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Dopamine Agonist-Induced Elevation of Striatal Acetylcholine: Relationship between Receptor Occupancy and Response in Normal and Denervated Rat Striatum

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SUMMARY

Unilateral denervation of the nigrostriatal dopamine (DA) pathway with 6-hydroxydopamine resulted in a supersensitive response for elevation of striatal acetylcholine concentrations by the full DA agonist (*R*)-(−)-*N*-*n*-propylnorapomorphine (NPA), reflected in a parallel 4-fold leftward shift in the dose-response curve (ED_{50}, intact, 8.8 μg/kg; denervated, 2.2 μg/kg). The maximal response, however, was not changed. In the intact striatum, irreversible DA receptor inactivation with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (6 mg/kg) produced a depression in the maximal acetylcholine increase elicited by NPA (control, 52.4%; EEDQ, 25.0%), without altering the ED_{50} for the agonist. In contrast, in the denervated striatum, EEDQ treatment produced a much smaller reduction in the maximal response (to 39.6%), as well as a small rightward shift in the ED_{50} (from 2.2 to 3.5 μg/kg). Double-reciprocal analysis of equieffective doses of NPA necessary to elicit response yielded similar values for the pseudo-dissociation constant (pseudo-K_A, in units of dose) in intact and denervated striatum (8.3 and 7.0 μg/kg, respectively). A plot of receptor occupancy versus response was linear for the intact striatum, indicating the absence of a receptor reserve. In contrast, a nonlinear relationship was obtained for the denervated side, and a small apparent receptor reserve for NPA of 25–30% was estimated to be present. The results suggest that 6-hydroxydopamine-induced supersensitivity reflects the generation of a postsynaptic D2 DA receptor reserve, which may account for the observation that weak partial agonists elicit measurable response in supersensitive animals (and at presynaptic DA receptors, which normally exhibit a receptor reserve for agonists) but not at normosensitive receptors devoid of spare receptors.

Cholinergic neurotransmission in the rat striatum is regulated by postsynaptic DA receptors (1–3) of the D2 type (4, 5). DA agonists inhibit the release (3, 6) and turnover (1) of ACh, resulting in an increase in ACh levels, which provides a reliable index of cholinergic tone (2). Other D2 receptors in rat striatum (autoreceptors) are found on terminals of nigrostriatal DA neurons and regulate the release (7) and synthesis (8, 9) of DA. An extensive and diverse body of evidence indicates that DA agonists are pharmacologically more potent in regulating autoreceptor- than postsynaptic-mediated functional effects (5, 10, 11).

We have recently provided evidence that this autoreceptor selectivity is due to a difference in the efficiency of receptor/effector coupling at these sites, manifested by an apparent receptor reserve for DA agonists at presynaptic but not postsynaptic D2 receptors. Thus, striatal DA autoreceptor-mediated inhibition of synthesis (12, 13) and release (14) of DA displayed a large receptor reserve for full agonists such as apomorphine and NPA, whereas a clear absence of a receptor reserve was found for a postsynaptic response (inhibition of cholinergic transmission) (15).

Unilateral 6-OHDA-induced degeneration of nigrostriatal DA neurons, DA depletion, or chronic receptor blockade elicit behavioral, biochemical, and radioligand binding manifestations of postsynaptic DA receptor supersensitivity (see Ref. 16 for review). Under such conditions, the potency of DA agonists for effecting behavioral (17) and biochemical (18) responses is increased, reflected in a leftward shift in the dose-response curve. Weak partial DA agonists such as the enantiomers of 3-PPP (19) and EMD 23,448 (20), which display full or nearly full intrinsic activity at DA autoreceptors (13, 21) because of efficient receptor/effector coupling at this site (12, 13), elicit very weak or no responses at normosensitive postsynaptic D2

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ABBREVIATIONS: DA, dopamine; ACh, acetylcholine; 6-OHDA, 6-hydroxydopamine; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; NPA, (*R*)-(−)-*N*-*n*-propylnorapomorphine; DOPAC, 3,4-dihydroxyphenylacetic acid; EMD 23,448, 3-[4-(4-phenyl-1,2,3,6-tetrahydropyridyl-1)-butyl]indole; HVA, homovanillic acid; 3-PPP, 3-(3-hydroxyphenyl)-*N*-*n*-propylpiperidine; SCH 23390, 8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzapin-7-ol; NEM, *N*-ethylmaleimide; G protein, guanine nucleotide regulatory protein.

