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Dopamine-Deficient Mice Are Hypersensitive to Dopamine Receptor Agonists

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Dopamine-deficient (DA−/−) mice were created by targeted inactivation of the tyrosine hydroxylase gene in dopaminergic neurons. The locomotor activity response of these mutants to dopamine D1 or D2 receptor agonists and L-3,4-dihydroxyphenylalanine (L-DOPA) was 3- to 13-fold greater than the response elicited from wild-type mice. The enhanced sensitivity of DA−/− mice to agonists was independent of changes in steady-state levels of dopamine receptors and the presynaptic dopamine transporter as measured by ligand binding. The acute behavioral response of DA−/− mice to a dopamine D1 receptor agonist was correlated with c-fos induction in the striatum, a brain nucleus that receives dense dopaminergic input. Chronic replacement of dopamine to DA−/− mice by repeated L-DOPA administration over 4 d relieved the hypersensitivity of DA−/− mutants in terms of induction of both locomotion and striatal c-fos expression. The results suggest that the chronic presence of dopaminergic neurotransmission is required to dampen the intracellular signaling response of striatal neurons.

Key words: c-Fos; D1 receptor; D2 receptor; caudate putamen; dopamine; dopamine transporter; haloperidol; knock-out mice; L-3,4-dihydroxyphenylalanine (L-DOPA); nucleus accumbens; quinpirole; SCH 23390; SKF 81297; striatum; substantia nigra; tyrosine hydroxylase

The requirement for dopamine secretion from neurons projecting from the midbrain to the corpus striatum for the initiation and organization of motor function has been recognized since the introduction of L-3,4-dihydroxyphenylalanine (L-DOPA) therapy for parkinsonism (Birkmayer and Hornykiewicz, 1961; Hornykiewicz, 1966; Cotzias et al., 1967). Lesioning experiments in rodents and primates using the catecholaminergic neurotoxins, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), or the monaminergic synaptic vesicle depleting agent, reserpine, have confirmed the specific role of dopamine in motor coordination (Fischer and Heller, 1967; Ungerstedt, 1968; Ungerstedt et al., 1974; Goldstein et al., 1975; Langston et al., 1983, 1984). In these neurotransmitter depletion experiments, it was also noted that the long-term removal of dopamine resulted in enhanced sensitivity to the neurotransmitter itself. When rats with unilateral 6-OHDA lesions of nigrostriatal neurons were treated with dopamine receptor agonists, rotational locomotion was induced away from the lesioned side (Ungerstedt and Arbuthnott, 1970; Ungerstedt, 1971a,b). Ungerstedt and coworkers hypothesized that removal of dopaminergic input to one side of the striatum sensitized ipsilateral postsynaptic neurons and led to an imbalanced response to dopaminergic compounds from the two sides of the striatum.

Mutant mice were created in which dopamine production is eliminated in dopaminergic neurons (Zhou and Palmiter, 1995). These DA−/− mice survive embryogenesis and early postnatal life, but by 3–4 weeks of age, they become hypoactive and hypophagic and die without intervention. DA−/− mutants can be maintained as adults with daily L-DOPA treatment. Characterization of dopaminergic sensitivity in these mutants readdresses the hypothesis that dopamine dampens the magnitude of the response to the neurotransmitter itself in three unexplored ways. First, DA−/− mice are deficient in the production of a single neurotransmitter. The specificity of the gene-targeting approach contrasts with previous models in which entire catecholaminergic nerve terminals were destroyed by chemical lesioning, contents of monoaminergic synaptic vesicles were depleted by reserpine administration, or production of both dopamine and norepinephrine were inhibited by α-methyltyrosine treatment (Rech et al., 1966; Moore and Rech, 1967). Second, the complete removal of dopaminergic function throughout the nervous system of DA−/− mice contrasts with the lesioned models in which new dopaminergic nerve terminals can reinnervate the striatum and promote recovery from various behavioral deficiencies (Zigmond and Stricker, 1973; Dravid et al., 1984; Choulli et al., 1987). DA−/− mice provide a stable model to examine the sensitivity to dopaminergic compounds under dopamine-depleted conditions. Third, specific inactivation of dopamine production by gene targeting eliminates dopaminergic function from the time midbrain catecholaminergic markers are normally first expressed at embryonic day 11.5 (Foster et al., 1988). The specific removal of dopamine from developing dopaminergic circuits of the embryonic brain has not been achieved by any method except targeted mutagenesis (Zhou and Palmiter, 1995; Fon et al., 1997; Wang et al., 1997; Zetterström et al., 1997; Castillo et al., 1998; Saucedo-Cardenas et al., 1998). This report addresses how long-term loss of dopamine during development affects expression of compo-

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nents of the dopaminergic neurotransmission machinery and behaviors elicited by dopaminergics in adulthood.

MATERIALS AND METHODS

Behavioral studies. All mice were maintained and used in accordance with guidelines for animal care and experimentation established by the National Institutes of Health and the University of Washington Animal Care Committee. DA−/− mice were created as described (Zhou and Palmiter, 1995). Loss-of-function alleles at the tyrosine hydroxylase (*Th*) gene locus, whose product is rate-limiting for catecholamine biosynthesis, were introduced by gene targeting. TH function was restored in noradrenergic cells of $Th^{-/-}$ mice by introducing the *Th* coding region downstream of the transcriptional regulatory elements of the dopamine β-hydroxylase (*Dbh*) gene locus. DA−/− and wild-type (WT) cage-mate mice used in behavioral experiments were 3- to 12-month-old, C57BL/6 × 129/SvEv hybrids. WT mice included animals that were $Th^{+/+}$, $Th^{+/-}$, $Dbh^{+/+}$, $Dbh^{TH/+}$, and all combinations of these genotypes. Activity was measured in cages (20 × 20 × 40 cm) equipped with four infrared photobeams (San Diego Instruments) that were arrayed 8.8 cm apart along the long axis of the cage walls. Photobeam interruptions were recorded by a computer running PASF software (San Diego Instruments), and only consecutive interruptions of adjacent photobeams were counted as an ambulation. Distance traveled was calculated by multiplying the number of ambulations by the distance between photobeams. Food pellets (Purina, St. Louis, MO; 5015 chow, 11% fat, 4.35 kcal/gm) and tap water were available to the animals *ad libitum*. The light cycle was maintained on a 12 hr light/dark schedule with the lights turning on at 7:00 A.M.

