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Dopamine D1 Receptor Mutant Mice Are Deficient in Striatal Expression of Dynorphin and in Dopamine-Mediated Behavioral Responses

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Summary

The brain dopaminergic system is a critical modulator of basal ganglia function and plasticity. To investigate the contribution of the dopamine D1 receptor to this modulation, we have used gene targeting technology to generate D1 receptor mutant mice. Histological analyses suggested that there are no major changes in general anatomy of the mutant mouse brains, but indicated that the expression of dynorphin is greatly reduced in the striatum and related regions of the basal ganglia. The mutant mice do not respond to the stimulant and suppressive effects of D1 receptor agonists and antagonists, respectively, and they exhibit locomotor hyperactivity. These results suggest that the D1 receptor regulates the neurochemical architecture of the striatum and is critical for the normal expression of motor activity.

Introduction

Dopamine is the principal neurotransmitter in four major neural systems in the brain (Dahlström and Fuxe, 1964; Fallon and Moore, 1978; Graybiel and Ragsdale, 1983; Lindvall and Björklund, 1983). The largest of these is the nigrostriatal pathway, which originates from dopamine-synthesizing neurons of the midbrain substantia nigra complex and innervates the dorsal striatum (caudoputamen). Degeneration of this pathway leads to Parkinson's disease (Hornykiewicz, 1966). The mesolimbic system, relatively spared in Parkinson's disease, arises in the midbrain ventral tegmental area and innervates the ventral striatum (nucleus accumbens and olfactory tubercle) and parts of the limbic system. This system is thought to influence motivated behaviors, including activity related to reward (Koob and Bloom, 1988; Koob, 1992a, 1992b). The ventral tegmental area also gives rise to the smaller mesocortical pathway, which innervates part of the frontal cortex, and may be involved in certain aspects of learning and memory (Le Moal and Simon, 1991). Together, these dopaminergic systems are major targets for the neuropharmacological actions of psychomotor stimulants such as cocaine and amphetamine (Graybiel et al., 1990a; Koob, 1992a; Nestler et al., 1993). The fourth dopamine-containing pathway is the hypothalamic tuberoinfundibular system, which projects mostly to the pituitary stalk and regulates prolactin secretion (Fuxe et al., 1969).

Dopamine receptors are strongly expressed in the targets of these pathways and on many dopamine-containing neurons as well. The dopamine receptors so far cloned fall into two classes, the D1-like ($D1_A$ and D5 or $D1_B$) and the D2-like (D2, D3, and D4) receptors (Gingrich and Caron, 1993; Civelli et al., 1993). These classes were originally differentiated by the pharmacological approach and in terms of their positive (D1-like) or negative (D2-like) coupling to cAMP. Other second messenger systems have also been implicated in their actions (Sibley et al., 1993; Kebabian and Calne, 1979).

Although there is some differential distribution of these dopamine receptor subtypes in the brain (e.g., Sokoloff et al., 1990), the distributions overlap extensively, especially in the main dopamine-containing systems arising in the midbrain. The overlap extends to the cellular level (e.g., Meador-Woodruff et al., 1991; Surmeier et al., 1993). This intermixing of expression patterns and the lack of antagonists uniquely selective for each of the cloned receptors have hampered attempts to establish differential functions for the different receptors. For example, selective antagonists of D1-like and D2-like receptors block the psychomotor activation produced by indirect sympathomimetics (Mailman et al., 1984; Cabib et al., 1991) and the reinforcing effects of cocaine (Woolverton, 1986; Koob et al., 1987; Bergman et al., 1990; Caine and Koob, 1994) and produce decreases in locomotor behavior, in exploration, and in activation associated with rewards in general (Koob, 1992a, 1992b). Further, there is some evidence of more mesolimbic-like actions of D1 antagonists and more nigrostriatal-like actions of D2 antagonists, but any such differentiation is observed only at the low end of the dose effect functions (Amalric et al., 1993). We therefore turned to the gene targeting technique (Capecchi, 1989) for a more precise and selective means of assessing the specific actions of particular dopamine receptor subtypes.

To study the functions of the D1 receptor, we generated D1-deficient mice and studied the effects of the mutation on the anatomy of the brain and on locomotor function. We found that the brains of the mutants were generally well formed, but that abnormalities were present in the expression of dynorphin in the striatum and its output pathways and in the neurochemical compartmentalization of the striatum into striosomes and matrix. In addition, the mutant mice did not respond to the stimulant and suppressive effects of D1 agonists and antagonists, respectively, and exhibited increased locomotor activity. These findings suggest that the D1-deficient mouse could serve as a valuable tool in analyzing the functions of dopamine neurotransmission in the brain.

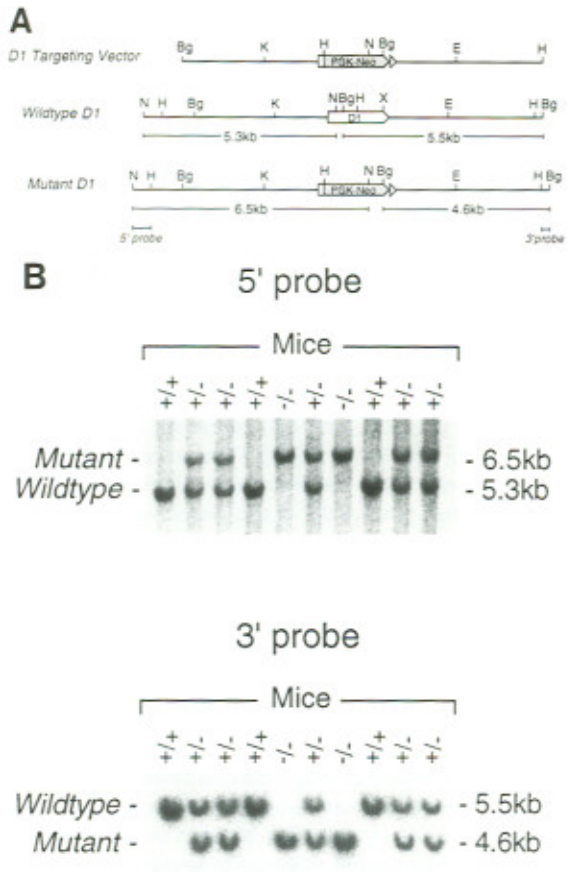

Figure 1. Generation of D1-Deficient Mice

(A) The D1 targeting construct, wild-type, and mutant loci of the mouse D1 gene. The open box represents the entire D1 gene. The stippled box depicts the *neo* gene driven by a phosphoglycerate kinase (PGK) promoter. The solid line represents extragenic sequences of the D1 gene. 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: Bg, BglII; E, EcoRI; H, HindIII; K, KpnI; N, NcoI; X, XbaI.

(B) Genomic Southern blot analyses of tail biopsies. Genomic DNA was isolated from a litter of ten pups from one heterozygous breeding pair. DNA was digested either with NcoI and hybridized with a 5′ probe (top) or with BglII and hybridized with a 3′ probe (bottom). The genotype of each pup is indicated.

Results

Generation of D1-Deficient Mice

Oligonucleotide primers with sequences chosen from the rat and human D1 gene sequences were used to clone a piece of the mouse D1 gene (Dearry et al., 1990; Monsma et al., 1990; Sunahara et al., 1990; Zhou et al., 1990). An 880 bp DNA fragment was judged to be part of the mouse D1 gene sequence (data not shown). We performed Southern blot analysis of mouse genomic DNA using this mouse D1 DNA fragment as the probe and found that D1 is a single copy gene in the mouse genome (data not shown). The D1 probe was also used to screen a mouse 129 genomic library to obtain the entire mouse D1 gene and its flanking sequences. A restriction map of the mouse D1 gene is shown in Figure 1A. Sequence analysis indicates that the mouse D1 gene, like its rat and human counterparts, is intronless (data not shown).

The D1 gene targeting construct used is shown in Figure 1A. This targeting construct was designed to delete 95% of the D1 gene coding sequence and to replace it with a *neo* gene. Five embryonic stem (ES) cell clones harboring the desired homologous recombination were identified, ES cells amplified from three of the homologous recombinants were used to generate male chimeric mice, and these were bred extensively with C57B6 females to obtain mice homozygous for D1 deletion, as identified by genomic Southern blot analysis (Figure 1B).

