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Differential Fos-protein induction in rat forebrain regions after acute and long-term haloperidol and clozapine treatment

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Abstract

Both acute and long-term effects of haloperidol and clozapine on Fos-like immunoreactive nuclei in several rat forebrain areas were quantified. Rats were treated with saline (1 ml/kg · day, control), haloperidol (1 mg/kg · day) and clozapine (20 mg/kg · day) i.p. for 21 days. Two hours before perfusion fixation a single (acute treatment) or last (long-term treatment) dose of the drug was given. Drug-induced catalepsy and gain in body weight were also measured. A single dose of haloperidol produced large increases in Fos-like immunoreactive nuclei in the striatum, the nucleus accumbens and central amygdala. Following long-term treatment these increases were reduced in all nuclei studied, except the lateral septum. Acute clozapine treatment had slight (if any) effects on the number of Fos-like immunoreactivity-expressing nuclei in the striatum, but the increases in the nucleus accumbens, the lateral septum, the paraventricular and supraoptic nuclei of the hypothalamus and the central amygdala were substantial. Long-term clozapine treatment reduced the acute response significantly in all the areas except the nucleus accumbens. Both haloperidol and clozapine treatment reduced the weight gain of the rats. Haloperidol, but not clozapine, induced catalepsy that remained maximal during the long-term haloperidol treatment. These results indicate that in most brain areas high Fos-protein levels are not necessary to maintain antipsychotic activity or side-effects. The persisting effect of clozapine in the nucleus accumbens may be of significance to the efficacy of this drug in treatment-refractory schizophrenia.

Keywords: Haloperidol; Clozapine; Fos protein; Tolerance; Nucleus accumbens; Striatum; Amygdala; (Long-term treatment)

1. Introduction

Typical (e.g. haloperidol) and atypical (e.g. clozapine) antipsychotics have different antipsychotic effects and acute side-effects. Whereas the extrapyramidal side-effects (particularly parkinsonism, characteristic of typical antipsychotics) may disappear during chronic medication, the antipsychotic effects persist (Baldessarini, 1985; Lickey and Gordon, 1991; Campbell and Baldessarini, 1981).

Acute administration of antipsychotics induces c-fos expression in several areas of the rat forebrain as was shown with immunocytochemical methods (Dragunow et al., 1990; MacGibbon et al., 1994; Nguyen et al., 1992; Robertson and Fibiger, 1992). The proto-oncogene c-fos encodes a nuclear phosphoprotein, Fos, that, after binding to DNA, modulates the transcription of target genes (Curran and Franza, 1988). Fos protein is believed to act as an initiator of long-term cellular changes (neural plasticity) in response to a variety of extracellular stimuli, including drugs (e.g. Graybiel et al., 1990; Robertson et al., 1992; Rogue and Vincendon, 1992). Changes in the expression of c-fos, or the protein product, Fos, may therefore reveal the cerebral site of action of antipsychotics. The patterns of Fos-like immunoreactive nuclei induced by acute haloperidol and clozapine treatment are different. After acute haloperidol treatment many Fos protein-positive nuclei are found in the striatum, a region where a single dose of clozapine has little or no effect (Dragunow et al., 1990; MacGibbon et al., 1994; Robertson and Fibiger, 1992). A single dose of either drug induces Fos protein in the nucleus accumbens, but whereas the haloperidol-induced Fos-like immunoreactivity was seen throughout the nucleus, the effect

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of clozapine administration was most marked in the medial part (Deutch et al., 1992; MacGibbon et al., 1994; Robertson and Fibiger, 1992). Long-term treatment with antipsychotics has been shown to alter several biochemical parameters, including up-regulation of dopamine D_2 receptors (Seeman, 1980; Leysen et al., 1987). Thus far, the changes in proto-oncogene products after long-term haloperidol treatment have been studied only as c-fos mRNA expression with Northern blotting on homogenized tissues (Miller, 1990), but no histochemical study has appeared.

