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Dopamine receptors in the substantia nigra are involved in the regulation of muscle tone

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ABSTRACT **The aim of the present study was to localize the dopamine receptors involved in the regulation of muscle tone. A strategy was used whereby the effects on muscle tone of injecting the irreversible dopamine receptor antagonist *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) in discrete brain regions were assessed. Increases in muscle tone were measured as changes in electromyographic activity of the gastrocnemius and tibialis muscles of conscious, unrestrained rats. No increases in muscle tone were found after injections of EEDQ into the anterior and posterior striatum, which produced marked reductions in dopamine receptor concentration. The effects on muscle tone of injecting EEDQ into the substantia nigra pars reticulata were also assessed. Large increases in muscle tone were observed associated with inactivation of either D_1 or D_2 dopamine receptors in the substantia nigra. The increased muscle tone was not reduced by subcutaneous administration of apomorphine, despite the presence of a normal population of striatal dopamine receptors. These findings provide evidence that dopamine receptors in the substantia nigra play an important role in the regulation of muscle tone. Further, they challenge the hypothesis that the muscle rigidity of Parkinson disease results primarily from loss of striatal dopamine receptor stimulation.**

The association of the characteristic symptoms of Parkinson disease—bradykinesia, tremor, and rigidity—with degeneration of nigrostriatal neurons (1) has led to the hypothesis that the neurochemical mechanism underlying these symptoms is a loss of dopamine release in the striatum. The success of L-dihydroxyphenylalanine (L-dopa) in alleviating the motor symptoms of Parkinson disease is attributed to augmentation of dopamine stores and the maintenance of striatal dopamine receptor stimulation (2). Experimental findings have also shown increased muscle tone (muscle rigidity) associated with reductions in striatal dopamine after lesioning of nigrostriatal neurons by the neurotoxin 6-hydroxydopamine (3) or after treatment with reserpine (3, 4).

Although these findings have been interpreted as support for the striatal dopamine hypothesis, they have not taken into account that nigral dopaminergic neurons release dopamine from both their dendrites (5) and their terminals, so that dopamine concentration in the substantia nigra, as well as the striatum, will be reduced in Parkinson disease and after reserpine or 6-hydroxydopamine treatment. The aim of the present study was to investigate the role of striatal and nigral dopamine receptors in the regulation of muscle tone. The irreversible dopamine receptor antagonist *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was injected into these regions, and the effects on muscle tone, measured as changes in the electromyographic (EMG) activity of the antagonistic muscles of the hind limb, were recorded. The area and extent of the resulting dopamine receptor inactivation were assessed using quantitative autoradiography so that the area(s) associated with changes in muscle tone could be identified.

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METHODS

EMG Measurements. Male Sprague–Dawley rats were anesthetized (sodium pentobarbitone at 45 mg/kg and sodium methohexitone at 10 mg/kg, i.p.) and placed in a stereotaxic frame. A pair of stainless steel electrodes was implanted into the gastrocnemius and anterior tibialis muscles, and a fifth wire (earth) was laid on the surface of the tibialis muscle. The five wires were threaded under the skin and joined to a five-pin socket attached with dental cement to the surface of the skull. After recovery from surgery, the animal was connected via a headset containing an amplifier (6) to a Grass polygraph (model 7D). The EMG signal was amplified, filtered (10 Hz–10 kHz), rectified, and integrated over 10-sec periods; the resultant signal was recorded at 10 Hz for 20-min periods on a computerized recording system (CODAS; Dataq, Akron, OH). EMG is expressed as mean tonic EMG activity (mV/10 sec) (3). Phasic activity resulting from animal movement was excluded from analysis.

Intracerebral Injections of EEDQ. Bilateral intrastriatal injections of EEDQ (1 μmol in 1 μl; Sigma) dissolved in dimethyl sulfoxide (DMSO) were made in conscious animals via guide cannulae (7). Control rats were injected with 1 μl of DMSO. Bilateral intranigral injections of EEDQ were made into the substantia nigra pars reticulata (SNr) via 27-gauge stainless steel needles, at the same time the EMG electrodes were inserted. Rats were killed 10 or 24 hr after intracerebral injection, as described in the text. The coordinates of the three striatal injection sites were anterior sites: A, 1.0 mm; L, 2.5 mm; D, −5.0 and −7.4 mm; posterior site: P, −1.0 mm; L, 4.0 mm; V, −5.0 mm; and for the nigral site: P, −5.3 mm; L, 2.4 mm, V, −7.9 mm, according to the atlas of Paxinos and Watson (8). Selective protection of either D_2 or D_1 receptors was achieved by subcutaneous injections of raclopride (75 μmol/kg) or SCH 23390 (1.7 μmol/kg), respectively, given 1 hr before EEDQ, as described (9, 10).