The D1-deficient mice appeared healthy and had no gross abnormalities. They, however, were smaller than their wild-type littermates. For instance, 10-week-old adult mice (n = 46) weighed, on the average, 30% less than the wild types (n = 46). Because of the reduced body weight, the mutant mice were weaned 3–5 days later than their wild-type littermates. The litter sizes of the mutants seemed normal, and there was no obvious sex bias in the offspring. However, the genotype distribution of the first 100 offspring produced by heterozygous breeding was 38 wild-type mice, 37 heterozygous mutants, and 25 homozygous mutants, suggesting a significant deviation from Mendelian segregation.

Brain Architecture in the Mutant Mice

The general anatomy of the mutant brains appeared normal in Nissl-stained sections. The hindbrain and forebrain were well developed, and the major forebrain targets of the dopaminergic pathways were without obvious anatomical defects. The size of the mutant brains was reduced relative to that of the wild types, however, and the striatum, the target of the nigrostriatal tract, was also reduced in size (e.g., Figures 2 and 4).

Dopamine-Containing Systems Survive in D1 Mutant Mice

Autoradiographic ligand binding with a selective D1 receptor antagonist, [^{3}H]SCH23390, showed that D1-binding sites were absent in the brains of the D1-deficient mice (Figures 2A–2D). Despite the absence of D1 ligand binding, the main dopamine-containing systems of the brain were present in the mutant mice, as judged by immunostaining for the catecholamine synthesizing enzyme, tyrosine hydroxylase (Figures 2E and 2F). Autoradiographic [^{3}H]mazindol labeling for dopamine uptake sites confirmed the persistence of a dense nigrostriatal innervation (Figures 2G and 2H).

Abnormal Dynorphin Expression in the Mutant Striatum

Because the striatum normally contains the highest concentrations of D1-like receptors in the brain, we analyzed this region in particular detail. We used three approaches to determine the effects of the mutation. We first stained sections for dopamine and adenosine 3′,5′-monophos-

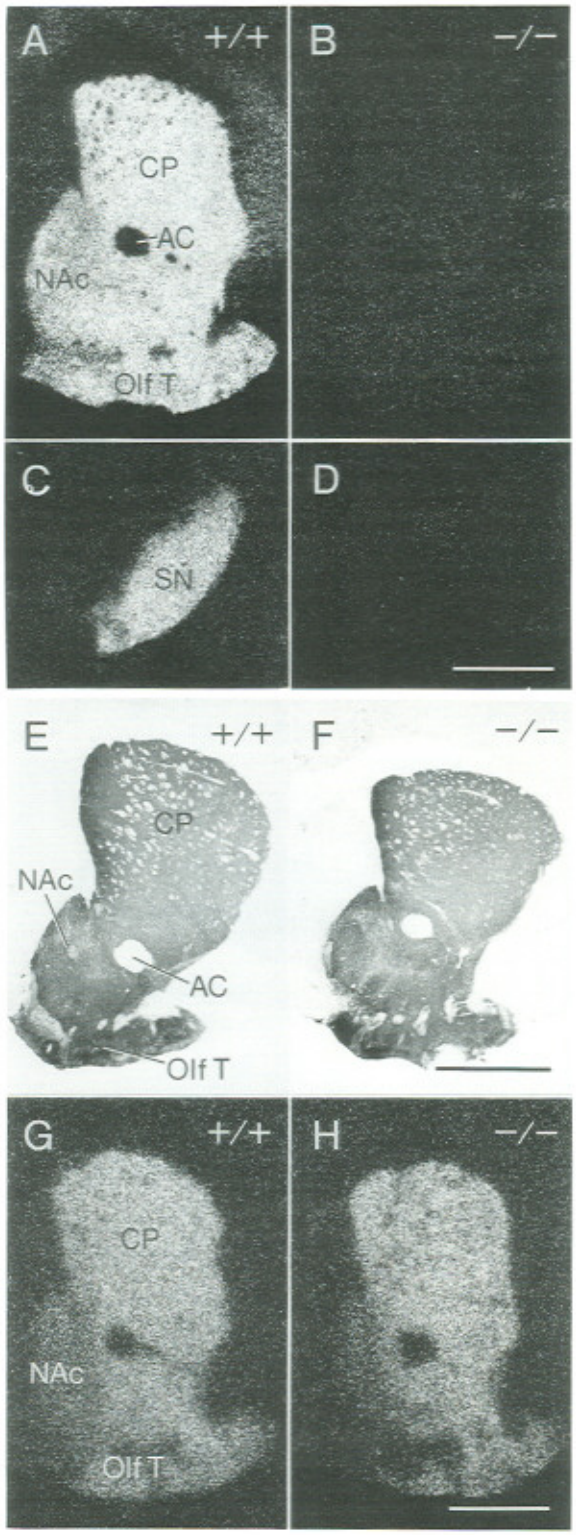

Figure 2. D1 Ligand Binding Autoradiography in the Striatum and Substantia Nigra and Histological Markers for the Dopamine-Containing Innervation of the Striatum

(A–D) Transverse brain sections through wild-type (+/+) (left) and mutant (−/−) (right) brains processed with the D1-like receptor-preferring ligand [^{3}H]SCH23390. (A) and (B) show sections through the striatum. (C) and (D) illustrate sections through the substantia nigra.

(E–H) Sections through the striatum of wild-type (+/+) (left) and mutant (−/−) (right) brains showing tyrosine hydroxylase immunoreactivity (E and F) and [^{3}H]mazindol binding for dopamine uptake sites (G and H).

Abbreviations: CP, caudoputamen; AC, anterior commissure; NAc, nucleus accumbens; Olf T, olfactory tubercle; SN, substantia nigra. Scale bars indicate 1 mm.

phate–regulated phosphoprotein (DARPP-32), a molecule expressed by many striatal projection neurons and considered as a relatively selective marker for striatal neurons bearing D1-like receptors (Walaas and Greengard, 1984; cf. Snyder et al., 1992; Le Moine et al., 1991; Anderson and Reiner, 1991). There was widespread DARPP-32 staining in the mutant striatum, as well as in other sites of known DARPP-32 expression, such as the deep cortical layers (data not shown). The levels of DARPP-32 expression were not analyzed quantitatively. However, this result suggests that many striatal neurons of the type that normally bear D1 receptors survive in the mutant mice and can express a molecule regulated by D1 receptors.

D1-like and D2-like receptors are thought to be expressed preferentially, but probably not exclusively, in different subsets of γ-aminobutyric acidic (GABAergic) striatal projection neurons (Gerfen, 1992; Gerfen et al., 1990; Meador-Woodruff et al., 1991; Steiner and Gerfen, 1993; Le Moine et al., 1991; cf. Surmeier et al., 1992, 1993). D1-like receptors are preferentially expressed on the projection neurons coexpressing the neuropeptides dynorphin and substance P. D2-like receptors are strongly expressed by projection neurons coexpressing the neuropeptide enkephalin. We stained sets of striatal sections for dynorphin immunoreactivity and others for enkephalin immunoreactivity to differentiate between these cell types.

There was a striking difference between the mutant and wild-type brains in the immunostaining for dynorphin in the striatum (Figures 3–5). In the wild-type mice, the caudoputamen showed characteristic clusters of dynorphin-immunoreactive neurons (Figure 4A; Figure 5A). These clusters correspond to the neurochemically specialized striosomes of the striatum (Graybiel, 1990; Gerfen, 1992). There were also dispersed dynorphin-immunoreactive neurons in the matrix. These were especially clear in the medial and caudolateral caudoputamen and in the ventral part of the nucleus that merges with the strongly dynorphin-positive ventral striatum.

