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Differential expression of c-Fos and Zif268 in rat striatum after haloperidol, clozapine, and amphetamine

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ABSTRACT **Antipsychotic drugs are monoamine receptor antagonists. However, the mechanisms by which these direct actions are translated into therapeutic effects are unknown. Candidate mechanisms include receptor-mediated regulation of gene expression in target neurons. Inducible transcription factors, including certain immediate early genes (IEGs), may mediate between receptor-activated second messenger systems and expression of genes involved in the differentiated functions of neurons. We examined the specificity of induction of the IEGs c-*fos* and *zif268* after acute administration of several antipsychotic drugs and, for comparison, the stimulant amphetamine, which has pharmacologic effects relatively opposite to those of antipsychotics. Antipsychotic drugs with potent dopamine D_2 receptor antagonist properties, such as haloperidol, induced both c-fos and zif268 mRNA in the caudate–putamen; however, the atypical antipsychotic drug clozapine induced zif268 but not c-fos mRNA in that region. Similarly, haloperidol, but not clozapine, induced c-Fos-like immunoreactivity in the caudate–putamen. In contrast, both drugs induced c-Fos-like immunoreactivity in the nucleus accumbens. Like haloperidol, amphetamine induced both c-fos and zif268 mRNA in the caudate–putamen, but the anatomic patterns of induction of c-Fos-like immunoreactivity by the two drugs were dramatically different. Haloperidol and amphetamine induced AP-1 binding activity in cell extracts from the caudate–putamen, indicating that drug-induced IEG expression results in protein products that may function in the regulation of target gene expression. Thus these data demonstrate that inductions of IEG expression by haloperidol, clozapine, and amphetamine are specific, may be biologically relevant, and suggest avenues for further investigation.**

The antipsychotic drugs are effective in the treatment of severe psychiatric disorders. However, their serious side effects and limitations in efficacy have spurred a search for better compounds. A more complete understanding of the pharmacology of antipsychotic drugs would provide important clues for the development of improved treatments. Because the full therapeutic effects of antipsychotic drugs generally take several weeks to emerge, it has been postulated that their interactions with neurotransmitter receptors are merely the initial step in their actions and that the therapeutic effects result from adaptive processes that may occur in response to repetitive or chronic receptor occupancy by the drugs. An important candidate mechanism for such drug-induced neural plasticity is receptor-mediated regulation of neuronal gene expression. The induction of cellular immediate early genes (IEGs) may be a critical signal transduction step in neural plasticity induced by neurotransmitters and drugs, with the protein products of IEGs functioning to activate or repress genes that encode proteins involved in the differentiated functions of target neurons. In particular, it has been reported that the IEG c-*fos* is induced in rat striatum by acute doses of haloperidol and other D_2 receptor antagonists (1, 2). However, D-amphetamine and cocaine, drugs with markedly different pharmacologic and clinical properties, have also been reported to activate striatal c-*fos* expression by a mechanism probably involving stimulation of dopamine D_1 receptors (3–5). Since c-*fos* is induced by both dopamine agonists and antagonists, a major challenge in relating IEG activation to downstream biological effects is to understand how specificity is achieved. The goal of this study was to examine the specificity of activation of the IEGs c-*fos* and *zif268* (6) [also known as NGFI-A (7), *Egr-1* (8), and *Krox-24* (9)] by haloperidol and clozapine in relation to their therapeutic actions. As a first step in determining the biological significance of the observed IEG induction, we also undertook to determine whether drug-induced c-Fos expression could be correlated with increased AP-1 binding activity within the caudate–putamen, since c-Fos and related proteins exert their known actions on transcription as components of AP-1 complexes binding to DNA.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (175–200 g) were used for all experiments. All drugs were administered intraperitoneally.

Northern Blot Analysis. Animals were sacrificed by rapid decapitation 45 min after injection. Total RNA was prepared as described (10). Total RNA (20 μg) was electrophoresed on each lane of a formaldehyde gel, transferred to GeneScreen, (DuPont), UV cross-linked, and hybridized with nick-translated probes for c-*fos*, *zif268*, or cyclophilin (11).

