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Dopamine-Deficient Mice Are Severely Hypoactive, Adipsic, and Aphagic

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Summary

Mice unable to synthesize dopamine (DA) specifically in dopaminergic neurons were created by inactivating the tyrosine hydroxylase (TH) gene then by restoring TH function in noradrenergic cells. These DA-deficient ($DA^{-/-}$) mice were born at expected frequency but became hypoactive and stopped feeding a few weeks after birth. Midbrain dopaminergic neurons, their projections, and most characteristics of their target neurons in the striatum appeared normal. Within a few minutes of being injected with L-dihydroxyphenylalanine (L-DOPA), the product of TH, the $DA^{-/-}$ mice became more active and consumed more food than control mice. With continued administration of L-DOPA, nearly normal growth was achieved. These studies indicate that DA is essential for movement and feeding, but is not required for the development of neural circuits that control these behaviors.

Introduction

Dopamine (DA) is one of the classical neurotransmitters of the central nervous system (CNS). About 75% of the dopaminergic neurons have their cell bodies in the substantia nigra of the midbrain and project to the striatum (Carlsson, 1959; Dahlstrom and Fuxe, 1964; Lindvall and Bjorlund, 1983). These neurons have been implicated in the regulation of motor behavior in part because their degeneration is thought to underlie Parkinson's disease (Hornykiewicz, 1966). Another distinct dopaminergic system arises in the ventral tegmental area of midbrain and projects to the nucleus accumbens, olfactory tubercule, and frontal cortex. These pathways are thought to influence motivated behaviors, including activities related to reward (Koob, 1992; Self and Nestler, 1995). Dysfunction of this system has been implicated in depression and schizophrenia (Seeman et al., 1993).

DA is synthesized from tyrosine by the sequential actions of tyrosine hydroxylase (TH), which generates L-dihydroxyphenylalanine (L-DOPA), and L-aromatic amino acid decarboxylase (AADC). DA is actively transported into synaptic vesicles by a vesicular amine transporter (Liu et al., 1992b). When DA is released, it can bind to membrane receptors that are coupled to trimeric G proteins; the most prominent are the D1 and D2 receptors, which stimulate and inhibit adenylyl cyclase, respectively (Civelli et al., 1993). DA is cleared from synapses by plasma membrane transporters and then either degraded by monoamine oxidases or recycled into synaptic vesicles (Amara and Kuhar, 1993). The membrane transporters are the primary site of action of antidepressants and addictive drugs such as cocaine and amphetamine.

Our knowledge of the behavioral functions of DA in the CNS is derived primarily from studies with DA receptor ligands and neurotoxins. For example, blocking or stimulating postsynaptic DA receptors reduces or increases locomotion and exploratory behavior, respectively (Arnt, 1987; Clark and White, 1987). Likewise, increasing synaptic DA concentration by inhibiting DA transporters or blocking DA autoreceptors stimulates locomotion (Arnt, 1987; Woolverton and Johnson, 1992). Blocking DA receptors attenuates the rewarding actions of food, intracranial self-stimulation, and psychomotor stimulants (Fibiger and Phillips, 1988; Koob, 1992; Wise and Hoffman, 1992). Bilateral lesions of midbrain dopaminergic pathways of adult rats with 6-hydroxydopamine (6-OHDA) result in long-lasting adipsia, aphagia, hypoactivity, and loss of exploratory behavior (Ungerstedt, 1971). The neurotoxin 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) preferentially destroys DA neurons in the substantia nigra and produces symptoms that resemble Parkinson's disease (Kopin and Markey, 1988). While these approaches have produced a general understanding of DA function, they have limitations due to incomplete blockade, nonspecific effects on other neuronal systems, or loss of cellular components in addition to DA. Furthermore, most of these studies have been restricted to adult animals. Relatively little is known about the role of DA in development. DA and its receptors appear in the brain between embryonic day 10 (E10) and E15 in rodents (Voorn et al., 1988; Schambra et al., 1994), and a number of experiments suggested that DA signaling could play a role in establishing synaptic connections (Lankford et al., 1988; Rodrigues and Dowling, 1990; Todd, 1992; Lauder, 1993).

We provide genetic evidence in this report that DA is essential for proper CNS functions such as drinking, feeding, and movement, but is not required for the development of neural circuits involved in these behaviors.

Results

Targeting TH-Coding Sequence to Dopamine-β-Hydroxylase Promoter and Generation of DA- and Norepinephrine-Deficient Mice

We previously disrupted the TH gene in mice (Zhou et al., 1995), which resulted in deficiency in both DA and norepinephrine (NE). To restore the expression of the TH gene in noradrenergic neurons, the TH coding sequence was targeted to the noradrenergic-specific dopamine-β-hydroxylase (DBH) promoter by homologous recombination in embryonic stem (ES) cells. Figure 1A shows the structure of the targeting vector, pDBH-TH. Out of 660 ES cell clones, two clones with predicated genomic structure were identified by Southern blot analysis (data not shown). Both ES cell clones were separately microinjected into

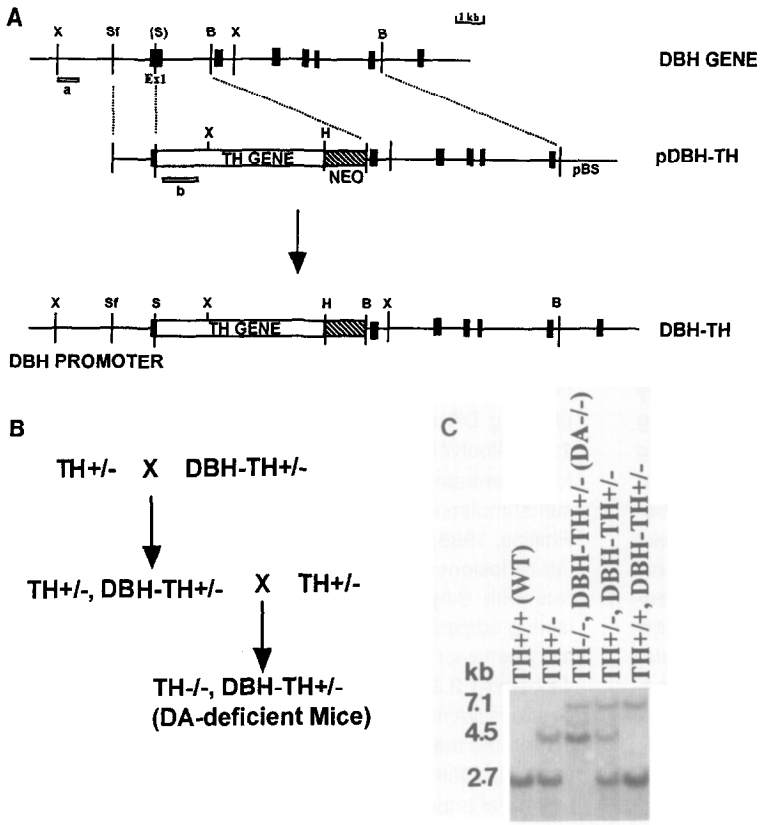

Figure 1. Gene Targeting, Mating Strategy, and Genetic Diagnosis of $DA^{-/-}$ Mice

(A) The murine DBH gene and targeting vector pDBH-TH. A region of the DBH gene that includes the proximal promoter is shown. The entire TH coding region, including 1 kb sequence after the polyadenylation site and a *neo* cassette, was inserted between exons 1 and 2 of the DBH gene. Locations of probes from DBH gene (box a) and the TH gene (box b) that were used for screening ES cell clones and mice are indicated. Abbreviations: B, BamHI; H, HindIII; Sf, Sfi; X, Xba; S, artifical SalI; pBS, pBluescript (Strategene).