In the mutant mice, dynorphin-positive cell clusters in the caudoputamen were undetectable, or nearly so (Figure 4B; Figure 5B). The loss of dynorphin-positive cell clusters seemed particularly pronounced because there were dynorphin-immunoreactive neurons medially and caudolaterally in the caudoputamen (Figure 4B). However, there were also fewer than normal dynorphin-positive neurons dispersed through the matrix of the mutant caudoputamen (Figures 4A and 4B). Dynorphin immunoreactivity appeared lowered in the entopeduncular nucleus and substantia nigra, known targets of the dynorphin D1 receptor–expressing neurons of the striatum (see Figure 6). Levels of dynorphin immunostaining in the ventral striatum were difficult to judge because of the high intensity of the stain-

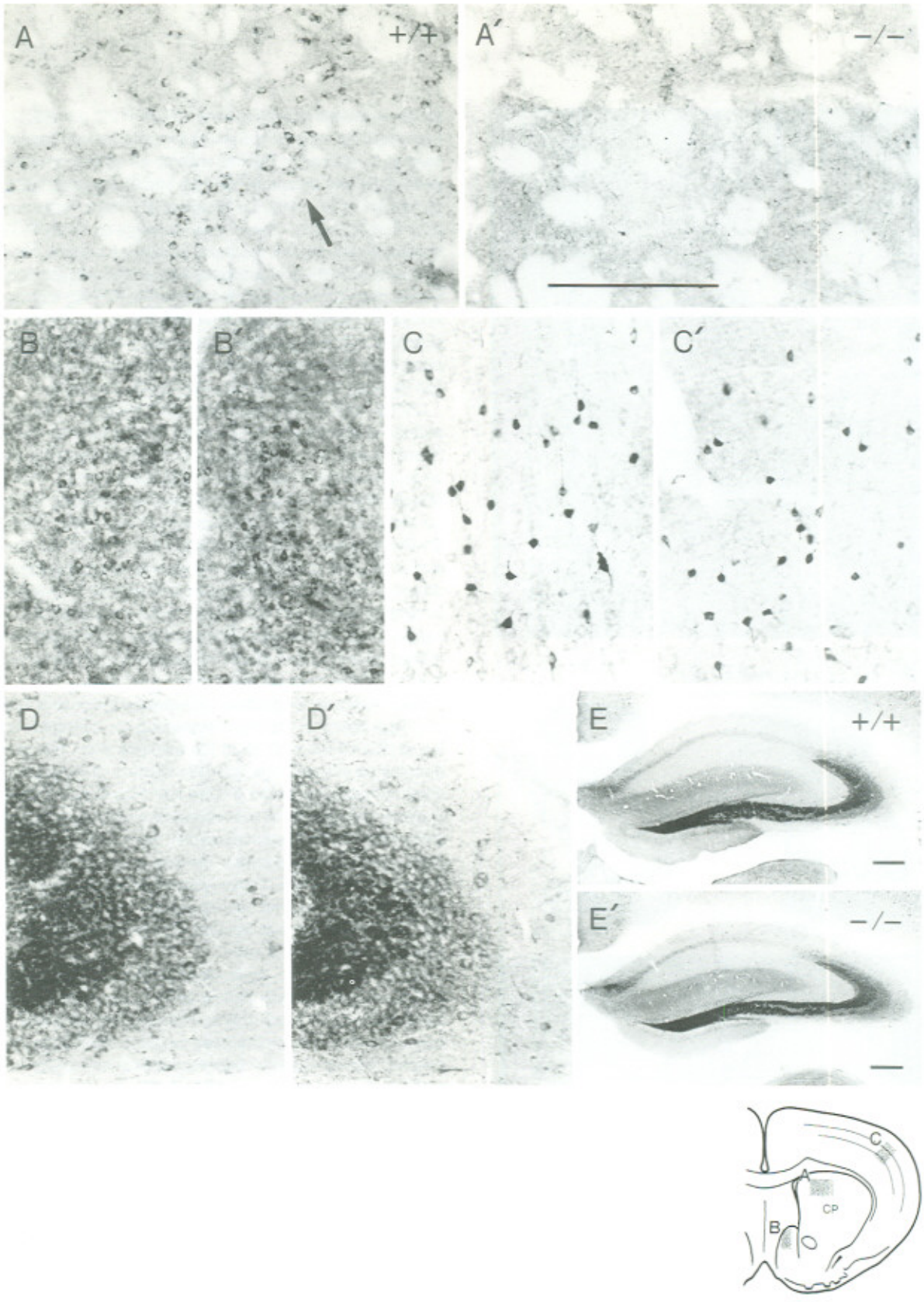

Figure 3. Staining for Dynorphin Immunoreactivity in the Striatum and Other Brain Regions of Mutant and Wild-Type Brains Obtained with Polyclonal Antiserum Raised against Dynorphin B1-13

The diagram at lower right shows approximate sites illustrated in the caudoputamen (A and A′), nucleus accumbens (B and B′), and cerebral cortex (C and C′). The hippocampus is shown in (D) and (D′) and at lower magnification in (E) and (E′). In all but (E) and (E′), wild type (+/+) is on the left, mutant (−/−) on the right. In (E) and (E′), wild type is on the top. Sections selected from the wild-type and the mutant brains were at approximately matched levels. Photomicrograph in (A) illustrates a cluster of dynorphin-immunoreactive neurons in the caudoputamen (arrow). Such clusters were not detected in the mutant caudoputamen (A′). Scale bar for (A)–(D′) (shown in [A′]) is 200 μm; scale bar for (E) and (E′) (shown in [E′]) is also 200 μm.

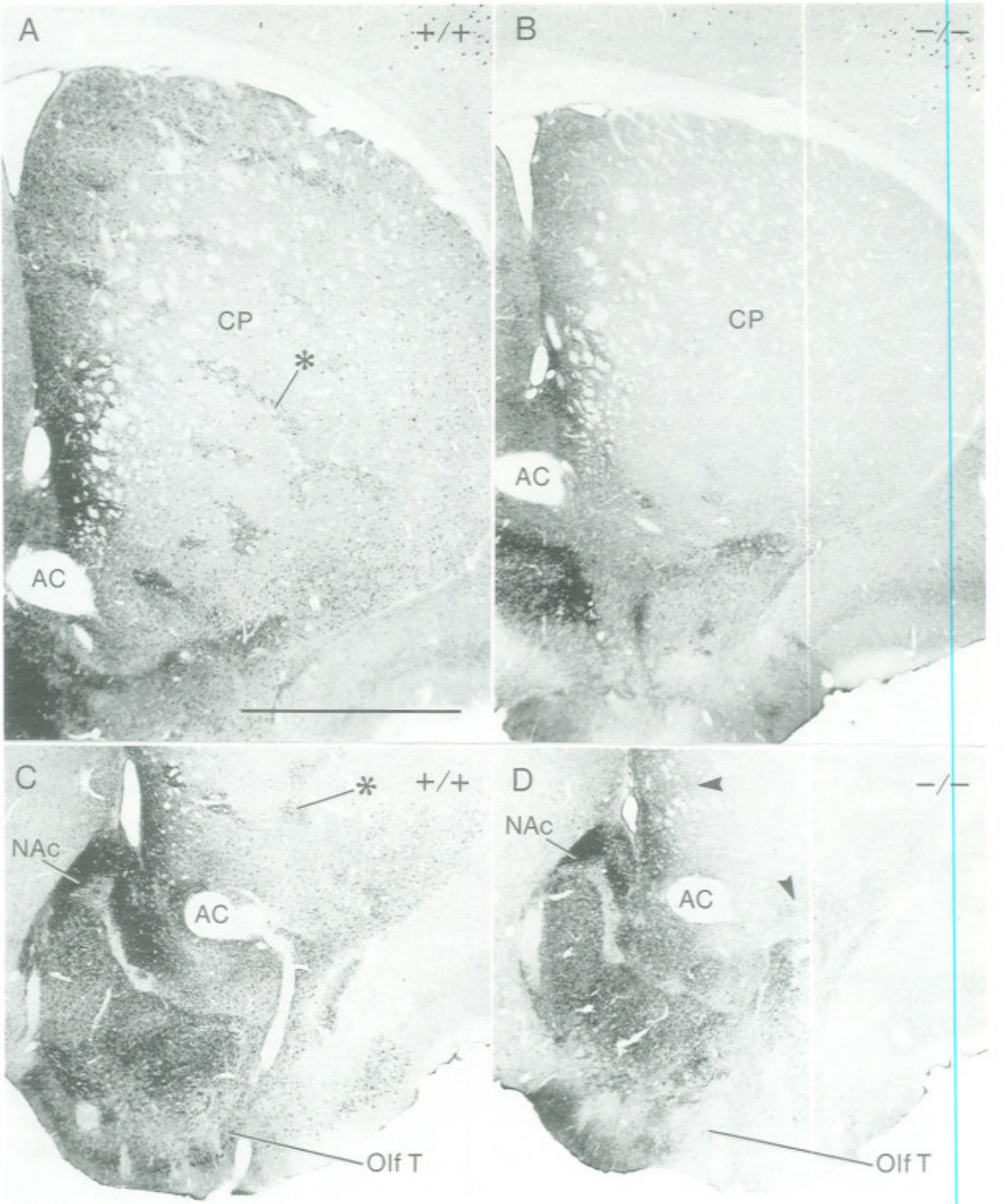

Figure 4. Reduction of Dynorphin B1-29 Immunostaining in Striatum of D1-Deficient Mouse

(A and B) Low power photomicrographs illustrating dynorphin-positive cell clusters (striosomes; example at asterisk) in the caudoputamen of wild-type (+/+) mouse (A) and lack of detectable dynorphin-positive striosomes in the caudoputamen of D1-deficient (−/−) mouse (B). Dynorphin immunostaining was also reduced in the matrix.