Immunocytochemistry. Two hours after drug treatment animals were deeply anesthetized and transcardially perfused, first with chilled 0.1 M phosphate-buffered saline (PBS, pH 7.4) and then with 4% (wt/vol) paraformaldehyde in PBS. Brains were removed, immersed in perfusion solution for 4 h, and then placed in cryoprotectant. Sections (50 μm) were incubated for 1 h in 10% (vol/vol) normal goat serum (GIBCO) and then incubated free-floating in affinity-purified rabbit IgG polyclonal antibody against the peptide (SGFNADYEASSSRC) corresponding to residues 4–17 of human c-Fos protein (Oncogene Science, Manhasset, NY, antibody PC-05) for 48 h at 4°C (1:500 dilution). Sections were washed, incubated with peroxidase-conjugated goat anti-rabbit IgG (Boehringer Mannheim; 1:300 dilution in PBS with 1% normal goat serum), and then treated with 3,3′-diaminobenzidine. Every third section was immunostained. Nissl-stained sections adjacent to immunostained sections were used to identify five AP levels through the striatum (12) (Bregma 1.7, 1.2, 0.75, 0.2, −0.3 mm). Doses were as follows:

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Abbreviations: IEG, immediate early gene; IR, immunoreactive.
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haloperidol at 0.5 mg/kg (n = 2), 1 mg/kg (n = 4), or 5 mg/kg (n = 2); clozapine at 10 mg/kg (n = 2) or 20 mg/kg (n = 2); amphetamine at 2 mg/kg (n = 2), 4 mg/kg (n = 4), 8 mg/kg (n = 2), 12 mg/kg (n = 2), or 16 mg/kg (n = 4).

Gel-Shift Assays. Two hours after drug treatment, animals were sacrificed; the caudate–putamen (100 mg) was dissected out, suspended in 1 ml of 0.5 M sucrose/10 mM Hepes·KOH, pH 7.9/1.5 mM $MgCl_2$/10 mM KCl/10% (vol/vol) glycerol/1 mM EDTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride, and homogenized (10 strokes in a Dounce B homogenizer). The homogenate was centrifuged at 4000 × g for 5 min at 4°C. The pellet was resuspended in 0.1 ml of 20 mM Hepes, pH 7.9/25% glycerol/0.5 M KCl/1.5 mM $MgCl_2$/0.4 mM EDTA/0.5 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride, incubated for 10 min at 4°C, and then centrifuged at 14,000 × g for 10 min at 4°C. The supernatant was used for binding. *In vitro*-translated proteins were prepared from RNA produced by SP6 RNA polymerase using rabbit reticulocyte lysates (Promega).

Protein extracts (5 μg) or *in vitro*-translated proteins (1 μl of lysate containing c-Fos and/or JunD) were incubated in 20 μl of 10 mM Hepes, pH 7.9/15% glycerol/0.1 mM EDTA/80 mM KCl/2 mM $MgCl_2$/1 mM dithiothreitol/poly (dI-dC) (10 μg/ml) at 4°C with or without unlabeled competitor (100 ng). After a 15-min incubation, 1 ng of ^{32}P-labeled double-stranded AP-1 oligonucleotide was added. Samples were incubated for 15 min at 23°C and then electrophoresed through a nondenaturing 4% polyacrylamide gel. The AP-1 sequence was GATCGGCTGAGTCAGGG; AP-2 was GATCCAGCCCGCCGGCGATTG.

RESULTS

The Effects of Haloperidol, Clozapine, and D-Amphetamine on Expression of c-fos and zif268 mRNA. Haloperidol (1 mg/kg) or D-amphetamine (4 mg/kg) produced a rapid and transient induction of c-fos and zif268 mRNA in the caudate–putamen as determined by Northern blot analysis. The increase in mRNA of each IEG peaked 30–45 min after injection and returned to baseline by 2 h after injection (data not shown). No effect of clozapine (20 mg/kg) on c-fos mRNA was observed; its effect on zif268 mRNA peaked at 45 min. Based on this observed time course of mRNA induction, rats were sacrificed 45 min after drug injection for Northern blot analysis in subsequent experiments.