receptors (see Ref. 21 for review). However, in animals with supersensitive DA receptors, weak partial agonists elicit measurable response (21, 22), although the affinities of both full and partial agonists for D2 binding sites are unchanged (23, 24). These results suggested to us (13) that the increased density of D2 receptor binding sites produced by chronic receptor blockade, DA depletion, or denervation may, in effect, increase receptor/effector coupling; such an effect should be demonstrable as the generation of a receptor reserve for full agonists at postsynaptic D2 receptors ordinarily devoid of spare receptors. The present study was undertaken to examine this issue by determining the relationship between receptor occupancy and response for the full DA agonist NPA in normal and denervated rat striatum. In these studies, DA receptors were partially and irreversibly inactivated with EEDQ (25, 26).

Experimental Procedures

Animals and surgery. Groups of male OFA rats (200–250 g; KFM, Fuellinsdorf, Switzerland) were unilaterally lesioned with 6-OHDA, as described by Hefti *et al.* (27). The animals were anesthetized with sodium hexobarbital (50 mg/kg, intraperitoneally) and placed in a Kopf stereotaxic frame. In order to obtain an almost total nigrostriatal lesion, 12 μg of 6-OHDA (in 4 μl of saline containing 0.2 mg/ml ascorbic acid) were injected into the right anteromedial substantia nigra [A 2400, V 2.6, L 1.6, according to the atlas of Koenig and Klippel (28)].

Drug treatments. Twenty-one days after the lesion, groups of animals were treated with vehicle or EEDQ (6 mg/kg, subcutaneously; Fluka, Buchs, Switzerland). On day 22, animals were further subdivided into groups that received either vehicle or various doses of (*R*)-(−)-NPA (1–100 μg/kg, subcutaneously; synthesized at Sandoz, Ltd.) 40 min before sacrifice by microwave irradiation focused on the head (6 kW, 2.45 GHz, 1.7 sec; Pueschner Mikrowellen-Energietechnik, Bremen, FRG). The brains were immediately removed and dissected on ice and tissues were frozen and stored at −70° until analyzed.

In another experiment, groups of unilaterally lesioned rats were treated with the D1 antagonist SCH 23390 (1 mg/kg, subcutaneously; Schering-Plough Corp., Bloomfield, NJ) 30 min before EEDQ, the D2 antagonist sulpiride (60 mg/kg, intraperitoneally; Ravizza, Milan, Italy) 3 hr before EEDQ, or vehicle only. The next day, animals in each pretreatment group received NPA (0.1 mg/kg, subcutaneously) or vehicle, followed by sacrifice and analysis as described above.

Biochemical analyses. ACh and choline were determined by the gas chromatography-mass fragmentography method of Jenden *et al.* (29), as described previously (15). In brief, striatal tissues were homogenized in 0.1 M perchloric acid, containing deuterated internal standards of ACh-d_9 and choline-d_9, and centrifuged. Endogenous ACh and choline and their deuterated analogs were extracted from the supernatant with dipicrylamine in dichloromethane, as ion pairs. Choline and its deuterated analog were derivatized with propionyl chloride and the resulting mixture was demethylated with sodium benzenethiolate and subjected to gas chromatography-mass fragmentography.

Dopamine and its acidic metabolites DOPAC and HVA were determined in supernatant aliquots of striatal homogenates (0.1 N HCl containing 0.05 mM ascorbic acid and deuterated internal standards) after centrifugation, using a gas chromatography-mass fragmentography technique as described by Karoum *et al.* (30).

Data analysis. Dose-response curves for NPA-induced elevation of ACh levels in the intact and lesioned striatum after vehicle or EEDQ pretreatment were simultaneously analyzed for best fit using the ALLFIT computer program of De Lean *et al.* (31), as described previously in detail (12–15). In brief, the program provided statistical tests of the goodness of fit after the curves were constrained to share one or more parameters (response at zero dose, slope factor, 50% of maximally effective dose or ED_{50}, and response at "infinite" dose). In practice, the response at zero dose for all curves was set to zero. Curves were analyzed first without constraints and then by successively constraining them to share a common slope factor, ED_{50}, or maximal response. The best fit was that analysis which permitted one or more parameters to be shared without a significant increase in the residual variance (13, 31).

