ELECTROPHYSIOLOGICAL CONSEQUENCES OF D_2 AND/OR D_3 RECEPTOR KNOCKOUT BY ANTISENSE OLIGONUCLEOTIDES IN NIGROSTRIATAL DOPAMINERGIC NEURONS

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INTRODUCTION

Mesencephalic dopaminergic neurons possess receptors for their own neurotransmitter, dopamine, at both somatodendritic as well as axon terminal regions. These receptors are termed autoreceptors and act to modulate dopaminergic synaptic transmission in two ways. Activation of the somatodendritic autoreceptors produces a membrane hyperpolarization by increasing a potassium conductance (Lacey, 1993) which leads to an inhibition of spontaneous activity (Groves et al., 1975). Activation of the terminal autoreceptors produces a decrease in terminal excitability which is presumed to reflect a membrane hyperpolarization (Tepper et al., 1985) which leads to a reduction in dopamine synthesis and in calcium- and impulse-dependent release of dopamine from nerve terminals (Starke et al., 1989; Wolf and Roth, 1987).

Both types of dopamine autoreceptors were originally identified as dopamine D2 receptors on pharmacological, biochemical and electrophysiological grounds (e.g., Boyar et al., 1987; Morelli et al., 1988; Starke et al., 1989; Tepper et al., 1984; Wolf and Roth, 1987). However, this identification was based on the concept that there exist two subtypes of dopamine receptors: D1 receptors, activation of which stimulates the production of cAMP, and D2 receptors, activation of which leads to inhibition of cAMP synthesis (Kebabian and Calne, 1979). More recently, molecular cloning experiments have revealed that there are actually 2 *families* of dopamine receptors, termed D1 and D2 (Schwartz et al., 1992; Sibley and Monsma, 1992). The D1 family comprises D_1 and D_5 receptors and the D2 family comprises D_2, (of which there are two isoforms, D_{2S} and D_{2L} which arise as a result of post-transcriptional modification of a single gene product (Giros et al., 1989; Monsma et al.,

The Basal Ganglia V, Edited by Ohye et al.
Plenum Press, New York, 1996

1989), D_3 and D_4 receptors. Studies of the distribution of mRNAs for the different dopamine receptors have revealed that both D_2 and D_3 mRNA exist in substantia nigra, where both have been localized to dopaminergic neurons (Sokoloff et al., 1990). Thus, although the functional dopamine autoreceptor (i.e., the autoreceptor that mediates the membrane hyperpolarization at the somatodendritic and terminal regions of the dopamine neuron and the corresponding inhibition of firing and transmitter release) is a member of the D2 family, it is no longer clear whether it is a D_2 or a D_3 receptor, or whether the somatodendritic and terminal autoreceptors are identical.

Conventional pharmacological approaches cannot resolve these questions because most D2 class agonists and antagonists have relatively high affinity for both D_2 and D_3 receptors. However, it is possible to produce loss of a given dopamine receptor subtype with great specificity by the administration of short length antisense oligodeoxynucleotides (AON) that are complementary to the mRNA that codes for a given receptor (Zhang and Creese, 1993). This technique, known as antisense knockout, can be applied *in vivo* by administering specific oligodeoxynucleotides intraventricularly or directly into the brain to produce widespread or highly localized decreases in specific dopamine receptors. We used local infusion of dopamine receptor AONs into substantia nigra to determine the functional effects of the loss of the dopamine D_2 and/or D_3 autoreceptors on nigrostriatal dopaminergic neurons on their electrophysiological properties and response to administration of the mixed autoreceptor agonist, apomorphine.

METHODS

Antisense Treatment

The AON and random oligodeoxynucleotide control sequences and the methods for chronic intranigral administration have already been described (Zhang and Creese, 1993; Martin et al., 1994; Sun et al., 1996) In brief, male Sprague-Dawley rats weighing between 150 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 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 the appropriate substance, inserted into the guide cannula and lowered so that the tip was 500 μm dorsal to 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, D_2, D_3 or D_2+D_3 AON (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 AON was a 19-mer complementary to codons 2-8 of the D_2 receptor mRNA with sequence 5′-AGGACAGGTTCAGTGGATC-3′ and the D_3 AON, also directed against codons 2-8, had the sequence 5′-TTATCTGGCTCAGAGGTGC-3′. A D_2 random oligodeoxynucleotide control consisted of the same bases as the D_2 AON with 11 of the 19 bases mismatched from the sense mRNA: 5′-AGAACGGCACTTATGGGTG-3′. All AONs 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. The AONs 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 (Trent and Tepper, 1991). The firing pattern of each neuron was classified as pacemaker, random or bursty on the basis of the neuron's autocorrelation histogram (Tepper et al., 1995) 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 (Tepper et al., 1985) To obtain an estimate of the excitability of the somatodendritic region of the dopaminergic neurons (Trent and Tepper, 1991), the proportion of striatal-evoked antidromic responses consisting of the full initial segment-somatodendritic spike was counted while each neuron was stimulated at threshold.

