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Phenotypic Characterization of Neuroleptic-Sensitive Neurons in the Forebrain: Contrasting Targets of Haloperidol and Clozapine

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The prototypical neuroleptic haloperidol and the atypical antipsychotic clozapine induce distinctly different patterns of c-fos expression in the forebrain. While haloperidol appears to increase c-fos expression via its D2 dopamine receptor antagonist properties, the receptor mechanisms by which clozapine produces its unique pattern of c-fos expression are not known. The present experiments sought to address this question by determining the phenotypes of neurons in which clozapine increases Fos-like immunoreactivity (FLI). Fos immunostaining combined with in situ hybridization histochemistry using a cDNA oligonucleotide probe for D3 receptor mRNA indicated that the great majority (95%) of clozapine-induced FLI neurons in the major island of Calleja (ICjM) express D3 receptors. Similarly, in the nucleus accumbens (NAc) and lateral septal nucleus (LSN), the majority of clozapine-induced FLI neurons express D3 receptor mRNA (NAc 69%; LS 73%). In marked contrast, haloperidol-induced FLI neurons failed to express D3 receptors in any brain region. Studies with oligonucleotide probes for enkephalin (ENK) and dynorphin (DYN) indicated that clozapine increases c-fos expression in both ENK and DYN containing neurons in the NAc (ENK 40%, DYN 53%) and LSN (ENK 32%, DYN 59%). Haloperidol also increases c-fos expression in ENK and DYN containing neurons, albeit in a different pattern (striatum: ENK 93%, DYN 20%; nucleus accumbens: ENK 46%, DYN 36%; lateral septum: ENK 29%, DYN 18%). The present results demonstrate that haloperidol and clozapine target different populations of neurons even in regions such as the NAc and LSN, where they both increase c-fos expression. In addition, the fact that the majority of clozapine-sensitive neurons in NAc, LSN, and ICjM express D3 receptors suggests that activity at these receptors may contribute to the unique clinical profile of this antipsychotic agent. These data indicate that D3 receptors may represent novel targets in the pharmacotherapy of schizophrenia.

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KEY WORDS: *Clozapine; Haloperidol; D3 dopamine receptors; c-fos; Nucleus accumbens; Striatum; Enkephalin; Dynorphin*

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Clozapine has been termed an "atypical" antipsychotic agent in part because at therapeutic doses it fails to produce the extrapyramidal, Parkinsonian-like side effects that are associated with the use of "typical" neuroleptics such as haloperidol and chlorpromazine. While the mechanisms by which clozapine generates its unique clinical profile are not established, recent studies from several laboratories have demonstrated that, compared to typical neuroleptics, clozapine produces a rather unique regional pattern of Fos-like immunoreactive

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(FLI) neurons in the forebrain (Deutch et al. 1992; Nguyen et al. 1992; Robertson and Fibiger 1992; MacGibbon et al. 1994), Fos being the protein product of the immediate early gene c-fos which is thought to be an activity marker in some neurons (Sagar et al. 1988; Dragunow and Faull 1989). Thus, while haloperidol and clozapine both increase c-fos expression in the nucleus accumbens (NAc) and lateral septal nucleus (LSN), haloperidol also potently targets the dorsal striatum, where clozapine is essentially without effect, whereas clozapine also targets the islands of Calleja and the medial prefrontal cortex (PFC), where haloperidol is inactive. In the striatum, haloperidol is thought to increase c-fos expression by blocking D2 dopamine receptors on enkephalin-containing medium spiny neurons that project to the globus pallidus (Robertson et al. 1992). Dopaminergic mechanisms have also been implicated in the NAc by virtue of the fact that extensive lesions of the mesotelencephalic dopaminergic system block the effects of both haloperidol and clozapine on c-fos expression in this structure (Robertson and Fibiger 1992). Despite this, there are reasons to believe that haloperidol and clozapine may increase Fos-like immunoreactivity in different populations of accumbal neurons. For example, the distribution of FLI neurons within the NAc is not the same after the two drugs (Robertson and Fibiger 1992), clozapine being relatively more active in the so-called shell region, and haloperidol being approximately equally active in the core and shell (Deutch et al. 1992).

In a recent study, Guo et al. (1995) investigated the receptor mechanisms by which clozapine produces its unique pattern of c-fos expression in the forebrain and concluded that while its serotonergic (5-HT_2) and noradrenergic (α1) antagonist properties do not contribute, actions at dopamine D3 receptors may be responsible for this pattern of effects. The present study, therefore, combined in situ hybridization histochemistry for D3 receptor mRNA with Fos immunohistochemistry to determine if D3 receptors are expressed in neurons in which clozapine increases FLI. For comparative purposes, identical experiments were conducted with haloperidol.

Medium spiny projection neurons, which represent 90–95% of all neurons in the dorsal striatum, can be subdivided into two major populations: 1) enkephalinergic, D2-expressing cells with projections to the external division of the globus pallidus; and 2) D1-expressing dynorphin/substance P-containing neurons which innervate entopeduncular nucleus and the substantia nigra pars reticulata (Beckstead and Kersey 1985; Gerfen and Young 1988; Albin et al. 1989; Alexander and Crutcher 1990; Anderson and Reiner 1990; Gerfen et al. 1990; Le Moine et al. 1990, 1991; Gerfen 1992). While haloperidol selectivity increases c-fos expression in striato-pallidal, enkephalinergic neurons via a D2 receptor mechanism (Robertson et al. 1992), at present, the peptidergic phenotype of neurons in the NAc that are targeted by haloperidol is not known. Similarly, the extent to which clozapine increases FLI in enkephalinergic vs. dynorphinergic neurons in the NAc has not been investigated. To determine the peptidergic nature of neurons in the NAc which increase c-fos expression in response to clozapine or haloperidol, Fos immunohistochemistry was combined with in situ hybridization histochemistry using cDNA oligonucleotide probes for enkephalin or dynorphin. A similar analysis was applied to the lateral septal nucleus, major island of Calleja, medial prefrontal cortex and dorsal striatum.

MATERIALS AND METHODS

Adult male Wistar rats (280–320 gm) were maintained on a 12-hr light/12-hr dark cycle, with free access to food and water. The rats were handled periodically for 4–5 days prior to the experiment.

Drug Administration and Brain Section Preparation

Clozapine (H. Lundbeck, Kobenhavn-Valby) and haloperidol (McNeil Pharmaceutical, Stouffville, Canada) were dissolved in 40 μl of 20% acetic acid and brought to final volume (1 ml) with 0.9% saline. Rats were injected subcutaneously with clozapine (20 mg/kg) or haloperidol (1 mg/kg). Two hours after the injection, the rats were deeply anesthetized with pentobarbital and perfused with 0.9% saline (200 ml) followed by 150 ml 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). The brains were removed and placed in fresh fixative (4% PFA) for at least 12 hours. Twenty or 25 μm sections were cut from each brain using a Vibratome. The sections were collected in 0.02 M PBS for immunostaining and in situ hybridization.