Pseudo-dissociation constants (K_A values, in units of dose; see Ref. 13) for NPA-induced elevation of ACh were obtained by the method of Furchgott and Bursztyn (32), using the equation

$$\frac{1}{[A]} = \frac{1}{q[A']} + \frac{1-q}{qK_A}$$

where $[A]$ is the concentration of agonist necessary to produce a specific level of response before inactivation, $[A']$ is the concentration needed to produce the same response after inactivation, and q is the fraction of receptors left intact (i.e., not inactivated). The pseudo-K_A values were obtained by plotting the reciprocals of the equieffective NPA doses after inactivation, $1/[A']$, against the reciprocals of the doses before inactivation, $1/[A]$, for each pair of dose-response curves in the intact and denervated striatum. The equieffective doses were determined at five levels of response (corresponding to 30, 40, 50, 60, and 70% of the maximum effect after EEDQ treatment) (13, 32) from the ALLFIT-derived best fit dose-response curves. Each resulting straight line had a slope of $1/q$ and pseudo-K_A equal to (slope − 1)/y − intercept.

The pseudo-K_A values, in units of dose, were used to calculate fractional receptor occupancy (f) at a particular dose $[A]$, from the law of mass action:

$$f = [RA]/[R_T] = [A]/K_A + [A]$$

where $[RA]$ is the concentration of receptor-agonist complex and $[R_T]$ is the initial or total concentration of active receptors. Fractional receptor occupancy at a particular dose was then plotted against fractional response at that dose (obtained from the control best fit dose-response curve).

Results

Effects of 6-OHDA lesion and EEDQ treatment on striatal ACh, choline, DA, and DA metabolite levels. The effects of 6-OHDA and EEDQ treatment, singly or in combination, on striatal levels of ACh, choline, DA, and the DA metabolites DOPAC and HVA are shown in Table 1. 6-OHDA lesion did not significantly alter basal ACh levels in either vehicle- or EEDQ-treated rats, whereas DA levels were drastically reduced (>98% mean reduction); DOPAC and HVA concentrations were also very low. Individual animals that did not show at least 95% loss of striatal DA were discarded from analysis. EEDQ treatment significantly reduced basal ACh levels in both intact and lesioned striatum, an effect observed with neuroleptics (2, 4) and, therefore, consistent with the DA receptor-inactivating (or irreversible blocking) action of EEDQ (25, 26). NPA dose-dependently increased ACh levels in all pretreatment groups (see Fig. 1). NPA also produced a small dose-dependent increase in DA levels (maximal increase, ~20%) and dose-dependent reductions in DOPAC and HVA concentrations (data not shown). These effects were apparent only in the intact striatum; the basal levels of DA and its metabolites were so low in the lesioned striatum that clear effects of NPA could not be discerned (data not shown).

NPA-induced elevation of striatal ACh after 6-OHDA and EEDQ treatment. Dose-response curves for NPA-induced elevation of striatal ACh levels after all four pretreatments are shown in Fig. 1. In 6-OHDA-lesioned striatum, the dose-response curve for NPA was shifted 4-fold to the left [ED_{50} (μg/kg): vehicle/vehicle, 8.8; lesioned/vehicle, 2.2], without a change in the maximal response (shared maximum =

TABLE 1

ACh, choline, DA, and DA metabolite concentrations in striata of 6-OHDA- and EEDQ-treated rats

Data shown (in pmol/mg of tissue ± standard error) are for the four non-drug-treated control groups (i.e., not treated with NPA). NPA dose-dependently increased ACh levels in all four groups (see Fig. 1). See text for discussion of the effects of NPA on the other neurochemical parameters.

Pretreatment	n	ACh	Choline	DA	DOPAC	HVA
				pmol/mg of tissue		
Vehicle/Vehicle	9	73.5 ± 1.5	35.3 ± 1.8	54.9 ± 1.6	4.14 ± 0.16	2.88 ± 0.12
Lesion/Vehicle	9	76.5 ± 2.0	37.9 ± 2.4	1.03 ± 0.26[a]	0.12 ± 0.02[a]	0.26 ± 0.06[a]
Vehicle/EEDQ	8	44.7 ± 1.5[b]	33.8 ± 1.9	43.7 ± 1.6[b]	12.32 ± 1.06[b]	8.23 ± 0.30[b]
Lesion/EEDQ	8	48.6 ± 2.0[b]	36.3 ± 2.3	0.62 ± 0.10[a]	0.09 ± 0.01[a]	0.18 ± 0.03[a]

[a] Significantly different from corresponding nonlesioned group, $p < 0.001$.
[b] Significantly different from corresponding non-EEDQ-treated group, $p < 0.001$.

