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Limited participation of 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors in the clozapine-induced Fos-protein expression in rat forebrain regions

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Abstract

Through the development of tolerance following long-term clozapine treatment, we investigated whether 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors participate in the clozapine-induced Fos-protein expression in the rat forebrain. Tolerance exists when the acutely increased Fos responses to a challenge dose of the 5-HT_{1A} and 5-$HT_{2A/2C}$ agonists 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane-hydrochloride (DOI) and 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), respectively, given simultaneously to rats, are attenuated after 3-week clozapine (20 mg kg^{-1} day^{-1} i.p.) pretreatment. As compared to the acute effects of clozapine, the Fos responses to concomitant administration of the 5-HT receptor agonists DOI (2.5 mg kg^{-1} i.p.) and 8-OH-DPAT (2.5 mg kg^{-1} i.p.) were more pronounced in the prefrontal cortex, the nucleus accumbens core and the dorsomedial and ventromedial striatum, areas in which clozapine (20 mg kg^{-1} i.p.) exhibited marginal effects. In the hypothalamic paraventricular nucleus, both clozapine and DOI/8-OH-DPAT induced a remarkably high number of Fos-positive nuclei. Long-term clozapine pretreatment attenuated the acutely induced Fos expression of the 5-HT receptor agonists in the nucleus accumbens core, the dorsomedial and ventromedial parts of the striatum and the lateral septum, indicating (partial) common sites of action of the agents in these brain regions. No tolerance was found in the nucleus accumbens shell and the hypothalamic paraventricular nucleus and the central amygdala, suggesting that the clozapine-induced Fos responses, though distinct in these regions, are independent of 5-HT receptors. The prefrontal cortex and the dorsolateral striatum indicated only a tendency towards tolerance. In addition, the involvement of the tested 5-HT receptor agonists in the clozapine-enhanced release of plasma corticosterone became apparent. The present results indicate that the clozapine-induced patterns of Fos expression in the rat forebrain can only be in part attributed to an interaction with 5-$HT_{1A/2A/2C}$ receptors.

Keywords: Clozapine; Fos-protein; Tolerance; DOI; 8-OH-DPAT

1. Introduction

The clinical effects of atypical antipsychotic drugs differ from those of typical neuroleptics in, e.g. the production of less prominent extrapyramidal side effects (Coward et al., 1989; Gerlach and Peacock, 1994) and, in some cases, in their effects on negative symptoms of schizophrenia (see Meltzer and Gudelsky, 1992). Clozapine, an atypical antipsychotic, has the potential to interfere with several cerebral receptors, including dopamine and 5-HT receptor types, as shown both in vitro and in vivo (Bymaster et al., 1997). Consequently, the clinical profile of clozapine has been attributed to a mild blockade of the dopamine D_2 type receptors in the basal ganglia, resulting in low extrapyramidal side effects (Gerlach and Peacock, 1994). Furthermore, a pronounced blockade of prefrontal 5-HT_2 receptors and activation of 5-HT_{1A} receptors resulted in alleviation of negative symptoms (Meltzer, 1999). Conversely, haloperidol, a typical antipsychotic drug, is associated with a high incidence of extrapyramidal side effects at therapeutic doses and accordingly blocks dopamine D_2 receptors in vivo up to 85% (Farde et al., 1992). In particular, the ratio of 5-HT_2/dopamine D_2 receptor blockade is believed to determine the clinical profile of clozapine (Kapur and Remington, 1996).

Antipsychotic agents induce a specific distribution pattern of brain regional Fos-protein expression following acute as well as long-term treatment in rats. Acutely

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administered clozapine induces high levels of Fos-protein in several limbic areas, including the prefrontal cortex, the nucleus accumbens shell, the lateral septum, the central amygdala and the hypothalamic paraventricular nucleus and low expression in the striatum. Haloperidol, however, exhibits its major acute Fos effects in the basal ganglia, the nucleus accumbens and the lateral septum (Dragunow et al., 1990; Deutch and Duman, 1996; Robertson and Fibiger, 1992; Robertson et al., 1994; Sebens et al., 1995, 1996). The difference in patterns of Fos distribution between the two antipsychotics in vivo has been attributed to the previously mentioned affinities for the dopamine and 5-HT receptor subtypes. In line with the possible involvement of the latter receptors are the results reported by Tremblay et al. (1998). They found that concomitant administration of the 5-HT receptor agonists 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane-hydrochloride (DOI) and 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), a 5-$HT_{2A/2C}$ and a 5-HT_{1A} agonist, respectively, transforms the typical pattern of brain regional c-*fos* expression generated by haloperidol into a pattern resembling that of the atypical antipsychotic clozapine. These studies concern the acute effects only, whereas chronic alterations of receptor properties may be more relevant for the clinical effects.

