CHAPTER 7

ELECTROPHYSIOLOGICAL CORRELATES OF IN VIVO ANTISENSE KNOCKOUT OF DOPAMINE D2 AUTORECEPTORS ON SUBSTANTIA NIGRA DOPAMINERGIC NEURONS (DSG)

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FROM:
ANTISENSE STRATEGIES FOR THE STUDY OF RECEPTOR MECHANISMS
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INTRODUCTION

The midbrain dopaminergic system is of critical importance to normal cognitive and motor functioning. The loss of projections from substantia nigra pars compacta dopaminergic neurons to the neostriatum produces the tremor, rigidity and akinesia that is characteristic of Parkinson's disease. A disturbance in the regulation of dopaminergic transmission in the forebrain is believed to underlie the devastating cognitive and behavioral impairments in schizophrenia and perhaps other psychoses, and the effectiveness of antipsychotic drugs correlates directly with their affinity for dopamine receptors.[8] Many drugs of abuse, particularly stimulants such as cocaine and amphetamine, produce their

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euphoric effects through interaction with telencephalic projections of the dopamine neurons of the substantia nigra and ventral tegmental area.[16] Understanding the mechanisms that control dopamine release and its consequent physiological effects in forebrain terminal fields is therefore of great importance to our understanding of the biological bases of a number of different normal and abnormal behaviors.

AUTORECEPTOR MODULATION OF DOPAMINERGIC NEURONAL ACTIVITY

The most basic mechanism by which dopaminergic transmission is regulated is by the rate and pattern of activity of dopaminergic neurons. Increases in impulse flow along dopamine axons lead to increases in dopamine release in terminal fields. This has been verified a number of times by several different techniques including in vitro release studies (see ref. 36 for review), and more recently, by in vivo microdialysis[40] or voltammetric[14] detection of dopamine in the neostriatum, nucleus accumbens or neocortex. One of the more unusual properties of dopaminergic neurons is that they possess receptors for their own neurotransmitter, dopamine, in the somatodendritic region as well as in their axon terminal regions. These receptors are termed "autoreceptors" and serve to modulate dopaminergic neurotransmission through at least two different mechanisms. The somatodendritic autoreceptors are stimulated by dopamine released from presynaptic dendrites of dopaminergic neurons[6,11,30] at dendrodendritic synapses and perhaps at other sites as well.[15,51] These autoreceptors are believed to play a role in self-inhibition of dopamine neurons[17] by opening a potassium conductance that hyperpolarizes dopaminergic neurons[22,23] thereby reducing their spontaneous firing rate. The nerve terminal autoreceptors are stimulated by dopamine released from nearby terminals and act to reduce subsequent impulse dependent dopamine release and dopamine synthesis.[36,52] In a similar manner to that of the somatodendritic autoreceptor, terminal autoreceptor activation produces a hyperpolarization and decrease in excitability of the nerve terminal.[44,45]

It has been known since the late 1970s that there are two major subtypes of dopamine receptors, termed D_1 and D_2 receptors. These subtypes were originally defined on the basis of their differential affinities for various ligands and linkage to intracellular second messenger pathways.[20] The D_1 subtype is linked to stimulation of adenylate cyclase, whereas the D_2 receptor is negatively or not coupled to this enzyme. The binding sites of these two classes of receptors are sufficiently different that specific agonists and antagonists exist, making it possible to demonstrate with electrophysiological or biochemical techniques which of these receptor subtypes mediates a given physiological response. On the basis of these criteria, the dopamine somatodendritic autoreceptor was originally identified as a dopamine D_2 receptor on pharmacological,[29] electrophysiological[23] and molecular biological[27]

grounds. Similarly, the autoreceptor on the axon terminals of dopaminergic neurons was identified as a D_2 receptor on pharmacological[4,36] and electrophysiological[44,45] grounds.

Modern View of Dopamine Receptor Classification

More recently, advances in molecular biology have indicated that there are at least five different genes coding for six different subtypes of dopamine receptor.[33] Two of these receptor subtypes (D_{2S}, D_{2L}) consist of isoforms that arise as a result of post-transcriptional modification of a single gene product.[13,28] Thus, rather than two different dopamine receptors, there exist two families of dopamine receptors. The D1 family is comprised of the D_1 and D_5 receptors and the D_2 family of the D_{2L}, D_{2S}, D_3 and D_4 receptors. Although these different dopamine receptor subtypes are differentially distributed throughout the central nervous system, in some areas mRNAs for two or three of them are found within the same region, and sometimes even within the same neuron.[3,7,32,39] This new knowledge forces a re-examination of the "identification" of specific subtypes of dopamine receptors with physiological effects, particularly with respect to the dopamine autoreceptor.