The patterns of Fos immunoreactivity in the forebrain of the rat were compared after acute and chronic haloperidol and clozapine treatments. The density of Fos-immunoreactive nuclei was quantified in the following forebrain regions: the dorsomedial, dorsolateral and ventrolateral striatum, the nucleus accumbens, the lateral septum, the paraventricular and supraoptic nuclei of the hypothalamus and the central amygdala.

Catalepsy (the animal equivalent of parkinsonism in humans, Ezrin-Waters and Seeman, 1977) and weight gain were scored for the long-term treatment.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 180–220 g at the start of the experiment were kept in a 12-h light/dark cycle environment with free access to food and water during the treatment period. Experiments were performed during the light period.

2.2. Design of the study

Five groups of rats, consisting of 3–5 animals each, received once a day for 21 days a dose of either saline (1 ml/kg, 'control'), haloperidol (1 mg/kg) or clozapine (20 mg/kg) by intraperitoneal injection. Saline injections were given to control stress effects caused by e.g. handling and the injections. Stress is known to induce Fos-like immunoreactivity in e.g. limbic brain regions. The acute and control experiments consisted of 21 days' saline treatment, followed by a single dose of either haloperidol, clozapine or saline given i.p. 2 h before perfusion, respectively. The chronically treated groups received daily haloperidol or clozapine for the same time period and the last dose was given 2 h before perfusion. Twice a week the animals were weighed and catalepsy was scored (1 h after the injection). Two hours after the last injection the animals were deeply anaesthetized with sodium pentobarbital (100 mg/kg i.p.) and perfused transcardially for fixation within 15 min.

2.3. Drugs and reagents

Haloperidol (Janssen Pharmaceutica, Beerse, Belgium) for intravenous use was obtained commercially and diluted with saline. Clozapine, a gift of Sandoz (Basel, Switzerland) was dissolved in slightly acidified (pH 6.2) saline. Neither saline nor drug solutions were buffered; the injection of the solutions did not produce apparent discomfort. All reagents used were of analytical quality and purchased from Merck (Darmstadt, Germany). Sources of the other reagents are mentioned in the appropriate sections.

2.4. Catalepsy testing

Rats were tested for catalepsy in a cardboard box with the forelimbs gently put on a wooden bar located 12 cm above the bottom. The time to step-down was recorded, with a maximum of 60 s. One minute later the rats were retested. This procedure was repeated 5 times, taking the mean of the 3 longest recordings as catalepsy score. A similar procedure was used in previous studies (De Graaf and Korf, 1986; Yntema and Korf, 1987).

2.5. Immunohistochemistry

Perfusion was performed with saline (100 ml) followed by 300 ml of 4% paraformaldehyde dissolved in 0.1 M sodium phosphate buffer pH 7.4. The brains were removed and postfixed overnight at 4°C in a 4% paraformaldehyde solution containing 0.05% glutardialdehyde and then stored in 50 mM Tris-HCl buffer pH 7.4, with 0.1% Na-azide. Coronal sections of 50 μm were cut from the postfixed brains, using a Vibratome. Immunostaining was performed on free-floating sections, under continuous agitation, with a polyclonal antiserum (Oncogene Science, Ab-2, Uniondale, NY, USA) raised in rabbit against Fos peptide (4–17 amino acids of human Fos). In brief: sections were rinsed twice with 50 mM Tris-buffered saline (pH 7.4) and then pretreated for 10 min at room temperature with the same buffer containing 0.3% hydrogen peroxide. Thereafter they were rinsed in Tris-buffered saline 5 times and placed for 72 h at 4°C in the Fos primary antiserum (1:250) solution, in saline buffer containing 1% bovine serum albumin (BDH Laboratory Supplies, Poole, UK) and 0.5% Triton X-100 (Baker grade). Next the sections were rinsed in Tris-buffered saline (3×10 min each) and incubated for 1 h at room temperature with a biotinylated rabbit anti-goat secondary antibody (1:400, Vector Laboratories, Burlingame, CA, USA). After rinsing in buffered saline (2×15 min each) the sections were incubated with avidin-biotinylated horseradish peroxidase complex (1:125, Vector Laboratories) for 1 h at room tempera-