Quantitative Autoradiography. After measurement of EMG activity, animals were killed by decapitation, the brains were removed, and sagittal sections (20 μm) were cut by a cryostat. Thaw-mounted sections were incubated with either the D_1 receptor ligand [^{3}H]SCH 23390 (3 nM) or the D_2 ligand [^{3}H]sulpiride (15 nM) for 1 hr at room temperature (7). Nonspecific binding was defined using 1 μM unlabeled SCH 23390 (D_1) and 1.7 μM sulpiride (D_2), respectively. In experiments involving intranigral EEDQ injections, sections were incubated with the D_2 ligand [^{125}I]iodosulpiride according to the method of Morelli *et al.* (11). Sections were exposed to tritium-sensitive film (Hyperfilm; Amersham) for 5 days

Abbreviations: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; EMG, electromyographic; DMSO, dimethyl sulfoxide; GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine.

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([^{125}I]iodosulpiride), 3 weeks ([^{3}H]sulpiride), or 8 weeks ([^{3}H]SCH 23390). Autoradiographs were analyzed using a computerized densitometry system (MD20; Flinders Imaging, Adelaide, South Australia), and optical density values of selected brain regions were converted to fmol/mg of tissue by reference to tritium and ^{125}I standards (Amersham). Receptor concentration was calculated from the average optical density of two or three sections from each rat.

RESULTS

Inactivation of Central Dopamine Receptors. Initial experiments demonstrated that an intraperitoneal injection of EEDQ (60 μmol/kg), a dose previously shown to produce marked reduction in dopamine receptor concentration throughout the brain (9, 10), was associated with significant increases in EMG activity of both gastrocnemius and tibialis muscles. These increases occurred 2 hr after injection, reached a maximum of 287% in gastrocnemius and 284% in tibialis of vehicle-injected control values, and persisted for 48 hr. After EEDQ injection, striatal D_1 and D_2 dopamine receptor concentrations were reduced to 12.1% ± 3.5% and 11.8% ± 1.9% of control values, respectively. The findings established that inactivation of central dopamine receptors was associated with increased muscle rigidity, and experiments were carried out next to localize the site of the dopamine receptors involved.

Inactivation of Striatal Dopamine Receptors. EEDQ or DMSO was injected bilaterally into one of the three striatal sites described in *Methods*, and EMG activity was recorded for 8–24 hr postinjection. No increases in EMG activity were observed, despite quantitative autoradiographic confirmation of significant dopamine receptor loss at all injection sites (anterior sites: D_1, 19.2% ± 2.4%; D_2, 23.4% ± 3.6%; posterior site: D_1, 21.8% ± 1.4%; D_2, 29.7% ± 5.2% compared with vehicle-injected controls). Fig. 1 shows the extent of D_1 receptor loss 10 hr after EEDQ injection into each of the three sites, compared with a DMSO-injected control.

Inactivation of Nigral Dopamine Receptors. To investigate the hypothesis that nigral dopamine receptors were involved in the regulation of muscle tone, bilateral injections of EEDQ were made into the substantia pars reticulata. Ten hours after injection there was a significant increase in EMG activity in the gastrocnemius (218%) and anterior tibialis (138%) muscles compared with vehicle-treated controls (Table 1). This effect persisted for at least 24 hr. Behaviorally, the animals were akinetic; they exhibited a hunched posture and increases in muscle tone (rigidity) determined by limb palpation. These changes were associated with reductions in D_1 and D_2 receptor concentrations, averaged for the whole of the substantia nigra, to 14.6% ± 2.3% and 24.3% ± 6.4%, respectively, of vehicle-injected controls, assessed 10 hr after injection of EEDQ.

To determine if receptor inactivation along the needle track contributed to increases in EMG activity, experiments were conducted in which EEDQ was injected at sites dorsal to the nigra (coordinates: P, −5.3 mm; L, 2.4 mm; V, −6.2 mm). These injections had no effect on EMG activity, which remained at control levels.