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 (50-200 μg/kg, i.v.) was subsequently administered in an attempt to reverse the inhibition.

Controls consisted of cells recorded ipsilateral to infusions of saline or D_2 random oligodeoxynucleotide, contralateral to D_2 or D_3 AON infusions, or from untreated rats. Since none of the parameters measured differed among these different control groups, the data were pooled into a single control group against which neurons recorded from D_2, D_3 and D_2+D_3 AON treated rats were compared by analysis of variance.

RESULTS

Spontaneous Activity

Neither D_2, D_3 nor D_2+D_3 AON infusion had any effect on the mean spontaneous firing rate, the coefficient of variation of the interspike intervals, or the distribution of firing patterns of nigrostriatal neurons as shown in Figure 1. Although there was a tendency for rats treated with D_3 antisense (D_3 or D_2+D_3 AON treated) to show a larger proportion of neurons firing in the pacemaker mode, this was not statistically significant (χ^2=6.6, df=6, p=0.360).

Antidromic Responses

Neurons recorded ipsilateral to D_2, D_3 or D_2+D_3 AON infusions exhibited significantly lower thresholds for antidromic responding from the ipsilateral neostriatum (F=4.38, df=3, 76, $p<0.05$). Each of the AON treatments was significantly different from the control group (Fisher's PLSD, $p<0.05$), but there were no significant differences among the different treatment groups.

Treatment with D_2, D_3 or D_2+D_3 AON also increased the proportion of antidromic spikes consisting of the full initial segment-somatodendritic spike (F= 3.49, df= 3, 61, $p<0.05$). As was the case with antidromic threshold currents, each of the AON treatments was significantly different from the control group (Fisher's PLSD, $p<0.05$), but there were no significant differences among the different treatment groups. These data are summarized in Figure 2.

