/-}$ mice 2 hr after saline (i.p.), 6 mg/kg clozapine, or 0.6 mg/kg haloperidol. Bars represent the mean ± SEM of at least six mice. Data were analyzed by ANOVA followed by Dunnett's t test. (asterisk) $p < 0.05$ compared to the same genotype receiving saline; (double asterisk) $p < 0.01$ compared to the same genotype receiving saline; (section mark) $p < 0.01$ compared to $D4R^{+/+}$ mice receiving saline. (C) L-DOPA accumulation in the CPU of $D4R^{+/+}$ and $D4R^{-/-}$ mice treated for 30 min with NSD. The mean values ± SEM for ten mice of each genotype are shown. The data were analyzed by Student's t test: (triple asterisk) $p < 0.001$ versus the $D4R^{+/+}$ group.

coordinated movements over time. Male mice of both genotypes were given a single, brief training session after which the number of falls, time between falls, and
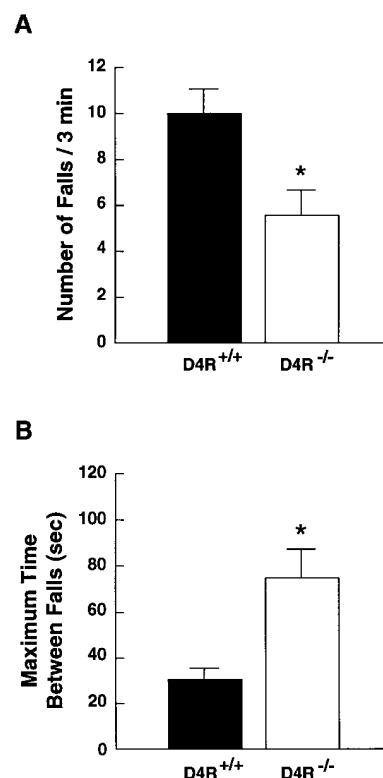
A

B

Figure 4. Mutant Mice Outperform Their $D4R^{+/+}$ Littermates on the Rotarod

The number of falls (A) and maximum time between falls (B) were counted during the 3 min test period. (-/-, n = 25; +/+, n = 27). The data were analyzed by Student's t test. (asterisk) $p < 0.01$.

length of time on the rotarod were recorded for each mouse during a 3 min test session. Interestingly, the $D4R^{-/-}$ mice outperformed their $D4R^{+/+}$ littermates in this test, experiencing 50% fewer falls (Figure 4A) and remaining on the rotating rod 2.5 times longer than their wild-type siblings (Figure 4B).

D4 Receptor-Deficient Mice Are More Responsive to the Locomotor-Stimulating Effects of Ethanol

Dopamine levels in brain fluctuate in response to activity (Freed and Yamamoto, 1985) and following the administration of various drugs of abuse. Acute doses of ethanol induce changes in DA levels in mouse (Dar and Wooles, 1984) and rat brain (Imperato and Di Chiara, 1986), and its effect on DA release has been associated with ethanol-stimulated activity (Imperato and Di Chiara, 1986). Ethanol-induced locomotor stimulation in rodents has been suggested as an animal model of ethanol-induced euphoria, and DA receptor antagonists have been found to block ethanol's stimulant effects (Phillips and Shen, 1996). These findings, along with results from studies of ethanol self-administration (Rassnick et al., 1992), have been interpreted to support a role for midbrain DA pathways in the rewarding properties of ethanol. Therefore, we investigated the responses of $D4R^{-/-}$ and $D4R^{+/+}$ mice to ethanol using a simple test of the drug's effect on spontaneous horizontal locomotion.

To correct for individual and genotype-dependent differences in basal locomotion, the day 2 baseline score for each mouse was subtracted from its ethanol response on day 3. We have found this subtraction method of estimating strain sensitivity to drug stimulant effects to provide extremely reliable results (Phillips et al., 1991, 1995) and have successfully used similar procedures to breed mice selectively for increased and reduced sensitivity to the locomotor stimulant effects of ethanol (Phillips et al., 1991).