EEDQ inactivates both D_1 and D_2 receptors (12, 13), so it is not possible to conclude if one or both receptor subtypes are

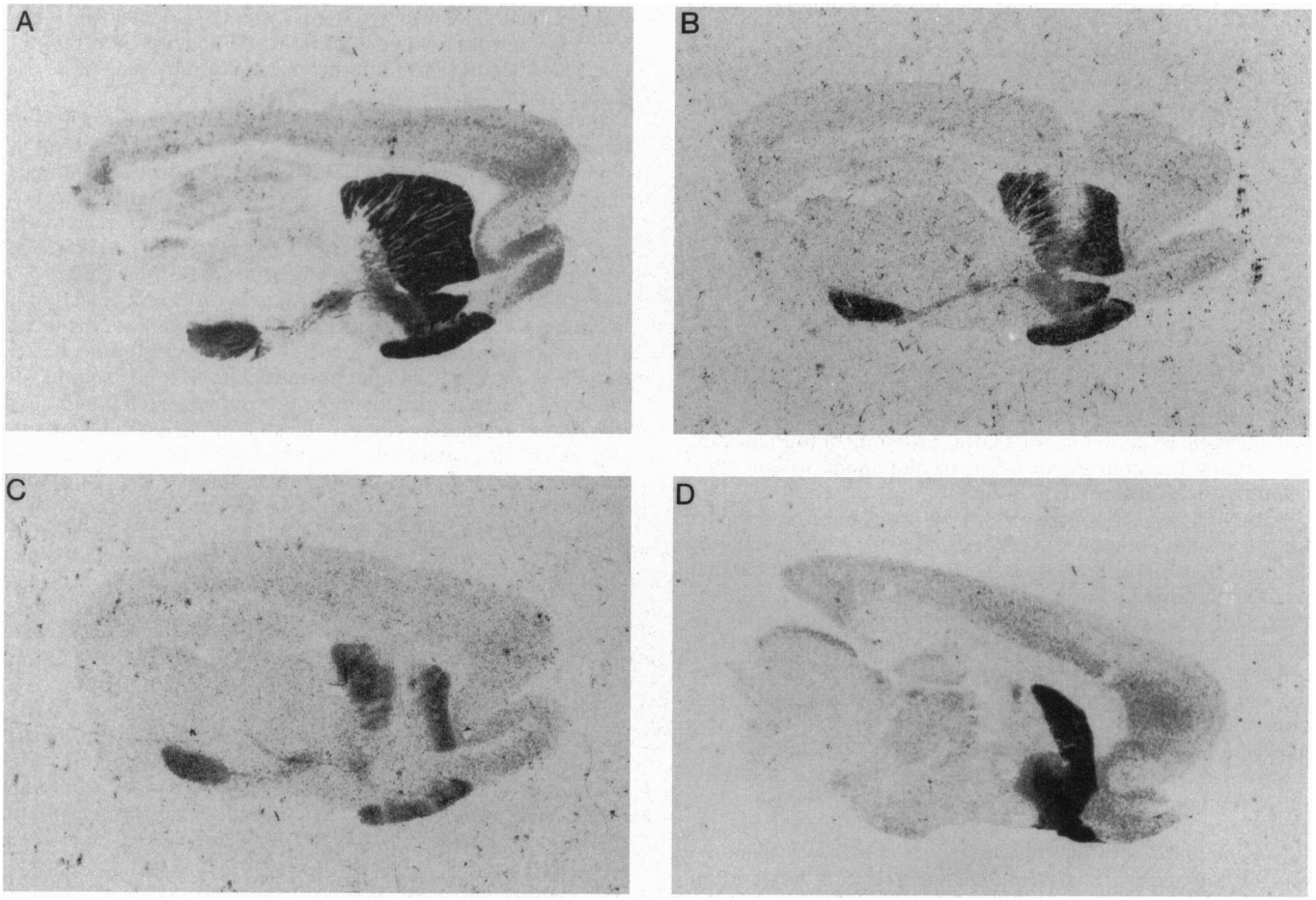

FIG. 1. Representative autoradiographs of D_1 receptor binding in sagittal brain sections after intrastriatal injections of EEDQ or DMSO. D_1 receptor distribution in striatum and substantia nigra of a DMSO-injected animal (*A*) and after intrastriatal injection of EEDQ into the dorsoanterior (*B*), ventroanterior (*C*), or lateroposterior (*D*) striatum is shown. Injection coordinates of the three sites were anterior sites: A, 1.0 mm; L, 2.5 mm; D, −5.0 or −7.4 mm; posterior site: P, −1.0 mm; L, 4.0 mm; V, −5.0 mm.

