

Facilitated Glutamatergic Transmission in the Striatum of D₂ Dopamine Receptor-Deficient Mice

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Cepeda, C., R. S. Hurst, K. L. Altemus, J. Flores-Hernández, C. R. Calvert, E. S. Jokel, D. K. Grandy, M. J. Low, M. Rubinstein, M. A. Ariano, and M. S. Levine. Facilitated glutamatergic transmission in the striatum of D₂ dopamine receptor-deficient mice. *J Neurophysiol* 85: 659–670, 2001. Dopamine (DA) receptors play an important role in the modulation of excitability and the responsiveness of neurons to activation of excitatory amino acid receptors in the striatum. In the present study, we utilized mice with genetic deletion of D₂ or D₄ DA receptors and their wild-type (WT) controls to examine if the absence of either receptor subtype affects striatal excitatory synaptic activity. Immunocytochemical analysis verified the absence of D₂ or D₄ protein expression in the striatum of receptor-deficient mutant animals. Sharp electrode current- and whole cell patch voltage-clamp recordings were obtained from slices of receptor-deficient and WT mice. Basic membrane properties were similar in D₂ and D₄ receptor-deficient mutants and their respective WT controls. In current-clamp recordings in WT animals, very little low-amplitude spontaneous synaptic activity was observed. The frequency of these spontaneous events was increased slightly in D₂ receptor-deficient mice. In addition, large-amplitude depolarizations were observed in a subset of neurons from only the D₂ receptor-deficient mutants. Bath application of the K⁺ channel blocker 4-aminopyridine (100 μM) and bicuculline methiodide (10 μM, to block synaptic activity due to activation of GABA_A receptors) markedly increased spontaneous synaptic activity in receptor-deficient mutants and WTs. Under these conditions, D₂ receptor-deficient mice displayed significantly more excitatory synaptic activity than their WT controls, while there was no difference between D₄ receptor-deficient mice and their controls. In voltage-clamp recordings, there was an increase in frequency of spontaneous glutamate receptor-mediated inward currents without a change in mean amplitude in D₂ receptor-deficient mutants. In WT mice, activation of D2 family receptors with quinpirole decreased spontaneous excitatory events and conversely sulpiride, a D2 receptor antagonist, increased activity. In D₂ receptor-deficient mice, sulpiride had very little net effect. Morphologically, a subpopulation of medium-sized spiny neurons from D₂ receptor-deficient mice displayed decreased dendritic spines compared with cells from WT mice. These results provide evidence that D₂ receptors play an important role in the regulation of glutamate receptor-mediated activity in the corticostriatal or thalamostriatal pathway. These receptors may function as

gatekeepers of glutamate release or of its subsequent effects and thus may protect striatal neurons from excessive excitation.

INTRODUCTION

Dopamine (DA) receptors play an important role in the modulation of excitability and the responsiveness of neurons to excitatory and inhibitory amino acids in the striatum (Calabresi et al. 1997a, 2000; Cepeda and Levine 1998; Konradi et al. 2000; Levine and Cepeda 1998). The effects of DA are mediated by at least five identified receptor subtypes classified into two families according to their pharmacological profiles (Civelli et al. 1991; Creese and Fraser 1997; Sibley and Monsma 1992). This classification scheme recognizes a D1 family composed of D_{1A} and D_{1B} subtypes in rat or their corresponding D₁ and D₅ forms in human, and a D2 family composed of three subtypes, D₂, D₃, and D₄. To minimize confusion, we will use D1 and D2 to refer to the families of DA receptors and subscript notation (e.g., D₂ and D₄) to refer to subtypes within each family.

The effects of DA on the excitability of striatal cells are complex. The outcome of DA modulation depends on a number of factors such as the DA receptor subtype preferentially activated, the receptor location at pre- or postsynaptic sites, the concentration of ambient DA, and the activity-state of the neuron subject to DA modulation (Cepeda and Levine 1998). We have proposed a relatively simple scheme to explain some of DA's modulatory actions in which its effects are a function of the specific receptor subtypes with which it interacts (either the DA receptor subtypes or the neurotransmitter receptors that it will modulate). In the striatum, D1 receptor activation enhances responses elicited by glutamate receptors, particularly those mediated by *N*-methyl-D-aspartate (NMDA) receptors. In contrast, D2 receptor activation reduces glutamate receptor responses, particularly those mediated by activation of non-NMDA receptors. The enhancing effects of D1 receptor activation appear to involve postsynaptic actions (Cepeda et al.

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1993, 1998a; Flores-Hernández et al. 1999). The attenuating effects mediated by D2 receptors may involve both presynaptic actions on corticostriatal terminals and/or postsynaptic actions (Cepeda et al. 1993; Flores-Hernández et al. 1997; Levine et al. 1996b).

Although there is considerable evidence for the importance of functional D2 receptors in the striatum, their electrophysiological roles and their cellular location still remain areas of debate. One major controversial area is whether D2 receptors are located presynaptically on corticostriatal axons regulating glutamate release (Schwarcz et al. 1978) or are located postsynaptically on striatal neurons regulating excitability. Pharmacological studies have been the most consistent in supporting presynaptic DA receptor regulation of glutamate release via D2 receptors (Godukhin et al. 1984; Kornhuber and Kornhuber 1986; Maura et al. 1988, 1989; Mitchell and Dogett 1980; Rowlands and Roberts 1980; Yamamoto and Davy 1992). Studies examining receptor binding and anatomical localization have provided conflicting outcomes. In some cases, the existence of presynaptic D2 receptors was supported, whereas in others it was not (Fisher et al. 1994; Garau et al. 1978; Hersch et al. 1995; Joyce and Marshall 1987; Schwarcz et al. 1978; Sesack et al. 1994; Trugman et al. 1986).

Electrophysiological studies demonstrating pre- or postsynaptic actions of D2 family receptors have also been inconclusive. In the first studies examining effects of DA, both pre- and postsynaptic mechanisms were implicated in the reduction of striatal excitability (Arbuthnott et al. 1987; Brown and Arbuthnott 1983; Garcia-Muñoz et al. 1991; Mercuri et al. 1985). *In vitro* experiments initially suggested that the reduction of excitatory postsynaptic potentials by DA was mediated exclusively by postsynaptic D1 receptors (Calabresi et al. 1987a). D2 receptor-mediated effects were not observed unless these receptors were rendered hypersensitive by DA-depleting lesions (Calabresi et al. 1988, 1993). Subsequent studies have revealed that D2 receptor-mediated effects were not always dependent on rendering these receptors hypersensitive, and electrophysiological evidence has been obtained for both pre- and postsynaptic actions of D2 receptors in the striatum (Cepeda et al. 1994; Hsu et al. 1995; Jiang and North 1991).

Another approach to examine DA's role in presynaptic modulation of glutamatergic activity involves use of the K⁺ channel blocker 4-aminopyridine (4-AP) (Flores-Hernández et al. 1994). This blocker causes release of excitatory and inhibitory neurotransmitters. Addition of bicuculline (BIC), a GABA_A receptor antagonist, isolates excitatory synaptic activity. Under these conditions, D2 receptor activation reduces excitatory synaptic activity in a subset of neurons, probably via presynaptic modulation (Flores-Hernández et al. 1997).

The reasons for the inconsistencies in electrophysiological findings probably stem from differences in experimental approach and recording conditions. For example, modulation by D2 receptors *in vitro* has been more difficult to demonstrate than *in vivo* (Brown and Arbuthnott 1983; Calabresi et al. 1996; however, see Hsu et al. 1995). On the other hand, acute effects of DA mediated by activation of D2 receptors are mild at best (Cepeda et al. 1994; Flores-Hernández et al. 1994) but can be enhanced after chronic DA depletion (Calabresi et al. 1988, 1993), hence the conclusion that the control of glutamate release by presynaptic D2 receptors might operate *in vivo* under physiological conditions, whereas *in vitro* it requires

supersensitivity of D2 receptors (Calabresi et al. 1996). One overriding factor that might help unravel the inconsistencies is examining differences between acute effects of application of D2 receptor agonists and antagonists and the more chronic effects of long-term alterations in receptor function like pharmacological blockade or lesions of DA inputs. The present experiments address these differences by examining chronic effects of genetic deletion of specific DA receptors as well as acute treatment with agonists and antagonists.