Both wild-type and mutant female mice were exposed to three sessions of locomotor testing over as many days in automated Omnitech digiscan monitors. On the first 2 days, mice were tested after being injected with saline. On the third day, they were tested after receiving a 2 g/kg injection of ethanol (20% v/v). Animals were continuously monitored for 30 min postinjection with data collected every 5 min (Figure 5A). A 2-way analysis of variance (ANOVA) grouped on genotype and time indicated significant changes in ethanol response over time (F[5,105] = 31.6, p < 0.001), with a greater stimulation of locomotor activity in $D4R^{-/-}$ than $D4R^{+/+}$ mice (F[1,21] = 8.9, p < 0.01).

D4 Receptor-Deficient Mice Are More Responsive to the Locomotor-Stimulating Effects of Cocaine and Methamphetamine than Wild-Type Mice

The psychostimulants cocaine and methamphetamine raise DA levels in the NAc and frontal cortex, albeit by different mechanisms (Maisonneuve et al., 1990; Weiss et al., 1992; Camp et al., 1994) and produce dramatic increases in locomotor activity. As with other drugs of abuse, including ethanol, the ability of cocaine and methamphetamine to increase DA levels in mesolimbic and mesocortical structures is believed to underlie some of their positive reinforcing properties (Kuhar et al., 1991). Intrigued by our observation that ethanol stimulated locomotor activity in the $D4R^{-/-}$ mice to a greater extent than it did in the $D4R^{+/+}$ mice, we investigated the responsiveness of the mutant mice to cocaine and methamphetamine.

For each drug tested, two groups of wild-type and mutant mice were evaluated over 3 consecutive days. All mice received an i.p. injection of saline on the first 2 days, and their locomotor behavior was then recorded in 5 min time blocks for a total time of 30 min in the cocaine study and 15 min in the methamphetamine study. Due to the availability of larger groups of wild-type and mutant mice at the time these studies were conducted, we were able to include a saline-treated control group on the drug treatment day (day 3). Therefore, on this third test day mice were randomly distributed into saline, cocaine (15 or 30 mg/kg), or methamphetamine (1 and 2 mg/kg) groups.

Differences between saline-treated groups of both genotypes persisted on day 3 so that simple comparisons of group means on this day did not provide a good measure of stimulant response relative to baseline. Therefore, as in our ethanol study, estimates of drug sensitivity were derived by subtracting day 2 from day 3 scores. These doses of cocaine (Figure 5B) and methamphetamine (Figure 5C) stimulated locomotor activity

A   Ethanol
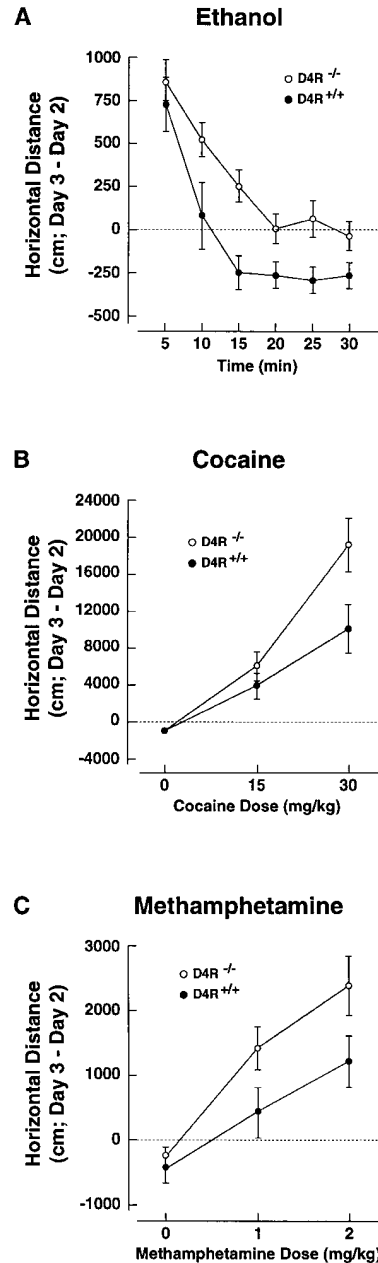
B   Cocaine

C   Methamphetamine

Figure 5. Mutant Mice Are More Sensitive to the Stimulating Properties of Ethanol, Cocaine, and Methamphetamine

