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Lack of cross-tolerance between haloperidol and clozapine towards Fos-protein induction in rat forebrain regions

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Abstract

We investigated whether the acute effects of haloperidol and clozapine on Fos expression in the rat forebrain are mediated by the same receptors through evaluation of cross-tolerance, particularly in the commonly affected areas. Acutely administered haloperidol (1 mg/kg, i.p.) and clozapine (20 mg/kg, i.p.) induce regionally different (e.g., the striatum, the hypothalamic paraventricular and supraoptic nuclei, and the central amygdala) and overlapping (e.g., the nucleus accumbens and the lateral septum) patterns of Fos-protein distribution in the rat forebrain. After long-term treatment, part of the acute effects of these drugs disappears in most brain areas, except in the lateral septum, the hypothalamic paraventricular and supraoptic nuclei and the amygdala following haloperidol administration. Cross-tolerance between haloperidol and clozapine was determined by administering a challenge dose of the one antipsychotic, following a 21-day pretreatment with the same or the other drug or saline. In none of the investigated brain regions was cross-tolerance towards Fos-protein induction found after haloperidol challenge in the clozapine-treated rats. Conversely, a competitive dose of clozapine in long-term haloperidol-treated rats showed cross-tolerance in the lateral septum, while the common effect of the drugs in both the dorsomedial and the dorsolateral parts of the striatum was very small. These findings indicate that, for the major part, the responses to haloperidol and clozapine are mediated by different receptors, even in brain areas that are affected by both drugs.

Keywords: Haloperidol; Clozapine; Fos-protein; Cross-tolerance; Nucleus accumbens; Septum, lateral

1. Introduction

Antipsychotic drugs may cause long-lasting alterations in brain functioning by changing the pattern of gene expression, such as c-fos. Fos, the protein product of the c-fos gene, has been proposed as a marker of metabolically activated neurons (Sheng and Greenberg, 1990). Recent studies have shown that the antipsychotics haloperidol and clozapine, when given acutely, induce different patterns of Fos-like immunoreactivity in the rat forebrain. The most marked effects of haloperidol were found in the striatum, the nucleus accumbens and the lateral septum. Clozapine exerts its major effects in the nucleus accumbens, the lateral septum, the hypothalamic paraventricular- and supraoptic nuclei and the central amygdala (Dragunow et al., 1990; Deutch et al., 1992; Nguyen et al., 1992; Fink-Jensen and Kristensen, 1994; MacGibbon et al., 1994; Merchant et al., 1994; Robertson et al., 1994; Sebens et al., 1995; Wan et al., 1995). In rats treated acutely with either classical or atypical antipsychotics, the nucleus accumbens appears to be the brain area where the increase in Fos-positive nuclei is most consistent (Robertson et al., 1994; Wan et al., 1995). After long-term treatment, such a Fos response was attenuated in several brain areas when rats were exposed to an additional dose of haloperidol or clozapine (Sebens et al., 1995). Apparently, the target cell recognizes whether it has been exposed to either antipsychotic, thus indicating the development of tolerance.

In the present study we investigate whether the actions of haloperidol and clozapine are mediated by common or by different mechanisms in vivo. If, in rats that develop tolerance following long-term drug treatment, a challenge dose of the test drug given 24 h after the last injection of the long-term treatment with the same or another antipsychotic leads to a comparable increase in Fos-protein induction that differs from the effect of a single dose in drug-naive animals, common mechanisms of action are involved, possibly but not necessarily through identical receptor types. Conversely, if the antipsychotics have different mechanisms of action regarding the observed Fos-pro-

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tein response, the number of Fos-positive nuclei, induced by a challenge dose of the test drug after long-term treatment with either the same or a competitive substance, will be different, whereas the number in response to a challenge with one drug following pretreatment with the other will be equal to the number seen after a single dose of the test drug. So our paradigm reveals possible cross-tolerance between classical and atypical antipsychotics at a brain regional level.

