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Dopamine D3 Receptor Mutant Mice Exhibit Increased Behavioral Sensitivity to Concurrent Stimulation of D1 and D2 Receptors

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Summary

The dopamine D3 receptor is expressed primarily in regions of the brain that are thought to influence motivation and motor functions. To specify in vivo D3 receptor function, we generated mutant mice lacking this receptor. Our analysis indicates that in a novel environment, D3 mutant mice are transiently more active than wild-type mice, an effect not associated with anxiety state. Moreover, D3 mutant mice exhibit enhanced behavioral sensitivity to combined injections of D1 and D2 class receptor agonists, cocaine and amphetamine. However, the combined electrophysiological effects of the same D1 and D2 agonists on single neurons within the nucleus accumbens were not altered by the D3 receptor mutation. We conclude that one function of the D3 receptor is to modulate behaviors by inhibiting the cooperative effects of postsynaptic D1 and other D2 class receptors at systems level.

Introduction

The brain dopamine (DA) system is a critical modulator of voluntary movement and motivated behaviors and is known to influence neural functions ranging from endocrine and somatomotor control to learning and memory (White, 1989; Robbins, 1992; Graybiel, 1995). A central problem in understanding the DA system is to link various dopaminergic functions to different members of the two DA receptor classes, the D1 class (D1 and D5) and the D2 class (D2, D3, and D4) receptors (Civelli et al., 1993; Gingrich and Caron, 1993). Complicating such understanding is the fact that there is a great deal of regional overlap of the receptor distributions, making it difficult to associate specific functions to particular receptor subtypes (Surmeier et al., 1992; Civelli et al., 1993; Gingrich and Caron, 1993). For example, D1, D2, and D5 receptors are all well-represented in the neocortex, and D1 and D2 receptors are both strongly concentrated in the striatum. The D3 receptor is largely expressed in the limbic system, including the nucleus accumbens, olfactory tubercle, the ventral pallidum, and the amygdala, and to a lesser extent, the striatum (Sokoloff et al., 1990; Murray et al., 1994).

The nucleus accumbens has been implicated in motivated behaviors such as drug self-administration (Olds, 1979, 1982; Hoebel et al., 1983) and conditioned cue preference (CCP) (Kelsey et al., 1989; Everitt et al., 1991; Hiroi and White, 1991a; White et al., 1991; White and Hiroi, 1993), which are thought to depend on reward (White et al., 1987). The limbic system-selective expression of the D3 receptor has led to particular interest in this receptor as a potential mediator of some of the psychoaffective functions of DA neurotransmission. Pharmacological studies generally support this view. For example, cocaine self-administration is attenuated by the coadministration of moderately D3 receptor-selective agonists, leading to the suggestion that the D3 receptor may be important for this behavior (Caine and Koob, 1993; Parsons et al., 1996). However, the in vivo selectivity of these and other "D3-selective" ligands has been questioned (Large and Stubbs, 1994; Burris et al., 1995; Gonzalez and Sibley, 1995) and thus severely limits conclusions about the in vivo functions of the D3 receptor.

D3 receptors have been implicated in the regulation of motor behavior by the finding that a reduction in spontaneous locomotion is produced by 7-OH-DPAT, a D3 receptor agonist with moderate selectivity (Daly and Waddington, 1993; Svensson et al., 1994b). Presynaptic DA autoreceptors were originally thought to mediate these behavioral effects (Clark et al., 1985), but a series of studies have attributed them to postsynaptic D3 receptors, because they are observed in the absence of neurochemical alterations known to be mediated by DA autoreceptors including DA release or synthesis (Waters et al., 1993, 1994; Svensson et al., 1994a, 1994b; Sautel et al., 1995).

In order to investigate key issues of whether the D3 receptor is involved in motor behavior and responses to psychostimulants and to explore the underlying mechanisms of D3 receptor function, we have used the gene-targeting approach to generate mice lacking D3 receptors (Drago et al., 1994; Xu et al., 1994a, 1994b, 1996; Baik et al., 1995; Accili et al., 1996; Calabresi et al., 1997). We report here that the D3 receptor mutant mice exhibit no obvious changes in the general anatomy of the brain DA system, but show behavioral abnormalities. They are more active when both D1 and D2 receptors are simultaneously stimulated. We demonstrate that the effect is attributable to a mechanism other than the

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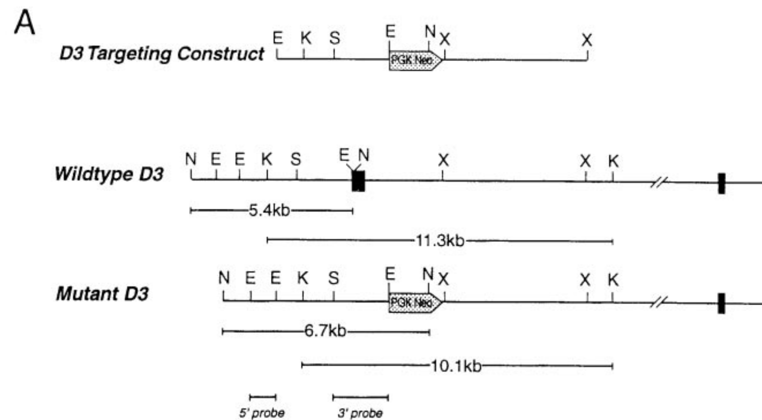

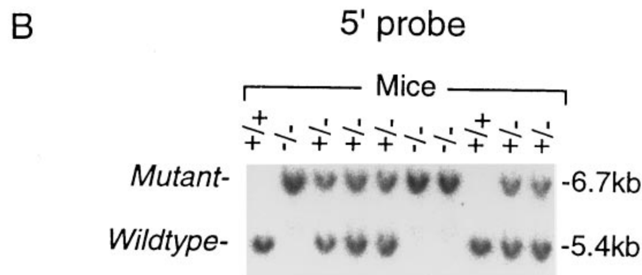

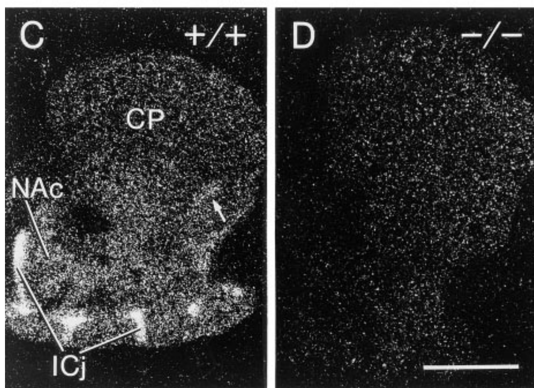

Figure 1. Generation of D3 Receptor Mutant Mice

(A) The D3 targeting construct, wild type, and mutant loci of the mouse D3 receptor gene. The black boxes represent the first and the second exon of the D3 receptor gene. The shaded box depicts the neo gene driven by a PGK promoter. The solid line represents extragenic sequences. The expected sizes of the hybridizing restriction fragments for both the wild type and the mutant alleles are indicated under the corresponding wild type and the mutant loci sequences. Abbreviations for restriction enzyme sites are: E, Eco RI; K, KpnI; N, NcoI; S, SacI; X, XbaI.