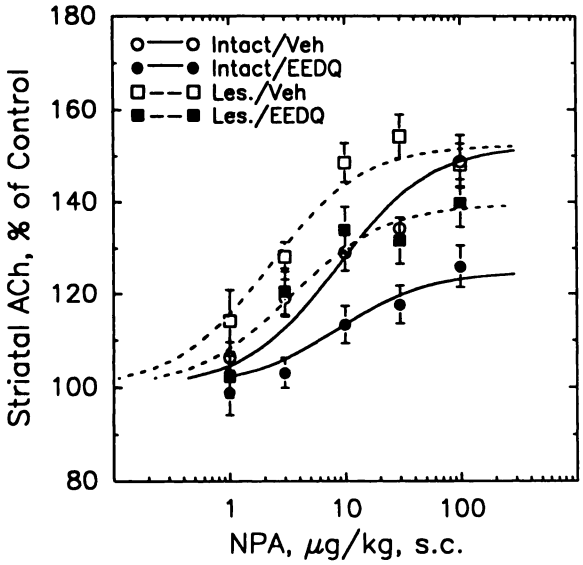

Fig. 1. Dose-response curves for NPA elevation of striatal ACh in intact and 6-OHDA-lesioned (*Les.*) striatum 24 hr after vehicle or EEDQ (6 mg/kg) treatment. ALLFIT analysis indicated that all four curves could be constrained to share a common slope factor (1.07) without a significant increase in the residual variance ($F(3,11) = 1.79$, $p > 0.05$). Inspection of the curves suggested that the maximal response did not differ for the intact/vehicle and lesioned/vehicle groups. Furthermore, we previously demonstrated that the ED_{50} values in intact vehicle- and EEDQ-treated rats were not different. Simultaneous ALLFIT analysis with these additional constraints did not significantly increase the residual variance ($F(3,13) = 1.92$, $p > 0.05$); this analysis was, therefore, considered to be the best fit for the data. Constraining the maximal response for either EEDQ curve to be shared with the maximum for the vehicle curves, or with each other, resulted in significantly poorer fits ($p < 0.025$ or better), indicating that each EEDQ maximum is different from the shared vehicle maximum and from each other. Each point is the mean ± standard error of six to nine rats.

52.4%). A similar shift (3-fold) was reported by Paturle *et al.* (18) for the DA agonist pergolide, also without a significant change in the maximal response. In the intact striatum, EEDQ treatment reduced the maximal response (vehicle/vehicle, 52.4%; vehicle/EEDQ, 25.0%) but did not alter the ED_{50} (8.8 μg/kg), as we reported previously (15). In the lesioned striatum, EEDQ treatment reduced the maximal response to a considerably smaller degree (lesioned/vehicle, 52.4%; lesioned/EEDQ, 39.6%) and also elicited a small rightward shift in the dose-response curve (ED_{50}, 2.2 and 3.5 μg/kg, respectively).

Double-reciprocal Furchgott analysis (13, 32) of equieffective doses of NPA required to elicit equivalent levels of response in vehicle and EEDQ-treated rats, for both intact and denervated striatum, is shown in Fig. 2. As predicted by receptor theory, the pseudo-K_A values for NPA in both intact and denervated striatum were very similar (8.3 and 7.0 μg/kg, respectively), whereas the ED_{50} values were not. The K_A/ED_{50} ratio (8.3/8.8 = 0.94) was near unity in the intact striatum, indicating the absence of a receptor reserve for NPA; however, this ratio was substantially different from unity (7.0/2.2 = 3.2) in 6-OHDA-lesioned striatum, suggesting the presence of a small receptor reserve for the agonist.

Relationship between receptor occupancy and response for NPA in intact and denervated striatum. Using the pseudo-K_A values obtained as described above, fractional receptor occupancy was calculated for each control (i.e., non-EEDQ) best fit dose-response curve and plotted against the corresponding increase in ACh levels, as shown in Fig. 3; experimental points are also shown. In intact striatum, there was a strictly linear relationship between receptor occupancy and response, i.e., there is no apparent receptor reserve for NPA-induced elevation of striatal ACh levels; the same result was obtained previously (15). In contrast, this relationship was clearly nonlinear in denervated striatum; a small receptor reserve for NPA of about 25–30% can be estimated from Fig. 3, because 95% of the maximal response (experimentally indistinguishable from 100%) was obtained at 70–75% receptor occupancy.

