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Functional Roles of Dopamine D_2 and D_3 Autoreceptors on Nigrostriatal Neurons Analyzed by Antisense Knockdown *In Vivo*

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Two different 19-mer antisense oligodeoxynucleotides complementary to the initial coding regions of dopamine D_2 or D_3 receptor mRNA were infused unilaterally into the substantia nigra of rats for 3–6 d to suppress synthesis of D_2 and/or D_3 receptors on substantia nigra dopaminergic neurons, thereby producing specific reductions of D_2 and/or D_3 receptors. Autoradiographic receptor binding revealed that D_2 and D_3 antisense oligodeoxynucleotides specifically and significantly reduced D_2 or D_3 binding in the ipsilateral substantia nigra, respectively, without affecting dopamine receptor binding in the neostriatum. Either D_2 or D_3 antisense oligodeoxynucleotides greatly attenuated the ability of apomorphine to inhibit dopaminergic neurons *in vivo*, an effect that was potentiated by simultaneous administration of D_2 and D_3 antisenses. Despite these effects, neither the rate nor the pattern of spontaneous activity of antisense-treated nigrostriatal neurons differed from those in the control groups. The proportion of antidromic responses consisting of full spikes from antisense-treated rats was significantly greater, and the mean antidromic threshold was significantly lower than in controls, indicating that autoreceptor knockdown increased both somatodendritic and terminal excitability. These data demonstrate that selective reduction of specific dopamine receptor subtypes by antisense infusion can be effected *in vivo*, and that nigrostriatal neurons express both D_2 and D_3 autoreceptors at their somatodendritic and axon terminal regions. Although the somatodendritic and terminal autoreceptors modulate dendritic and terminal excitability, respectively, the interaction of endogenously released dopamine with somatodendritic autoreceptors does not appear to exert a significant effect on spontaneous activity in anesthetized rats.

Key words: antisense oligodeoxynucleotide; autoreceptor; dopamine receptor subtype; D_2 receptor; D_3 receptor; electrophysiology; substantia nigra

Mesencephalic dopaminergic neurons express receptors for their own neurotransmitter, dopamine (DA), in their somatodendritic and axon terminal regions. Stimulation of somatodendritic autoreceptors inhibits the spontaneous activity of dopaminergic neurons (Bunney et al., 1973; Groves et al., 1975), whereas stimulation of the axon terminal autoreceptors reduces the excitability of DAergic axon terminals and inhibits DA synthesis and release (Tepper et al., 1987; Starke et al., 1989; Wolf and Roth, 1990).

DA receptors were originally classified as D_1 or D_2 receptors based on their differing affinities for various ligands and linkage to intracellular signaling pathways (Kebabian and Calne, 1979). Within this classification, the somatodendritic autoreceptor was identified as a D_2 receptor on pharmacological (Morelli et al., 1988), electrophysiological (Lacey et al., 1987), and molecular biological (Meador-Woodruff et al., 1991) grounds. The axon terminal autoreceptor was similarly identified as a D_2 receptor pharmacologically (Boyar and Altar, 1987; Starke et al., 1989) and electrophysiologically (Tepper et al., 1984a,b).

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However, advances in molecular biology have rendered this "identification" uncertain, because it is now clear that two *families* of DA receptors exist (Sibley and Monsma, 1992). The D1-class family consists of D_1 and D_5 receptors and the D2-class family of D_{2L} or $_S$ isoforms and D_3 and D_4 receptors. (Nonsubscripted receptor subtypes, e.g., D1-class, refer to the receptor family, whereas subscripted receptor subtypes, e.g., D_2, refer to the molecular biologically defined specific receptor subtypes.)

Relatively large amounts of D_2 mRNA exist in the midbrain, localized to dopaminergic neurons; however, the existence of D_3 receptors in midbrain is more controversial. Some studies report D_3 mRNA in the substantia nigra–ventral tegmental area (Sokoloff et al., 1990; Bouthenet et al., 1991), whereas others failed to find D_3 mRNA or found it only in trace amounts (Valerio et al., 1994; Diaz et al., 1995; Le Moine and Bloch, 1996). On the other hand, it has recently been argued on pharmacological grounds that some DA autoreceptors may, in fact, be D_3 receptors (Meller et al., 1993; Tang et al., 1994; Chesi et al., 1995; Kreiss et al., 1995; Lejeune and Millan, 1995; Nissbrandt et al., 1995). Because the K_Ds for most agonists and antagonists are within one order of magnitude between D_2 and D_3 receptors (Gingrich and Caron, 1993; Seeman and Van Tol, 1994; Burris et al., 1995), they cannot be used to discriminate, at least in a physiologically useful manner, among members within the D2-class family. Thus, the precise subtype(s) of the somatodendritic and terminal autoreceptors remains unknown.

Antisense knockdown refers to the ability of specifically designed short sequences of oligodeoxynucleotides to bind to their complementary mRNA and stop translation, thereby preventing

the expression of the protein that the mRNA codes for. It is highly specific, and can be used to probe the functions of receptor subtypes for which specific and selective agonists or antagonists do not exist (Zhang and Creese, 1993). Here we describe the electrophysiological consequences of antisense knockdown of DA D_2, and/or D_3 receptors in DAergic nigrostriatal neurons.

MATERIALS AND METHODS

Subjects. Eighty-three male Harlan Sprague Dawley rats (Zivic-Miller) weighing between 150 and 250 gm at the time of initial surgery were used in these experiments. All animals were treated in strict accordance with guidelines set forth in the Public Health Service manual "Guide for the Care and Use of Laboratory Animals."

Antisense treatment. Rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (15 mg/kg) intraperitoneally and placed in a stereotaxic apparatus. Under aseptic conditions, the scalp was reflected and a small burr hole was drilled overlying the left substantia nigra [anterior: 2.0 (from lambda); lateral: 4.7 (at a 20° angle; see below)]. A 28 ga stainless steel infusion guide cannula (Plastics One, Roanoke, VA) was lowered 5.9 mm from the cortical surface and affixed with cyanoacrylate glue and dental cement. After a 24 hr recovery period, the inner 33 ga injection cannula, which was 1 mm longer than the guide, was filled, inserted into the guide, and connected to a length of Teflon tubing connected through an Instech Lab fluid swivel to a Harvard Apparatus Model digital 22 syringe pump. To minimize mechanical damage to the pars compacta, the nigral cannula was inserted at an angle of 20° to the midline such that the tip of the inner cannula was positioned ~500 μm dorsal and lateral to the center of the pars compacta. Oligodeoxynucleotides (10–20 μg/μl) or saline vehicle were infused continuously at 0.1 μl/hr for 3–6 d while the animals were housed in individual circular Plexiglas cages with access to food and water *ad libitum*. During the course of the antisense treatment, some animals were observed for two 30 min periods each day (at approximately 10:00 A.M. and 7:00 P.M.), and rotational behavior was recorded manually. A rotation was scored when a rat traversed 360° in either direction, and the total number of rotations was calculated by subtracting the number of ipsilateral rotations from the number of contralateral rotations that occurred during both 30 min periods.

Antisense oligodeoxynucleotides. The D_2 antisense oligodeoxynucleotide (AODN) was a 19-mer complementary to codons 2–8 (nucleotides 4–22) of the D_2 receptor mRNA with sequence 5′-AGGACAGGTTC-AGTGGATC-3′. The D_3 AODN, also directed against codons 2–8 (nucleotides 4–22), had the sequence 5′-TTATCTGGCTCAGAGGTGC-3′. There were two AODN controls: a D_2 random AODN consisted of the same bases as in the D_2 AODN in pseudorandom order with 11 of the 19 bases mismatched from the sense mRNA (5′-AGAACGGC-ACTTAGTGGGT-3′) and a D_3 random oligodeoxynucleotide that consisted of the same bases as in the D_3 antisense AODN but with 14 bases mismatched (5′-ATTCTGAGTTCGGTCAGCG-3′). Neither of the two random oligodeoxynucleotides is complementary to any other known sequence. All oligodeoxynucleotides consisted of *S*-oligodeoxynucleotides, in which the phosphodiester backbone of the nucleotide was modified by the inclusion of a phosphorothioate to increase the resistance of the nucleotide to degradation by endogenous nucleases (Agrawal et al., 1991; Sklarczyk and Kaczmarek, 1995). All AODNs were purchased from Oligos, Etc. (Wilsonville, OR).

Stimulating and recording. For acute recording experiments, rats were anesthetized with urethane (1.3 gm/kg, i.p.), the left femoral vein or a lateral tail vein was cannulated, and the rat was installed into a stereotaxic frame. Body temperature was maintained at 37 ± 1°C with a thermostatically controlled solid-state heating device. All wound edges and contact points between the animal and the stereotaxic frame were infiltrated with lidocaine ointment (5%) or solution (2%).

