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Dopamine D_3 receptors are not involved in the induction of c-*fos* mRNA by neuroleptic drugs: comparison of the dopamine D_3 receptor antagonist GR103691 with typical and atypical neuroleptics

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Abstract

The effect of acute and chronic administration of dopamine receptor antagonists on the expression of mRNA encoding the cellular immediate-early gene c-*fos* was investigated in rat brain by in situ hybridization using ^{35}S-labelled oligonucleotide probes. The selective dopamine D_3 receptor antagonist GR103691 had no effect on the level of c-*fos* mRNA after acute or chronic treatment. Acute treatment with haloperidol increased the level of c-*fos* mRNA in the caudate-putamen, nucleus accumbens shell and core, olfactory tubercle and parietal cortex. After chronic treatment with haloperidol increases in the level of c-*fos* mRNA in the caudate-putamen and nucleus accumbens core were no longer observed. The increase in the level of c-*fos* mRNA in the nucleus accumbens shell was attenuated but still significantly elevated above the level measured in vehicle-treated animals. In the olfactory tubercle, parietal cortex, frontal cortex and cingulate cortex the level of c-*fos* mRNA was decreased after chronic haloperidol treatment. Acute sulpiride treatment reduced the level of c-*fos* mRNA in the olfactory tubercle, parietal cortex and cingulate cortex. After chronic treatment with sulpiride the level of c-*fos* mRNA was reduced in the dorsal caudate-putamen only. Acute clozapine treatment increased the level of c-*fos* mRNA in the nucleus accumbens shell and islands of Calleja. After chronic treatment with clozapine the level of c-*fos* mRNA remained elevated in the islands of Calleja but not in the nucleus accumbens shell. These results indicate that acute and chronic blockade of dopamine D_3 receptors does not cause induction of c-*fos* transcription in limbic, striatal or cortical regions of rat brain. This study also demonstrated that acute blockade of dopamine receptors with haloperidol, sulpiride and clozapine induced different regionally specific patterns of c-*fos* expression which were altered after chronic blockade.

Keywords: Dopamine D_3 receptor; c-*fos*; Hybridization in situ; Neuroleptic; (Rat)

1. Introduction

The application of molecular cloning techniques to the study of dopamine receptors has expanded the classical dopamine D_1/D_2 receptor classification scheme to include five dopamine receptor subtypes. These may be divided into D_1-like (D_1 and D_5) and D_2-like (D_2, D_3 and D_4) based upon their predicted transmembrane topologies and functional and pharmacological properties when expressed in cells (Sibley and Monsma, 1992). The dopamine D_3 receptor is of particular interest because of its potential involvement in the genesis and treatment of psychiatric and neurological disease.

Alterations in receptor expression are believed to be mediated, at least in part, through sustained changes in the rate of gene transcription. Changes in the rate of gene transcription are under the control of transcriptional regulatory proteins. Activation of one group of transcriptional regulatory proteins occurs within minutes of cellular stimulation. Hence, genes encoding these proteins are called cellular immediate-early genes (Morgan and Curran, 1989). Chronic blockade of dopaminergic neurotransmission with dopamine receptor antagonists is known to increase the level of dopamine D_2 receptor expression in the striatum

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and to result in a functional supersensitivity (Burt et al., 1977; Rupniak et al., 1985a,b; Savasta et al., 1988). This increase is believed to contribute to some of the motor side-effects of neuroleptic treatment. One method of identifying where these transcriptional changes might occur after manipulations of dopaminergic neurotransmission is to examine the expression of immediate-early genes. One immediate-early gene, c-*fos*, is affected by dopaminergic neurotransmission and is induced in a regionally specific manner by typical and atypical neuroleptics (Miller, 1990; Robertson and Fibiger, 1992). It has been demonstrated that haloperidol and raclopride increase c-*fos* expression in the caudate-putamen while clozapine increases c-*fos* expression in the nucleus accumbens. It has been suggested that differences in the pattern of c-*fos* expression account for the varying propensity of neuroleptics to induce extrapyramidal side-effects (Dragunow et al., 1990; Merchant and Dorsa, 1993; Miller, 1990; Nguyen et al., 1992; Robertson and Fibiger, 1992). Nothing is known about the contribution of the dopamine D_3 receptor to the changes in expression of c-*fos* induced by neuroleptic drugs.

In this study the effect of acute and chronic administration of the selective dopamine D_3 receptor antagonist GR103691 (Murray et al., 1995) on c-*fos* expression was compared to those of the typical and atypical neuroleptics haloperidol, clozapine and sulpiride. Since these neuroleptics have relatively high affinity at the dopamine D_3 receptor (Table 1) such a comparison may enable elucidation of any role the dopamine D_3 receptor has in the effects of neuroleptics on c-*fos* induction.

2. Materials and methods

2.1. Housing of animals

Adult male Sprague-Dawley rats (Bantin and Kingman, Hull, UK) were housed in groups of six with free access to rat chow and water. The animals were kept in a temperature controlled environment (21 ± 2°C) on a 12 h light/dark cycle (light period 07:00–19:00 h).

Table 1
Comparison of the receptor affinities (K_i) for antagonists at dopamine D_1, D_2 and D_3 receptors

	K_i value (nM)		
	D_1	D_2	D_3
GR103691	398	40	0.3
Haloperidol	~ 80	1.2	~ 7
Sulpiride	~ 45000	~ 15	~ 13
Clozapine	~ 170	~ 230	~ 170

Data taken from Seeman and Van Tol (1994) and Murray et al. (1995) (GR103691).

2.2. Drug administration

Rats were randomly allocated to one of five treatment groups. The treatments consisted of once daily subcutaneous injections of GR103691 (1 mg/kg), haloperidol (5 mg/kg), (−)-sulpiride (100 mg/kg), clozapine (20 mg/kg) or vehicle (deionized water pH 5.0) in an injection volume of 1 ml/kg. Rats in each treatment group received either a single injection of antagonist (acute treatment) or a series of once daily injections for 21 days (chronic treatment). The rats receiving chronic treatment were weighed every third day and the injection volume adjusted accordingly. The doses of drugs used (haloperidol, sulpiride and clozapine) in this study were relevant to those used clinically for the treatment of schizophrenia but increased by an arbitrary factor of 5 to account for the generally lesser pharmacological sensitivity of rats to such compounds (Jenner and Marsden, 1987).

2.3. Tissue preparation

Rats were killed by cervical dislocation 30 min after the last injection. Brains were removed and frozen in isopentane (−45°C). Frozen brains were sectioned (12 μm) with a cryostat (Bright Instruments), thaw-mounted onto chrome alum/gelatin-coated slides, allowed to dry, and stored at −70°C until use.