We compared the number of Fos-positive nuclei in the rat forebrain after a single dose of haloperidol or clozapine with that induced by a challenge dose, administered after long-term pretreatment with the same or the competitive drug. Fos-positive nuclei were quantified in the following rat forebrain regions: the dorsomedial, dorsolateral and ventrolateral striatum, the nucleus accumbens, the lateral septum, the paraventricular and supraoptic nuclei of the hypothalamus and the amygdala.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 200–220 g at the start of the experiment were maintained on a 12-h light/dark cycle, with free access to food and water during the treatment period. All experiments were performed during the light period.

2.2. Design of the study

Nine groups of rats were treated once daily for 21 days with either saline (1 ml/kg), haloperidol (1 mg/kg) or clozapine (20 mg/kg) by intraperitoneal injection.

To study the effects of acute treatment, 2 groups of rats received saline injections for 21 days, followed by a dose of haloperidol (Sal + Hal) or clozapine (Sal + Cloz) on the subsequent day. The long-term treated groups were administered either haloperidol (Hal + Hal) or clozapine (Cloz + Cloz), for 22 days. For cross-tolerance experiments 2 groups of rats were injected with haloperidol or clozapine for 21 days, followed on the next day by a single dose of the competitive drug, clozapine (Hal + Cloz) or haloperidol (Cloz + Hal). To control for interference of residual haloperidol or clozapine, persisting 24 h after the last injection, 2 groups of rats were treated with either drug for 21 days, and a dose of saline was injected 24 h later (Hal + Sal and Cloz + Sal). Control animals received saline injections during the same time period (Sal + Sal). All groups consisted of 5–6 animals. Two hours after the last injection rats were perfused under pentobarbital anaesthesia. The animal experiments were approved by the Committee on Animal Bio-ethics of the University of Groningen.

2.3. Drugs

Haloperidol (Janssen Pharmaceutica, Beerse, Belgium) for intravenous use was obtained commercially and diluted with saline. Clozapine, generously supplied by 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.

2.4. Immunohistochemistry

Animals were perfused under deep anaesthesia (pentobarbital 100 mg/kg i.p.) with saline (100 ml) followed by 300 ml of 4% paraformaldehyde dissolved in 0.1 M sodium phosphate buffer pH 7.4. Fixation was performed within 15 min. The brains were removed and postfixed overnight at 4°C in a 4% paraformaldehyde solution containing 0.05% glutaraldehyde and then stored in 50 mM Tris-buffered saline pH 7.4, with 0.1% Na-azide. Coronal sections of 50 μm were cut from the postfixed brains, using a Vibratome. The immunohistochemical procedure was performed on free-floating sections, according to the previously described procedure (Sebens et al., 1995). In the present study, a sheep polyclonal antiserum (Cambridge Research Biochemicals, CRB, OA-11-824, UK) against Fos-peptide (16 amino acids of mouse and human c-fos) was used. Briefly, sections were preincubated for 1 h at room temperature in 4% normal rabbit serum (Sigma Immuno Chemicals, St. Louis, MO, USA), to decrease background staining. Subsequently, the Fos primary antiserum was added, diluted (1:2000) in Tris-buffered saline, containing 2% bovine serum albumin (BDH Laboratory Supplies, Poole, UK), 2% normal rabbit serum and 0.5% Triton X-100 (Baker Grade). The other components used in the immunohistochemical procedure were: a biotinylated anti-sheep secondary antibody (1:800, Pierce, Rockford, IL, USA) and an avidin-biotinylated horseradish peroxidase complex (1:125, Vector Laboratories). The peroxidase reaction was developed with DAB-nickel and H_2O_2. To control for the specificity of immunoreactivity, some of the sections were incubated with omission of the primary or the secondary antibody.