Before locomotor activity was measured, mice were placed in activity cages for 3 d for acclimatization. For acute behavioral studies, adult mice were treated with 0.9% (w/v) saline (10 μl/gm, i.p.), (±)-SKF 81297 (10 μl/gm, i.p.; Research Biochemicals International, Natick, MA) diluted at various concentrations (0.125, 0.5, or 0.75 mg/ml) in 0.9% saline, quinpirole (10 μl/gm, i.p.; Research Biochemicals International) diluted in 0.9% saline at 0.005 or 0.01 mg/ml, or 1.5 mg/ml L-DOPA (33 μl/gm, i.p.; Sigma, St. Louis, MO) diluted in PBS (in mM: 137 NaCl, 3 KCl, 8 Na_2HPO_4, and 2 KH_2PO_4, pH 7.4) and 0.25% (w/v) ascorbic acid. Antagonists that were administered at the same time as L-DOPA included SCH 23390 (10 μl/gm, i.p.; 0.02 mg/ml; Research Biochemicals International) and haloperidol (10 μl/gm, i.p.; 0.06 mg/ml; McNeil Pharmaceutical, Spring House, PA). DA−/− mice used in experiments had been treated with their last 50 mg/kg L-DOPA injection 24 hr before any experiment began, and the mutants were maintained between experimental treatments with daily doses of 50 mg/kg L-DOPA. For chronic behavioral studies, two different groups of adult DA−/− mice were treated with 100 mg/kg L-DOPA (66 μl/gm, i.p.; 1.5 mg/ml) at 11:00 A.M., 3:00 P.M., 7:00 P.M., 11:00 P.M., and 7:00 A.M. for 4 d. For the chronic immediate early gene induction studies, a third group of adult DA−/− mice was treated in the group's home cages with 100 mg/kg L-DOPA (66 μl/gm, i.p.; 1.5 mg/ml) at 7:00 P.M., 11:00 P.M., 7:00 A.M., 11:00 A.M., and 3:00 P.M. for 38 hr. Subsets of these mice were killed by rapid asphyxiation, and brains were dissected at 9:00 P.M., 9:00 A.M., 9:00 P.M. (second day), and 9:00 A.M. (second day) for c-fos immunohistochemistry. For brain and striatal dopamine measurements, another group of adult DA−/− mice was treated in the group's home cages with 100 mg/kg L-DOPA (66 μl/gm, i.p.; 1.5 mg/ml) at 7:00 A.M., 11:00 A.M., 3:00 P.M., 7:00 P.M., and 11:00 P.M. for 4 d.

Brain and striatal dopamine measurements. WT and DA−/− mice were killed, and half-brains or striata were dissected and stored at −70°C. Measurements were performed as described (Thomas et al., 1998).

Dopamine receptor and transporter autoradiography. Mice that had never been treated with L-DOPA were killed on postnatal day 20 or 21. Brains were dissected and were frozen in cold isopentane. To measure D1-like receptor densities, slide-mounted sections (10 μm) were incubated at 22°C for 1 hr in the presence of 1 nM [^{125}I]-(+)-SCH 23982 (2200 Ci/mmol; DuPont NEN, Boston, MA), which has a reported K_d of 93 pM in similar assays (Altar and Marien, 1987), and 5 μM ketanserin (Research Biochemicals International), a serotonin receptor antagonist, in (mM): 120 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 0.01% (w/v) ascorbic acid, and 50 Tris-HCl, pH 7.4. Adjacent sections were also incubated with 5 μM fluphenazine (Research Biochemicals International) to assess nonspecific binding. To measure D2-like receptor densities, sections were incubated at 22°C for 4 hr in the presence of 100 pM [^{125}I]-epidepride (2000 Ci/mmol; Amersham, Buckinghamshire, England), which has a reported K_d of 78 pM in similar assays (Janowsky et al., 1992), and 100 nM idazoxan (Sigma), an α-adrenergic receptor antagonist, in 120 mM NaCl and 50 mM Tris-HCl, pH 7.4. Adjacent sections were also incubated with 1 μM haloperidol (Research Biochemicals International) to assess nonspecific binding. To measure dopamine transporter (DAT) densities, sections were pre-incubated at 22°C for 30 min in PBS, pH 7.4, and then were incubated at 22°C for 1 hr in the presence of 500 pM [^{125}I]-RTI-121 (2200 Ci/mmol; DuPont NEN), which has a reported K_d of 250 pM in homogenate binding assays (Staley et al., 1995), in PBS. Adjacent sections were also incubated with 30 μM (−)-cocaine (Research Biochemicals International) to assess nonspecific binding. After incubation with radioligands, sections were rinsed twice at 4°C for 2 to 5 min in incubation buffer, once in water, and dried. Slides were apposed to Hyperfilm β_{max} (Amersham) for 6 hr for [^{125}I]-(+)-SCH 23982 and [^{125}I]-epidepride and 16 hr for [^{125}I]-RTI-121. Autoradiograms were digitized using an imaging densitometer (model GS-700; Bio-Rad, Hercules, CA) and Multi-Analyst software (version 1.0.2; Bio-Rad). Densities were quantified by comparing autoradiographic signals to plastic polymer standards containing known concentrations of ^{125}I; tissue equivalent values, calculated by embedding ^{125}I in rat brain gray matter, represent 47% of the activity exhibited by polymer standards (Amersham).

Receptor radioligand binding in homogenates. Adult mice that had been treated daily with 50 mg/kg L-DOPA (33 μl/gm, i.p.; 1.5 mg/ml) were killed, and striata (including the caudate putamen, nucleus accumbens, and olfactory tubercule) were isolated and frozen on dry ice. Striatal tissue from two mice was disrupted in a Dounce homogenizer in 2 ml 50 mM Tris, pH 7.5. The homogenates were centrifuged at 18,000 × *g* for 30 min at 4°C. The pellet was rinsed in 50 mM Tris, pH 7.5, and centrifuged again. The pellet was resuspended in 100 ml of Tris-buffered saline (TBS; 50 mM Tris, 120 nM NaCl, pH 7.5). To measure D1-like receptor binding, ~100 μg of protein from the striatal homogenates was incubated in 0.01–1.00 nM [^{3}H]-SCH 23990 (89.0 Ci/mmol; Amersham) in a total volume of 3 ml TBS for 1 hr at 30°C. Nonspecific binding was estimated by including 5 μM fluphenazine in a separate set of samples. To measure D2-like receptor binding, ~100 μg of protein from the striatal homogenates was incubated in 0.02–1.60 nM [^{3}H]-spiperone (25.0 Ci/mmol; Amersham) in a total volume of 3.5 ml TBS containing 20 nM ketanserin for 30 min at 30°C. Nonspecific binding was estimated by including 20 μM haloperidol (McNeil Pharmaceutical) in a separate set of samples. At the end of the incubation, samples were filtered onto glass-fiber disks (GF/C; 24 mm; Whatman, Maidstone, UK), presoaked with TBS under reduced pressure, and then rinsed in TBS. Filters were dried, placed into 2.5 ml scintillation fluid (EcoLume; ICN Biomedicals, Costa Mesa, CA), and counted. For each experiment, determinations were made from triplicate samples. Results from three independent experiments were averaged. Protein content in striatal homogenates was determined using bicinchoninic acid and cupric sulfate (Pierce, Rockford, IL) and bovine serum albumin (BSA) as a standard.