Effects of protecting D1 or D2 DA receptors from inactivation by EEDQ. EEDQ irreversibly inactivates D1 DA receptors, as well as subtypes of serotonin and α-adrenergic receptors (25, 26). Moreover, NPA is a mixed D1/D2 agonist (33). Although striatal cholinergic neurotransmission appears to be regulated by D2 but not D1 receptors (4, 5), it is, nevertheless, possible that the effects of NPA in EEDQ-treated animals could be modulated by concomitant loss of D1 and other receptors. The effects of NPA were, therefore, compared in rats treated with either SCH 23390 (1 mg/kg) or sulpiride (60 mg/kg) before EEDQ to protect D1 or D2 receptors, respectively, from inactivation. Fig. 4 shows that sulpiride pretreatment completely prevented the EEDQ-induced reduction in response to NPA (0.1 mg/kg) in the intact striatum. Although the protective effect of sulpiride was less pronounced in the lesioned striatum, the mean NPA-induced increase in ACh was not significantly different from that in the vehicle-treated group. Thus, the reduction in response after EEDQ is due to inactivation of D2 receptors and not to nonspecific effects. In contrast, selective protection of D1 receptors by pretreatment with SCH 23390 did not prevent the EEDQ-induced attenuation of response (Fig. 4), indicating that the effect of NPA on ACh levels is due to its interaction with D2 sites.

Discussion

The present results demonstrate that unilateral denervation of the nigrostriatal DA pathway with 6-OHDA elicits a super-

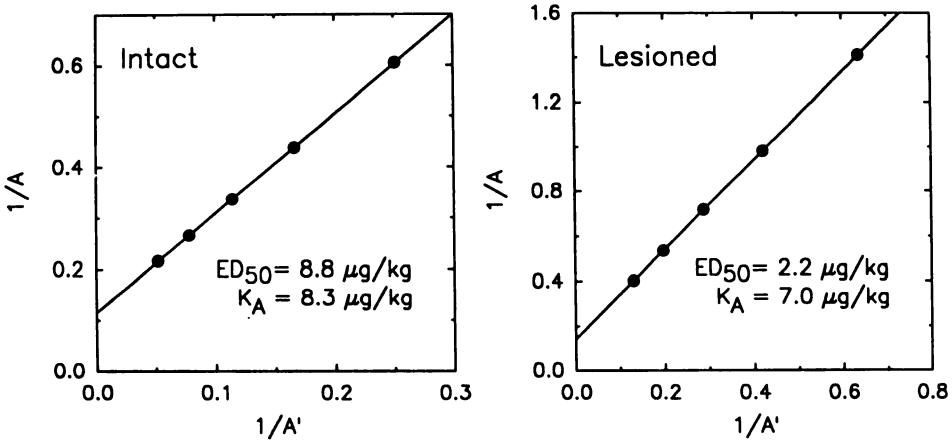

Fig. 2. Double-reciprocal plots of the equieffective dose of NPA required for elevation of striatal ACh levels in intact and 6-OHDA-lesioned striatum. Doses were obtained at five levels of effect, corresponding to 30, 40, 50, 60, and 70% of the maximal response in EEDQ-treated rats.