Although there is certainly a receptor of the D_2 family on the cell bodies and at the nerve terminals of dopaminergic neurons, both D_2 and D_3 mRNA have been identified in substantia nigra and ventral tegmental area [3,33] where they are localized to dopaminergic neurons.[35] Dopamine and most "D_2-selective" agonists and antagonists bind to both the D_2 and the D_3 receptor with relatively high affinity.[33] For instance, studies comparing the distribution of D_2 and D_3 receptor mRNA with [^{125}I]iodosulpiride binding reveal that sulpiride labels both D_2 and D_3 receptors.[3] Thus, the identification of the nigral autoreceptor as a D_2 (and exclusively a D_2) receptor is no longer certain.

Although many "selective" dopamine agonists and antagonists have been synthesized and/or identified in recent years, these drugs are effectively selective only between receptors of the D_1 and D_2 families. Although some ligands (e.g., quinpirole, 7-OH DPAT) show differential binding to D_2 and D_3 receptors under optimal in vitro conditions, the Kds for most agonists and antagonists under physiological conditions are within one order of magnitude between D_2 and D_3 receptors.[12,35] Because of this, they cannot be used to discriminate, at least in a physiologically useful manner, among receptor subtypes that share the same or nearly the same binding sites, i.e., members within the D1 or D2 families. The greatest sequence homology among D_{2S}, D_{2L}, D_3 and D_4 receptors and between D_1 and D_5 receptors lies within the transmembrane spanning regions of the proteins, the region that is involved with ligand binding.[33] Thus, although sulpiride serves as a "selective" antagonist at D_2 receptors and SCH23390 as a "selective" antagonist at D_1 receptors, sulpiride cannot be used to discriminate between physiological effects mediated by D_2 or D_3 receptors,[35] and

SCH23390 similarly is not useful for determining if an effect is mediated by a D_1 or a D_5 receptor.

In Vivo Antisense Oligodeoxynucleotide Knockout

Antisense knockout refers to the ability of specifically designed short sequences of oligodeoxynucleotides (single-stranded DNA) to bind to their complementary mRNA and stop translation, thereby preventing the expression of the protein that the mRNA coded for. Recent studies have demonstrated the feasibility of using in vivo administration of antisense oligodeoxynucleotides to produce knockouts of specific receptor subtypes and/or subunits including muscarinic m_2, $GABA_B$, $NMDA_{R1}$, neuropeptide Y-Y1 and dopamine D_2 receptors.[19,47,48,50,54,55] Antisense oligodeoxynucleotides enter cells both in vitro or in vivo by receptor-mediated endocytosis or nonselective pinocytosis,[25] and bind specifically to their target mRNA and stop protein translation.[53] As the protein is degraded during the course of normal cellular activity, it is not replaced, resulting in a lack of that protein in the cells which had taken up the antisense oligodeoxynucleotide. The double-stranded DNA/RNA hybrid is also a substrate for ribonuclease H-mediated degradation.[49] Regardless of the precise molecular mechanism of action, the antisense knockout technique offers, for the first time, the ability to reduce or eliminate a particular receptor subtype with absolute specificity. Furthermore, because of the limited spread of antisense oligodeoxynucleotides in the brain, it is possible, for the first time, to selectively knock out either pre- or postsynaptic receptors. This technique has recently been successfully applied to the dopamine D_2 receptor to study the behavioral[50,54,55] and electrophysiological effects[26,37,38] of dopamine D_2 receptor knockout. In this chapter we will summarize the electrophysiological consequences of the knockout of dopamine D_2 autoreceptors by in vivo administration of an antisense oligodeoxynucleotide directed against the dopamine D_2 mRNA.

METHODS

Antisense Treatment

The antisense oligodeoxynucleotide and random oligodeoxynucleotide control sequences, and the methods for chronic intranigral administration have already been described.[26,37,38,54] In brief, male Sprague-Dawley rats weighing between 150 g and 250 g were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (15 mg/kg) i.p. and placed in a stereotaxic apparatus. The scalp was reflected and a small burr hole drilled in the skull overlying and lateral to the left substantia nigra. A 28 g stainless steel infusion guide cannula was lowered at a 20° angle and affixed in place with cyanoacrylate glue and dental cement. Following a 24 hour recovery period, a 33 g injection cannula, 1 mm longer than the guide, was filled with saline vehicle, D_2 ran-

dom oligodeoxynucleotide control sequence or D_2 antisense oligodeoxynucleotide, inserted into the guide cannula and lowered to a position just above the substantia nigra pars compacta. The cannula was joined to a length of teflon tubing connected through a fluid swivel to a microsyringe pump and saline, D_2 random antisense, or D_2 antisense oligodeoxynucleotide (10-20 μg/μl) was infused continuously at 0.1 μl/hour for 6 days while the animals were housed in individual circular Plexiglas cages with ad libitum access to food and water.