The recent generation of DA receptor-deficient mutant mice has provided another tool to examine hypotheses concerning the specific role of each DA receptor subtype using electrophysiological analyses (Levine et al. 1996a). In the present studies, we used D₂ and D₄ DA receptor-deficient mutant mice to assess whether the removal of these receptor subtypes alters DA's ability to modulate glutamate synaptic responses in the striatum. Although D₂ receptors have generally been hypothesized as the prominent D2 receptor localized in the striatum, the D₄ receptor has also been localized on corticostriatal terminals (Tarazi et al. 1998) as well as on striatal neurons (Ariano et al. 1997). In the present study, we utilized intracellular and whole cell patch-clamp recording techniques to examine spontaneous depolarizations (current clamp with sharp electrodes) and spontaneous excitatory synaptic currents (voltage clamp with patch electrodes) in slices from D₂ and D₄ DA receptor-deficient mutant mice. We hypothesized that if D₂ receptors are responsible for inhibitory regulation of glutamate responses in the striatum, then there should be an increase in spontaneous synaptic activity in the striatum in D₂ receptor-deficient mice. If, on the other hand, D₄ receptors are responsible for inhibitory regulation, then there should be an increase in spontaneous synaptic activity in D₄ receptor-deficient mice.

METHODS

Animals

D₂ and D₄ receptor-deficient mutant mice were obtained from the colony of Dr. David K. Grandy at the Oregon Health Sciences University, Portland, OR. The methods used for the generation and identification of these mice have been described (Kelly et al. 1997, 1998; Rubinstein et al. 1997). The D₂ mice (129S4/SvEv; C57BL/6) were incipient congenics after five backcrosses to C57BL/6J and the D₄ mice (129P2/OlaHsd, C57BL/6) were F₂ hybrids. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at UCLA. The total number of mice used in these experiments was 60 [20 D₂ receptor-deficient, 18 wild-type (WT) littermates, 12 D₄ receptor-deficient, 10 WT littermates]. Ages ranged from 3 mo to up to 1 yr. For whole cell recordings with patch electrodes, young animals (3 mo) were used because visualization of individual neurons with infrared videomicroscopy and the quality of the patches were better than in older animals. For intracellular recordings with sharp electrodes and anatomical studies, we had no age limitations and animals spanned the range of 3 mo to 1 yr. There were no consistent differences in data due to age, and results were pooled according to genotype.

Preparation of slices

Our procedures for brain slice preparation have been described (Cepeda et al. 1998a; Levine et al. 1996a,b). Briefly, mice were anesthetized with halothane and then euthanized by decapitation.

After dissection, brains were placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) [which contained (in mM) 130 NaCl, 26 NaHCO₃, 3 KCl, 5 MgCl₂, 1.25 NaH₂PO₄, 1.0 CaCl₂, and 10 glucose 10 (pH 7.2–7.4)]. Striatal sections were cut (350 μ m) in the coronal plane and placed in oxygenated (95% O₂-5% CO₂) ACSF (same as preceding except 2 mM CaCl₂ and 2 mM MgCl₂). After at least 1 h the slice was transferred to a Haas-type recording chamber for standard intracellular recordings. For patch recordings from visualized neurons, the slice was placed in a perfusion chamber attached to the fixed-stage of an upright microscope (Zeiss Axioskop) and submerged in continuously flowing oxygenated ACSF (25°C, 4 ml/min). Cells were visualized with a 40 \times water-immersion lens and illuminated with near infrared (IR) light (790 nm, Ealing Optics, Holliston, MA), and the image was detected with an IR-sensitive CCD camera. Cells were typically visualized from 30 to 100 μ m below the surface of the slice. It was possible to distinguish medium- and large-sized striatal cells. In the present study, only medium-sized neurons were examined.

Current-clamp recordings

To evaluate electrophysiological properties, standard current-clamp recordings were obtained using high-impedance sharp electrodes (3 M K-acetate, 80–120 M Ω). Signals were amplified (Axoclamp-2A, Axon Instruments, Foster City, CA), displayed on an oscilloscope, and digitized for subsequent computer analysis (pClamp 6.0.1, Axon Instruments). All data were obtained from neurons with resting membrane potentials (RMP) of at least -60 mV and action potentials exceeding 55 mV. Membrane properties (RMP, action potential amplitude and duration, input resistance, and rheobase) were measured.

Whole cell voltage clamp

The whole cell patch technique was used for voltage-clamp recordings. Patch electrodes (3–6 M Ω) were filled with the following internal solution (in mM): 130 Cs-methanesulfonate, 10 CsCl, 4 NaCl, 1 MgCl₂, 5 MgATP, 5 EGTA, 10 HEPES, 0.5 GTP, 10 phosphocreatine, and 0.1 leupeptin (pH 7.25–7.3, osmolality 280–290 mOsm). Axopatch 200A or 1D amplifiers were used for voltage-clamp recordings. A 3 M KCl agar bridge was inserted between the extracellular solution and the Ag-AgCl indifferent electrode. Tight seals (2–10 G Ω) from visualized medium-sized cells were obtained by applying negative pressure. The membrane was disrupted with additional suction and the whole cell configuration was obtained. The access resistances ranged from 8 to 15 M Ω and were compensated 60–85%. Cells were held at -60 mV.

Drug application

The K⁺ channel blocker 4-AP (100 μ M) was added to the ACSF to induce spontaneous synaptic activity (Flores-Hernández et al. 1994). BIC (10 μ M) was used to block spontaneous activity caused by activation of GABA_A receptors. In these conditions, the remaining activity was due to activation of glutamate receptors. A DA D₂ agonist (quinpirole, 10–20 μ M) or antagonist (sulpiride, 10 μ M) was used to study the modulation of 4-AP-induced synaptic activity. Spontaneous or evoked synaptic activity was analyzed using the Mini Analysis Program (Synaptosoft, Leonia, NJ). Only events clearly distinguishable from background noise, usually larger than 3 mV (current clamp) or 5 pA (voltage clamp) were included in the analysis.

Cell identification

In all experiments, sharp electrodes were filled with 2% biocytin and patch electrodes were filled with 0.2% biocytin (Sigma, St Louis, MO) to label neurons. After the experiment, the slice was fixed in 4% paraformaldehyde overnight and then processed according to pub-

lished protocols (Kita and Armstrong 1991). For the morphological analysis, only cells obtained from sharp electrode intracellular recordings were examined. Recovered neurons from experiments using patch electrodes were only identified as medium-sized spiny cells. Their morphology or the existence of dye-coupling was not examined in these cells because of the possibility of dye leakage when the seal was made.