ture. Both the second and the third incubation steps were repeated. After the last incubation containing the peroxidase complex the sections were rinsed twice with Tris-buffered saline and then with 50 mM Tris-HCl buffer (pH 7.4). Fos immunoreactivity was revealed by placing the sections in a solution containing 0.05% 3,3'-diaminobenzidine (Pierce Chemical Company, Rockford, IL, USA), 0.2% ammonium nickel sulphate (BDH) and 0.01% H_2O_2 and the reaction was stopped by rinsing in Tris-buffered saline. The sections were mounted on gelatin/chrome-alum coated slides, air dried, dehydrated and coverslipped with DePeX mounting medium (BDH). Sections from control and experimental groups were processed simultaneously using the same solutions.

2.6. Quantification, statistics and presentation of the results

Fos-immunoreactive nuclei were counted within a 400×400 μm grid at a magnification of $125\times$ in the dorsomedial, dorsolateral and ventrolateral striatum, the nucleus accumbens and lateral septum and in the paraventricular and supraoptic hypothalamic nuclei and the central nucleus of the amygdala. Camera lucida drawings illustrating the anterior-posterior position of the counted sections, according to the atlas of Paxinos and Watson (1982) and the areas used for counting the Fos-containing nuclei (shaded squares) are shown in Fig. 1. The mean number ($\pm$S.E.M.) of bilateral Fos-positive nuclei is shown in the figures. Weight gain (in g) is shown as mean $\pm$ S.E.M. The significance of the differences in the number of Fos-positive nuclei or in weight gain was determined with the Wilcoxon-Mann-Whitney test (Siegel and Castellan, 1988).

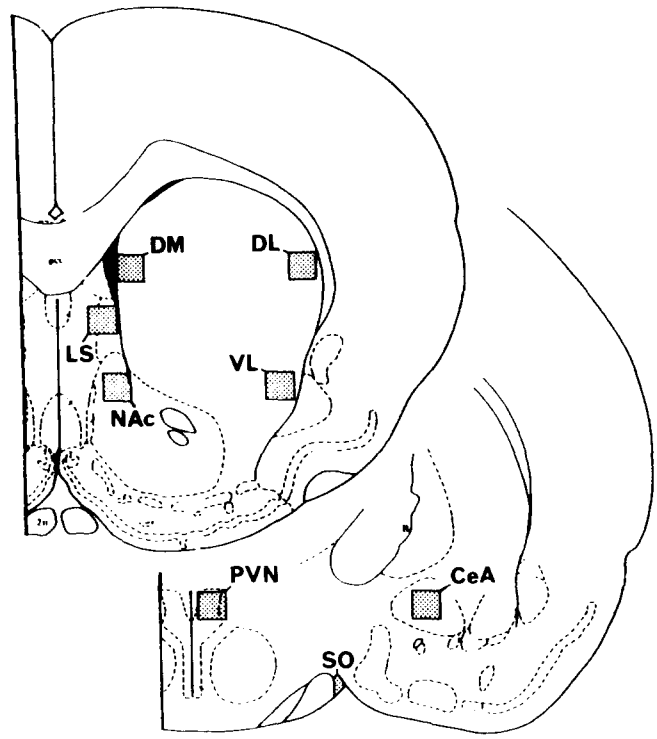

Fig. 1. Camera lucida drawings of representative sections used for quantification of Fos-positive nuclei. Shadings indicate the 400×400 μm areas counted. DM, dorsomedial striatum; DL, dorsolateral striatum; VL, ventrolateral striatum; NAc, nucleus accumbens; LS, lateral septum; PVN, paraventricular nucleus of the hypothalamus; SO, supraoptic nucleus of the hypothalamus; CeA, central amygdala.