(B) Genomic Southern analyses of tail biopsies from a litter of pups of one heterozygous breeding pair. Genomic DNA was isolated from the tails of pups, digested with NcoI, and hybridized with a 5′ probe. The resulting genotype of each pup is indicated.

(C and D) Autoradiographic ligand binding for DA D3 receptors in wild type and D3 mutant mice. Autoradiograms illustrating distributions of [^{125}I]iodosulpride binding in the presence of domperidone to label D3 receptor binding sites in the control (+/+) (C) and mutant (−/−) (D) mice. ICj, Islands of Calleja; CP, caudoputamen; NAc, nucleus accumbens. The arrow in (C) points to a putative striosome. Scale bar indicates 1 mm.

synergistic effects of D1 and D2 receptor stimulation on the firing of single neurons within the ventral striatum. The D3 mutant mice also show increased sensitivity to amphetamine in the CCP paradigm. We conclude that one role of DA D3 receptors is to down-regulate excessive transmission at postsynaptic D1 and D2 class receptors, which jointly control motor and reward behaviors.

Results

Generation of DA D3 Receptor Mutant Mice

A fragment of the mouse D3 receptor gene was cloned by the use of oligonucleotide primers with sequences chosen from the rat D3 receptor gene sequence (Sokoloff et al., 1990). A 330 base pair DNA fragment, judged to be part of the mouse D3 receptor gene sequence (data not shown), was chosen to perform Southern blot analysis of mouse genomic DNA. The results showed that the D3 receptor is encoded by a single gene in the mouse genome (data not shown). We screened a genomic library (strain 129) with the D3 gene probe in order to obtain the first exon of the mouse D3 receptor gene and its flanking sequences. Figure 1A shows a restriction map of the mouse D3 receptor gene.

To inactivate the D3 receptor gene, we designed a targeting construct to delete the entire first exon of the D3 gene coding sequence and to replace it with a neo gene that encodes a selectable marker for G418 resistance (Figure 1A). Thirty embryonic stem (ES) cell clones containing the intended homologous recombination were identified, and four of them were amplified

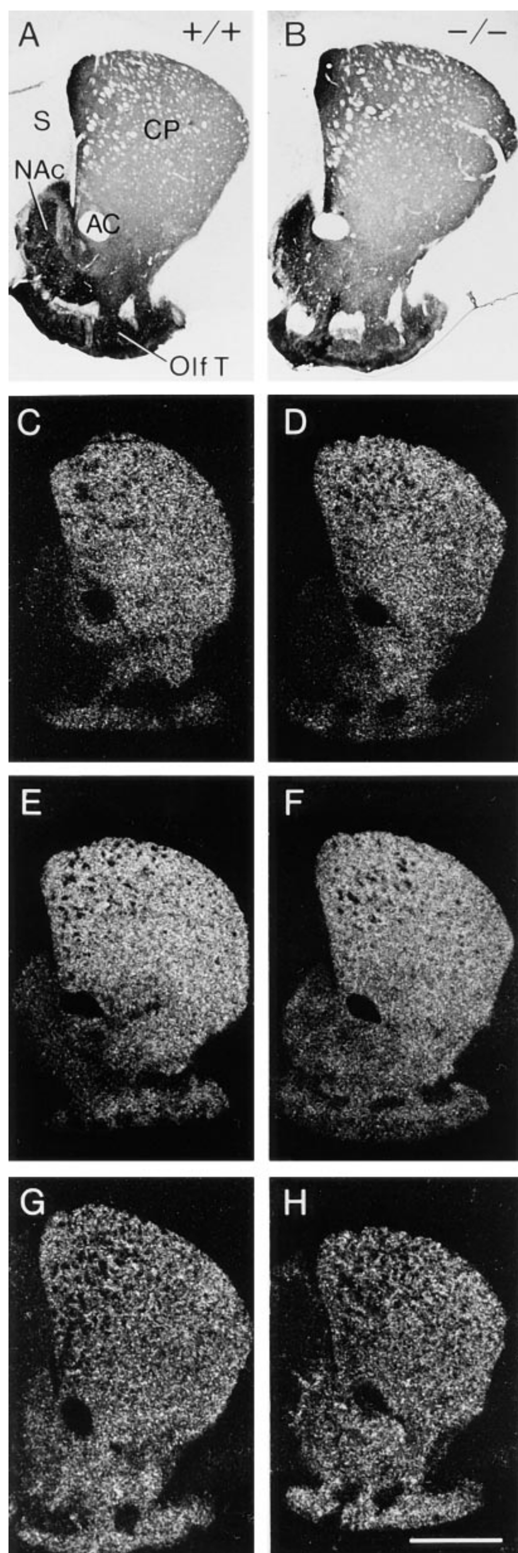

Figure 2. Immunostaining and Ligand-Binding Markers for the DA-Containing Innervation of the Striatum

Left (A, C, E, and G): transverse sections through the striatum of wild-type (+/+) mice. Right (B, D, F, and H): matched levels through the striatum of D3 receptor mutants (−/−). (A) and (B) show tyrosine hydroxylase immunoreactivity. (C) and (D) illustrate [^{3}H]mazindol binding for DA uptake sites. (E) and (F) show D1 receptor binding with [^{3}H]SCH23390. (G) and (H) illustrate D2 receptor binding with [^{3}H]spiroperidol. CP, caudoputamen; NAc, nucleus accumbens; AC, anterior commisure; Olf T, olfactory tubercle; S, septum. Scale bar indicates 1 mm.

and used to generate male chimeric mice. Extensive breeding of these mice with C57BL/6 females was carried out in order to obtain mice homozygous for D3 gene mutation.

The deletion was confirmed by genomic Southern analysis (Figure 1B). In addition, ligand binding was carried out with a D3 and D2 receptor-selective compound, [^{125}I]iodosulpride, in the presence of a D2-selective displacer, domperidone. Autoradiographic analysis (Figure 1C) showed that there were abundant D3-binding sites in the wild-type brains. The highest density of D3 receptor expression was found in the islands of Calleja and to a lesser extent the nucleus accumbens, and substantial binding occurred in other main limbic system sites, as originally reported by Sokoloff et al. (1990). Some D3 binding also occurred in the caudoputamen, with heightened expression in ventral striosomes, as reported for the human (Murray et al., 1994). D3-binding sites were undetectable in the D3 mutants (Figure 1D). These data confirmed that the removal of the critical part of the D3 receptor gene made the expression of the DA D3 receptor completely absent in the mutant mice.

The D3 receptor mutants appeared healthy and had no gross physical abnormalities. The mutant mice were fertile, their litter sizes were normal, and there was no obvious sex bias in their offspring. For all the subsequent studies, male D3 mutant mice were used, with their male wild-type littermates as controls. All the mice ranged from 9–16 weeks of age at the time of study.