2.5. Quantification and statistical analysis

Schematic drawings of the representative sections used for counting Fos-positive nuclei are shown in Fig. 1. The counted areas are indicated by shaded squares. Fos-immunoreactive nuclei were counted within a 400 × 400 μm grid at a magnification of 125 × in the dorsomedial, dorsolateral and ventrolateral striatum, the nucleus accumbens, the lateral septum, the paraventricular hypothalamic nucleus and the central nucleus of the amygdala. In the supraoptic nucleus the total number of Fos-positive nuclei in the cross-section was determined. Fos-positive nuclei

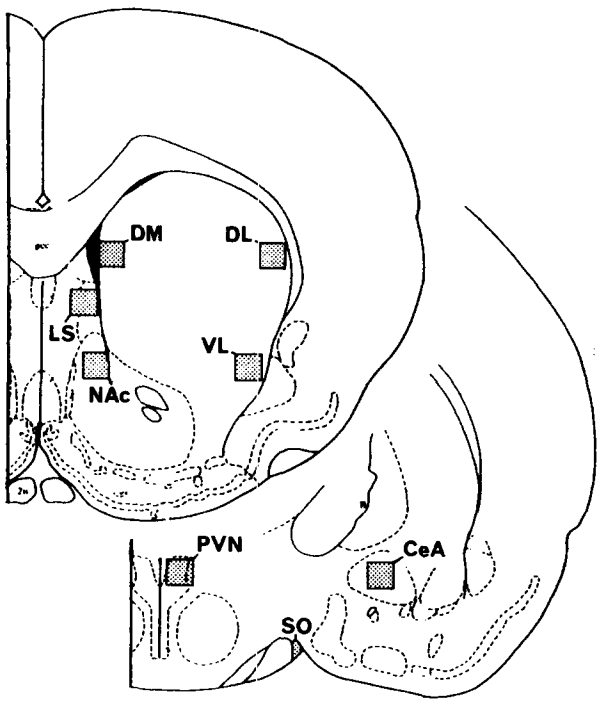

Fig. 1. Schematic representations of the 2 levels used for the counting of Fos-positive nuclei. Shadings indicate the counted areas. DM, dorsomedial striatum; DL, dorsolateral striatum; VL, ventrolateral striatum; NAc, nucleus accumbens; LS, lateral septum; PVN, paraventricular nucleus of the hypothalamus; SO, supraoptic nucleus of the hypothalamus; CeA, central amygdala.

were counted bilaterally and averaged per animal. The mean number ($\pm$S.E.M.) of Fos-positive nuclei per experimental group was determined. As the nuclei counts can be considered to be distributed according to a Poisson distribution, Poisson regression analysis (with likelihood-ratio tests, Kleinbaum et al., 1988) was used for comparison of various groups. Because of the fairly large number of comparisons and also in view of the approximate nature of likelihood-ratio tests, the differences were considered significant if $P < 0.001$.

3. Results

3.1. Staining procedure and Fos-positive nuclei in controls

The expression of Fos-positive nuclei was generally lower than that obtained in previous work, probably because a different antibody was used. The c-fos antibody PCO5 (Oncogene Science) used previously recognises Fos as well as Fos-related antigens (MacGibbon et al., 1994). The presently used antibody (CRB, OA-11-824) is more specific for Fos.

In control rats hardly any Fos-positive nuclei could be detected in the striatal regions, but limbic areas, like the nucleus accumbens, the lateral septum and the central amygdala, as well as the paraventricular nucleus of the hypothalamus exhibited a slight to moderate Fos-protein content (Fig. 2).

3.2. Haloperidol: acute- and long-term treatment, cross-tolerance

The pattern of Fos expression induced by haloperidol administration in this experiment is in general agreement with our previous study. After a single dose of haloperidol the number of Fos-positive nuclei increased in the dorsomedial, the dorsolateral and the ventrolateral striatum, in the nucleus accumbens and in the lateral septum. The striatal effect could be observed along the entire rostrocaudal axis. A minor effect was found in the paraventricular and supraoptic nuclei of the hypothalamus and the central amygdala (Fig. 2, Table 1).

Long-term haloperidol treatment decreased the acutely induced number of Fos-positive nuclei by 46% in the dorsomedial, 80% in the dorsolateral, 70% in the ventrolateral part of the striatum and by 65% in the nucleus accumbens. In the lateral septum, the hypothalamic paraventricular and supraoptic nuclei and the amygdala Fos-protein induction was found to persist to the full extent (Fig. 2, Table 1).