Table 1. EMG activity in anterior tibialis and gastrocnemius muscles

Group*	Injection	n	EMG activity, mV/10 sec	
			Tibialis	Gastrocnemius
A	Vehicle	8	0.282 ± 0.032	0.207 ± 0.027
	EEDQ	8	0.388 ± 0.047†	0.452 ± 0.027†
	EEDQ + 1 μmol Apo	6	0.275 ± 0.059‡	0.319 ± 0.059
	EEDQ + 10 μmol Apo	6	0.252 ± 0.072	0.313 ± 0.115
B	Vehicle	7	0.277 ± 0.037	0.200 ± 0.045
	EEDQ + SCH	7	0.456 ± 0.041†	0.388 ± 0.054†
	EEDQ + SCH + 1 μmol Apo	7	0.400 ± 0.051†	0.374 ± 0.057†
	EEDQ + SCH + 10 μmol Apo	7	0.498 ± 0.039†	0.490 ± 0.043†
C	Vehicle	5	0.225 ± 0.063	0.261 ± 0.053
	EEDQ + RAC	5	0.467 ± 0.039†	0.517 ± 0.034†
	EEDQ + RAC + 1 μmol Apo	5	0.471 ± 0.045†	0.423 ± 0.080
	EEDQ + RAC + 10 μmol Apo	5	0.470 ± 0.042†	0.453 ± 0.049†

Apo, apomorphine; SCH, SCH 23390; RAC, raclopride.
*Group A, EMG activity 10 hr after intranigral injection of EEDQ; group B, EMG activity 24 hr after intranigral injection of EEDQ and pretreatment with SCH 23390; group C, EMG activity 24 hr after intranigral injection of EEDQ and pretreatment with raclopride.
†$P < 0.01$ compared with vehicle-injected control.
‡$P < 0.05$ compared with EMG activity prior to apomorphine injection, by Bonferroni's t statistic.

involved in the regulation of muscle tone. To identify the subtype, rats were injected with raclopride or SCH 23390 1 hr prior to intranigral injection of EEDQ to prevent inactivation of D_2 or D_1 receptors, respectively (9, 10, 12). After pretreatment with SCH 23390 and 24 hr after intranigral injection of EEDQ, there were significant increases in EMG activity (Fig. 2) associated with a selective reduction in D_2 receptor binding of 43% compared with controls. D_1 receptors were maintained at control levels by SCH 23390 pretreatment. Significant increases in EMG activity were also observed 24 hr after EEDQ injection, following raclopride pretreatment. These increases were associated with a selective 40% loss of D_1 receptors in the absence of changes in D_2 receptor concentration (Fig. 2). In both these experiments, quantitative autoradiography of nigral D_1 and D_2 dopamine receptors confirmed that increased EMG activity was associated with a large area of receptor loss (Fig. 3).

To investigate if EMG increases were due to nonspecific effects of EEDQ or effects at other receptors, rats were injected with both raclopride and SCH 23390 prior to nigral EEDQ injection to protect both dopamine receptor subtypes (Fig. 2). Twenty-four hours after this treatment, no changes in EMG activity were observed and the animals were behaviorally indistinguishable from vehicle-injected controls. The effectiveness of the antagonist pretreatments was confirmed by quantitative autoradiographic analysis, which showed that nigral D_1 and D_2 receptor concentrations were not changed from control levels (D_1, 92.7% ± 6.8%; D2, 93.9% ± 17.3% of vehicle-injected control values). These results clearly implicate inactivation of nigral dopamine receptors in the regulation of EMG activity.

Effects of Apomorphine on Increased EMG Activity. In most experiments, administration of the mixed D_1/D_2 dopamine agonist apomorphine (1 and 10 μmol/kg, s.c.) did not reduce the increased EMG activity associated with reductions in nigral dopamine receptor concentration (Table 1). For example, after selective inactivation of nigral D_2 (Table 1, group B) or D_1 (Table 1, group C) receptors, the significantly increased EMG activity was not reduced by either dose of apomorphine. Quantitative autoradiographic analysis confirmed that striatal dopamine receptors were unaffected by the experimental procedures (see Fig. 3). However, in the group receiving intranigral injection of EEDQ alone (Table 1, group A), increased EMG activity was reduced by apomorphine exclusively in the tibialis. Inspection of the individual data for the EEDQ group showed that apomorphine reduced EMG activity in three of six rats and that they had less loss of nigral dopamine receptors than the three nonresponding rats.