After removal of the scalp, a small burr hole was drilled overlying the neostriatum (1.0 mm anterior to bregma, 3.7 mm lateral to the midline) for the insertion of stimulating electrodes. Bipolar stimulating electrodes with a tip separation of ~150 μm and *in vitro* impedances of ~10 kΩ were formed from 100-μm-diameter enamel-coated stainless steel wires (California Fine Wire). After releasing some CSF by puncturing the atlanto-occipital membrane, stimulating electrodes were lowered 4.0 mm below the cortical surface and cemented in place with cyanoacrylate glue and dental cement. For recording, holes ~3.0 mm in diameter were drilled above the substantia nigra at coordinates 2.1 mm anterior to lambda and 2.0 mm lateral to the midline, and the infusion cannulae were carefully removed.

Recording electrodes were fabricated from 2.0 mm outer diameter capillary tubing (World Precision Instruments, Kwik-filr) on a Narishige PE-2 vertical pipette puller and possessed *in vitro* impedances of ~20 MΩ when filled with 2 M NaCl. The electrode impedance was lowered to between 4 and 10 MΩ by passing 500 msec 150 V direct current pulses (Grass stimulator, model S-48) through the electrode *in vitro*.

Constant current electrical stimuli were generated with a Winston A-65 timer/stimulator and SC-100 constant current stimulus isolators. Neostriatal stimuli consisted of monophasic square-wave pulses of 0.2–3.0 mA intensity and 100–500 μsec duration and were delivered at a rate of 0.67 Hz. The antidromic threshold current was defined as the minimum current sufficient to elicit antidromic responses on 100% of the trials on which a collision with a spontaneous spike did not occur (Tepper et al., 1984a). Single unit extracellular recordings were amplified with a Neurodata IR-183 preamplifier and displayed on a Tektronix 5113A storage oscilloscope. All data were recorded on magnetic tape for off-line analysis on a Macintosh IIfx computer with a National Instruments MIO16L multifunction board and custom-designed software.

DAergic neurons were identified by their extracellular waveforms, often characterized by a prominent notch in the initial positive phase and having a duration of 2–5 msec, slow spontaneous activity, and long latency antidromic responses evoked from neostriatum that consisted mostly of initial segment (IS) only spikes (Deniau et al., 1978; Guyenet and Aghajanian, 1978; Tepper et al., 1984a,b). The pattern of firing of each neuron was classified as pacemaker, random, or bursty based on the neuron's autocorrelation histogram. Neurons showing three or more peaks in the autocorrelogram were classified as pacemaker, those showing an initial peak followed by a decay to a steady state were classified as bursty, and the remainder as random (Tepper et al., 1995). The proportion of antidromic spikes evoked at the threshold current for each neuron that consisted of the full spike (initial segment plus somatodendritic components) was taken as an index of somatodendritic excitability (Trent and Tepper, 1991).

Inhibitory responses after striatal stimulation were analyzed by constructing peristimulus time histograms and cumulative sum histograms (CUSUMs) (Ellaway, 1977) generated from spike trains. Changes in the slope of CUSUMs were used to delineate the onset, offset, and duration of stimulus-evoked effects as described in detail by Tepper et al. (1995). The average firing rate during the interval so defined was calculated by dividing the average number of spikes per bin during this interval by the prestimulus baseline activity.

Apomorphine dose–response measurements. After the establishment of a stable baseline firing rate for at least 5 min, a dose of apomorphine HCl that was double the previous dose was injected intravenously every 2 min, starting with either 1 or 2 μg/kg. This was continued until complete inhibition of spontaneous activity was obtained, a cumulative dose of 2048 μg/kg was reached, or until the cell was lost. In some cases in which complete inhibition was obtained, haloperidol lactate (50–200 μg/kg, i.v.) was subsequently administered in an attempt to reverse the inhibition. In most cases, the effects of apomorphine were studied on only one cell per animal. However, in a few cases, dose–response experiments were conducted on two cells, one ipsilateral to the infused side and one on the contralateral side. In these cases, haloperidol was not administered to the first cell studied, and a minimum of 3 hr elapsed between the last injection of apomorphine for the first cell and the beginning of the collection of baseline data for the second neuron.

Autoradiography. For receptor autoradiography, animals were euthanized by overdose of urethane, and the brains were rapidly removed and either frozen in isopentane cooled with dry ice or placed in a −80°C freezer. Subsequently, 20 μm coronal sections were taken on a cryostat at −18°C, thaw-mounted on gelatin-subbed slides, and stored at −80°C. Immediately before beginning the labeling incubation, the sections were gradually brought to room temperature, preincubated for 30 min in 50 mM Tris-HCl buffer, pH 7.4, containing 120 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, and 0.001% ascorbic acid (for D2-class binding) or 20 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2, containing 50 mM NaCl, 1 mM EDTA, 10 μM pargyline, and 0.1% ascorbic acid (for D_3 binding), and then incubated for 60 min at 22°C in the same buffer containing 1.2 nM ^{3}H-spiperone for D2-class binding or 3 nM ^{3}H-7-OH-*N,N*-di-*n*-propyl-2-aminotetralin (7-OHDPAT) for D_3 binding. Nonspecific D2-class binding was determined by incubation in the presence of 1 μM (+) flupentixol, and nonspecific D_3 binding was determined in the presence of 1 μM eticlopride. Incubation was terminated by rinsing the

slides twice for 5 min with ice-cold buffer. After washing, the slides were dipped quickly in ice-cold water and dried under a stream of cold air. For D1-class binding, the buffer, incubation, and exposure times were the same as for D2-class binding, except that 1 nM ^{3}H-SCH23390 was used in place of ^{3}H-spiperone. Slides were then placed in x-ray cassettes, together with [^{3}H] microscales (Amersham), and exposed to Hyperfilm-[^{3}H] (Amersham) for 2 weeks (for ^{3}H-spiperone binding) or for 2 months (for ^{3}H-7-OHDPAT binding) at 4°C. Average binding densities were determined with a computerized image analysis system (Microcomputer Imaging Device, Imaging Research, St. Catharine's, Ontario, Canada).

Histological analyses. At the end of the recording experiment, animals that were not used for autoradiographic determination of the extent of receptor knockdown were deeply anesthetized with urethane and perfused intracardially with 50 ml of saline followed by 250 ml of 4% paraformaldehyde. The brain was then removed and placed into fixative overnight. Frozen coronal sections (60–80 μm) through substantia nigra and neostriatum were cut and processed either for tyrosine hydroxylase (TH) immunocytochemistry using a primary antibody from Eugene Tech International (Allendale, NJ) and the ABC protocol from Vector Laboratories (Burlingame, CA) as described previously (Tepper et al., 1994) or stained with neutral red for verification of recording and stimulating sites.

Data analysis. There were five types of controls for the local AODN infusions: recordings from substantia nigra DAergic neurons ipsilateral to saline infusions, ipsilateral to D_2 random AODN infusions, ipsilateral to D_3 random AODN infusions, contralateral to infusion of AODNs, and from untreated control animals. Most parametric electrophysiological data were analyzed with ANOVA followed by Fisher's protected least significant difference (PLSD) *post hoc* test at the $p < 0.05$ level of significance. Numbers in the text are expressed as mean ± SEM. Because these initial ANOVAs showed no significant differences in any of the parameters measured among the five different control groups, they were pooled into a single control group to increase the power of the statistical analyses. The distribution of firing pattern was analyzed with the χ^2 test. Dose–response and rotation data were first analyzed with a repeated-measures ANOVA followed by a factorial ANOVA to determine specific differences among the different treatment groups by dose or day of treatment. ED_{50}s were log-transformed before ANOVA because the raw data exhibited heterogeneity of variance. Binding data were analyzed with paired *t* tests.

RESULTS

Neuronal identification

This report is based on data obtained from 175 neurons recorded from 83 rats. All neurons selected for study were presumed to be substantia nigra DAergic neurons on the basis of long duration bi- or triphasic extracellularly recorded waveform (>2.0 msec); spontaneous firing rate between 0.7 and 10.7 spikes/sec; pacemaker, random, or bursty firing pattern; and location in the midbrain. Of these neurons, 91 (52%) could be driven antidromically from neostriatum with characteristics (antidromic latency range, 7.70–23.5 msec; mean latency, 14.18 ± 0.38 msec; 66.4 ± 3.1% of all antidromic action potentials consisting of initial segment-only spike) that identify them conclusively as DAergic neurons (Deniau et al., 1978; Guyenet and Aghajanian, 1978). Representative examples of antidromically identified nigrostriatal DAergic neurons from an untreated control rat, a D_2 random AODN-, a D_2 AODN-, and a D_3 AODN-treated rat are shown in Figure 1.