Fos-Like Immunohistochemistry

All solutions were prepared with pyrogen-free, ultrapure water and autoclaved before using. Brain sections were processed for Fos immunostaining (Guo et al. 1992b) using the avidin-biotin-complex (ABC) method (Guo et al. 1992a) under RNAase-free conditions. Briefly, the free-floating sections were preincubated with 0.5% H_2O_2 for 15 min to remove endogenous peroxidase activity, and then incubated with the Fos primary antibody (Cambridge Research Biochemicals, CRB OA-11-824; 1:3000 dilution), a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA; 1:200), and avidin-biotinylated horseradish peroxidase complex (Vector Laboratories; 1:200). The sections were stained with 0.1% 3,3′-diaminobenzidine (DAB) in

0.002% H_2O_2, and then mounted on double chrome-alum-coated, diethyl pyrocarbonate-treated slides.

Oligonucleotide Probes

For D3 receptor mRNA in situ hybridization, three 35-mer oligonucleotides, derived from the putative third cytoplasmic loop of rat D3 receptor were synthesized (Biotechnology Laboratory, University of British Columbia), and purified by the butanol extraction method (Sawadogo and Van Dyke 1991). The three antisense oligonucleotides were complementary to bases 470–504, 901–935, or 1163–1197 of the rat D3 receptor (Sokoloff et al. 1990) and the sense of oligonucleotide was identical to the 901–935 base sequence. Although the data presented are based on the 901–935 probe, all three probes generated the same pattern of results. The ENK and DYN oligonucleotides (Young et al. 1986) were synthesized to be complementary to bases 382–421 and 870–909, respectively of preproenkephalin A (Yoshikawa et al. 1984) and prodynorphin (Civelli et al. 1985).

Oligonucleotides were tailed with ^{33}P-dATP (NEN Research Products, Boston, MA) or ^{35}S-dATP (NEN Research Products, Boston, MA) on the 3′-OH end of each oligonucleotide by terminal deoxynucleotidyl transferase (TdT) (NEN Research Products, Boston MA; Gibco BRL, Gaithersburg, MD). The reaction mixture contained 5 μl of 2 nmol/ml oligonucleotide, 7 μl 5 × TdT buffer (0.5 M potassium cacodylate, pH 7.2, 10 mM $CoCl_2$, 1 mM DTT), 5 μl 12.5 mCi/ml ^{33}P-dATP or ^{35}S-dATP, 15 μl H_2O, and 3 μl TdT. The reaction was carried out at 37°C for 30 min, and terminated by adding 100 μl reagent A (NEN Research Products, Boston, MA) on ice. The labeled oligonucleotide probes were purified with NENSORB purification cartridges (NEN Research Products, Boston, MA).

In Situ Hybridization

For each hybridization experiment, brain sections from 5–6 drug-treated rats were used. Hybridizations were carried out as described by Lewis et al. (1988), with minor modifications. Briefly, sections were hybridized with one of the three probes described above ($5 \times 10^5 - 1 \times 10^6$ cpm/section) at 37°C overnight in buffer containing 5 × SSPE (0.75 M NaCl, 0.05 M NaH_2PO_4, 0.0055 M EDTA, pH 7.4), 50% (vol/vol) deionized formamide, 10% (wt/vol) dextran sulfate, 1 × Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin), 0.25 mg/ml tRNA and 0.2 mg/ml salmon sperm DNA. The hybridization solution also contained 100 mM DTT (dithiothreitol) when a ^{35}S-labeled probe was used. After hybridization, the sections were washed at room temperature with 1 × SSC (saline sodium citrate buffer, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2) for 15 min × 4, then with 0.5 × SSC (4 × 15 min), followed by 4 × 15 min at 55–60°C with 0.5 × SSC. For ^{35}S-labeled sections, washing solutions contained 14 mM β-mercaptoethanol. The sections were air dried and examined autoradiographically by apposition to X-ray film (X-Omat, Kodak). The exposures were 3–5 days for ^{33}P- and 10–14 days for ^{35}S-labeled slides. The slides were then dipped into Kodak NTB2 nuclear emulsion (1:1 diluted with 0.6 M ammonium acetate) at 42°C, and exposed for 12–16 days at −70°C (^{33}P) or 3–5 weeks at room temperature (^{35}S). After being developed in Kodak D-19 at 16°C for 4 min, the sections were fixed, dehydrated, and mounted for microscopic image analysis.

Data Analyses

The particular area of each brain region that was studied in the present experiments corresponded to those delineated in Robertson and Fibiger (1992). To determine whether clozapine- or haloperidol-induced Fos-positive neurons colocalized with D3 receptor, ENK or DYN mRNA, these in situ mRNA signals in different brain regions were digitized using image quantification analysis with a MCID program (Imaging Research Inc., St. Catharines, Ontario). The density of in situ mRNA signals was measured as proportional grain area, i.e., grain area/scan area. For the major island of Calleja, in which cells are smaller than those in the striatal complex, a 91.9 μm^2 circle was used as the scanning area for each measured cell. For the other brain regions, the scan area/cell was 153.8 μm^2. Circles with the same area were placed in the vicinity of FLI neurons in different brain regions to obtain background measures (Figure 1). For each brain region, the measured neurons were classified into two groups: 1) Neurons with a grain ratio (proportional grain area) at least two times higher than the mean background grain ratio were assigned to the $mRNA^+/Fos^+$ (mRNA-positive/Fos-positive) group; and 2) FLI neurons which failed to meet this criterion were assigned to the FOS+ (mRNA-negative/Fos-positive group).

Between-group differences in the mean grain ratio of the two groups of neurons and background within specified brain regions were evaluated by one way ANOVA. Newman-Keul's post hoc test was performed to compare the density of in situ grains in Fos-positive neurons and background in each brain area.