The D_2 antisense oligodeoxynucleotide was a 19-mer complementary to codons 2-8 of the D_2 receptor mRNA with sequence 5'-AGGACAGGTTCAGTGGATC-3'[54] The D_2 random oligodeoxynucleotide control consisted of the same bases as in the D_2 antisense in pseudo-random order with 11 of the 19 bases mismatched from the sense mRNA: 5'-AGAACGGCACTTATGGGTG-3'. Both oligodeoxynucleotides consisted of modified "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, but which does not prevent uptake into cells.[1] The oligodeoxynucleotides were synthesized by Oligos Inc., (Wilsonville, OR).

Electrophysiological Measurements

On the 7th day after the start of the infusion, rats were anesthetized with urethane (1.3 g/kg, i.p.), the left femoral vein or a lateral tail vein was cannulated, and the rat installed into a stereotaxic frame. A bipolar stimulating electrode was placed in the ipsilateral neostriatum and extracellular recordings of antidromically identified substantia nigra dopaminergic neurons were obtained by conventional means as described previously.[46]

Dopaminergic 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 only spikes.[9,18,44] The firing pattern of each neuron was classified as pacemaker, random or bursty on the basis of the neuron's autocorrelation histogram.[43] The threshold current for each neuron was defined as the minimum stimulating current that evoked antidromic responses from neostriatum to 100% of the stimulus deliveries.[44]

Following the establishment of a stable baseline firing rate for at least 5 minutes, a dose of apomorphine hydrochloride that was double the previous dose was injected intravenously every two minutes, 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 μg/kg, i.v.) was subsequently administered in an attempt to reverse the inhibition.

Autoradiography and Histology

For D_2 receptor autoradiography, animals were euthanized by overdose of urethane and the brains rapidly removed and frozen in liquid nitrogen. Subsequently, 20 μm coronal sections were taken on a cryostat at -18°C, thaw-mounted on gelatin-subbed slides and stored at -80°C. Sections were gradually brought to room temperature and incubated for 30 minutes in 0.2 nM [^{3}H] spiperone in 50 mM Tris-HCl buffer, containing 120 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl and 1 mM $MgCl_2$. The incubation period was terminated by rinsing the slides twice for five minutes with ice-cold buffer. Nonspecific binding was determined by incubation in the presence of 1 μM (+) flupentixol. After washing the slides were dipped quickly in ice-cold water and dried under a stream of cold air. Slides were then placed in X-ray cassettes together with [^{3}H] microscales (Amersham) and exposed to Hyperfilm-[^{3}H] (Amersham) for a period ranging from 4 days to 6 weeks at 4°C. Average binding densities were determined with a computerized image analysis system (MCID).

In order to determine if there were any nonspecific neurotoxic effects of the antisense treatment, some brains were sectioned at 60 μm and processed for Nissl staining (Neutral Red) in order to compare the cytoarchitecture of the infused side with that of the contralateral control. In other cases, immunostaining for tyrosine hydroxylase, a marker for dopaminergic neurons in the midbrain, was performed to examine dopaminergic neurons specifically by procedures previously described.[41]

RESULTS

Spontaneous Activity and Antidromic Responses

Spontaneous activity was assessed by measuring spontaneous firing rate, mean interspike interval (ISI), coefficient of variation of the ISI (CV), and by constructing autocorrelation histograms from spontaneous spike trains. The pattern of firing of each neuron was categorized as pacemaker, random, or bursty according to the autocorrelation histogram as described previously.[43] Treatment with D_2 antisense did not significantly alter the mean firing rate, coefficient of variation or the firing pattern of dopaminergic neurons as shown in Table 7.1.

In all neurons that were antidromically activated from ipsilateral neostriatum, the threshold current was measured. In contrast to the other parameters measured, antisense treatment produced a significant reduction in threshold current (Table 7.1), signifying an increase in dopamine terminal excitability, a parameter that we have previously shown is inversely related to the degree of terminal autoreceptor activation.[42]

Response to Administration of Apomorphine

Administration of the D_2 antisense oligo for six days markedly attenuated the ability of intravenously administered apomorphine to in-

Table 7.1. Effects of D_2 antisense treatment on electrophysiological properties of nigrostriatal dopaminergic neurons