The number of Fos-positive nuclei induced by a challenge dose of haloperidol in long-term clozapine-treated rats and that induced in animals pretreated with haloperidol were the same only in the paraventricular nucleus of the hypothalamus. The different Fos expression observed after haloperidol challenge following clozapine pretreatment and a single haloperidol injection was limited to the dorsomedial and dorsolateral striatum, the nucleus accumbens, the supraoptic nucleus and the amygdala. Considering both findings, no cross-tolerance between the 2 drugs occurred in any of the investigated brain regions after haloperidol challenge. Interference of residual clozapine can be excluded as no Fos-protein induction could be detected 24 h after clozapine injection, except in the nucleus accumbens, where the effect was very small (Fig. 2, Table 2).

3.3. Clozapine: acute- and long-term treatment, cross-tolerance

Acute clozapine treatment caused a slight increase in Fos-positive nuclei in the dorso- and ventrolateral striatum, while in the dorsomedial striatum the increase was moderate. A more pronounced effect on Fos-protein induction was found in limbic areas, particularly in the nucleus accumbens, the lateral septum, the central amygdala and the supraoptic nucleus. Prominent was the increase in the paraventricular nucleus of the hypothalamus, an area that is, like the supraoptic nucleus, extensively interconnected with limbic structures (Fig. 2, Table 2).