Immunohistochemistry. Slide-mounted, frozen sections (20 μm) were thawed and fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Sections were preincubated in blocking buffer containing 8% (v/v) normal goat serum (Vector Laboratories, Burlingame, CA) and 3% (w/v) BSA (Sigma) diluted in PBS, pH 7.4. Tissue was incubated at 4°C overnight with a polyclonal rabbit anti-c-fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 in blocking buffer. Sections were incubated at 22°C for 2 hr in biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories) diluted 1:200 in 3% (w/v) BSA in PBS. Tissue was incubated at 22°C for 30 min in streptavidin-conjugated horseradish peroxidase (Zymed Laboratories, San Francisco, CA) diluted 1:20 in 3% (w/v) BSA in PBS. Immunoreactivity was revealed by developing at 22°C for 5 min in 0.03% (v/v) hydrogen peroxide and aminoethyl carbazole chromagen (Zymed Laboratories).

RESULTS

Locomotor responses to dopamine receptor agonists

Adult WT and DA−/− mice were treated with saline or increasing amounts of the dopamine D1 receptor-selective agonist SKF 81297, and locomotor activity was monitored for 4 hr in activity cages. Saline-elicited activity of DA−/− mice was equivalent to that of WT mice (Fig. 1*A*). SKF 81297 induced activity for 5–6 hr that was characterized by early stereotyped grooming motions that later gave way to locomotion, rearing, and jumping in both WT and mutant mice. Increasing doses of SKF 81297, ranging from 1.25 to 7.5 mg/kg, induced increasing amounts of locomo-

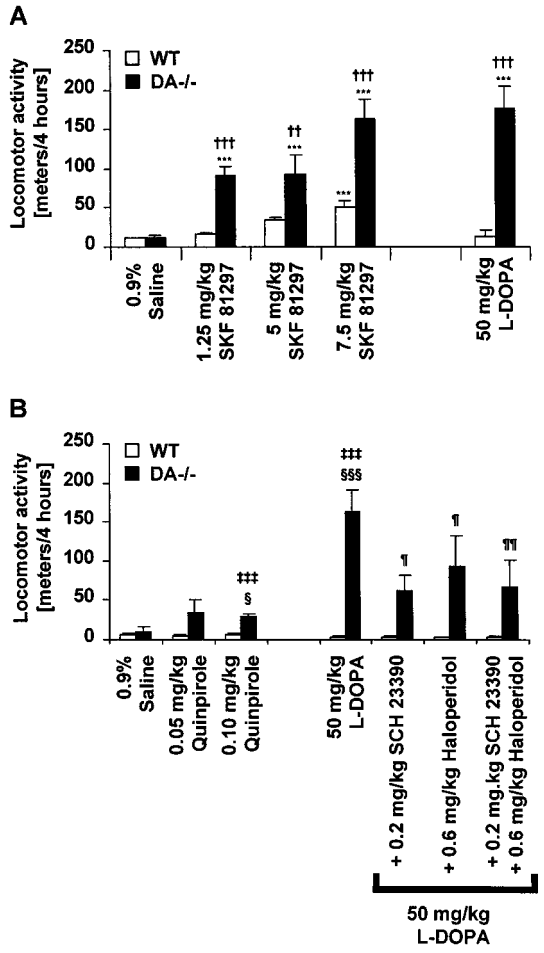

Figure 1. Locomotor response of DA−/− mice to dopamine receptor agonists and antagonists. Locomotor activity is reported as distance traveled in 4 hr in activity cages (mean ± SEM). *A*, SKF 81297- and L-DOPA-elicited locomotor activity. WT mice (*white bars; n* = 8), DA−/− mice (*black bars; n* = 5–8). ***$p < 0.001$, by Student's *t* test between saline and drug treatments. ††$p < 0.01$, †††$p < 0.001$, by Student's *t* test between WT and DA−/− groups. *B*, Quinpirole-induced activity and effects of SCH 23390 and haloperidol on L-DOPA-elicited locomotion. WT mice (*white bars; n* = 4–12), DA−/− mice (*black bars; n* = 4–12). §$p < 0.05$, §§§$p < 0.001$, by Student's paired *t* test between saline and drug treatments. ‡‡‡$p < 0.001$, by Student's *t* test between WT and DA−/− groups. ¶$p < 0.05$, ¶¶$p < 0.01$, by Student's paired *t* test between L-DOPA and L-DOPA + antagonist treatments.

tion in WT mice, and the maximum activity was threefold greater than saline treatment. These doses were chosen because they had no significant effect on the locomotor activity of D1 receptor knock-out mice (Xu et al., 1994). At each dose, SKF 81297 induced threefold to sixfold greater locomotor responses in DA−/− mutants compared to WT mice, and the maximum activity achieved was 16-fold greater than saline treatment.

Administration of the product of tyrosine hydroxylation, L-DOPA (50 mg/kg), to WT mice had no significant effect on locomotor activity as compared to saline treatment (Fig. 1*A*). In contrast, L-DOPA-treated DA−/− mutants traveled 13 times farther than similarly treated WT mice in 4 hr. This dose of L-DOPA (50 mg/kg) restores a maximum of 9% of normal dopamine content in the mutant striatum but does not alter dopamine content in WT striatum (Szczypka et al., 1999).

Injection of the D2 receptor-selective agonist quinpirole at 0.05 and 0.10 mg/kg induced fivefold to sixfold greater locomotor responses in DA−/− mutants compared to WT mice, and the maximum activity achieved was threefold greater than saline treatment over 4 hr in DA−/− mice (Fig. 1*B*). Quinpirole did not have a significant effect on locomotion in WT mice. Higher doses of quinpirole (1 or 2 mg/kg) induced less locomotion and more stereotypic movements in DA−/− mice (data not shown).

Administration of the D1 receptor-selective antagonist SCH 23390 (0.2 mg/kg) reduced L-DOPA-induced activity in DA−/− mice by 60%. Similarly, administration of the D2 receptor-selective antagonist haloperidol (0.6 mg/kg) reduced L-DOPA-elicited locomotion in DA−/− mice by 40%. Coadministration of both antagonists reduced L-DOPA-stimulated activity by 60%. These doses of SCH 23390 and haloperidol were used because they did not affect the activity of D1 and D2 receptor knock-out mice, respectively (Xu et al., 1994; Kelly et al., 1998).

Whereas daily treatment of DA−/− mice with 50 mg/kg L-DOPA stimulated feeding comparable to that of WT mice and was adequate to support survival (Szczypka et al., 1999), none of the doses of the D1 agonist SKF 81297 induced significant feeding in the mutants (data not shown). Although low doses (0.05 or 0.1 mg/kg) of the D2 agonist quinpirole were optimal for stimulating locomotion, they had little effect on feeding behavior of DA−/− mice. A higher dose (2 mg/kg) of quinpirole induced feeding that was initially near WT levels, but repeated daily administration was insufficient to sustain normal feeding levels (data not shown).