(B) Breeding strategy for generating $DA^{-/-}$ mice.

(C) Southern blot analysis of representative tail DNA samples. DNA was digested with XbaI and hybridized with probe b from the TH gene. The 2.7 kb band is the wild-type (WT) TH allele, the 4.5 kb band is the disrupted TH allele, and the 7.1 kb band represents the DBH-TH allele. The faint 5.5 kb band is due to partial digestion.

blastocysts, and one produced chimeras that transmitted the DBH-TH transgene via the germline.

DBH-TH transgenic mice were used to produce offspring with selective NE or DA deficiency. When heterozygous DBH-$TH^{+/-}$ mice were intercrossed, the homozygous DBH-$TH^{+/+}$ mice were deficient in DBH function and had a phenotype indistinguishable from $DBH^{-/-}$ mice (Thomas et al., 1995). Figure 1B shows the strategy of generating $DA^{-/-}$ mice. DBH-$TH^{+/-}$ mice were first crossed with $TH^{+/-}$ mice to generate $TH^{+/-}$DBH-$TH^{+/-}$ mice. These compound heterozygotes were then crossed with $TH^{+/-}$ mice to generate mice of six different genotypes that can be distinguished by Southern blots of XbaI-digested DNA using probe b from the TH gene (Figure 1A). Figure 1C shows the pattern of hybridization bands for five of the six genotypes. The $TH^{-/-}$ genotype is not represented because most $TH^{-/-}$ mutants die in utero (Zhou et al., 1995). For simplicity, we refer to mice with the genotype of $TH^{-/-}$DBH-$TH^{+/-}$ as $DA^{-/-}$.

Restoration of NE in Noradrenergic Cells of $TH^{-/-}$ Mice

We predicted that the DBH-TH gene would restore the ability to synthesize DA and NE in cells that normally express DBH (noradrenergic cells) when introduced into the $TH^{-/-}$ genetic background. In peripheral organs that are innervated by sympathetic neurons such as the heart, there are normally high levels of NE but little DA (Figure 2A). In $TH^{-/-}$ mice, neither DA nor NE was detected. However, NE levels were restored in heart (Figure 2A), salivary glands, and adrenal glands (data not shown) of $DA^{-/-}$ mice. Both DA and NE are abundant in the brains of wild-type mice but absent in $TH^{-/-}$ mice (Figure 2B). NE levels were restored in the brains of $DA^{-/-}$ mice, but DA remained low (Figure 2B).

To determine where TH was expressed in the brain of $DA^{-/-}$ mice, we performed immunocytochemistry with an antibody to TH. Figure 3F shows that TH immunoreactivity was observed only in noradrenergic neurons of the locus ceruleus in $DA^{-/-}$ mice, but not in midbrain DA neurons (Figure 3D), nor in their terminals in the telencephalon, including caudate putamen, nucleus accumbens, and olfactory tubercle (Figure 3B). In wild-type brains, TH immunoreactivity was present in all three regions (Figures 3A, 3C, and 3E). TH immunoreactivity and catecholamine histofluorescence were also restored in the adrenal medulla of $DA^{-/-}$ mice (data not shown).

The DBH-TH Transgene Prevents the Embyonic Fatality of $TH^{-/-}$ Mice

In crosses of $TH^{+/-}$DBH-$TH^{+/-}$ mice with $TH^{+/-}$ mice, we expected 25% of the offspring to be $TH^{-/-}$ and 50% of them to have the DBH-TH gene. Because most $TH^{-/-}$ mice die in utero (Zhou et al., 1995), we expected one seventh of mice born (see Figure 1B) to be $DA^{-/-}$, if dopaminergic function was not required prior to birth. In fact, 114 out of 789 mice born were $DA^{-/-}$, indicating that the DBH-TH transgene rescued the embryonic fatality of $TH^{-/-}$ mutants.

Early Postnatal Fatality of $DA^{-/-}$ Mice and Rescue by L-DOPA

Newborn $DA^{-/-}$ mice were grossly indistinguishable from their littermates. But by postnatal day 10–15 (P10–P15),

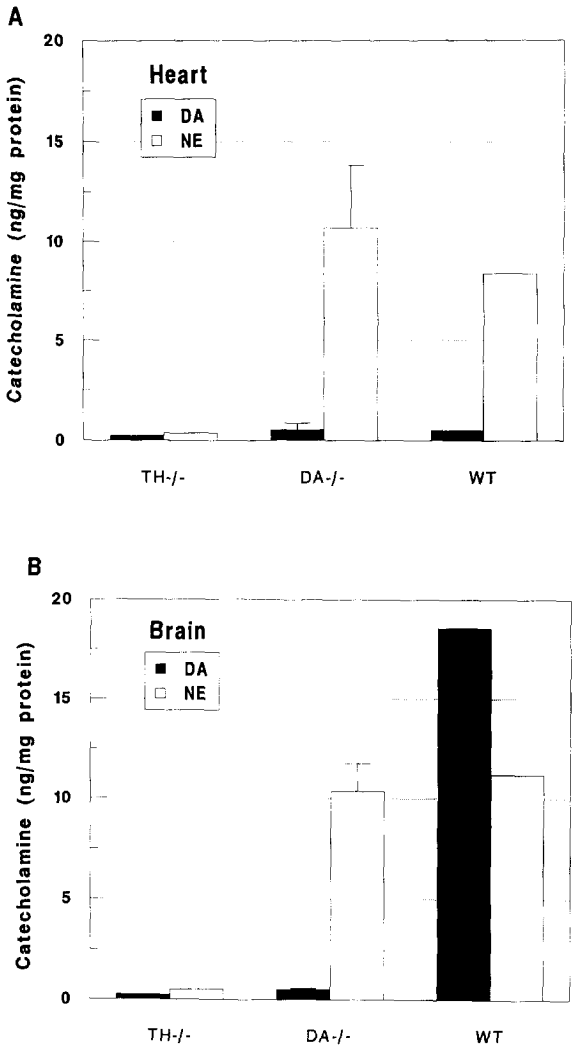

Figure 2. Catecholamine Levels in the Heart and the Brain

DA and NE levels from the heart (A) and brain (B) of P16 wild-type (n = 1), $TH^{-/-}$ (n = 1), and untreated $DA^{-/-}$ (n = 2) mice were measured by high pressure liquid chromatography and electrochemical detection (Thomas et al., 1995).

they were recognizable by their smaller size, hypoactivity, poorly groomed coats, and hunched posture. Neurological tests revealed that 16-day-old $DA^{-/-}$ mice had normal reflexion, corneal, startle, righting, grasping, and placing reflexes (assessed according to Drago et al., 1994). $DA^{-/-}$ mice could balance on a stationary bar, and no tremor was observed. At P16, their weight was 70% of littermates, but their growth rate became negative after P17, and they all died by 4 weeks of age (Figures 4A and 4B).

We attempted to rescue $DA^{-/-}$ mice with L-DOPA, the product of TH enzymatic activity. L-DOPA was administered intraperitoneally, usually beginning at P15, when $DA^{-/-}$ pups were easily recognized. L-DOPA, at a dose of 50 mg/kg twice a day, rescued all $DA^{-/-}$ mice to adulthood (Figure 4A). Such treatment restored nearly normal growth rate of $DA^{-/-}$ mice (Figure 4A). At 7 weeks of age, $DA^{-/-}$ female mice weighed 17.6 ± 0.77 g (mean ± SEM; n = 9) while their littermates weighed 20.9 ± 1.2 g (n = 7). To date, more than 50 $DA^{-/-}$ mice have been rescued to adulthood.