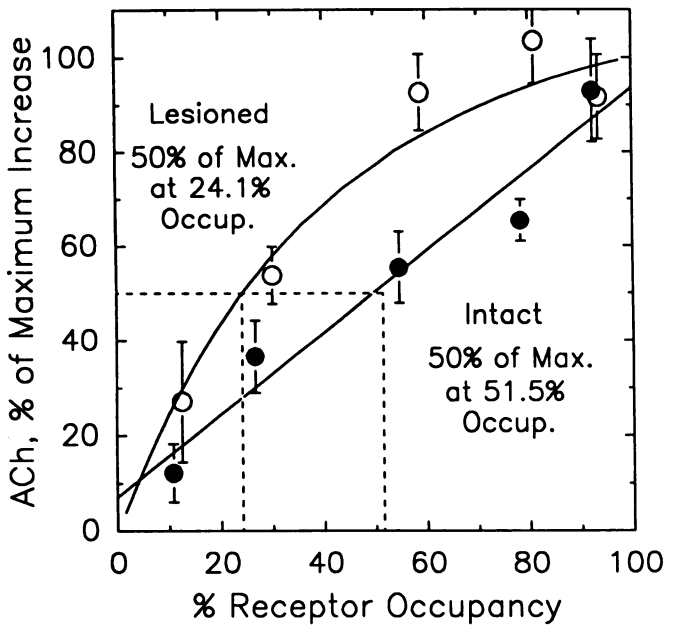

Fig. 3. Maximal elevation of striatal ACh levels as a function of receptor occupancy in intact and lesioned striatum. For the lesioned group, receptor occupancy was calculated from the law of mass action and the pseudo-K_A value obtained as described in Experimental Procedures; for the intact group, the *straight line* represents a least squares fit through the data points. The *symbols* represent values calculated for the actual data points in the experimental dose-response analyses.

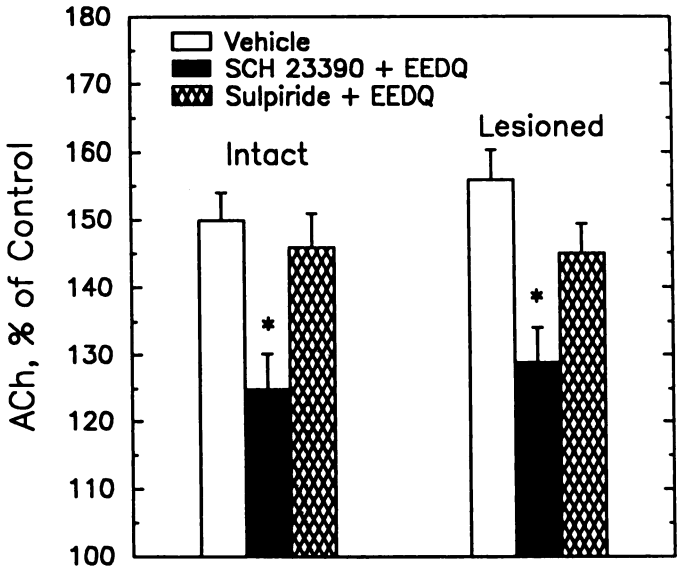

Fig. 4. Effects of protecting D1 or D2 receptors on the ACh response to NPA (0.1 mg/kg, subcutaneously) in intact and 6-OHDA-lesioned striatum. Rats were treated with vehicle alone, SCH 23390 (1 mg/kg, 30 min), or sulpiride (60 mg/kg, 3 hr) before EEDQ (6 mg/kg). The response to NPA was measured 24 hr later. Basal ACh levels (no NPA) were 66.5 ± 2.4 (n = 7) and 68.3 ± 2.7 (n = 7) pmol/mg of tissue in the intact and lesioned striatum, respectively. Each value is the mean ± standard error of eight rats. *Significantly different from respective vehicle-pretreated group, $p < 0.01$ (Dunnett's test). Neither of the sulpiride-pretreated groups was significantly different from its corresponding vehicle-pretreated group.

sensitive response to NPA-induced elevation of striatal ACh levels. This supersensitivity is reflected in a 4-fold increase in the potency of NPA, without a change in the maximal response. These results are very similar to those obtained by Paturle *et al.* (18), using the DA agonist pergolide. Furthermore, although there is no receptor reserve for this response in the intact striatum, 6-OHDA denervation in effect generates a receptor reserve of 25–30%, as estimated from the occupancy/response plot (Fig. 3). It is interesting to note that radioligand binding experiments indicate that 6-OHDA denervation increases striatal D2 receptor density to a similar extent (see Ref. 16 for review).

An important assumption in these studies is that the penetration of NPA into the brain is not significantly affected by the lesion. Although we have been unable to find any report comparing the distribution of NPA or other DA agonists in lesioned and intact striatum, it is noteworthy that, although the blood-brain barrier is compromised shortly after 6-OHDA infusion into the substantia nigra, this effect disappears by 21 days after lesion (34). It is, therefore, unlikely that differences in central NPA accumulation account for the present results determined at this time point.