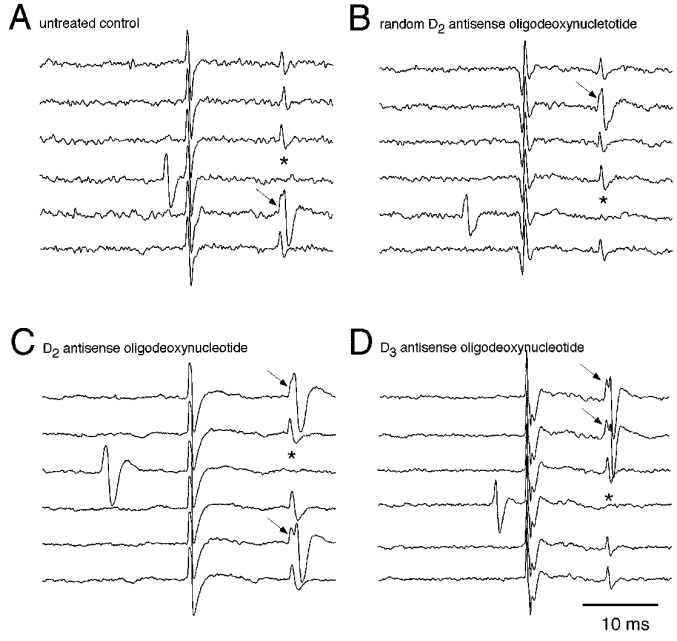

Figure 1. Representative antidromic responses of substantia nigra DAergic neurons to striatal stimulation. Each group consists of six consecutive stimulus deliveries recorded from an untreated control (*A*), ipsilateral to a 6 d infusion of a control oligodeoxynucleotide (*B*), ipsilateral to a 6 d infusion of D_2 AODN (*C*), and ipsilateral to a 6 d infusion of D_3 AODN (*D*). The majority of the antidromic action potentials consist of the initial segment spike only, but some consist of a full initial segment-somatodendritic spike (*arrows*). The antidromic nature of the responses is verified by collision (*asterisks*) with spontaneous action potentials in the fourth, fifth, third, and fourth traces in *A*, *B*, *C*, and *D*, respectively. Positivity is upward.

Apomorphine dose–response

Administration of either the D_2 or the D_3 AODN for 3–6 d produced a marked attenuation in the ability of intravenously administered apomorphine to inhibit the spontaneous activity of nigral DAergic neurons. Representative examples of ratemeter recordings are shown in Figure 2. In cells recorded from untreated controls (Fig. 2*A*) or ipsilateral to control oligodeoxynucleotide infusions (Fig. 2*B*), sequential intravenous injections of apomorphine produced marked inhibition of firing of antidromically identified nigrostriatal DAergic neurons before eventually producing complete suppression of spontaneous activity, typically by the time the cumulative dose reached 16 μg/kg. This inhibition could be readily reversed by subsequent administration of haloperidol (50–200 μg/kg, i.v.), as shown in Figure 2*A*. In contrast, neurons ipsilateral to infusion of D_2 (Fig. 2*B*) or D_3 (Fig. 2*C*) AODN were far less susceptible to the inhibitory effects of apomorphine, and in some cases could not be completely inhibited even at a cumulative dose of 2048 μg/kg. Simultaneous infusion of both D_2 and D_3 AODN (Fig. 2*D*) produced an even greater attenuation of the inhibitory response to apomorphine. The dose–response curves illustrated in Figure 2*E* reveal that treatment with either AODN alone produced a dramatic shift to the right in the apomorphine dose–response relation, and that the effects of the D_2 and D_3 AODNs appeared to be additive (repeated measures $F = 11.73$; df = 3, 40; $p < 0.05$). Overall, the D_2 AODN dose–response curve ($p < 0.05$), the D_3 AODN dose–response curve ($p < 0.05$), and the D_2 + D_3 AODN dose–response curve ($p < 0.05$) were significantly different from the control dose–response curve. The D_2 + D_3 AODN dose–response curve also differed significantly from the D_2 AODN curve ($p < 0.05$).

ED_{50}s were estimated from the dose–response data. In the control groups, this was straightforward because all neurons were eventually completely inhibited by apomorphine, usually by the time the 16 μg/kg dose was reached. However, in the AODN-treated groups, many of the neurons could not be inhibited below 50% of baseline firing, even after the highest dose of apomorphine (1024 μg/kg). For these neurons, the ED_{50} was computed by fitting a log–probit curve to the data points and extrapolating. This was required for 6 of 14 D_2 AODN-treated neurons, 5 of 12

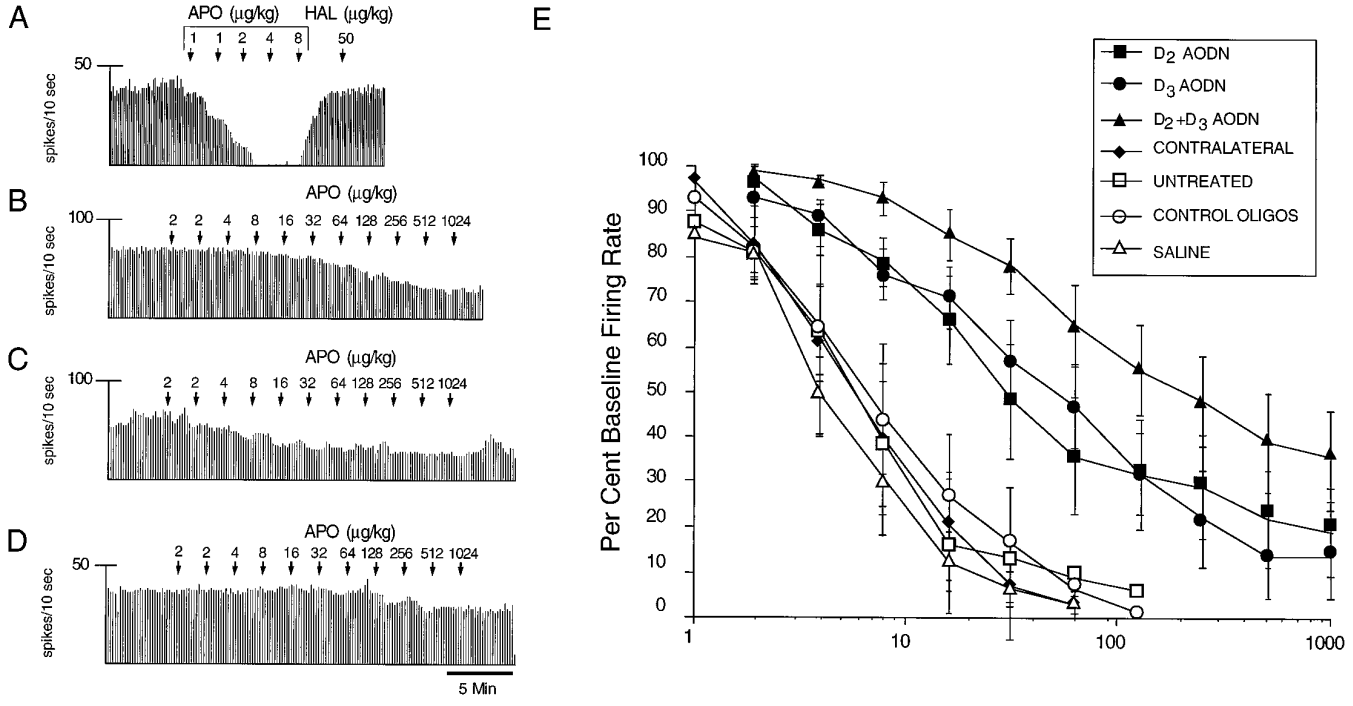

Figure 2. Individual ratemeter records of representative nigrostriatal neurons recorded from an untreated control animal (*A*), ipsilateral to a 6 d infusion of D_2 AODN (*B*), ipsilateral to a 6 d infusion of D_3 AODN (*C*), and ipsilateral to a 6 d infusion of both D_2 and D_3 AODNs (*D*). Apomorphine was administered intravenously every 2 min as indicated by the *arrows*. In the untreated (*A*) neuron, apomorphine produced complete inhibition of firing after the 8 μg/kg dose, and the inhibition was completely reversed by haloperidol, 50 μg/kg, intravenously. In contrast, neurons treated with D_2, D_3, or D_2 + D_3 AODN showed little or no effect at 8 μg/kg apomorphine and could not be completely inhibited even at cumulative doses in excess of 2000 μg/kg. *E*, Dose–response curves comparing the inhibition of firing among nigrostriatal neurons recorded ipsilateral to saline, random oligodeoxynucleotide, or D_2, D_3, or D_2 + D_3 AODN infusion, or contralateral to D_2 or D_3 AODN infusion or in untreated control rats. Note that all of the control groups are nearly identical, whereas the AODN antisense curves are shifted markedly to the *right*. Error bars indicate SEM. Each point is the mean of measurements from 3–6 cells for each of the different control groups, and from 7–10 cells for the 6 d D_2 AODN infusion, the D_3 AODN infusions, and the combined D_2 + D_3 AODN infusions.

D_3 AODN-treated neurons, and 4 of 8 neurons from animals treated with D_2 + D_3 AODN. The fact that so many AODN-treated neurons could not be completely inhibited, or even inhibited to 50% of baseline, generated considerable variability in their ED_{50} estimates, which resulted in very large SEMs. The values were: control = 10.05 ± 3.5 μg/kg; D_2 AODN = 475.6 ± 253.3 μg/kg; D_3 AODN = 341.2 ± 248 μg/kg; D_2 + D_3 AODN = 623.8 ± 297 μg/kg. The increased ED_{50}s after AODN treatment were not attributable to increases in baseline firing rate (see below); there were no significant correlations between ED_{50} and baseline firing within any of the AODN treatment groups, or overall when the AODN groups were pooled. However, there was a significant correlation between firing rate and ED_{50} with the pooled control group when one outlier was eliminated ($r = 0.62$; $F = 6.86$; df = 1, 11; $p < 0.05$), as has been described previously (Tepper et al., 1982; White and Wang, 1984).