RESULTS

Distribution of D3 mRNA

The major island of Calleja was heavily labeled by the D3 oligonucleotide antisense probe (Figure 2A). At low magnification, a moderate density of D3 mRNA signal was also evident in the lateral septal nucleus (Figures 2A, B). Labeling in the nucleus accumbens could only be resolved at higher magnification. In adjacent sec-

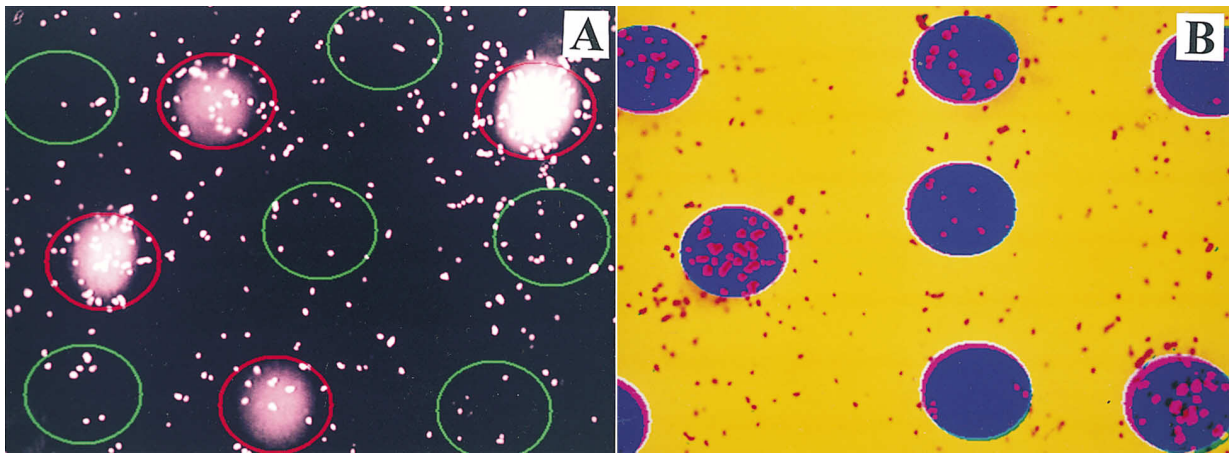

Figure 1. **(A)** Dark-field digitized image of clozapine-induced Fos-positive neurons in the NAc after in situ hybridization with the ^{33}P-labeled D3 antisense probe. Grain densities in individual neurons were measured with a defined area (red ovals). Circles with the same area (green) were placed in the vicinity of Fos-positive neurons to obtain the background measures. **(B)** After scanning, the density of in situ mRNA signals in each circle was measured as proportional grain area, i.e., grain area (red)/scan area (blue).

tions, neither ^{33}P- nor ^{35}S-labeled D3 sense oligonucleotide probes resulted in significant signal in these brain regions (Figure 2C).

Clozapine-Induced Fos Colonization. At high magnification, the great majority of FLI neurons in the ICjM met the criterion for positive labeling with the ^{33}P D3 antisense probe (Figure 3A). Similarly, some of the FLI neurons in the lateral septal nucleus (Figure 3C) and nucleus accumbens (Figure 3B) were significantly labeled with the D3 probe. These impressions were confirmed by quantitative analysis of the autoradiographic data wherein the criterion for positive labeling was set as being at least twice the level of background activity (see Materials and Methods). As shown in Table 1, the average density of D3 antisense labeling in neurons that met this criterion was about 4 times greater than grain ratios of either background or of FLI neurons that failed to meet the D3 positive criterion ($D3^+/Fos^+$ grain ratios 0.098–0.114; background grain ratios 0.022–0.025; $D3^-/Fos^+$ grain ratios 0.027–0.030). This 3–4 fold difference in grain density between double labeled and single labeled or background measures was seen in all of the experiments (Tables 1–6) and serves to confirm the validity of this quantitative approach.

Table 1 shows that nearly all (94.5%) of the clozapine-

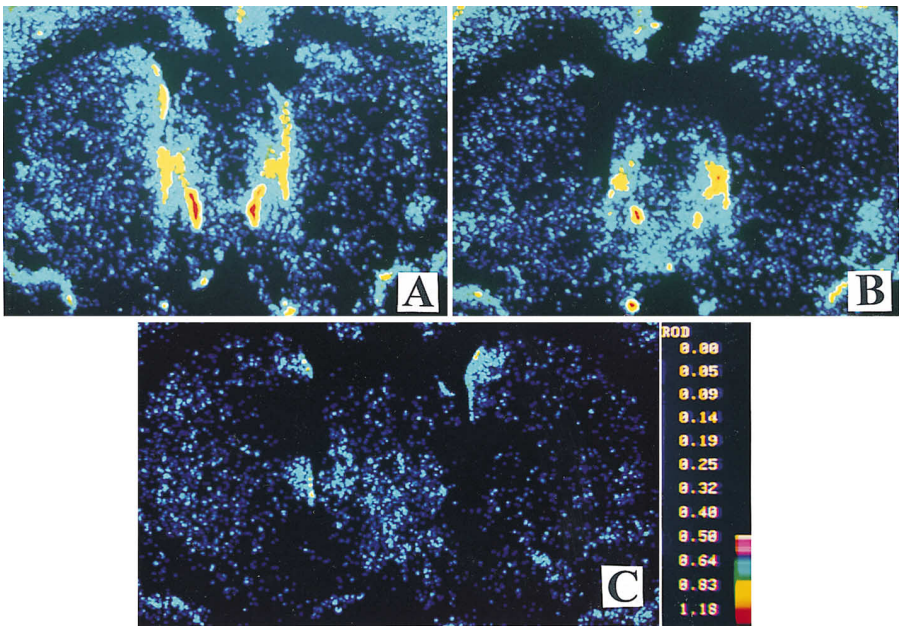

Figure 2. Pseudocolor coded dark-field digitized images of coronal sections showing the distribution of D3 receptor mRNA in rat brain by in situ hybridization. D3 receptor mRNA is expressed in the ICjM, and, less abundantly, in the LSN **(A, B)**. **(C)** A brain section between A and B hybridized with the ^{33}P-labeled D3 sense probe. The scale bar represents relative optical densities (ROD) of D3 receptor transcript levels in brain sections.

induced FLI neurons in the major island of Calleja express D3 mRNA. Similarly, in the nucleus accumbens the majority (69%) of FLI neurons in this structure also expressed above background levels of D3 mRNA (Table 1). In the lateral septal nucleus, many (73%) of the clozapine-induced FLI neurons were also positive for D3 message.

Haloperidol-Induced Fos Colocalization. In contrast to clozapine, haloperidol-induced FLI neurons very rarely colocalized with D3 message in the nucleus accumbens (Figure 3E), lateral septal nucleus (Figure 3F), or dorsal striatum. This was confirmed by the quantitative analysis (Table 2), which showed that only a small minority of haloperidol-induced FLI neurons met the criterion for being D3 positive in the striatum (4%), nucleus accumbens (9%), or lateral septal nucleus (6%).