2.4. In situ hybridization

The level of c-*fos* mRNA was assessed in rat brain sections by in situ hybridization using ^{35}S-labelled oligonucleotide probes. To increase the intensity of the hybridization signal, a mixture of two oligonucleotide probes were used to hybridize to the c-*fos* mRNA transcripts. The distribution of the c-*fos* mRNA was quantified in units of amol/mg tissue by the use of brain paste standards in a way analogous to quantitative receptor autoradiography (Nunez et al., 1989).

2.5. Probe design

Probes were selected by computer analysis of nucleotide sequences using the University of Wisconsin Genetics Computer Group Sequence Analysis Package (GCG) and by accessing the National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) program via electronic mail (Altschul et al., 1990; Devereux et al., 1984).

Putative probe sequences were screened against all known nucleic acid sequences held within the Genetic Sequence Data Bank (Genbank) and European Molecular Biology Laboratory (EMBL) databases, using the BLAST program. No probe showed significant homology with any sequence (other than itself) within the databases.

Two probes (bases 141–188, 5′ – act gca gcg gga gga tga cgc ctc gta gtc cgc gtt gaa acc cga gaa – 3′; and bases 1001–1048, 5′ – ggc tcc cag tct gct gca tag aag gaa cca gac agg tcc aca tct ggc – 3′) were selected from the c-*fos* sequence (accession No. X06769). The base numbers are those of the sequences as they appear in the GCG database not from the start codon. The probes were taken from areas which show little homology between immediate-early genes, i.e., not from either of the highly conserved homology regions of immediate-early genes which are thought to be involved in DNA-binding, dimerization and effector function (Ryder et al., 1989). Oligodeoxyribonucleotides were synthesized using an 'Expedite' oligonucleotide synthesis system (Millipore) at Glaxo Group Research.

2.6. Oligonucleotide labelling

To label each probe 15 pmol of oligonucleotide were incubated in 20 μl of labelling buffer (100 mM cacodylate buffer (pH 6.8), 1 mM $CoCl_2$, 0.1 mM dithiothreitol, 100 μg/ml bovine serum albumin containing 30 μCi [^{35}S]α-thio-deoxyadeninetriphosphate ([^{35}S]dATP) (1200–1400 Ci/mmol, NEN)). The reaction was initiated by the addition of 20 units of terminal deoxynucleotidyl transferase (Promega) and the mixture incubated for 1 h at 37°C. The reaction was terminated by a 10 min incubation at 70°C followed by brief vortexing. At this stage a 1 μl aliquot was removed for use in determination of the probes specific activity and 10 μg yeast tRNA (10 mg/ml) was added to the reaction mixture as a carrier. The probe was purified by size exclusion chromatography through a NICK column (Pharmacia) and ethanol precipitation. Labelled probes were resuspended in a Tris/EDTA buffer (pH 8.0) containing 10 mM dithiothreitol (Tris/EDTA buffer is 10 mM Tris-HCl containing 1 mM EDTA). The specific activity of the probes varied ($(0.5–2.0) \times 10^9$ cpm/μg), but was typically 10^9 cpm/μg.

2.7. Prehybridization treatments

Slides were removed from storage and the sections were immediately fixed, by immersion in PBS (pH 7.4) containing 4% paraformaldehyde, for 5 min at room temperature. The sections were rinsed three times in PBS for 5 min at room temperature and then acetylated in 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride for 10 min with occasional agitation. Sections were then rinsed in PBS for 5 min followed by dehydration through a graded series of ethanols (70% for 1 min, 80% for 1 min, 95% for 1 min and 100% for 2 min), delipidation with chloroform (two 5 min immersions) and rehydration through the graded ethanols (100% for 2 min and 95% for 1 min). Finally the sections were fully dried under a stream of cool air.

2.8. Hybridization

Radiolabelled oligonucleotide probes were diluted in hybridization buffer (1–2 μl/100 μl hybridization buffer ≅ 100 pg/μl) and 50–100 μl spread over the sections. The sections were encircled using a 'PAP' pen (Agar Scientific) to prevent the hybridization buffer running off the slides. Slides were then placed on an acrylic platform inside a plastic box and incubated at 37°C for 16–20 h. Hybridization buffer consisted of 50% deionized formamide, 4 × saline sodium citrate (pH 7.0) (1 × saline sodium citrate is 0.15 M sodium chloride and 0.015 M sodium citrate), 1 × Denhardt's solution (0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 250 μg/ml denatured salmon sperm DNA, 50 μg/ml yeast tRNA, 50 μM dATP, 10% (w/v) dextran sulphate and 100 mM dithiothreitol (added just before use).

2.9. Post hybridization washing

Much of the hybridization buffer was washed from the sections by agitating each slide in 2 × saline sodium citrate (pH 7.0) for approximately 10 s. The sections were then immersed in 1 × saline sodium citrate (pH 7.0) for 5–10 min followed by four 15 min washes at 55°C in 1 × saline sodium citrate and two 30 min washes at room temperature in 1 × saline sodium citrate. Sections were then briefly dipped into deionized water to remove buffer salts and dried under a stream of cool air.

2.10. Controls for hybridization specificity

The specificity of the in situ hybridization signal was determined by the following. (1) Elimination of hybridization signal by pretreatment of sections with RNase (20 μg/ml RNase A, in 10 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl, for 30 min at 37°C). (2) Elimination of hybridization signal by the presence of a 20-fold excess of unlabelled probe. (3) A correlation between the predicted hybrid melting temperature and the signal seen/abolished at different washing stringencies. (4) The use of multiple probes directed towards the same target mRNA yielding identical patterns of hybridization when used individually. (5) The observed pattern of hybridization corresponded to that previously published for c-*fos* mRNA (Merchant and Dorsa, 1993).

2.11. Preparation of autoradiographs

The sections were exposed, together with brain paste standards, to Hyperfilm β-max (Amersham) for 6 weeks. After the exposure period, the films were developed for 5 min in D19 developer (Kodak), fixed for 6 min in 'Fix 3000' (Kodak) containing 0.15% X-ray hardener (Kodak), rinsed for 15 min in running water and then dried.