To begin to estimate the minimum time required for AODN treatment to cause a functional knockdown of DA autoreceptors, a number of rats were given supranigral D_2 or D_3 antisense infusions for 3 d and prepared for recording on the fourth day. The response of these rats to apomorphine was indistinguishable from that in rats that had been treated with the AODN for 6 d (data not shown). All other electrophysiological parameters (see below) were likewise identical in the 3 and 6 d treated groups. Therefore, in all subsequent data analyses, data from animals treated for 3 d and those treated for 6 d were pooled.

Striatal-evoked inhibitory responses

To determine whether the reduction of the inhibitory response to apomorphine induced by the D_2 or D_3 AODNs was specific to DAergic inhibitory responses rather than an attenuation of inhibitory responses in general, the responses of DAergic neurons to single-pulse stimulation of neostriatum (0.67 Hz; 100–500 μsec duration; 0.2–3 mA) were examined. Striatal stimulation typically produces inhibition in DAergic neurons (Tepper et al., 1990). DAergic neurons from AODN-treated animals displayed inhibitory responses to striatal stimulation that were indistinguishable from those in controls in terms of onset latency, duration, and magnitude of inhibition, as illustrated in Figure 3.

Spontaneous activity

Spontaneous activity was assessed by measuring spontaneous firing rate and calculating the coefficient of variation of the interspike interval. In addition, the pattern of firing for each neuron was categorized based on the autocorrelation histogram of the neuron, as described above. Neither D_2, D_3, nor D_2 + D_3 AODN significantly altered mean firing rate or the coefficient of variation of DAergic neurons, as shown in Figure 4, *A* and *B*. Similarly, the relative proportions of neurons firing in the pacemaker, random, or bursty modes were not affected by any antisense treatment, as shown in Figure 4*C*.

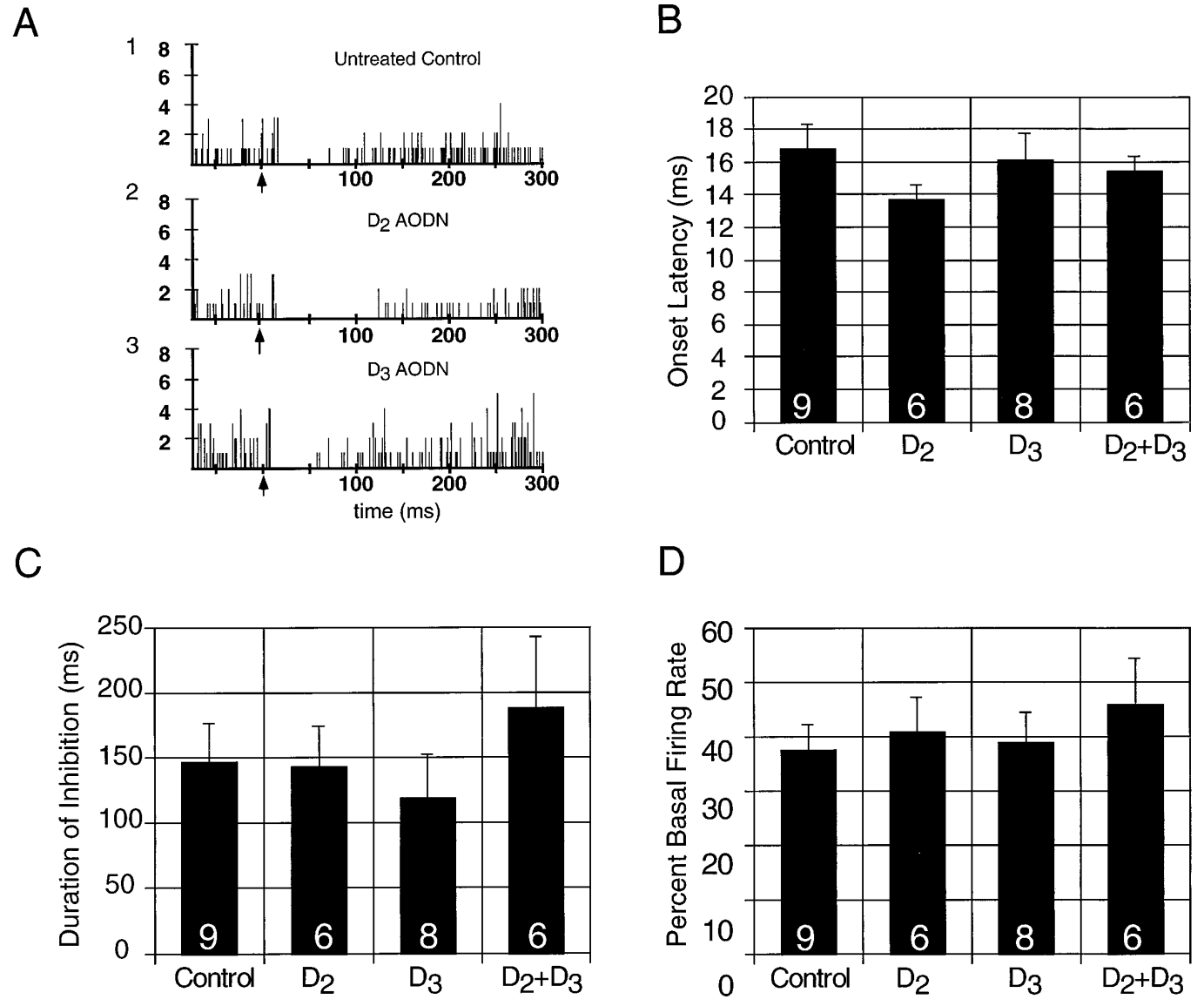

Figure 3. Striatal-evoked inhibitory responses of nigrostriatal neurons are not affected by AODN treatment. *A1,2,3*, Representative peristimulus time histograms showing inhibitory effect of striatal stimulation (*arrow* at time 0) in neurons from an untreated control, ipsilateral to D_2 AODN infusion, and ipsilateral to D_3 AODN infusion, respectively (bin width, 2 msec; 200 stimulus presentations in each case). Onset latency and duration of inhibition were computed by fitting a straight line through the prestimulus portion of the CUSUM histogram and another through the region where the slope changes. The intersection of these two lines represents the time at which the firing rate changes. The offset of stimulus-driven effects is similarly calculated by fitting a third line to the remainder of the CUSUM histogram, and the intersection of this line segment with the second line segment is the point at which the effect is no longer detectable. The magnitude of the inhibition was calculated by dividing the mean number of spikes per bin for the entire inhibitory period by the prestimulus control and expressing the ratio as the percent basal firing rate. Neither the onset latency (*B*), duration (*C*), nor magnitude of striatal-evoked inhibition (*D*) was significantly altered by AODN treatment. *Numbers* in *bars* indicate number of cells tested. Error bars denote SEM.

Antidromic response properties

The neostriatal threshold stimulating current was used as an index of the excitability of the terminals of DAergic nigrostriatal neurons (Tepper et al., 1984a). The mean threshold current in AODN-treated neurons was significantly lower than in controls ($F = 4.38$; df = 3, 76; $p < 0.05$), indicating an increase in nigrostriatal terminal excitability. *Post hoc* tests revealed significantly lower thresholds in DAergic neurons from D_2 AODN-treated, D_3 AODN-treated, and $D_2 + D_3$ AODN-treated rats than in controls (PLSD; $p < 0.05$). In addition, the proportion of antidromic spikes that consisted of the full spike [IS plus somatodendritic (SD) component] was calculated for each neuron as an index of somatodendritic excitability (Matsuda and Jinnai, 1980; Trent and Tepper, 1991). A significantly higher proportion of antidromic responses of neurons from AODN-treated rats consisted of a full spike than in control neurons ($F = 3.49$; df = 3, 61; $p < 0.05$), indicating a depolarization and increase in excitability of the somatodendritic membrane. As with antidromic excitability measurements, this could be attributed to a significantly higher proportion of full spike responses in each of the three AODN-treated groups compared with controls (PLSD; $p < 0.05$ for each comparison). These data are illustrated in Figure 5.