	Untreated	Saline	Contralateral	D_2 Random Antisense	D_2 Antisense
Mean Firing Rate	3.85 ± 0.32 (17)	3.09 ± 0.26 (10)	3.77 ± 0.50 (15)	3.29 ± 0.39 (11)	4.10 ± 0.27 (55)
Coefficient of Variation	0.342 ± 0.052 (17)	0.435 ± 0.093 (10)	0.504 ± 0.070 (15)	0.405 ± 0.036 (11)	0.439 ± 0.029 (55)
Firing Pattern (P/R/B)	4/6/7	3/5/2	5/6/4	3/6/2	12/27/16
Threshold Current (mA)	1.69 ± 0.31 (14)	1.19 ± 0.19 (6)	2.09 ± 0.35 (6)	1.58 ± 0.33 (11)	1.06 ± 0.17* (18)

* significantly different from pooled controls, Bonferroni/Dunn, $p < .05$

Effects of D_2 antisense treatment on electrophysiological properties of nigrostriatal dopaminergic neurons. Spontaneous firing rates (spikes/sec), coefficient of variation (standard deviation of the mean interspike interval/ the mean interspike interval) and threshold current are expressed as mean ± standard error. Numbers in parentheses refer to number of neurons per group. Firing patterns are expressed as the number of cells firing in (P)acemaker mode, (R)andom mode, and (B)ursty mode respectively for each treatment group and were analyzed by c^2 analysis.

hibit the firing of dopaminergic neurons as shown for three representative neurons in Figure 7.1. In neurons recorded from untreated controls (A) or ipsilateral to random D_2 oligodeoxynucleotide infusion (B), sequential intravenous injections of apomorphine produced marked inhibition of firing of antidromically identified nigrostriatal dopaminergic neurons before eventually producing complete suppression of spontaneous activity, typically by the time the dose reached 8 μg/kg. This inhibition could be readily reversed by subsequent administration of 50 μg/kg haloperidol, i.v. In contrast, neurons ipsilateral to infusion of D_2 antisense oligodeoxynucleotide were far less susceptible to the inhibitory effects of apomorphine, and in some cases could not be completely inhibited even at a cumulative dose over 2,000 μg/kg. The dose response curves in Figure 7.2 reveal that D_2 antisense treatment produced a dramatic shift to the right in the apomorphine dose-response relation, whereas neurons from rats treated with saline, the D_2 random oligodeoxynucleotide or recorded contralateral to D_2 antisense infusion were indistinguishable from neurons recorded from untreated control rats.

It is worth noting that there was some variability in the response of individual neurons from different antisense-treated animals. About half of the neurons showed a maximum inhibition of firing to about

