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Striatal Fos expression is indicative of dopamine D1/D2 synergism and receptor supersensitivity

(striatum immediate-early genes)

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ABSTRACT **Immediate-early genes, such as c-*fos*, are responsive to dopaminergic stimulation in the brain and can have prolonged effects on the transcription of other genes. Thus, they may mediate some of the long-term consequences of altered dopaminergic transmission on striatal neurons, such as the supersensitivity to dopamine and its agonists that occurs in response to dopamine denervation. The two dopamine receptor families, D1 and D2, interact synergistically under normal conditions but independently after treatments that induce pronounced supersensitivity to dopamine agonists. Using immunocytochemical methods in rats treated with directly acting selective dopamine agonists, we have determined that dopamine-mediated expression of Fos and Fos-like antigens in the striatum normally requires concomitant stimulation of D1 and D2 receptors. Separate administration of a high dose of a selective D1 (SKF 38393; 20 mg/kg) or D2 (quinpirole; 3 mg/kg) agonist induced Fos-like immunoreactivity in few neurons, whereas combined administration of the D1 and D2 agonists produced patches of intensely stained immunoreactive nuclei in the caudate-putamen. Repeated administration of reserpine (1 mg/kg per day for 5 days), which causes supersensitivity to dopamine agonists and a breakdown in D1/D2 synergism behaviorally, also causes a change in control of c-*fos*, such that independent stimulation of D1 receptors by SKF 38393 (20 mg/kg) elicited pronounced Fos-like immunoreactivity in the striatum; combined treatment with SKF 38393 (20 mg/kg) and quinpirole (3 mg/kg) in reserpine-treated rats elicited Fos-like expression in no more neurons than did D1 agonism alone. These data demonstrate that dopamine-mediated Fos expression in the striatum is indicative of the state of D1/D2 synergism and receptor supersensitivity.**

The immediate-early gene family is responsive to dopaminergic stimulation in the brain. *fos*, *jun*, and *zif* are all expressed in the rat striatum within minutes after systemic administration of the indirectly acting dopamine (DA) agonist amphetamine (1–6). These genes encode proteins with long half-lives that bind to regulatory sites on DNA and thereby alter transcription of other genes, such as preproenkephalin and prodynorphin (7–10). Thus, they have the potential to serve as intracellular intermediaries in the transduction of neuronal membrane signals into long-term adaptations within populations of DA-receptive neurons.

Two families of DA receptor have been discerned on the basis of pharmacology and deduced amino acid homology: the D1 family, composed of D_{1A} and D_{1B} subtypes (also known as D1 and D5, respectively), and the D2 family, composed of D_{2A}, D_{2B}, and D_{2C} subtypes (also known as D2, D3, and D4, respectively) (11). An intriguing aspect of DA receptor function is that, under normal conditions, concomitant stimulation of dopamine receptors of the D1 and D2 families is required for manifestation of many of the behavioral and electrophysiological effects of DA and DA-like drugs (for review, see ref. 12), a phenomenon we refer to as requisite D1/D2 synergism. For example, intense stereotyped sniffing, licking, and gnawing (13, 14) and disinhibition of globus pallidus neurons (13) are observed in rats following concurrent stimulation of D1 and D2 receptors but not following stimulation of either receptor subtype alone. In addition, D2-mediated inhibition of striatal neuron firing requires concomitant D1 stimulation (15, 16). In contrast, following destruction of the mesostriatal dopaminergic pathways with 6-hydroxydopamine (6-OHDA) or repeated treatment with the monoamine-depleting agent reserpine, the requirement for coactivation of D1 and D2 receptors is relieved. Behaviors that previously required concomitant D1/D2 stimulation (such as stereotyped sniffing) can be elicited by independent stimulation of either D1 or D2 receptors (17–21), and D2-mediated inhibition of striatal neurons (15) or disinhibition of pallidal neurons (22) no longer requires D1 costimulation. Additive or synergistic interactions between D1 and D2 receptors may still occur in some paradigms following 6-OHDA or repeated reserpine treatments (e.g., refs. 23 and 24; however, see ref. 15), but concurrent stimulation of the two receptor classes is clearly not required for DA-mediated behavioral and electrophysiological effects.