(C and D) Dynorphin immunostaining in the ventral striatum. Wild-type (+/+) (C) and mutant (−/−) (D) mice both had considerable dynorphin immunostaining in parts of the nucleus accumbens and olfactory tubercle. However, the immunostaining appeared weaker in the mutant and in some regions (e.g., olfactory tubercle) was considerably diminished. Immunostaining was sharply diminished in the overlying mutant caudoputamen, but medially and ventrolaterally there was still appreciable dynorphin immunoreactivity (arrowheads). Asterisk in (C) indicates a cluster of dynorphin-immunoreactive neurons in caudoputamen of the wild-type mouse. Abbreviations are as in Figure 2. Scale bar indicates 1 mm.

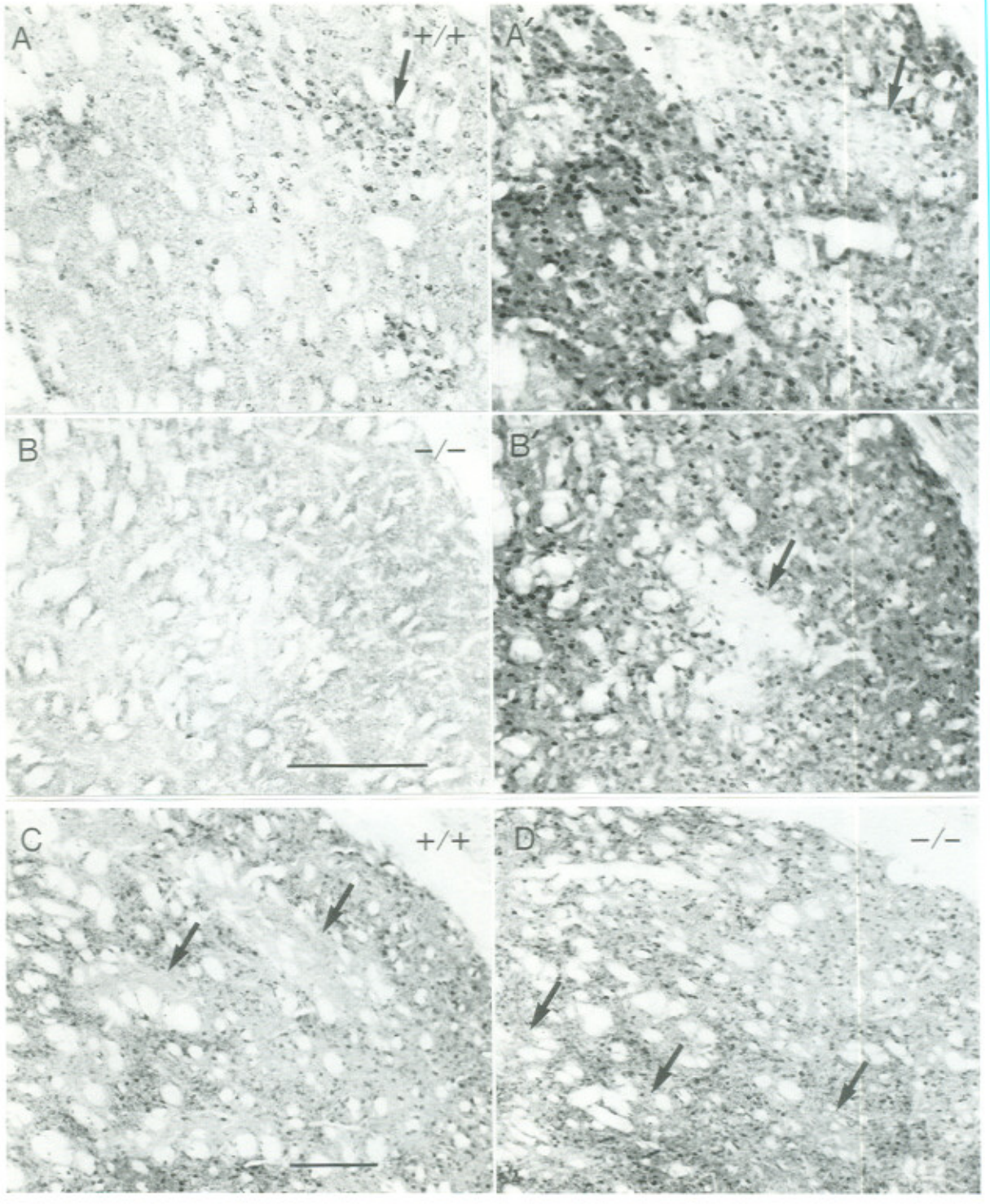

Figure 5. Striosomes in Wild-Type and D1-Deficient Mice

(A and A′) Photomicrographs showing that dynorphin-immunoreactive cell clusters in wild-type (+/+) striatum (A) (example at arrow) correspond to calbindin-poor cell clusters in serially adjacent section (A′). Arrows point to corresponding sites in the two sections.

(B and B′) There were calbindin-poor zones in the D1-deficient (−/−) mice (arrow in [B′]), but in adjacent sections stained for dynorphin immunoreactivity, dynorphin-positive cell clusters were not detectable. Sections illustrated in (A) and (B) were stained with a polyclonal antiserum raised against dynorphin B1-29. Scale bar in (B) indicates 200 μm for (A), (A′), (B), and (B′).

(C and D) Blurring of enkephalin-poor striosomes in D1-deficient mouse. Photomicrograph in (C) shows relatively clearly defined enkephalin-poor zones in wild-type (+/+) mouse (examples at arrows). (D) illustrates the lack of definition of such zones found in the D1 mutants (−/−) (arrows). Striosomes, marked by arrows, were identified by reference to calbindin-poor zones observed in adjacent sections in each mouse (data not shown). Scale bar in (C) indicates 200 μm for both photographs.

ing (Figures 3B and 3B′; Figures 4C and 4D), but dynorphin-like immunoreactivity in the mutants also appeared to be decreased (Figures 4C and 4D).

The loss of detectable dynorphin-positive cell clusters and down-regulation elsewhere in the striatum was observed with both of the peptide antisera we used (Figures 3–5). Immunostaining for dynorphin had apparently normal patterns in many other regions of the mutant brain (Figure 3). However, we noted, for example, the presence of dynorphin-immunoreactive neurons in such sites as the cerebral cortex (Figures 3C and 3C′) and intense dynorphin immunoreactivity in the hippocampus (Figures 3D, 3D′, 3E, and 3E′). We did not make a quantitative study of these regions and thus cannot judge whether the levels of dynorphin immunoreactivity were normal in the mutants.

Striosome–Matrix Compartments in Mutant Mice

Because the lack of dynorphin immunoreactive clusters could indicate that the striosomal compartment of the striatum failed to form in the mutant mice, we screened sections for two characteristic markers for the striosome–matrix compartments. Autoradiography for Mu opiate receptor-binding sites preferentially marks the striosomes (opiate patches) of the caudoputamen (Pert et al., 1976; Graybiel, 1990). The calcium-binding protein calbindin D_{28k} is a selective marker for the large matrix compartment surrounding the striosomes (Gerfen et al., 1983). This marker leaves striosomes almost unstained. Both opiate patches (data not shown) and calbindin-poor zones (Figures 5A′ and 5B′) were present in the mutants, as they were in the wild types. However, the calbindin-poor zones were not always as well delineated in the mutants as in the wild types.