Distribution of Enkephalin mRNA

At low magnification, it was evident that enkephalin mRNA was abundant in the striatum, nucleus accum-

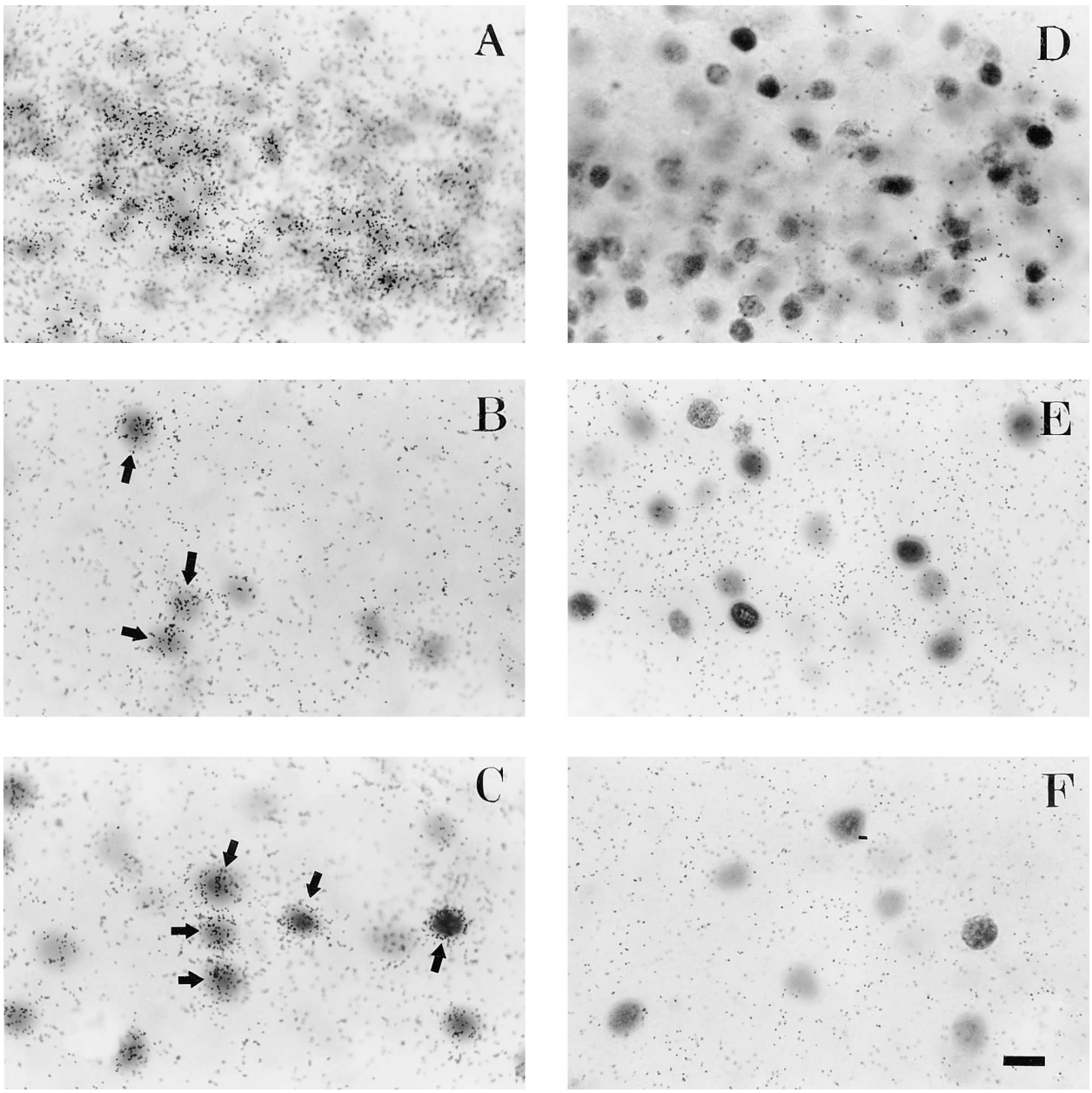

Figure 3. Bright-field views of rat brain sections after in situ hybridization with ^{33}P-labeled D3 probes. In rats treated with clozapine **(A-D)**, most Fos-positive neurons in the ICjM **(A)**, and many in the NAc **(B)** and LSN **(C)** were labeled by the D3 antisense probe, while the D3 sense probe only produced few autoradiographic grains in the ICjM **(D)**. In contrast, most haloperidol-induced Fos-positive neurons in the NAc **(E)** and LSN **(F)** did not contain D3 receptor mRNA.

Table 1. Proportion of Clozapine-Induced Fos-Positive Neurons that Express D3 Receptor mRNA in the Forebrain

	Prefrontal Cortex		Nucleus Accumbens		Lateral Septum		Major Island of Calleja	
Brain Region	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]
Total	127		312		291		127	
$D3^{+c}/Fos^{+}$	36	0.101 ± 0.005[e]	216	0.111 ± 0.005[e]	212	0.100 ± 0.004[e]	120	0.114 ± 0.005[e]
Fos^{+d}	91	0.029 ± 0.002	96	0.030 ± 0.003	79	0.029 ± 0.002	7	0.027 ± 0.003
Background	30	0.025 ± 0.002	60	0.025 ± 0.002	45	0.024 ± 0.003	30	0.022 ± 0.002
Colocalized neurons (%)	28.3		69.2		72.9		94.5	

[a]Grain ratio: proportional grain area, i.e., grain area/scan area. For the major island of Calleja, scan area/cell = 91.9 μm^2. For the other brain regions, scan area/cell or background area = 153.8 μm^2.
[b]Data represent the mean (± S.E.M.) proportional grain area of Fos-positive neurons or background areas.
[c]$D3^{+}$: D3 receptor mRNA-positive; Fos^{+}: Fos-positive. Neurons with the grain ratios two times higher than the mean proportional grain area of the background were assigned to this group.
[d]Neurons with grain ratios less than two times the mean proportional grain area of the background were assigned to the Fos^{+} group.
[e]Significantly different from background area and Fos^{+} neurons ($P < .001$).

bens and olfactory tubercles, and present, but less so, in the lateral septal nucleus.

Clozapine-Induced Fos Colocalization. While only a small minority (6%) of clozapine-induced FLI neurons in the major island of Calleja was labeled with the enkephalin mRNA probe (Figure 4C; Table 3), a significant percentage of FLI neurons in the nucleus accumbens (40%) and lateral septal nucleus (32%) met the criteria for being enkephalinergic (Figures 4A and B). The vast majority (90%) of FLI neurons in the medial prefrontal cortex of clozapine-treated animals failed to meet this criterion and were therefore classified as non-enkephalinergic (Table 3).