Dopamine receptor and transporter ligand binding

Brain sections from drug-naïve WT and DA−/− mice were incubated in the presence of [^{125}I]-SCH 23982, and the density of autoradiographic signals was visualized to estimate the steady-state levels of dopamine D1-like receptors. D1-like receptor binding in the caudate putamen (CPu) was remarkably similar in WT and DA−/− sections (Fig. 2*A,B*, Table 1). D1-like receptor densities were also similar in the nucleus accumbens (NAc) and substantia nigra pars reticulata (SNr).

Binding studies with the ligand [^{125}I]-epidepride were conducted to measure steady-state levels of dopamine D2-like receptors. D2-like receptor binding in the CPu was similar in WT and DA−/− sections (Fig. 2*C,D*, Table 1). D2-like receptor densities were also similar in the NAc and substantia nigra pars compacta (SNc).

[^{125}I]-RTI-121, a cocaine congener, was used to measure steady-state levels of the presynaptic dopamine plasma membrane transporter. DAT binding in the CPu was similar in WT and DA−/− sections (Fig. 2*E,F*, Table 1). DAT densities were also similar in the NAc and SNc.

Additional binding studies in striatal membrane homogenate preparations were performed to measure D1-like and D2-like receptor levels. Saturation receptor binding and Scatchard analyses using the radiolabeled D1-like receptor ligand [^{3}H]-SCH 23390 revealed similar binding parameters for WT and DA−/− striatal homogenates (Fig. 3*A,B*). The estimated maximal binding (B_{max}) values for WT and DA−/− striatal homogenates were 1435 ± 134 and 1608 ± 223 pmol/gm protein, respectively. The estimated dissociation constants (K_d) for WT and DA−/− striatal homogenates were 0.172 ± 0.013 and 0.144 ± 0.019 nM, respectively.

Saturation receptor binding and Scatchard analyses using the radiolabeled D2-like receptor ligand [^{3}H]-spiperone also revealed similar binding parameters for WT and DA−/− striatal homog-

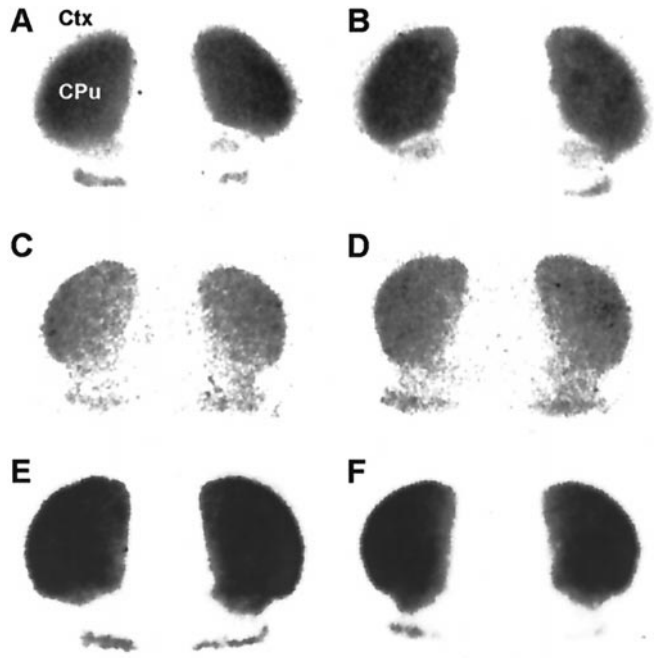

Figure 2. Total binding of dopamine receptor and transporter radioligands in WT and DA−/− striatum. *A*, *C*, *E*, Representative WT coronal sections through the striatum. *Ctx*, Cortex; *CPu*, caudate putamen. *B*, *D*, *F*, Representative DA−/− sections. *A*, *B*, D1-like receptor radioligand binding. *C*, *D*, D2-like receptor radioligand binding. *E*, *F*, DAT radioligand binding.

Table 1. Autoradiographic densities of dopamine D1-like and D2-like receptors and DAT

	Brain region	WT	DA−/−	Number of mice
D1	CPu	132.3 ± 3.2	137.3 ± 10.4	2 WT, 2 DA−/−
	NAc	84.7 ± 15.4	69.8 ± 1.4	
	SNr	157.8 ± 3.1	132.3 ± 5.8	
D2	CPu	68.4 ± 44.2	64.1 ± 28.8	3 WT, 3 DA−/−
	NAc	34.2 ± 26.1	37.3 ± 25.3	
	SNc	41.9 ± 33.0	33.5 ± 24.2	
DAT	CPu	23.4 ± 6.1	26.8 ± 6.9	3 WT, 3 DA−/−
	NAc	6.6 ± 1.9	5.0 ± 0.5	
	SNc	17.7 ± 0.4	19.6 ± 1.1	

Densities are reported as specific radioligand binding in nanocuries per milligram of tissue equivalent (mean ± range). For each brain region, 5–20 sections were examined for each mouse. CPu, Caudate putamen; NAc, nucleus accumbens; SNr, substantia nigra pars reticulata; SNc, substantia nigra pars compacta.

enates (Fig. 3*C,D*). The estimated B_{max} values for WT and DA−/− striatal homogenates were 494 ± 14 and 488 ± 44 pmol/gm protein, respectively. The estimated K_d values for WT and DA−/− striatal homogenates were 0.168 ± 0.022 and 0.125 ± 0.019 nM, respectively.

Induction of striatal c-fos immunoreactivity

Induction of the immediate early gene, *c-fos*, is often correlated with acute elevations of cAMP/Ca^{2+}-dependent signaling within neurons (Dragunow and Faull, 1989; Robertson et al., 1995; Fields et al., 1997; Rajadhyaksha et al., 1999). Adult WT and DA−/− mice were injected with either saline or 1.25 mg/kg SKF 81297, animals were killed after 2 hr, and induction of nuclear c-fos immunoreactivity was assessed in the striatum. Virtually no nuclear immunoreactivity was observed in saline-treated WT and DA−/− mice (Fig. 4*A,B*). SKF 81297 (1.25 mg/kg) induced abundant c-fos expression in the striatum of DA−/− mice at 2 hr (Fig. 4*D*) but had no effect in WT mice (Fig. 4*C*). Likewise, L-DOPA treatment induced c-fos expression in the striatum of DA−/− mice at 2 hr (Fig. 4*F*) but not in WT mice (Fig. 4*E*). Higher doses of SKF 81297 (3.75 mg/kg) or L-DOPA (100 mg/kg) induced greater amounts of c-fos-immunoreactive nuclei in DA−/− mutants but were without effect in WT mice (data not shown). Whereas SKF 81297 treatment induced uniformly distributed nuclear immunoreactivity across the DA−/− striatum, L-DOPA administration induced nuclear immunoreactivity that was enriched in the lateral CPu.