In six experiments, rats were treated with vehicle, haloperidol at 0.5 or 1.0 mg/kg, clozapine at 10 or 20 mg/kg, or D-amphetamine at 2 or 4 mg/kg. Haloperidol and D-amphetamine induced c-*fos* expression in the caudate–putamen in a dose-dependent fashion (Fig. 1), with c-*fos* induced 4- to 5-fold by the highest dose of haloperidol (1 mg/kg) and 3- to 4-fold by the highest dose of D-amphetamine (4 mg/kg). Clozapine had no effect on c-*fos* expression in the caudate–putamen. Haloperidol and D-amphetamine also induced *zif268* expression in a dose-dependent fashion (Fig. 2). Haloperidol (1 mg/kg) induced *zif268* ≈4-fold and D-amphetamine induced *zif268* ≈3-fold. In contrast to its lack of effect on c-*fos* expression, clozapine had a reproducible effect on expression of *zif268*, inducing it ≈2-fold.

Drug-Specific Patterns of c-Fos Protein Expression in Striatum. The observation that a dopamine antagonist (haloperidol) and an indirect agonist (amphetamine) activated expression of IEGs in the rat striatum prompted the question of whether the effects were occurring in the same cells. Expression of c-Fos was, therefore, studied anatomically by immunohistochemistry. Rats were treated with vehicle, clozapine, haloperidol, or D-amphetamine and killed 2 h after drug administration. Coronal sections immunostained for c-Fos demonstrated some variability in the density of c-Fos immunoreactive (c-Fos-IR) material between animals treated

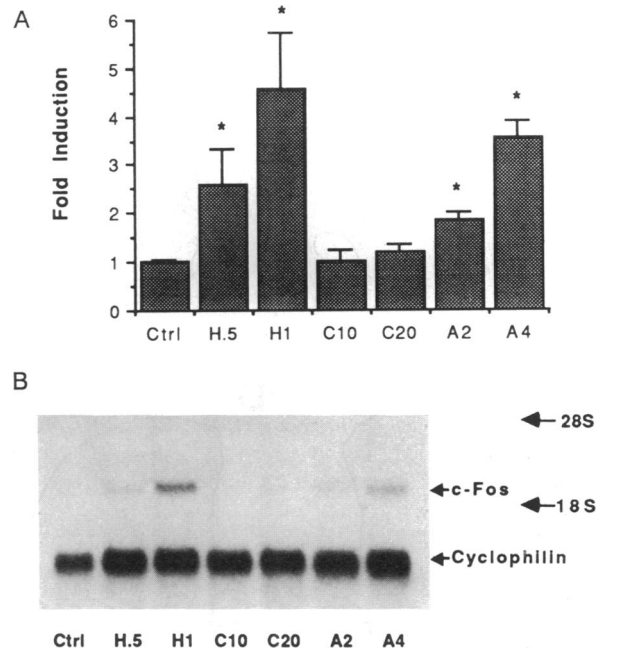

FIG. 1. (*A*) Fold induction of c-fos mRNA in rat striatum 45 min after intraperitoneal injection of drug (mean and SEM from six experiments). (*B*) Representative Northern blot. Bars and lanes: Ctrl, vehicle; H.5, haloperidol at 0.5 mg/kg; H1, haloperidol at 1 mg/kg; C10, clozapine at 10 mg/kg; C20, clozapine at 20 mg/kg; A2, D-amphetamine at 2 mg/kg; A4, D-amphetamine at 4 mg/kg. Values are determined densitometrically and normalized to cyclophilin mRNA. *, Statistically significant vs. control ($P < 0.05$) by two-tailed t test.

with the same drug and dose. However, for each drug condition, distinctive patterns of distribution of c-Fos-IR material were evident (Fig. 3). Where present, immunostaining was nuclear. The caudate–putamen and nucleus accumbens of vehicle-treated rats were nearly devoid of c-Fos-IR cells. The few observed were restricted to the medial wall of the striatum adjacent to the lateral ventricle (data not shown).