Immunohistochemistry

Anti-peptide antisera developed for the D₂ (McVittie et al. 1991) and D₄ (Ariano et al. 1997) receptor subtypes were used to evaluate receptor expression in the D₂ and D₄ receptor-deficient mice. Fresh-frozen brain sections (10 μ m thick) were cut in the coronal plane. Tissue sections from receptor-deficient and WT control mice were processed simultaneously to minimize potential differences in experimental processing. The antisera were applied to the slide-mounted section and incubated overnight at 4°C in a humidified environment. The following day, unbound primary anti-DA receptor antisera were rinsed off and secondary, fluorescently labeled anti-rabbit antisera were applied for 1–2 h at 4°C in a humidified environment. Sections were examined immediately using a scanning laser confocal microscope (BioRad MRC 600). Digitized images for control and mutant striata were matched from similar regions of the nucleus and obtained by 10 accumulated scans from the laser without any further image enhancement. Laser settings were identical for each antiserum detected in an experimental pair. Controls for the procedure included use

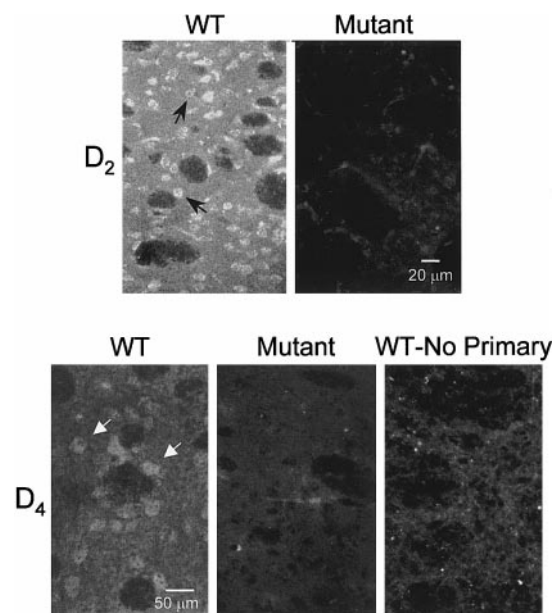


FIG. 1. *Top left*: cellular D₂ dopamine (DA) receptor protein localization in the striatum in a wild-type (WT) littermate of a D₂ receptor-deficient mutant. Many medium-sized neurons demonstrate immunofluorescence within the cytoplasm (\rightarrow). The neuropil is also stained and is contrasted with the unstained myelinated fiber bundles coursing through the nucleus. *Top right*: lack of staining for D₂ DA receptor protein in the striatum of a D₂ receptor-deficient mutant. *Bottom left*: cellular D₄ DA receptor protein localization in the striatum in a WT littermate of a D₄ receptor-deficient mutant. Fewer medium-sized neurons (\rightarrow) demonstrate immunofluorescence compared with the staining for the D₂ receptor in the WT and the intensity of the neuropil staining is reduced. *Bottom middle*: lack of staining for D₄ DA receptor protein in the striatum of a D₄ receptor-deficient mutant. *Bottom right*: lack of staining in the striatum of a WT littermate of a D₄ receptor-deficient mutant when the primary antibody is deleted. Note the similarity of staining pattern to the mutant. The panels for WT and each respective receptor-deficient mutant in this figure were generated using identical parameters to make direct comparisons between sections. Calibration at *top right* refers to *top panels* and in *bottom left* to *bottom panels*.

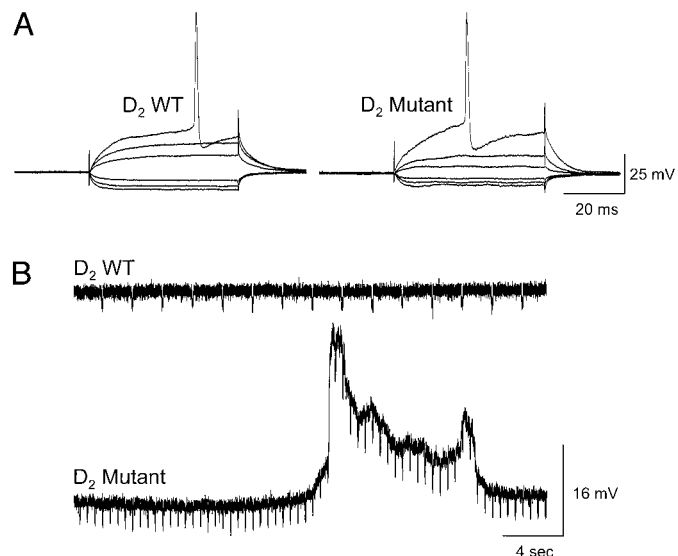


FIG. 2. *A*: examples of basic membrane properties in a WT and D₂ receptor-deficient mutant. Traces show voltage responses to a series of 3 hyperpolarizing and 3 depolarizing current pulses (0.2-nA steps from -0.6 to $+0.6$ nA, 50-ms duration). In each series, the highest amplitude depolarizing current pulse evoked an action potential. RMPs were -90 mV for each neuron. *B*, *top*: an example of spontaneous activity in a striatal neuron from a WT. *Bottom*: an example of a large spontaneous depolarization in a striatal neuron from a D₂ receptor-deficient mutant. This particular depolarization lasted about 10 s and displayed a maximum amplitude of about 20 mV. Downward deflections in both traces are hyperpolarizing current pulses injected through the recording electrode. Calibration refers to both traces.

of multiple antisera directed against different epitopes of the receptor protein sequence, use of preimmune sera, omission of primary antisera, and adsorption of the primary antisera with the peptide antigen.

Statistics

Differences between group means were analyzed with *t*-tests or the appropriate nonparametric statistic. In the text and tables, values are presented as mean \pm SE. Differences between means were considered statistically significant when $P < 0.05$.

RESULTS

Immunohistochemistry

D₂ receptor expression was examined in four D₂ receptor-deficient and four WT mice. Receptor protein staining was prevalent within medium- and large-diameter neurons in WT striata (Fig. 1, *top left*) while immunofluorescence was absent in the receptor-deficient mice (Fig. 1, *top right*). The cerebral cortex of WT animals also showed moderate levels of receptor expression. Receptor expression for D₄ receptors was unaltered in the D₂ receptor-deficient mice (data not shown), suggesting the genetic deletion did not change DA receptor staining for this closely related receptor protein. No staining was visible in the control experiments in D₂ WT mice in agreement with published results using this antibody (McVittie et al. 1991), indicating specificity for the D₂ reagent in mouse tissue.

D₄ receptor protein staining in striatum also was examined in four pairs of D₄ receptor-deficient and WT mice. In general, there was a reduced immunohistochemical reaction in the striata of WT mice stained for D₄ protein compared with WT striata stained for D₂ protein as demonstrated using these

antibodies in the rat (compare Fig. 1, *top left* and *bottom left*) (Ariano et al. 1997; McVittie et al. 1991). D₄ receptor expression was detected within medium-sized neurons and the surrounding neuropil within the striatum of the WT (Fig. 1, *bottom left*). This staining pattern was lost in the D₄ receptor-deficient animals (Fig. 1, *bottom middle*). The background in the D₄ receptor-deficient section represents nonspecific staining. When the primary antibody was omitted, the staining looked very similar to the D₄ staining in the receptor-deficient mutant (Fig. 1, *bottom right*). Immunohistochemical staining for D₂ and D₃ DA receptors was unchanged in the D₄ receptor-deficient mutants (data not shown).

Membrane properties

Intracellular recordings with sharp electrodes in current clamp were obtained from 55 striatal neurons from D₂ recep-