3. Results

3.1. Fos-immunoreactive nuclei in controls

In control rats Fos-positive nuclei were – among other – observed in the following forebrain areas: the nucleus accumbens, lateral septum, the frontal cortex, olfactory tubercle, claustrum, the medial rim of the striatum and the islands of Calleja (see also Fig. 2A). Investigations of the diencephalon included the paraventricular, the supraoptic, the dorsal and the ventromedial hypothalamic regions and the central amygdala. In the control experiments these areas exhibited some Fos immunoreactivity, with the exception of the ventromedial hypothalamic nucleus, where no positive nuclei were observed. The number of Fos-positive nuclei in the dorsal area of the hypothalamus was identical in saline-, haloperidol- and clozapine-treated rats. None of the treatments induced Fos protein in the ventromedial hypothalamus. Thus, a possible relation between the labelling in the dorsal area of the hypothalamus and the antipsychotic efficacy of these drugs is unlikely and therefore Fos-like immunoreactivity in these hypothalamic regions will not be reported here.

3.2. Acute treatment

The patterns of Fos-like immunoreactivity induced by acute haloperidol and clozapine administration were distinctly different. Fos-positive nuclei in the basal ganglia after acute haloperidol treatment in rats are shown in Fig. 2. Animals given a single dose of haloperidol had the most pronounced increase in Fos-containing nuclei in the nucleus accumbens, the dorsomedial, the dorsolateral and the ventrolateral striatum and the central amygdala. Little increase in Fos protein was observed in the lateral septum (Fig. 3). The number of Fos-positive nuclei in the paraventricular and the supraoptic nuclei of the hypothalamus was low and often not above basal levels, whereas in these acute haloperidol experiments Fos-like immunoreactivity in the frontal cortex was less than seen in control saline-treated animals (not shown in detail).

Fig. 2. Photomicrographs illustrating distribution of Fos-positive nuclei in response to haloperidol treatment (1 mg/kg i.p.), at the level of the striatum. (A) Control (saline injections for 21 days); (B) acute response (21 days saline and a single dose of haloperidol), (C) long-term treatment (21 days haloperidol administration). Str, striatum; LS, lateral septum; cc, corpus callosum; aca, anterior commissure. Scale bar: 1 mm.

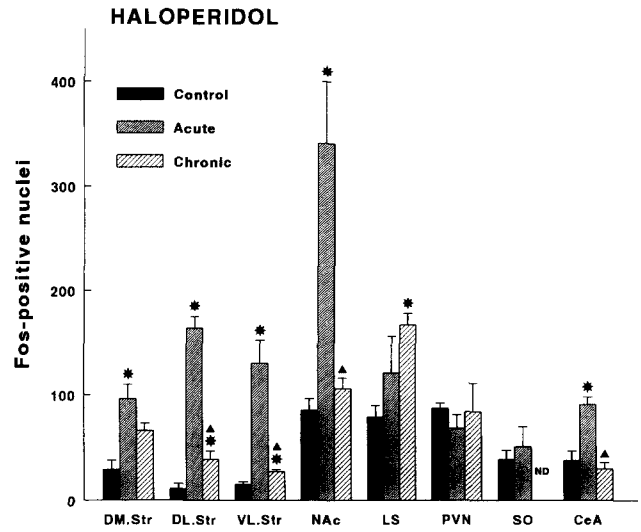

Fig. 3. Histogram showing the mean number of Fos-positive nuclei (±S.E.M.) in controls, after acute and long-term haloperidol treatment in several forebrain regions. Differences were statistically significant: between haloperidol-treated and control rats ($^{*}P < 0.05$) and between acute and long-term haloperidol treatment ($^{\blacktriangle}P < 0.05$). Brain areas include: DM.Str, dorsomedial striatum; DL.Str, dorsolateral striatum; VL.Str, ventrolateral striatum; NAc, nucleus accumbens; LS, lateral septum; PVN, paraventricular nucleus of the hypothalamus; SO, supraoptic nucleus; CeA, central amygdala; ND, not determined.