A number of investigators have attempted to quantify the supersensitivity produced by 6-OHDA lesion. These studies, using behavioral paradigms such as rotation, have shown that the potency of DA agonists such as apomorphine is increased 5–40-fold at supersensitive D2 receptors, as compared to normosensitive D2 receptors (17, 35–37). Seeman (16), in a theoretical analysis applying the concepts of receptor occupancy theory, showed that a relatively small (40%) increase in D2 receptor density, as is found in supersensitive animals, could increase the potency of an agonist approximately 4-fold. The present results constitute a direct experimental verification of

this analysis, albeit for a biochemical rather than a behavioral response.

There are, however, a number of differences in the dose-effect relationship for the biochemical supersensitivity described here and the behavioral supersensitivity reported previously (17, 35–37). First, although the extent of DA depletion and time after lesion are very similar in the present study and those reported earlier, the magnitude of the increase in agonist potency described here (4-fold) corresponds to the low end of the range reported by others; indeed, some have reported supersensitivity changes of 30–40-fold (17, 35). In addition, although we and others (18) found no change in the slope or maximum of the biochemical dose-response curve after denervation, an increase in both of these parameters has been observed in some (35, 36) but not all (17) behavioral studies. It is highly probable that such differences reflect additional steps in the mechanism(s) responsible for transduction of receptor/effector coupling into expression of behavior. It should also be noted that the time-course for peak rotational response differs markedly from that for the increase in D2 receptor density (38), again suggesting that supersensitive behavioral expression involves additional mechanisms unrelated to a simple increase in receptor density.

The present demonstration that 6-OHDA treatment generates an apparent postsynaptic D2 DA receptor reserve for the full agonist NPA may explain why weak partial agonists such as the enantiomers of 3-PPP and EMD 23,448 display measurable postsynaptic receptor activity in supersensitive but not normosensitive animals (see Introduction). Receptor occupancy theory (39) predicts that, for a weak partial agonist, the main effect of an increase in receptor reserve (or density) would be an increase in the maximal response (rather than a leftward shift in the dose-response curve). Thus, it should come as no surprise that such agonists do elicit response in supersensitive animals. Although neither 3-PPP (5) nor EMD 23,448 (40) increase striatal ACh levels in normal rats, it is predicted that they will significantly increase ACh levels in 6-OHDA-lesioned animals; such studies are in progress.

Although 6-OHDA denervation generated a postsynaptic D2 DA receptor reserve, its magnitude (25–30%) was much smaller than that found at normosensitive D2 autoreceptors modulating both synthesis and release of DA (about 70%). Although there is no *a priori* reason to expect that denervation should elicit the same degree of receptor reserve as is normally observed at these DA autoreceptors, it is interesting to speculate that the greater efficiency of receptor/effector coupling at D2 autoreceptors versus postsynaptic receptors may be the result of a number of different factors. Pre- and postsynaptic D2 receptors may be coupled to the same or different G proteins, which in turn may activate the same or different effector moieties (e.g., adenylate cyclase, phosphoinositide turnover, etc.); alternatively, the G protein and effector may be identical but the efficiency of receptor/effector coupling may be related to differences in receptor/G protein stochiometry. Interestingly, both presynaptic (synthesis-regulating) (41) and postsynaptic (42) striatal D2 receptors are pertussis toxin-sensitive and are, therefore, probably coupled to G_i and/or G_o. Recently, a subtype of G_i, $G_{i3\text{-}\alpha}$, has been found in association with neurons containing both DA and cholecystokinin (43) and it has been suggested that this subtype, which is implicated in regulating the opening of K^+ channels (44), may be coupled to presynaptic DA receptors. G proteins are apparently directly inactivated by treatment with NEM *in vitro* (45). However, whereas NEM treatment largely reduced a postsynaptic D2 DA receptor response (DA inhibition of adenylate cyclase), it had no effect on a presynaptic D2-mediated response (45) [DA inhibition of its own electrically evoked release, which we have previously shown exhibits a large receptor reserve (14)] Although the G proteins coupled to the D2 receptors regulating these responses may be different and, therefore, demonstrate differential sensitivity to NEM, these results are also consistent with the possibility that D2 pre- but not postsynaptic receptors possess a substantial "G protein reserve," as suggested by Herdon (45). Rapidly emerging developments in our knowledge and understanding of the specific G proteins responsible for coupling receptors to specific functional responses may provide a means for choosing among these and other possible mechanisms underlying the pharmacological demonstration of receptor reserve in the central nervous system.

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