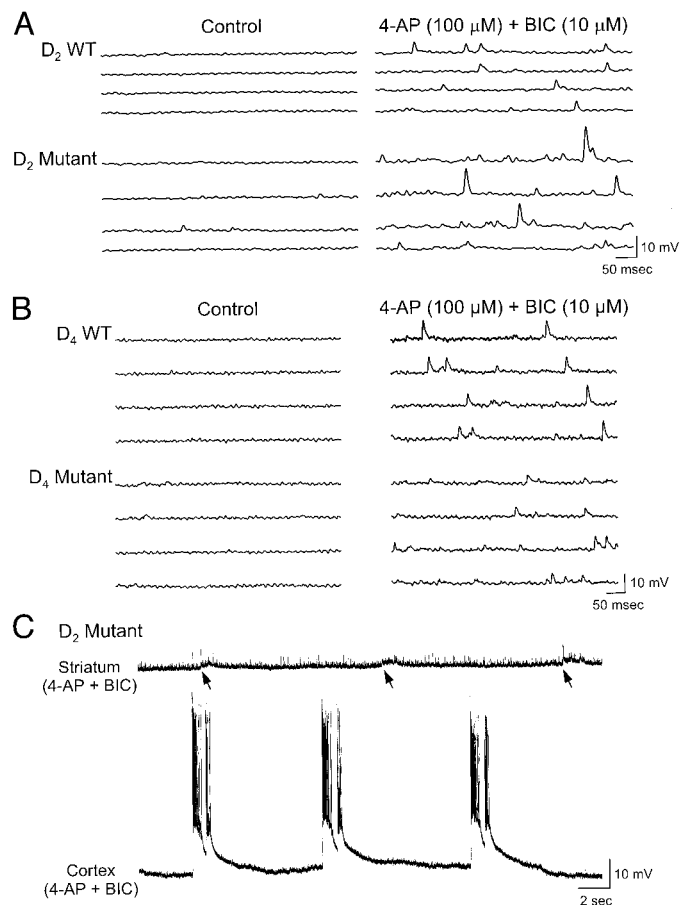


FIG. 3. *A*: examples of spontaneous activity before (control) and after bath application of 4-aminopyridine (4-AP) and bicuculline (BIC) in neurons from a D₂ WT and a D₂ receptor-deficient mutant. The 4 lines in *A* and *B* represent continuous traces. Note that application of 4-AP and BIC produced larger and more frequent depolarizations in the D₂ receptor-deficient mutant. *B*: examples of spontaneous activity before and after bath application of 4-AP and BIC in neurons from a D₄ WT and a D₄ receptor-deficient mutant. *C*, *top*: low-amplitude rhythmic depolarizations occur in a striatal neuron (\uparrow) from a D₂ receptor-deficient mutant in the presence of 4-AP (100 μ M) and BIC (10 μ M). *Bottom*: intracellular recording from a cortical pyramidal neuron in the cortex overlying the striatum from a D₂ receptor-deficient mutant in the presence of 4-AP (100 μ M) and BIC (10 μ M). Note that rhythmic depolarizing bursts have been evoked in the cortex at a frequency similar to the depolarizations in the striatum. Recordings in the *top* and *bottom* traces were not made simultaneously but sequentially (1st striatum and then cortex) in the same slice. Calibration refers to both traces.

tor-deficient, 35 striatal neurons from littermate WT control mice, 40 striatal neurons from D₄ receptor-deficient, and 38 striatal neurons from littermate WT controls. All but two striatal neurons recovered and stained with biocytin were medium-sized spiny neurons. These were large aspiny neurons and electrophysiological data from these two neurons were omitted.

In general, passive and active membrane properties measured from current-clamp recordings with sharp electrodes were similar in both D₂ and D₄ receptor-deficient and their respective WT controls (Fig. 2A). In D₂ mice, RMPs for neurons obtained from WT and receptor-deficient were

-80.2 ± 1.2 versus -79.1 ± 0.9 (SE) mV, respectively; action potential amplitudes were 77.8 ± 2.0 versus 75.5 ± 1.9 mV; half-amplitude durations for action potentials were 1.6 ± 0.1 versus 1.5 ± 0.1 ms; rheobases were 0.9 ± 0.1 versus 1.2 ± 0.1 nA; input resistances were 36.4 ± 2.9 versus 29.6 ± 2.9 M Ω .

Similarly in D₄ animals RMPs were -73.8 ± 3.1 versus -78.8 ± 1.9 mV; action potential amplitudes were 76.1 ± 5.3 versus 79.4 ± 2.4 mV; action potential half-amplitude durations were 1.5 ± 0.1 versus 1.8 ± 0.1 ms; rheobases were 0.9 ± 0.1 versus 0.9 ± 0.1 nA; and input resistances were 27.9 ± 2.3 versus 31.6 ± 3.1 M Ω in neurons from WT and receptor-deficient mice, respectively.

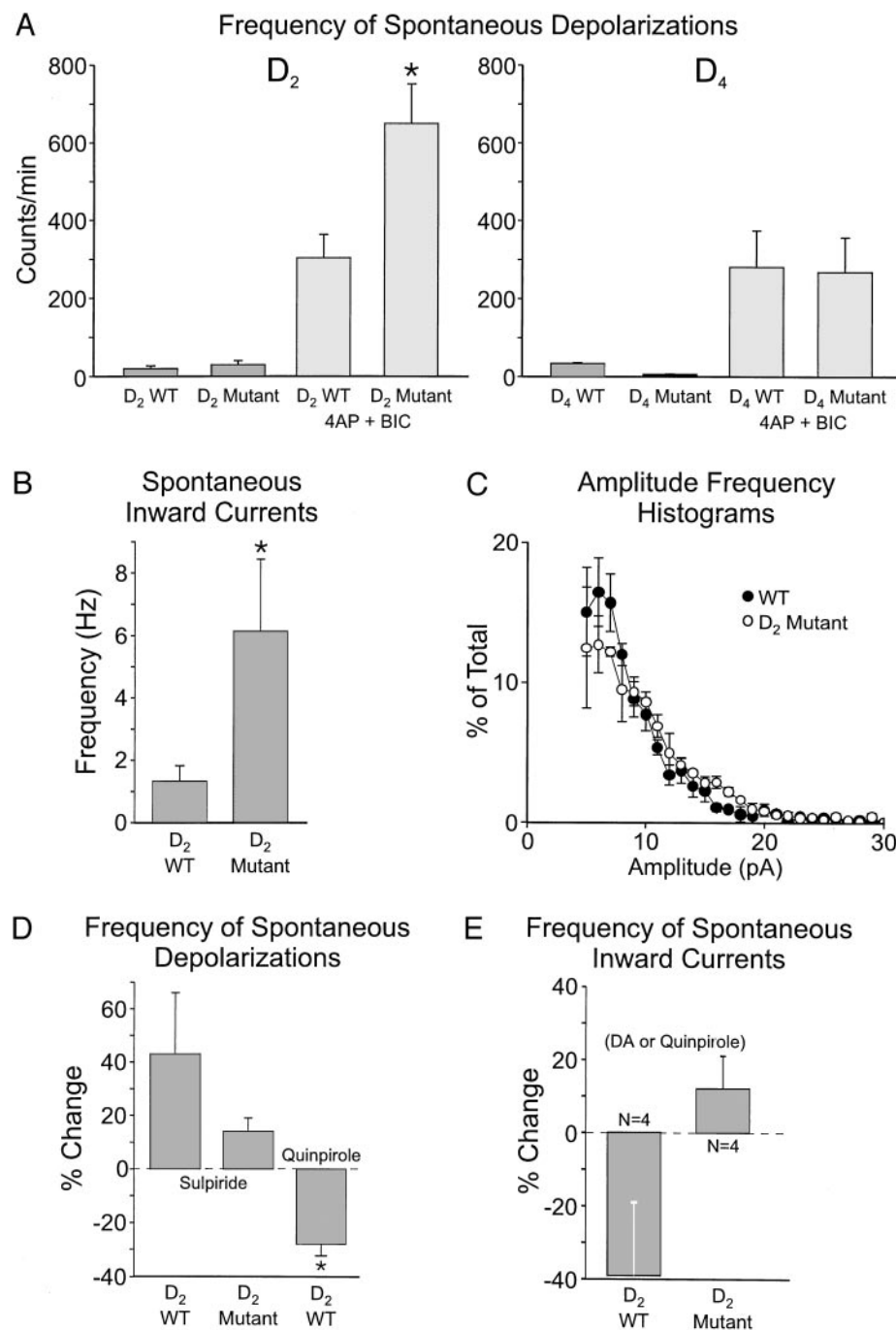


FIG. 4. *A*: mean (\pm SE) frequencies of spontaneous depolarizations before and after exposure to bath application of 4-AP and BIC in WT controls and D₂ and D₄ receptor-deficient mutants. The difference in the mean frequency of depolarizations after 4-AP and BIC between WT and D₂ receptor-deficient mutants was statistically significant (*). *B*: mean (\pm SE) frequencies of spontaneous inward currents in D₂ WT and D₂ receptor-deficient mutants. The difference in mean frequencies was statistically significant ($P < 0.05$; *). *C*: normalized amplitude frequency histograms for spontaneous inward currents for D₂ WT and D₂ receptor-deficient mutants. The frequencies were normalized for each cell, and all cells from mutant and WT were averaged for each amplitude bin. The histograms were not significantly different. *D*: mean (\pm SE) percent change in frequency of spontaneous depolarizations after sulpiride or quinpirole in D₂ WT and D₂ receptor-deficient mutants. *Left bar*: sulpiride increased the frequency of spontaneous depolarizations in neurons from WT mice ($n = 3$). *Middle bar*: little effect of sulpiride in the mutants ($n = 5$). *Right bar*: quinpirole decreased the frequency of spontaneous depolarizations in neurons from WT mice ($n = 6$). *, decrease is statistically significant ($P = 0.021$). *E*: mean (\pm SE) percent change in frequency of spontaneous EPSCs after DA or quinpirole in D₂ WT and D₂ receptor-deficient mutants. *Left bar*: DA or quinpirole decreased the frequency of excitatory postsynaptic currents (EPSCs) in neurons from WT mice ($n = 4$). *Right bar*: DA or quinpirole had little net effect on the frequency of spontaneous EPSCs in D₂ receptor-deficient mutants ($n = 4$). For *E*, cells that were exposed to DA or quinpirole were pooled in each group.