Autoradiography

Quantitative autoradiography was performed on selected sections from brains after supranigral infusion of D_2 AODN, D_3 AODN,

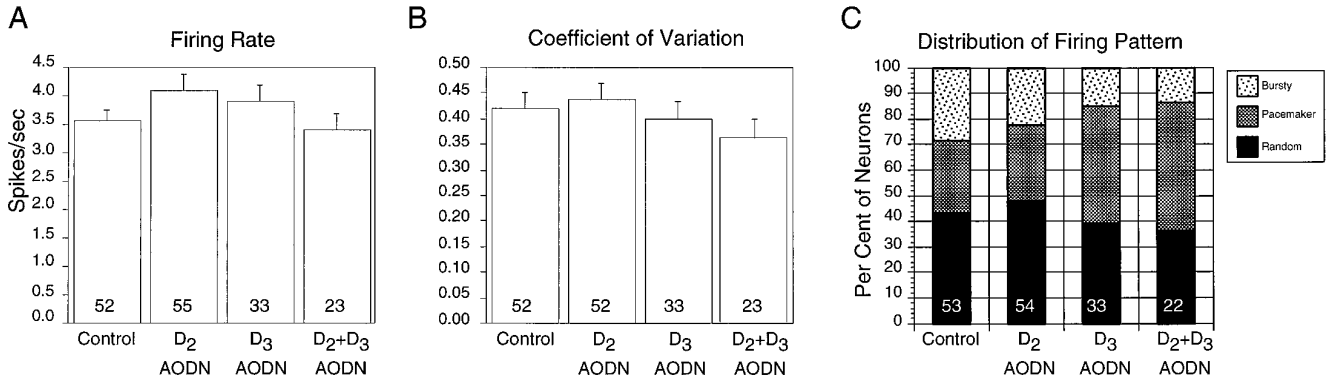

Figure 4. Lack of effect of D_2, D_3, or $D_2 + D_3$ AODN treatment on the rate or pattern of spontaneous activity of DAergic neurons. Firing patterns were classified from autocorrelograms as described in the text. *Numbers* in *bars* indicate the number of neurons measured. Error bars denote SEM.

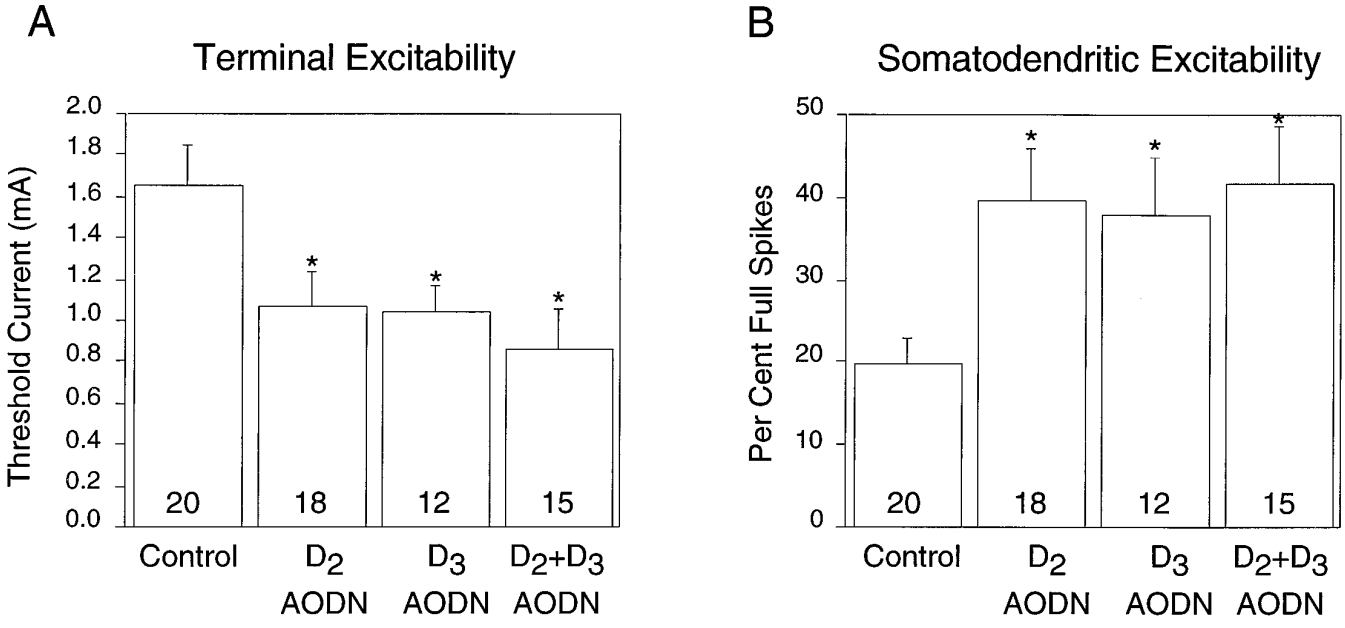

Figure 5. Effects of AODN treatment on terminal and somatodendritic excitability of nigrostriatal DAergic neurons. *A*, Minimum current necessary to activate an antidromic response on each noncollision trial (threshold) is significantly lowered by treatment with D_2, D_3, or $D_2 + D_3$ AODN, indicating an increase in terminal excitability. *B*, The proportion of antidromic responses at threshold that consists of the full, or initial segment and somatodendritic, spike components is significantly increased by D_2, D_3, or $D_2 + D_3$ AODN treatment, indicating increased somatodendritic excitability. *Numbers* in *bars* indicate the number of neurons measured. Error bars denote SEM. *, significantly different from control group at $p < 0.05$.

$D_2 + D_3$ AODN, and random oligo infusion. Representative autoradiograms are shown in Figure 6, and the quantitative analyses are summarized graphically in Figure 7. The specific binding of D2-class (^{3}H-spiperone), D_3 (^{3}H-7-OHDPAT), and D1-class receptors (^{3}H-SCH-23390) in the substantia nigra on the treated side was compared with that on the control side by averaging the binding density over the pars compacta on either side of selected sections and dividing the average for the treated side by that for the control side. The decrease in ^{3}H-spiperone binding on the D_2 AODN-treated side ranged from a low of 32.4% to a high of 76.1%. The mean decrease in ^{3}H-spiperone binding was 48.9 ± 3.3% ($t = 11.96$; df = 1, 15; $p < 0.05$).

Although present at considerably lower levels than ^{3}H-spiperone binding, ^{3}H-7-OH-DPAT binding clearly demonstrated the presence of D_3 receptors in the substantia nigra (Fig. 6*E,F*). Because the nonspecific binding of ^{3}H-7-OH-DPAT was so low, the measurement of the change in D_3 binding was made on total binding for ^{3}H-7-OH-DPAT rather than on specific binding, as was the case for ^{3}H-spiperone and ^{3}H-SCH-23390 binding, both of which showed considerable nonspecific binding. There was no significant decrease in ^{3}H-7-OH-DPAT binding after D_2 AODN treatment (see Fig. 6*E*). Conversely, D_3 AODN did not significantly decrease ^{3}H-spiperone binding in substantia nigra (−4.3 ± 2.8%; see Fig. 6*G,H*) but did decrease ^{3}H-7-OH-DPAT binding by 44.6 ± 5.8% ($t = 7.17$; df = 1, 12; $p < 0.05$; see Fig. 6*F*). Combined treatment with D_2 and D_3 AODNs decreased ^{3}H-spiperone binding by 56.2 + 4.7% ($t = 11.06$; df = 1, 11; $p < 0.05$) and ^{3}H-7-OH-DPAT binding by 49.8 + 6.2% ($t = 7.53$; df = 1, 11; $p < 0.05$). In contrast, there was no appreciable change in D1-class (^{3}H-SCH23390) binding in substantia nigra after D_2, D_3, or combined $D_2 + D_3$ AODN infusion. Thus, the effects of the D_2 and D_3 AODNs were specific to the receptors for which they were designed, and were localized to the substantia nigra and immediately surrounding regions on the ipsilateral side.