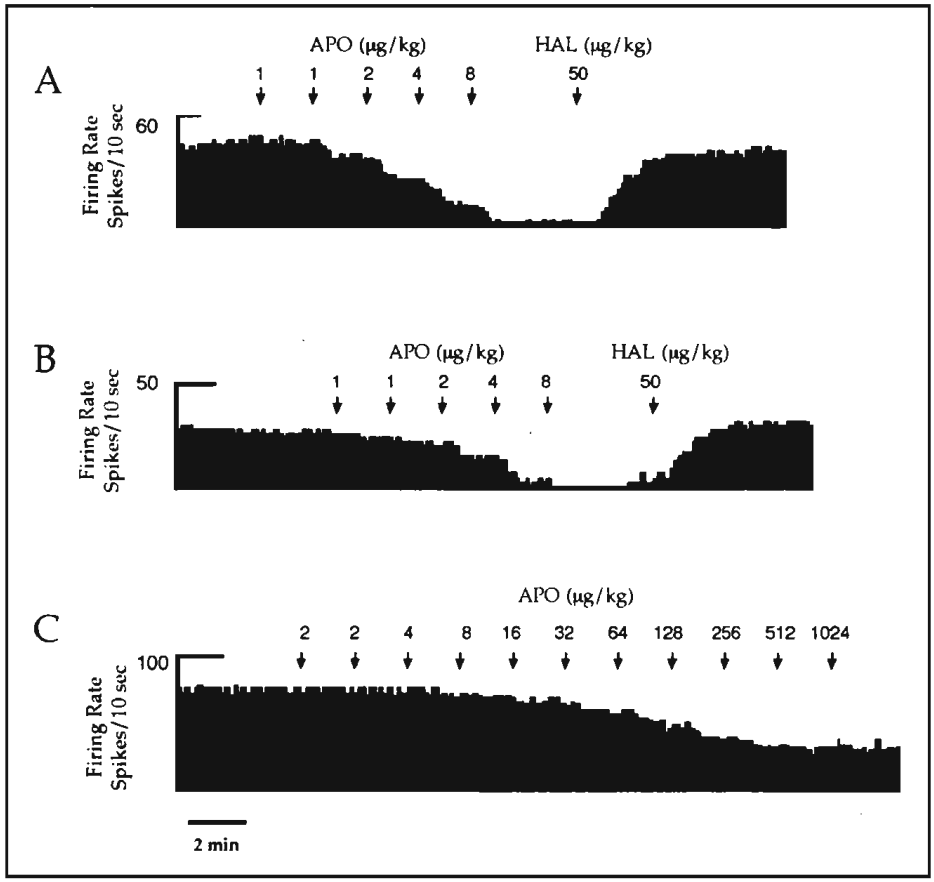

Fig. 7.1. Representative ratemeter plots showing the effects of intravenous administration of the dopamine receptor agonist, apomorphine, on the extracellularly recorded firing rate of antidromically identified nigrostriatal dopaminergic neurons in vivo from saline treated, random antisense treated and D_2 antisense treated rats. (A) Recording obtained from a neuron ipsilateral to saline control infusion for six days. The neuron is inhibited by the bolus of 8 mg/kg. The inhibition is completely reversed by 50 mg/kg of the dopamine D2 class antagonist, haloperidol. (B) Recording obtained from a neuron ipsilateral to D_2 random antisense control infusion for six days. The neuron is almost completely inhibited after 4 mg/kg is and completely inhibited by the bolus of 8 mg/kg. The inhibition is completely reversed by 50 mg/kg, haloperidol. (C) Recording obtained from a neuron ipsilateral to D_2 antisense infusion for six days. The neuron shows virtually no effect to the bolus of 8 mg/kg and is inhibited by less than 50% after a cumulative dose of 512 mg/kg. Complete inhibition could not be achieved even at a cumulative dose of 2,048 mg/kg. In each cell, apomorphine was injected at a dose doubling the previous dose every two minutes.

80% of the pre-drug control levels at the highest dose of apomorphine tested (a bolus of 1024 μg/kg), whereas other neurons could be inhibited to a greater extent, sometimes completely, albeit always at doses much greater than those required to completely inhibit control neurons.

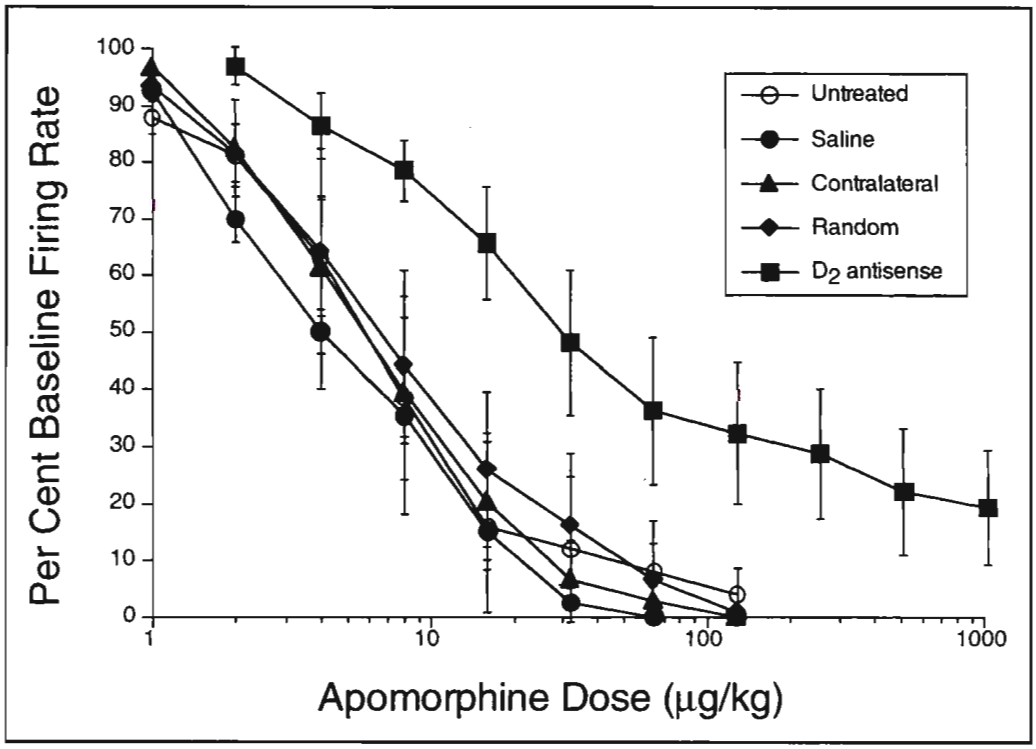

Fig. 7.2. Cumulative dose response curves of the firing rate response to intravenously administered apomorphine in nigrostriatal dopaminergic neurons recorded in untreated control animals, contralateral to D_2 antisense infusion, or ipsilateral to saline infusion, D_2 random antisense infusion or D_2 antisense. In all control groups, neurons exhibited an ED_{50} of approximately 6 mg/kg, whereas the treated neurons' dose response curve is shifted markedly to the right, with an ED_{50} of approximately 31 mg/kg. See text for further details.