Survival of DA-Containing Pathways in the Absence of DA

Do dopaminergic neurons survive in $DA^{-/-}$ mice? This question was addressed by immunostaining with an antibody against AADC, the enzyme that converts L-DOPA to DA. AADC-positive cells were found in the midbrain (Figure 5D), olfactory bulb, and hypothalamus (data not shown) of $DA^{-/-}$ mice, where dopaminergic neurons normally exist. AADC immunoreactivity was also found in the normal dopaminergic targets in the striatum, such as caudate putamen, nucleus accumbens, and olfactory tubercle (Figure 5B), while no TH immunoreactivity was observed in nearby sections from $DA^{-/-}$ mice (see Figure 3B). Sagittal sections were immunostained with both TH and AADC antisera to demonstrate the fiber pathway of midbrain dopaminergic neurons to their targets in the telencephalon. No TH immunoreactivity was observed in midbrain dopaminergic neurons, the medial forebrain bundle, or the striatum of $DA^{-/-}$ mice (Figure 5F). However, when the antibody against AADC was used, similar immunostaining patterns were observed in $DA^{-/-}$ and wild-type brains (Figures 5G and 5H). These studies demonstrate that both midbrain DA neurons and their terminals in the telencephalon appear normal in $DA^{-/-}$ mice.

Normal Neurogenesis of the Striatum in the Absence of DA

Several approaches were used to determine the effects of DA deficiency on neural differentiation of the striatum, a major target of midbrain DA neurons. First, sections were stained with cresyl violet (Figures 6A and 6B) and by the Nissl and Homer's silver methods (data not shown). There were no obvious anatomical changes in the brains of $DA^{-/-}$ mice as compared with wild-type mice, except that the size of the mutant brain was reduced and the neurons were slightly more compact. Second, the expression of D1 and D2 receptor mRNA was examined by in situ hybridization with digoxygenin-labeled probes. Figures 6F and 6H show that both D1 and D2 receptor mRNAs were expressed in the mutant striatum and that the pattern and intensities of hybridization were indistinguishable from wild-type striatum. Third, sections were stained for DARPP32 (DA- and cyclic AMP–regulated phosphoprotein), a protein whose phosphorylation is regulated by D1 receptor stimulation (Hemmings and Greengard, 1986). Figure 6D shows that there was widespread DARPP32 staining in the $DA^{-/-}$ striatum and that the intensity was similar to wild-type controls (Figure 6C). Other sites of DARPP32 expression, such as the deep layers of the cerebral cortex, were also similar (Figures 6C and 6D). Quantitative analysis of DARPP32 by Western blot revealed the same level of DARPP32 in $DA^{-/-}$ and wild-type striatum (data not shown). About 90% of striatal neurons use γ-aminobutyric acid (GABA) as their principal neurotransmitter (Kita and Kitai, 1988). Western blots revealed that the levels of GAD65 and GAD67, the enzymes responsible for the synthesis of GABA, in the mutant striatum were

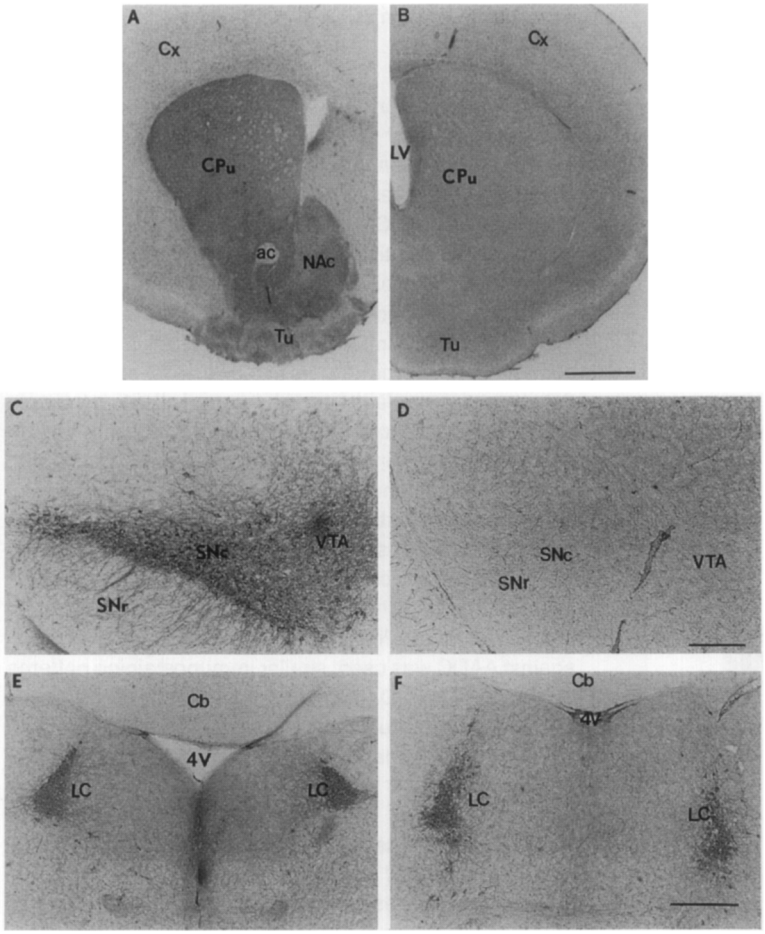

Figure 3. TH Immunostaining in the Frontal Brain, Midbrain, and Brainstem Region of Wild-Type and $DA^{-/-}$ Brains

Coronal sections at different levels from P16 wild-type brain (A, C, and E) and untreated $DA^{-/-}$ brain (B, D, and F) were stained with anti-TH antiserum. In wild-type brain, TH immunostaining was observed in the caudate putamen, nucleus accumbens, and olfactory tubercle (A) and in the substantia nigra and ventral tegmental area of the midrain (C) and locus ceruleus (E). In $DA^{-/-}$ brain, TH immunoreactivity was observed in the noradrenergic brainstem nuclei (F), but was undetectable in the midbrain DA neurons (B) and their terminals in the frontal brain (D). Abbreviations: 4V, fourth ventricle; ac, anterior commissure; Cb, cerebellum; CPu, caudate putamen; Cx, cerebral cortex; LC, locus ceruleus; LV, lateral ventricle; NAc, nucleus accumbens; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; Tu, olfactory tubercle; VTA, ventral tegmental area. Scale bars in (A) and (B), 1 mm; in (C) and (D), 250 μm; in (E) and (F), 500 μm.

the same as those of wild type (data not shown). Taken together, these results indicate that neurogenesis in the striatum is essentially normal in the absence of DA.

Reduced Substance P and Dynorphin Expression in $DA^{-/-}$ Striatum

Substance P, dynorphin, and enkephalin are the primary neuropeptides found in the striatal projection neurons that express either D1 or D2 receptors (Graybiel, 1990; Gerfen, 1992). Studies of rodents and primates treated with neurotoxins or with DA receptor antagonists indicated that the expression of neuropeptides in the striatum may be controlled by DA signaling (Anderson and Reiner, 1990; Gerfen et al., 1990). To assess whether the expression of neuropeptides is altered in $DA^{-/-}$ mice, we performed immunostaining. Figures 7B and 7D show that substance P immunoreactivity was reduced in both the cell bodies of striatal neurons and their terminals in substantia nigra pars reticulata relative to controls (Figures 7A and 7C). Similar results were observed for dynorphin (Figures 7E and 7F). However, we did not observe obvious change in intensity or pattern of enkephalin immunoreactivity in the $DA^{-/-}$ striatum (data not shown). These results suggest that DA signaling normally induces substance P and dynorphin expression in the striatum.