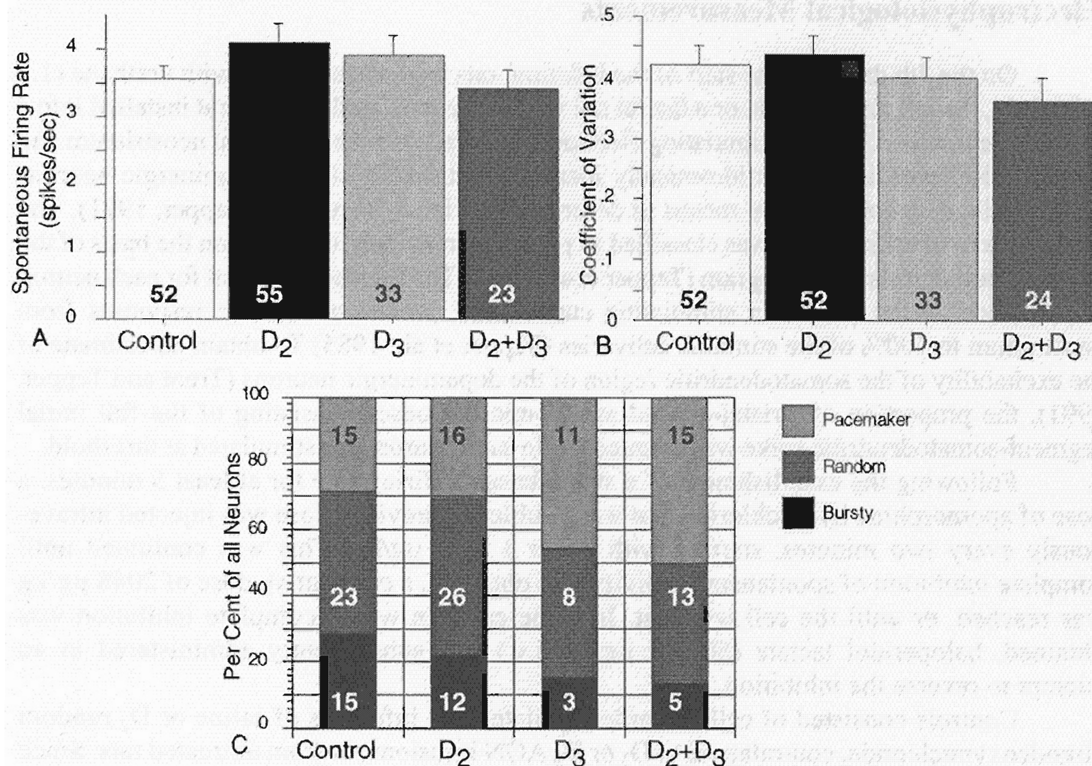

Figure 1. Effects of D_2, D_3 and D_2+D_3 antisense infusion into the substantia nigra on the spontaneous activity of nigrostriatal dopaminergic neurons. A. Lack of effect of any of the antisense treatments on the mean spontaneous firing rate. B. Lack of effect of any of the antisense treatments on the mean coefficient of variation of the interspike intervals (cv=standard deviation of the interspike intervals/mean interspike interval). C. Lack of effect of any of the antisense treatments on the distribution of spontaneous firing patterns as determined from autocorrelation histograms. Error bars represent the S.E.M. The numbers within the bars represent the number of neurons in each group.

Apomorphine Dose Response

Both D_2, D_3 and combined D_2+D_3 AON infusions produced a significant shift to the right in the apomorphine dose response curve. There was some variability in the response of individual neurons from the different AON-treated animals, as can be seen from inspection of the error bars in Figure 3, particularly at the higher doses. About half of the neurons showed a maximum inhibition of firing to about 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, although these always required doses much greater than those required to completely inhibit control neurons. In all cases in which it was administered, haloperidol completely reversed the apomorphine induced inhbition of firing.

DISCUSSION

Specificity of the Antisense Oligodeoxynucleotides

Although intraventricular administration of the D_2 AON significantly reduced D_2 receptor (^{3}H-spiperone) binding in dorsal and ventral striatum by 40-70% as measured by

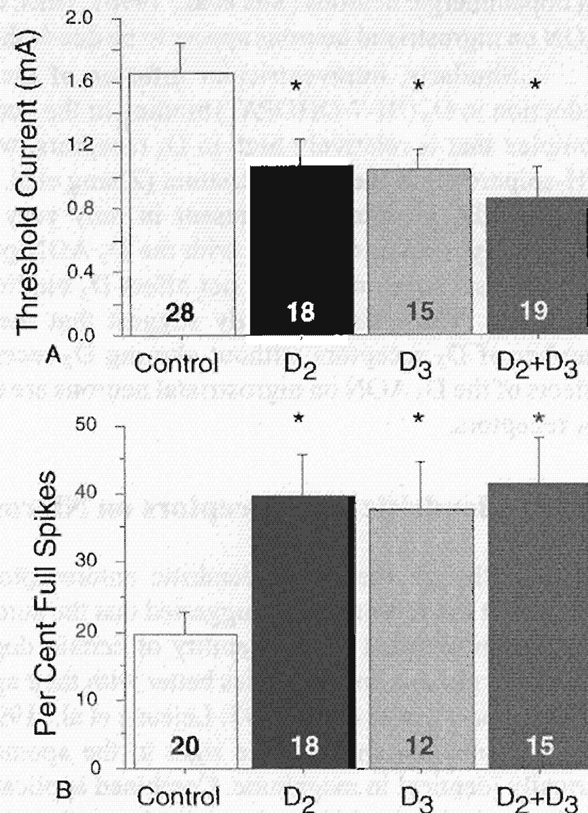

Figure 2. Effects of D_2, D_3 and D_2+D_3 antisense infusion into the substantia nigra on the antidromic response properties of nigrostriatal dopaminergic neurons. A. The current required to elicit antidromic responses to 100% of the stimulus deliveries (threshold) is significantly reduced by D_2, D_3 and D_2+D_3 antisense treatment ($F=4.38$, $df= 3, 76$, $p<0.05$). B. The proportion of antidromic action potentials consisting of both the initial segment and somatodendritic components (full spikes) was greatly increased by D_2, D_3 and D_2+D_3 antisense treatment ($F=3.49$, $df= 3, 61$, $p<0.05$). Error bars represent the S.E.M. The numbers within the bars represent the number of neurons in each group. Asterisk indicates significantly different from Control group (Fisher's PLSD, $p<0.05$).