DISCUSSION

The results of the present study are consistent with the hypothesis that D_1 and D_2 dopamine receptors in the substantia nigra play an important role in the regulation of muscle tone. Inactivation of these receptors by EEDQ consistently resulted in an akinetic state and a large increase in EMG activity in both gastrocnemius and anterior tibialis muscles. No increases in

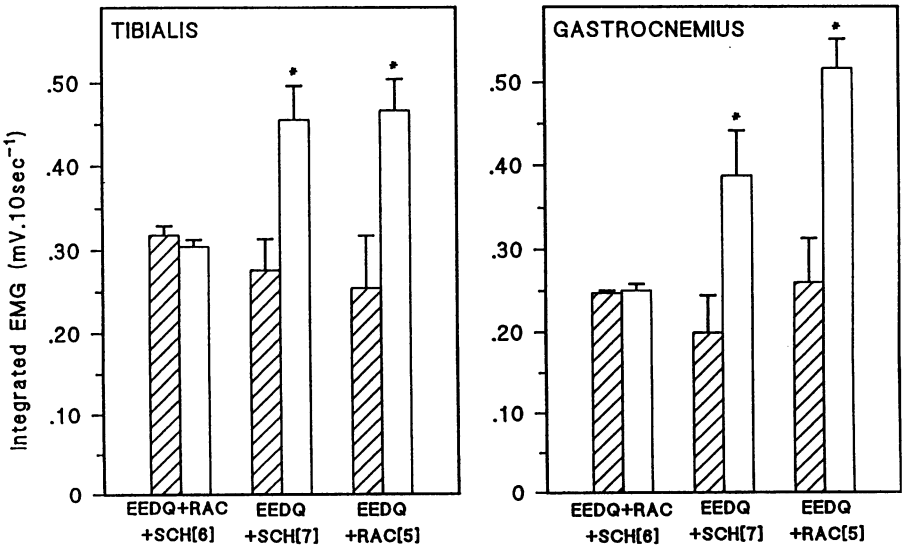

FIG. 2. The effects of intranigral injections of EEDQ or DMSO after pretreatment with raclopride and SCH 23390 (EEDQ + RAC + SCH), SCH 23390 alone (EEDQ + SCH), and raclopride alone (EEDQ + RAC) on EMG activity. EMG activity was measured 24 hr after injection of EEDQ or DMSO. Hatched bars represent DMSO-injected animals, and open bars represent EEDQ-injected animals; the number of animals in each group is given in brackets. Each bar represents the mean ± SEM. *, $P < 0.001$ Bonferroni's t statistic.