(A) Time course of locomotor activity for female $D4R^{-/-}$ and $D4R^{+/+}$ mice after 2 g/kg ethanol treatment. Day 2 saline scores during a 30 min test were subtracted from day 3 scores to correct for genotype-based differences in basal locomotion (n = 9 $D4R^{+/+}$ and 14 $D4R^{-/-}$).
(B) Locomotor responses of $D4R^{-/-}$ and $D4R^{+/+}$ mice to cocaine HCl (15 and 30 mg/kg). Day 2 saline scores were subtracted from day 3 cocaine scores to correct for genotype-based differences in basal locomotion (n = 10 $D4R^{+/+}$ and 10 $D4R^{-/-}$).
(C) Locomotor response of $D4R^{-/-}$ and $D4R^{+/+}$ mice to methamphetamine HCl (1 or 2 mg/kg). Day 2 saline scores were subtracted from day 3 drug scores to correct for genotype-based differences in basal locomotion (n = 8–10 mice per genotype, gender, and dose).

in both the wild-type and $D4R^{-/-}$ mice. However, the mutant mice displayed locomotor supersensitivity compared to the wild-type animals. An ANOVA revealed a

significantly greater locomotor stimulant response to cocaine treatment in mutant relative to wild-type mice (F[1,104] = 6.7, p < 0.01) that was dose-dependent (F[2,104] = 41.1, p < 0.001). Furthermore, there was also a significant genotype by dose interaction (F[2, 104] = 3.9, p < 0.05). Similarly, an ANOVA indicated significantly greater locomotion in response to methamphetamine treatment in mutant mice compared to wild-type animals (F[1,114] = 7.7, p < 0.01) that was dose-dependent (F[2,114] = 24.7, p < 0.001).

Discussion

Since its initial cloning and pharmacological characterization, there has been considerable speculation as to the physiological significance of the D4R and, in particular, its involvement in schizophrenia. In spite of what has been learned about D4R's pharmacology, anatomical distribution, genetics, and in vitro physiology, none of these efforts have directly assessed D4R function in vivo. We have used targeted mutagenesis to engineer a strain of mouse that completely lacks functional D4Rs. These mutant mice displayed less spontaneous locomotion and rearing activity than their wild-type littermates in novel and familiar surroundings. In addition, mutant mice consistently showed elevated DA synthesis and turnover in dorsal striatum, outperformed $D4R^{+/+}$ mice on the rotarod, and were supersensitive to cocaine, methamphetamine, and ethanol. Based on these findings, we speculate that D4Rs in vivo participate in the modulation of neurotransmission between the cortex, the basal ganglia, and the thalamus.

Clozapine Insensitivity
Historically the ability of a compound to antagonize DA agonist-stimulated locomotor behavior has been used to identify potentially useful antipsychotic drugs. Clozapine, for example, has been shown to block the hyperlocomotion produced by the direct DA agonist apomorphine (Krisch et al., 1994) and the indirect agonist methamphetamine (Arnt, 1995). Clozapine is receiving considerable clinical attention as an atypical antipsychotic medication because it does not produce Parkinson-like symptoms in schizophrenic patients.

Clozapine has been shown to act as an antagonist of the D4R-mediated inhibition of forskolin-stimulated cAMP production (Bouvier et al., 1995) and displays about a 10-fold higher affinity for the cloned D4R compared to D2R and D3R (Seeman and Van Tol, 1994). Clozapine also has high affinity for the M4 muscarinic receptor (Zorn et al., 1994) and 5HT2 serotonin receptor (Leysen et al., 1993). Consequently, it was of interest to evaluate the effect of clozapine on locomotion in D4R-deficient mice.