The effects of acute clozapine treatment on the expression of forebrain and diencephalic Fos-like immunoreactivity were different from the haloperidol ones. There was a marginal increase in labelled nuclei in the dorsomedial and the dorsolateral striatum, whereas no change was observed ventrolaterally. A conspicuous increase in nuclear Fos protein immunoreactivity was seen in the limbic areas, especially in the paraventricular and supraoptic nuclei of the hypothalamus and the central amygdala (Figs. 4 and 5). The lateral septum and nucleus accumbens had a significant but less pronounced increase as well (Fig. 5).

Both acute haloperidol and clozapine treatments were followed by a notably heterogeneous distribution of Fos-positive nuclei in the islands of Calleja.

One hour after injection of haloperidol a maximal catalepsy score (60 s) was seen, whereas controls and clozapine-treated animals showed no cataleptic symptoms. Instead, clozapine induced sedation.

3.3. Long-term treatment

Long-term haloperidol treatment gave a considerable reduction in Fos-positive nuclei compared to the effect of a single dose. The reductions were 76% in the dorsolateral and 79% in the ventrolateral part of the striatum, 69% in the nucleus accumbens and 67% in the central amygdala (see Figs. 2 and 3). No significant decreases were found after 21 days' haloperidol treat-

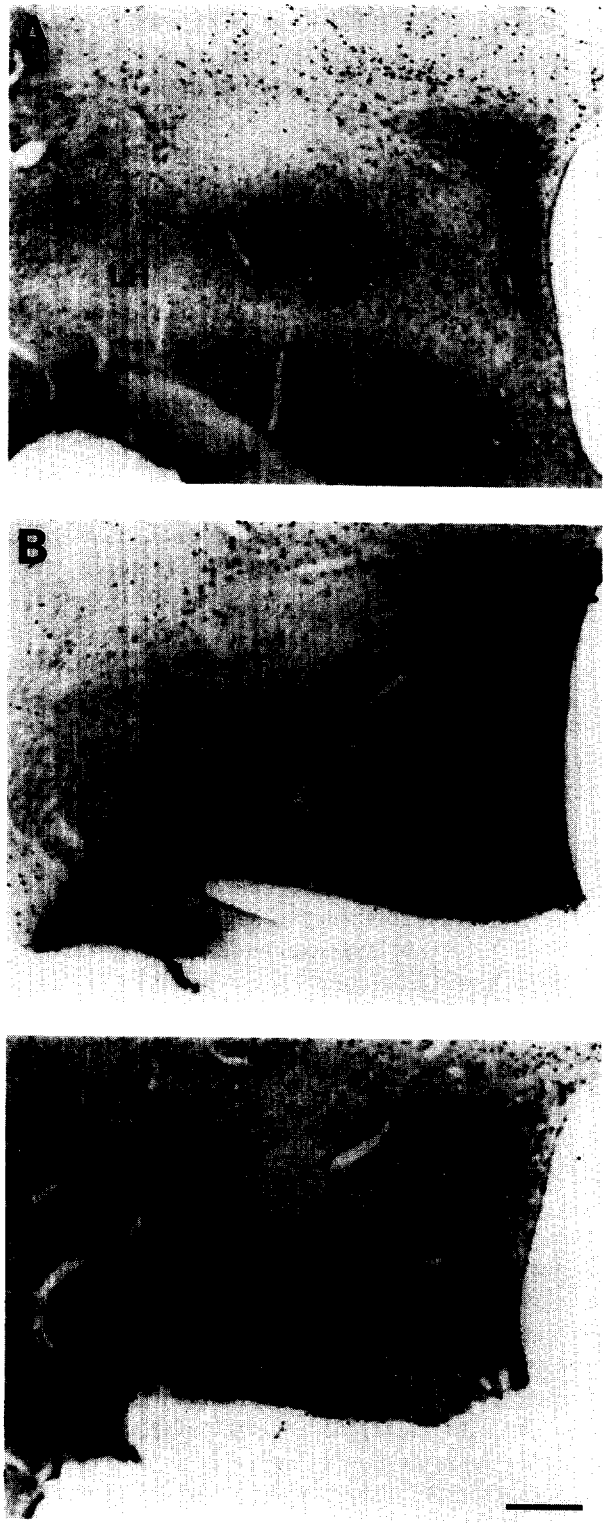

Fig. 4. Photomicrographs illustrating the Fos immunoreactivity in the paraventricular and supraoptic nuclei of the hypothalamus after clozapine administration. (A) Control; (B) single dose of clozapine; (C) 21 days clozapine treatment. LH, lateral hypothalamus; f, fornix; opt, optic tract. Scale bar: 250 μm.

ment in the other nuclei examined. Moreover, the lateral septum exhibited a small increase in Fos immunoreactivity.