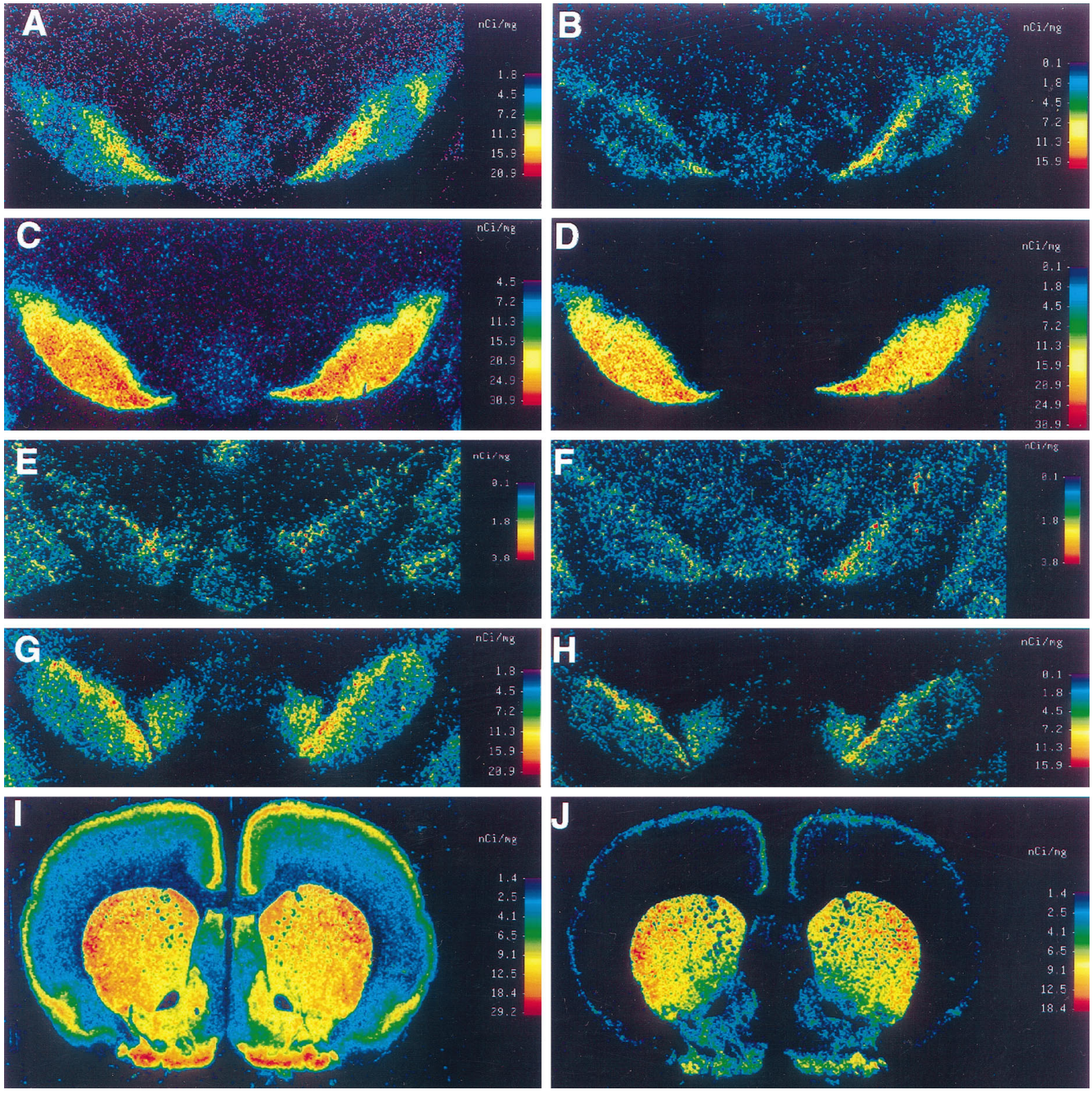

Figure 6. Representative autoradiograms of D2-class (^{3}H-spiperone), D3 (^{3}H-7-OHDPAT), and D1-class (^{3}H-SCH23390) receptor binding in substantia nigra and D2-class receptor binding in neostriatum after 6 d of infusion of AODNs into the left substantia nigra. *A*, Total D2-class binding in substantia nigra after infusion of D_2 AODN into the left substantia nigra. *B*, Specific D2-class binding in substantia nigra pars compacta ipsilateral to the infusion is reduced to 51.5% compared with the contralateral side. *C*, Total D1-class binding in substantia nigra from a nearby section. *D*, Specific D1-class binding in substantia nigra (pars compacta plus pars reticulata) on the ipsilateral side is unchanged compared with the contralateral control side, demonstrating the receptor specificity of the D_2 AODN knockdown. *E*, Total D_3 binding after infusion of D_2 AODN into left substantia nigra. There is no decrease in D_3 binding after D_2 AODN infusion, demonstrating the specificity of the D_2 AODN. *F*, Total D_3 binding after infusion of D3 AODN into the left substantia nigra. D_3 binding is reduced to 50% of the contralateral control side. *G*, Total D2-class binding after infusion of D_3 AODN into left substantia nigra. *H*, Specific D2-class binding is unchanged after D3 AODN. *H*, Total D2-class binding in neostriatum after infusion of D_2 AODN into left substantia nigra. *J*, Specific D2-class binding in neostriatum shows no significant change ipsilateral to the infusion, indicating that there is no retrograde knockdown after supranigral infusion of D_2 AODN.

There was apparently no retrograde transport of the D_2 AODN back to the neostriatum with subsequent postsynaptic receptor loss after supranigral infusion of DA receptor AODNs, because ^{3}H-spiperone binding in the neostriatum did not differ significantly between the control and treated sides, despite the large differences that were seen in substantia nigra (Fig. 6*I,J*).

Tyrosine hydroxylase immunocytochemistry

To determine whether the electrophysiological changes seen after AODN infusion could be attributable to nonspecific toxic effects on DAergic neurons, DAergic neurons in the ventral mesencephalon were visualized directly by TH immunocytochemistry. As can

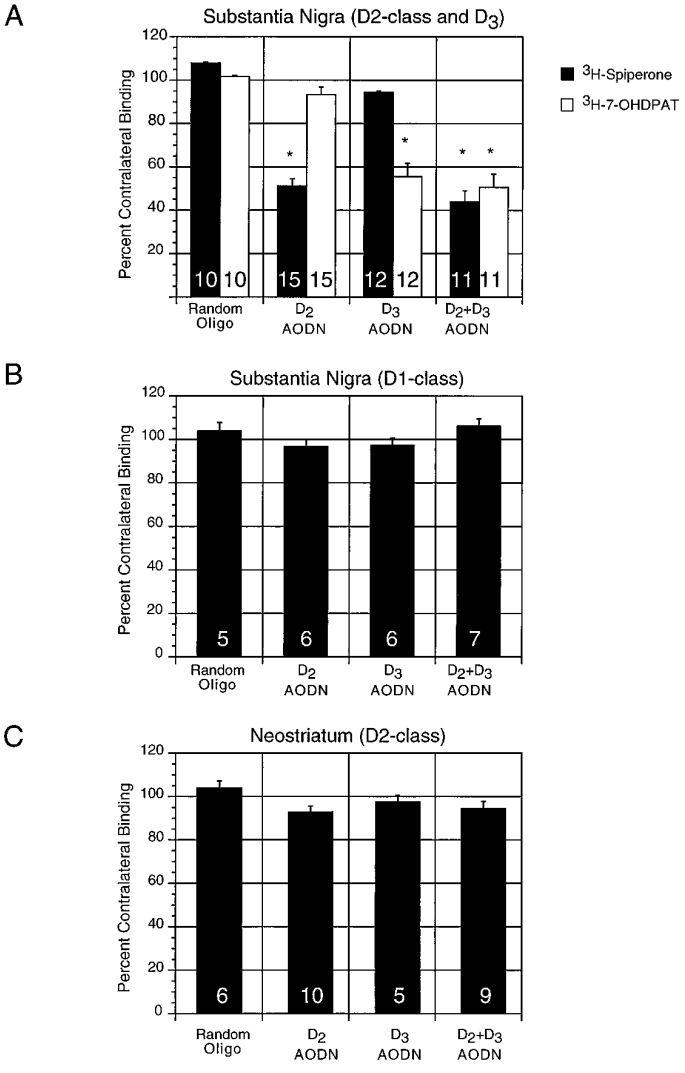

Figure 7. Mean effects of unilateral supranigral infusion of D_2, D_3, and D_2 + D_3 AODNs on DA receptor binding in substantia nigra and neostriatum. D2-class binding estimated from specific ^{3}H-spiperone binding and D_3 binding estimated from ^{3}H-7-OHDPAT binding. *A*, Random oligodeoxynucleotides do not affect either D2-class or D_3 receptor binding. D_2 AODN specifically reduces D2-class binding without affecting D_3 binding, whereas D_3 AODN reduces D_3 binding without significantly affecting D2-class binding. Combined administration of D_2 and D_3 AODNs produces reductions in D2-class and D_3 binding similar to that seen when either AODN is administered alone. Control values for ^{3}H-spiperone binding = 5.66 ± 0.4 nCi/mg; for ^{3}H-7-OHDPAT binding = 1.22 ± 0.15 nCi/mg. *B*, Neither random oligodeoxynucleotides nor any of the AODNs infused supranigrally affect D1-class binding in substantia nigra estimated from ^{3}H-SCH23390 binding. Control value for ^{3}H-SCH23390 binding = 18.02 ± 0.82 nCi/mg. *C*, Neither random oligodeoxynucleotides nor any of the AODNs infused supranigrally affects D2-class (^{3}H-spiperone) receptor binding in the ipsilateral neostriatum. Control value for ^{3}H-spiperone binding in neostriatum = 12.02 ± 0.82 nCi/mg. *Numbers* in *bars* indicate number of neurons measured. Error bars denote SEM. *Significantly different from control group at $p < 0.05$.