Our finding that apomorphine-induced locomotion in the mutant mice was insensitive to the 0.6 mg/kg dose of clozapine compared to wild-type animals, yet sensitive to 6 mg/kg, is consistent with the interpretation that the D4R was functionally eliminated in the mutant mice. This result also suggests that, in wild-type mice, a low dose of clozapine prevents the D4R-mediated contribution to the overall locomotor response whereas at higher doses its antagonism of the D2R results in akinesia.

Taken together, we interpret these clozapine data to support our hypothesis that the D4R may serve a facilitory role in terms of normal movement (see below).

Hypoactivity in Mice Lacking the D4 Receptor
Although the F2 mutant mice appeared normal in their home cages, when placed in both novel and familiar open field environments, they scored significantly lower than wild-type mice on several measures of locomotion. These findings support anecdotal observations that, in their home cages, $D4R^{-/-}$ mice were easier to catch than their wild-type littermates.

The reduced overall locomotor activity engaged in by the $D4R^{-/-}$ mice is in contrast to what has been reported for mice lacking D3Rs (Accili et al., 1996) but is similar to what we have observed in our strain of mouse that lacks D2Rs (unpublished data). Our findings suggest that the D4R is more involved in facilitating a variety of normal locomotor behaviors in the mouse than was originally predicted based on its distribution in the brain.

There are several possible explanations for why the mutant mice are less active in familiar surroundings. For example, the complete absence of D4Rs during brain development may result in unbalanced DA signaling in the cortex and basal ganglia that manifests itself as hypolocomotion. Another explanation may be that loss of the D4R from limbic brain regions has altered the motivational state of the mutant mice such that the time spent exploring their environment is reduced.

A third explanation relies on the recent report (Mrzljak et al., 1996) of D4R immunoreactivity in GABA neurons in the substantia nigra pars reticulata (SNr) and the globus pallidus (GP) of the monkey. These two structures are the major output nuclei through which the striatum communicates with the thalamus and cortex. As such, these two nuclei figure importantly in one widely cited model of basal ganglia function and dysfunction in Parkinsonism and Huntington's disease (Wichmann and DeLong, 1996). In this model, hypokinesis (e.g., Parkinson's disease) is associated with the loss of DA, in part, because the balance of striatal transmission is shifted to the so-called indirect striatal pathway. This has the net effect of increasing activity in SNr/GP neurons. An increase in SNr/GP neuronal firing would tend to inhibit thalamic signaling to the motor cortex, resulting in bradykinesis and akinesia in advanced forms of the disease. With this model in mind, the loss of D4R expression in the inhibitory GABAergic neurons of the SNr/GP may render them less sensitive to the inhibitory tone produced by DA activation of D4Rs coupled to potassium channels (Werner et al., 1996). As a result, the GABAergic SNr/GP neurons that project to the ventral lateral thalamic nucleus (VLT) would tend to be disinhibited in the D4R-deficient mice. The net effect of losing D4R-mediated inhibitory tone from SNr/GP neurons may be increased firing of these inhibitory cells, which, in turn, would inhibit the firing of excitatory VLT neurons, thereby disrupting stimulus–response coupling between the basal ganglia, thalamus, and cortex. Consequently, the behavioral manifestation of this disrupted circuit may be the reduced locomotion that characterizes the mutant mice in a familiar environment.

Dysregulation of Dopamine Synthesis in Mice Lacking D4 Receptors

An important observation made in the course of our characterization of the D4R-deficient mice was that both the biosynthesis and turnover of DA in the CPU were increased. The vast majority of DA in the striatum is produced in the terminals of neurons that project from cell bodies located in the SNc. The regulation of DA synthesis and release in the CPU is complex. Not only does glutamate have a stimulatory effect on DA levels in the CPU (Westerink et al., 1992), but modulation of tyrosine hydroxylase (TH) activity by cAMP-dependent kinases (Zigmond et al., 1989), Ca^{2+} − calmodulin protein kinase II (Ishii et al., 1991), L-DOPA, and DA (Ribeiro et al., 1992) have also been shown to be important.