Hypoactivity of $DA^{-/-}$ Mice

By P10–P15, spontaneous hypoactivity of $DA^{-/-}$ pups became apparent. Within 15 min of a single injection of L-DOPA to these naive mice, they became very active and began feeding, which continued for a few hours, but by 12 hr they were hypoactive again. To quantitate their motor activity, we placed rescued adult $DA^{-/-}$ mice in an activity chamber equipped with infrared beams, and their activity during the light phase of the light–dark cycle was measured. Control mice (those wild-type or heterozygous for TH) traveled ~23 m/hr, whereas untreated $DA^{-/-}$ mice traveled only ~3 m/hr. An intraperitoneal injection of L-DOPA, at a dose of either 25 or 50 mg/kg, increased locomotion of $DA^{-/-}$ mice 50- to 100-fold to a level that exceeded that for controls (Figure 8A). Robust activity was apparent within 15 min, peaked at ~1 hr, and then decayed. By 4 hr, activity of $DA^{-/-}$ mice was ~25% of maximum, and by 24 hr it was back to basal levels. The same doses of L-DOPA had no effect on locomotion of control mice (data not shown). The $DA^{-/-}$ mice responded similarly to repeated injections of L-DOPA (Figure 8A). Stereotypical movements such as grooming, sniffing, and rearing were quantified as single beam breaks in the activity cage. Normal mice produced ~700 stereotypical beam breaks per hour, whereas the $DA^{-/-}$ mice produced ~280 breaks

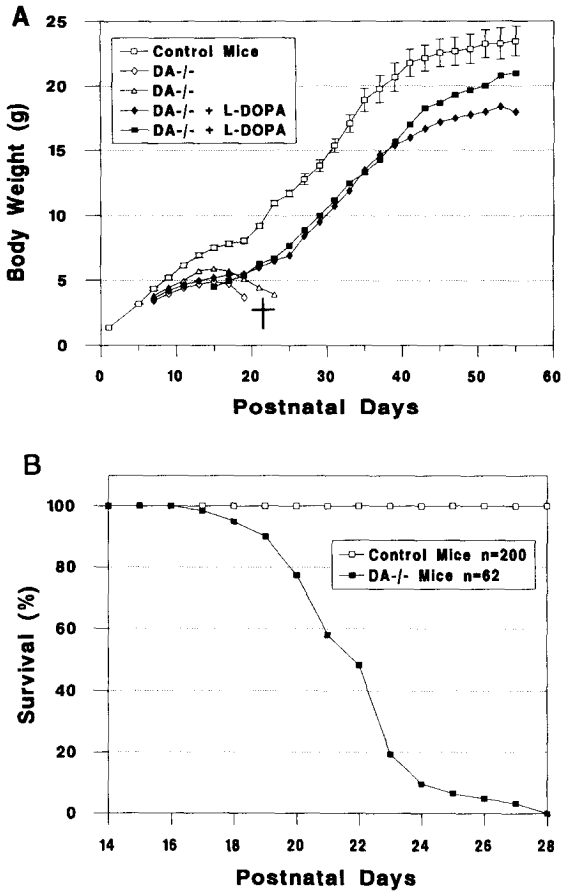

Figure 4. Postnatal Growth and Survival of DA $^{-/-}$ Mice and Rescue by L-DOPA

(A) Body weights of two untreated DA$^{-/-}$ mice and two DA$^{-/-}$ mice injected twice daily with 50 mg/kg L-DOPA, starting at day 15, are compared with weight of control mice (mean ± SEM; n = 6). Death is indicated by a cross.

(B) Survival of control and untreated DA$^{-/-}$ mice.

(Figure 8C). L-DOPA produced a slight increase in stereotypical behavior in control mice (data not shown), but a 4- to 5-fold increase in DA$^{-/-}$ mice (Figure 8C).

The amount of DA in the brains of DA$^{-/-}$ mice 1 hr after injection of L-DOPA was ~38% of that in control mice; it fell to ~16% of controls by 4 hr and was back to basal levels by 24 hr (Figure 8B). Thus, the half-life of DA in DA$^{-/-}$ mice is less than 3 hr.

The DA$^{-/-}$ mice were also tested for their ability to respond to the D2-specific agonist quinpirole and to the D1-specific agonist SKF 81297. Both compounds, at doses that either slightly increased (SKF 81297) or decreased (quinpirole) locomotion of control mice, increased locomotion of DA$^{-/-}$ mice ~40-fold, but not as much as L-DOPA (Figure 8A). Both compounds also increased stereotypical activity of DA$^{-/-}$ mice (Figure 8C). Higher doses of both quinpirole and SKF 81297 did not increase motor output further (data not shown). D1 receptor–selective antagonist SCH 23390 did not block the stimulatory effect of quinpirole in DA$^{-/-}$ mice, which dispels the possibility that the D2 agonist might be activating both D1 and D2 receptors in these mice (data not shown). Instead, these results suggest that either D1 or D2 receptor stimulation increases motor activity in DA$^{-/-}$ mice.

To assess the motor activity of DA$^{-/-}$ mice further, we tested them on a Rota rod. They were able to balance on a stationary rod for at least 3 min, even when untreated. However, when the rod was rotating at 10 rpm, they fell off within 5 s even after many trials, whereas control mice could stay on a rotating rod for 90 s (the duration of the trial) after five trials. Injection of DA$^{-/-}$ mice with L-DOPA improved their ability to balance on the rotating rod, although they were never as good as controls (Figure 8D). These results suggest that execution of more complicated motor behaviors that require integration of sensory inputs is impaired in DA$^{-/-}$ mice.

Reversal of Adipsia and Aphagia of DA$^{-/-}$ Mice by L-DOPA

Untreated DA$^{-/-}$ mice became runted and succumbed to death during postnatal week 3. DA$^{-/-}$ mice would neither drink nor eat wet or dry food even when it was placed near them. As shown in Figures 9A and 9C, DA$^{-/-}$ mice would virtually cease eating or drinking ~12 hr after the last L-DOPA injection. There was an attendant 25% loss of body weight during the next 3 days, and, if untreated, the mice would die during the next day or two (Figure 9B). Readministration of L-DOPA after 3 days of withdrawal resulted in intense consumption of food and water, which exceeded that of similarly treated control mice by ~50% during the first few days (Figures 9A and 9C), and within 3 days they regained their previous body weight (Figure 9B). Food consumption of normal mice was unaffected by L-DOPA, but it reduced water intake by approximately the amount of fluid injected (Figures 9A and 9C). For these experiments, L-DOPA was administered twice a day at 50 mg/kg. The mice consumed considerably less food when the drug was given only once a day, but they could survive on that regimen. The rates of food and water consumption were maximal during the first 2 hr after L-DOPA administration, declined to ~40% of that rate during the next 4 hr, and were virtually nil thereafter (Figure 9D). These rates parallel brain DA$^{-/-}$ content (see Figure 8B). We conclude that DA is required for ingestive behaviors.