The loss of requisite D1/D2 synergism observed following 6-OHDA or repeated reserpine treatments represents a qualitative difference from the normal state, and, in addition, is associated with a supersensitivity to DA or other DA agonists (20). Recent findings strongly refute the widely held belief that this supersensitivity can be explained by increased DA receptor density (20, 21, 25–27). For example, profound supersensitivity can be observed in rats without increased striatal D2 density, and increased striatal D2 density alone produces a relatively small enhancement of sensitivity (20). These findings have created a void in the search for neural mechanisms underlying this type of plasticity. Application of the concepts of D1/D2 synergism to the study of other properties of basal ganglia neurons, such as immediate-early gene expression, may help to elucidate further the mechanism(s) of dopaminergic supersensitivity.

The goal of this study was to determine whether striatal immediate-early gene response is indicative of the state of D1/D2 synergism and supersensitivity to DA agonists. Although several studies have attempted to assess the relative importance of D1 and D2 receptors in mediation of DA-stimulated immediate-early gene response, none has addressed the possibility that qualitative differences in the state of interaction between the two DA receptor families occur in normosensitive versus supersensitive conditions. The exper-

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Abbreviations: DA, dopamine; 6-OHDA, 6-hydroxydopamine; CPu, caudate-putamen.

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iments reported here used directly acting selective DA agonists to test the hypothesis that striatal c-*fos* expression is elicited by synergistic activation of D1 and D2 receptors under normal conditions but not following treatments that induce a profound supersensitivity to dopamine agonists.

MATERIALS AND METHODS

Drug Treatments. In experiment 1, adult male Sprague–Dawley rats (200–300 g) were injected i.p. with saline (1 ml/kg), the selective D1 agonist SKF 38393 (20 mg/kg), the selective D2 agonist quinpirole (3 mg/kg), or the combination of SKF 38393 and quinpirole (20 and 3 mg/kg, respectively). These are high doses of agonists that are not fully effective behaviorally and electrophysiologically when given separately but are highly effective when given in combination (13, 20). In experiment 2, rats were injected once daily for 5 days with vehicle or reserpine (1 mg/kg, s.c.), a treatment known to induce DA receptor supersensitivity and a breakdown in the requirement for D1/D2 coactivation with respect to behavioral and electrophysiological phenomena (15, 17, 20). Two hours after the last vehicle or reserpine injection, rats were injected i.p. with saline, SKF 38393 (20 mg/kg), or quinpirole (3 mg/kg). In experiment 3, rats were injected once daily for 5 days with reserpine (1 mg/kg, s.c.) and then injected i.p. with SKF 38393 (20 mg/kg) or the combination of SKF 38393 (20 mg/kg) and quinpirole (3 mg/kg) 2 h after the last reserpine injection.

Immunocytochemistry. Two hours after DA agonist or saline injections, the animals were deeply anesthetized and perfused transcardially with 0.1 M phosphate-buffered saline (PBS; pH 7.4) followed by 4% (wt/vol) paraformaldehyde in PBS (pH 7.4). Brains were removed, immersed in 4% paraformaldehyde for 45 min, and placed in 30% sucrose for ≈18 h at 4°C. Frozen coronal sections (40 μm) through the striatum were cut on a microtome and incubated sequentially in 5% normal rabbit serum (1 h) and 1% normal rabbit serum containing polyclonal sheep antibodies generated against amino acid residues 2–17 of human and rat Fos peptide (Cambridge Research Biochemicals, OA-11-823; 1:1000) for 42 h. The specificity of this antiserum has been demonstrated by the fact that immunoreactivity is abolished by preadsorption with the peptide against which the antibody was raised (2, 28); nonetheless, cross-immunoreactivity with related yet unidentified proteins cannot be ruled out (29). Sections were then incubated sequentially in biotinylated rabbit anti-sheep IgG (1:200; 1 h), avidin–biotin–horseradish peroxidase complex (ABC; Vector Laboratories; 1 h), and 3,3′-diaminobenzidine (0.05%) in Tris·HCl (pH 7.4) containing 0.03% H_2O_2 and 0.04% NiCl.

Quantification of Fos-like Immunoreactivity. Immunostained sections were mounted, dehydrated, coverslipped, and viewed through a Nikon microscope attached to a computerized image analysis system (MCID; Imaging Research, St. Catherines, ON, Canada). Target criteria for area and optical density were set on the basis of visual identification of Fos-immunoreactive neurons. Targets within a 1.26-mm^2 area of the caudate-putamen (CPu) were automatically counted. Areal contribution of white matter was similarly quantified and found to be very consistent from one section to the next. Data are therefore presented as number of Fos-immunoreactive neurons per mm^2 of striatal tissue and were analyzed by analysis of variance and Newman–Keuls *post hoc* tests.