After long-term clozapine treatment a small, but signifi-

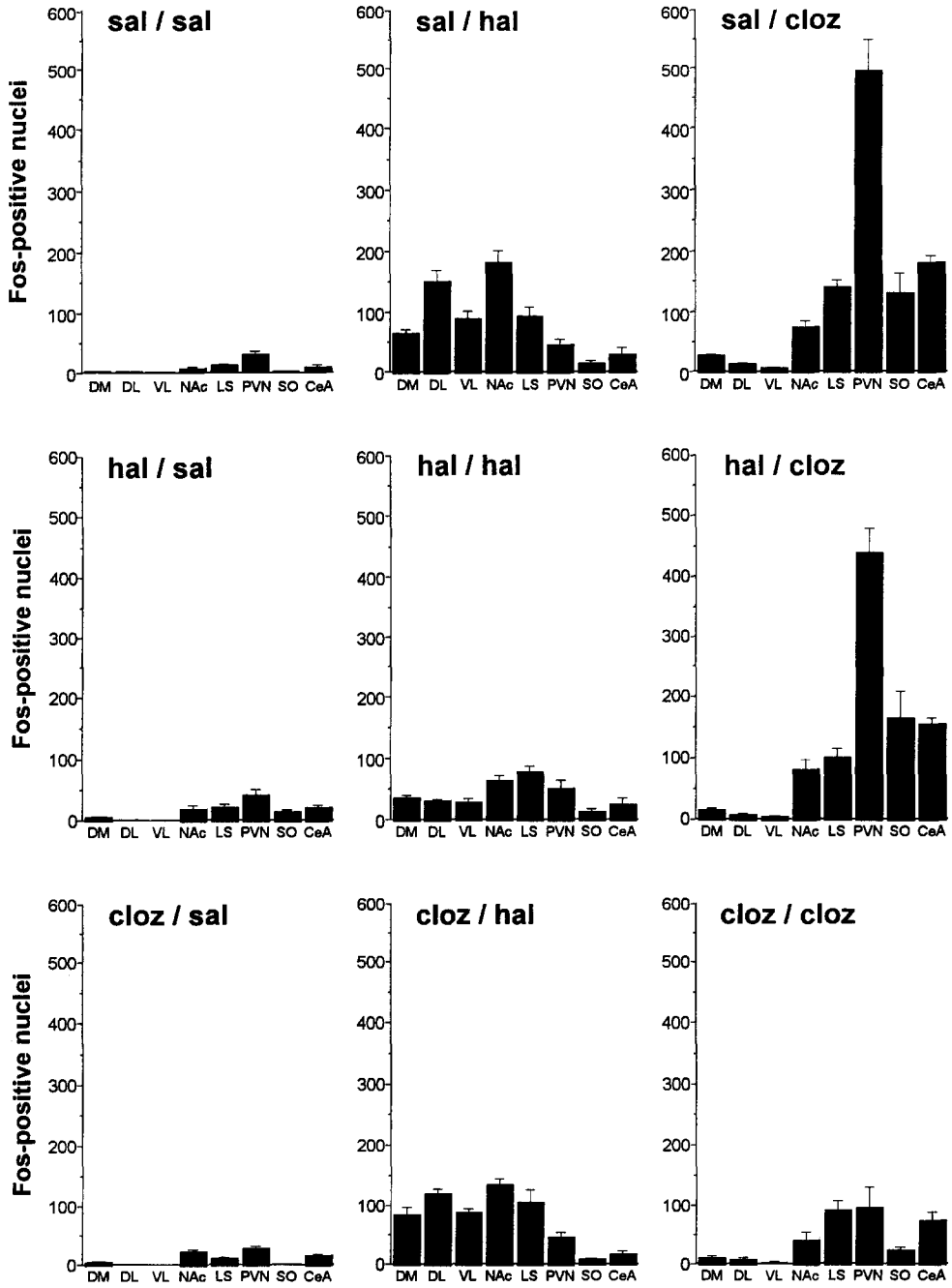

Fig. 2. Histogram showing the mean number (±S.E.M.) of Fos-positive nuclei in controls (Sal + Sal), after acute (Sal + Hal, Sal + Cloz) and long-term haloperidol (Hal + Hal) or clozapine (Cloz + Cloz) treatment, 24 h after long-term haloperidol (Hal + Sal) or clozapine (Cloz + Sal) treatment and after a challenge dose of haloperidol or clozapine in long-term clozapine- or haloperidol-treated rats, respectively (Cloz + Hal, Hal + Cloz). Brain areas include: DM, dorsomedial striatum; DL, dorsolateral striatum; VL, ventrolateral striatum; NAc, nucleus accumbens; LS, lateral septum; PVN, paraventricular nucleus of the hypothalamus; SO, supraoptic nucleus; CeA, central amygdala. For statistical significance of the differences, see Tables 1 and 2.

cant decrease in the number of Fos-positive nuclei was seen, with respect to the acutely induced Fos expression, both in the dorsomedial and the dorsolateral striatum, while in the nucleus accumbens and the lateral septum the decrease was more obvious. The reduction was substantial in the paraventricular (81%) and supraoptic nuclei (83%) of the hypothalamus and in the amygdala (59%, Fig. 2, Table 2).

The effect of long-term pretreatment with either haloperidol or clozapine on the induction of Fos-positive nuclei by a subsequent clozapine challenge was similar in the dorsomedial, the dorsolateral and the ventrolateral parts

Table 1
Statistical significance of the differences in number of Fos-positive nuclei between different experimental groups

	DM	DL	VL	NAc	LS	PVN	SO	CeA
Sal + Hal/Sal + Sal	>	>	>	>	>	>	>	>
Hal + Hal/Sal + Sal	>	>	>	>	>	>	>	>
Hal + Hal/Hal + Sal	>	>	>	>	>	NS	NS	NS
Hal + Hal/Sal + Hal	<	<	<	<	NS	NS	NS	NS
Hal + Sal/Sal + Sal	NS	NS	NS	>	NS	NS	>	>
Cloz + Hal/Hal + Hal	>	>	>	>	>	NS	<	<
Cloz + Hal/Sal + Hal	>	<	NS	<	NS	NS	<	<
Cloz + Hal/Hal + Cloz	>	>	>	>	NS	<	<	<
Cloz + Hal/Cloz + Cloz	>	>	>	>	NS	<	<	<

Groups are defined as: (Sal + Sal) controls, (Sal + Hal) acute and (Hal + Hal) long-term haloperidol-treated, (Hal + Sal) saline challenged and (Hal + Cloz) clozapine challenged long-term haloperidol-treated and (Cloz + Hal) haloperidol challenged long-term clozapine-treated rats. Brain regions include: DM, dorsomedial striatum; DL, dorsolateral striatum; VL, ventrolateral striatum; NAc, nucleus accumbens; LS, lateral septum; PVN, paraventricular nucleus of the hypothalamus; SO, supraoptic nucleus; CeA, central amygdala. First group/second group: < or > indicates the first group is statistically significant smaller or larger than the second group at $P < 0.001$. NS not significant.