Chronic administration of L-DOPA

The feeding and drinking behaviors and hence the viability of adult DA−/− mice were maintained by daily administration of 50 mg/kg L-DOPA (Zhou and Palmiter, 1995; Szczypka et al., 1999). Each L-DOPA treatment at this dose induced locomotor hyperactivity (200–700 m) and feeding behavior (3–5 gm food intake) in DA−/− mice that lasted for 6–9 hr. Thereafter, the activity and ingestive behavior subsided. By 24 hr after L-DOPA administration, brain dopamine levels were at the limits of detection. The daily locomotor activity and food intake of L-DOPA-treated WT mice were 50–100 m and 3–5 gm, respectively. The hyperactivity induced by daily acute administration of 50 mg/kg L-DOPA could be observed from the very first injection of DA−/− mice, and this hypersensitive response was similar in magnitude throughout the lifetime of the mutants (data not shown).

To address the hypothesis that the hyperactive locomotor response to dopamine receptor agonists observed in the mutants results from the absence of continuous dopaminergic signaling, adult DA−/− mutants were subjected to near-continuous L-DOPA treatment for 4 d, and then the behavioral sensitivity to L-DOPA was assessed. A standard dose of 50 mg/kg L-DOPA was administered on the first day to establish a baseline locomotor response for each group of DA−/− mice (Fig. 5*A,B*). On the second through fifth days, 500 $mg \cdot kg^{-1} \cdot d^{-1}$ L-DOPA (five injections of 100 mg/kg L-DOPA, once every 4 hr) was administered. This treatment initially led to robust locomotor activity on the second day with some mutants traveling in excess of 1 km. However, on subsequent days the mutants' locomotor response to additional L-DOPA treatment was attenuated. During this time, the feeding levels of the mutants remained above those observed on the first day (Fig. 5*C,D*). On the sixth day, the locomotor response to a single 100 mg/kg L-DOPA challenge was <20% of the activity elicited by 50 mg/kg L-DOPA on the first day. On the seventh, eighth, and ninth days, acute challenges of 50 mg/kg L-DOPA (Fig. 5*A*) induced locomotor activity levels that were far below those achieved on the first day.

After chronic administration of 500 $mg \cdot kg^{-1} \cdot d^{-1}$ L-DOPA, complete restriction of L-DOPA treatment on the seventh day resulted in near total cessation of both locomotor activity and food intake (Fig. 5*B,D*). L-DOPA restriction for 24 hr did not reinstate the hypersensitive response of the mutants to 50 mg/kg L-DOPA in terms of their locomotor activity on the eighth and ninth days (Fig. 5*B*).

Dopamine levels in the whole brain and striatum were measured at various times during the chronic L-DOPA treatment course by HPLC and electrochemistry (Table 2). As shown previously, DA−/− mice have 1% of WT dopamine content (Zhou

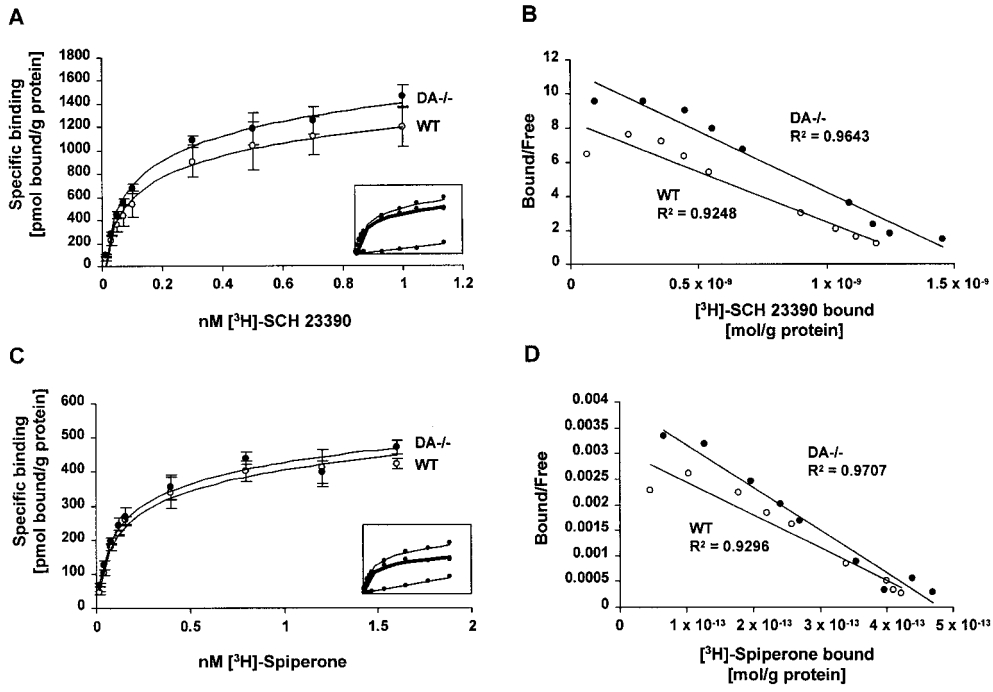

Figure 3. Saturation analysis of D1-like and D2-like receptor binding in striatal membrane homogenates. Striatal tissue from two mice was combined for each genotype for each experiment. Saturation curves represent the average curves from three independent experiments. *A*, Specific [^{3}H]-SCH 23390 binding. WT homogenates (*white circles; n* = 3), DA−/− homogenates (*black circles; n* = 3). *Inset,* Total binding (*top curve*), specific binding (*middle curve*), and nonspecific binding in the presence of 5 μM fluphenazine (*bottom curve*) from a typical experiment. *B,* Scatchard transformation of data in *A* (mean ± SEM). B_{max}: WT = 1435 ± 134 pmol/gm protein, DA−/− = 1608 ± 223 pmol/gm protein. K_d: WT = 0.172 ± 0.013 nM, DA−/− = 0.144 ± 0.019 nM. *C*, Specific [^{3}H]-spiperone binding. WT homogenates (*white circles; n* = 3), DA−/− homogenates (*black circles; n* = 3). *Inset,* Total binding, specific, nonspecific binding in the presence of 20 μM haloperidol from a typical experiment. *D,* Scatchard transformation of data in *C*. B_{max}: WT = 494 ± 14 pmol/gm protein, DA−/− = 488 ± 44 pmol/gm protein. K_d: WT = 0.168 ± 0.022 nM, DA−/− = 0.125 ± 0.019 nM.

and Palmiter 1995; Szczypka et al., 1999). Treatment with L-DOPA had no significant effect on brain or striatal dopamine content in WT mice, but it raised dopamine content in DA−/− mice to 30–50% of WT levels. This level of dopamine was maintained for the duration of the chronic treatment (Table 2, 98 hr). Cessation of L-DOPA treatment resulted in a near complete loss of dopamine in the brain and striatum of DA−/− mice (Table 2, 96 hr + 24 hr with no L-DOPA).