Autoradiography, Histology and Immunocytochemistry

Estimates of the extent of D_2 receptor loss in the pars compacta of the substantia nigra ipsilateral to D_2 antisense infusion obtained from quantitative autoradiography ranged from a low of 40% to a high of approximately 80%, and varied from animal to animal and section to section. The mean changes in binding over the entire pars compacta region for all brains analyzed are shown in Table 7.2. In some cases there appeared to be a near-total absence of D_2 binding near the infusion site in substantia nigra. This was not due to a nonspecific loss of neurons due to neurotoxicity or to nonspecific loss of other receptor binding sites since both Nissl stain and tyrosine hydroxylase immunocytochemistry revealed a normal complement of dopaminergic neurons on the infused side, as shown in Figure 7.3. Furthermore, in other studies, D_1 receptor binding on the infused side was shown to be normal.[37] There were no cases in which the knockout spread to the contralateral noninfused side, and the autoradiograms

Table 7.2. Effects of D_2 antisense treatment on dopamine D_2 receptor binding in substantia nigra pars compacta

	Contralateral	Ipsilateral	Per Cent Change
D_2 Random Antisense	6.53 ± 0.61	6.61 ± 0.72	+ 1.0%
D_2 Antisense	6.58 ± 0.55	3.10 ± 0.49	– 52.9%*

* significantly different from pooled controls, Bonferroni/Dunn, $p < .05$

Effects of D_2 antisense treatment on dopamine D2 receptor binding in substantia nigra pars compacta. D2 receptors were labeled by 1.2 nM ^{3}H-spiperone with or without 1 mm flupentixol. Numbers under contralateral (control) and ipsilateral (D_2 antisense treated) refer to mean density + standard error in nCi/mg-tissue.

showed a rather sharp delineation of receptor loss near the infusion site in the ipsilateral substantia nigra.

DISCUSSION

These results demonstrate that in vivo supranigral infusion of a phosphorothioated antisense oligodeoxynucleotide directed against the 2nd through 8th codons of the D_2 dopamine receptor mRNA for six days produces a marked reduction in dopamine D_2 receptor binding largely constrained to the substantia nigra with no accompanying signs of neurotoxicity. This reduction in D_2 receptor binding was associated with significant changes in some of the electrophysiological properties of nigrostriatal dopaminergic neurons.

The marked changes in the autoreceptor-mediated properties of dopaminergic neurons after D_2 antisense treatment demonstrates that at least some of the autoreceptors are of the D_2 subtype. Whereas this finding is certainly not unexpected based on previous pharmacological studies of midbrain dopaminergic neurons,[22,23] as mentioned in the introduction, the knowledge that midbrain dopamine neurons also express D_3 receptor mRNA, as well as recent reports that putative D_3-preferring agonists were more potent than D_2-preferring agonists at inhibiting dopaminergic neurons[21,24] left open the possibility that the principal functional autoreceptor on dopamine neurons would prove to be a D_3 receptor. Whereas the present results do not rule out the possibility that there may also be a D_3 autoreceptor on dopaminergic neurons, they confirm that there is certainly a D_2 autoreceptor, at both the somatodendritic and nerve terminal regions.

That there is a D_2 somadendritic autoreceptor is demonstrated by the marked shift to the right in the apomorphine dose-response curve. Previous studies have shown that the inhibition of dopaminergic neurons by low doses of systemically administered apomorphine is due to