The calbindin immunostaining, unlike the Mu opiate–binding site autoradiography, could be carried out on sections adjacent to those stained for dynorphin immunoreactivity. This permitted direct comparisons of the dynorphin-positive clusters and striosomes. In the wild-type mice, there was good correspondence between the dynorphin-positive clusters and the calbindin-poor zones, confirming that the clusters were striosomes (Figures 5A and 5A′). In the mutants, calbindin-poor striosomes could be found, but there were not corresponding dynorphin-positive clusters (Figures 5B and 5B′). The lack of dynorphin immunostaining therefore did not reflect a total absence of the striosomal compartment.

Staining for enkephalin immunoreactivity also appeared abnormal in the mutants (Figures 5C and 5D). In normal mice, striosomes have relatively little enkephalin immunostain, whereas the matrix is heavily stained (Graybiel et al., 1990b). This pattern was present in the wild-type mice (Figure 5C), but in the mutants (Figure 5D), enkephalin-poor striosomes were much more difficult to detect. Thus, the expression of a second neuropeptide appeared to be altered, although the defect was subtle compared with that seen for dynorphin expression.

Immunostaining for substance P, known to be coexpressed with dynorphin in striatal neurons (Anderson and Reiner, 1990; Besson et al., 1990), was not obtained in neural perikarya. However, it was possible to detect substance P immunostaining in axons, and in the mutant brains, substance P immunostaining appeared diminished in the output targets of the striatum (Figures 6C and 6D).

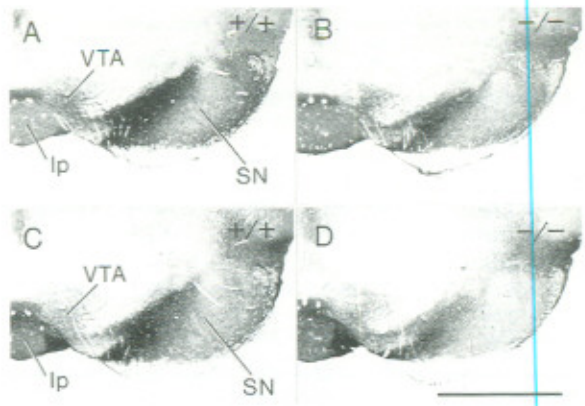

Figure 6. Immunostaining for Dynorphin and for Substance P in the Substantia Nigra

(A and B) Dynorphin. (C and D) Substance P. Sections from wild-type brains (+/+) are shown on left, sections from mutant brains (−/−) on right. Both dynorphin and substance P immunoreactivities are reduced in the substantia nigra of the mutants relative to levels visible in the wild types. Abbreviations: SN, substantia nigra; VTA, ventral tegmental area; lp, interpeduncular nucleus. Scale bar shows 1 mm.

Striatal Interneurons in the Mutant Mice

There are distinct classes of interneurons in the striatum in addition to the projection neurons, and these neurons also express dopamine receptors (Weiner et al., 1990; Bernard et al., 1992). We checked for the presence of three such classes of interneuron with stains for choline acetyltransferase immunoreactivity to mark cholinergic interneurons, nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase (nitric oxide synthase) activity to mark somatostatin-containing interneurons (Vincent et al., 1983), and parvalbumin and GABA immunoreactivities to mark the parvalbumin/GABA interneurons (Kubota et al., 1993). We found that each of these classes of striatal interneuron were present in the mutants, as they were in the wild types (data not shown). Counts of the interneurons were not carried out, but these data suggest that there was not a failure of intrinsic neuronal development in the striatum in the absence of D1 receptor modulation.

Behavioral Nonresponsiveness to D1 Agonists and Antagonists

As a functional assay of the D1-like receptor mutation, the effects of D1 receptor agonist and antagonist drugs on motor activity were measured by the photocell beam interruption method (Moorcroft et al., 1971; Koob et al., 1978). The effects of SKF81297, a full D1 receptor agonist, were studied during the light phase of the light–dark cycle. Data for the 2 hr habituation sessions preceding each drug injection are shown in Table 1. SKF81297 at doses of 1.22, 4.82, and 7.41 mg/kg produced dose-dependent hyperactivity in the wild-type mice, whereas no increases in locomotion were observed in mutant mice (main effect of group, $F[1,18] = 19.2$; interaction, $F[3,54] = 17.7$]; Figure

Table 1. Total Beam Breaks during 2 hr of Habituation

	Wild Type		Knockout	
Agonist/Antagonist	Mean	SEM	Mean	SEM
SKF81297				
Saline	3,135	379.2	5,609.9	1,240.6
1.22 mg/kg	2,485.7	428.8	6,438.3	1,315.5
4.82 mg/kg	3,019.9	378.6	5,757.8	1,285.2
7.41 mg/kg	2,552.9	303.7	7,371.9	1,623.5
SCH23390				
Vehicle	7,179.6	1,401.6	12,299.1	1,377.7
10 μg/kg	4,471.1	764.6	12,417	1,490.7
30 μg/kg	4,497.6	996.1	12,344.7	1,652.1

Values represent mean and SEM total photocell beam interruptions (horizontal and vertical activity combined) for 2 hr habituation sessions; n = 10 mice per group for SKF81297 treatments and n = 10 mutants and n = 9 wild-type mice per group for SCH23390 treatments.

7A). In the wild-type mice, all doses of SKF81297 also significantly increased photobeam interruptions compared with saline-treated controls (F[3,27] = 77.8). Thus, the locomotor stimulant effect of SKF81297 was undetectable in the D1-deficient mice.

The effects of SCH23390, a D1 receptor antagonist, were studied during the dark phase of the light–dark cycle to capitalize on the natural increase in activity of the dark period. Data for the 2 hr habituation sessions preceding each drug injection are shown in Table 1. SCH23390 at doses of 10 and 30 μg/kg produced significant dose-dependent reduction in photobeam interruptions in the wild-type mice, while having no effect in the mutant mice (main effect of group, F[1,17] = 15.2; interaction, F[2,34] = 12.1; Figure 7B). In the wild-type mice, SCH23390 produced a dose-dependent reduction in photobeam interruptions compared with vehicle in these mice (F[2,16] = 8.8]. The D1-deficient mice thus responded neither to the motor-stimulant effects of a D1 receptor agonist nor to the motor-suppressive effects of a D1 receptor antagonist.

The lack of D1-mediated responsiveness in the mutants was confirmed in the ring procedure test of drug-induced catalepsy (Pertwee, 1972). This test employed doses of SCH23390 (50, 100, and 200 μg/kg) higher than those required in the locomotor activity assay. SCH23390 produced a dose-dependent increase in duration of time cataleptic in the wild-type mice (F[3, 18] = 90) but had no effect in the mutant mice [main effect of group, F[1,13] = 286]; interaction, F[3,39] = 103; Figure 7C]. Taken together, these data demonstrate a lack of response of the D1-deficient mice to D1 receptor ligands over a wide range of doses.

Hyperactivity in the D1-Deficient Mice

To investigate the effects of the D1 receptor deficiency on locomotion, we measured basal levels of motor activity during both phases of the light–dark cycle in mutant and wild-type mice. Photocell beam interruptions measured during a 3 hr test session conducted during the dark phase of the light–dark cycle demonstrated a significant hyperactivity in mutant mice compared with wild-type control mice

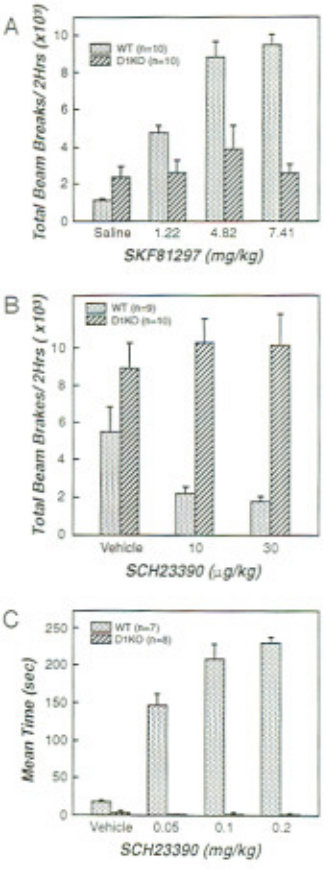

Figure 7. Effects of D1 Agonist and Antagonist on Locomotor Activity and Catalepsy

(A) Following a 2 hr habituation period, mice were injected with saline or increasing doses of SKF81297. Values represent mean + SEM total photocell beam interruptions (horizontal and vertical activity combined) for 2 hr test sessions tested during the light phase of the light–dark cycle.