Our observation that the mutant mice accumulate more L-DOPA following DOPA decarboxylase inhibition and produce more DOPAC than their wild-type littermates is consistent with the loss of an inhibitory D2-like autoreceptor. However, to date there has been no convincing demonstration of D4R immunoreactivity or mRNA in the SNc of rodents or primates. Rather, Mrzljak et al. (1996) have demonstrated D4R immunoreactivity in glutamatergic pyramidal cells of the monkey frontal cortex, Ariano et al. (1997) recently reported D4R immunoreactivity in the frontal cortex of the rat, and we have observed relatively abundant D4R transcripts in mouse frontal cortex (see Figure 1C). Taken together, we propose that rather than acting as an autoreceptor, activated D4Rs in the cortex modulate glutamate release, which affects DA synthesis and turnover in the CPU.

In support of our hypothesis, there is evidence that glutamatergic pyramidal cells project to the CPU (Carter, 1982) as well as to the substantia nigra (Carter, 1982) and that DA release is stimulated by glutamate acting on either nigrostriatal nerve terminals within the CPU (Westerink et al., 1992) or cell bodies in the substantia nigra (Overton and Clark, 1992; Christoffersen and Meltzer, 1995). Given that expression of D4Rs is highest in the cortex (Mrzljak et al., 1996; Ariano et al., 1997), we propose that populations of corticostriatal and corticonigral pyramidal cells in D4R-deficient mice may no longer experience the inhibitory tone normally provided by mesocortical DA. Consequently, they should release more glutamate into their synaptic fields. Increased glutamate in the CPU should stimulate DA synthesis and release from nigrostriatal nerve terminals in the CPU whereas glutamate released in the substantia nigra should stimulate nigrostriatal cell firing and the elevation of DA in the CPU. Based on the biochemical evidence and our behavioral findings (see below), we suggest that certain selective compounds for the D4R may have some utility in the treatment of Parkinson's disease.

Enhanced Rotarod Performance by Mutant Mice

Our observation that mice lacking the D4R outperformed their wild-type littermates on the rotarod was unexpected because they were less active in the open field studies. However, we propose that the mutant mice are more adept at this complex motor task, in part, because of increased DA synthesis in their CPU.

It is well-documented in the rat that significant changes in DA metabolism are required for coordinated voluntary motor behavior (Freed and Yamamoto, 1985). Furthermore, patients with Parkinson's disease are able to accomplish complex motor tasks following L-DOPA therapy and initiate coordinated movement when confronted with an appropriate stimulus. Consequently, the D4R-deficient mice may be more adept at this complex motor task because their striatal DA reserves are elevated and the rotarod constitutes a significant challenge to move or fall.

Mutant Mice Are Supersensitive to the Locomotor-Stimulating Effect of Ethanol, Cocaine, and Methamphetamine

With the discovery that the human *Drd4* gene is highly polymorphic within its coding region, there has been widespread interest in determining whether there is an association between various *Drd4* alleles and the development of mental illness or predisposition to drug abuse–related behaviors. Although some of the results are controversial, recent reports have documented the association of various *Drd4* alleles with alcoholism (George et al., 1993; Muramatsu et al., 1996; Geijer et al., 1997), risk-taking behaviors (Benjamin et al., 1996; Ebstein et al., 1996), and opiate addiction (Kotler et al., 1997) in humans. Unfortunately it is not possible to extend these findings to the mouse because the 48 base pair sequence, whose copy number varies in humans, is not repeated in the mouse. However, the extensive catalog of murine genetic markers, coupled with quantifiable behavioral measures, has made possible the identification of candidate genes associated with alcoholism and drug abuse (Crabbe et al., 1994). In fact, Phillips et al. (1994) have demonstrated that the murine *Drd4* gene lies within 13 cM of a provisional quantitative trait locus associated with ethanol-drinking behavior. Extensive behavioral (Wise and Bozarth, 1987; Robinson and Berridge, 1993), biochemical (Maisonneuve et al., 1990), and electrophysiological (White, 1996) testing has revealed that habitually abused drugs possessing positive reinforcing properties elevate DA levels in the nucleus accumbens and the frontal cortex. Consequently, our observation that locomotor activity in mice lacking D4Rs was stimulated by ethanol, cocaine, and methamphetamine to a greater extent than in wild-type mice revealed an unanticipated involvement of D4Rs in drug-induced locomotor activity. Further studies will be required to determine if classical sensitization to repeated drug administration occurs in these mutant mice as well as whether higher doses of these drugs produce stereotypy.