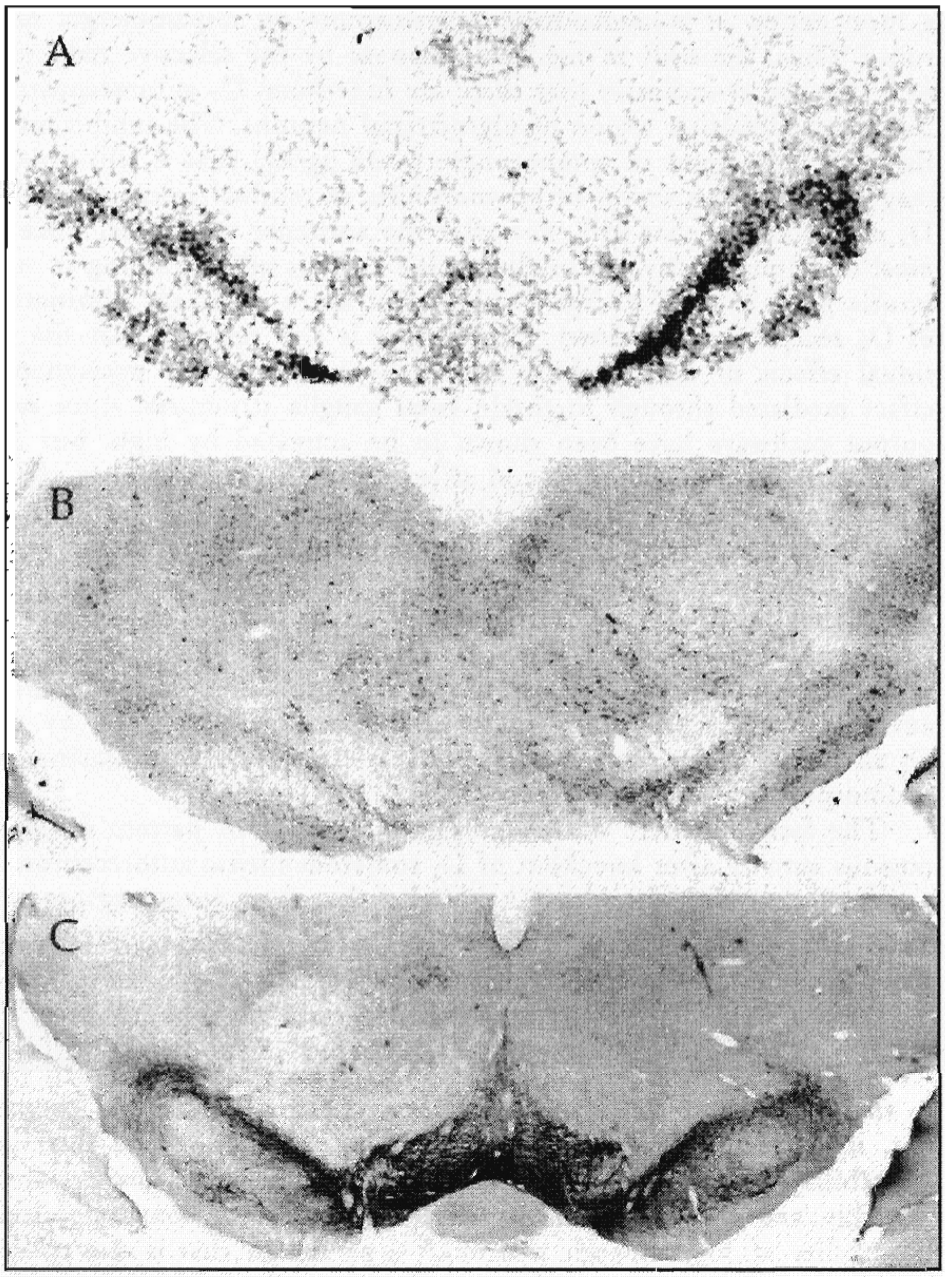

Fig. 7.3. Autoradiographic, histological and immunocytochemical sequela of infusion of D_2 antisense into the left substantia nigra. (A) Specific D2 binding (^{3}H-spiperone which also labels D_3 receptors) is reduced by approximately 70% overall in the substantia nigra (pars compacta plus pars reticulata) of the substantia nigra ipsilateral to the infusion compared to the contralateral side. (B) Nissl staining reveals no apparent damage to neurons in the vicinity of the substantia nigra on the infused (left) side of the brain. (C) Tyrosine hydroxylase immunostaining of a section near to that shown in B from the same brain reveals no loss of dopaminergic (TH positive) neurons on the infused. The lightly stained circular area over the substantia nigra on the left side is nonspecific mechanical damage resulting from the infusion cannula.

a local action at somadendritic autoreceptors on dopaminergic neurons.[2] Thus, the shift in the dose response by the selective reduction in D_2 receptors indicates that there are functional D_2 autoreceptors at the somatodendritic region of nigrostriatal neurons. The inhibition of firing to large doses of apomorphine (> 32 μg/kg) that is still present may represent the action of apomorphine at residual somatodendritic D_2 autoreceptors that still exist after the antisense treatment, since in most cases quantitative autoradiographic analysis of D_2 binding in substantia nigra showed a large decrease, but not a complete elimination of D_2 receptors (see below). However, it is also possible that the residual effects of apomorphine were due to a long-loop postsynaptic effect mediated through forebrain basal ganglia structures, since such output pathways have been shown to be activated by high, but not low doses of apomorphine,[34] and at least part of the inhibitory effects on dopaminergic cell firing of high dose amphetamine, another substance that acts at dopaminergic autoreceptors, has been shown to be due to mediated through these long-loop pathways.[5,31] It seems most likely that both of these explanations contribute to the effects of apomorphine following D_2 antisense treatment. It is also possible that some of the inhibition arises from the activation of D_3 somadendritic autoreceptors, since these would not have been affected by the D_2 antisense treatment, but would be effectively stimulation by apomorphine.