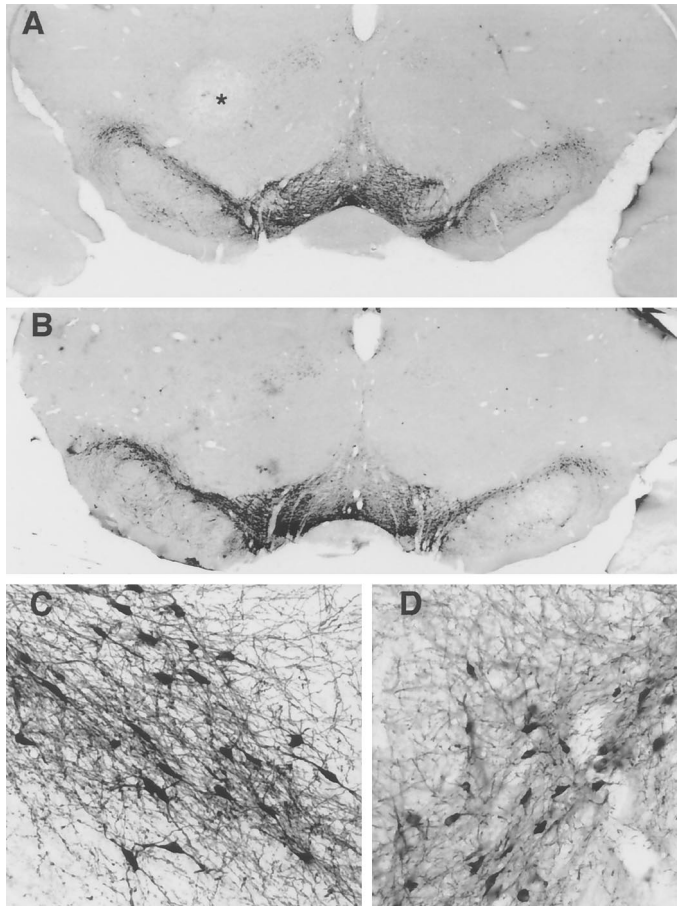

Figure 8. Effects of unilateral supranigral D_2 AODN infusion on TH immunoreactivity in substantia nigra. *A*, Sixty micrometer coronal section through the midbrain of an animal in which 10 μg/μl D_2 AODN was infused above the left substantia nigra for 6 d. The *pale area* marked by the *asterisk* is a small, nonspecific lesion made by the infusion cannula. Note that there is no apparent damage to DAergic (TH-immunopositive) substantia nigra neurons ipsilateral to the infusion. If anything, the density of TH immunostaining appears greater on the infused side. *B*, Sixty micrometer coronal section 180 μm posterior to that shown in *A*. An increased density of TH immunostaining is still apparent on the infused (*left*) side. *C, D*, Higher magnification photomicrographs through the central region of pars compacta ipsilateral (*C*) and contralateral (*D*) to D_2 AODN infusion. Although the cell bodies also appear darker, the increased dendritic staining on the treated side is particularly noticeable.

be seen in Figure 8, after a 6 d infusion of D_2 AODN, there was no apparent loss of TH immunoreactive neurons ipsilateral to the AODN infusion, demonstrating that the D_2 AODN did not produce nonspecific damage to DAergic neurons. Although no attempt was made to quantify these data, it is interesting that the DAergic neurons ipsilateral to the infusion appear to stain more intensely than those on the contralateral side.

Behavioral observations during AODN treatment

The behavior of rats receiving unilateral intranigral infusion of D_2 and/or D_3 AODN was, for the most part, unremarkable. However, AODN-treated rats exhibited a modest contralateral postural deviation during rest, and intermittent or continuous spontaneous contralateral rotations at a relatively low rate, as shown in Figure 9. Both the contralateral postural deviation and the spontaneous contralateral rotation were evident by 24 hr after the start of the infusion, and became maximal after 3 d of infusion. Repeated measures ANOVA revealed a significant increase in rotations among both AODN-treated groups ($F = 6.10$; df = 3, 26; $p < 0.05$). A subsequent factorial ANOVA revealed that all treated groups differed from the random oligodeoxynucleotide-treated controls on all days of treatment ($p < 0.05$), and that the

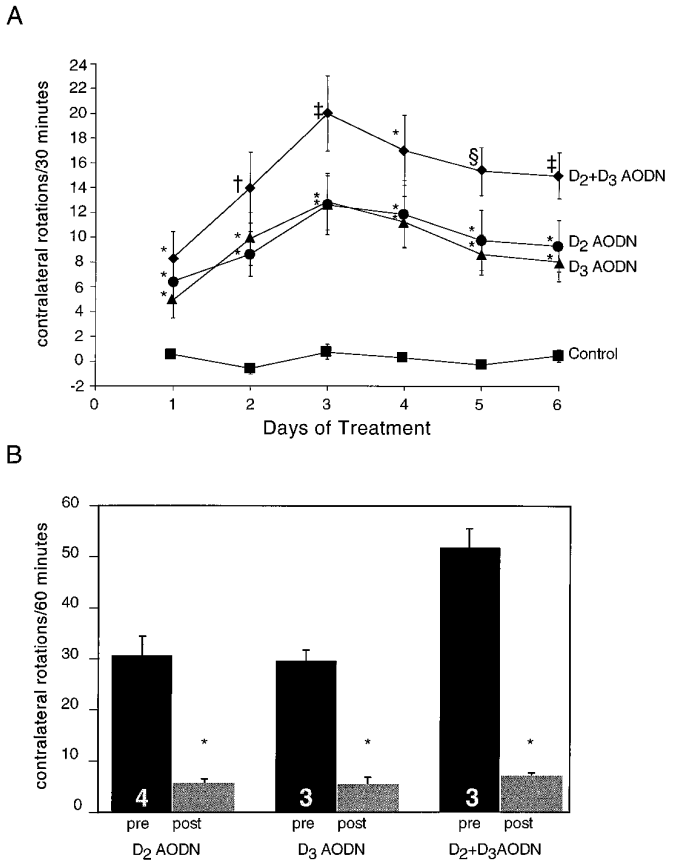

Figure 9. *A*, Spontaneous contralateral rotational behavior induced by unilateral supranigral infusion of 10 $\mu g/\mu l$ D_2, D_3, random oligodeoxynucleotide (control), or $D_2 + D_3$ (20 $\mu g/\mu l$ total) AODN (100 nl/hr). Each point represents the mean of two daily 30 min measurement periods from 9 to 16 animals. Error bars represent SEM. *Significantly different from control group at $p < 0.05$; †, significantly different from control and from D_2 AODN-treated group at $p < 0.05$; §, significantly different from control and D_3 AODN-treated group at $p < 0.05$; ‡, significantly different from controls and from D_2 and D_3 AODN-treated group at $p < 0.05$. *B*, Challenge with apomorphine (1 mg/kg, i.p.) reduces rotational behavior in each of the treated groups. Data from 3- and 6-d treated rats combined; preinjection (*pre*) data taken from −60 to 0 min before injection and postinjection (*post*) data from 5 to 65 min after injection. *Significantly different from respective preinjection control.

combined $D_2 + D_3$ AODN-treated group also differed significantly ($p < 0.05$) from either the D_2 AODN-treated or the D_3 AODN-treated groups on days 2, 3, and 5, suggesting an additivity in the effects of D_2 and D_3 receptor knockdown on spontaneous rotation. These asymmetric postural and locomotor behaviors were never observed in either saline- or random oligodeoxynucleotide-infused rats, and suggest an increased DA release in the ipsilateral striatum as a result of D_2 and/or D_3 autoreceptor knockdown. When challenged with the DA agonist apomorphine, no additional rotational behavior was induced in these rats, but the postural and locomotor asymmetries were almost completely abolished, as shown in Figure 9*B*.

DISCUSSION

Specificity and extent of *in vivo* DA receptor AODN knockdown

The autoradiographic data demonstrate that the receptor knockdown produced by local infusion of DA receptor AODNs into the brain is relatively localized around the infusion site on the ipsilateral side. This is consistent with data showing that the maximal spread of an *S*-oligodeoxynucleotide of similar size injected intracerebrally was between 0.7 and 1.3 mm (Sklarczyk and Kaczmarek, 1995). Furthermore, D_2 AODN reduced specifically ^{3}H-spiperone (D2) binding in the substantia nigra without affecting D_3 binding, and D_3 AODN reduced specifically ^{3}H-7OH DPAT (D_3) binding without affecting D2 binding.

Supranigral infusion of D_2 receptor AODN did not affect striatal ^{3}H-spiperone binding. This observation is particularly important because it indicates that there is no retrograde knockdown of DA receptors. This is presumably because there is no uptake of the AODNs by striatonigral nerve terminals in substantia nigra. Thus, local infusion of AODNs can be used to selectively inactivate presynaptic or postsynaptic DA receptors, something that has not been possible by the use of conventional receptor antagonists or transgenic knockouts.

The maximal extent of the receptor loss, estimated from autoradiography, was always less than 100%. However, because the pars compacta is a relatively poorly defined structure and because the areas included in the autoradiographic comparisons unavoidably contained regions of low DA receptor density, averaging over the entire pars compacta would tend to underestimate the maximal knockdown in the most affected areas. In addition, ^{3}H-spiperone can also label D_3 receptors (Sokoloff et al., 1990; Bouthenet et al., 1991). D_2 AODN does not affect D_3 binding, and thus some of the residual ^{3}H-spiperone binding after D_2 AODN infusion is likely attributable to D_3 receptors. Finally, because there is no retrograde uptake of D_2 AODN, any D2-class receptors that may exist on nigral afferents would also be unaffected, and might contribute to the remaining ^{3}H-spiperone binding. Thus, it is difficult to specify the upper limit of the knockdown potential of AODNs infused into the brain, but our data suggest that in the best cases there is an extensive reduction in DA receptors.

There were no obvious toxic effects of D_2 AODN. The substantia nigra on the infused side appeared indistinguishable from its contralateral control in Nissl-stained sections, and TH immunocytochemistry failed to reveal any loss of DAergic neurons. Indeed, if there was any difference at all, the TH immunostaining appeared to be *darker* on the treated side than on the contralateral control. This may reflect a disinhibition of TH synthesis caused by the loss of D_2 somatodendritic autoreceptors (Gauda and Gerfen, 1995).