Spontaneous membrane depolarizations

In 55% of the neurons examined from D₂ receptor-deficient mutants, large-amplitude (24 ± 2.7 mV) long-duration (26 ± 7 s) spontaneous membrane depolarizations occurred (Fig. 2B). These events never occurred in WT mice ($P = 0.003$, Fisher exact test for difference between frequencies in mutants and WT). These potentials were blocked by application of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; $10 \mu\text{M}$; data not shown), a non-NMDA receptor antagonist. The spontaneous depolarizations appeared similar to membrane depolarizations observed *in vivo* intracellular recordings (Wilson and Kawaguchi 1996). However, in contrast to the *in vivo* depolarizations, these events were more sporadic and irregular occurring on average about once per minute. They only rarely reached the threshold for firing. These events also were specific to neurons recorded from D₂ receptor-deficient animals as they were not observed in any of the striatal neurons recorded from D₄ receptor-deficient mice.

Spontaneous synaptic activity

During current-clamp recordings with sharp electrodes, low-amplitude (up to 10 mV) spontaneous synaptic activity is rarely observed in slices under the present recording conditions and

was infrequent in WT mice. In contrast, in D₂ receptor-deficient mice spontaneous activity was present (Fig. 3A, left). Spontaneous activity was also infrequent in the D₄ receptor deficient mutant (Fig. 3B). To quantify these events, we counted the number of depolarizations exceeding 3 mV in amplitude for a 90-s epoch. Although there were slight increases in the mean frequency of these events in the D₂ receptor-deficient mutants (29 ± 11 vs. 20 ± 7 events/90 s in D₂ mutants and WTs, respectively), the difference was not statistically significant. The neurons obtained from the D₄ receptor-deficient mutants displayed less frequent spontaneous synaptic events than those obtained from their WT controls (3.1 ± 1.5 vs. 34 ± 20.4 events/90 s in D₄ mutants and WTs, respectively).

4-AP-induced synaptic activity

In the presence of 4-AP and BIC, cells from both receptor-deficient and WT mice displayed marked increases in the frequency of synaptic events (Fig. 3, A and B, right). However, the D₂ receptor-deficient mutants displayed significantly more events than their WT controls ($P = 0.007$; Figs. 3A, right, and 4A). Although the frequency of spontaneous events was increased markedly in the D₄ receptor-deficient mutants, there was no difference with respect to values obtained from WT

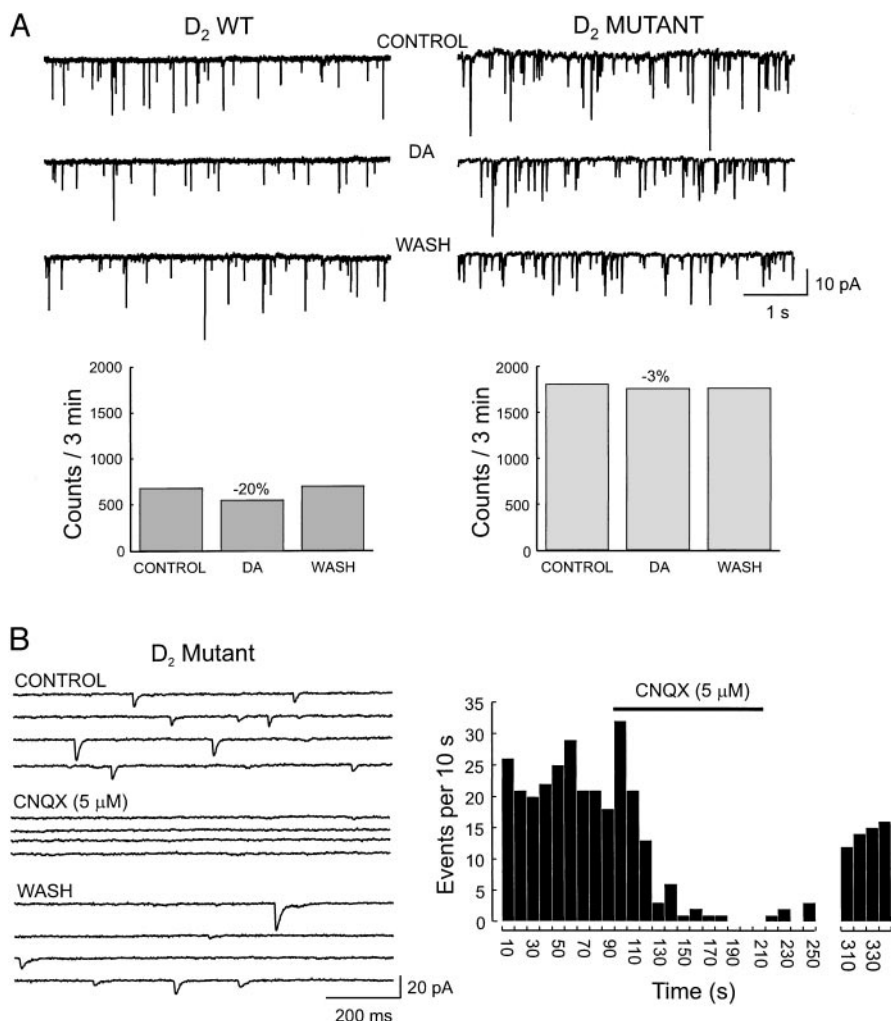
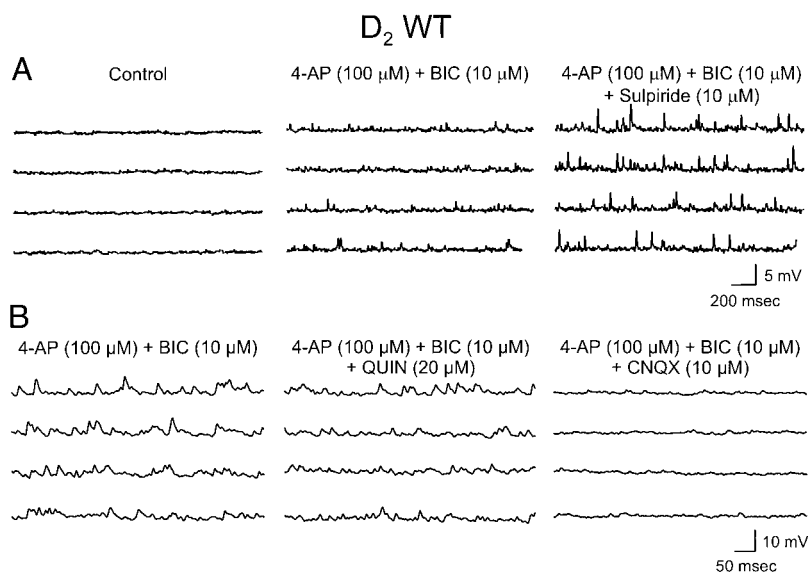


FIG. 5. *A*: spontaneous synaptic currents recorded from neurons from a WT and D₂ receptor-deficient mutant. Currents were recorded at a holding potential of -60 mV in the presence of BIC ($10 \mu\text{M}$). *Top 3 traces* from each cell show examples of membrane current recorded over a 5-s period before (control), during bath application of DA ($20 \mu\text{M}$), and after its wash-out (wash). Graphs show plots of the total number of events detected in each condition during a 3-min period. In the neuron from the WT, DA produced a 20% decrease in the frequency of events while in the D₂ receptor-deficient mutant there was virtually no change (3% decrease). *B*: spontaneous synaptic currents are blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a non-NMDA receptor antagonist. *Left traces*: continuous records before (control), during, and after (wash) application of CNQX. *Right*: summed events in 10-s epochs. Holding potential was -60 mV, and all recordings were made in the presence of $10 \mu\text{M}$ BIC.



controls (Figs. 3B, right, and 4A). Spontaneous and 4-AP-induced synaptic activity was completely blocked by CNQX (10 μM, $n = 5$), a non-NMDA receptor antagonist in neurons from both receptor-deficient mutants and WT mice (Fig. 6B, right).