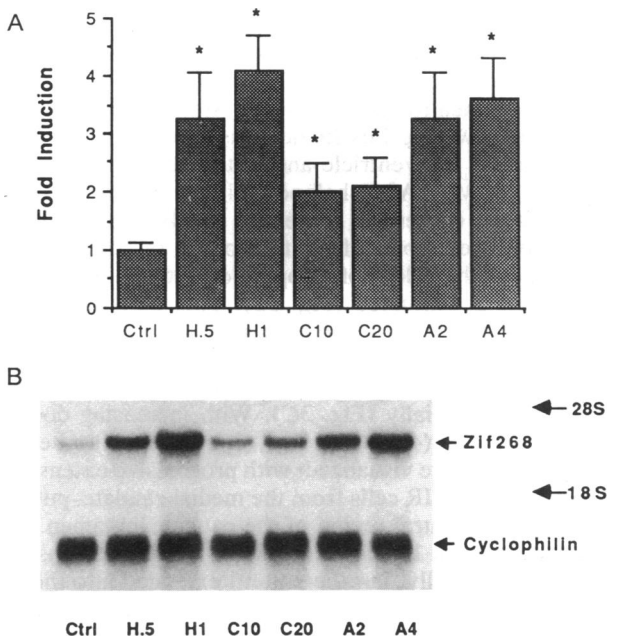

FIG. 2. (*A*) Fold induction of zif268 mRNA in rat striatum 45 min after intraperitoneal injection of drug. Drugs and methodology are as in Fig. 1 (six experiments). (*B*) Representative Northern blot.

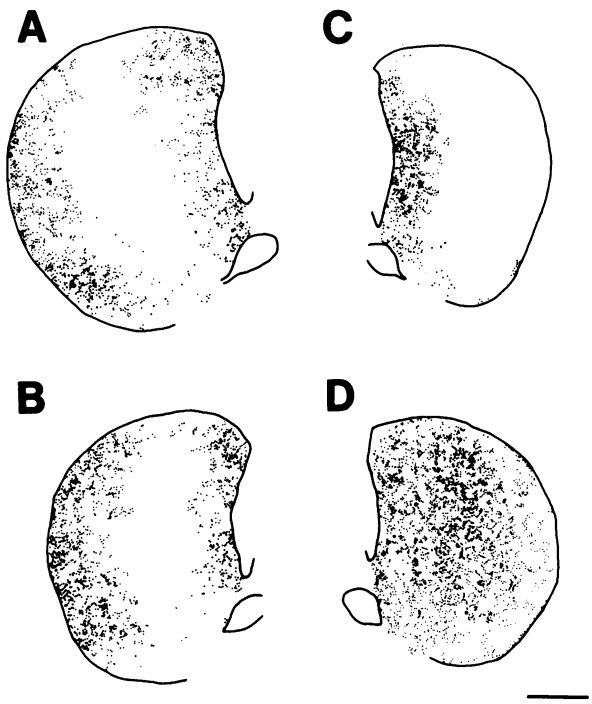

FIG. 3. Camera lucida representations of c-Fos-IR neurons evident in the caudate–putamen of rats 2 h after intraperitoneal injection of haloperidol at 1 mg/kg (*A*), haloperidol at 5 mg/kg (*B*), D-amphetamine at 4 mg/kg (*C*), D-amphetamine at 12 mg/kg (*D*). (AP = Bregma 0.2 mm for each case.) Each dot represents a single c-Fos-IR nucleus. Light- and dark-staining cells are identically represented. (Bar = 1.0 mm.)

After clozapine at 20 mg/kg, markedly increased numbers of c-Fos-IR cells were seen within the dorsal and medial regions of the nucleus accumbens (Fig. 4*B*), including, but not restricted to, the shell region (13). At this midlevel through the nucleus accumbens, a similar pattern of increased c-Fos-IR material was observed after D-amphetamine at 4 mg/kg (Fig. 4*C*) and haloperidol at 1 mg/kg (Fig. 4*D*).