of the striatum and in the lateral septum, which suggests the involvement of common mechanisms of action of the 2 drugs in these regions. Another indication that cross-tolerance occurred is the somewhat reduced effect of a challenge dose of clozapine in haloperidol-pretreated animals versus saline-pretreated rats in both the dorsomedial and the dorsolateral striatum and a more distinct reduction in the lateral septum. Of all nuclei investigated, the existence of cross-tolerance between the two antipsychotics was apparent only in the lateral septum, while the common effect in both the dorsomedial and the dorsolateral parts of the striatum was very small though significant (see Fig. 2, Table 2).

Table 2
Statistical significance of the differences in number of Fos-positive nuclei between different experimental groups.

	DM	DL	VL	NAc	LS	PVN	SO	CeA
Sal + Cloz/Sal + Sal	>	>	>	>	>	>	>	>
Cloz + Cloz/Sal + Sal	>	>	NS	>	>	>	>	>
Cloz + Cloz/Cloz + Sal	NS	>	NS	>	>	>	>	>
Cloz + Cloz/Sal + Cloz	<	<	NS	<	<	<	<	<
Cloz + Sal/Sal + Sal	NS	NS	NS	>	NS	NS	NS	NS
Hal + Cloz/Cloz + Cloz	NS	NS	NS	>	NS	>	>	>
Hal + Cloz/Sal + Cloz	<	<	NS	NS	<	<	>	<
Hal + Cloz/Cloz + Hal	<	<	<	<	NS	>	>	>
Hal + Cloz/Hal + Hal	<	<	<	>	>	>	>	>

Groups are defined as: (Sal + Sal) controls, (Sal + Cloz) acute and (Cloz + Cloz) long-term clozapine-treated, (Cloz + Sal) saline challenged and (Cloz + Hal) haloperidol-challenged long-term clozapine-treated and (Hal + Cloz) clozapine-challenged long-term haloperidol-treated rats. For abbreviations of the counted forebrain regions, see legend to Table 1. First group/second group: < or > indicates the first group is statistically significant smaller or larger than the second group at $P < 0.001$. NS not significant.

4. Discussion

The patterns of Fos-protein expression following acute treatments observed here were similar to those of our previous paper (Sebens et al., 1995) and most other reports (e.g., Dragunow et al., 1990; Nguyen et al., 1992; Robertson and Fibiger, 1992; MacGibbon et al., 1994; Fink-Jensen and Kristensen, 1994; Robertson et al., 1994; Wan et al., 1995). The Fos response to haloperidol, as compared to clozapine, was much more pronounced in the striatum, whereas clozapine elicited Fos expression exclusively in the hypothalamus and in the amygdala. In most brain areas investigated, the acute response had attenuated after a 3-week treatment period. In none of the investigated brain areas cross-tolerance between haloperidol and clozapine towards Fos-protein induction was observed, except for a significant interaction in the lateral septum and in both the dorsomedial and dorsolateral parts of the striatum.

Acute haloperidol administration induced a striatal pattern of Fos distribution that correlates with the distribution of dopamine D_2 receptors (Boyson et al., 1986; Loopuyt, 1989). Thus, both dopamine D_2 receptors and haloperidol-induced Fos-positive nuclei have their highest densities in the dorsolateral part of the striatum, a region that is involved in motor functions through its connections with the sensorimotor cortex (McGeorge and Faull, 1989). High occupancy of striatal dopamine D_2 receptors is thought to cause extrapyramidal side-effects (Farde et al., 1992). In line with this are the in vivo estimations of dopamine D_2 receptor occupancies of 78% by haloperidol and 47% by clozapine, as assessed by positron emission tomography (Farde et al., 1992, 1994). Accordingly, clozapine is associated with a low incidence of parkinsonism (Kane et al., 1988; Coward et al., 1989) and with only a few (yet significant) Fos-positive nuclei in various parts of the striatum. Although the Fos response was minimal, cross-tolerance could be observed in both the dorsolateral and the dorsomedial striatum. Despite the fact that clozapine may block a substantial number of the dopamine D_2 receptors, no conspicuous amounts of Fos-protein were found in the striatum, not even in the dorsolateral part. Unlike haloperidol, clozapine has affinity for dopamine D_1 (Farde et al., 1994) and muscarinic receptors (Bolden et al., 1991), which may explain the small number of Fos-positive nuclei in the striatal regions. The significant cross-tolerance between haloperidol and clozapine towards Fos-protein induction in the dorsomedial tip of the striatum – a limbic region – may reflect common dopamine D_2 or D_3 receptor antagonism (Richelson, 1985; Sokoloff et al., 1992).