Table 2
Levels of c-*fos* mRNA in rat brain after acute treatment (30 min) with dopamine receptor antagonists

	Vehicle (water, pH 5)	GR103691 (1 mg/kg)	Haloperidol (5 mg/kg)	Sulpiride (100 mg/kg)	Clozapine (20 mg/kg)
NAs	0.59 ± 0.07	0.83 ± 0.11	2.08 ± 0.11 [b]	0.60 ± 0.07	1.05 ± 0.13 [a]
NAc	0.45 ± 0.02	0.45 ± 0.04	2.01 ± 0.18 [b]	0.31 ± 0.02	0.37 ± 0.03
OT	0.46 ± 0.02	0.47 ± 0.02	0.62 ± 0.05 [b]	0.35 ± 0.03 [a]	0.40 ± 0.03
ICj	1.13 ± 0.22	1.00 ± 0.16	0.75 ± 0.14	0.78 ± 0.09	3.13 ± 0.47 [b]
ICjM	0.46 ± 0.04	0.57 ± 0.07	0.52 ± 0.05	0.47 ± 0.04	2.48 ± 0.15 [b]
CPdl	0.42 ± 0.03	0.43 ± 0.04	4.03 ± 0.18 [b]	0.33 ± 0.02	0.54 ± 0.05
CPdm	0.48 ± 0.03	0.50 ± 0.01	2.78 ± 0.18 [b]	0.39 ± 0.03	0.48 ± 0.03
CPvl	0.40 ± 0.08	0.40 ± 0.03	3.77 ± 0.11 [b]	0.31 ± 0.03	0.37 ± 0.02
CPvm	0.50 ± 0.03	0.52 ± 0.07	3.08 ± 0.18 [b]	0.42 ± 0.08	0.41 ± 0.04
FrCx	0.70 ± 0.09	0.90 ± 0.15	0.50 ± 0.07	0.63 ± 0.08	0.64 ± 0.09
PrCx	0.43 ± 0.04	0.46 ± 0.03	0.30 ± 0.01 [b]	0.32 ± 0.03 [a]	0.33 ± 0.02 [a]
CgCx	1.71 ± 0.22	1.35 ± 0.25	1.27 ± 0.06	0.95 ± 0.12 [a]	1.01 ± 0.10

The mRNA encoding c-*fos* was visualized in coronal striatal rat brain sections by hybridization of ^{35}S-labelled oligonucleotide probes specific for the c-*fos* mRNA transcript and subsequent autoradiography. Values are means ± S.E.M. amol/mg ($n = 5$–6). [a] = $P < 0.05$, [b] = $P < 0.01$, Dunnett's test. Abbreviations: NAs, nucleus accumbens shell; NAc, nucleus accumbens core; OT, olfactory tubercle; ICj, islands of Calleja; ICjM, major islands of Calleja; CPdl, dorsolateral caudate-putamen; CPdm, dorsomedial caudate-putamen; CPvl, ventrolateral caudate-putamen; CPvm, ventromedial caudate-putamen; FrCx, frontal cortex; PrCx, parietal cortex; CgCx, cingulate cortex.

Brain paste standards were prepared by mixing varying quantities of [^{35}S]dATP with homogenized brain tissue. The brain homogenate was frozen and 12 μm sections cut using a cryostat (Bright Instruments). Sections of each concentration were thaw-mounted onto slides so that all concentrations were present on each slide.

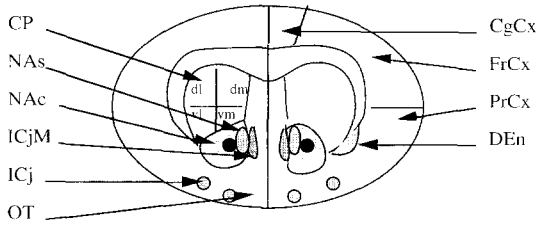
2.12. Quantification of autoradiograms

Images were digitized and quantified using MCID-M1 image analysis software (Imaging Research). The amount of radioactivity bound to brain sections was assessed by the use of the [^{35}S]dATP brain paste standards. A standard curve relating the radioactivity contained within the standards and their corresponding film optical density values was generated using the image analysis software. The specific activity of the probes was used to convert the values into μmol/mg tissue. Specific hybridization signals were measured by subtraction of images in adjacent control (20-fold excess of cold probe) sections. The level of c-*fos* mRNA was evaluated in coronal sections of rat brain between levels 1.0 and 1.6 mm rostral to bregma (Paxinos and Watson, 1986).

2.13. Statistical analysis

Statistical analysis between treatment groups was performed for each brain region by one-way analysis of variance. Where an overall significant difference between treatment groups occurred Dunnett's multiple comparison *t*-test was used to compare each treatment with the control group. A drug effect was considered significant if P was < 0.05.

Fig. 1. Digitized autoradiographic images showing hybridization of ^{35}S-labelled oligonucleotide probes to c-*fos* mRNA in coronal sections of rat brain cut at the level of the posterior nucleus accumbens (1.2 mm rostral to bregma, Paxinos and Watson, 1986). Rats received a single injection of vehicle (a), GR103691 (b), haloperidol (c), sulpiride (d) or clozapine (e) and were killed 30 min later. Coronal sections of their brains were processed for in situ hybridization and labelled with [^{35}S]oligonucleotide probes specific for c-*fos* mRNA. Abbreviations: NAs, nucleus accumbens shell; NAc, nucleus accumbens core; OT, olfactory tubercle; ICj, islands of Calleja; ICjM, major islands of Calleja; CPdl, dorsolateral caudate-putamen; CPdm, dorsomedial caudate-putamen; CPvl, ventrolateral caudate-putamen; CPvm, ventromedial caudate-putamen; DEn, dorsal endopiriform nucleus; FrCx, frontal cortex; PrCx, parietal cortex; CgCx, cingulate cortex.



2.14. Drugs

The selective dopamine D_3 receptor antagonist 4″-acetyl-*N*-[4-[4-(2-methoxyphenyl)-1-piperazinyl]butyl][1,1″-biphenyl]-4-carboxamide (GR103691; Murray et al., 1995) was obtained from Glaxo Group Research, Ware. Haloperidol was purchased from Sigma, while clozapine (Sandoz) and (−)-sulpiride (SESIF) were supplied as gifts. The drugs were initially dissolved in glacial acetic acid and then made up to volume with deionized water. The pH of all drug solutions was adjusted to 5.0 with NaOH.