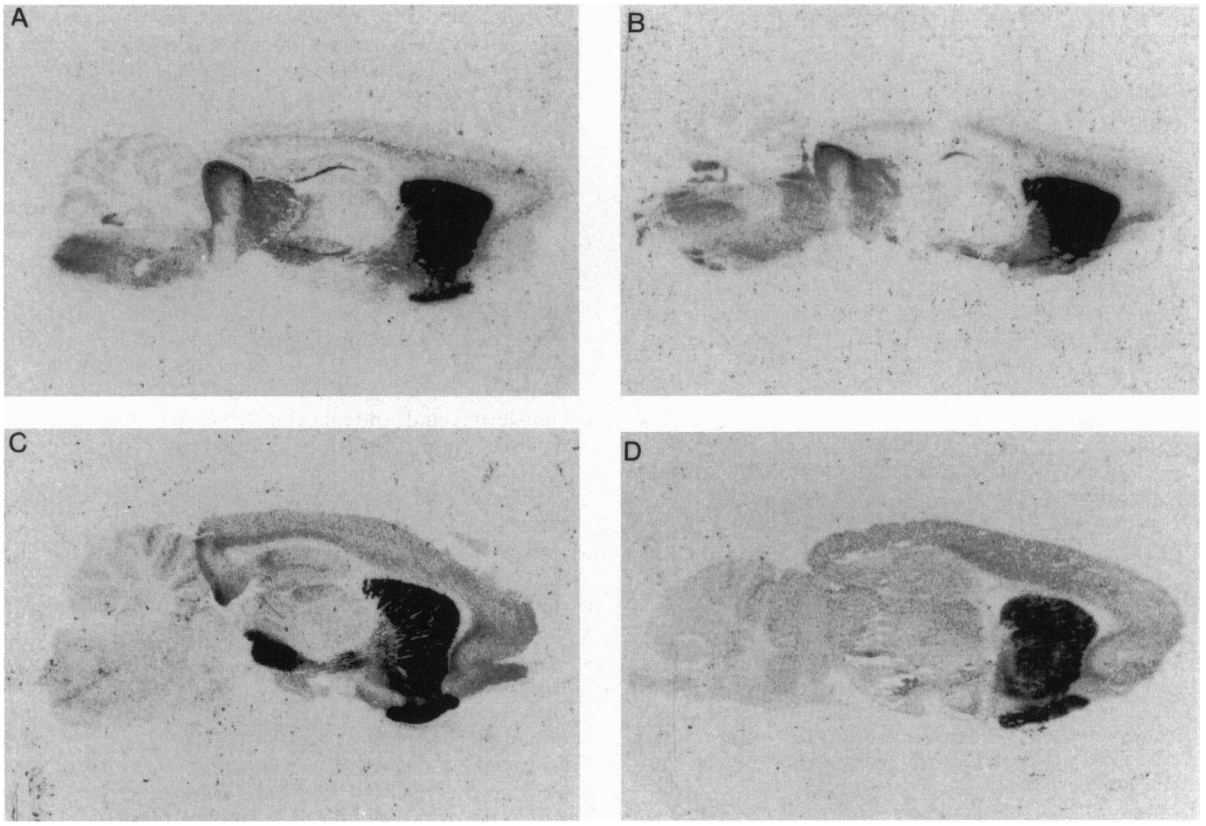

FIG. 3. Representative autoradiographs showing dopamine receptor binding in sagittal sections of rat brain after nigral injections of EEDQ or DMSO (injection coordinates: P, −5.3 mm; L, 2.4 mm; V, −7.9 mm). D_2 receptor binding in DMSO-injected controls (*A*) and after the selective inactivation of nigral D_2 receptors (EEDQ + SCH 23390 pretreatment) (*B*) is shown. D_1 receptor binding in DMSO-injected controls (*C*) and after selective inactivation of nigral D_1 receptors (EEDQ + raclopride pretreatment) (*D*) is shown.

EMG activity were observed after dopamine receptor loss in the striatum. Doses of apomorphine of up to 10 μmol/kg failed to reduce the EMG increases associated with large losses of nigral dopamine receptors, despite the demonstration that striatal dopamine receptors were not reduced. The failure of apomorphine to reduce EMG activity is interesting and may result from its interaction with the small remaining population of nigral dopamine receptors being insufficient to reverse the functional effects after EEDQ injection. This hypothesis is supported by the finding that in one experiment (Table 1, group A) apomorphine did reduce EMG activity in three of six rats after intranigral injection of EEDQ. The magnitude of their response to apomorphine appeared to be related to the size of the population of dopamine receptors remaining in the substantia nigra. We have previously shown apomorphine to be effective in reducing increased EMG activity associated with dopamine loss after 6-hydroxydopamine lesions, when the population of dopamine receptors was intact (3). Overall, the findings support an important role for nigral dopamine receptors in muscle tone regulation and suggest that the magnitude of nigral dopamine receptor stimulation is an important determinant of EMG activity.

Besides dopamine receptors, EEDQ also inactivates α-adrenoceptors (14), 5-hydroxytryptamine (5-HT) receptors (12), and muscarinic receptors (15), so it was possible that the increases in EMG activity were attributable to inactivation of these nondopamine receptors or to nonspecific effects. This possibility was investigated by pretreating rats with both raclopride and SCH 23390 prior to intranigral injections of EEDQ to achieve selective protection of D_2 and D_1 receptors. No increase in EMG activity was seen in the pretreated rats at times when increased EMG activity was consistently observed in nonpretreated rats (Fig. 2). This finding clearly implicates dopamine receptor inactivation in EMG increases and confirms that inactivation of nondopamine receptors in the substantia nigra has no effect on EMG activity. Although high doses of SCH 23390 have been reported to protect 5-HT_{2C} receptors partially from inactivation by EEDQ (12), the low dose used in the present study (1.7 μmol/kg) selectively protected D_1 receptors because of the 10-fold greater affinity of SCH 23390 *in vivo* for D_1 compared with 5-HT_{2C} receptors (16). Further, subsequent studies showed that protection of 5-HT_{2c} receptors by pretreatment with mesulergine did not prevent increases in EMG activity after intranigral injection of EEDQ (A.D.C., unpublished observations). Overall, these findings exclude a role for 5-HT_{2c} receptors and support the conclusion that nigral dopamine receptors are involved in the regulation of muscle tone.