The similarity of c-Fos-IR cell distribution at midnucleus accumbens levels with all three drugs was in sharp contrast to the drug-specific patterns seen at mid to posterior striatal levels (AP, Bregma 0.5 to −0.5 mm). After clozapine at 20 mg/kg, there were very few c-Fos-IR neurons evident in the caudate–putamen (data not shown). After haloperidol at 0.5 mg/kg, there were c-Fos-IR neurons evident in the medial striatum along the ventricle and in the ventrolateral region (data not shown). After haloperidol at 1 mg/kg, c-Fos-IR neurons were evident in increased numbers medially and laterally but were absent from the core of the striatum (Fig. 3*A*). At the highest dose of haloperidol tested (5 mg/kg), this pattern was accentuated (Fig. 3*B*). This pattern contrasts markedly with that produced by D-amphetamine. D-Amphetamine at 4 mg/kg produced an increased number of c-Fos-IR neurons medially along the ventricular surface, with a lower density dorsomedially (Fig. 3*C*). With increasing doses of D-amphetamine (4–16 mg/kg), increased numbers of c-Fos-IR cells were visualized, with progressive extension of the zone of c-Fos-IR cells from the medial caudate–putamen to include the central region of the caudate–putamen. With D-amphetamine at 12 and 16 mg/kg, c-Fos-IR neurons were most dense medially, but the pattern extended into the core of the striatum (Fig. 3*D*). Even at these doses, however, there was a paucity of c-Fos-IR neurons in the lateral region of the striatum at this level (Fig. 3*D*). At high doses, the complementarity between the patterns of haloperidol- and D-amphetamine-induced c-Fos-IR material was dramatically evident in that haloperidol markedly induced c-Fos-IR material in a zone of lateral neurons, relatively devoid of c-Fos-IR material after D-amphetamine (Fig. 3 *B* vs. *D*). At more anterior striatal levels, differences between patterns of haloperidol- and amphetamine-induced c-Fos-IR material were still present, but the complementarity was less dramatic. At all doses of D-amphetamine studied, the pattern of c-Fos-IR material was patchy, in agreement with Graybiel *et al.* (4).

Immunostaining of untreated and saline-injected animals with antibody to Egr-1, equivalent to Zif268 (gift of Vikas Sukhatme, University of Chicago), revealed high basal levels of expression throughout the striatum (data not shown). This precluded interpretation of drug-induced alterations in the pattern of Egr-1/Zif268 immunoreactivity by visual inspection.

The Role of Dopamine Receptor Types in IEG Activation. To define the receptors by which antipsychotic drugs and amphetamine produced IEG activation, we compared the effects of the typical (mixed D_2 and D_1 receptor antagonist) antipsychotic drug *cis*-flupentixol, its relatively inactive stereoisomer *trans*-flupentixol (which has a 100-fold lower affinity for both D_1 and D_2 receptors), the selective D_1 antagonist SCH-23390, and the selective D_2 antagonist sulpiride. As seen in Fig. 5, *cis*-flupentixol and sulpiride activated c-*fos* and *zif268* expression as determined by Northern blot analysis, whereas *trans*-flupentixol and SCH-23390 did not. By comparison, and consistent with previous reports (4, 5), the effects of amphetamine on c-*fos* and *zif268* expression in the striatum were completely inhibited by pretreatment with SCH-23390 (data not shown).

Induction of Biologically Active AP-1 Binding Proteins by Haloperidol and D-Amphetamine. To determine whether the Fos protein observed immunohistochemically (and related proteins) could be biologically active, we studied the induction of AP-1 binding activity in the caudate–putamen after haloperidol and amphetamine administration by a gel-shift assay (Fig. 6). Both haloperidol (1 mg/kg) and D-amphetamine (4 mg/kg) produced an ≈2-fold induction of an activity that specifically bound a consensus AP-1 sequence. The binding was determined to be specific for the AP-1 sequence based on competition with a 100-fold excess of an unlabeled AP-1 oligonucleotide (Fig. 6*A*, lanes 7 and 11) and on lack of competition with unrelated sequences including an AP-2 oligonucleotide (Fig. 6*A*, lanes 8 and 12). In addition, the retarded band comigrated with a band produced by incubation of the AP-1 probe with a mixture of *in vitro*-translated c-Fos and JunD. Both haloperidol and D-amphetamine induced AP-1 activity within 2 h (corresponding temporally with the appearance of immunohistochemically detectable c-Fos). A similar induction of AP-1 binding by haloperidol was seen in the nucleus accumbens (C. Konradi and S.E.H., unpublished data).

DISCUSSION

These studies were undertaken to evaluate the biological specificity of c-*fos* induction previously reported for haloperidol and psychostimulant drugs (1, 2, 4, 5) and to investigate the possible relevance of IEG induction to the clinical actions of antipsychotic drugs. It has been reported that haloperidol and other D_2 antagonists activate expression of c-*fos* in striatum (1, 2). Amphetamine and cocaine also induce c-*fos* expression in striatum, although this effect appears to occur through a D_1 receptor-linked pathway (4, 5). We report that D_2 receptor antagonists and amphetamine, but not D_1 receptor antagonists, regulate not only c-fos but also zif268 mRNA in the striatum. However, drug-regulated expression of these two genes was not identical: the atypical antipsychotic drug clozapine, a weak D_2 receptor antagonist, induced *zif268* but not c-*fos* in the caudate–putamen. The lack