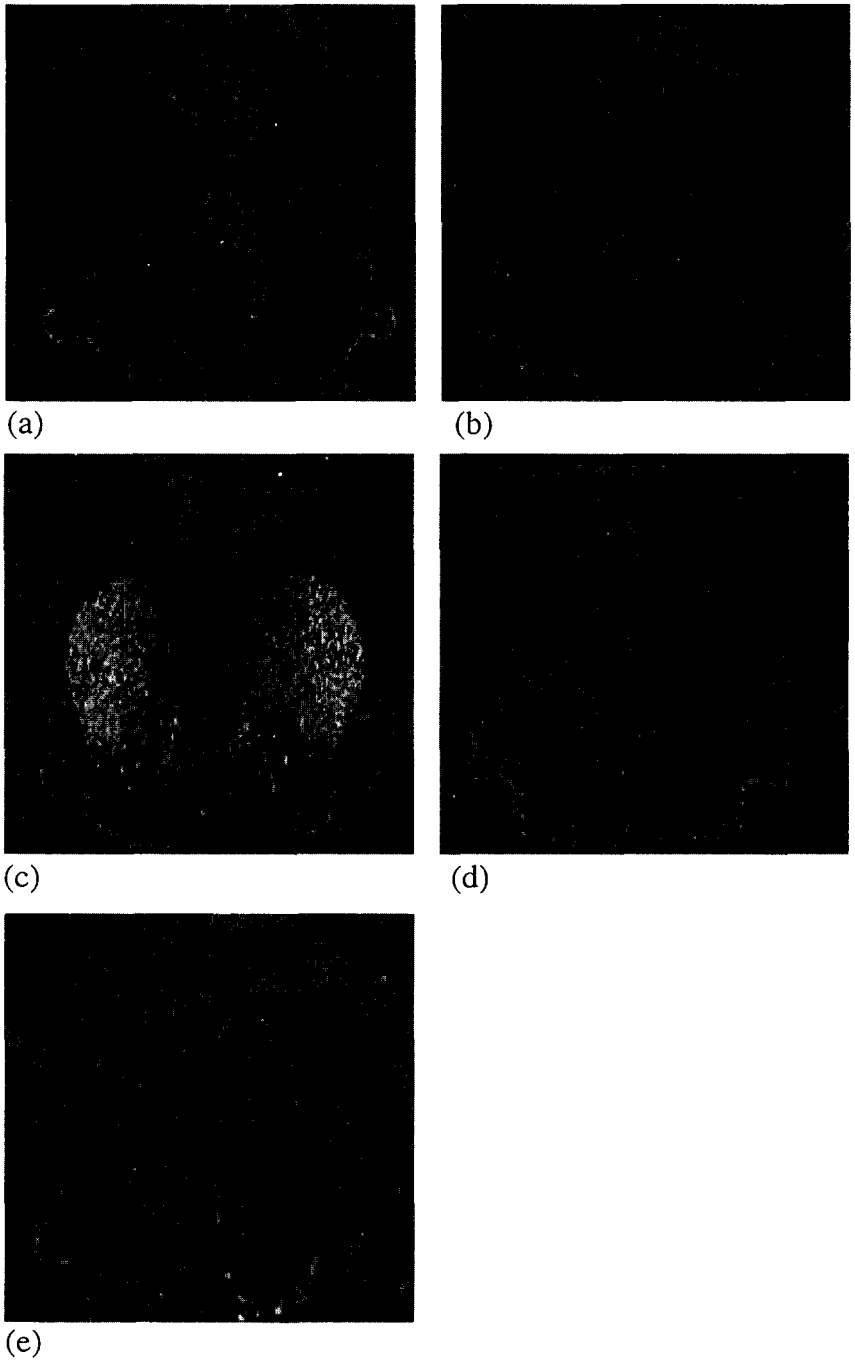
3. Results

3.1. Acute dopamine antagonist administration

The levels of c-*fos* mRNA in different brain regions of control animals and after acute administration of the drugs are shown in Table 2 and depicted in Fig. 1.

Acute treatment with GR103691 had no significant effect on the level of c-*fos* mRNA in any of the brain regions examined.

In striatal regions, haloperidol caused a large increase in



the level of c-*fos* mRNA in the dorsolateral, dorsomedial, ventrolateral and ventromedial quadrants of the caudate-putamen. The increase was greatest in the lateral quadrants. In limbic regions, haloperidol increased the level of c-*fos* mRNA in the nucleus accumbens shell and core and olfactory tubercle, but had no effect in the islands of Calleja or major islands of Calleja. In cortical regions, haloperidol caused a decrease in the level of c-*fos* mRNA in the parietal cortex.

In contrast to haloperidol, sulpiride had no effect on c-*fos* expression in striatal regions. In limbic regions it caused a decrease in c-*fos* mRNA levels in the olfactory tubercle only. In cortical regions, sulpiride, like haloperidol, caused a decrease in the level of c-*fos* mRNA in the parietal cortex. Sulpiride also caused a reduction in the level of c-*fos* mRNA in the cingulate cortex.

Clozapine had no effect on c-*fos* expression in the striatum. In limbic regions, clozapine, like haloperidol, caused an increase in the level of c-*fos* mRNA in the nucleus accumbens shell but, unlike haloperidol, did not in the nucleus accumbens core. In the islands of Calleja and major islands of Calleja clozapine caused a large increase in the level of c-*fos* mRNA. In cortical regions, clozapine, like haloperidol and sulpiride, reduced the level of c-*fos* mRNA in the parietal cortex.

3.2. Chronic dopamine antagonist administration

The pattern and level of c-*fos* mRNA transcription after chronic administration of haloperidol and sulpiride, and to a lesser extent clozapine, was different to that seen after acute administration. The levels of c-*fos* mRNA in different brain regions of control animals and after chronic administration of the drugs are shown in Table 3 and depicted in Fig. 2. In general there was less of an effect on c-*fos* expression following chronic treatment than was observed after acute treatment.

Chronic treatment, like acute treatment, with GR103691 had no significant effects on the level of c-*fos* mRNA in any of the brain regions examined.

After chronic treatment with haloperidol, the large increase in the level of c-*fos* mRNA that acute haloperidol administration produced in the striatum no longer occurred. Neither was there any stimulation of c-*fos* transcription in the nucleus accumbens core. Indeed the level of c-*fos* mRNA appeared to be reduced (though not significantly) in these areas. An increase still occurred in the nucleus accumbens shell, but was greatly attenuated in comparison to acute treatment. The level of c-*fos* mRNA was reduced rather than increased in the olfactory tubercle after chronic treatment. The reduction in the level of c-*fos* mRNA in the parietal cortex was greater and significant reductions in the level of c-*fos* mRNA were also measured in frontal and cingulate cortex.

In striatal regions, in contrast to the effect of acute administration, chronic sulpiride treatment caused a reduction in the level of c-*fos* mRNA in the dorsal quadrants. There was a trend for a reduction in the ventral quadrants also, but this was not significant. In limbic regions the reduction of c-*fos* in the olfactory tubercle no longer occurred, the level of c-*fos* mRNA was unchanged in the remaining areas. In cortical regions the reduction of c-*fos* mRNA that acute treatment stimulated in the parietal cortex and cingulate cortex was not evident.