The Brain DA System Appears Normal in the D3 Receptor Mutant Mice

To investigate the effect of D3 gene mutation on the development and maintenance of the DA system, ligand binding and immunostaining experiments were performed. Despite the absence of D3 ligand binding, the main DA-containing systems of the brain were preserved in the mutant mice, as judged by immunostaining for tyrosine hydroxylase (TH), the synthetic enzyme for catecholamines (Figures 2A and 2B), and by autoradiographic [^{3}H]mazindol ligand binding for the DA transporter (Figures 2C and 2D). There were no evident differences between the mutant and the wild-type mice. To test whether the deletion of the DA D3 receptor altered the expression of D1 class receptors or other D2 class receptors, we carried out ligand binding with the selective D1 receptor antagonist, [^{3}H]SCH23390 (Figures 2E and 2F) and the selective D2 receptor antagonist, [^{3}H]spiroperidol (Figures 2G and 2H). The results demonstrated that both D1- and D2-binding sites are present in the dorsal and ventral striatum of the D3 mutant mice and that the distributions and the densities of both binding sites in the mutants and controls are qualitatively and quantitatively similar (Table 1). Therefore, despite the absence of the D3 receptors during the development of the mutants, we found no detectable changes in their mesostriatal DA systems.

Table 1. Density of D1 Class and D2 Class Dopamine Receptor Ligand-Binding in the Striatum of D3 Mutant and Wild-Type Mice

			Caudoputamen		
		Nucleus Accumbens	Rostral	Middle	Caudal
[^{3}H]SCH23390	Wild Type	17.0 ± 1.0	46.5 ± 1.3	44.6 ± 0.8	39.7 ± 0.9
(D1 Class Receptor)	D3 Mutant	18.3 ± 0.9	49.8 ± 1.7	46.4 ± 1.4	42.5 ± 1.2
[^{3}H]spiroperidol	Wild Type	9.4 ± 0.4	9.6 ± 0.5	12.7 ± 0.6	11.7 ± 0.6
(D2 Class Receptor)	D3 Mutant	10.1 ± 0.5	11.1 ± 0.8	13.3 ± 0.8	13.3 ± 0.7

Values represent calculated amounts of ligand bound. Densitometry was performed on autoradiograms of brain sections and tritiated standards, and optical densities were converted to nCi/mg brain tissue equivalent (mean ± SEM) by reference to the standards.

DA D3 Receptor Mutant Mice Exhibit Enhanced Locomotor Responses to a Novel Environment

To evaluate the role of D3 receptors in modulating baseline motor activity, we determined the activity of both the mutant and the wild-type mice in our automated locomotor activity chambers. Although there was no significant difference between the two groups of mice when the entire 30 min period was analyzed, examination of the time course of activity indicated that the D3 mutants were significantly more active during the first 5 min of the test (Figure 3A; $p < 0.01$, Dunnett's test). This suggested that D3 mutants may be more responsive to a novel environment. We next determined whether repeated exposure to the test environment would reduce the initial period of greater activity in the D3 mutant mice. Groups of 12 mutant and wild-type mice were tested for locomotor activity on five occasions, separated by 7 days. The heightened response of the D3 mutant mice during the first 5 min period of the initial test was completely absent in all subsequent tests, suggesting that the behavior was elicited in response to the novel conditions of the test apparatus and exhibited rapid habituation both within and between sessions (Figure 3B; $p < 0.05$, Dunnett's test).

One possible explanation for the transiently enhanced behavioral responsiveness to a novel environment by the D3 mutants is that the D3 receptor mutation altered the anxiety state of the mice. We therefore tested mutants and wild-types in an "elevated plus" maze, which scores exploration of the two open arms of the maze to indicate reduced anxiety and time spent in enclosed arms of the maze to indicate heightened anxiety (Lister, 1987; Dawson and Tricklebank, 1995). In this behavioral model, which used a 5 min test duration, D3 mutant mice were again more active than the wild-type mice, as indicated by a greater number of total arm entries (Table 2). This effect bordered on statistical significance [$F(1,11) = 4.27$, $p = 0.06$]. There were no significant differences between the mice with respect to the number of entries into or the amount of time spent within the open or closed arms of the maze. There were only trends toward greater preference for the closed arms (Table 2). We thus conclude that alterations in anxiety state in the D3 receptor mutant mice were unlikely to be related to the greater activity displayed in novel environments.

The D3 Receptor Mutation Causes Enhanced Locomotor Activation in Response to Combinations of D1 and D2 Class Receptor Agonists

In rodents, stimulation of both D1 and D2 class receptors is required for eliciting responses to drugs that enhance synaptic concentrations of DA, such as cocaine and amphetamine, which produce unconditioned behavioral effects, including enhanced locomotor activity, sniffing, licking, and biting (Waddington and Daly, 1993). This requirement is most obviously observed when selective agonists for D1 and D2 class receptors are tested in animals acutely depleted of DA such that endogenous activation of DA receptors is abolished (Jackson and Hashizume, 1986; Clark and White, 1987; Walters et al., 1987; White et al., 1988). Recent evidence indicates that the brain region involved in mediating these unconditioned behaviors, the striatum, contains neurons that coexpress different combinations of D1 and D2 class receptors, including D3 receptors (Surmeier et al., 1992; Le Moine and Bloch, 1996). In order to help identify the role of D3 receptors in unconditioned behaviors, we compared the effects of simultaneous administration of D1 class receptor-selective and D2 class receptor-selective agonists in D3 mutant mice and in wild-type mice.

Groups of mutant and wild-type mice received injections of saline, the D1 class agonist SKF 81297 (3.0 mg/kg), the D2 class agonist PD 128907 (1.5 mg/kg), or the combination of these two drugs, with each injection separated by 7 days. The putative D3 receptor-selective agonist PD 128907 suppressed locomotor activity equally well in mutant and wild-type mice, indicating that this effect results from non-D3, D2 class receptors, namely D2 or D4 (Figure 4). In contrast to PD 128907, SKF 81297 produced a marked hyperactivity that was also identical in the two groups of mice (Figure 4). When the two agonists were coadministered, locomotor activity was also increased, albeit less so than when SKF 81297 was administered alone. In this protocol, D3 mutant mice were more active than wild-type mice ($p < 0.05$, Dunnett's test). This finding suggests that during coactivation of D1 and D2 class receptors, stimulation of D3 receptors by PD 128907 in the wild-type mice caused a suppression of locomotion as compared to the mutant mice.