After application of 4-AP and BIC, in addition to small-amplitude membrane depolarizations, some cells from receptor-deficient and WTs displayed rhythmic, large amplitude, long duration depolarizations (Fig. 3C, top). In D₂ receptor-deficient mutants, more cells displayed this type of activity (42 vs. 15% in mutant vs. WT, respectively). It is possible that this activity was a reflection of epileptiform activity evoked in cortical pyramidal neurons by 4-AP and BIC, since we observed such activity in the cortex (Fig. 3C, bottom).

Spontaneous inward currents

Spontaneous synaptic activity is better observed using whole cell patch-clamp recordings because the resolution (signal-to-noise ratio) improves considerably. Whole cell patch-clamp recordings were obtained from visualized medium-sized neurons in D₂ receptor-deficient mutants ($n = 6$) and WT controls ($n = 5$). Input resistances were not significantly different between WT and D₂ receptor-deficient mutants (197 ± 29 vs. 179 ± 50 MΩ, respectively).

Spontaneous excitatory postsynaptic currents (EPSCs) were more frequent in D₂ receptor-deficient mice than in their WT controls (Fig. 5A). To quantify these events, we counted the number of EPSCs exceeding 5 pA in amplitude for 3–5 min epochs. The average frequency of spontaneous events was

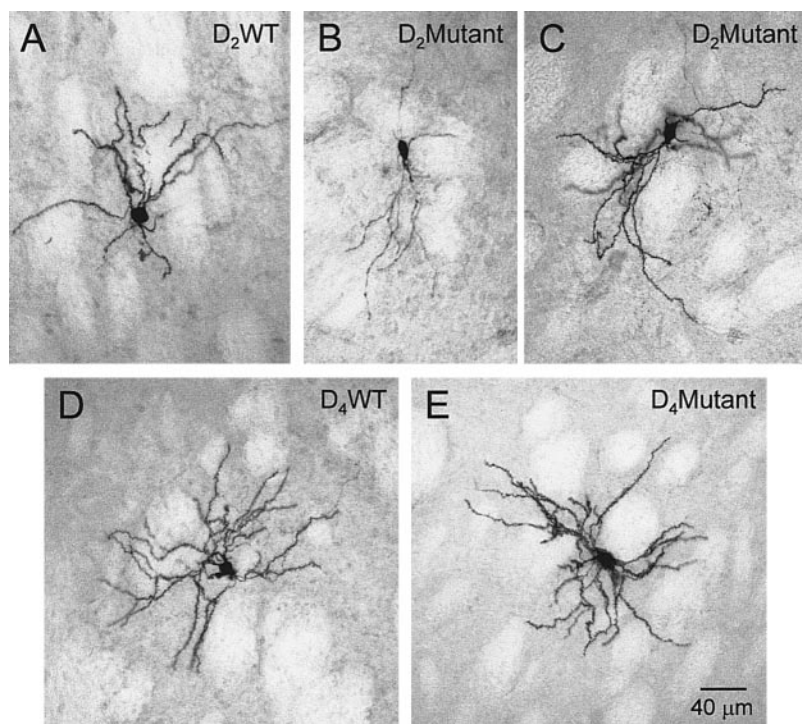


FIG. 7. Low-magnification micrographs of biocytin-filled medium-sized spiny striatal neurons. *A*: example of a neuron from a D₂ WT. *B*: example of a neuron from a D₂ receptor-deficient mutant that displayed thin dendrites and decreased spine densities. *C*: example of a neuron from a D₂ receptor-deficient mutant that had a dendritic appearance similar to that of WTs. *D* and *E* show medium-sized spiny neurons from a D₄ WT and a D₄ receptor-deficient mutant. Note that dendritic appearances were similar. Calibration in *E* refers to all panels.

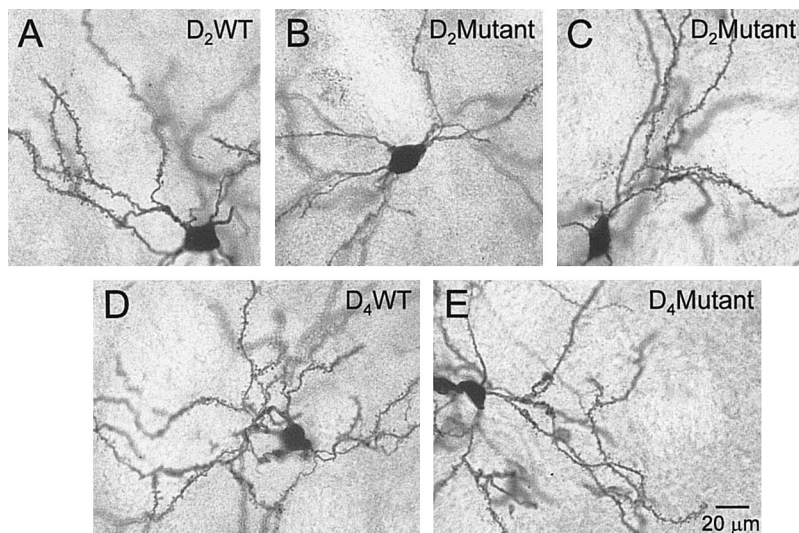


FIG. 8. High-magnification micrographs of biocytin-filled medium-sized spiny striatal neurons. *A*: example of a neuron from a D_2 WT. *B*: example of a neuron from a D_2 receptor-deficient mutant that displayed thin dendrites and decreased spine density. *C*: example of a neuron from a D_2 receptor-deficient mutant that had a dendritic appearance and spine density similar to that of WTs. *D* and *E* show medium-sized spiny neurons from a D_4 WT and a D_4 receptor-deficient mutant. Note that dendritic appearance and spine densities were similar. Calibration in *E* refers to all panels.

significantly higher in D_2 receptor-deficient mutants compared with WTs ($P < 0.05$; Fig. 4*B*). In contrast, the mean amplitudes of spontaneous events were similar (10.6 ± 0.7 pA in D_2 receptor-deficient mice vs. 9.7 ± 0.6 pA in WTs). Normalized amplitude frequency histograms were also similar (Fig. 4*C*). Spontaneous EPSCs were blocked by CNQX ($5 \mu\text{M}$; $n = 4$) indicating they were mediated by activation of non-NMDA receptors (Fig. 5*B*).

Effects of DA and D_2 receptor agonists

The effects of D_2 receptor antagonists and agonists were tested in current clamp with sharp electrodes and voltage clamp with patch electrodes in WT and D_2 receptor-deficient neurons. In current clamp conditions spontaneous or 4-AP-induced synaptic activity was increased by sulpiride ($10 \mu\text{M}$), the D_2 receptor antagonist (Figs. 4*D* and 6*A*) in neurons from WT mice. In contrast, in all WT neurons it was reduced by quinpirole ($10\text{--}20 \mu\text{M}$), the D_2 agonist (Figs. 4*D* and 6*B*). In D_2 receptor-deficient mutants, sulpiride ($10 \mu\text{M}$) had inconsistent effects from cell to cell, either increasing ($n = 3$) or decreasing ($n = 2$) frequencies of events by small amounts (Fig. 4*D*). Quinpirole was not tested in current-clamp conditions in D_2 receptor-deficient mutants. The effects of DA, quinpirole, and sulpiride were not examined in the D_4 receptor-deficient mutants because there were no differences between mutant and WT mice on basal measures of spontaneous activity.