RESULTS

In experiment 1, saline-injected rats displayed very low levels of Fos-like immunoreactivity in the CPu, as did rats injected with either SKF 38393 or quinpirole alone. By contrast, rats injected with the combination of the D1 and D2 agonists exhibited clusters of intensely stained immunoreactive nuclei in the CPu (Figs. 1 and 2). These clusters resembled in size

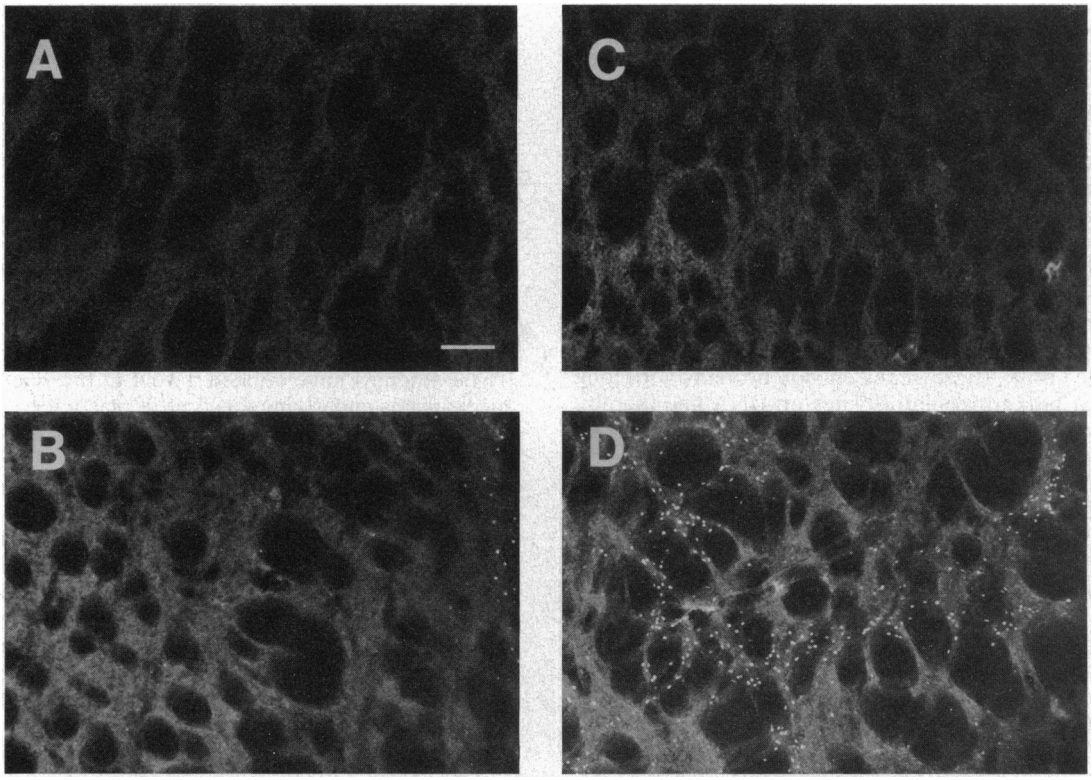

FIG. 1. Reverse-image photomicrographs of Fos-like immunoreactivity in CPu. Rats treated acutely with saline (*A*), SKF 38393 (20 mg/kg, i.p.) (*B*), or quinpirole (3 mg/kg, i.p.) (*C*) showed very low levels of immunoreactivity, whereas rats treated with SKF 38393 and quinpirole (*D*) showed patches of densely immunoreactive nuclei. (Bar = 0.1 mm.)