Chronic clozapine administration also had no effect in striatal or cortical regions. The effects of chronic clozapine treatment contrasted with those of acute administration in limbic areas. There was no longer an increase in the level

Table 3
Levels of c-*fos* mRNA in rat brain after chronic treatment (21 days) with dopamine receptor antagonists

	Vehicle (water, pH 5)	GR103691 (1 mg/kg)	Haloperidol (5 mg/kg)	Sulpiride (100 mg/kg)	Clozapine (20 mg/kg)
NAs	0.74 ± 0.03	0.79 ± 0.08	1.02 ± 0.06 [b]	0.52 ± 0.06	0.84 ± 0.10
NAc	0.71 ± 0.04	0.73 ± 0.10	0.51 ± 0.06	0.47 ± 0.04	0.74 ± 0.10
OT	1.00 ± 0.08	0.95 ± 0.13	0.52 ± 0.11 [b]	0.83 ± 0.07	1.14 ± 0.08
ICj	1.46 ± 0.16	1.09 ± 0.18	1.28 ± 0.18	0.72 ± 0.05	3.59 ± 0.46 [b]
ICjM	0.52 ± 0.08	0.49 ± 0.07	0.54 ± 0.06	0.46 ± 0.07	0.56 ± 0.10
CPdl	0.74 ± 0.03	0.73 ± 0.09	0.56 ± 0.08	0.46 ± 0.04 [a]	0.76 ± 0.10
CPdm	0.80 ± 0.04	0.80 ± 0.12	0.58 ± 0.08	0.48 ± 0.04 [a]	0.69 ± 0.08
CPvl	0.67 ± 0.04	0.70 ± 0.10	0.52 ± 0.08	0.44 ± 0.05	0.70 ± 0.10
CPvm	0.72 ± 0.04	0.79 ± 0.10	0.64 ± 0.11	0.54 ± 0.06	0.73 ± 0.10
FrCx	0.98 ± 0.08	0.95 ± 0.13	0.52 ± 0.11 [b]	0.83 ± 0.07	1.14 ± 0.08
PrCx	0.81 ± 0.06	0.80 ± 0.10	0.41 ± 0.06 [a]	0.65 ± 0.10	0.75 ± 0.10
CgCx	1.25 ± 0.11	1.31 ± 0.17	0.71 ± 0.11 [a]	1.11 ± 0.20	1.35 ± 0.10

The mRNA encoding c-*fos* was visualized in coronal striatal rat brain sections by hybridization of ^{35}S-labelled oligonucleotide probes specific for the c-*fos* mRNA transcript and subsequent autoradiography. Values are means ± S.E.M. amol/mg ($n = 5$–6), [a] $P < 0.05$, [b] $P < 0.01$, Dunnett's test. Abbreviations: NAs, nucleus accumbens shell; NAc, nucleus accumbens core; OT, olfactory tubercle; ICj, islands of Calleja; ICjM, major islands of Calleja; CPdl, dorsolateral caudate-putamen; CPdm, dorsomedial caudate-putamen; CPvl, ventrolateral caudate-putamen; CPvm, ventromedial caudate-putamen; FrCx, frontal cortex; PrCx, parietal cortex; CgCx, cingulate cortex.

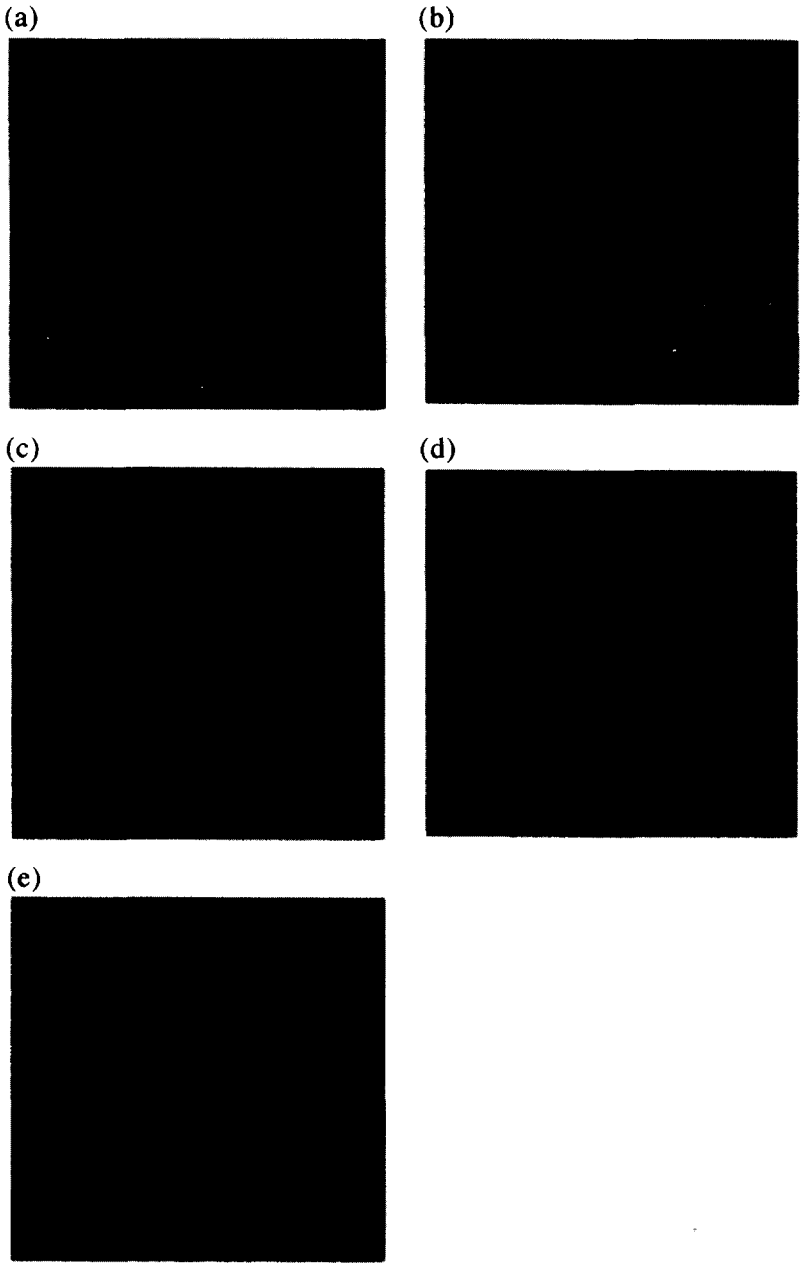


Fig. 2. Digitized autoradiographic images showing hybridization of ^{35}S-labelled oligonucleotide probes to c-*fos* mRNA in coronal sections of rat brain cut at the level of the posterior nucleus accumbens (1.2 mm rostral to bregma, Paxinos and Watson, 1986). Rats received injections of vehicle (a), GR103691 (b), haloperidol (c), sulpiride (d) or clozapine (e) for 21 days. They were killed 30 min after their last injection. Coronal sections of their brains were processed for in situ hybridization and labelled with [^{35}S]oligonucleotide probes specific for c-*fos* mRNA. Abbreviations: see legend to Fig. 1.

of c-*fos* mRNA in the nucleus accumbens shell. The rise in the c-*fos* mRNA level that acute clozapine treatment caused in the islands of Calleja was not attenuated. However, in the major islands of Calleja the level of c-*fos* mRNA was not increased.