Normalization of striatal c-fos response in DA−/− mice

Chronic L-DOPA (500 mg · kg^{-1} · d^{-1}) treatment also resulted in reduction of sensitivity of DA−/− mice to L-DOPA in terms of their striatal c-fos response. Another group of adult DA−/− mutants was injected with 100 mg/kg L-DOPA once every 4 hr, and mice were killed at 2, 14, 26, and 38 hr after the treatments began. At 2 hr, c-fos induction was observed in the striatum, and nuclear immunoreactivity was enriched in the lateral CPu (Fig. 6*A*), as observed with 50 mg/kg L-DOPA treatment (Fig. 4*F*). At 14 hr, the immunoreactivity was reduced, particularly in the lateral CPu (Fig. 6*B*). At 26 hr, the immunoreactive response was reduced even further throughout the striatum (Fig. 6*C*) and by 38 hr, striatal c-fos immunoreactivity was almost completely abolished (Fig. 6*D*).

DISCUSSION

The results suggest that the chronic presence of dopamine is required to dampen the response to dopaminergic agonists, both in terms of locomotor activity and striatal intracellular signaling. Mice that developed with a complete loss of dopaminergic neurotransmission displayed an enhanced behavioral response to D1-like or D2-like receptor agonists. A dose of L-DOPA that restored only 9% of normal striatal dopamine content (Szczypka et al., 1999) induced locomotion in the mutants that far exceeded the activity of treated WT mice. D1 and D2 receptor antagonists both reduced L-DOPA-induced activity suggesting that activation of both receptor subtypes is important in the L-DOPA-induced behavioral response.

D1-like and D2-like receptor ligand binding in the WT and DA−/− striatum were similar, both in terms of maximal binding and affinity. The observations suggest that dopamine is not required during embryonic and postnatal development for adequate expression of D1-like and D2-like receptors, which is consistent with previous observations that D1 and D2 receptor mRNA levels are normal in the DA−/− striatum (Zhou and Palmiter, 1995). Dopamine D3 receptor mRNA levels also appeared normal in the striatum of both drug-naïve and L-DOPA-treated mutants (D. S. Kim and M. S. Szczypka, unpublished observations). In 6-OHDA-lesioned rats, many studies have described dopaminergic hypersensitivity resulting from dopamine depletion that is independent of changes in D1-like receptor levels (Altar and Marien, 1987; Luthman et al., 1990; Morelli et al., 1990; Duncan et al., 1993). However, in some reports, striatal D2-like receptor levels were enhanced (Dewar et al., 1990; Bordet et al., 1997). The discrepancy may reflect differences between selectively inactivating dopamine production versus eliminating entire dopaminergic nerve terminals.

Although receptor levels appeared normal in DA−/− mice, accessibility of receptors to ligands or to relevant heterotrimeric G-proteins *in vivo* could be enhanced in the DA−/− striatum. For example, desensitization of dopamine receptors by G-protein-coupled receptor kinases (GRKs) has been described (Tiberi et al., 1996; Ito et al., 1999). Steady-state DAT levels were unaltered in the DA−/− striatum, suggesting that dopamine is not required to regulate transporter expression. It is possible, however, that downregulation of presynaptic DAT activity, which is responsible for reuptake of dopamine from the synaptic cleft, could underlie the hypersensitivity to L-DOPA. With less DAT activity, dopamine would be recycled less efficiently and have greater opportunity to signal through its receptors.

The D1 receptor agonist SKF 81297 induced a fourfold to

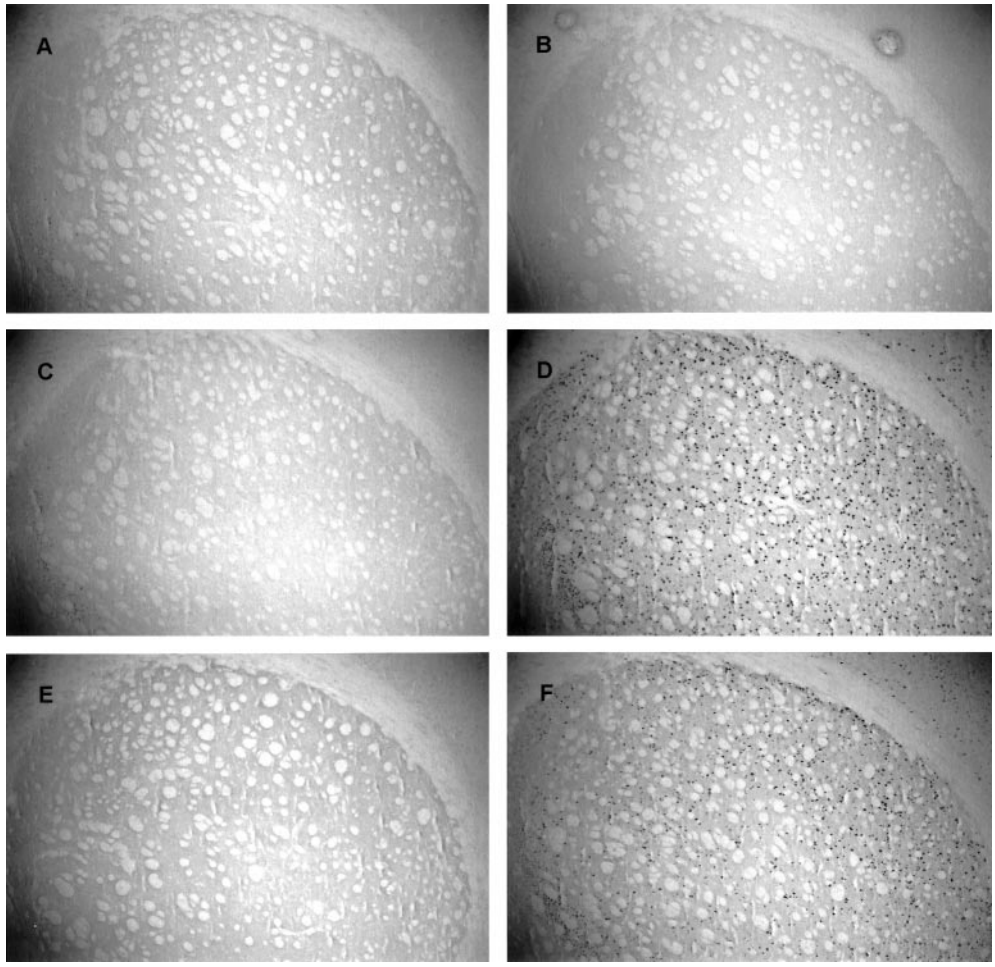

Figure 4. Acute induction of c-fos immunoreactivity in WT and DA−/− striatum after 2 hr of treatment. *A*, *C*, *E*, Representative WT coronal sections showing the dorsal caudate putamen (*top,* dorsal; *right,* lateral). *B*, *D*, *F*, Representative DA−/− sections. *A*, *B,* Sections after 0.9% saline treatment. *C*, *D,* Sections after 1.25 mg/kg SKF 81297 treatment. *E*, *F,* Sections after 50 mg/kg L-DOPA treatment.

fivefold greater locomotor response in DA−/− mutants as compared to WT mice. The hypersensitive behavioral response was correlated with induction of striatal c-fos immunoreactivity in DA−/− mice. c-fos induction has been observed in other models of dopaminergic hypersensitivity (Paul et al., 1992; LaHoste et al., 1993) and reflects elevated phosphorylation and activation of the cAMP/Ca^{2+}-response element-binding (CREB) protein, which can activate *c-fos* gene expression (Cole et al., 1994; Konradi et al., 1994; Liu and Graybiel, 1996, 1998). Dopamine appears to dampen the intracellular signaling response downstream of D1 receptors in postsynaptic striatal neurons. Increased G_s/G_{olf} protein levels and thus increased coupling between D1 receptors and adenylyl cyclase have been observed in 6-OHDA-lesioned rats (Luthman et al., 1990; Cowburn et al., 1991; Hervé et al., 1993).