Following an additional 7 day period, the same mice were pretreated with reserpine to disrupt vesicular stores of DA and thereby to deplete acutely DA-synthesizing neurons of the transmitter. To avoid possible DA receptor supersensitivity and disappearance of D1:D2 class receptor interactions, we used a 4 hr pretreatment protocol in which over 95% of tissue DA was depleted (data not shown). After reserpine treatments, mice were again tested with the combination of SKF 81297 and PD 128907. Reserpine abolished all activity, producing akinesia. As in the nonreserpinized condition, locomotor activation produced by the costimulation of D1 and D2 class receptors was significantly greater than with either

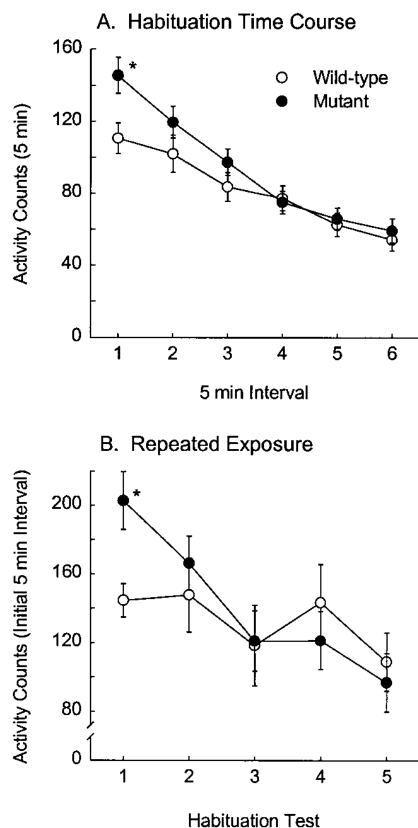

Figure 3. Baseline Motor Activity of the D3 Receptor Mutant Mice in a Novel Environment

The locomotor activity of (A) D3 mutants (n = 62) and wild-type (n = 63) mice during the first 30 min of exposure to the testing environment and (B) a separate set of mutant and wild-type mice (n = 12 each) tested repeatedly for responses to the activity chambers. Data points represent mean ± SEM.

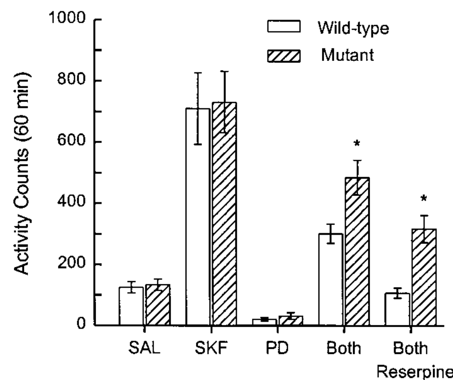

Figure 4. Effects of D1 and D2 Class Receptor-Selective Agonists on Locomotor Activity in D3 Mutant and Wild-Type Mice

Mutant and wild-type mice (n = 8) received injections of saline, PD 128907, SKF 81297, and the combination of these two drugs. Data from the saline test in reserpinized mice are not shown because the mice were completely immobile, and thus the results are not visible on this scale (mutant = 1.23 ± 0.15 and wild-type = 0.96 ± 0.85 counts). All bars represent mean ± SEM.

drug alone (Figure 4; $p < 0.05$, Dunnett's test) in the mutants than in the wild-type mice. The latter effect was confirmed in separate groups of reserpinized mutant and wild-type mice (n = 6 each) tested with the combination of SKF 81297 (1.0 mg/kg) and 0.5 mg/kg quinpirole, another D2 class agonist (502 ± 57 counts for wild types versus 730 ± 123 counts for mutants; $p < 0.05$, Dunnett's test). In the quinpirole experiment, as in many previously published studies (Clark and White, 1987; Jackson et al., 1988), we also demonstrated that neither agonist alone produced motor stimulation in reserpinized mice, whether mutant or wild type (total counts for all conditions were below 70). These findings confirm that combined stimulation of postsynaptic D1 class and D2 class receptors is required for motor activity and suggest that concomitant D3 receptor stimulation (by PD 128907 or quinpirole in the wild-type mice) reduces the normal cooperative effects of D1 class and D2 (or D4) receptors on locomotor activity, as indicated by the greater motor activity in mice lacking the D3 receptor.

D3 Receptor Mutant Mice Exhibit Greater Hyperactivity to Low But Not High Doses of Cocaine

If postsynaptic D3 receptors suppress locomotor activity when it is induced by simultaneous activation of D1 and D2 class receptors, we would then expect that enhancing synaptic concentrations of DA, and thus stimulating all DA receptor subtypes, might expose significant differences between the D3 mutant and the wild-type mice. Considerable evidence indicates involvement of both D1 and D2 class receptors in the locomotor stimulant effects of cocaine (Cabib et al., 1991; Tella, 1994), which increases synaptic DA levels by preventing DA reuptake into DA nerve terminals. Using a randomized design, we tested groups of mutant and wild-type mice with saline and four doses of cocaine. Cocaine elicited dose-dependent increases in locomotor activity in both groups of mice [Figure 5; $F(1,4) = 6.6$, $p < 0.001$]. However, the effects of the two lowest doses of cocaine were considerably greater in the D3 mutant mice at the 5.0 mg/kg dose ($p < 0.05$, Dunnett's test) and bordered on significance at the 10 mg/kg dose ($p < 0.10$, Dunnett's test). This finding is consistent with a normal dampening effect of D3 receptors on motor activity produced by concurrent stimulation of D1 and D2 class

Table 2. Performance of D3 Receptor Mutant Mice in the Elevated Plus Maze

Group	Open Arm Entries	Closed Arm Entries	Time in Open Arms	Time in Closed Arms
Wild Type	4.33 ± 0.96	8.33 ± 1.77	75.0 ± 29.75	135.33 ± 31.74
Mutant	4.86 ± 0.83	12.0 ± 1.23	46.57 ± 18.08	163.86 ± 16.31

Mutant (n = 7) and wild-type (n = 6) mice were tested in an elevated plus maze and were scored for number of entries into and the time spent within the open and closed arms. All values represent mean ± SEM.

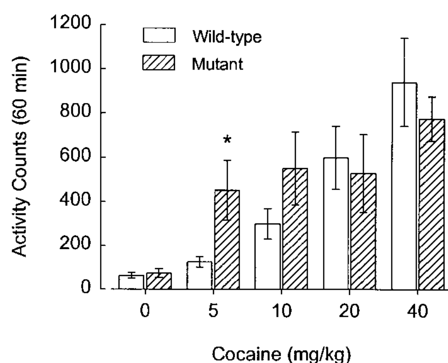

Figure 5. Effects of Cocaine on Motor Activity of D3 Receptor Mutant ($n = 9$) and Wild-Type ($n = 10$) Mice
Each bar represents the mean ± SEM.

receptors but also suggests that such a dampening effect can be overcome with sufficient stimulation of D1 and other D2 class receptors by synaptic DA.