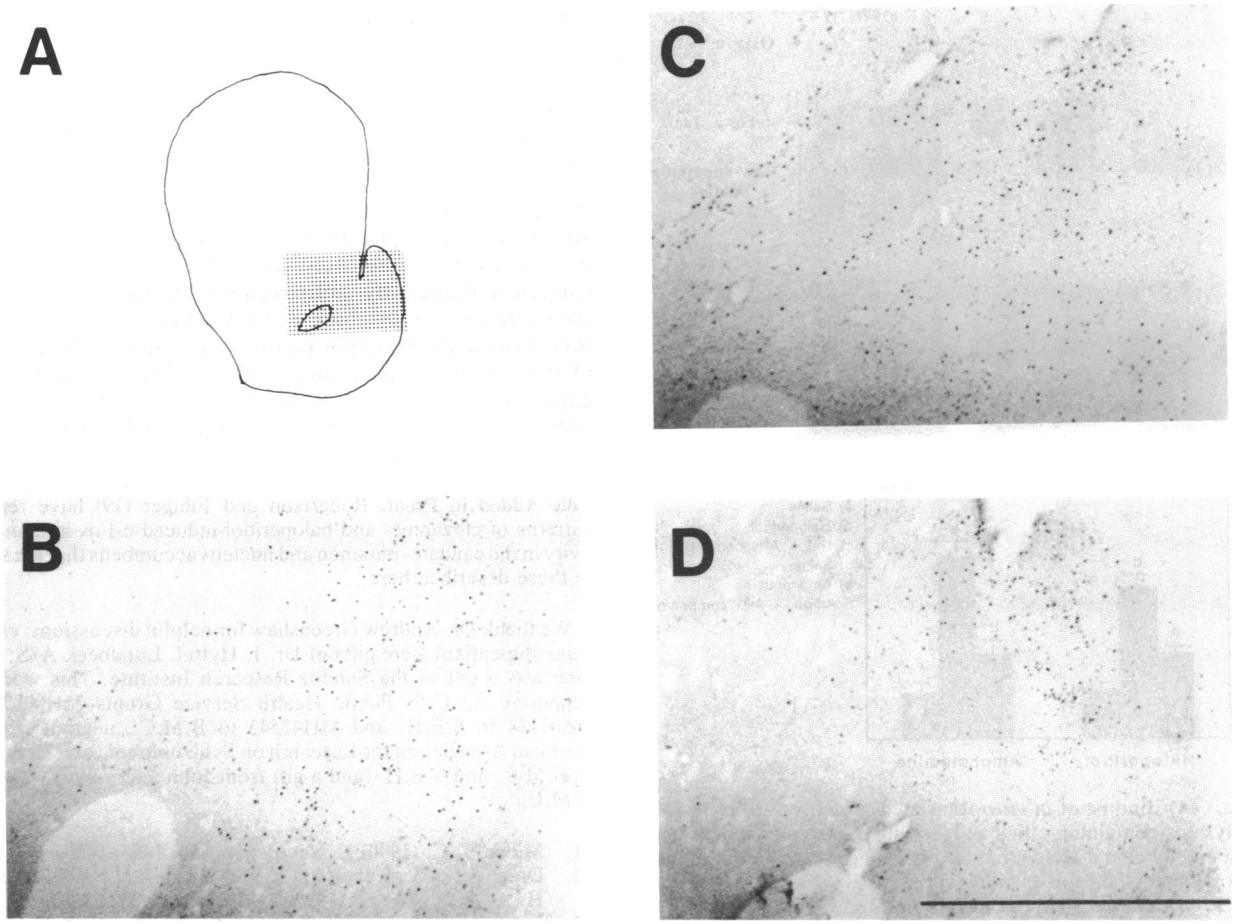

FIG. 4. (*A*) Schematic diagram depicting the dorsomedial region of the nucleus accumbens (stippled) (AP level Bregma = 1.7 mm) at which photomicrographs in *B–D* were taken. Photomicrographs show c-Fos-IR material 2 h after intraperitoneal injection of clozapine at 20 mg/kg (*B*), D-amphetamine at 4 mg/kg (*C*), and haloperidol at 1 mg/kg (*D*). (Bar = 1.0 mm.)

of c-*fos* induction in the caudate–putamen by clozapine and the lesser activation of *zif268* in comparison with haloperidol are not likely to be due to a dose–response effect since the clozapine induction of zif268 mRNA appears to have reached a plateau at the doses used. Differential regulation of c-*fos* and *zif268* has also been reported in the dentate gyrus of the hippocampus after high-frequency stimulation of the perforant path (14).