quantitative autoradiography (Zhang and Creese, 1993) and significantly reduced D_2 binding in substantia nigra by up to 90% following supranigral infusions (Martin et al., 1994, Sun et al., 1995, 1996), there was no effect on D_1 binding in adjacent sections from any of these regions. Tyrosine hydroxylase immunostaining and Nissl staining after supranigral administration of D_2 AON failed to reveal any evidence of a non-specific toxic effect of the antisense

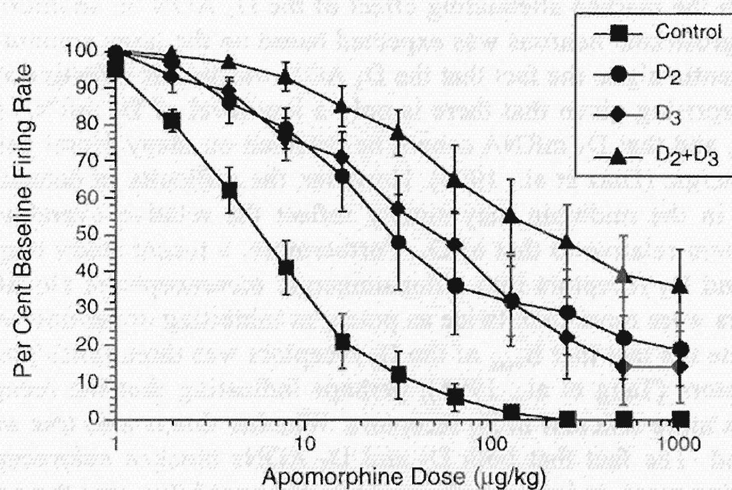

Figure 3. Cumulative dose response curve for apomorphine induced inhibition of spontaneous firing of nigrostriatal dopaminergic neurons. Error bars represent ± 1 SEM. N= 8 to 20 neurons per point.

on dopaminergic neurons (Sun et al., 1996). Thus, the electrophysiological effects of the D_2 AON on nigrostriatal neurons appear to be due to the specific loss of dopamine D_2 receptors.

Similarly, intraventricular infusion of the D_3 AON for 3 days produced a 47% reduction in D_3 (^{3}H-7-OHDPAT) binding in the nucleus accumbens, a region of the striatal complex that is relatively high in D_3 receptors, while this AON did not alter D_2 binding (^{3}H-spiperone) in the dorsal striatum (Zhang et al., 1996), a region of the striatal complex in which D_3 receptors are present in only very low amounts (Sokoloff et al., 1990). Conversely, similar treatment with the D_2 AON produced a 50% reduction in D_2 binding in the dorsal striatum but did not affect D_3 binding in the nucleus accumbens (Zhang et al., 1996). These data strongly suggest that the D_3 antisense selectively reduced the number of D_3 receptors without altering D_2 receptors and that the electrophysiological effects of the D_3 AON on nigrostriatal neurons are thus due to the specific loss of dopamine D_3 receptors.

Somatodendritic Autoreceptors on Nigrostriatal Neurons

Although the somatodendritic autoreceptor had long been assumed to be a D_2 receptor, it has recently been suggested that the autoreceptor may be of the D_3 subtype since it has been reported that the ability of certain dopamine agonists to inhibit the firing of dopaminergic neurons correlates better with their apparent affinity for D_3 receptors than for D_2 receptors (Kreiss et al., 1995; Lejeune et al., 1995). Nevertheless, both D_2 and D_3 AONs produced marked shifts to the right in the apomorphine dose response curve that were virtually identical in magnitude. Combined application of both D_2 and D_3 AON produced a qualitatively larger shift to the right than either AON alone. Since previous studies have shown that the inhibition of firing of dopaminergic neurons by low doses of systemically administered apomorphine is due to a local action at somadendritic autoreceptors (Akaoka et al., 1992), these data suggest that the majority of nigrostriatal dopaminergic neurons posses both D_2 and D_3 somatodendritic autoreceptors. In some neurons there was an almost complete blockade of inhibition except at very high apomorphine doses. At these high doses apomorphine is no longer acting locally, and some or all of the residual inhibition could have resulted from stimulation of postsynaptic dopamine receptors and consequent activation of descending inhibitory pathways from the forebrain (Skirboll et al., 1979).