Discussion

DA neural circuits have been studied intensively because of their involvement in movement control, drug abuse, and cognition. Our current understanding of the functions mediated by DA stems largely from studies involving lesions of specific neural tracts along with the responses of animals to DA agonists and antagonists. Classical genetics has not provided much insight. However, transgenic technologies provide new ways to apply genetics to this complicated system. Recently, mice lacking the D1 or D2 receptors have been reported (Drago et al., 1994; Xu et al., 1994a; Baik et al., 1995). Systematic inactivation of the genes for all the DA receptors will ultimately reveal the

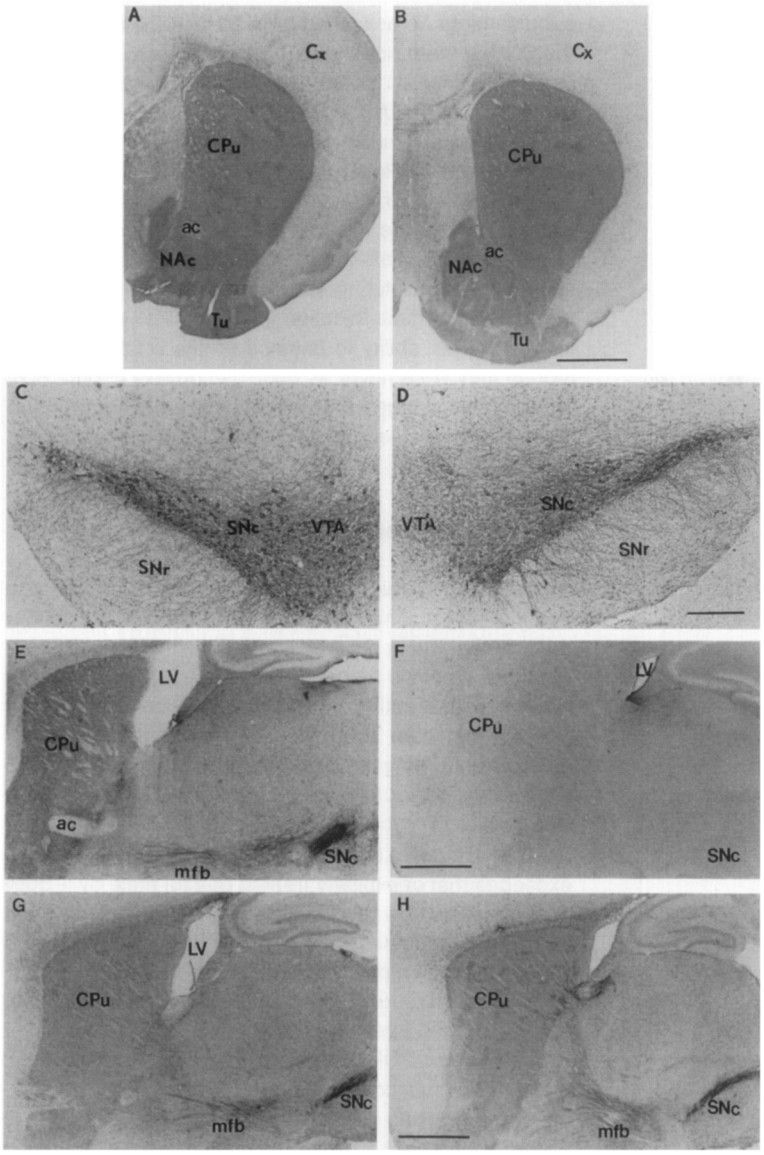

Figure 5. TH and AADC Immunostaining of Wild-Type and Mutant Brains

Sections on left are controls, and those on right are from an untreated $DA^{-/-}$ mice (P16). (A)–(D) are coronal, while (E)–(H) are sagittal. Sections were stained with antisera against AADC, except for (E) and (F), which were stained with antisera against TH. Note that normal AADC immunoreactivities were observed in both coronal and saggital sections of $DA^{-/-}$ mutant brains (B, D, and H). Abbreviations are the same as Figure 3; mfb, medial forebrain bundle. Scale bar in (A) and (B), 1 mm; in (C)–(H), 250 μm.

contribution of each receptor subtype. However, one limitation of receptor inactivation is that it is an irreversible process. Hence, functional changes in the animals could reflect either deficits in signal transduction or developmental abnormalities. As an alternative, we eliminated the ability of the mice to synthesize DA, reasoning that we might be able to rescue some or all behavioral defects by providing agonists to activate various DA receptor subtypes.

Targeting of TH Gene to the DBH Promoter

We used a gene targeting approach rather than random integration of transgenes because our previous experience with human DBH–*lacZ* transgenes suggested that, while the expression was observed in all expected cell types, there was some apparently inappropriate expression in midbrain DA neurons (Mercer et al., 1991). Our analysis of mice with TH driven by the DBH promoter indicates that TH expression is restricted to cells that are known to synthesize NE. The amount of NE produced in both the periphery and the brain is approximately normal despite the presence of only a single copy of the TH gene. Mechanisms involving transcription, stabilization of TH mRNA, or activation of TH enzymatic activity could account for the adequacy of a single DBH-TH allele. In principle, $DA^{-/-}$ mice could have been made in one step, by replacing the transcriptional regulatory elements of the TH gene with those from the DBH gene; however, the successful execution of that strategy requires knowing all the relevant regulatory elements of the DBH gene. There is a further advantage of the two-step strategy because in subsequent experiments TH can be targeted only to adrenergic or dopaminergic cells.

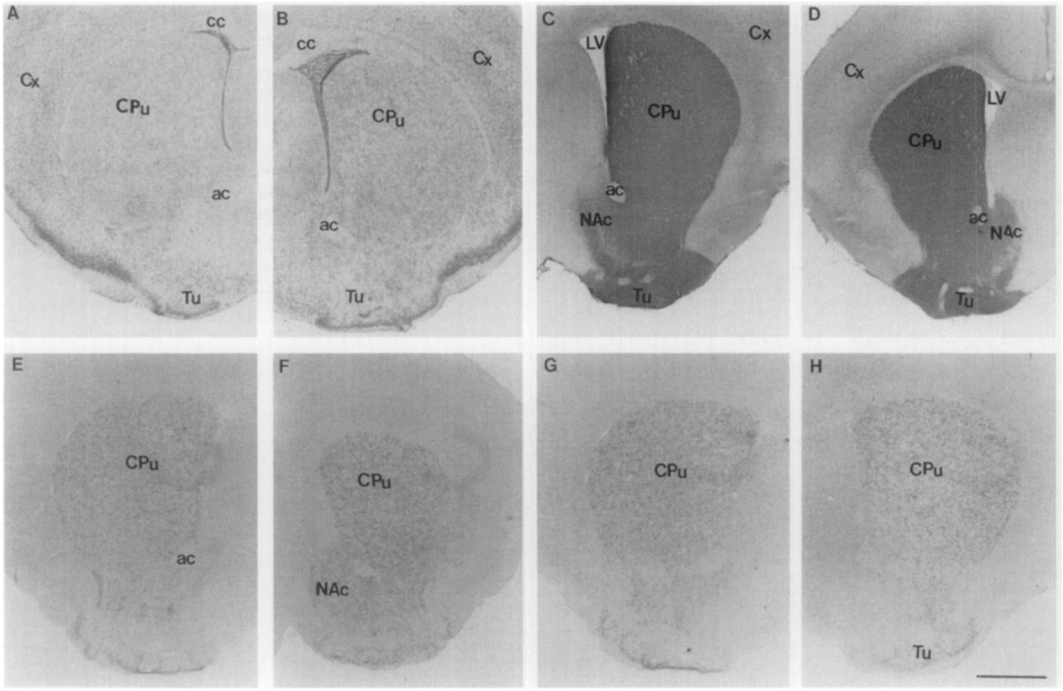

Figure 6. The Striatum of Wild-Type and DA$^{-/-}$ Mice

Low power view of the striatum from coronal brain sections of a P16 wild-type mouse (A, C, E, and G) and an untreated DA$^{-/-}$ mouse (B, D, F, and H). Sections were stained with cresyl violet (A and B), with antisera against DARPP32 (C and D), and with digoxygenin-labeled DA D1 (E and F) and D2 receptor (G and H) RNA antisense probes. The structure and morphology of the striatum are preserved in DA$^{-/-}$ mice (B, D, F, and H). The expression of DA D1 and D2 receptor mRNA and the immunostaining of DARPP32 are normal (D, F, and H). Abbreviations: cc, corpus callosum; other abbreviations as Figure 3. Scale bar, 1 mm.