The behavioral and c-fos responses of DA−/− mutants induced by a direct D1 receptor agonist support the notion that the hypersensitivity results from changes that are independent of the manner in which dopamine is synthesized, released, recycled, and degraded by presynaptic neurons. Although SKF 81297 administration was sufficient to induce locomotor hyperactivity and striatal c-fos immunoreactivity, the possibility that alterations in nondopaminergic presynaptic inputs to the striatum, such as glutamate, GABA, and acetylcholine, may underlie the hypersensitivity cannot be excluded. Postsynaptic changes in the activity of receptors for other neurotransmitters could also be altered. Blockade of striatal NMDA receptors can potentiate or reduce the acute hypersensitive behavioral response to dopaminergic agonists in unilaterally 6-OHDA-lesioned animals (Morelli and Di Chiara, 1990; Morelli et al., 1992; Paul et al., 1992; Morelli, 1997), but it is unclear whether the hypersensitive response in lesioned animals in which dopaminergic nerve terminals and co-released factors have been removed is identical to the hypersensitivity observed in DA−/− mice.

SKF 81297 induced c-fos immunoreactivity that was uniformly distributed throughout the DA−/− striatum, whereas immunoreactive nuclei were enriched in lateral cells of the CPu with L-DOPA administration. L-DOPA is probably taken up into all presynaptic neurons, converted into dopamine by aromatic L-amino acid decarboxylase, and transported into synaptic vesicles. Nonuniform induction of c-fos after L-DOPA administration may reflect the regulated release of dopamine from dopaminergic neurons preferentially in the lateral CPu. The uniform induction of c-fos immunoreactivity by SKF 81297 suggests that D1 receptor-expressing neurons are equally sensitized throughout the DA−/− striatum.

Chronic L-DOPA administration reduced the locomotor hyperactivity of DA−/− mice. Maximal striatal dopamine levels achieved during chronic treatment were only 50% of WT levels, and the mutants retained the ability to convert L-DOPA to dopamine in the striatum, suggesting that a small amount of relatively constant dopaminergic release is sufficient to reverse the hypersensitivity of DA−/− mice. The attenuation of the hyperactivity was correlated with reduction of L-DOPA-elicited striatal c-fos

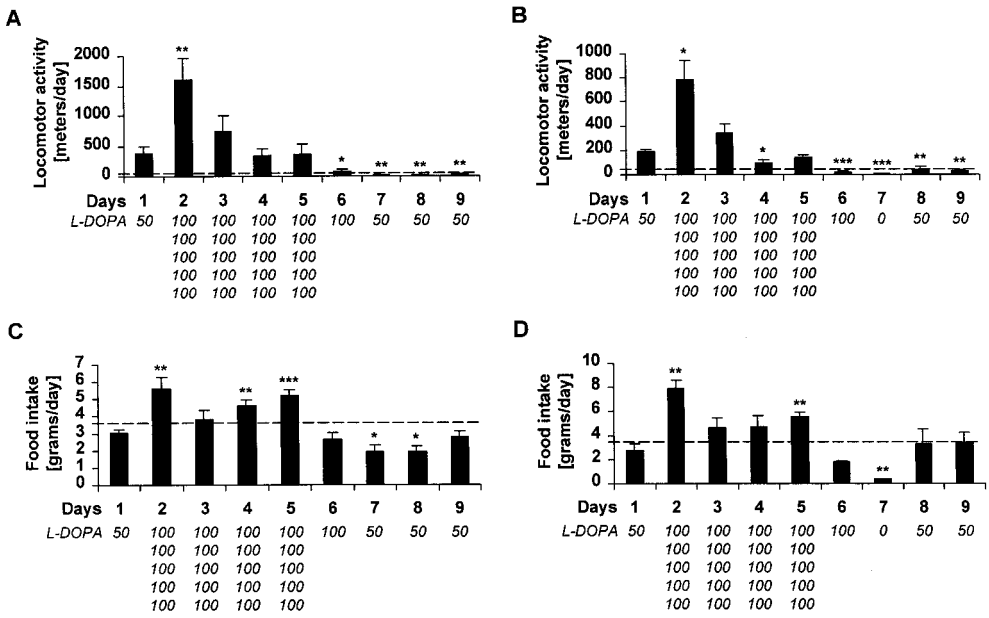

Figure 5. Locomotor and feeding responses of DA−/− mice after chronic L-DOPA administration over 4 d. Locomotor activity is reported as distance traveled in meters, and feeding is shown as grams of food intake in 24 hr (mean ± SEM). The *horizontal dashed lines* indicate daily locomotor activity (45.1 ± 7.6 m; $n = 8$) and food intake (3.6 ± 0.2 gm; $n = 8$) of saline-treated WT mice. *Numbers in italics* indicate daily doses of L-DOPA. *A*, Locomotor response of DA−/− mice ($n = 4$) to an initial dose of 50 mg/kg L-DOPA, chronic doses of 500 $mg \cdot kg^{-1} \cdot d^{-1}$, and subsequent challenge doses of 100 mg/kg and 50 mg/kg. *B*, Locomotor response of DA−/− mice ($n = 3$) as treated in *A*, except no L-DOPA treatment for 24 hr on seventh day. *C*, Feeding responses of DA−/− mice ($n = 4$) described in *A*. *D*, Feeding responses of DA−/− mice ($n = 3$) described in *B*. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; by Student's *t* test comparing each response to initial response on first day.

induction, which is consistent with the reduction in effective dopaminergic signaling observed in chronically treated, unilaterally 6-OHDA-lesioned rats (Hossain and Weiner, 1993). Because the time required for normalization of locomotor behavior (4 d) and intracellular cAMP/Ca^{2+}-mediated signaling (36 hr) was relatively long, the decline in dopaminergic sensitivity may reflect transcriptional alterations, circuit-level adaptations, or other changes that can only be corrected over a period of days.

The normalization of activity resulting from chronic L-DOPA administration to DA−/− mice was surprisingly stable. The locomotor response to L-DOPA challenges remained at low levels for at least 3 d after chronic treatment. Even after omission of one day of L-DOPA treatment, which depleted brain dopamine almost completely, 50 mg/kg L-DOPA administration did not restore a locomotor response comparable to that observed before the chronic course. Omission of L-DOPA treatment for >24 hr was not possible because the mutant mice depend on L-DOPA for feeding behavior and survival. The observation that behavioral resensitization requires >1 d of dopamine depletion suggests that the initial process of sensitization in the mutants occurs over a period of days. The hypersensitivity is likely established during the embryonic and perinatal stages when there is a prolonged absence of mesostriatal dopaminergic input. One prediction of this interpretation is that postsynaptic striatal neurons of WT animals normally reduce their sensitivity to dopamine as dopaminergic synapses arise during development.