The fact that there was no change in the rate or pattern of spontaneous activity after knockout of D_2 somatodendritic autoreceptors is not consistent with the widely-held belief that these autoreceptors play a role in the self-inhibition of the firing rate of dopaminergic neurons.[17] It is possible that some compensatory changes in the dopaminergic neurons or their afferents occurred that mask the increase in spontaneous firing rate that would be predicted on the basis of the self-inhibition hypothesis. However, this explanation seems unlikely due to the rather short times involved, and because other related properties, including the response to apomorphine and the basal terminal excitability were markedly altered. In addition we have also reported that this same antisense treatment causes an increase in somatodendritic excitability of dopaminergic neurons,[37] a parameter that is also modulated by somatodendritic autoreceptors. Taken together, these data support our previous suggestion, derived from an independent line of evidence, that the activation of somatodendritic autoreceptors on dopaminergic neurons by endogenously released dopamine in vivo may have more to do with local regulation of dendritic excitability than with the direct modulation of spontaneous firing rate.[46]

The decrease in antidromic threshold current in D_2 antisense-treated neurons provides evidence that autoreceptor known to exist on the dopaminergic nerve terminals is a D_2 receptor. Previous studies have shown that acute local administration of the dopamine D_2 class recep-

tor antagonists, haloperidol or sulpiride, into the terminal regions of nigrostriatal neurons causes a reduction in antidromic threshold currents[42,44] because they block the inhibitory effects of endogenous dopamine on the dopaminergic nerve terminals. The decrease in threshold found after antisense treatment in the present study presumably results from a similar loss of D_2 receptor stimulation that arises from the ongoing release of endogenous dopamine. Although these findings need to be confirmed with further experiments in which autoreceptor-mediated changes in terminal excitability and modulation of evoked release of dopamine are measured in antisense treated animals, the present data suggest that there exists an autoreceptor of the D_2 subtype on nigrostriatal terminals, and that application of D_2 antisense oligodeoxynucleotide to the cell body of a neuron results in a loss of D_2 receptors at the nerve terminal as well as the somatodendritic region. As with the somatodendritic autoreceptor data, these findings do not rule out the possibility that there may also be a D_3 autoreceptor at terminal regions of nigrostriatal neurons, but they do demonstrate that a D_2 autoreceptor is present.

While the quantitative autoradiography showed that D_2 receptors in substantia nigra were markedly reduced after nigral infusion of the D_2 antisense, they were not eliminated. The simplest explanation for this is that it may not be possible to achieve complete antisense knockout of dopamine receptors (and perhaps any protein) due to compensatory cellular mechanisms. This might account for the failure of the antisense treatment to completely eliminate the inhibitory effects of apomorphine in the electrophysiological experiments. However, as stated above, different autoradiographic sections through substantia nigra from different animals varied in their degree of receptor loss. The average estimate of the degree of receptor loss (~50%) was determined by comparing the entire area of the substantia nigra pars compacta from many animals. Inspection of individual autoradiograms (see Fig. 7.3A for example) often revealed regions on the antisense-treated side in which binding was nearly indistinguishable from background. This could be due to a highly localized effect of the antisense, in which case averaging over the entire pars compacta would tend give an underestimate of the true degree of knockout at the most strongly affected sites nearest the infusion. In addition, it is certain that some of the bound radioligand represents binding to D_3 receptors, since spiperone labels D_3 receptors with almost the same affinity as D_2 receptors. Thus the true maximal extent of D_2 receptor knockout possible with antisense remains to be determined, but it is certain to be greater than our estimate of 50% based on the quantitative autoradiography reported here.

In summary, these data verify the effectiveness of the technique of in vivo local administration of antisense oligodeoxynucleotides to markedly reduce or eliminate dopamine D_2 receptors in specific cell groups in the brain. Another approach to the problem of identifying

the functions of specific dopamine receptor subtypes is the use of transgenic animals in which genes coding for certain receptors have been knocked out.[10] However, the antisense knockout technique may have significant advantages over the use of transgenic knockouts for identifying and studying receptor subtype function when subtype specific agonists or antagonists do not exist. Although the question of the maximal extent of receptor knockout possible with in vivo antisense administration still remains to be determined, because antisense knockout can be directed towards specific areas in the brain, and because the antisense can be administered at any developmental stage or after the brain is fully mature, antisense knockout of CNS receptors avoids the compensatory changes that are likely to occur when a particular receptor is missing from the entire brain from the moment of conception, as is the case with current transgenic models.

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