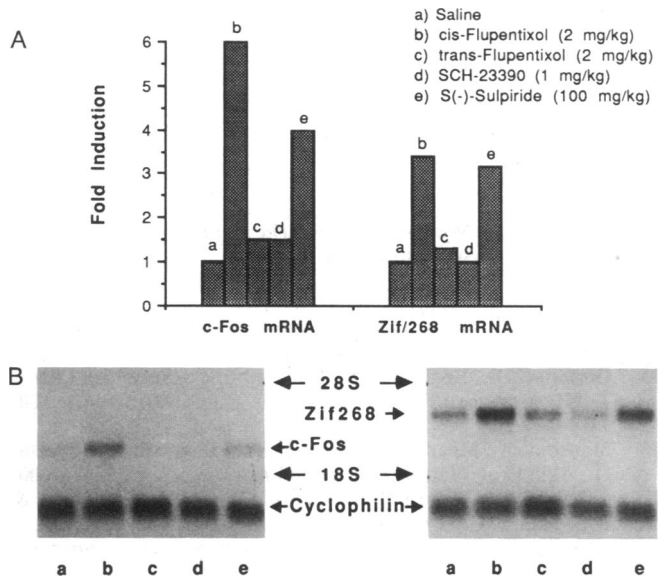

FIG. 5. (*A*) Fold induction of c-fos and zif268 mRNA 45 min after intraperitoneal injection of drugs (two experiments). The graph is a densitometric analysis of the Northern blot in *B*. (*B*) Upper band in the blot to the left is c-fos mRNA. Upper band in the blot to the right is zif268 mRNA. In both blots, the lower band is cyclophilin mRNA.

The distinct patterns of c-Fos-IR material observed in the striatum in response to haloperidol, clozapine, and amphetamine begin to address the specificity of c-Fos induction but raise interesting questions concerning how these patterns were generated. For example, the extent to which D_1 and D_2 receptors are segregated on different cells remains uncertain (15, 16). However, even if D_1 and D_2 receptors are largely found on different cells, additional mechanisms for drug-induced c-Fos activation must be hypothesized because the observed patterns of amphetamine and haloperidol-induced c-Fos-IR material do not correspond precisely with the known distributions of D_1 and D_2 receptors, respectively (16). The observed patterns of c-Fos-IR material induced by all three drugs also do not appear to correspond with the distributions of D_3 or D_4 receptors as reported to date (17, 18).

It is noteworthy that in contrast with the distinct patterns of expression in the caudate–putamen, haloperidol, clozapine, and amphetamine produced similar patterns of c-Fos-IR material at midlevels of the nucleus accumbens, a limbic region in which all three drugs are thought to exert important effects on motor activity and mood. However, it cannot be assumed that these drugs activated identical cells within the accumbens.

Dragunow *et al.* (2) have reported haloperidol-induced c-Fos immunoreactivity in striatum and showed a region of the striatum with relatively homogeneous immunostaining (6). Whereas they reported a rostral to caudal decline in