(B) Following a 2 hr habituation period, mice were injected with vehicle or increasing doses of SCH23390. Values represent mean + SEM total photocell beam interruptions (horizontal and vertical activity combined) for 2 hr test sessions tested during the dark phase of the light–dark cycle.

(C) Catalepsy testing was conducted 15 min following injection with vehicle or SCH23390. Values represent mean + SEM time (seconds) immobile during a 5 min test. In all cases, WT represents the wild-type mice and D1KO represents the mutants.

(F[1,18] = 14.5; Figure 8]. Mutant mice generated more than twice as many photobeam interruptions as the wild-type mice. During the habituation sessions before SCH23390 treatments, the mutant mice were also significantly more active than the wild types (F[1,17] = 15). When tested during the light phase, the mutant mice exhibited a trend toward increased activity compared with the control mice, but this result did not reach statistical significance. However, during the habituation sessions before SKF81297 treatment, also carried out during the light phase, the mutant mice were significantly more active than the wild-type controls (F[1,18] = 8.3). Thus, D1-deficient mice appear to exhibit a general behavioral hyperactivity.

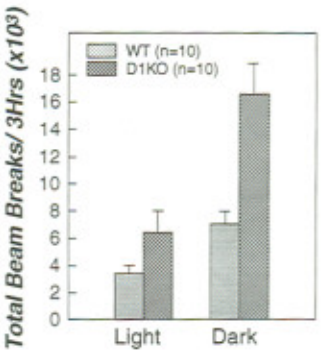

Figure 8. Basal Locomotor Activity Tested during Light or Dark Phases of the Light–Dark Cycle

Values represent mean + SEM total photocell beam interruptions (horizontal and vertical activity combined) for 3 hr test sessions. WT represents wild-type mice; D1KO represents the mutants.

Discussion

Previous studies on the function of the D1 receptor have relied primarily on the pharmacological approach of applying agonists and antagonists of D1-like receptors (Koob, 1992b). More recent experiments have incorporated the use of antisense constructs against a dopamine receptor subtype to address this issue (Weiss et al., 1993; Zhang and Creese, 1993). In the present study, we used a genetic approach to generate mice that do not carry the D1 gene in their genome. The absence of the D1 receptor in the mutant mice was demonstrated by genomic Southern blotting and was confirmed by ligand binding studies and behavioral tests. Our initial study of these mice suggests that loss of the D1 receptor affects the neurochemical phenotype of neurons in the striatum and produces changes in the locomotor behavior of the mutant mice.

Reduced Dynorphin Expression in the Striatum

A major abnormality that we found in the mutant brains was in the expression of dynorphin immunoreactivity in the striatum. The loss in dynorphin immunostaining in striosomes was obvious at a glance. There was also a more generalized decrease in dynorphin expression in the matrix compartment of the striatum, in parts of the ventral striatum, and in sites receiving dynorphin-immunoreactive inputs from the striatum, including the entopeduncular nucleus and the substantia nigra. In many other regions of the brain, however, dynorphin staining survived in qualitatively normal distributions. We emphasize that quantitative studies of the dynorphin immunostaining have not been carried out. Our results do suggest, however, that D1 receptors exert an influence on the expression of the dynorphin gene in striatal projection neurons.

One possibility suggested by these findings is that D1 gene expression is crucial for the normal development of the striatum and its striosome–matrix compartments. The loss of the D1 gene might result in the loss of many dynorphin-expressing neurons, which in typical immunostains are particularly evident in striosomes. Developmentally, D1 receptor mRNA is expressed early during the formatic of the striatum. During the time of striosome formatio D1 ligand–binding sites are preferentially associated wit this compartment (Murrin and Zeng, 1989; Schambra e al., 1994). Neurons of the striosomal compartment ar born early during striatal neurogenesis, and the majorit of matrix cells are born subsequently in the second wav of neurogenesis (Graybiel and Hickey, 1982; Graybie 1984b; van der Kooy and Fishell, 1987; van der Kooy e al., 1987). As the matrix cells invade the striatum, cluster of striosomal cells form. It is during this time that the firs dopamine fibers of the future nigrostriatal system form "dopamine islands," which correspond to the developing striosomes (Voorn et al., 1988; Graybiel, 1984a). It is a this time that D1 ligand–binding sites become strongly and preferentially expressed in the proto-striosomes (Murrin and Zeng, 1989; Schambra et al., 1994). The D1 receptor-linked DARPP-32 also is first expressed in the developing striosomal system (Foster et al., 1987). The especially marked loss of dynorphin expression in striosomes could reflect such differentially strong developmental expression of the D1 receptor mechanism in striosomes.

Evidence also suggests that there may be differentially strong coupling of D1 receptors to cAMP in striosomes during the first days of postnatal development (Liu et al., 1994). This is a time during which programmed cell death occurs in the striatum (Fentress et al., 1981; Fishell and van der Kooy, 1991) and during which the striosome–matrix compartments are remodeled (Graybiel, 1984b; van der Kooy et al., 1987). The lack of D1 receptors could destabilize this program of striatal development and of striosome–matrix formation in particular. Our evidence clearly showed, however, that the striosomal system survived in the D1 mutants. Calbindin-poor patches and opiate patches, both markers of striosomes, were present; we confirmed in serial sections that the calbindin-poor zones contained neurons (stained for DARPP-32), and some striosomes could be found in the sections immunostained for enkephalin. These experimental findings argue against massive cell death as the principal source of the dynorphin deficit.

Even though striosomes were present in the mutants, they were less crisply delineated (and sometimes even difficult to detect) in the enkephalin-immunostained sections. Even in the calbindin material, which gave unequivocal evidence for the presence of striosomes, these compartments were less crisply defined in some of the mutant mice than in their wild-type littermates. It is unclear whether these changes in enkephalin and calbindin immunostaining were linked to the deficit in dynorphin expression or occurred independently. They do suggest, however, that in addition to a deficit in dynorphin expression in the striatum, there may be a more general developmental abnormality in the striosomal system. We were unable to achieve reliable perikaryal staining for substance P, which is coexpressed with dynorphin in striosomal neurons, but one possibility suggested by our findings is that a defect in substance P might also occur in the mutants. This possibility is supported by the decline in substance P fiber staining that we found in the mutant substantia nigra.

Another leading possibility to account for our findings is that the D1 gene may tightly control the expression of the dynorphin gene in striatal neurons and that the loss of the D1 gene leads to inactivation of the dynorphin gene or a down-regulation or dysregulation of its expression. This view accords well with our finding that, although the dynorphin defect was particularly obvious in striosomes, it was not limited to them, but was also present in the matrix and in the ventral striatum. There is strong evidence that, at adulthood, striatal dynorphin expression is regulated by dopamine receptors (Sivam, 1989; Jiang et al., 1990; Li et al., 1986, 1988; Hanson et al., 1987; Hurd et al., 1992), that D1 receptors are crucial for this regulation, and that the regulation is particularly pronounced in striosomal neurons (Gerfen et al., 1990, 1991; Daunais et al., 1993; Hurd and Herkenham, 1993). Most, though not all, experiments further suggest that D1 receptors in the striatum are primarily expressed by dynorphin-containing neurons (Le Moine et al., 1991; Gerfen et al., 1990; Surmeier et al., 1992, 1993). The explicit suggestion has been made that this regulation of dynorphin occurs through a D1 receptor–cAMP pathway by inducing Fra-like immediate-early genes (Zhang et al., 1992; Daunais et al., 1993; Bronstein et al., 1994). This suggestion is in good accord with the finding that the induction by psychomotor stimulants of several immediate-early genes in the striatum can be blocked by dopamine D1 receptor antagonists (Graybiel et al., 1990a; Young et al., 1991) and with the observation that such D1 receptor–mediated induction can occur differentially in striosome and matrix compartments (Graybiel et al., 1990a; Pennypacker et al., 1992; Moratalla et al., 1992, 1993; Dilts et al., 1993). Our preliminary results with in situ hybridization suggest that the level of prodynorphin mRNA is down-regulated in the striatum of the D1 mutants.