4. Discussion

4.1. Acute treatment

Regional differences in the induction of c-*fos* mRNA and Fos protein by typical and atypical neuroleptics have been demonstrated before (Nguyen et al., 1992; Robertson and Fibiger, 1992; Miller, 1990; Merchant and Dorsa, 1993; Dragunow et al., 1990). The aim of this study was to evaluate whether the dopamine D_3 receptor has a role in the induction of c-*fos* expression by neuroleptics, using the putative dopamine D_3 receptor antagonist GR103691. For comparison the effects of other typical and atypical neuroleptics were also studied. In addition the effect of chronic treatment versus acute treatment with each drug was investigated since clinically these drugs are used long-term.

The results of this study were in agreement with the majority of previous studies. Thus, there was a large

increase in c-*fos* expression in the striatum and nucleus accumbens after acute administration of haloperidol but only a rise in the nucleus accumbens after acute administration of clozapine. In addition, in this study acute administration of clozapine caused a large increase in the level of c-*fos* mRNA in the islands of Calleja and major islands of Calleja. Sulpiride was reported to increase the level of c-*fos* mRNA in the striatum (Nguyen et al., 1992), however no increase in the level of c-*fos* mRNA after sulpiride treatment was detected in these experiments. Differences in the design of the studies is a possible reason for this discrepancy. Nguyen et al. (1992) killed their animals 45 min after the injection (cf. 30 min in these experiments); it is possible, therefore, that this extra time allowed enough sulpiride to penetrate the brain to increase levels of c-*fos* significantly. However, sufficient sulpiride penetrated the brain to cause significant reductions in the level of c-*fos* mRNA in the olfactory tubercle, parietal cortex and cingulate cortex. Also, acute administration of sulpiride can displace [^{3}H]spiperone within 30 min of an s.c. injection (unpublished observations). This suggests that a substantial concentration of sulpiride does reach striatal synapses within this time.

The dopamine D_3 receptor ligand GR103691 had no effect on the level of c-*fos* mRNA at the single dose and time point used in this study. The use of a range of doses and time-points was unfortunately precluded by a limited supply of the compound. This indicates that the dopamine D_3 receptor is not coupled to c-*fos* transcription in vivo. Which immediate-early gene is linked to dopamine D_3 receptor occupation remains to be determined. Although Pilon et al. (1994) have demonstrated in vitro that stimulation of dopamine D_3 receptors expressed in NG108-15 cells with quinpirole leads to an induction of c-*fos*. Systemically administered GR103691, at the dose used in this study, can inhibit behaviours produced by muscimol stimulation (Oakley et al., 1991) of mesolimbic dopamine pathways (C.M. Stubbs, personal communication). The inability of GR103691 to alter c-*fos* expression is therefore unlikely to be a result of inadequate brain penetration. The absence of an effect of GR103691 in the islands of Calleja and major islands of Calleja, where dopamine D_3 receptors are most highly expressed and where the level of dopamine D_2 receptor expression is comparatively low, suggests that the effect of clozapine in the islands of Calleja and major islands of Calleja is not due to an interaction with dopamine D_3 receptors. The islands of Calleja and major islands of Calleja also contain high levels of dopamine D_1 and 5-HT receptors. Clozapine has a high affinity for the dopamine D_1 receptor and 5-HT_2 and 5-HT_{1C} receptor subtypes. It is therefore possible that the c-*fos* induction in the islands of Calleja and major islands of Calleja may be through blockade of the dopamine D_1 receptor or through non-dopaminergic mechanisms. The former seems unlikely though since the dopamine D_1-like antagonist SCH23390 does not elevate c-*fos* expression in rat striatum (Nguyen et al., 1992; Merchant and Dorsa, 1993; Robertson and Fibiger, 1992). Alternatively, since clozapine has a relatively high affinity for the dopamine D_4 receptor, the effects of clozapine may be due to an action at dopamine D_4 receptors. However, this is also unlikely because dopamine D_4 receptors are present at an extremely low level throughout the striatum and are not present in the islands of Calleja or major islands of Calleja (Van Tol et al., 1991; O'Malley et al., 1992).

Recently, Guo et al. (1995) examined the contribution of other neurotransmitter systems to clozapine-induced c-*fos* expression. Destruction of noradrenaline and 5-HT input to the striatum had no effect on the pattern of Fos immunoreactivity induced by clozapine. Similarly, administration of the 5-HT_2 antagonist ritanserin in combination with haloperidol did not mimic clozapine's effects. Thus, noradrenergic neurons, 5-HT neurons and 5-HT receptors were not involved in clozapine's unique pattern of c-*fos* expression. In contrast, quinpirole or 7-OH-DPAT prevented Fos induction by clozapine in the nucleus accumbens, major islands of Calleja, lateral septum and medial prefrontal cortex. From these results they concluded that clozapine induces c-*fos* expression in limbic areas by antagonism of dopamine D_3 receptors. The results of this study contradict such a conclusion and instead suggest that clozapine induces c-*fos* mRNA via an indirect action. However, Guo et al. (1995) looked at Fos protein and we examined c-*fos* mRNA. It is conceivable that this contributed to the observed differences because the c-*fos* mRNA and Fos protein in any one area may be on different neuronal populations. A study combining both immunohistochemistry and in situ hybridization would help to resolve this issue.

The results of these experiments are supported by recent reports that atypical and typical neuroleptics produce a different pattern of c-*fos* induction after acute administration. MacGibbon et al. (1994) demonstrated that haloperidol induced c-*fos* mRNA and Fos protein in both striatal and limbic areas while clozapine only had an effect in limbic areas. Sebens et al. (1995) have also shown that acute administration of haloperidol induced Fos protein in striatal and limbic areas with clozapine only eliciting Fos protein production in limbic areas. These workers examined the effect of chronic treatment as well and found that the increases were greatly attenuated in all areas for both drugs with the exception of clozapine's effect in the nucleus accumbens. Simpson and Morris (1994) have also found that acute haloperidol treatment induces c-*fos* mRNA in striatal and limbic areas of rat brain. In contrast to this study and others (see above) they found that clozapine had no effect on c-*fos* expression. The low dose of clozapine that Simpson and Morris (1994) used may explain this discrepancy.

4.2. Chronic treatment

The mechanisms underlying the extrapyramidal side-effects of neuroleptic treatment are unclear, but may relate to

adaptive changes in receptor density and/or coupling efficiency. If alterations in dopamine receptor populations are involved, c-*fos* expression might be expected to change since its induction is under dopaminergic control. In this study, like that of Merchant et al. (1994), chronic haloperidol administration did not cause the large increases in c-*fos* mRNA in the striatum that were produced by acute administration. Whereas in the nucleus accumbens shell c-*fos* levels were elevated after both acute and chronic treatment, albeit to a lower extent following chronic administration. Miller (1990) found no attenuation in the level of c-*fos* induction caused by haloperidol after chronic treatment. However, the duration of treatment used was shorter (12 days) and the dose of haloperidol lower (2.5 mg/kg) in Miller's study. It is possible that these methodological differences are the cause of this discrepancy. Dragunow et al. (1990) also found no reduction in Fos-like immunoreactivity in the striatum after 7 days treatment with 2 mg/kg haloperidol. This was contrary to their expectations as they believed that c-*fos* transcription was negatively regulated by Fos protein. However the antibody used was not specific for Fos but could detect many immediate-early genes. Thus it is possible that the signal detected after 7 days was not Fos protein.