Essentially Normal Development of Dopaminergic and Striatal Neurons in DA$^{-/-}$ Mice

Dopaminergic neurons arise in the ventral midbrain of the mouse at E10 in response to an inductive signal (Sonic hedgehog) from the underlying floorplate (Hynes et al., 1995). During the next few days, DA neurons extend axons toward the developing striatum. By E14 (in the rat), this nigrostriatal tract reaches the striatal anlage at a time when only a few of the striatal neurons have been formed (Bayer, 1984; van der Kooy and Fishell, 1987). mRNAs for both D1 and D2 receptors and D2 ligand binding are already evident in the striatal primordium (Schambra et al., 1994). Thus, the ability to synthesize DA and a receptor system capable of responding to DA are present at the time that nigrostriatal connections are developing. Because dopaminergic neurons also express D2 receptors, ablating DA signaling could affect the development of dopaminergic neurons as well as striatal neurons or nigrostriatal synaptogenesis.

The initial impression is that the number of DA neurons, their projections to striatum, and the morphological organization of the striatum are normal, except that the levels of substance P and dynorphin in the striatum are reduced. Most importantly, when L-DOPA is administered to naive DA$^{-/-}$ mice, they respond dramatically, suggesting that functional connections have been established. Thus, we tentatively conclude that DA-mediated signaling is not required for either neurogenesis of the striatum or the establishment of functional nigrostriatal connections. This conclusion is reinforced by previous studies on D1 and D2 receptor knockout mice, perinatally 6-OHDA-lesioned rats, and transplantation of embryonic striatum into DA-depleted adult brain of mice (Liu et al., 1992a; Lauder, 1993; Drago et al., 1994; Xu et al., 1994a; Baik et al., 1995). However, it must be tempered by the possibility that some DA may reach the developing brain. Small amounts of circulating maternal DA could enter fetal circulation (Thomas et al., 1995) and be taken up by developing DA neurons. Alternatively, because DA$^{-/-}$ mice produce DA as a precursor of NE in noradrenergic neurons, some might be secreted and utilized by DA neurons. Our direct chemical measurements of DA in the brain of untreated P16 DA$^{-/-}$ mice indicate that it is <3% of that in normal mice.

DA and Activity

The observation that DA$^{-/-}$ mice are severely hypokinetic is consistent with akinesia in Parkinson's disease, hypoactivity resulting from blockade of DA receptors, or destruction of DA neurons by neurotoxins (Hornykiewicz, 1966; Ungerstedt, 1971; Arnt, 1987; Clark and White, 1987; Kopin and Markey, 1988; Zigmond and Stricker, 1989). The observation that neonatal DA$^{-/-}$ mice are indistinguishable from their littermates by postnatal week 1 indi-

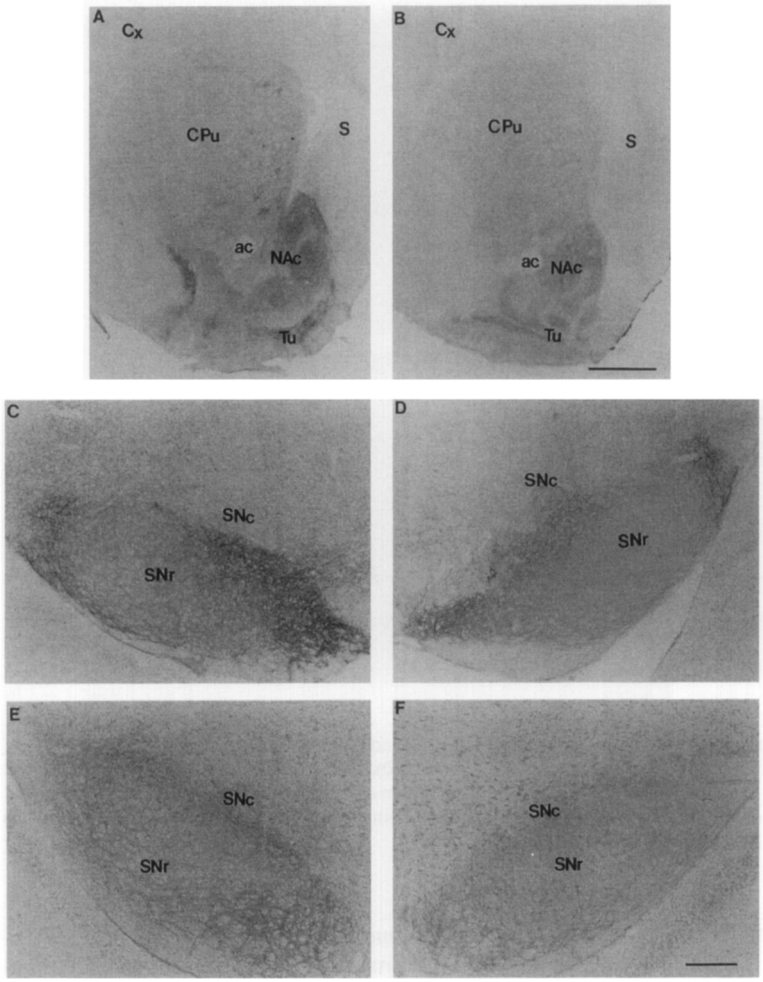

Figure 7. Substance P and Dynorphin Immunostaining of Wild-Type and Mutant Brains

Coronal sections of P16 wild-type (A) and untreated $DA^{-/-}$ (B) brain were cut at the striatal level, whereas the wild type (C and E) and $DA^{-/-}$ (D and F) were at midbrain level. (A)–(D) were stained with antisera against substance P, whereas (E) and (F) were stained with antisera against dynorphin B. Abbreviation: S, septum. Other abbreviations as in Figure 3. Scale bar in (A) and (B), 1 mm; in (C)–(F), 250 μm.

cates that the limited movements that neonates make are not dependent upon DA. Thus, dopaminergic pathways achieve dominant control of activity only after the first week or so. Although detailed dose response experiments have not been performed with dopaminergic agonists, we predict that DA receptors in $DA^{-/-}$ mice will be hypersensitive compared with control mice because they would not be down-regulated. The excessive motor activities of $DA^{-/-}$ mice during the first hour after treatment with dopaminergic agonists could reflect an increased number of DA receptors that can be activated, as well as increased efficacy of G protein activation.

Despite considerable evidence supporting the requirement of D1 receptor stimulation for D2 receptor–mediated neurophysiological and behavioral responses (Clark and White, 1987; Waddington and Daly, 1993; Xu et al., 1994b), we observed a significant improvement of locomotion and stereotypy in $DA^{-/-}$ mice with low doses of a D2 agonist, indicating that D1 stimulation is not required for D2-mediated behaviors in these mice. The similar response of these mice to both D1 and D2 agonists is consistent with models in which D1 and D2 receptors are incorporated into parallel pathways (striatonigral and striatopallidal) such that opposite biochemical effects upon their activation result in similar neurophysiological and behavioral outputs (Albin et al., 1989; Alexander and Crutcher, 1990; Gerfen, 1992). This model, with two parallel output pathways, predicts that mice lacking either D1 or D2 receptors would have essentially normal basal motor activities. The D1 receptor knockout mice display either normal (Drago et al., 1994) or 2- to 3-fold enhanced activity (Xu et al., 1994a, 1994b), whereas D2 receptor knockout mice manifest 4-fold reduced locomotor activity (Baik et al., 1995). The combined results suggest that both motor output pathways can work independently, but synergy is achieved by coordinated activation of both pathways. The performance of $DA^{-/-}$ mice in more complicated tasks, such as balance on a Rota rod, which requires the integration of sensory inputs, was improved by the drug treatments, but not to normal levels. Perhaps this reveals an underlying developmental abnormality caused by DA deficiency before treatment with L-DOPA began.