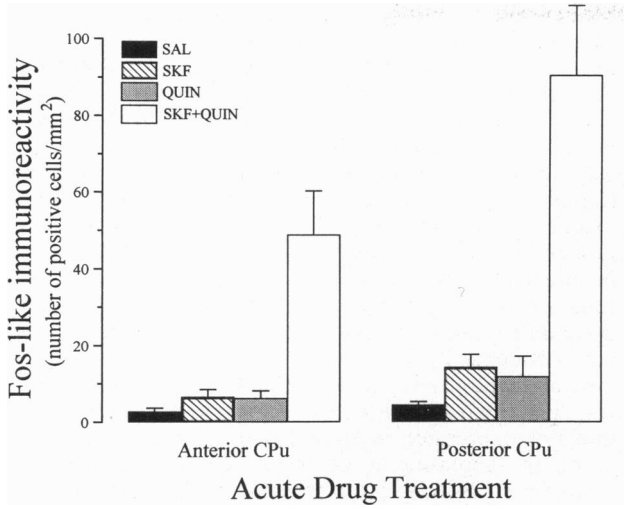

FIG. 2. Fos-like immunoreactivity in anterior and posterior CPu of rats treated acutely with D1 or D2 agonists alone or in combination (see Fig. 1 legend for doses). Anterior CPu corresponds to plates 13–15 and posterior CPu corresponds to Plates 18–20 of Paxinos and Watson (30). Rats treated with the combination of SKF 38393 and quinpirole had significantly higher Fos counts (anterior CPu, $P < 0.05$; posterior CPu, $P < 0.01$) than rats in any of the other treatment groups, none of which differed significantly from each other. Number of Fos-immunoreactive nuclei was significantly higher in posterior relative to anterior CPu ($P < 0.001$).

and shape those observed after amphetamine administration, which have been shown to correspond to calbindin-poor striosomal compartments (2, 3). Analysis of variance yielded a significant main effect for acute drug treatment ($F_{(3,16)} = 25.98$; $P < 0.001$). Newman–Keuls *post hoc* tests showed that rats treated with the combination of SKF 38393 and quinpirole had significantly higher Fos counts (anterior CPu, $P < 0.05$; posterior CPu, $P < 0.01$) than rats in any of the other treatment groups, none of which differed significantly from each other. In addition, Fos-like immunoreactivity was higher in posterior relative to anterior striatum for all treatment groups ($F_{(1,16)} = 18.53$; $P < 0.001$) but especially for the combined SKF 38393 and quinpirole treatment (Fig. 2).

In experiment 2, rats pretreated with reserpine and injected with SKF 38393 displayed pronounced Fos-like immunoreactivity in the CPu (Fig. 3; Table 1). Although there was no anterior–posterior gradient of Fos induction in reserpine/SKF 38393 rats, immunoreactivity was higher in lateral relative to medial CPu (Table 1). Overall, however, the distribution of Fos-like immunoreactive neurons was much more homogeneous than that observed in normal rats treated with the combination of SKF 38393 and quinpirole; that is, there was no observable clustering. Rats pretreated with reserpine and injected with saline, or vehicle pretreated rats injected with either SKF 38393 or saline showed very low levels of immunoreactivity, comparable to experimentally naive rats injected with either saline or SKF 38393 alone. Quinpirole did not induce striatal Fos-like immunoreactivity regardless of whether rats were pretreated with reserpine or vehicle (Table 1).

In experiment 3, rats were treated for 5 days with reserpine and then injected acutely with SKF 38393 (20 mg/kg) ($n = 8$) or the combination of SKF 38393 (20 mg/kg) and quinpirole (3 mg/kg) ($n = 7$). The number of striatal Fos-like immunoreactive nuclei and the pattern of Fos expression did not differ between these two groups ($F_{(1,13)} = 0.19$; $P = 0.67$) (Table 1). A reserpine regimen identical to that used here was shown previously to deplete striatal DA by 81.4% (20).