Compared to the acute treatment, long-term treatment with clozapine induced no reduction in Fos-positive nuclei in the striatum and nucleus accumbens. Reductions of 60% and 67% were found in the paraventricular and supraoptic nucleus of the hypothalamus, respectively, whereas the amygdala had a 62% reduction in immunoreactivity (Figs. 4 and 5). A marginal decrease was seen in the lateral septum (21%).

A notably different response for the acute and the long-term effects of either drug was found in the nucleus accumbens. Whereas haloperidol reduced acute immunoreactivity greatly (69%), chronic clozapine treatment did not change the acutely induced number of Fos-positive nuclei. Despite the numerous reductions in immunoreactive nuclei in the forebrain and diencephalic areas as a result of long-term treatment, the remaining number of stained nuclei was still above baseline in these areas.

Also remarkable was the difference in weight gain of the experimental groups; at the end of the experiment the gain in weight of the control animals was about twice that of the long-term haloperidol- and 4 times that of clozapine-treated groups (Fig. 6). The decreased growth of both the haloperidol and clozapine groups was already evident on the fourth day of treatment. In the present experiments catalepsy scores were not decreased below 60 s during the long-term haloperidol treatment (data not shown).

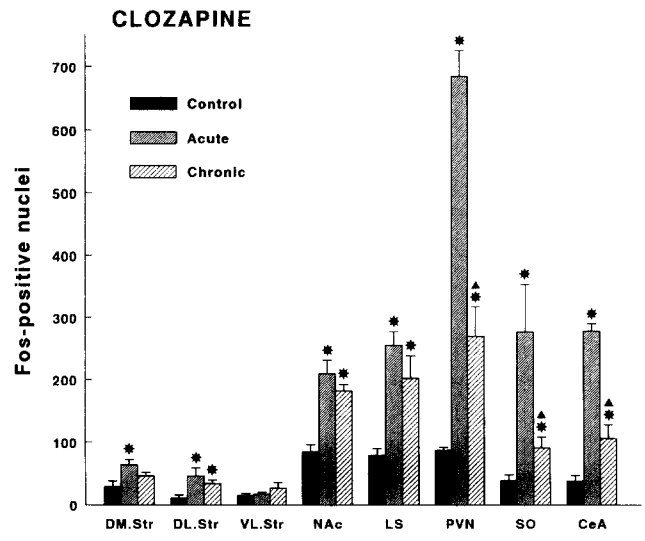

Fig. 5. Histograms showing the mean number (±S.E.M.) of Fos-positive nuclei in controls, after acute and long-term clozapine treatment in several forebrain regions. Differences were statistically significant: between clozapine-treated and control rats ($^{*}P < 0.05$) and between acute and long-term clozapine treatment ($^{\blacktriangle}P < 0.05$). For abbreviations see legend to Fig. 3.