D3 Receptor Mutant Mice Exhibit Increased Sensitivity to Amphetamine in the Conditioned Cue Preference Paradigm

To investigate the role of D3 receptor in mediating the positive reinforcing effects of psychostimulants, we tested mutant and wild-type mice in a CCP paradigm with amphetamine as the reinforcing drug. Amphetamine is an indirect DA receptor agonist that is self-administered by both humans and animals (Pickens and Thompson, 1971; Le Moal and Simon, 1991). The CCP paradigm exploits the natural tendency of mammals to form conditioned approach or escape responses to neutral cues in the presence of rewarding or aversive events (White et al., 1987; Carr et al., 1989). This paradigm has been used widely to study the neurobiological basis of behavioral changes elicited by amphetamine (Reicher and Holman, 1977; Mackey and van der Kooy, 1985; Carr et al., 1988; Bechara and van der Kooy, 1989; Hiroi and White, 1991a, 1991b; Beninger, 1992; Markou et al., 1993). The amphetamine CCP is well documented with both inbred and outbred strains of rats (Schechter and Calcagnetti, 1993). Moreover, Laviola et al. (1994) used an outbred strain of mice, CD-1, and demonstrated it could acquire CCP readily over a wide range of amphetamine doses. We replicated this result in preliminary experiments (data not shown).

As shown in Figure 6, the D3 mutants exhibited significant preferences for the test compartment paired with amphetamine at the 0.1–5.0 mg/kg dose range during the entire 20 min test session. By contrast, the wild-type mice exhibited significant preferences for the test compartment only at doses higher than 0.5 mg/kg. Furthermore, during the first 2 min of the test session, the mutant mice showed significant preferences at the highest dose of amphetamine (5 mg/kg) and exhibited a tendency toward preference at other doses (0.04–0.5 mg/kg). No such tendency or preference was exhibited by the wild-type mice during the first 2 min. An analysis of variance (ANOVA) showed a significant interaction between dose and compartment [$F(4,76) = 3.86$, $p < 0.01$] and a significant main effect of compartment [$F(1,76) = 26.39$, $p < 0.001$] for the full 20 min test. For the mutant mice, least significant difference (LSD) tests indicated significant preferences at the three highest doses: 0.1 mg/kg [$t(7) = 2.05$]; 0.5 mg/kg [$t(7) = 2.95$, $p < 0.03$]; 5.0 mg/kg [$t(7) = 3.38$, $p < 0.005$]. For the wild-type mice, LSD tests indicated significant preferences at 0.5 mg/kg [$t(7) = 2.87$, $p < 0.03$] and 5.0 mg/kg [$t(7) = 2.88$, $p < 0.03$]. The ANOVA computed on the data for the first 2 min showed significant interactions between group and compartment [$F(1,76) = 5.48$, $p < 0.03$] and significant main effects of dose [$F(4,76) = 2.56$, $p < 0.05$] and compartment [$F(1,76) = 5.20$, $p < 0.03$]. For the mutant mice, only those tested with 5 mg/kg of d-amphetamine showed a significant preference for the paired compartment [$t(7) = 2.30$, $p < 0.05$]. For the wild-type mice, none of the doses produced a significant preference during the first 2 min of the test session.

Synergistic Electrophysiological Effects of D1 and D2 Class Agonists on Ventral Striatal Neurons Are Not Altered in D3 Receptor Mutant Mice

Extracellular single-cell recordings from striatal neurons have demonstrated that both D1 and D2 class agonists can suppress both spontaneous and glutamate-evoked firing. In addition, coadministration of D1 and D2 class agonists produces an inhibition that is synergistic in nature that is greater than the additive effects of the two agonists given alone (White and Hu, 1993). We have previously demonstrated similar interactions in the mouse nucleus accumbens (Xu et al., 1994b), in which D3 receptors are densely expressed. To determine whether the D3 receptor mutation altered the synergistic electrophysiological effects mediated by simultaneous activation of D1 and D2 class receptors, we compared the capacity of various D1 and D2 class agonists, administered alone and in combination, to suppress the firing of nucleus accumbens neurons in the D3 receptor mutant and wild-type mice.

PD 128907 and quinpirole inhibited glutamate-induced activation of nucleus accumbens neurons to a nearly identical extent in the two groups of mice (Figure 7). When PD 128907 was coadministered with an inactive iontophoretic current of SKF 81297 (98% ± 3% of control firing rate), the D1 class agonist markedly potentiated the inhibitory effects of PD 128907 and did so equally well in both the mutant and wild-type mice (Figure 7B). These findings suggest that the enhanced locomotor stimulation observed when D3 mutant mice are tested with combinations of D1 and D2 class agonists may not be mediated by alterations at the level of single neurons within the nucleus accumbens.

Discussion

To explore the functions of the DA D3 receptors and the underlying mechanisms, we generated mice lacking this receptor. Our genomic Southern analysis and ligand-binding experiments demonstrated that the D3 receptor gene was successfully inactivated and that the

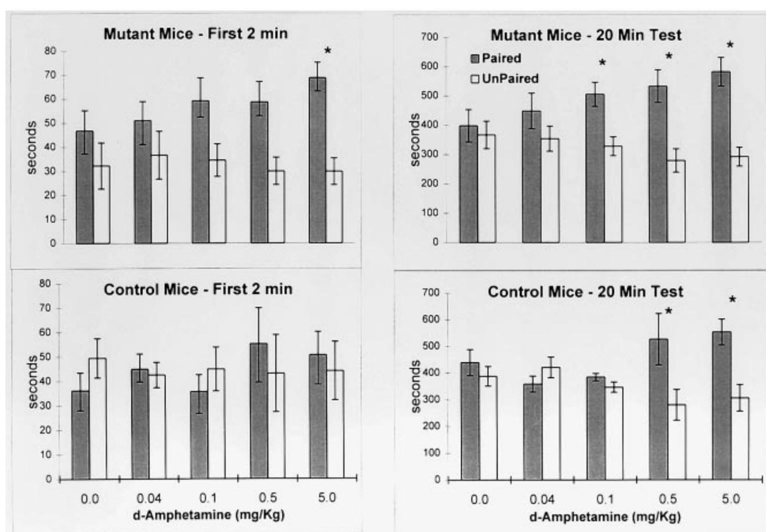

Figure 6. Effects of D-Amphetamine Sulfate across a Range of Doses on Conditioned Cue Preference in D3 Receptor Mutant and Control Mice

Each bar represents the mean amount of time spent by a group of mice (n = 8) in the compartment paired with drug (black) and the compartment paired with saline (white) during the first 2 min (left panels) and the full 20 min (right panels) of the tests. The error bars indicate SEM. Asterisks indicate significant preferences for the drug-paired compartment.

expression of the D3 receptor was abolished in the mutant mice. Because the inactivation of the D3 receptor gene could lead to developmental changes that could complicate our interpretations regarding its function, we carefully screened the brain DA system with neurochemical markers. Our analysis indicates that in the brains of the D3 mutants, D1 class and D2 class DA receptor ligand-binding sites are expressed in normal patterns, as are binding sites for the DA transporter. Moreover, we found no abnormality in TH immunostaining patterns. These results indicate that the dopaminergic components of the brain can develop and persist in the absence of D3 receptor function with apparently normal anatomical distributions. Our behavioral analysis demonstrates that the D3 mutant mice exhibit increased behavioral sensitivity to concurrent stimulation of D1 and D2 DA receptors, increased sensitivity to low-dose positively rewarding stimuli, and heightened locomotor activity in response to novel environments.