Haloperidol-Induced Fos Colocalization. In the striatum, nearly all (93%) haloperidol-induced FLI neurons were also labeled with the enkephalin mRNA probe (Table 4; Figure 5A). In the nucleus accumbens (Figure 5B) and lateral septal nucleus (Figure 5C), the number of FLI neurons meeting the criterion for being enkephalinergic was much smaller, being 46% in the nucleus accumbens and 29% in the lateral septum (Table 4).

Distribution of Dynorphin mRNA

In accordance with previous reports (Young et al. 1986; Gerfen and Young 1988), the macroscopic distribution of dynorphin mRNA was similar to that of enkephalin except that dynorphin transcripts were also found in the cerebral cortex and higher levels were observed in the ventral than in the dorsal striatum.

Clozapine-Induced Fos Colocalization. The majority (61%) of FLI neurons met the criterion for being dynorphinergic in the major island of Calleja (Table 5; Figure 5F). In addition, more than half of the clozapine-induced FLI neurons in the nucleus accumbens (53%) and lateral septal nucleus (59%) met this criterion (Table 5; Figures 5D and E). In the medial prefrontal cortex about one-

Table 2. Proportion of Haloperidol-Induced Fos-Positive Neurons that Express D3 Receptor mRNA in the Forebrain

	Striatum		Nucleus Accumbens		Lateral Septum	
Brain Region	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]
Total	215		220		191	
$D3^{+}/Fos^{+c}$	9	0.088 ± 0.011[e]	19	0.105 ± 0.009[e]	11	0.096 ± 0.008[e]
Fos^{+d}	206	0.024 ± 0.002	201	0.020 ± 0.002	180	0.020 ± 0.002
Background	60	0.022 ± 0.002	60	0.021 ± 0.002	40	0.026 ± 0.002
Colocalized neurons (%)	4.2		8.6		5.8	

[a]Grain ratio: proportional grain area, i.e., grain area/scan area. For all measured brain regions, scan area/cell or background area = 153.8 μm^2.
[b]Data represent the mean (± S.E.M.) proportional grain area of Fos-positive neurons or background areas.
[c]$D3^{+}$: D3 receptor mRNA-positive; Fos^{+}: Fos-positive. Neurons with the grain ratios two times higher than the mean proportional grain area of the background were assigned to this group.
[d]Neurons with grain ratios less than two times the mean proportional grain area of the background were assigned to Fos^{+} group.
[e]Significantly different from background area and Fos^{+} neurons ($P < .001$).

Table 3. Proportion of Clozapine-Induced Fos-Positive Neurons that Express Enkephalin mRNA in the Forebrain

	Prefrontal Cortex		Nucleus Accumbens		Lateral Septum		Major Island of Calleja	
Brain Region	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]
Total	129		304		284		128	
Enk^+/Fos^{+c}	13	0.091 ± 0.007[e]	122	0.118 ± 0.006[e]	92	0.101 ± 0.004[e]	8	0.089 ± 0.006[e]
Fos^{+d}	116	0.027 ± 0.002	182	0.030 ± 0.002	192	0.029 ± 0.002	120	0.025 ± 0.002
Background	30	0.026 ± 0.002	55	0.035 ± 0.003	45	0.023 ± 0.003	30	0.025 ± 0.002
Colocalized neurons (%)		10.1		40.1		32.4		6.3

[a]Grain ratio: proportional grain area, i.e., grain area/scan area. For the major island of Calleja, scan area/cell = 91.9 μm^2. For the other brain regions, scan area/cell or background area = 153.8 μm^2.
[b]Data represent the mean (± S.E.M.) proportional grain area of Fos-positive neurons or background areas.
[c]Enk^+: enkephalin mRNA-positive; Fos^+: Fos-positive. Neurons with the grain ratios two times higher than the mean proportional grain area of the background were assigned to this group.
[d]Neurons with grain ratios less than two times the mean proportional grain area of the background were assigned to Fos^+ group.
[e]Significantly different from background area and Fos^+ neurons ($P < .001$).

fifth (19%) of the FLI neurons showed significant labeling with the ^{35}S dynorphin probe.

Haloperidol-Induced Fos Colocalization. About one-fifth of the FLI neurons in the striatum (20%) and lateral septal nucleus (18%) showed significant labeling for dynorphin mRNA (Table 6; Figures 5D and F). In the nucleus accumbens, the percentage of haloperidol-induced FLI neurons that also met the criterion for being dynorphinergic was higher, reaching 38% (Table 6; Figure 5E).

DISCUSSION

Distribution of D3 Receptor mRNA

The present results confirm previous reports that granule cells in the islands of Calleja express D3 receptor mRNA in high abundance (Sokoloff et al. 1990; Bouthenet et al. 1991; Landwehrmeyer et al. 1993; Diaz et al. 1995). Although labeling in the nucleus accumbens was not obvious at low magnification, when viewed under higher power it was clear that significant numbers of neurons in the nucleus accumbens also contained above background levels of D3 mRNA (Figure 3b; Table 1), this being consistent with these earlier reports. The present experiments also indicate that there is a moderate level of D3 mRNA expression in the caudal aspect of the lateral septal nucleus and prefrontal cortex (Figures 2A and B), this also being in agreement with previous communications (Sokoloff et al. 1990; Le Moine and Bloch 1996; Diaz et al. 1997). The presence of D3 mRNA in the lateral septal nucleus and prefrontal cortex was confirmed when the brain sections were examined under higher power magnification as many neurons in this structure showed above background labeling with the D3 probe (Figure 3C; Table 1).

Table 4. Proportion of Haloperidol-Induced Fos-Positive Neurons that Express Enkephalin mRNA in the Forebrain

	Striatum		Nucleus Accumbens		Lateral Septum	
Brain Region	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]
Total	284		256		201	
Enk^+/Fos^{+c}	263	0.180 ± 0.009[e]	118	0.129 ± 0.006[e]	58	0.119 ± 0.052[e]
Fos^{+d}	24	0.050 ± 0.003	138	0.041 ± 0.002	143	0.034 ± 0.002
Background	60	0.036 ± 0.003	50	0.038 ± 0.002	40	0.030 ± 0.002
Colocalized neurons (%)		92.6		46.1		28.9

[a]Grain ratio: proportional grain area, i.e., grain area/scan area. For all measured brain regions, scan area/cell or background area = 153.8 μm^2.
[b]Data represent the mean (± S.E.M.) proportional grain area of Fos-positive neurons or background areas.
[c]Enk^+: enkephalin mRNA-positive; Fos^+: Fos-positive. Neurons with the grain ratios two times higher than the mean proportional grain area of the background were assigned to this group.
[d]Neurons with grain ratios less than two times of the mean proportional grain area of the background were assigned to Fos^+ group.
[e]Significantly different from background area and Fos^+ neurons ($P < .001$).