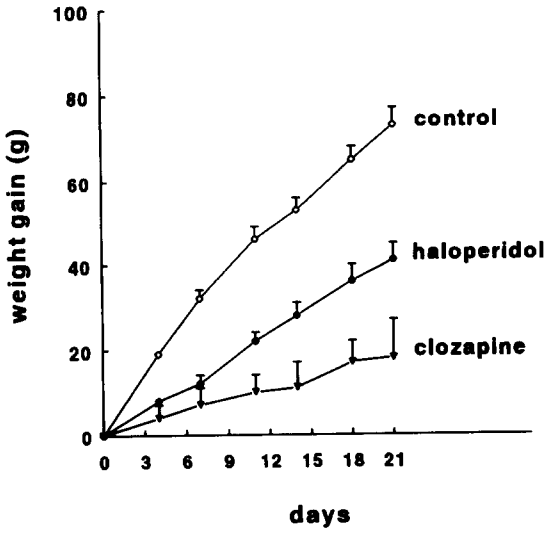

Fig. 6. Weight gain (mean ± S.E.M.) of the control rats and during long-term haloperidol and clozapine treatment. Growth of controls (saline; number of animals 15) was significantly greater than that of haloperidol-treated (number of rats = 3) or clozapine-treated (number of rats = 5) rats ($P < 0.05$).

4. Discussion

The different patterns of Fos-like immunoreactivity in the rat forebrain and diencephalon seen after acute haloperidol and clozapine treatment persisted after 21 days' treatment with either drug. Both drugs, however, induced attenuation of the response in several, but not in all, brain regions investigated after the long-term treatment. Both neuroleptic treatment protocols led to a diminished weight gain over the 21 days. Haloperidol-induced catalepsy did not decrease during the long-term treatment.

The results for striatal Fos expression after acute treatment with either drug are in agreement with other reports (e.g. Robertson and Fibiger, 1992; Rogue and Vincendon, 1992; Dragunow et al., 1990; MacGibbon et al., 1994; Nguyen et al., 1992). The pattern of striatal Fos expression after a single haloperidol injection, which is presumably caused by the blockade of dopamine D_2 receptors, correlates with their striatal distribution, as characterized by a declining gradient both in the rostral/caudal and lateral/medial direction (Boyson et al., 1986; Joyce et al., 1985; Loopuyt, 1989). Blockade of the striatal dopamine D_2 receptors is thought to cause extrapyramidal side-effects and catalepsy (Richelson, 1984; Silverstone and Turner, 1988; Farde et al., 1992). After a single dose of haloperidol the time course of evolving catalepsy and Fos immunoreactivity is about the same (e.g. Yntema and Korf, 1987; Dragunow et al., 1990). Moreover, clozapine, that induces a low incidence of parkinsonism (Borison et al., 1983; Kane et al., 1988; Coward et al., 1989) and no catalepsy, evoked a low or no increase of striatal Fos protein (e.g. this study; Robertson and Fibiger, 1992; MacGibbon et al., 1994). Thus, after a single dose of the neuroleptics, there is an apparent relation between striatal Fos protein induction and catalepsy. In the present long-term treatment study, however, there developed a dissociation between catalepsy and Fos protein induction, suggesting that, over the long term, the relation may become less obvious than that seen with acute treatment.

The acute drug treatment effects in the mesolimbic system, besides appearing in the lateral septum and the nucleus accumbens (present study; Robertson and Fibiger, 1992; Robertson et al., 1992), are also seen in the amygdala, which is part of the mesocortical limbic circuitry (present study; MacGibbon et al., 1994). These structures are innervated by the dopaminergic ventral tegmental area (A10; Beckstead et al., 1979), emphasizing the importance of the limbic dopaminergic circuitry. The effects of repeated haloperidol and clozapine administration were different in the nucleus accumbens, where clozapine did not lead to tolerance towards Fos expression. Tolerance was marginal in the lateral septum with both drugs, but was significant in the central amygdala. Clinical studies showed that clozapine is therapeutically effective in patients that benefit insufficiently from classical antipsychotics (Kane et al., 1988). It is tempting to suggest that the lack of tolerance in the nucleus accumbens after clozapine, as compared to after haloperidol, is related to this clinical efficacy in chronic patients. Accordingly, the lack of clinical effects of the classical neuroleptics in therapy-resistant patients could be due to the development of tolerance towards drug effects or to the suppression of stress-related events, particularly in the limbic areas.