In voltage-clamp conditions using patch electrodes, addition of DA ($20 \mu\text{M}$) (Fig. 5*A*) or the D_2 agonist quinpirole ($20 \mu\text{M}$) reduced the frequency of EPSCs in WT [data for DA ($n = 2$) and quinpirole ($n = 2$) were pooled because there were no differences] (Fig. 4*E*). In D_2 receptor-deficient mutants, both DA ($20 \mu\text{M}$; $n = 2$) and quinpirole ($20 \mu\text{M}$; $n = 2$) had little or no consistent effect on spontaneous EPSCs (2 cells increased slightly in frequency to quinpirole, 1 cell decreased, and 1 cell increased to DA; Fig. 4*E*).

Morphology

Medium-sized neurons filled with biocytin during intracellular recordings with sharp electrodes (50 from D_2 receptor-deficient and 22 from WT) did not show gross morphological

abnormalities (Figs. 7, *A–C*, and 8, *A–C*). Cross-sectional somatic areas were similar (135 ± 5 vs. $133 \pm 6 \mu\text{m}^2$ in D_2 receptor-deficient mutants and WTs, respectively). The circumference of the dendritic field was estimated for cells that did not have truncated dendrites by measuring the linear distance between dendritic endings. These values were also similar (585 ± 22 vs. $577 \pm 20 \mu\text{m}$ in D_2 receptor-deficient mutants and WTs, respectively). However, a subpopulation of cells from D_2 receptor-deficient mice displayed subtle changes in spine densities and dendritic appearance (Figs. 7*B* and 8*B*). Spine densities and dendritic appearance were rated by multiple observers blinded to genotype according to a semi-quantitative three-point scale [1 = thin dendrites, few spines ($<2/10 \mu\text{m}$); 2 = thick dendrites, moderate spine density ($3\text{--}5/10 \mu\text{m}$); 3 = thick dendrites, high spine density (>5 spines/ $10 \mu\text{m}$)]. There was a statistically significant increase in the proportion of neurons with thin dendrites and spine densities $<0.2/10 \mu\text{m}$ in D_2 receptor-deficient mutants (32% in mutants vs. 6% in WT mice; $P < 0.01$, χ^2 statistic). In this subset of neurons dendrites sometimes displayed varicosities instead of spines. Dye-coupling occurred in both WT and receptor-deficient animals. The incidence of coupling was slightly higher in WT animals (12% in D_2 receptor-deficient compared with 19% in WT), but the difference was not statistically significant.

Medium-sized neurons from D_4 receptor-deficient mutants ($n = 31$) and WT controls ($n = 32$) were similar in somatic cross-sectional area (120 ± 6 vs. $132 \pm 5 \mu\text{m}^2$ for mutant vs. WT, respectively), dendritic field circumference (593 ± 29 vs. $617 \pm 33 \mu\text{m}$ for mutant vs. WT, respectively), dendritic appearance and spine density estimates (Figs. 7, *D* and *E*, and 8, *D* and *E*). The incidence of dye-coupling was also similar (19% in mutants vs. 22% in WT).

DISCUSSION

The results of these experiments indicate that genetic deletion of the D_2 receptor produces a series of chronic electrophysiological alterations consistent with an inhibitory role of this DA receptor subtype in the intact striatum. First, high-amplitude, long-duration spontaneous depolarizing events that were mediated by activation of glutamate receptors occurred only in neurons from D_2 receptor-deficient mice. Second, in the

presence of 4-AP and BIC, the frequency and amplitude of glutamate receptor-mediated spontaneous depolarizations were increased. Third, there was an increase in spontaneous glutamate receptor-mediated inward membrane currents.

In addition, acute modulation of D₂ family receptors in WT controls altered spontaneous membrane depolarizations and inward currents mediated by activation of glutamate receptors in a manner consistent with an inhibitory function of such receptors. Interestingly, such acute modulation tended to produce milder effects than genetic deletion of the receptor.

Taken together these observations argue for an increase in glutamate transmission at the corticostriatal and possibly the thalamostriatal pathway in D₂ receptor-deficient animals. Concomitant morphological changes, though subtle, suggest that the increased excitatory transmission in the D₂ receptor-deficient mutant may be deleterious to a subpopulation of medium-sized striatal spiny neurons. These effects did not occur in D₄ receptor-deficient mice, indicating specificity of function of D₂ receptors in regulation of striatal glutamate transmission.

As pointed out in the preceding text, there is considerable evidence for both pre- and postsynaptic localization of D₂ receptors in striatum. Although our findings demonstrate an inhibitory role for this receptor subtype in modulating glutamate receptor-mediated responses, we cannot exclusively discriminate between pre- and postsynaptic function. Increases in frequency of inward currents without changes in average amplitude of events is typically taken as evidence for a change in presynaptic modulation (Dudel and Kuffler 1961; Takahashi et al. 1996). Previously, using a similar paradigm (4-AP and BIC), D₂ receptors were shown to presynaptically modulate glutamate receptor-mediated responses in a subset of striatal neurons (Flores-Hernández et al. 1997). The present data on the effects of quinpirole and sulpiride in WT mice support these findings, demonstrating similar acute effects of pharmacological manipulations. Furthermore increases in synaptic activity in the D₂ receptor-deficient mutants would indicate the inhibitory modulation is absent. Although we could not directly compare amplitudes of spontaneous depolarizations in current-clamp experiments (because RMPs and input resistances were not always the same), in voltage-clamp experiments, average amplitudes and normalized amplitude frequency histograms of spontaneous inward currents were similar in mutant and WT mice, providing at least some supporting evidence that the missing inhibition could have been of presynaptic origin. The demonstration of D₂ receptors on at least a subpopulation of corticostriatal terminals (Fisher et al. 1994; Sesack et al. 1994) provides additional morphological support for a presynaptic modulatory role of D₂ receptors. Presynaptic inhibition of glutamate release may involve DA's actions on voltage-gated calcium channels at the presynaptic terminal. D₂ receptors may be activated presynaptically to reduce calcium currents involved in the release of glutamate (Bargas et al. 1998; Lovinger et al. 1994). Indeed, D₂ receptors have been shown to decrease calcium currents in a number of systems (Berkowicz and Trombley 2000; Koga and Momiyama 2000; Miyazaki and Lacey 1998; Shoji et al. 1999). Thus lack of D₂ receptors signals the loss of an important negative regulatory mechanism controlling glutamate release.

Previously we demonstrated that activation of D₂ receptors attenuates synaptic responses mediated by glutamate receptors in the striatum (Levine et al. 1996b). At that time, we inter-

preted the data to indicate that the effects of D₂ receptors were primarily postsynaptic. Using paired-pulse facilitation as an index of presynaptic effects, we demonstrated that application of DA did not alter paired-pulse facilitation. However, in that study, quinpirole produced a nonsignificant increase in paired-pulse facilitation, suggesting that there may have been a presynaptic component to D₂ activation in a subset of neurons, a conclusion similar to the one reached by Flores-Hernández and colleagues (1997).

The failure to demonstrate presynaptic effects by activation of D₂ receptors may depend on the experimental approach. Clear presynaptic effects have been observed using chronic depletions of DA (Calabresi et al. 1993). This is probably because after DA-depletion D₂ receptors become supersensitive. Similarly, the present study demonstrated a presynaptic inhibitory role of D₂ receptors on glutamate release because the mutant animals have been chronically deprived of D₂ receptors.