The nucleus accumbens is an important locus of the antipsychotic actions of neuroleptics whilst striatal areas are probably involved in extrapyramidal side-effects. No tolerance occurs to the antipsychotic action of neuroleptics but extrapyramidal side-effects can subside with time (Crow, 1982). The attenuation of the induction of c-*fos* expression in the striatum after chronic treatment with haloperidol but not in the nucleus accumbens shell conforms with these clinical observations. However, the increase in the level of c-*fos* in the nucleus accumbens shell, induced by acute clozapine treatment, was attenuated after chronic treatment. Also sulpiride had no effect on c-*fos* induction in the nucleus accumbens shell. Since clozapine and sulpiride are both effective antipsychotics these observations suggest that the induction of c-*fos* transcription may not be a good indication of the anatomical sites where neuroleptics exert their antipsychotic action.

Acute administration of clozapine caused an increase in the level of c-*fos* mRNA in both the islands of Calleja and major islands of Calleja, but after chronic treatment the induction of c-*fos* was only maintained in the islands of Calleja and not the major islands of Calleja, despite both areas expressing a similar complement of receptors. This implies that the action of clozapine is indirect and that the islands of Calleja and major islands of Calleja function independently. This is supported by evidence that the islands of Calleja receive more afferent innervation from the A9 cell group (substantia nigra pars compacta) whereas the major islands of Calleja have a greater proportion of innervation from the A10 cell group (ventral tegmental area). However whilst there is a distinct topographical organization of projections from dopaminergic neuron cell bodies to the islands of Calleja and major islands of Calleja, overlap does occur (Fallon et al., 1978) indicating that their functions are not entirely independent.

4.3. Mechanisms of c-fos induction

The mechanism(s) by which dopamine antagonists stimulate c-*fos* transcription are at present unclear. A number of explanations have been hypothesized, they are: (1) a direct effect through blockade of postsynaptic dopamine D_2 receptors on striatopallidal neurons; (2) blockade of presynaptic autoreceptors resulting in increased dopamine release which then acts upon dopamine D_1 receptors; (3) blockade of inhibitory dopamine D_2 receptors on the terminals of corticostriatal glutaminergic fibres; (4) actions upon other neurotransmitter systems (Cole and Di Figlia, 1994; Dragunow et al., 1990; Fu and Beckstead, 1992; MacGibbon et al., 1994; Robertson and Fibiger, 1992; Robertson et al., 1992; Simpson and Morris, 1994). In summary, dopamine D_1 and D_2 receptors appear to have opposing effects upon c-*fos* expression (activation of D_1 stimulates, activation of D_2 inhibits). Thus, blockade of dopamine D_1 receptors should decrease c-*fos* levels while blockade of dopamine D_2 receptors should increase c-*fos* levels. Haloperidol and clozapine, which both induce c-*fos* transcription, have in addition to their high affinity for dopamine D_2 receptors (1.2 nM and 230 nM, respectively) an appreciable affinity for dopamine D_1 receptors (80 nM and 170 nM, respectively), while sulpiride is very selective for dopamine D_2 receptors (15 nM, D_2; 45 μM, D_1) (Seeman and Van Tol, 1994). From the above sulpiride, like haloperidol and clozapine, might be expected to induce c-*fos* transcription by blockade of dopamine D_2 receptors. The absence of such an effect indicates that dopaminergic control of c-*fos* induction is complex and seems to require blockade of both dopamine D_1 and D_2 receptors.

The reduction of c-*fos* induction in the dorsal striatum after chronic haloperidol treatment, corresponds with the increase in striatal dopamine D_2 receptors that chronic haloperidol treatment causes (Burt et al., 1977; Rupniak et al., 1985a,b; Savasta et al., 1988). The attenuation in c-*fos* induction after chronic haloperidol treatment may therefore be a result of the greater density of inhibitory (on c-*fos* transcription) dopamine D_2 receptors. Maintenance of c-*fos* expression would therefore appear not to be essential for continuance of the antipsychotic effects of neuroleptics. As stated above, in addition to their actions at dopamine D_1 and D_2 receptors all three drugs have high affinity for dopamine D_3 receptors, thus a contribution of the dopamine D_3 receptor in the induction of c-*fos* (and other immediate-early genes) cannot be excluded. But the absence of an effect by GR103691 on c-*fos* transcription does not support this notion. However, even if the dopamine D_3 receptor is not directly stimulating the changes in the level of c-*fos* expression, the presence of

the dopamine D_3 receptor in the nucleus accumbens shell and core, islands of Calleja and major islands of Calleja make it a possible target for regulation by c-*fos* and other immediate-early genes which may be induced by other dopaminergic or non-dopaminergic receptors.

The results of these experiments demonstrate that: (1) concomitant blockade of dopamine D_1 and D_2, but not D_3, receptors causes induction of c-*fos* in rat striatum; (2) typical and atypical neuroleptics induce a different pattern of c-*fos* transcription; (3) the pattern of c-*fos* transcription induced by acute administration of neuroleptics is different to that induced by chronic treatment. It has also been demonstrated that typical and atypical neuroleptics induce different patterns of FosB, c-jun, JunB, JunD, and Krox24 (also known as zif268) in the striatum (MacGibbon et al., 1994; Nguyen et al., 1992; Rogue and Vincendon, 1992; Simpson and Morris, 1994). The contribution of the dopamine D_3 receptor in the induction of these immediate-early genes remains to be investigated. Immediate-early genes are considered to be messengers responsible for long term changes in gene expression. The differences in immediate-early gene induction between typical and atypical neuroleptics may explain their different effects on the positive and negative symptoms of schizophrenia and distinct extrapyramidal side-effect profiles.

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References

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990, Basic local alignment search tool, J. Mol. Biol. 215, 403.

Burt, D.R., I. Creese and S.H. Snyder, 1977, Antischizophrenic drugs: Chronic treatment elevates dopamine receptor binding in brain, Science 196, 326.

Cole, D.G. and M. Di Figlia, 1994, Reserpine increases fos activity in the rat basal ganglia via a quinpirole-sensitive mechanism, Neuroscience 60, 115.

Crow, T.J., 1982, The biology of schizophrenia, Experientia 38, 1276.