Very different responses to the two antipsychotics were seen in hypothalamic nuclei, including the paraventricular and the supraoptic nuclei. The clozapine-induced Fos expression in these hypothalamic nuclei was the highest observed in the rat brain. In the paraventricular nucleus high immunoreactivity was found in both the magno- and parvocellular parts of the nucleus. The response was reduced after 21 days' treatment with clozapine, but remained significantly elevated. Clozapine may block a variety of receptors in vivo, including the dopamine D_4 (Van Tol et al., 1991), the serotonin-2 (Coward, 1992; Morilak et al., 1993) and the histamine H_1 (Leysen et al., 1993; Richelson, 1984) type receptor. Of interest are the effects of intracerebroventricular application of histamine on c-fos expression in the hypothalamic nuclei, including the paraventricular and supraoptic nuclei (Kjaer et al., 1994). Clozapine, a potent antagonist of histamine H_1 receptors, may activate Fos expression in these nuclei as well, just as both the agonist and antagonist of

dopamine receptors evoke c-fos in similar brain areas (e.g. Robertson et al., 1992). On the other hand, the receptors mentioned are also located in other brain regions, where no strong responses could be demonstrated. Stress may also cause Fos expression in the paraventricular nucleus (Sawchenko and Swanson, 1983), but in the present protocol, we tried to reduce the stress due to handling and injections. The paraventricular nucleus receives innervation from several hypothalamic nuclei, most intense from the dorsomedial hypothalamic nucleus (Ter Horst and Luiten, 1986), from nuclei in the brainstem (e.g. nucleus of the solitary tract, Ter Horst et al., 1989) and the forebrain (including bed nucleus of the stria terminalis and subfornical organ, Sawchenko and Swanson, 1983). Some parvocellular neurons of the paraventricular nucleus contain corticotropin releasing hormone and its increase in the brain ventricular system modifies several metabolic and autonomic effects, such as mean arterial pressure, heart rate and plasma glucose and glucagon levels (Dunn and Berridge, 1990). Gudelsky et al. (1989) reported marked increases in serum concentrations of corticosterone and adrenocorticotropic hormone after clozapine treatment, whereas haloperidol had no such effects. Interestingly, the effects of clozapine medication on the autonomic functions, including heart rate, in schizophrenic patients differed from those of conventional neuroleptics (Zahn and Pickar, 1993). The high Fos response in the supraoptic and magnocellular paraventricular nuclei seen after an acute dose of clozapine may be due to increased activity of vasopressinergic and oxytocinergic neurons. Regions that preferentially link up with either the oxytocinergic or vasopressinergic neurons in the paraventricular nucleus also preferentially innervate the same cell type in the supraoptic nucleus (Sawchenko and Swanson, 1983). The paraventricular nucleus is known to be involved in the regulation of food intake (Leibowitz and Stanley, 1986), which was obviously reduced in the present experiments and the clozapine-treated group showed the clearest decline in body weight gain. Since food and water intake are linked in rats, the reduced food intake will activate vasopressinergic neurons in the paraventricular and supraoptic nuclei.

This study, based on Fos protein production as a marker for neuronal activity, has demonstrated that typical (haloperidol) and atypical (clozapine) antipsychotics act at quite different loci in the forebrain and diencephalon; such a differential expression persists after chronic medication. Acute haloperidol has the most pronounced effects in the extrapyramidal regions and nucleus accumbens, whereas clozapine is particularly active in hypothalamic areas. Long-term treatment with either drug leads to the development of differential biochemical tolerance, which may contribute to their differential clinical profile.

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