One explanation of our results may be that ethanol, cocaine, and methamphetamine are stimulating DA-producing neurons in the CPU, neurons whose DA synthesis and turnover is already elevated in mutant, drug-naive mice. Consequently, an additional release of striatal DA in response to these drugs may be sufficient to produce the apparent locomotor supersensitivity observed in the $D4R^{-/-}$ mice. Another possible explanation for the drug supersensitivity we have observed is based on immunohistochemical, electrophysiological, and molecular data. As mentioned previously, D4R immunoreactivity

has been detected in both excitatory pyramidal cells and GABA interneurons in monkey frontal cortex, and D4Rs have been shown to activate inwardly rectifying potassium channels that can hyperpolarize cells and inhibit their firing. Therefore, when drugs such as cocaine, methamphetamine, and ethanol elevate the concentration of mesocortical DA, (Maisonneuve et al., 1990; Fadda et al., 1991) this increase may have a greater excitatory effect on pyramidal cells expressing D1-like receptors (Bergson et al., 1995) in the cortex since the D4R-mediated inhibitory tone normally present on these neurons has been lost. This loss of inhibitory tone may disrupt the normal balance between D1-like receptor-mediated excitation and D4R-mediated inhibition of cortical cells. Behaviorally this imbalance may manifest itself as an apparent supersensitivity, that is, increased locomotion relative to the response of wild-type mice. A third plausible explanation for the drug supersensitivity displayed by the mutant mice is that the functional coupling of a particular dopamine receptor subtype may be altered in mice lacking D4Rs.

Conclusion

The D4R-deficient mouse provides a new and useful model system in which to explore the in vivo role of the D4R in normal and drug-induced behaviors. Based on the phenotypes we have observed and what is known about the mesolimbic, mesocortical, and nigrostriatal DA pathways, we propose that the D4R acts at the cellular level as an inhibitory postsynaptic receptor that primarily modulates the firing of neurons in the frontal cortex and the basal ganglia. As a consequence of its restricted anatomical distribution, this DA receptor subtype is well-positioned to influence neurotransmission between the cortex, the basal ganglia, and the thalamus. Yet more definitive studies designed to evaluate the role of D4Rs in the intact adult mouse will benefit from receptor-specific antagonists or a temporal conditional knockout mouse model once these resources become available.

Experimental Procedures

Cloning and Targeted Disruption of the *D4R* Gene
A 129SvEv mouse genomic library (generously provided by P. Soriano) was screened with a human D4.4 receptor probe. Positive phages were mapped and partially sequenced. CsCl banded targeting vector (25 μg) was linearized (NotI) and electroporated into $\sim2 \times 10^7$ 129/Ola Hsd E14TG2A embryonic stem cells (provided by R. Murray) and maintained under double selection (300 μg/ml G418, 2 μM gancyclovir) as previously described (Rubinstein et al., 1996). Total RNA was prepared from mouse tissue using the RNeasy Mini Kit (Qiagen) and used for first strand cDNA synthesis (Superscript II, GIBCO-BRL). An aliquot of this cDNA was then subjected to amplification by PCR (94°C, 1.5 min; 60°C, 2.0 min; 72°C, 2.5 min $\times$ 30) with primers C and A and Southern blotted and probed with a ^{32}P-labeled fragment of the mouse D4 receptor cDNA. RT-PCR products from all three genotypes were subcloned into pAMP (GIBCO-BRL) and subjected to automated DNA sequence analysis.