The injection volume used in our study was large (1 μl), and leakage from the injection site could have resulted in dopamine receptor inactivation in dorsal areas surrounding the injection track, including the superior colliculus, a major projection area of the nigra involved in motor control. This possibility was ruled out by observations that EEDQ injections dorsal to the nigra did not affect EMG activity.

The substantia nigra provides both inputs to the striatum, via dopaminergic neurons originating in the pars compacta, and receives outputs from the striatum to the pars reticulata. These include a direct inhibitory pathway, mediated by D_1 receptors, and an indirect pathway, mediated by D_2 receptors, via the subthalamic nucleus (reviewed in ref. 17). It has been shown in monkeys rendered parkinsonian by treatment with the dopamine neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine that imbalance of these nigral inputs results in akinesia and increased muscle tone (18). These effects were associated with increases in the activity of nigrothalamic ef-

ferents and increased inhibition of the thalamus and thalamocortical neurons.

Our results suggest that dopamine receptor stimulation in the nigra plays an important role in the regulation of motor control. Dopamine is released from the dendrites of dopaminergic neurons (5) and therefore can regulate the activity of dopaminoceptive neurons in the substantia nigra. Autoradiographic and immunohistochemical studies have shown substantial concentrations of D_1 and D_2 receptors in the nigra (19, 20). D_2 receptors are found in the pars reticulata and the pars compacta of the substantia nigra, where they are located on the cell bodies (19) and the dendrites (20) of dopaminergic neurons and nondopaminergic neurons (20). Their role as autoreceptors is well documented (21), but they also have effects on nondopaminergic neurons, inhibiting γ-aminobutyric acid (GABA) release (22) and regulating the rate of firing of efferent GABAergic neurons (23). D_1 receptors are located on the terminals of striatonigral GABAergic neurons, and their activation increases GABA release from these neurons (24). Increases in EMG activity were observed after selective inactivation of either dopamine receptor subtype alone, indicating that both receptor subtypes are involved in the regulation of muscle tone, as shown for many other behavioral and electrophysiological effects mediated by dopamine receptors (reviewed in ref. 25). We hypothesize that a decrease in either nigral receptor subtype leads to increased firing of GABAergic nigrothalamic neurons, which has been associated with increases in muscle tone (18). The hypothesis that both D_1 and D_2 receptors are involved in muscle tone regulation is consistent with clinical reports that the D_2 agonist bromocriptine is more effective in controlling the symptoms of Parkinson disease, if coadministered with L-dopa (26).

The findings of the current study challenge the view that dopamine agonists reduce increased muscle tone by interacting only with striatal dopamine receptors, which is widely held to be the basis of L-dopa's therapeutic effects in the treatment of Parkinson disease. The hypothesis that nigral dopamine receptors play an important role in the regulation of muscle tone is compatible with existing knowledge concerning the neurochemistry and treatment of Parkinson disease. For example, it is well known that there is a marked decrease in nigral, as well as striatal, dopamine concentration in Parkinson disease (27).

The success of L-dopa therapy, therefore, may be primarily dependent on replacement of dopamine at nigral sites. This view is consistent with the findings of experimental studies showing that the behavioral effects of L-dopa correlated better with nigral rather than striatal concentrations of dopamine and were blocked by intranigral administration of SCH 23390 (28, 29).

The failure of dopamine receptor inactivation in large areas of the striatum to result in increased muscle tone raises important questions about the relative roles of nigral and striatal dopamine receptors in motor control. However, our findings do not rule out a role for striatal dopamine receptors in the regulation of muscle tone because the receptor reserve in the striatum may be larger than the reductions in D_1 and D_2 receptor concentration of 75–80% achieved in the present study.

In conclusion, the hypothesis that nigral dopamine receptors play a key role in the regulation of muscle tone has important implications for understanding the neurochemical changes underlying the symptoms of Parkinson disease and the rational development of new drugs to treat it. Significantly, the nigral hypothesis may explain the limited success of transplantation into the striatum for the treatment of this disabling condition (30).

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