After the chronic course, removal of L-DOPA treatment for 24 hr resulted in a near total loss of locomotion and feeding. This observation underscores the notion that dopaminergic signaling is acutely required for the initiation and maintenance of voluntary locomotor and feeding behavior. The severe hypoactive and hypophagic phenotypes of DA−/− mice (Zhou and Palmiter, 1995) likely result because of an acute requirement for dopamine and not because of inadequate previous conditioning of these behaviors. Recovery from hypophagia in adult 6-OHDA-lesioned rats occurs after feeding behavior is reconditioned using highly palatable food, and the recovery also requires dopaminergic reinnervation of postsynaptic cells (Zigmond and Stricker, 1973). In DA−/− mice, dopaminergic function was completely eliminated when L-DOPA treatment was removed and thus the mutants returned to their hypophagic state.

Even though chronic L-DOPA administration led to normalization of the hyperactivity, the feeding response to L-DOPA was not attenuated. The daily 50 mg/kg L-DOPA regimen used to maintain DA−/− mice before the experiment does not support normal growth or adiposity (Zhou and Palmiter, 1995). Body weight

Table 2. Brain and striatal dopamine levels during chronic L-DOPA administration

Time during chronic L-DOPA treatment*	WT mice ($n = 3$)		DA−/− mice ($n = 3$)	
	Brain	Striatum	Brain	Striatum
0 hr	22.8 ± 4.1	71.3 ± 2.2	0.303 ± 0.090	0.333 ± 0.069
2 hr	25.0 ± 1.9	71.0 ± 8.0	7.60 ± 0.17	22.9 ± 2.6
14 hr	21.7 ± 2.8	95.5 ± 6.3	9.6 ± 1.6	54.6 ± 8.2
98 hr	27.0 ± 3.5	80.8 ± 14	7.02 ± 2.4	29.5 ± 11
96 hr + 24 hr with no L-DOPA	16.8 ± 1.2	70.3 ± 6.0	0.393 ± 0.032	0.890 ± 0.082

Values are reported as nanogram per milligram of protein (mean ± SEM). Dopamine concentrations were measured by HPLC and electrochemical detection.

*Adult WT and DA−/− mice were treated with 100 mg/kg L-DOPA at 7:00 A.M., 11:00 A.M., 3:00 P.M., 7:00 P.M., and 11:00 P.M. for 4 d. On the fifth day, no further L-DOPA was administered for 24 hr. DA−/− mice were last treated with 50 mg/kg L-DOPA 24 hr before the chronic treatment began.

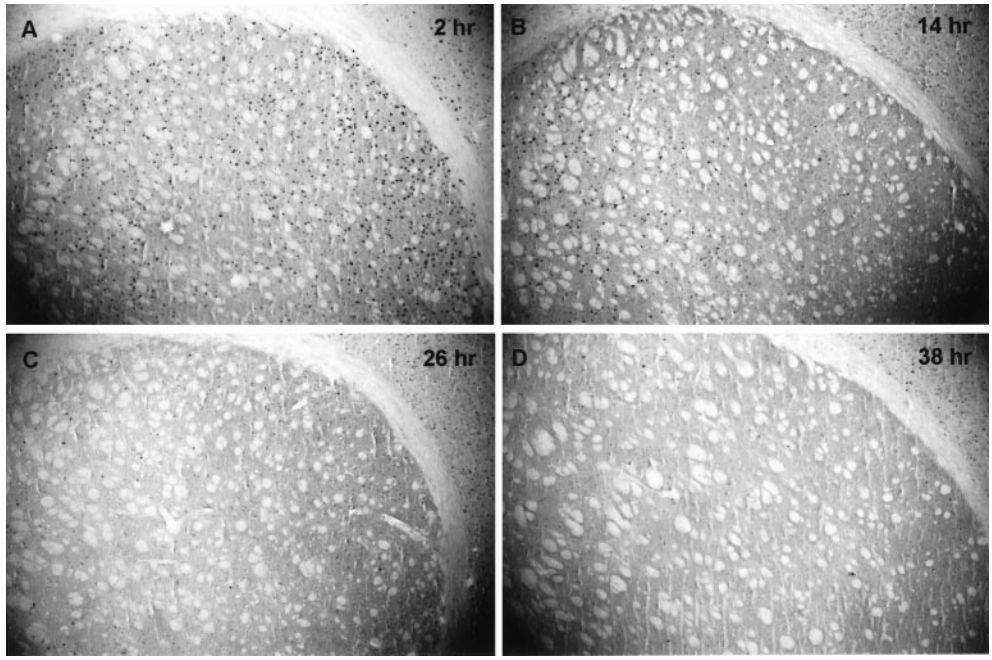

Figure 6. c-fos immunoreactivity response of DA−/− mice after chronic L-DOPA (500 $mg \cdot kg^{-1} \cdot d^{-1}$) treatment. Representative DA−/− coronal sections showing the dorsal caudate putamen (*top,* dorsal; *right,* lateral). *A*, 2 hr. *B*, 14 hr. *C*, 26 hr. *D*, 38 hr.

increased during the chronic L-DOPA treatment course but never reached normal levels (data not shown). The observation that feeding was only slightly impaired after chronic treatment suggests that dopaminergic pathways involved in feeding were differentially affected as compared to those required for locomotion.

We have described the hypersensitivity to dopamine receptor agonists in genetically altered mice that cannot produce dopamine in dopaminergic neurons. The results suggest that the chronic presence of dopamine dampens the behavioral and neuronal responses to the neurotransmitter itself. The observations extend the conclusions reached by others (Ungerstedt and Arbuthnott, 1970; Ungerstedt, 1971a,b; Kostrzewa, 1995; Moy et al., 1997) by demonstrating that the reversal of the hypersensitivity that occurs with near-continuous dopamine replacement takes place over a period of days and that resensitization requires restriction of brain dopamine for >24 hr. Enhanced sensitivity to dopaminergic agonists in terms of locomotor activity can also occur after repeated exposure to psychostimulants, such as cocaine and amphetamine (Pierce and Kalivas, 1997; White and Kalivas, 1998). This contrasts sharply with the enhanced sensitivity of DA−/− mice to dopamine receptor agonists and the reversal of sensitivity observed with repeated L-DOPA administration. Sensitization of neuronal circuits as a consequence of neurotransmitter depletion has also been described in other experimental systems (Cangiano, 1985; Walters et al., 1991; Cho et al., 1999). The sensitivity that develops in the absence of dopaminergic input reflects the ability of striatal neurons to adapt to insufficient dopamine release, as occurs in parkinsonism, and reveals another example of the remarkable plasticity of the mammalian brain.

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