Table 5. Proportion of Clozapine-Induced Fos-Positive Neurons that Express Dynorphin mRNA in the Forebrain

	Prefrontal Cortex		Nucleus Accumbens		Lateral Septum		Major Island of Calleja	
Brain Region	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]
Total	149		298		264		134	
Dyn^+/Fos^{+c}	29	0.092 ± 0.004[e]	158	0.129 ± 0.006[e]	155	0.100 ± 0.004[e]	82	0.096 ± 0.004[e]
Fos^{+d}	120	0.023 ± 0.002	140	0.040 ± 0.003	109	0.028 ± 0.002	52	0.028 ± 0.002
Background	30	0.021 ± 0.002	50	0.037 ± 0.003	40	0.026 ± 0.003	30	0.023 ± 0.003
Colocalized neurons (%)	19.4		53.0		58.7		61.2	

[a]Grain ratio: proportional grain area, i.e., grain area/scan area. For the major island of Calleja, scan area/cell = 91.9 μm^2. For the other brain regions, scan area/cell or background area = 153.8 μm^2.
[b]Data represent the mean (± S.E.M.) proportional grain area of Fos-positive neurons or background areas.
[c]Dyn^+: dynorphin mRNA-positive; Fos^+: Fos-positive. Neurons with the grain ratios two times higher than the mean proportional grain area of the background were assigned to this group.
[d]Neurons with grain ratios less than two times of the mean proportional grain area of the background were assigned to Fos^+ group.
[e]Significantly different from background area and Fos^+ neurons ($P < .001$).

Phenotypes of Clozapine-Induced Fos-Positive Neurons

Nucleus Accumbens. The majority (69%) of neurons in which clozapine increased FLI expressed above background levels of D3 mRNA (Table 1). Furthermore, 40% of the FLI neurons in the NAc were positive for enkephalin mRNA, while 53% were positive for dynorphin (Tables 3 and 4). As is the case in the dorsal striatum (Penny et al. 1986; Gerfen and Young 1988; Gerfen 1992), enkephalin and dynorphin/substance P are largely found in different populations of medium spiny neurons in the nucleus accumbens (Le Moine and Bloch 1995). Both populations of neurons express D3 receptor mRNA, although this is more common in the dynorphin/substance P cells (Le Moine and Bloch 1996). It appears, therefore, that clozapine increases c-fos expression in both of these populations.

Diaz et al. (1995) raised the possibility that some neurons in the nucleus accumbens co-express D3 and D1 receptors and this has now been documented (Le Moine and Bloch 1996). In view of the fact that in the nucleus accumbens D1 receptors are expressed primarily in dynorphin-containing neurons (Le Moine et al. 1991), it is possible that some clozapine-sensitive neurons in this structure consists of medium-spiny dynorphinergic cells that express both D1 and D3 receptors. The projections of these cells are not known but could include the ventral pallidum and the midbrain (Robertson and Jian 1995).

Major Islands of Calleja. Nearly 95% of the granule cells in the ICjM were positive for D3 message. In addition, the majority were positive for dynorphin but negative for enkephalin. Previous studies have shown that the ICjM contains substance P (Beckstead and Kersey 1985; Gerfen and Young 1988; Harlan et al. 1989). This suggests that ICjM neurons contain both dynorphin and substance P as is the case for some medium spiny neurons in the dorsal striatum. Granule cells in the

Table 6. Proportion of Haloperidol-Induced Fos-Positive Neurons that Express Dynorphin mRNA in the Forebrain

	Striatum		Nucleus Accumbens		Lateral Septum	
Brain Region	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]	Number of Neurons	Grain Ratio[a,b]
Total	315		456		246	
Dyn^+/Fos^{+c}	64	0.123 ± 0.005[e]	175	0.132 ± 0.008[e]	44	0.112 ± 0.008[e]
Fos^{+d}	251	0.026 ± 0.002	281	0.034 ± 0.002	202	0.028 ± 0.002
Background	60	0.020 ± 0.002	60	0.028 ± 0.002	45	0.027 ± 0.002
Colocalized neurons (%)	20.3		38.4		17.9	

[a]Grain ratio: proportional grain area, i.e., grain area/scan area. For all measured brain regions, scan area/cell or background area = 153.8 μm^2.
[b]Data represent the mean (± S.E.M.) proportional grain area of Fos-positive neurons or background areas.
[c]Dyn^+: dynorphin mRNA-positive; Fos^+: Fos-positive. Neurons with the grain ratios two times higher than the mean proportional grain area of the background were assigned to this group.
[d]Neurons with grain ratios less than two times of the mean proportional grain area of the background were assigned to Fos^+ group.
[e]Significantly different from background area and Fos^+ neurons ($P < .001$).

ICjM have also been shown to express D1 (Fremeau et al. 1991; Mengod et al. 1991) but not D2 dopamine receptors (Landwehrmeyer et al. 1993). Since nearly all granule cells in the ICjM express D3 receptors (Table 1), it seems certain that at least some of these neurons co-express both D1 and D3 receptors and utilize dynorphin and substance P as peptide transmitters as reported by Le Moine and Bloch (1996).

Lateral Septal Nucleus. The majority (73%) of clozapine-induced FLI neurons in the lateral septum expressed D3 receptor mRNA. In addition, the majority (59%) were also positive for dynorphin while a minority (32%) expressed enkephalin mRNA. The latter results are consistent with previous reports that there are enkephalinergic (Kivipelto and Panula 1986) and dynorphinergic (Young et al. 1986; Neal and Newman 1989) neurons in the lateral septal nucleus. The extent to which these markers identify the same or different populations of clozapine-sensitive neurons in the lateral septal nucleus remains to be determined. In addition, the afferent and efferent connections of the clozapine-sensitive neurons in the lateral septal nucleus are not presently known.