L-DOPA-responsive dystonias (DRDs) result from point mutations in either the TH gene or the cyclohydrolase gene required for synthesis of the biopterin cofactor (Ludecke et al., 1995; Nygaard, 1995). These mutations impair DA synthesis and thus reflect milder forms of the murine deficiency we have created. Like $DA^{-/-}$ mice, but unlike Par-

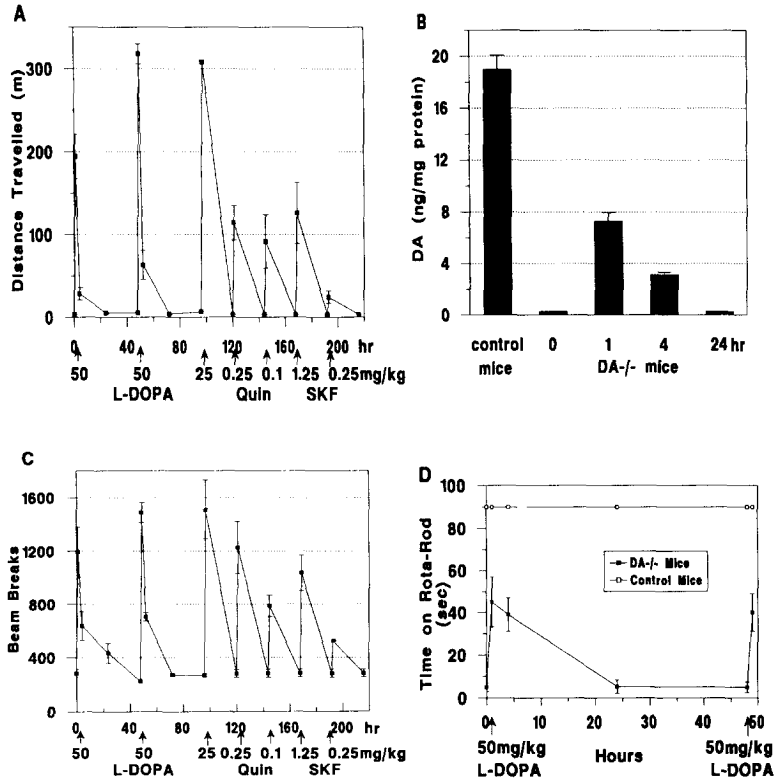

Figure 8. Locomotor and Stereotypical Activity of $DA^{-/-}$ Mice in Response to L-DOPA, Quinpirole, and SKF 81297

(A) Ambulation. Locomotion of $DA^{-/-}$ mice (mean ± SEM; n = 6) in a new environment is represented as the total distance traveled in 60 min during the light phase. Drugs administered are indicated by arrows. The effects of L-DOPA were measured 1, 4, 24, and 48 hr after drug administration, whereas the effects of quinpirole (Quin) and SKF 81297 were measured 15 min after injection because these agonists do not require metabolic activation.

(B) Stereotypy. Stereotypy index was calculated by subtracting ambulatory counts from the total activity counts.

(C) DA content. DA contents of whole brain of untreated control mice (n = 6) and $DA^{-/-}$ mice before (n = 1) and 1 hr (n = 2), 4 hr (n = 2), and 24 hr (n = 1) after 50 mg/kg L-DOPA injection were measured as in Figure 2.

(D) Rota rod. The time that control mice (n = 6) and $DA^{-/-}$ mice (n = 6) stayed on the Rota rod during a 90 s trial (10 rpm) was recorded.

kinson's patients, DRD patients respond to low doses of L-DOPA over a long period of time because DA neurons are intact. However, the twisting postures and movements that are characteristic features of DRD are not apparent in the $DA^{-/-}$ mice. Likewise, tremor, one of the hallmarks of Parkinson's disease, is present in MPTP-treated primates, but is not recognized in $DA^{-/-}$ mice or in MPTP- or 6-OHDA-treated rodents (Bergman et al., 1990). These observations probably reflect underlying differences in the utilization of dopaminergic pathways in rodents and primates.

DA and Feeding Behavior

Pioneering studies by Anand and Brobeck (1951) revealed that bilateral electrolytic lesions of the lateral hypothalamus (LH) in rats produced profound aphagia, adipsia, and akinesia. The hypothesis that LH is an important center for feeding was further supported by the observations that lesions of intrinsic LH glutamatergic neurons by kainate or ibotenic acid produced persistent adipsia and aphagia, that electrical stimulation of LH stimulated feeding in satiated rats, and that electrical activities of hypothalamic neurons was correlated with feeding and drinking behavior (Grossman et al., 1978; Rolls, 1981; Winn et al., 1984). Early evidence that DA might be a critical player in the LH syndrome came from the demonstration that most symptoms of LH ablation could be reproduced by injecting 6-OHDA into the LH or midbrain dopaminergic neurons of adult rats (Ungerstedt, 1971; Zigmond and Stricker, 1972; Fibiger et al., 1973). Surprisingly, especially in view of our results, neonatal lesions of rats with 6-OHDA have relatively mild effects on behavior and electrophysiology, despite >99% depletion of DA (Onn et al., 1990). Pharmacological experiments tend to support a role for DA in feeding in that DA agonists stimulate, whereas antagonists inhibit, feeding and drinking (Phillips and Nikaido, 1978; Dourish, 1983; Salamone et al., 1990), although contrary results have been obtained (Cooper and Al-Nasar, 1993). Relatively mild feeding deficits were observed in D1 or D2 receptor–deficient mice, and they grew to 70% or 85% of normal body weight, respectively (Drago et al., 1994; Xu et al., 1994a; Baik et al., 1995). We provide compelling evidence that DA is essential for feeding and drinking behavior. When $DA^{-/-}$ mice are born, they initiate suckling behaviors and nurse effectively enough to almost triple their birth weight. However, after 2 weeks, when normal mice would begin to explore other sources of nourishment, the $DA^{-/-}$ mice become lethargic and fail to eat and drink.

The role that DA plays in feeding and drinking behaviors is not established. Dopaminergic pathways could be required for the perception of hunger and thirst. Multiple connections between LH and midbrain DA neurons have been identified (Bunney and Aghajanian, 1976; Phillipson, 1979; Wright et al., 1980). Dopaminergic innervation might control the responsiveness of LH to interoceptive stimuli (Marrocco et al., 1994); i.e., dopaminergic tone could determine the threshold of hunger and thirst. In the absence of dopaminergic stimulation, the threshold may become so high that $DA^{-/-}$ mice do not perceive a need to eat or drink. There is evidence supporting a role of DA in determining the responsiveness to external stimuli (Braff and Geyer, 1990).