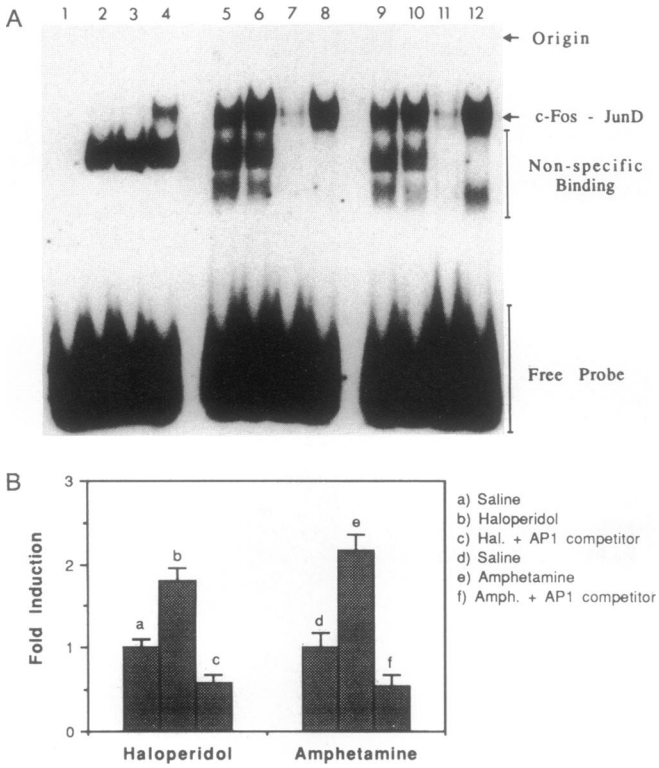

FIG. 6. (*A*) Binding of *in vitro*-translated proteins (1 μl of reticulocyte lysates containing c-Fos and/or JunD) or striatal extract (5 μg) to an end-labeled double-stranded AP-1 oligonucleotide. Conditions of binding by lane are as follows. Lanes: 1, AP1 probe without protein; 2, lysates containing *in vitro*-translated c-Fos; 3, JunD; 4, cotranslated c-Fos and JunD; 5, striatal extract from saline-treated rat; 6, haloperidol at 1 mg/kg; 7, haloperidol with unlabeled AP-1 competitor; 8, haloperidol with unlabeled AP-2 competitor; 9, saline; 10, D-amphetamine at 4 mg/kg; 11, D-amphetamine with AP-1 competitor; 12, D-amphetamine with AP-2 competitor. The arrow labeled c-Fos–JunD indicates the position of the specific band produced by binding of *in vitro*-translated protein to AP-1 probe (lane 4). (*B*) Densitometric analysis of specific bands produced by binding of striatal protein extracts to AP-1 oligonucleotide probe. Data are the mean and SEM from four experiments.

c-Fos-IR material in striatum after haloperidol, they did not comment on whether they observed a pattern of predominantly medial and lateral staining with a paucity of staining in the core of the mid- to posterior striatum, such as we observe. Graybiel *et al.* (4) have reported patchy expression of c-Fos induced by amphetamine, especially in the rostral striatum. We also observe patchy induction of c-Fos-IR material by amphetamine but find an increased density of cells medially. We observed central extension of the pattern of Fos-immunopositive cells with higher doses of amphetamine, although even at high doses of D-amphetamine (12–16 mg/kg), there was still heavier Fos immunostaining medially than laterally. Even at the highest doses of haloperidol (5 mg/kg) and D-amphetamine (16 mg/kg) tested, the patterns of c-Fos expression remained markedly different.

We studied AP-1 binding activity by gel-shift assay at a time (2 h) that corresponded to the maximal induction of c-Fos-IR material. The presence of substantial basal AP-1 binding activity (Fig. 6) is likely to result from constitutively expressed members of the *fos* and *jun* gene families (e.g., *junD*) in the striatum. The increases in AP-1 binding activity that we observe are likely to be due not only to c-*fos* induction but also to induction of the protein products of other IEGs as well.

In summary, our data reveal specificity of IEG activation in rat striatum in response to antipsychotic and stimulant drugs in two regards. (*i*) Different classes of antipsychotic drugs, with relative specificity for different dopamine and other monoamine receptors, do not activate all IEGs in concert. (*ii*) The effects of the D_2 receptor antagonist haloperidol, the atypical antipsychotic drug clozapine, and the indirect agonist D-amphetamine on c-Fos induction reveal anatomic specificity within the striatum. It will be interesting to determine whether activation of c-*fos* expression in the caudate–putamen by antipsychotic drugs correlates with their tendency to produce extrapyramidal side effects. Further investigation comparing the shared and unshared effects of typical and atypical antipsychotic drugs on neural gene expression at this cellular level of analysis is likely to provide important insights into the therapeutic mechanisms of action of these drugs.

Note Added in Proof. Robertson and Fibiger (19) have reported patterns of clozapine- and haloperidol-induced c-Fos immunoreactivity in the caudate–putamen and nucleus accumbens that are similar to those described here.

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