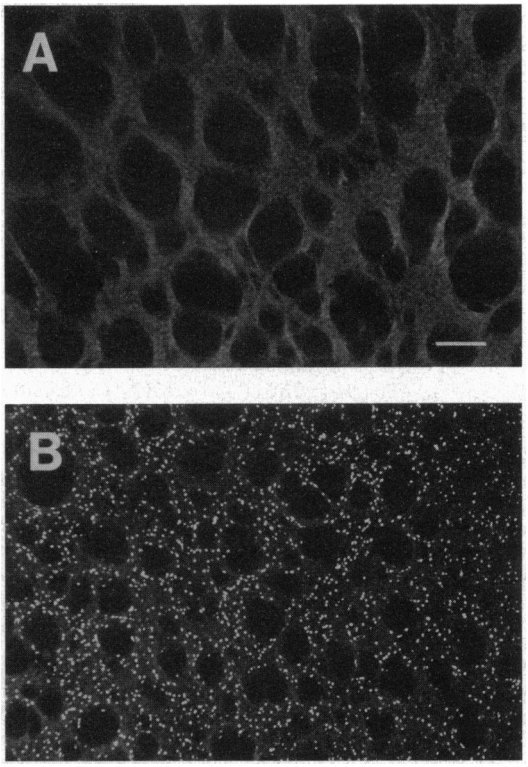

FIG. 3. Reverse-image photomicrographs of Fos-like immunoreactivity in CPu of rats injected daily with reserpine (1 mg/kg, s.c.) for 5 days. Few Fos-immunoreactive neurons were seen in reserpine-treated rats injected on day 5 with saline (*A*), but pronounced Fos-like immunoreactivity was observed in reserpine-treated rats injected on day 5 with SKF 38393 (20 mg/kg, i.p.) alone (*B*). Rats injected for 5 days with vehicle and then injected with saline or SKF 38393 showed very low levels of Fos-like immunoreactivity (data shown in Table 1). (Bar = 0.1 mm.)

DISCUSSION

The present results show that concomitant stimulation of D1 and D2 receptors is normally required for manifestation of a DA-mediated increase in Fos-like immunoreactivity in the intact striatum. This conclusion diverges from earlier interpretations, which emphasized D1 receptor involvement; however, all previous data on DA-mediated c-*fos* expression in neurologically intact rats is explicable under the presently espoused hypothesis of D1/D2 synergism (1–6, 31). For example, the present hypothesis predicts that c-*fos* transcription or Fos translation elicited in striatum by a mixed D1/D2 agonist, such as amphetamine, would be antagonized by a selective D1 or D2 antagonist. This has clearly been shown for the D1 antagonist SCH 23390 (2, 3, 5, 6). However, the results of studies with D2 antagonists have been more equivocal, with some investigators finding clear evidence for antagonism of amphetamine-induced Fos expression (2) and others not (31). Because D2 antagonist drugs administered alone can also elicit c-*fos* or Fos expression (2, 5, 6, 32–34), the interpretation of their effects on amphetamine-induced c-*fos* is necessarily difficult. Through the use of directly acting selective D1 and D2 agonists, the present experiments clarify the issue of D1/D2 interactions in striatal Fos expression, clearly supporting a role for D2 receptors in this DA agonist-mediated cellular response. Consistent with the present studies is the recent demonstration that the directly acting mixed D1/D2 agonist apomorphine elicits patchy Fos expression in the striatum (35). These findings, taken together with recent behavioral and electrophysiologic data, give rise to the conclusion that D1/D2 synergism is a fundamental

Table 1. Striatal Fos-like immunoreactivity following reserpine or vehicle pretreatment

Treatment, 5-day/acute	No. of Fos-immunoreactive nuclei per mm² of CPu		
	Medial	Lateral	*n*
	Experiment 2		
V/S	3.83 (±1.63)	2.08 (±0.55)	6
V/Q	1.20 (±0.76)	2.00 (±0.65)	5
V/SK	4.10 (±1.36)	7.40 (±2.67)	5
R/S	6.90 (±3.74)	8.30 (±2.97)	5
R/Q	2.60 (±0.52)	3.30 (±0.64)	5
R/SK	867.92 (±229.93)	1214.33 (±279.73)	6
	Experiment 3		
R/SK	904.94 (±72.31)	1212.77 (±85.30)	8
R/SK + Q	1045.38 (±73.76)	1227.98 (±48.77)	7

Mean (±SEM) number of Fos-immunoreactive nuclei per mm² in medial and lateral CPu of rats treated for 5 days with vehicle (V) or reserpine (R) and then injected acutely with saline (S), SKF 38393 (20 mg/kg) (SK), or quinpirole (3 mg/kg) (Q) alone (Exp. 2) or in combination (Exp. 3). In Exp. 2, analysis of variance yielded significant effects for drug treatment ($F_{(5,26)} = 11.71$; $P < 0.001$) and the medial–lateral variable ($F_{(1,26)} = 21.83$; $P < 0.001$). Newman–Keuls *post hoc* tests showed that rats pretreated with reserpine and injected acutely with SKF 38393 had significantly higher ($P < 0.01$) Fos counts than rats in any of the other treatment groups, none of which differed significantly from each other. In Exp. 3, rats treated repeatedly with reserpine and injected acutely with SKF 38393 did not differ from reserpine-pretreated rats injected with the combination of SKF 38393 and quinpirole with respect to the number of Fos-like immunoreactive neurons ($F_{(1,13)} = 0.19$; $P = 0.67$).

property of normal dopaminergic action within the basal ganglia that is evident in a wide range of functions.