The marked increase in the number of Fos-positive nuclei in both the nucleus accumbens and the lateral septum after haloperidol as well as after clozapine treatment suggests a crucial role of these limbic nuclei in mediating some of the antipsychotic actions of both drugs. But, the existence of a significant interaction between the 2

drugs in the lateral septum, as revealed by Fos-protein expression and the lack of cross-tolerance in the nucleus accumbens, points to an important regional difference in the mechanisms of action of both antipsychotics in these areas. It has been suggested that the antipsychotic actions of neuroleptics are mediated by dopamine D_3 receptors, which are expressed in both the nucleus accumbens and the lateral septum (Sokoloff et al., 1990; Bouthenet et al., 1991). Whatever mechanism, it is obvious that the apparently common actions of haloperidol and clozapine are limited to the lateral septum.

In the central amygdala haloperidol had almost no effect, whereas the Fos response to acute clozapine treatment was substantial. Clozapine preferentially inactivates dopaminergic neurons of the A10 area (Todorova and Dimpfel, 1994), by which the amygdala is innervated (Beckstead et al., 1979). Interesting is the relatively high density of dopamine D_4-type receptors in the amygdala (Van Tol et al., 1991). Since the affinity of clozapine is about 10 times higher for the dopamine D_4 receptor than for the dopamine D_2 receptor (Van Tol et al., 1991, 1992), the dopamine D_4 receptor could be the main target for the antipsychotic actions of clozapine in this region and as such play a role in the therapeutic effects of clozapine in treatment-resistant schizophrenic patients.

A considerable Fos response to clozapine was observed in the hypothalamic paraventricular and supraoptic nuclei, where haloperidol had minimal effect. This observation is in accordance with a number of clinical effects of clozapine. For instance, heart rate and blood pressure are known to be influenced by clozapine (Zahn and Pickar, 1993; Naber et al., 1992). In addition, polydipsia and associated hyponatremia have often been diagnosed in schizophrenic patients (Illowsky and Kirch, 1988). Clozapine, not haloperidol, appears to improve both these disorders (Lee et al., 1991; Henderson and Goff, 1994; Leadbetter and Shutty, 1994). Furthermore, a clinical dose of clozapine, but not of haloperidol, increases serum cortisol levels in humans (Gudelsky et al., 1989) and corticosterone levels in rats (Albinsson et al., 1993). Such increases may be related to the fact that some atypical antipsychotics, including clozapine, stimulate the hypothalamic-pituitary-adrenocortical axis more potently than classical drugs do (Nash et al., 1988). This effect of a higher cortisol excretion might be mediated by the serotonergic receptor subtypes 5-HT_{2A} and 5-HT_{2C} (Fuller, 1990), which are supposedly blocked by clozapine. The potent antihistaminic properties of clozapine may also contribute to the significant Fos response in the hypothalamic nuclei, since intracerebroventricularly infused histamine also increases Fos-protein levels in both the paraventricular and supraoptic nuclei (Kjaer et al., 1994).

In conclusion, the present study suggests that cross-tolerance through Fos-protein induction allows one to assess the different actions of antipsychotic drugs in vivo. There is a partial overlap in the regional distribution of Fos-protein observed in the rat forebrain after acute treatment with either haloperidol or clozapine. The present long-term experiments indicate that the two antipsychotics affect most brain structures by different receptor types, even in brain areas where both haloperidol and clozapine are active.

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