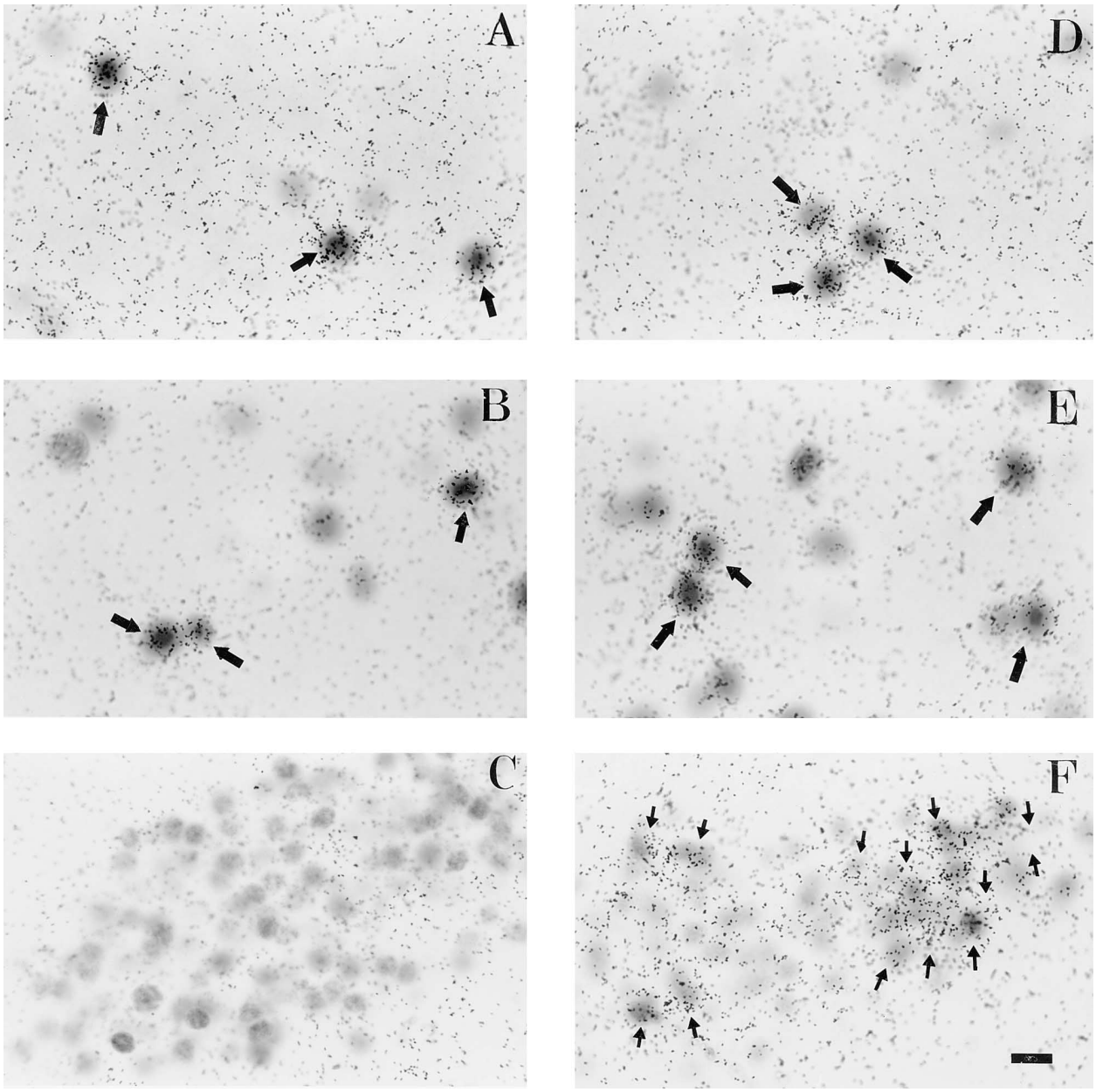

Figure 4. Bright-field views of brain sections after in situ hybridization with ^{35}S-labeled enkephalin **(A-C)** or dynorphin **(D-F)** probes. Many clozapine-induced Fos-positive neurons in the NAc **(A, D)** and LSN **(B, E)** express either Enk or Dyn mRNA. In the ICj, most clozapine-induced Fos-positive neurons do not express Enk mRNA **(C)**, whereas many contain Dyn mRNA **(F)**.

Phenotypes of Haloperidol-Induced Fos-Positive Neurons

Striatum. In accordance with previous results (Robertson et al. 1992), the large majority (93%) of haloperidol-induced FLI neurons in the dorsal striatum was enkephalinergic. The fact that nearly none of these neurons (4%) contained message for D3 receptors is also consistent with earlier work indicating that enkephalinergic neurons in the dorsal striatum express D2 receptors (Gerfen et al. 1990; Le Moine et al. 1990; Gerfen 1992) and that haloperidol increases c-fos expression in this structure via its antagonist properties at D2 receptors (Robertson and Fibiger 1992; Robertson et al. 1992). A small percentage (20%) of haloperidol-induced FLI neurons also met the criterion for being classified as dynorphinergic. Inasmuch as there is evidence that a subpopulation of neurons in the dorsal striatum coexpress both dynorphin and enkephalin (Penny et al. 1986; Gerfen and Young 1988), it seems likely that the