As reviewed earlier, repeated reserpine or 6-OHDA treatment causes a breakdown in requisite D1/D2 synergism with respect to behavior (17–21) and electrophysiology (15, 16). The present study extends these findings to immediate-early gene expression by showing that, as in 6-OHDA-treated rats (1), striatal Fos expression was elicited by independent stimulation of D1 receptors following a regimen of reserpine that substantially depletes striatal DA and results in supersensitivity and a breakdown in requisite D1/D2 synergism with respect to both electrophysiologic (15) and behavioral (20) phenomena. In addition, in reserpine-pretreated rats, combined treatment with both D1 and D2 agonists produced no greater striatal Fos-like expression than did independent stimulation of D1 receptors. Thus, striatal Fos expression is a reliable *ex vivo* indicator of DA supersensitivity and of the state of D1/D2 synergism. The fact that the concordance among the various manifestations of D1/D2 interaction is maintained under conditions in which these receptors exert either synergistic or independent effects strengthens our conclusion that D1/D2 synergism is a fundamental process in the dopaminergic modulation of basal ganglia activity. Furthermore, the present data strengthen the hypothesis that major supersensitivity is associated with a breakdown in requisite D1/D2 synergism (20).

Although the evidence indicates that combined D1/D2 stimulation is not required for DA-mediated effects following 6-OHDA or repeated reserpine treatment, submaximal doses of D1 and D2 agonist may still exert additive or synergistic effects in supersensitive animals (e.g., refs. 23 and 24; however, see ref. 15), a property shared with normosensitive animals. However, it is the response of these animals to either D1 or D2 stimulation alone that most clearly distinguishes them from normosensitive animals and, in our view, is most likely to provide insights concerning the neural changes associated with the supersensitive condition. In the present studies, combined administration of D1 and D2 agonists in rats treated repeatedly with reserpine did not elicit greater numbers of Fos-like immunoreactive neurons in striatum than did administration of a D1 agonist. This does not preclude the possibility that additive effects might have been obtained had lower doses of the agonists been used, but it does suggest that a maximal response is possible with independent D1 stimulation.

Regional heterogeneities within the striatum are manifold (for review, see ref. 36) and have recently been shown to extend to Fos expression (2, 3). The clustering of Fos-like immunoreactive neurons observed in the present experiment in intact rats treated with combined D1/D2 agonists was reminiscent of that previously reported for amphetamine-treated rats and corresponding to calbindin-poor striosomal compartments (2, 3). In addition, we observed more Fos-like immunoreactivity in the posterior than in the anterior CPu, which is consistent with the observation of Graybiel *et al.* (2) that Fos expression in striatal neurons of the matrix compartment increased at posterior levels in amphetamine-treated rats. In contrast to intact animals, D1-mediated Fos expression in reserpine-treated rats appeared more homogeneous but displayed a lateral–medial without an anterior–posterior gradient. At least two factors may contribute to these differing patterns. First, since Fos expression in normal rats requires both D1 and D2 receptor stimulation, while that in supersensitive rats is mediated solely through D1 sites, regional differences in the distribution of neurons controlled by these two receptor families may contribute to the observed patterns of Fos-like immunoreactivity. Second, regional variations in the extent of DA depletion induced by reserpine treatment, and thus in the magnitude of the resulting supersensitivity, could also contribute to the pattern of Fos-like immunoreactivity in reserpine-treated animals.

In addition to differences in the pattern, substantial differences in the magnitude of Fos expression occurred between reserpine-treated rats injected with SKF 38393 and naive rats given combined SKF 38393 and quinpirole, suggesting that the populations of neurons activated under the two treatment conditions may overlap but are not coincident. The greater number of Fos-immunoreactive neurons observed under supersensitive versus normosensitive conditions may relate to inequalities in the effective dose of DA agonists used in the two paradigms. Differing potencies of the DA agonists inducing Fos may result, at least in part, from enhanced coupling of D1 receptors to stimulatory guanine nucleotide-binding protein in reserpine-treated rats relative to controls (37). Thus, higher doses of SKF 38393 and/or quinpirole might have resulted in densities of Fos-positive cells in normal striatum similar to those observed in reserpine-treated animals given SKF 38393. In support of this, Cenci *et al.* (38) showed that treatment of normosensitive rats with the DA uptake inhibitor cocaine elicits Fos expression in an equivalent number of striatal neurons as does the mixed D1/D2 agonist apomorphine in supersensitive striatum.