Devereux, J., P. Haeberli and O. Smithies, 1984, A comprehensive set of sequence analysis packages for the vax, Nucl. Acids Res. 12, 387.

Dragunow, M., G.S. Robertson, R.L.M. Faull, H.A. Robertson and K. Jansen, 1990, D_2 dopamine receptor antagonists induce fos and related proteins in rat striatal neurons, Neuroscience 37, 287.

Fallon, J.H., J.N. Riley, J.C. Sipe and R.Y. Moore, 1978, The islands of Calleja: organization and connections, J. Comp. Neurol. 181, 375.

Fu, L. and R.M. Beckstead, 1992, Cortical stimulation induces fos expression in striatal neurons, Neuroscience 46, 329.

Guo, N., M.A. Klitenick, C.-S. Tham and H.C. Fibiger, 1995, Receptor mechanisms mediating clozapine-induced c-*fos* expression in the forebrain, Neuroscience 65, 747.

Jenner, P. and C.D. Marsden, 1987, Chronic pharmacological manipulation of dopamine receptors in brain, Neuropharmacology 26, 931.

MacGibbon, G.A., P.A. Lawlor, R. Bravo and M. Dragunow, 1994, Clozapine and haloperidol produce a differential pattern of immediate early gene expression in rat caudate-putamen, nucleus accumbens, lateral septum and islands of Calleja, Mol. Brain Res. 23, 21.

Merchant, K.M. and D.M. Dorsa, 1993, Differential induction of neurotensin and c-*fos* gene expression by typical versus atypical antipsychotics, Proc. Natl. Acad. Sci. USA 90, 3447.

Merchant, K.M., D.J. Dobie, F.M. Filloux, M. Totzke, M. Aravagiri, D.M. Dorsa, 1994, Effects of chronic haloperidol and clozapine treatment on neurotensin and c-*fos* mRNA in rat neostriatal subregions, J. Pharmacol. Exp. Ther. 271, 460.

Miller, J.C., 1990, Induction of c-*fos* m1RNA expression in rat striatum by neuroleptic drugs, J. Neurochem. 54, 1453.

Morgan, J.I. and T. Curran, 1989, Stimulus-transcription coupling in neurons: role of cellular immediate-early genes, Trends Neurosci. 12, 459.

Murray, P.J., L.A. Harrison, M.R. Johnson, G.M. Robertson, D.I.C. Scopes, D.R. Bull, E.A. Graham, A.G. Hayes, G.J. Kilpatrick, C. Large, M.J. Sheehan, C.M. Stubbs and M.P. Turpin, 1995, A novel series of arylpiperazines with high affinity and selectivity for the dopamine D_3 receptor, Bioorg. Med. Chem. Lett. 5, 219.

Nguyen, T.V., B.E. Kosofsky, R. Birnbaum, B.M. Cohen and S.E. Hyman, 1992, Differential expression of c-*fos* and zif268 in rat striatum after haloperidol, clozapine and amphetamine, Proc. Natl. Acad. Sci. USA 89, 4270.

Nunez, D.J., A.P. Davenport, P.C. Emson and M.J. Brown, 1989, A quantitative 'in-situ' hybridization method using computer-assisted image analysis, Biochem. J. 263, 121.

Oakley, N.R., A.G. Hayes and M.J. Sheehan, 1991, Effect of typical and atypical neuroleptics on the behavioural consequences of activation by muscimol of mesolimbic and nigro-striatal dopaminergic pathways in the rat, Psychopharmacology 105, 204.

O'Malley, K.L., S. Harmon, L. Tang and R.D. Todd, 1992, The rat dopamine D_4 receptor: sequence, gene, structure and demonstration of expression in the cardiovascular system, New Biol. 4, 137.

Paxinos, G. and C. Watson, 1986, The Rat Brain in Stereotaxic Coordinates (Academic Press Inc., London).

Pilon, C., D. Lévesque, V. Dimitriadou, N. Griffon, M.-P. Martres, J-C. Schwartz and P. Sokoloff, 1994, Functional coupling of the human dopamine D_3 receptor in a transfected NG 108-15 neuroblastoma-glioma hybrid cell line, Eur. J. Pharmacol. 268, 129.

Robertson, G.S. and H.C. Fibiger, 1992, Neuroleptics increase c-*fos* expression in the forebrain: contrasting effects of haloperidol and clozapine, Neuroscience 46, 315.

Robertson, G.S., S.R. Vincent and H.C. Fibiger, 1992, D_1 and D_2 dopamine receptors differentially regulate c-*fos* expression in striatonigral and striatopallidal neurons, Neuroscience 49, 285.

Rogue, P. and G. Vincendon, 1992, Dopamine D_2 receptor antagonists induce immediate early genes in the rat striatum, Brain Res. Bull. 29, 469.

Rupniak, N.M.J., M.D. Hall, E. Kelly, S. Fleminger, G. Kilpatrick, P. Jenner and C.D. Marsden, 1985a, Mesolimbic dopamine function is not altered during continuous chronic treatment of rats with typical or atypical neuroleptic drugs, J. Neural Transm. 62, 249.

Rupniak, N.M.J., M.D. Hall, S. Mann, S. Fleminger, G. Kilpatrick, P. Jenner and C.D. Marsden, 1985b, Chronic treatment with clozapine, unlike haloperidol, does not induce changes in striatal D_2 receptor function in the rat, Biochem. Pharmacol. 34, 2755.

Ryder, K., A. Lanahan, E. Perez-Albuerne and D. Nathans, 1989, Jun D, a third member of the jun gene family, Proc. Natl. Acad. Sci. USA 86, 1500.

Savasta, M., A. Dubois, J. Benavides and B. Scatton, 1988, Differential plasticity changes in D_1 and D_2 receptors in striatal subregions following impairment of dopaminergic transmission, Neurosci. Lett. 85, 119.

Sebens, J.B., T. Koch, G.J. Ter Horst and J. Korf, 1995, Differential

fos-protein induction in rat forebrain regions after acute and long-term haloperidol and clozapine treatment, Eur. J. Pharmacol. 273, 175.

Seeman, P. and H.H.M. Van Tol, 1994, Dopamine receptor pharmacology, Trends Pharmacol. Sci. 15, 264.

Sibley, D.R. and F.J. Monsma, 1992, Molecular biology of dopamine receptors, Trends Pharmacol. Sci. 13, 61.

Simpson, C.S. and B.J. Morris, 1994, Haloperidol and fluphenazine induce jun B gene expression in rat striatum and nucleus accumbens, J. Neurochem. 63, 1955.

Van Tol, H.H.M., J.R. Bunzow, R.K. Guan, R.K. Sunahara, P. Seeman, H.B. Niznik and O. Civelli, 1991, Cloning of the gene for a human dopamine D_4 receptor with high affinity for the antipsychotic clozapine, Nature 350, 610.