D3 Receptors Normally Dampen Locomotor Behavior Induced by Combined Stimulation of D1 and D2 Class Receptors

The motor stimulant effects observed when DA neurotransmission is increased require stimulation of both D1 and D2 class receptors, which can interact at the single-cell level or at the systems level in circuits including the nucleus accumbens and cortico-basal ganglia function (Waddington and Daly, 1993; White et al., 1993). Our findings demonstrate that mice lacking the DA D3 receptor are more active than wild-type mice when both D1 and D2 class receptors are stimulated either by combinations of selective D1 and D2 class agonists or by the DA uptake inhibitor cocaine but not when either class of receptor is activated alone. Thus, in normal mice, the D3 receptor can limit the expression of motor behavior mediated by cooperative activation of D1 and D2 class receptors. Interestingly, as the dose of cocaine increased, the differences between the mutant and wild-type mice disappeared. This suggests that the dampening effect of D3 receptor stimulation on motor behavior can be overcome with sufficient stimulation of other D1 and D2 class receptors by DA. It is also possible that enhanced activation of serotonin and norepinephrine receptors produced by cocaine may have influenced the behavior.

The fact that such differences between the D3 mutant

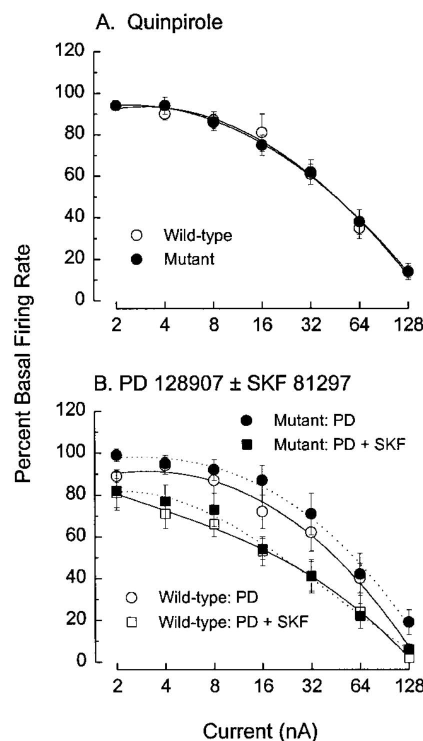

Figure 7. Effects of D1 and D2 Class Receptor-Selective Agonists on the Activity of Nucleus Accumbens Neurons

(A) Mutant (n = 12) and wild-type (n = 14) mice were injected with quinpirole.

(B) Mutant (n = 15) and wild-type (n = 12) mice were injected with the D2 class receptor agonist PD 128907 (PD). These mice were also coinjected with SKF 81297 (SKF) at a low iontophoretic current (4 nA), which by itself did not alter firing. All points represent mean ± SEM.

and wild-type mice were also observed in DA-depleted mice indicates that the D3 receptors relevant to such damping activity are likely to be postsynaptic, as autoreceptor activation would not be able to reduce DA release when there is no DA to be released. Moreover, the finding of similar behaviors in both reserpinized and nonreserpinized mice favors the view that D3 receptors are not massively occupied by endogenous DA under normal conditions (Schotte et al., 1996).

In normal rats and mice, concurrent administration of D1 and D2 class receptor agonists produces synergistic inhibition of normal activity in the nucleus accumbens (White and Wang, 1986; Hu and Wang, 1988; Hu and White, 1994; Xu et al., 1994b). Our results clearly implicate D2 (or D4) receptors, rather than D3 receptors, in this synergistic inhibitory effect, as both quinpirole and PD 128907 suppressed the firing of nucleus accumbens neurons equally well in D3 mutant and wild-type mice. Our studies also raise the possibility that the dampening effect of D3 receptors on combined D1 and D2 class receptor-mediated motor activity may not be paralleled at the single-cell level, because the effects of combined administration of SKF 81297 and PD 128907 were identical in D3 mutant and wild-type mice. We cannot be certain that our sample of neurons included those that expressed the D3 receptor, but over 80% of the neurons recorded were within the anterior ventromedial shell region, in which 40%–46% of the neurons express the D3 receptor mRNA. The D3 receptor responsible for dampening D1:D2 motor behavior may exist on a separate population of neurons that modulate those exhibiting synergism between D1 and D2 receptors. Obvious candidates for the latter role are neurons from the ventral pallidum area, which receives massive inputs from the nucleus accumbens (Zahm and Brog, 1992) and which expresses D3 receptors (Bouthenet et al., 1991; Diaz et al., 1995).

DA D3 Receptor and Reward-Related Behavior

D3 receptors are highly expressed in the terminal sites of the mesolimbic dopaminergic pathway, which is centrally involved in reward-related activities including addictive responses to psychostimulants (Self and Nestler, 1995; Hyman, 1996; Koob, 1996). We asked whether mutation of the D3 receptor in mice would produce changes in behavioral responsiveness in the CCP paradigm thought to measure reward-related behavior. Even though various CCP paradigms have been subject to different interpretations (Carr et al., 1989), the use of unbiased procedures in the present experiment is likely to exclude interpretations for the observed preference other than those based on a rewarding or positive affective property of amphetamine.

Significant place preferences were observed in the mutant mice at 0.1 mg/kg amphetamine for the full 20 min of testing, whereas no preferences were seen in the control groups at this low dose. Furthermore, the mutant mice showed significant place preferences at the highest dose of amphetamine (5 mg/kg) during the first 2 min of the test session, and they exhibited a strong tendency toward preferences at other doses (0.04–0.5 mg/kg). There was no indication of a preference in the wild-type mice during the first 2 min of the test sessions. To our knowledge, no significant CCP has been reported for amphetamine at a dose as low as 0.1 mg/kg, and no CCP has been reported for this or any other drug during a test as short as 2 min. The dose–response relationship for the behavior of the mutant mice during the 20 min test showed a monotonic increase in the size of the preference with increasing dose. By contrast, the control group exhibited no sign of a preference at doses up to and including 0.1 mg/kg of amphetamine. At the next higher dose (0.5 mg/kg), the wild-type mice did show a large preference, similar in amplitude to those of both the mutants and controls at the two highest doses we administered.

The existence of an abnormally strong conditioning effect in the D3 mutants suggests that in wild-type mice, there is a mechanism involving the D3 receptor that inhibits the expression of CCP at low doses of amphetamine. This could reflect D3 receptor-mediated inhibition of D1 and D2 receptor coactivation, which could be overcome with sufficient stimulation of other D1 and D2 class receptors by DA, as discussed above for cocaine-induced locomotion. Other mechanisms could result in increased sensitivity to amphetamine in the CCP paradigm as well. Regardless, our data suggest that functional expression of D3 receptor is involved in regulating behavioral responsiveness to the rewarding action of amphetamine and that D3 receptor-linked mechanisms can either attenuate positive effects or disrupt the conditioning process whereby the neutral cues in the conditioning environment become associated with this effect (White and Carr, 1985; White and Milner, 1992).