Since we did not obtain evidence for increases in glutamate receptor-mediated activity in D₄ receptor-deficient mutants, we can conclude that the D₄ receptor probably does not have an inhibitory role in the striatum, at least with respect to responses mediated by activation of glutamate receptors. The D₄ receptor is more prevalent in the cortex than in the striatum (Ariano et al. 1997). Interestingly, we have shown that in the cortex of D₄ receptor-deficient mice, there is an increase in glutamate receptor-mediated synaptic activity (Altemus et al. 1998), suggesting an inhibitory role of this DA receptor subtype in a different neural area.

4-AP has been shown to enhance synaptic transmission via multiple mechanisms, depending on its concentration (Rutecki et al. 1987; Thesleff 1980). In the hippocampus, it produces epileptiform activity (Rutecki et al. 1987). In the striatum, the effects of 4-AP have been hypothesized to be due to calcium-dependent glutamate release (Flores-Hernández et al. 1997). Application of 4-AP will also release other striatal transmitters like GABA, acetylcholine, and dopamine. In the present study, we used BIC to block effects of activation of GABA_A receptors and demonstrated that CNQX blocks the remaining depolarizations produced by the 4-AP. Thus it is highly probable that the increase in frequency of depolarizations after 4-AP and BIC was due to release of glutamate and activation of glutamate receptors. One caveat though is whether spontaneous DA release is altered in the D₂ mutant. If less DA is released by 4-AP, then one could argue that there might be less influence by DA on the remaining DA receptor subtypes. Although DA levels do not appear altered in D₂ receptor-deficient mutants (Dickinson et al. 1999), additional work will have to be performed to examine this issue in more detail. However, the observation that even in the absence of 4-AP spontaneous synaptic events were significantly increased in whole cell recordings demonstrates that glutamate release is facilitated in D₂ receptor-deficient mice. Application of 4-AP only makes this effect more distinct.

The results of the present study may now provide an electrophysiological basis for some of the previously reported phenotypes displayed by D₂ receptor-deficient mice. For example, reduced regulatory function of DA has been demonstrated in the substantia nigra of these mice (Mercuri et al. 1997). In nucleus accumbens, D₂ receptors have been shown to play an important role in prepulse inhibition (Ralph et al.

1999). Alterations in the expression of synaptic plasticity have also been observed in the D₂ receptor-deficient mouse (Calabresi et al. 1997b). In these mice, tetanic stimulation of the corticostriatal pathway in slices induces long-term potentiation instead of long-term depression. Calabresi et al. (1997a,b, 2000) have shown that such long-term potentiation occurs in the striatum when the NMDA receptor is relieved of its Mg²⁺ block. In light of the present findings, the change in synaptic plasticity in D₂ receptor-deficient mutants may be interpreted as an effect of dysregulated glutamate release in the striatum and the concomitant release of the NMDA receptor from its Mg²⁺ block. Thus D₂ receptors could play an important role in controlling glutamate-evoked responses in the striatum and the resulting direction of synaptic plasticity. The present findings also support our proposed scheme of DA's actions that predicts a negative regulation of glutamate receptor activation by D₂ receptors (Cepeda and Levine 1998; Cepeda et al. 1993; Levine and Cepeda 1998). Thus the absence of D₂ receptors in the mutant mice should favor the enhancement of excitatory synaptic activity.

Presynaptic regulation of glutamate release by DA acting on D₂ receptors may also help understand the low levels of spontaneous activity in the striatum. Although intrinsic potassium currents undoubtedly help keep medium-sized spiny neurons hyperpolarized (Calabresi et al. 1987b), the role of DA acting on D₂ receptors should not be neglected. DA released tonically could activate D₂ receptors located on corticostriatal terminals (possibly in a paracrine fashion) to prevent excessive release of glutamate and keep the postsynaptic membrane hyperpolarized (down-state). When highly synchronized inputs occur, the membrane would depolarize (up-state) (see Wilson and Kawaguchi 1996). As we have shown, under these circumstances, NMDA receptor blockade is relieved, and now DA, acting on D1 receptors, will facilitate glutamate responses (Cepeda and Levine 1998; Cepeda et al. 1993).

The presynaptic regulation of glutamate release by D₂ receptors is better appreciated after unilateral lesions of the substantia nigra that deplete the striatum of DA. One of the chronic effects of such lesions is to increase spontaneous synaptic activity and cell firing in striatal neurons (Cepeda et al. 1989; Galarraga et al. 1987; Schultz 1982). This activity can be modulated by D2 receptor agonists, possibly via presynaptic mechanisms (Calabresi et al. 1993).

If increased release of glutamate occurs in D₂ receptor-deficient mice as we propose, structural alterations in striatal neurons over long time periods could be expected due to increased exposure to glutamate. The present experiments indicate that a subpopulation of medium-sized spiny neurons labeled with biocytin have reduced dendritic spines. These findings are consistent with a degenerative process. In support of this interpretation, we have shown previously that striatal cell swelling, the first step in the excitotoxic cascade induced by exposure to NMDA or kainate, is reduced by quinpirole in a dose-dependent fashion (Cepeda et al. 1998b).

Although the majority of evidence favors D₂ receptor-mediated inhibition of corticostriatal glutamate-evoked activity, there is another interpretation that should be considered. Previously it had been shown that glutamate and DA are potentially co-localized in nigrostriatal neurons (Sulzer et al. 1998). Therefore it is a possibility that DA, acting via D2 autoreceptors, could inhibit glutamate release from endings of nigrostri-

atal neurons. If DA and glutamate are also colocalized in nigrostriatal neurons in the mouse, then the deletion of inhibitory D₂ autoreceptors may predispose DA-containing neurons to release increased quantities of glutamate and DA. As pointed out in the preceding text, recent evidence has been provided to the contrary. DA levels in the striatum of D₂ receptor-deficient mice and their WT controls are not different (Dickinson et al. 1999).

Receptor-deficient mouse mutants offer many advantages for the study of the molecular and cellular mechanisms underlying receptor function. They are especially valuable when pharmacological tools are either unavailable or they lack the specificity to discriminate among closely related receptor subtypes. However, as with any technique, there are limitations. One problem with such mice has to do with the developmental compensation that occurs in animals lacking a particular receptor subtype (Holsboer 1997). Consequently the effects observed in the receptor-deficient animals could be due to changes in other remaining receptors as well as the deleted receptor. There is no way to completely control for this possibility with the animals currently available, but it should be minimized in future conditional D₂ receptor-deficient mutant mice (Crawley et al. 1997; Pich and Epping-Jordan 1998). One approach that has been used to address the issue of compensation at the molecular level has been to examine mice for significant changes in the expression of the remaining DA receptor subtypes (Saiardi et al. 1998) as well as other relevant proteins (Murer et al. 2000). Another caveat associated with mutants concerns the genetic background of the strain. Behavioral studies using one D₂ receptor-deficient mutant suggested that this mouse was a potential model of Parkinson's disease (Baik et al. 1995). However, behavioral studies using the strain examined in the present experiments showed mild motor impairments in D₂ mutants (Kelly et al. 1998). Recent studies by other investigators suggest that there is little difference in the unconditioned locomotor behaviors between the two strains of D₂ receptor-deficient mutant mice (Boulay et al. 2000; Clifford et al. 2000). Moreover, the two WT background strains used in the present studies were similar to each other in every quantified electrophysiological parameter despite large differences in genetic background. Additional experiments will need to be performed to determine precisely how strain differences may affect electrophysiological analyses.

In conclusion, two main functions can be envisaged for D₂ receptors. First, by inhibiting glutamate responses, they could act as a filtering device to select synaptic inputs and increase the signal-to-noise ratio (Cepeda et al. 1993; Flores-Hernández et al. 1997). This function emphasizes acute effects of DA at D₂ receptors. Second, D₂ receptors could function as a gatekeeper and protect striatal neurons from excessive glutamate stimulation (Cepeda and Levine 1998; Godukhin et al. 1984). Such a function would emphasize more chronic, long-term effects of DA release and provide evidence for a paracrine role of DA.

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