Behavioral Methods
All spontaneous open field locomotion and rearing behavior of F2 mice of both genders was measured in an Omnitech digiscan activity monitor (40 cm²) on two consecutive days for 30 min each day immediately following the saline injection. Activity studies were performed between the hours of 10 A.M. and 2 P.M.

To assess clozapine's effect on locomotion, both wild-type and mutant genotypes were rendered akinetic by a treatment consisting of α-methyl-p-tyrosine (200 and 100 mg/kg, i.p. 3 hr and 1 hr, respectively) and a single injection of 5 mg/kg, i.p. reserpine (2 hr before testing). Mice exhibiting more than 20 counts in the first 5 min of the test period were excluded from the study. The remaining akinetic mice received either vehicle or clozapine, i.p., and 40 min later all mice were given 0.25 mg/kg apomorphine, i.p., and placed immediately inside a transparent acrylic box (30 $\times$ 20 $\times$ 20 cm) on top of an activity meter (Animex type DSE-LKB, Farad, Sweden). Locomotor activity was measured in 5 min intervals during 1 hr (0–5 min, 20–25 min, 40–45 min, and 60–65 min) and cumulative counts over these four periods were taken for data analysis.

Rotarod sessions were conducted in a room next to the vivarium. F2 mice of both genders and genotypes were given 60 min to adjust to their new surroundings prior to being individually placed in a neutral position on the immobile rotarod treadmill (Ugo Basile, Milan, IT). The speed was increased to 16 revolutions/min, and each mouse was given a 10 min training session. After each fall, the animal would be repositioned on the rod. The mice were retested 2 hr later for 3 min.

To evaluate the effects of ethanol, cocaine, and methamphetamine on locomotor behavior, mice were tested on 3 consecutive days in Omnitech digiscan activity monitors. On days 1 and 2, each animal received an i.p. injection of saline, and activity was recorded for 15 min (methamphetamine) or 30 min (ethanol and cocaine). On day 3 of the ethanol studies, all animals received an ethanol injection (2 g/kg; 20% v/v). In the experiments involving cocaine, mice received saline or cocaine HCl (15 or 30 mg/kg) on day 3. Similarly, mice in the methamphetamine studies were treated with saline or methamphetamine HCl (1 or 2 mg/kg) on day 3.

Electrochemical Detection of L-DOPA, DA, and Metabolites
HPLC-coupled electrochemical detection (Heikkila et al., 1984) of DA, DOPAC, HVA, 5-HT, and 5-HIAA was achieved using a Varian 5000 liquid chromatograph coupled to an electrochemical detector (BAS LC-4C). Clozapine (6 mg/kg) or haloperidol (0.6 mg/kg) brains were collected 2 hr after receiving saline, and the striatum and nucleus accumbens were dissected, weighed, homogenized, and deproteinized in 0.2 M perchloric acid (1/40 w/vol). Homogenates were centrifuged, and the supernatants were injected onto a 15.0 cm $\times$ 3.9 mm Nova-Pak C18 reverse phase column (Waters) developed in 250 ml of mobile phase (2.62 g NaH$_2$PO$_4$, 92 mg EDTA, 1.31 ml PICB8, 2 ml methanol) at 1.1 ml/min. The electrode potential was set at +0.7 V. Peak heights were measured by DATA Jet Integrator (Spectra-Physics) and quantified based on standard curves using DATAFIT.

L-DOPA (L-3,4-dihydroxyphenylalanine) accumulation (Carlsson et al., 1975) was measured electrochemically (Pugsley et al., 1995) in dorsal striatum of both sexes and genotypes after administration of 100 mg/kg, NSD 1015 (3-hydroxybenzylhydrazine dihydrochloride).

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