Alternatively, DA could be required for the motor execu-

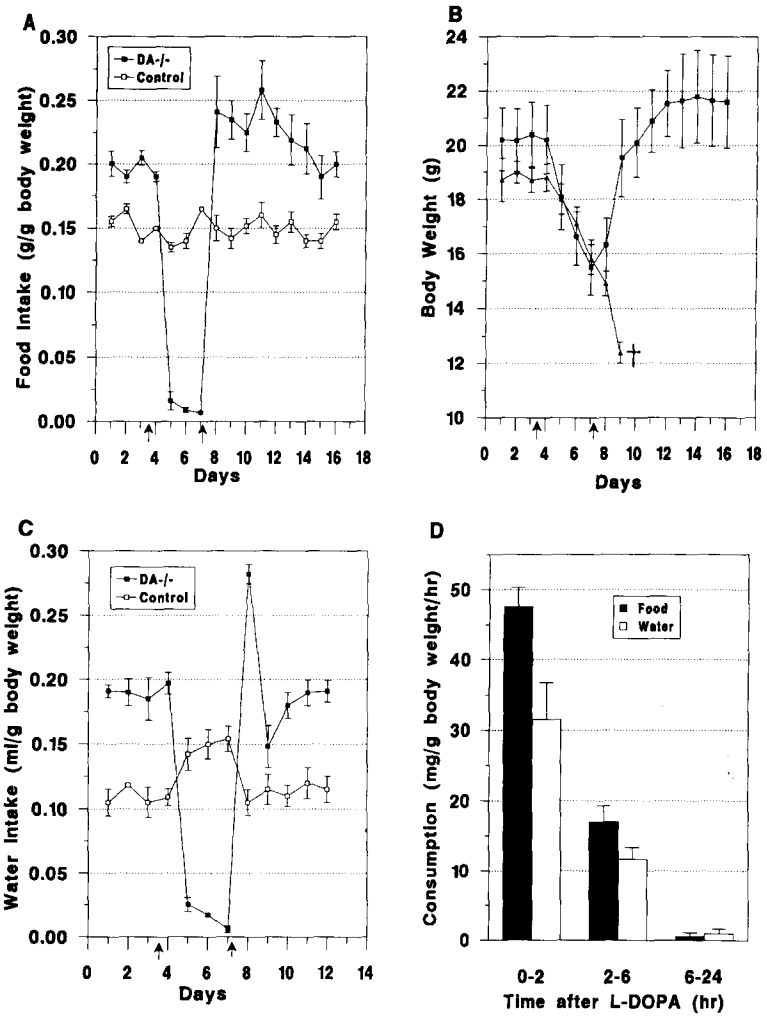

Figure 9. Adipsia and Aphagia of $DA^{-/-}$ Mice and Reversal by L-DOPA

(A) Comparison of food intake of $DA^{-/-}$ mice (mean ± SEM; n = 6) and control mice (n = 6) with or without L-DOPA. L-DOPA was not administered between the two arrows. Vehicle injection did not stimulate feeding in $DA^{-/-}$ mice.

(B) Body weights of the same group of $DA^{-/-}$ mice represented in (A) are shown. A different group of mice (n = 5) was tested for their ability to survival without L-DOPA. The cross indicates their death.

(C) Comparison of water intake of $DA^{-/-}$ mice (n = 6) and control mice (n = 6) with or without L-DOPA. L-DOPA was not given between the two arrows. Vehicle injection did not stimulate drinking in $DA^{-/-}$ mice.

(D) Consumption of food and water by $DA^{-/-}$ mice (n = 6) after single L-DOPA injection.

tion of feeding in response to signals from the hypothalamus. Regional 6-OHDA lesions in the ventrolateral striatum (but not other regions of the striatum) depress feeding transiently (Jicha and Salamone, 1991). Transplantation of nigral tissue into the striatum of neonatal rats protected against adipsia and aphagia when the endogenous nigrostriatal tracts were subsequently lesioned by 6-OHDA in adults (Schwarz and Freed, 1987). However, the placement of the grafts was critical, presumably to allow effective connections with the hypothalamus (Rogers et al., 1990). Furthermore, partial lesions of the striatum by kainate depressed eating and drinking, and bilateral lesions of nuclei globus pallidus and mesencephalic reticular formation caused aphagia and adipsia (Morgane, 1961; Parker and Feldman, 1967; Pettibone et al., 1978). The combined results of these experiments suggest that normal execution of feeding and drinking behavior requires the interaction of intrinsic LH neurons, dopaminergic neurons, striatal neurons, and neurons of midbrain reticular formation. An important question is whether adipsia or aphagia is simply secondary to motor deficits. Although we cannot answer this question definitively, we think it is unlikely because $DA^{-/-}$ mice can grasp food and swallow liquid food when put in their mouths.

A third possibility is that the adipsia and aphagia in the $DA^{-/-}$ mice is related to deficiency in reward mechanism mediated by the mesolimbic dopaminergic systems. The application of three reward study procedures (intracranial self-stimulation, intravenous self-administration, and conditioned place preference) has generated a substantial body of evidence indicating that mesolimbic DA neurons are an important link in the neural circuitry of reward (reviewed by Fibiger and Phillips, 1988; Koob, 1992; Wise and Hoffman, 1992). Hebb (1955) suggested that brain reward circuits may have evolved to reinforce survival behaviors such as feeding. Supporting evidence exists that the mesolimbic dopaminergic system is involved in food-rewarded behavior. For example, feeding stimulates DA turnover in the nucleus accumbens (Hernandez and Hoebel, 1988a, 1988b; Smith and Schneider, 1988; Joseph and Hodges, 1990), DA receptor blockers impair the reinforcement value of food (Wise et al., 1978), and inhibition of TH activity in the ventral tegmental area with antisense oligonucleotides inhibits food pellet–conditioned operant behavior (Skutella et al., 1994). Thus, in this scenario, eating and drinking are not perceived as rewarding experiences and are subsequently extinguished in $DA^{-/-}$ mice.

Other techniques will be necessary to determine which dopaminergic pathways are involved in the various behavioral deficits; however, these $DA^{-/-}$ mice provide ideal recipients for intracranial delivery of DA agonists, DA-producing cells, or TH genes to specific brain regions.

Experimental Procedures

Immunohistochemistry and In Situ Hybridization

For immunohistochemical studies, untreated mice 15–21 days old were sacrificed by CO_2 inhalation. Brains were fixed in 4% paraformaldehyde for 6–24 hr at 4°C and soaked in 20% sucrose overnight, frozen, and cut on a freezing microtome at 20–30 μm. Free-floating sections were stained according to standard streptoavidin immunohistochemical procedures (Xu et al., 1994a). Primary antisera included the following: monoclonal mouse antiserum against TH diluted 1:1000 (IncStar Corporation), monoclonal mouse antiserum against DARPP32 diluted 1:10,000, polyclonal rabbit antiserum against substance P diluted 1:5,000, polyclonal rabbit antiserum against rat dynorphin B diluted 1:2,000, polyclonal rabbit antiserum against met-enkephalin diluted 1:1000, and polyclonal rabbit antiserum against AADC diluted 1:2000. If no primary antibody was applied, no specific immunostaining was observed. In situ hybridization with digoxygenin-labeled D1 and D2 riboprobes was performed according to the method of Wilkinson and Nieto (1993).

Rescue, Feeding, and Drinking Experiments

L-DOPA (Sigma) was dissolved in saline solution (1.5 mg/ml) containing 0.25% ascorbic acid as antioxidant, filtered, aliquoted, and kept at −20°C. $DA^{-/-}$ pups, which were usually noticed by P14, were injected intraperitoneally twice a day at a dose of 50 mg/kg or 33 ml/kg body weight. Body weights were measured daily, and genotypes were determined by Southern blotting. Food and water were available ad lib; water and food consumption during were calculated from differential weights.

Motor Behavior

For locomotion activities, two to four mice (7–11 weeks of age) were placed in a transparent Plexiglas cage (28 cm × 17 cm × 11.5 cm) for 1 hr of habituation. Animal activity was monitored in an Opto-Vari system (Columbus Instruments) during the light phase of the light–dark cycle. Stereotypy was calculated by subtracting the ambulatory counts from the total counts. Distance traveled was calculated by multiplying the ambulatory counts by 2.65 cm (the distance between beams). The motor stimulatory effects of L-DOPA, quinpirole, and SKF 81297 (Research Biochemical Incorporated) were tested at various times after injection. The same group of mice was used for all three drugs. Experiments with each drug were separated by at least 3 days, and the mice were maintained with L-DOPA between experiments. Testing was begun 16 hr after last injection of L-DOPA. Quinpirole and SKF 81297 were administered intraperitoneally in 10 ml/kg body weight. For the Rota rod (rod, 4 cm in diameter; manufactured by Jones and Roberts) experiments, control and $DA^{-/-}$ mice were injected with L-DOPA and trained four times during the next hour at 10 rpm prior to the experiment.

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