Effects of DA receptor AODN treatment on apomorphine-induced inhibition of firing

Treatment with D_2 or D_3 AODN produced equivalent shifts to the right in the apomorphine dose–response curve. Combined treatment with both AODNs produced a significantly greater attenuation of the inhibitory effect of apomorphine. Although there was significant variability in the degree of attenuation, it is important that virtually all of the cells that were treated with either D_2 or D_3 AODN exhibited significantly less inhibition than control neurons. Thus, the variability did not arise because some neurons were essentially unaffected by D_2 or D_3 AODN treatment while others showed a near-complete blockade of apomorphine inhibition to one or the other AODN, which would suggest that some DA neurons express only D_2 receptors, whereas others express only D_3 receptors. Rather, the data suggest that virtually all neurons tested expressed both D_2 and D_3 somatodendritic autoreceptors.

The inhibition of nigrostriatal neurons in response to systemic

administration of apomorphine at low doses has been shown to be attributable to a local action on somatodendritic autoreceptors (Akaoka et al., 1992; Pucak and Grace, 1994). Although it has long been known that DAergic neurons possess an autoreceptor of the D2 family (Groves et al., 1975; Aghajanian and Bunney, 1977; Lacey et al., 1987), these data provide direct electrophysiological evidence that DAergic neurons express both D_2 and D_3 somatodendritic autoreceptors, and that both participate in the inhibition of firing caused by DA agonists.

The decrease in spontaneous activity after administration of higher doses of apomorphine that persisted in many neurons from D_2 or D_3 AODN-treated animals could have resulted from incomplete knockdown of D_2 or D_3 autoreceptors. However, some of the inhibition resulting from systemic administration of other DAergic agonists (e.g., amphetamine) results from effects on forebrain structures (Bunney and Aghajanian, 1978; Sasaki et al., 1990), and whereas low doses of apomorphine (<16 μg/kg, i.v.) affect only DAergic neurons, higher doses can affect neostriatal neurons (Skirboll et al., 1979). Thus, it may be that a significant component of the inhibition that persists at high doses after AODN treatment is attributable to long-loop postsynaptic effects rather than local autoreceptor stimulation.

Time course of DA receptor AODN knockdown

Neither the apomorphine dose–response curves nor any of the other electrophysiological properties of DAergic neurons differed between animals treated with D_2 AODN for 3 or 6 d, suggesting that the functional knockdown is near maximal by 3 d. This is consistent with our observations that AODN-treated animals began to exhibit spontaneous rotation after only 24 hr of treatment that reached a maximum at 3 d. Previous studies of the rate of DA receptor turnover based on the recovery of ^{3}H-spiperone binding after irreversible receptor alkylation showed that the estimated $t_{1/2}$ for D2-class receptor turnover depends on a number of factors, including age, DA content, and the precise methods used to inactivate the receptors (McKernan and Campbell, 1982; Hall et al., 1983; Leff et al., 1984; Norman et al., 1987), and can range from 8 hr to more than 6 d. Our electrophysiological and behavioral data favor the lower range of these turnover estimates.

Effects of DA receptor AODN treatment on spontaneous activity and somatodendritic excitability

Despite the marked effects of D_2 and/or D_3 AODN on the inhibition produced by apomorphine, neither AODN altered the spontaneous firing rate or pattern of nigrostriatal neurons. It is possible that compensatory changes in DAergic neurons or in their afferents masked the increase in spontaneous firing rate that would be predicted on the basis of the self-inhibition hypothesis. However, this explanation seems unlikely because of the rather short times involved (3 d) and because other related properties, including the response to apomorphine and the terminal and somatodendritic excitability, were markedly altered.

Changes in antidromic invasion of the somatodendritic region seen in extracellular recordings have been shown to correspond to changes in the level of membrane polarization of the somatodendritic region (Matsuda and Jinnai, 1980). Using this as an index of somatodendritic excitability, we have proposed previously that the physiological role of DAergic somatodendritic autoreceptors is more subtle than that of directly controlling the firing rate of the neuron as a whole (Trent and Tepper, 1991). In the present study, although D_2 and/or D_3 knockdown failed to increase the firing rate, the proportion of striatal-evoked antidromic responses that consisted of IS–SD spikes more than doubled. This was not attributable to the decreased threshold current in the treated cells (which might lead to a decrease in orthodromic inhibition from striatum and hence increase the excitability of the somatodendritic region; see Grace and Bunney, 1985; Grace, 1987; Trent and Tepper, 1991), because there was no correlation between the IS–SD percentage and the antidromic threshold current within any treatment group. Rather, the change appears attributable to a reduction in a tonic depression of somatodendritic excitability mediated by D_2 and D_3 autoreceptors interacting with endogenous DA. These data provide another example of an uncoupling of events in the somatodendritic and initial segment regions of the DAergic neuron (Grace, 1990; Hausser et al., 1995) and reinforce our previous suggestion (Trent and Tepper, 1990) that, at least in anesthetized rats, somatodendritic autoreceptors may not function to control the overall firing rate of the cell directly, but rather function to modulate somatodendritic excitability in a more subtle and local way.

Effects of DA receptor AODN treatment on antidromic thresholds

Axon terminal autoreceptors have been shown to modulate the stimulus-evoked release of DA in a number of different paradigms *in vitro* and *in vivo* (for review, see Starke et al., 1989). The threshold current has been used as an index of the excitability of DAergic nerve terminals and has been shown to vary depending on the extent of terminal autoreceptor stimulation; autoreceptor antagonists reduce the threshold current, whereas autoreceptor agonists increase threshold (Tepper et al., 1984a,b, 1987). In the present experiments, threshold currents of nigrostriatal neurons that were recorded ipsilateral to nigral infusion of D_2 or D_3 AODN were significantly lower than those in controls, demonstrating that DAergic neurons possess both functional D_2 and D_3 autoreceptors at the axon terminals, as well as in the somatodendritic region. The identification of a D_2 terminal autoreceptor is consistent with a previous study using D_2 AODN knockdown (Silvia et al., 1994), and the finding that there is also a D_3 terminal autoreceptor is consistent with claims from biochemical studies (Meller et al., 1993; Nissbrandt et al., 1995). *In vivo*, terminal autoreceptors are sufficiently occupied by endogenous DA that administration of antagonists (Tepper et al., 1984a) or AODN knockdown significantly decreases the inhibitory tone on the terminal, resulting in increased terminal excitability and, presumably, increased DA release. It is likely this effect that is responsible for the spontaneous contralateral rotation observed during AODN treatment. These data also demonstrate that it is possible to knock out receptors on axon terminals of neurons that originate in the infusion site but extend several millimeters away.

Relative roles of D_2 and D_3 autoreceptors on substantia nigra neurons

The attenuating effects of D_2 AODN on apomorphine-induced inhibition of nigrostriatal neurons and the increases in terminal and somatodendritic excitability were expected based on the large amount of D_2 mRNA and D2-class binding present in substantia nigra. However, that D_3 AODN was as effective as D_2 AODN, and that the effects of D_2 and D_3 AODN treatment were additive were somewhat surprising given recent reports that there is only a low level of D_3 mRNA present in the mesencephalon and that D_3 mRNA was below detectable levels on many DAergic nigral neurons (Diaz et al., 1995). However, the difficulty in detecting D_3 mRNA in the midbrain may simply reflect the relative overabun-

dance of D_2 mRNA and protein relative to that of D_3 mRNA (Griffon et al., 1995). Our autoradiographic data suggest the presence of D_3 binding in substantia nigra, albeit at relatively low levels. It may be that lower, but still physiologically functional, levels of D_3 receptors are present on most or all mesencephalic DAergic neurons, but these are associated with lower levels of mRNA that require more sensitive PCR or related techniques for detection (Sokoloff et al., 1990; Valerio et al., 1994) and that are missed by *in situ* hybridization. A recent study using transfection of D_2 and D_3 receptors into a DAergic mesencephalic clonal line showed that D_3 receptors were more than twice as potent at inhibiting DA release than D_2 receptors, despite the fact that B_{max} of the D_2 receptors was three times greater than that of the D_3 receptors (Tang et al., 1994), perhaps indicating that the receptor coupling mechanism(s) is more efficient for D_3 receptors. Nevertheless, the fact that both D_2 and D_3 AODNs attenuated autoreceptor function at both the terminal and the somatodendritic regions of the neuron more or less equally suggests that the normal electrophysiological response attributed to somatodendritic and terminal autoreceptor stimulation may require coactivation of both D_2 and D_3 receptors.

Conclusion

In vivo knockdown of specific DA receptors by injection of AODNs into discrete brain regions is a valuable technique for assessing the functional roles and sites of action of different DA receptor subtypes for which selective ligands do not yet exist. The lack of retrograde knockdown suggests that this technique offers a method for selective inactivation of either pre- or postsynaptic receptors, something that has not previously been possible. The data presented here demonstrate that DAergic neurons possess functional D_2 and D_3 autoreceptors at both their axon terminal and somatodendritic regions. Both types of terminal autoreceptors modulate terminal excitability and presumably the release of DA from the nerve terminals and/or its synthesis, whereas somatodendritic D_2 and D_3 autoreceptors play a role in modulating the excitability of local dendritic regions.

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