In contrast with dynorphin- and substance P–expressing neurons, enkephalin-immunoreactive striatal neurons mainly express D2-like dopamine receptors. This suggests that the decline in clarity of striosomes in the enkephalin stains may be an indirect effect of the D1 receptor loss, but at least some of the enkephalinergic neurons do express D1 receptors (Surmeier et al., 1993). Moreover, D1 receptors and D2 receptors are thought to interact in some aspects of intracellular regulation in the striatum (Piomelli et al., 1991; Kebabian and Calne, 1979; Ariano et al., 1989; Surmeier et al., 1992, 1993; Paul et al., 1992). Dysregulation of such interactions could occur with the deletion of D1 receptors and could account for our results.

We emphasize that other receptors and molecules could be affected by the D1 receptor loss also and could contribute to the staining abnormalities we found. For example, in the mutant mouse brains, we found an apparently complete absence of ligand binding with the ligand [^{3}H]SCH23390. The precise reason for this finding is unclear, for this D1-like receptor ligand binds both to D1 and to D5 dopamine receptors (Sunahara et al., 1990). It could be that [^{3}H]SCH23390 had higher affinity for the D1 receptor than for the D5 receptor under the binding conditions employed in the present study so that most D5 binding was below the threshold of detection. Alternatively, this finding could mean that the absence of the D1 receptor down-regulates or otherwise changes the D5 receptor.

Behavioral Changes in the D1 Mutants

Both dopamine receptors and dynorphin are strongly expressed by the striatal projection neurons that give rise to the so-called direct output pathway of the striatum. This output system is thought to have a general movement-enhancing function (Albin et al., 1989; Alexander and Crutcher, 1990). The direct pathway largely arises from the matrix compartment, in which dynorphin did appear decreased in the mutants. Moreover, we noted subnormal levels of dynorphin immunostaining in the entopeduncular nucleus and the substantia nigra, targets of the direct pathway, decreased substance P immunostaining in these direct pathway targets. Thus, the D1-deficient mutants showed at least two neurochemical abnormalities that might be linked with changes in locomotion: lack of the D1 receptor itself, which promotes locomotion (Dreher and Jackson, 1989), and dysregulation of dynorphin and apparently also substance P, neuropeptides expressed along with GABA in the neurons of the movement-enhancing direct striatal output pathway. Some decline in dynorphin apparently also occurred in the nucleus accumbens, which has been shown in the rat to be an important regulator of locomotor activity (Sharp et al., 1987).

Our behavioral studies suggested two changes in the locomotor behavior of the mutant mice. First, the mutant mice exhibited complete behavioral nonresponsiveness to D1 agonist and antagonist treatments. This observation confirmed the absence of D1 receptors in the mutants. It also suggests that, in the dose ranges employed in this study, these D1 ligands may indeed be selective for D1-like receptors. We note, however, that such selectivity is not assured by our findings, because the D5 receptor, on which these ligands also are thought to act, may not have a critical role in locomotor activity or catalepsy when exposed to the doses of ligand used in our behavioral tests.

The second behavioral abnormality we noted in the mutants was locomotor hyperactivity. At first glance, this seems counter to our finding of decreased immunodetectable dynorphin and substance P in the direct movement-enhancing pathway of the basal ganglia. Compensatory mechanisms may have been activated by the lack of D1 receptors and the decline in these peptides, however, and interactions with the D2 receptor movement-diminishing system may also have occurred.

Interestingly, it is thought that striosomes may contain the striatal cells that innervate the dopamine-containing cells of the substantia nigra. Thus, they may be part of a dynamic regulatory system controlling striatal function. Our experiments, in indicating a clear change in neurochemical phenotype of the striosomal system in the mutants, suggest the possibility that this regulatory loop, like the direct output pathway of the striatum, may be disordered. More generally, because our experiments suggest that gross structural changes due to the loss of the D1 gene are relatively circumscribed and because we have found systematic changes in striosomes, in direct pathway neurons, and in parts of the ventral striatum of the mutants,

the D1-deficient mice should be valuable for exploring dopaminergic D1 functions related not only to general motor behavior but also to other behaviors, including those associated with natural rewards and learning.

Experimental Procedures

D1 Gene, Targeting Construct, and ES Homologous Recombinants

Six oligonucleotide primers were used in PCRs with DNA isolated from mouse D3 ES cells as template. The sequences of these primers are 5′-TCCAAGGTGACCAACTTCTTTGT, 5′-TGGGCTATCTCCAGCCCTTTCC, 5′-GCCTTGGAGAGGGCAGCAGTCCA, 5′-GCCCACCCAAACCACACAAACAC, 5′-CTGTAGCATCCTAAGAGGGTTGA, and 5′-TTCAGGTCCTCAGAGGAGCCCAC. One of the PCR products contained part of the mouse D1 gene sequence and was used to clone the entire mouse D1 gene from a mouse 129 genomic library.

To make a D1 gene targeting construct, four-piece DNA ligation was performed. The following DNA fragments were used: a 3.9 kb BglII–NcoI fragment containing DNA from mostly 5′ of the D1 gene, a 1.8 kb fragment containing a *neo* gene driven by the PGK promoter, a 4.2 kb XbaI–HindIII fragment containing DNA mostly from 3′ of the D1 gene, and the plasmid pBluescript from Strategene.

To obtain homologous recombinants, mouse E14 ES cells were transfected with 50 μg of linearized targeting construct by electroporation using a Bio-Rad Gene Pulser at 800 V and 3 μF. G418 selection was applied 24 hr after transfection at 200 μg/ml. G418-resistant stable transfectants were isolated from day 7 to day 9 after the transfection. Genomic DNA were isolated and digested with NcoI and hybridized with a probe isolated from the 5′ flanking sequence of the D1 gene (see Figure 1A). Candidate homologous recombinants were confirmed by digesting their genomic DNA with BglII and hybridizing with a probe isolated from the 3′ flanking DNA sequence.

D1-Deficient Mice

Chimeric mice were generated as described by Bradley (1987). In brief, ES cells amplified from the homologous recombinants were injected into blastocysts isolated from C57B6 female mice. Then, the injected blastocysts were implanted back into the uteri of (C57B6 × DBA_2)F1 females. The resulting male chimeric mice were bred repeatedly with C57B6 females, and germline transmission was identified initially by screening for agouti offspring. Mice heterozygous for the D1 mutation were confirmed by genomic Southern blot analyses of DNA isolated from their tails. Finally, mice homozygous for D1 mutation were produced by crossing heterozygous mutant mice and were identified by Southern blotting of tail DNA. All breeding was carried out under standard animal housing conditions in the Massachusetts Institute of Technology animal facility.

Immunohistochemistry

For immunohistochemical studies, 11 wild-type and 13 mutant adult male mice were anesthetized with Nembutal and were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4). Brains were postfixed in 4% paraformaldehyde for ~2 hr, were cryoprotected in 20% glycerol overnight, and were cut coronally on a sliding microtome at 20 or 30 μm. Sections were stored in 0.1% sodium azide in 0.1 M phosphate buffer.

Primary antisera included the following: polyclonal rabbit antiserum against rat dynorphin B1-13 (rimorphin) diluted 1:2,000, provided by Drs. E. Weber (University of California, Irvine) and M. Sonders (Vollum Institute, Portland, OR); polyclonal rabbit antiserum against rat dynorphin B1-29 (leumorphin) diluted 1:10,000, provided by Dr. S. Watson (University of Michigan, Ann Arbor); polyclonal rabbit antiserum against substance P diluted 1:10,000, provided by Dr. J. S. Hong (National Institutes of Health, Research Triangle Park, NC); polyclonal rabbit antiserum against Met-enkephalin diluted 1:2,000 (INCSTAR Corporation, Stillwater, MN); polyclonal rabbit antiserum against tyrosine hydroxylase diluted 1:1,000 (Eugene Technology International, Ridgefield Park, NJ); polyclonal rabbit antiserum against calbindin D_{28k} diluted 1:3,000, provided by Dr. P. Emson (Medical Research Council, Cambridge, England); monoclonal mouse antiserum against DARPP-32 diluted 1:10,000, provided by Drs. E. L. Gustafson and P. Greengard (Rockefeller University, New York); monoclonal mouse antiserum against parvalbumin diluted 1:1,000 (Sigma); polyclonal goat antiserum against choline acetyltransferase diluted 1:100 (Chemicon International Incorporated, Temecula, CA).