D3 Receptor Mutant Mice Exhibit Greater Locomotor Activity Upon Exposure to a Novel Environment

Accili et al. (1996) reported that a targeted mutation of the D3 receptor gene is associated with motor hyperactivity in mice. Our results indicate that such an effect is transient, occurring immediately after the exposure to the test chamber, but habituating rapidly so that it is no longer evident when the mutant mice are repeatedly tested. Accordingly, we interpret the findings on increased locomotor activity as indicating that the D3 receptor mutation is not associated with hyperactivity per se but is associated with an enhanced responsiveness to a novel environment. This effect does not appear to be related to the altered anxiety state of the mutant mice, because in the elevated plus maze, an accepted rodent test of anxiety, the D3 mutant mice did not exhibit greater preference for open or closed arms of the maze. In fact, the results of this test also suggested a greater locomotor activity of the D3 mutants in a novel environment, in that the total number of arm entries increased but not the time spent within the arms. Consequently, the enhanced responsiveness to novel environments is likely to result from other altered processes. One intriguing possibility is that the loss of D3 receptors in olfactory tubercle and islands of Calleja compromises olfactory processes critical to the exploration of a new environment.

Conclusions

Our experiments indicate that mice lacking the D3 receptor exhibit behavioral differences from wild-type mice in their motor activity and their responses to the rewarding properties of amphetamine. We propose that one possible mechanism of D3 receptor function is to modulate behaviors by inhibiting the cooperative effects of postsynaptic D1 and other D2 class receptors. This inhibitory effect can be overcome with sufficient levels of synaptic DA or by the influence of other monoamines. The modulating property of the D3 receptors is likely to occur at a systems level as opposed to a cellular level, as our results show that synergistic electrophysiological effects of D1 and D2 class agonists on single neurons in the nucleus accumbens are not changed. In related work, we have found that the D3 mutant mice exhibit increased basal DA release (Koeltzow et al., submitted). Such a changed baseline of DA availability could also contribute to the effects we observed. Finally, it is important to point out that we used mice with a heterogeneous genetic background (129/SvxC57BL/6) in this work. In the future, to avoid possible contributions from genetic polymorphism, mice with an identical genetic background should be used for such behavioral studies. Nevertheless, our findings firmly place the DA D3 receptor as a key modulator of motor and reward-related behavior.

Experimental Procedures

D3 Receptor Gene, Targeting Construct, and ES Homologous Recombinants

PCR reactions were performed with two oligonucleotide primers and DNA isolated from mouse D3 ES cells. The primer sequences were: 5′-CGCGTTCCTCTGTGTGGGCCATG and 5′-CCAAGTACAC CACCCACGGCATC. The resulting PCR product containing sequence from the mouse D3 receptor gene was used to clone part of this gene from a mouse 129 genomic library.

To generate a D3 gene-targeting construct, four piece DNA ligation was performed with the following DNA fragments: a 3.7 kb EcoRI fragment containing DNA mostly from 5′ of the D3 receptor gene, a 1.8 kb fragment containing a neo gene driven by a PGK promoter, a 4.6 kb XbaI fragment containing DNA from the 3′ of the first exon of D3 gene, and the plasmid pBluescript from Stratagene.

Mouse D3 ES cells were transfected by electroporation with 50 μg of the linearized targeting construct (Bio-Rad Gene Pulser, 800 V, 3 μF). One day later, G418 selection was applied at 200 μg/ml, and 6–8 days later, G418-resistant stable transfectants were isolated. Genomic DNA from the transfectants was isolated, digested with NcoI, and then was hybridized with a probe. Candidate homologous recombinants identified by hybridization were tested further by digesting their genomic DNA with KpnI and hybridizing with a 3′ probe isolated from the DNA sequence just 5′ of the D3 receptor gene.

DA D3 Receptor Mutant Mice

To generate chimeric mice (Bradley, 1987; Xu et al., 1994a), ES homologous recombinants were injected into blastocysts isolated from female C57BL/6 mice. The injected blastocysts were implanted into the uteri of B6xDBA2 F1 females. The resulting male chimeric offspring were then bred repeatedly with C57BL/6 females, with screening for germ-line transmission by identification of agouti offspring. Confirmation of genetic transmission to identify mice heterozygous for the D3 mutation was accomplished by genomic Southern analyses of tail DNA. Heterozygous mutants were then crossed to generate mice homozygous for D3 receptor gene mutation, which were identified by Southern blotting of tail DNA. Breeding was carried out in the Massachusetts Institute of Technology animal facility.

For the motor behavior and the electrophysiological experiments, D3 mutant and control mice were transported to the Chicago Medical School. All mice were allowed 7–8 days to acclimate to the new surroundings prior to experimental testing. Mutant and wild-type mice were housed separately in groups of three to four with food and water available ad libitum in a temperature- and humidity-controlled room with a 12 hr light/dark cycle. For the CCP experiment, 40 D3 receptor mutants and 46 controls were shipped to McGill University. Upon arrival, all mice were housed in single plastic cages in a temperature-controlled room with the lights on from 7 a.m. to 7 p.m. All mice weighed approximately 30–45 g at the beginning of the experiments.

Ligand-Binding Autoradiography

Brains from seven mutant and eight wild-type F1 (129/SvxC57BL/6) mice were used. All mice were euthanized by decapitation. The brains were removed from the skulls, frozen, and stored at -80°C, and cut into 10 μm coronal sections. Thaw-mounted sections were stored at -20°C for a minimum of 2 days.

DA D3 receptor binding was carried out according to Landwehrmeyer et al. (1993). Sections were washed twice and were incubated for 30 min at RT with 0.1 nM of [^{125}I]iodosulpride (2,000 Ci/mmol, Amersham). Domperidone was added as a D2 receptor displacer in all incubations. D1 receptor binding was carried out according to Xu et al. (1994a). The incubations were carried out with 2.5 nM [^{3}H]SCH23390 (73 Ci/mmol, DuPont NEN) in 50 mM Tris-HCl buffer (pH 7.4) containing 10 μM mianserin to block serotonin receptor-binding sites. D2 receptor binding was carried out as described by Xu et al. (1994b) with 0.8 nM [^{3}H]spirosperidol (19 Ci/mmol, DuPont NEN) for 45 min at RT. Labeling of DA transporter-binding sites was carried out according to Graybiel and Moratalla (1989) with 15 nM [^{3}H]mazindol (DuPont NEN, 19 Ci/mmol) in 0.3 μM desimipramine to block the norepinephrine transporter. Sections were incubated for 40 min at RT. After incubation, sections were rinsed and dried.

For autoradiography, sections were apposed to Hyperfilm (Amersham) together with tritium standards ([^{3}H] Micro-scales, Amersham) to tritium-sensitive films (Hyperfilm, Amersham) for $\sim$4 weeks for [^{125}I]iodosulpride, $\sim$3 weeks for [^{3}H]SCH23390, $\sim$8 weeks for [^{3}H]spiroperidol, and $\sim$2 weeks for [^{3}H]mazindol. Films were developed in D-19 (Kodak).