Although the marked attenuating effect of the D_2 AON on apomorphine induced inhibition of nigrostriatal neurons was expected based on the large amount of D_2 mRNA present in substantia nigra, the fact that the D_3 AON was just as effective as the D_2 AON is somewhat surprising given that there is only a low level of D_3 mRNA present in the mesencephalon, and that D_3 mRNA cannot be detected on many nigral neurons that are clearly dopaminergic (Diaz et al., 1995). However, the difficulty in detecting D_3 mRNA and/or binding in the midbrain may simply reflect the relative overabundance of D_2 mRNA and protein relative to that of D_3. Furthermore, a recent study employing transfection of D_2 and D_3 receptors into a dopaminergic mesencephalic clonal line showed that D_3 receptors were more than twice as potent at inhibiting dopamine 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 in D_3 receptors. Whether this is also true *in situ* remains to be determined. The fact that both D_2 and D_3 AONs blocked autoreceptor-mediated inhibition of firing more or less equally suggests the possibility that the normal electrophysiological response attributed to somatodendritic autoreceptor stimulation may require coactivation of both D_2 and D_3 receptors.

Terminal Autoreceptors on Nigrostriatal Neurons

Previous biochemical studies concerning the existence of D_3 terminal autoreceptors on nigrostriatal neurons is contradictory. It has been reported that the efficacy of drugs acting at the striatal terminal autoreceptor to inhibit dopamine synthesis correlates better to D_3 binding affinity than to D_2 binding affinity (Meller et al., 1993). On the other hand, a D_3 AON infused into the lateral ventricle failed to affect dopamine synthesis in striatum or block the inhibitory effects of apomorphine on dopamine synthesis, although this oligodeoxynucleotide did elevate dopamine synthesis in nucleus accumbens (Nissbrandt et al., 1995). In the present experiments, both D_2 and D_3 AONs significantly reduced the threshold current for eliciting antidromic responses from neostriatum. Previous experiments have shown that the threshold can be modulated by terminal autoreceptors. Local infusion of D2 family antagonists like haloperidol or sulpiride *in vivo* reduces the threshold, indicating that the terminal autoreceptors are stimulated by endogenous dopamine under physiological conditions (Tepper et al., 1984). The results with D_2 and D_3 AONs exactly mimic the effects of administration of dopamine antagonists, which suggests that there exist both D_2 and D_3 autoreceptors on the axon terminals of nigrostriatal neurons.

Inferences about the Physiological Role of Somatodendritic Autoreceptors

It is interesting to note that although there were clear effects of both D_2 and D_3 AON treatment on the apomorphine dose response relation, on the terminal excitability, and on the proportion of antidromic responses consisting of the initial segment and somadendritic components, there was no effect of any AON treatment on the baseline spontaneous firing rate or pattern. Although it is possible that the lack of any detectable effect on spontaneous activity resulted from compensatory changes in the dopaminergic neurons or their afferents as a result of the loss of D_2 and/or D_3 receptors, this seems unlikely given the relatively short period of time of treatment (precisely the same effects were observed after only 3 days of treatment; Sun et al., 1995) and the fact that marked changes were observed in several other electrophysiological parameters.

The proportion of antidromic spikes consisting of the full spike is a measure of the level of excitability of the dendritic regions of the cell which is related to the local membrane potential (Matsuda and Jinnai, 1980). We have shown previously that this parameter can vary independently of the firing rate of the dopaminergic neuron (Trent and Tepper, 1991). The increased proportion of full spike antidromic responses coupled with the lack of change in the baseline firing rate after D_2 or D_3 AON treatment supports our previous suggestion that dopamine somatodendritic autoreceptors are effectively stimulated by endogenous dopamine under normal physiological conditions, but that the endogenous activation of these receptors does not normally inhibit the firing of these neurons as a whole, but rather modulates the excitability of certain restricted dendritic regions (Trent and Tepper, 1991).

CONCLUSIONS

Local administration of antisense oligodeoxynucleotides directed against the dopamine D_2 or D_3 receptor is a viable method for identifying the presence of and physiological consequences of these different receptor subtypes. The present results indicate that both D_2 and D_3 autoreceptors are present on nigrostriatal dopaminergic neurons and play important modulatory roles both at the somatodendritic and the axon terminal regions. There

was no indication of a differential distribution of the two subtypes to different parts of the neuron (i.e., nerve terminal vs. somatodendritic region). The fact that either AON alone was so effective at blocking or attenuating autoreceptor function at the cell body and axon terminal regions suggests the possibility that full expression of autoreceptor effects may require coactivation of both D_2 and D_3 receptor subtypes.

ACKNOWLEDGMENTS

This research was supported by MH-52383, MH-52450, a Johnson & Johnson Discovery Award and a Hoechst-Celanese Innovative Research Award.

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