Free-floating sections were stained according to standard avidin–biotin or streptavidin immunohistochemical protocols. Sections were pretreated for 10 min with 3% H_2O_2 in PBS containing 2% Triton X-100 (PBS–TX) and then for 30 min with 5% normal serum of the species in which the secondary antibody was raised, usually normal goat serum or normal horse serum, and then rinsed in PBS–TX. Incubations in primary antiserum were carried out at 4°C for 24–72 hr. Sections were then incubated for 1 hr in the secondary antiserum, biotinylated goat anti-rabbit IgG, or horse anti-mouse IgG (Vector Laboratories) diluted 1:500, and, containing 1% normal goat serum or 1% normal horse serum and 1% normal mouse serum, they were washed in PBS–TX and incubated for 1 hr in avidin–biotin–peroxidase complex (Vectastain, Vector Laboratories) or, in the case of immunostaining for dynorphin immunoreactivity, in peroxidase-conjugated streptavidin diluted 1:8,000 (Zymed Laboratories Incorporated). Finally, sections were treated with 0.05% diaminobenzidine containing 0.02 M sodium cacodylate, 0.1 N acetic acid with 0.002% H_2O_2, or, in the case of dynorphin, in 0.02% diaminobenzidine, 0.1 M PB buffer, 0.002% H_2O_2, and the staining was intensified with 0.08% nickel ammonium sulfate.

Sets of sections from several mutant and wild-type mice were also stained for NADPH diaphorase (NO synthase) activity by the protocol of Vincent et al. (1983) with modifications. Free-floating sections were incubated at 37°C in 0.1 M PB buffer containing 1 mM NADPH, 0.2 M nitroblue tetrazolium, and dimethyl sulfoxide. For general histologic study, other sets of sections were stained for Nissl substance with creoylecht violet.

Control experiments were carried out for the dynorphin immunohistochemistry by preabsorbing each dynorphin antiserum with the peptide against which it was raised. The dynorphin B1-13 antibody was preabsorbed with 50 μM dynorphin B1-13 synthetic peptide (Sigma), and the dynorphin B1-29 antibody was preabsorbed with 20 μM dynorphin B1-29 synthetic peptide (gift of Drs. S. Watson and A. Mansour). For each antiserum, staining was abolished or nearly so. We emphasize that both antisera may have detected the Leu-enkephalin sequence, as both dynorphin B1-13 and dynorphin B1-29 contain a Leu-enkephalin sequence. For all of the immunostaining results presented, we further stress that the immunostaining results described refer specifically to the patterns of immunoreactivity detected with the particular antiserum described above.

Ligand Binding

For ligand binding assays, three wild-type and three mutant adult male mice were sacrificed by cervical dislocation. Their brains were rapidly removed from the skulls, frozen in powdered dry ice, and stored at −80°C. Freshly frozen coronal sections were cut on a cryostat at 10 μm, thaw mounted onto slides (Probe On, Fisher Scientific), and stored at −20°C for at least 2 days, and then were brought to room temperature and washed for 5 min in the working buffer to remove endogenous ligands.

Ligand binding of D1 receptor–binding sites was carried out according to the procedure described by Savasta et al. (1986), with some modifications. Sections were incubated at room temperature for 1 hr with 2.5 nM [^{3}H]SCH23390 (specific activity, 73 Ci/mmol; Du Pont–New England Nuclear) in 50 mM Tris–HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. To block serotonin receptor-binding sites, 10 μM mianserin was added to the incubation buffer. After incubation, sections were rinsed twice for 2 min in fresh ice-cold working buffer, dipped in cold distilled water, and dried under a cool air stream.

Labeling of dopamine transporter-binding sites was carried out according to the protocol described by Graybiel and Moratalla (1989). In brief, sections were incubated for 40 min at room temperature with 15 nM [^{3}H]mazindol (specific activity, 19 Ci/mmol; Du Pont–New England Nuclear) in the working buffer described above containing, in this case, 0.3 μM desimipramine to block the norepinephrine transporter. After incubation, sections were rinsed twice (1 min each) in fresh ice-cold buffer, dipped in cold distilled water for 10 s, and dried.

All sections were dried for at least 2 hr before being exposed, to-

gether with tritium standards ([^{3}H] Microscales, Amersham), to tritium-sensitive film (Hyperfilm, Amersham). The exposure times varied with the ligand used: ~2 weeks for [^{3}H]mazindol, ~3 weeks for [^{3}H]SCH23390. At the appropriate times, films were developed in D-19 and analyzed on a Biocom 200 Image Analyzer (Les Ulis).

Locomotor Activity

For locomotor studies, two to four mice were housed in a Plexiglas cage (28 cm × 17 cm × 11.5 cm). Locomotor activity was measured in large Plexiglas cages that were placed into frames (29.2 cm × 50.5 cm) mounted with photocell beams (San Diego Instruments). The horizontal locomotion frames consist of a 4 × 8 array of beams. A second tier frame (7 cm in height) consisting of eight beams equally spaced along the long axis was used to measure rearing activity. For each mouse, a sequential record of beam breaks was recorded in a coordinate file consisting of time, duration, and location of horizontal beam breaks and duration of vertical beam breaks (rearing). When vertical activity data (rearing) were separated from horizontal locomotion (ambulation), the profile of results was qualitatively similar to that of total beam breaks (vertical and horizontal activity combined). Therefore, data are presented as combined beam breaks.

Basal activity levels were characterized during 3 hr sessions tested during the light phase of the light–dark cycle followed 1 week later by a 3 hr session tested during the dark phase of the light–dark cycle. The motor-stimulating effects of the D1 agonist SKF81297 (1.22 mg/kg [3.3 μM/kg], 4.82 mg/kg [13 μM/kg], and 7.41 mg/kg [20 μM/kg]) were tested (10 days later) during the light phase of the light–dark cycle, when basal levels of activity are lowest. The motor-suppressing effects of the D1 antagonist SCH23390 (10 μg/kg [24.5 nmol/kg] and 30 μg/kg [73.5 nmol/kg]) were tested (4–7 days later) during the dark phase of the light–dark cycle, when basal levels of activity are peaking. Mice were placed in the cages for a 2 hr habituation session, then removed from the cages, injected, and replaced in the cages for an additional 2 hr. SKF81297 was delivered in a saline vehicle. SCH23390 was dissolved in MeOH (1 μg/μl) and then diluted to the proper concentration with saline. Drug doses were administered in ascending dose order, starting with the respective vehicle, with 1–2 days between doses. All injections were administered in 1 ml per 100 g body weight volumes subcutaneously. SKF81297 hydrobromide was obtained from Research Biochemicals Incorporated and SCH23390 maleate was obtained from Schering.

Catalepsy

Following locomotor activity testing, a subset of the mice were evaluated for SCH23390-induced catalepsy using the mouse ring test (Pertwee, 1972). Mice were injected subcutaneously with SCH23390 (50 μg/kg [120 nmol/kg], 100 μg/kg [245 nmol/kg], or 200 μg/kg [490 nmol/kg]) or with vehicle and tested 15 min later. For testing, mice were placed on a metal ring (5.5 cm in diameter) attached to a ring stand 17 cm above the base. Each mouse was observed for a test duration of 5 min, and the time immobile, defined as absence of all voluntary movements except those associated with breathing, was recorded.

Data Analysis

Locomotor activity and catalepsy data were analyzed using a one-way (basal activity) analysis of variance (ANOVA) or two-way ANOVA (group and dose) with repeated measures on the dose factor. The locus of significant between group interactions was determined by tests for simple effects. Within-group comparisons were analyzed by one-way ANOVAs followed by Newman–Keuls post hoc tests. For all analyses, significance was assigned at $p < 0.05$ level.

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