Immunohistochemistry

Nine control and 9 mutant F1 mice were processed as described in Xu et al. (1994a) with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). Brains were briefly postfixed, cryoprotected, and cut at 20 μm on a sliding microtome. Free-floating sections were pretreated consecutively with 3% H_2O_2 in PBS containing 2% Triton X-100 (PBS-TX) for 10 min and with 5% normal goat serum (NGS) for 30 min, rinsed in PBS-TX, and incubated with polyclonal rabbit anti-TH (1:1000, Eugene Tech International, Ridgefield Park, NJ) for 24–72 hr at 4°C. Sections were then processed with ABC kits (Vector Laboratories) and were then developed with 0.05% diaminobenzidine (DAB) containing 0.02 M sodium cacodylate, 0.1 N acetic acid, and 0.002% H_2O_2.

Motor Behavioral and Anxiety Test Procedures

Locomotor activity experiments were conducted during the light portion of the light/dark cycle using previously described procedures (Xu et al., 1994b). Prior to behavioral testing, F2 animals were allowed to habituate to the testing environment for at least 30 min except when noted. Tests were generally conducted for a period of 1 hr. Drugs were administered intraperitoneally except where noted in volumes of 1 ml/100 mg immediately following the habituation period. All drugs were dissolved in saline with the exception of reserpine, which was dissolved in glacial acetic acid and administered with distilled water as vehicle.

A nonrandomized repeated measures design was employed to assess the effects of selective and combined stimulation of D1 and D2 class receptors. Following habituation and saline tests, F2 mice were tested once a week for 1 hr. For the first test, each mouse received SKF 81297. For the second and third tests, mice received PD 128907 and a cocktail of SKF (3.0 mg/kg) plus PD 128907 (1.5 mg/kg), respectively. For the fourth test, mice were reserpinized (5

mg/kg) 4–6 hrs prior to challenge with the same SKF 81297/PD 128907 cocktail.

Dose–response curves for cocaine (5.0, 10.0, 20.0, 40 mg/kg) were generated using experimenter-blind, repeated measures designs. In each experiment, saline was injected on the first day, and locomotor activity was assessed to establish baseline levels. On subsequent test days, spaced 1 week apart, each animal was randomly exposed to each challenge dose until each subject had been assessed with all doses. Equal numbers of F1 and F2 mice were used in this experiment.

To assess anxiety-related behaviors, we used an elevated plus maze constructed of 1/8′′ polypropylene plastic. Each of four arms (10×40 cm) are adjoined by a 10×10 cm intersection. The base of the maze was constructed such that the arms are elevated 30 cm above ground level. The walls of the two enclosed arms extend 15 cm above the base of each arm. At the beginning of the 5 min test, F2 mice were placed in the center of the apparatus facing an open arm. Entries into each arm and the amount of time spent on each arm were recorded manually by experimenters blind to conditions. An arm entry was recorded whenever an animal placed all four paws within a particular arm.

Differences between D3 mutant and wild-type mice in motor and anxiety behavioral tests were conducted either with independent t tests (single tests) or repeated measures ANOVA for dose–response determinations. Individual planned comparisons following ANOVAs were conducted with Dunnett's test with $\alpha = 0.05$.

Conditioned Cue Preference Apparatus

The testing apparatus consisted of two large compartments ($19 \times 19 \times 20$ cm) separated by a common wall and the entrances connected by a tunnel ($9 \times 13 \times 20$ cm). Mice could be confined in the large compartments by closing the doors to the tunnel. One large compartment was painted black, with a 1.2 cm grid wire mesh on the floor. The other was painted white, with a 0.6 cm grid wire mesh on the floor. The tunnel was painted gray and had a smooth floor. The entire apparatus was enclosed in a soundproof container ($86 \times 86 \times 1.36$ cm) lit with five incandescent bulbs. In a preliminary test, a group of eight F2 mice tested by the procedure described below with no drug treatment associated with either compartment did not exhibit a significant preference for either compartment.

Conditioned Cue Preference Procedure

All mice (F2) were handled daily for 4 consecutive days. On the fifth day, each mouse was placed in the tunnel and allowed to explore all three compartments freely for 10 min. Then training began. Each training required 2 days. On the first day, by random assignment, half of the mice in each experimental group were confined in the white compartment; the other half were confined in the black compartment for 30 min. On the second day, each mouse was confined to the other compartment for 30 min. These subgroups were further subdivided randomly so that half of the mice in each received a drug injection immediately before confinement on the first day; the other half received a saline injection. The injections were reversed on the second training day. This design, sometimes called the "unbiased CCP paradigm," counterbalanced both the compartment paired with the drug and the injection order within each experimental group. Two mice in each group received each of the four possible treatment combinations, giving a total of eight mice per group. The control group had 14 mice. Counterbalancing was maintained in this group.

No injections were given on the test day. Each mouse was placed into the tunnel and allowed to move freely in the three compartments for 20 min. Event times were recorded whenever the mouse entered or left one of the large compartments (defined as having all four paws in or out of the compartment). The total amount of time each mouse spent in each compartment during each consecutive 2 min period was calculated. Data were processed by ANOVA using group (mutant versus control) and dose as independent factors and compartment (paired versus unpaired) as a repeated measure. The dependent variable was time spent in the paired and unpaired compartments. Two separate analyses, on the time spent in the compartments during the first 2 min and one on total times for the full 20 min session, were computed. LSD tests using the error term from the ANOVAs were used to determine the significance of each treatment.

D-amphetamine sulfate was dissolved in 0.9% NaCl for intraperitoneal injections in concentrations of 5.0, 0.5, 0.1, and 0.04 mg/ml. Control injection solutions contained 0.9% NaCl. Four doses of d-amphetamine were tested: 5.0, 0.5, 0.1, and 0.04 mg/kg. Each dose was tested on different groups of mutant and control mice.

Electrophysiology

Electrophysiological procedures were conducted as detailed previously (Xu et al., 1994b). F2 mice were anesthetized and mounted in a stereotaxic apparatus. The coordinates for recording were: 5.6–5.8 mm anterior (A) to lambda, 0.5–0.9 mm lateral (L) to the midline suture, and 3.6–4.7 mm ventral (V) to the cortical surface. Nucleus accumbens neurons were activated to fire at rates of 4–5 spikes/s by iontophoretic administration of glutamate. Electrical signals were amplified, displayed on an oscilloscope, monitored by an audio amplifier, and led into a window discriminator for detection of individual action potentials. Integrated rate histograms were plotted on-line, while digital counts of action potentials were also obtained for permanent storage. The responses of nucleus accumbens neurons to microiontophoretic administration of drugs were determined by comparing the total number of spikes occurring during administration of the test compound to the basal firing rate. Current–response curves were determined by administering increasing currents (2–128 nA) through the drug barrel. At the end of the experiment, routine histological procedures were used to determine recording sites. All recorded neurons were verified to lie within the established borders of the nucleus accumbens and surrounding ventral striatal regions, including areas densest in D3 receptor mRNA, i.e., the anterior–ventral regions including the shell.

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