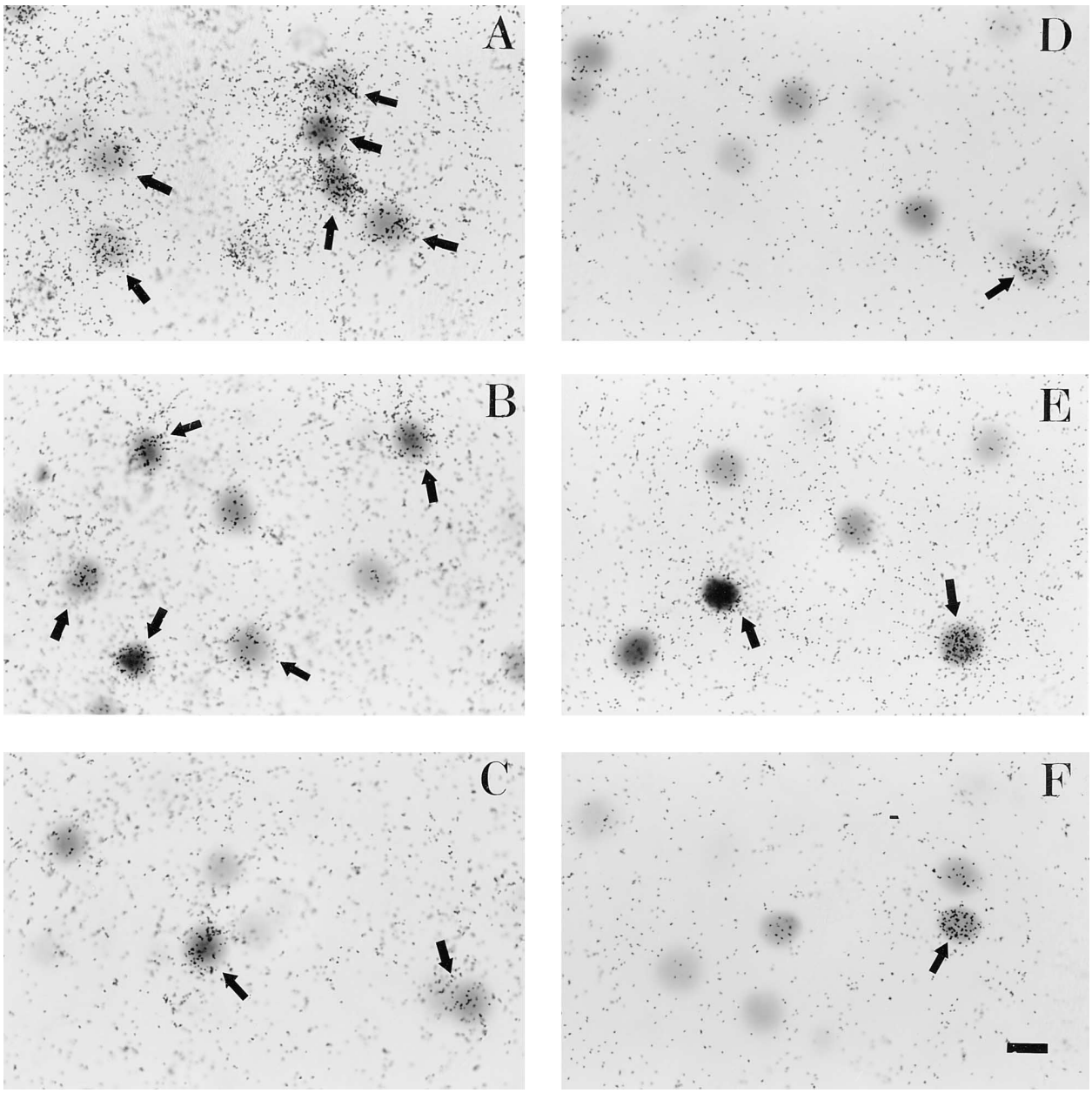

Figure 5. Bright-field views of brain sections from haloperidol-treated rats after in situ hybridization with the enkephalin **(A-C)** or dynorphin **(D-F)** probe. Most haloperidol-induced Fos-positive neurons in the striatum **(A)**, and fewer in the NAc **(B)** and LSN **(C)** express Enk mRNA. Some haloperidol-induced Fos-positive neurons in the striatum **(D)**, NAc **(E)**, and LSN **(F)** contain Dyn mRNA.

dynorphinergic neurons were in fact dynorphin/enkephalin co-expressing cells.

Nucleus Accumbens. Like the dorsal striatum, the great majority (91%) of haloperidol-induced FLI neurons in the nucleus accumbens failed to express significant D3 receptor message. However, 46% of the haloperidol sensitive neurons reached the criterion for being classified as enkephalinergic, while 38% were dynorphinergic. As was the case for clozapine-responsive neurons, the majority of haloperidol-responsive neurons in the nucleus accumbens appears to project to the ventral pallidum; however, a few innervate the midbrain (Robertson and Jian 1995).

The failure of haloperidol-induced FLI neurons to express significant D3 receptor message indicates that clozapine and haloperidol target different populations of neurons in the nucleus accumbens and although direct evidence is lacking, it seems reasonable to hypothesize that as is the case in the dorsal striatum, haloperidol increases c-fos expression in the nucleus accumbens via its D2 receptor antagonist properties. This is consistent with the findings that there are different populations of medium spiny neurons in this structure. All of the enkephalin neurons appear to express the D2 receptor, but a subpopulation also expresses D3 receptor mRNA (Le Moine and Bloch 1996). Furthermore, all of the substance P/dynorphin neurons express D1 receptors, while a subgroup of them also expresses the D3 receptor (Le Moine and Bloch 1996). Thus, both haloperidol and clozapine may each target at least two distinct subpopulations (D3 positive/enkephalin positive and D3 positive/dynorphin positive in the case of clozapine, and D2 positive/enkephalin positive and D3 negative/dynorphin positive in the case of haloperidol).

Lateral Septal Nucleus. As was the case for the other brain regions, haloperidol-induced FLI neurons in the lateral septal nucleus did not express D3 receptors. However, some (29%) met the criterion for being classified as enkephalinergic and a few (18%) were positive for dynorphin message. Clozapine and haloperidol therefore appear to target different populations of neurons in the lateral septal nucleus as most (73%) of the clozapine-sensitive cells express D3 receptors, while haloperidol-sensitive cells do not. Furthermore, there is a large difference in the percentage of dynorphin-positive cells that show increased c-fos expression in response to the two antipsychotic agents (59% for clozapine vs. 18% for haloperidol). Therefore, while many clozapine-sensitive cells in the lateral septal nucleus express D3 receptors and are dynorphinergic, the phenotype(s) of the haloperidol-sensitive cells remains to be determined.

Concluding Remarks

Recent pharmacological studies have provided evidence that clozapine-induced c-fos expression in the nucleus accumbens, major island of Calleja and lateral septal nucleus is mediated by antagonist actions of this antipsychotic at D3 dopamine receptors (Guo et al. 1995). The colocalization of D3 receptor mRNA and Fos-like protein following clozapine in these limbic brain regions is consistent with this hypothesis (Table 1). However, treatment with the D3 antagonist GR103691 does not appear to induce c-fos (Hurley et al. 1996b). Furthermore, the failure of haloperidol to increase FLI in D3 expressing neurons (Table 2), despite the fact that it has relatively high affinity for D3 receptors, suggests that although D3 receptor blockade may play a role in clozapine-induced c-fos expression, other actions of clozapine also likely contribute. The recent report that chronically administered clozapine and haloperidol have markedly different effects on the expression of D2 and D3 receptors in the striatum, nucleus accumbens, and major island of Calleja is consistent with the hypothesis that these two antipsychotic agents differ with respect to their actions on D2- and D3-expressing neurons in vivo (Hurley et al. 1996a).

The mechanisms by which clozapine produces its unique clinical profile (i.e., lack of extrapyramidal side effects in the presence of antipsychotic action, and superior activity against negative symptoms in schizophrenia) remain to be determined. On the basis of the restricted "limbic" distribution of D3 receptors, as well as the fact that some clinically effective antipsychotic agents have reasonably high affinities for these receptors, Schwartz and colleagues have hypothesized that activity at D3 receptors may contribute to the therapeutic actions of these compounds (Bouthenet et al. 1991; Sokoloff et al. 1990). The present finding that the majority of clozapine-sensitive neurons in the nucleus accumbens, lateral septal nucleus, and major island of Calleja express D3 receptors is consistent with this hypothesis and suggests, in particular, that clozapine may produce some of its unique clinical effects by actions at D3 as opposed to D2 receptors in vivo. The results of clinical trials with D3 selective antagonists will be informative in this regard.

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