Recent studies of the mechanisms by which D1/D2 synergism is maintained and broken down show that changes in DA receptor density cannot account for changes in the state of synergism. Treatments that up-regulate striatal D1 and/or D2 receptor density do not alter the normal pattern of synergism, whereas 5 days of reserpine treatment induces a breakdown in requisite synergism without altering D1 or D2 receptor density (20). Recent data indicate that both supersensitivity and a breakdown in synergism can be detected as early as 24–48 h after reserpine or 6-OHDA treatment, a time frame that limits the possible neural mechanisms that can be involved (21, 39–42).

Further insights into the neuronal basis of D1/D2 synergism and its breakdown may be revealed by considering the hypothesized cellular localizations of D1 and D2 receptors within distinct basal ganglia neurons. According to a recent model (43–45), DA is believed to exert an inhibitory influence

on striatopallidal neurons via D2 receptors, and an excitatory influence on striatonigral neurons via D1 receptors. On the assumption that c-*fos* expression is elicited by stimuli that result in neuronal depolarization (10), D1 receptor stimulation would be expected to induce c-*fos* expression in striatonigral neurons. In fact, Robertson *et al.* (46, 47) have recently shown that D1-stimulated Fos in the striata of 6-OHDA-treated rats occurs primarily in striatonigral neurons. The induction of c-*fos* expression in a population of striatal neurons by a selective D1 agonist in the reserpine experiments reported here is consistent with this interpretation. The model also predicts that agonist stimulation of D2 receptors would inhibit striatopallidal neurons and therefore not induce striatal c-*fos*, an expectation confirmed by the present findings as well as by the findings of others (1). In addition, it predicts that selective D2 antagonists would disinhibit the activity of D2-containing striatal neurons and thereby result in Fos expression, a prediction confirmed by several studies (2, 5, 6, 32–34). To determine whether D2 agonist-mediated c-*fos* expression is normally dependent on concomitant D1 stimulation, it may be necessary to examine other brain areas, such as the globus pallidus, where neurons are disinhibited by striatal D2 activity (13). Consistent with this rationale is the recent finding that quinpirole elicits Fos expression in the globus pallidus ipsilateral to a 6-OHDA lesion in rats (47).

The neuronal model outlined above appears most applicable to those conditions in which D1 and D2 receptor-mediated influences on striatum are independent. But, as the present work shows, in normosensitive animals D1-mediated activation of striatal neurons requires D2 costimulation to be manifest. Because c-*fos* cannot normally be induced in striatal neurons by D1 stimulation alone, the present data suggest that D1/D2 synergism occurs at the level of the D1- or D2-containing medium spiny striatal output neurons or upstream from them. Short-latency (i.e., 24–48 h) changes in the activity of axon collaterals from neighboring D1- and D2-containing striatal neurons or in the activity of long (i.e., striatothalamocortical) feedback loops are two plausible but presently untested mechanisms for altered D1/D2 interactions. In addition, it should be pointed out that SKF 38393, although widely used, is a partial D1 agonist, raising the possibility that different results might emerge from the use of full D1 agonists.

Although the present study has focused on the stimulant effects of DA agonists, previous work has shown that the treatment conditions that initiate a breakdown in requisite D1/D2 synergism, such as reserpine or D2 antagonist treatment (ref. 21; however, see ref. 20), induce c-*fos* expression in the striatum (2, 5, 6, 32–34). These treatments initiate a cascade of effects including translation of Fos and Fos-related proteins, which complex with Jun (the protein product of c-*jun*) to form the transcription factor AP-1 (10). Binding of AP-1 to DNA, which regulates the expression of other genes, persists for at least 8 h (48), a time frame consistent with that of the causal events leading to supersensitivity and a breakdown in requisite D1/D2 synergism (see above). Thus, immediate-early gene response may be not only an indicator of the state of DA receptor interaction and sensitivity but also a mediator of the cellular changes underlying a breakdown in requisite D1/D2 synergism and supersensitivity.

The identification in this report of an *ex vivo* indicator of the state of D1/D2 synergism may help to elucidate the cellular mechanisms underlying DA receptor plasticity. Furthermore, since DA receptor supersensitivity may be dependent on a breakdown in requisite D1/D2 synergism (20), the present findings may be important in understanding neurological and psychiatric conditions involving altered sensitivity to DA agonists.

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