In previous studies (Sebens et al., 1995, 1996, 1998), we have shown that long-term treatment and cross-tolerance are powerful paradigms to determine whether or not drugs induce Fos-protein expression via common sites of action. Accordingly, we observed that following long-term treatment with clozapine, the acute effects of haloperidol on the expression of Fos-protein remained virtually maximal in a number of limbic structures, thereby indicating the involvement of different mechanisms of action, e.g. receptors. On the other hand, the limbic Fos effects of olanzapine, atypical like clozapine, were significantly decreased following clozapine pretreatment, while the effects of olanzapine in the rat striatum remained nearly unaffected (Sebens et al., 1996, 1998). Such studies suggest, that although both haloperidol and clozapine (or olanzapine) induce similar distributions of Fos-protein expression in some limbic brain areas, the involved receptors must be different.

In the present study, we used a long-term treatment paradigm to investigate in vivo the role of the 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors in the brain regional pattern of Fos-protein expression as induced by clozapine. Rats were treated with either saline or clozapine for 3 weeks, followed by a single injection of saline, clozapine or a combination of DOI and 8-OH-DPAT. Thus, if the long-term effects of clozapine on Fos-expression in a particular brain region can be attributed, at least in part, to the involvement of either 5-HT receptors, then the Fos responses to a supplemental dose of the agonists DOI and 8-OH-DPAT should be attenuated, compared to the acute Fos effects of the agonists. In this study, we quantified the number of Fos-positive nuclei following the various treatment protocols in several regions of the rat forebrain. Since acute administration of clozapine or the 5-HT receptor agonists results in a several fold increase in circulating corticosterone levels, presumably mediated by 5-HT_{1A} receptors (Compaan et al., 1996; Fuller and Snoddy, 1990; Meltzer et al., 1989), we also measured plasma corticosterone levels.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 200–220 g at the start of the experiment were housed individually in a 12-h light/dark cycle environment 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

The acute experiments consisted of three groups of rats, which received saline (1 ml kg^{-1} day^{-1}) for 7 days prior to the challenge in order to minimize stress-induced Fos expression and enhancement of plasma corticosterone levels. The following day, a single dose of saline (1 ml kg^{-1}, $n = 5$), clozapine (20 mg kg^{-1}, $n = 4$) or a combination of the 5-HT receptor agonists DOI (2.5 mg kg^{-1}) and 8-OH-DPAT (2.5 mg kg^{-1}, $n = 6$) was administered. All injections were given intraperitoneally. The long-term experiments were performed in two groups of rats that received clozapine (20 mg kg^{-1} day^{-1}) for 3 weeks followed by a challenge dose of clozapine ($n = 6$) or the agonists DOI/8-OH-DPAT ($n = 5$). All animals were perfused transcardially under pentobarbital anaesthesia 2.5 h after the final drug injection. Before perfusion, a blood sample was taken from the heart (left ventricle) to determine plasma corticosterone levels. The blood samples were transferred to a centrifuge tube containing 10 μl heparin solution (500 U ml^{-1}) and centrifuged for 15 min at $500 \times g$. The plasma was separated from the blood cells and stored at −20°C for the corticosterone assay. All experimental procedures were approved by the Committee on Animal Bioethics of the University of Groningen.

2.3. Drugs

Clozapine, generously supplied by Novartis Basel (Switzerland), was dissolved in slightly acidified saline and pH adjusted to 5–6 with NaOH. DOI and 8-OH-DPAT were obtained commercially from Research Biochemicals (Natick, MA, USA) and Sigma (St. Louis, MO, USA), respectively. Both substances were dissolved in saline.

None of the solutions were buffered; the injections of the solutions did not produce any apparent discomfort.

2.4. Corticosterone assay

Corticosterone was extracted from 75 μl plasma using a liquid extraction method. Quantification of corticosterone was performed with High-Performance Liquid Chromatographic (HPLC) separation in combination with ultraviolet detection (Dawson et al., 1984). The absolute detection threshold for corticosterone in plasma was 0.8 μg/dl.

2.5. Immunohistochemistry

Animals were perfused under deep anaesthesia (pentobarbital 100 mg kg^{-1}) with saline for 1 min followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.4 for 15 min. Brains were removed and post-fixed overnight at 4°C in 4% paraformaldehyde solution before being stored in 50 mM Tris-buffered saline pH 7.4 containing 0.1% Na-azide. After cryoprotection by overnight immersion in a buffered (50 mM Tris/HCl buffer pH 7.4) 30% sucrose solution at room temperature, the brains were sliced into 30 μm coronal sections using a cryostat microtome. Immunostaining was performed on free-floating sections, according to the previously described procedure (Sebens et al., 1995). Briefly, sections were pretreated with 0.3% H_2O_2 and preincubated in 4% normal goat serum before the Fos primary antiserum (1:10,000, Oncogene Science, Ab-5, Cambridge, MA, USA) was added. A biotinylated anti-rabbit secondary antibody (1:800, Vector Laboratories, Burlingame, CA, USA) was used followed by an avidin–biotinylated horseradish peroxidase complex (1:125, Vector Laboratories). Intermittent washing was done with Tris-buffered saline. The peroxidase reaction was developed with DAB (3,3′ diaminobenzidine)-Ni (ammonium nickel sulphate)/H_2O_2 in Tris buffer. To control for the specificity of immunoreactivity, some of the sections were incubated with omission of the primary or the secondary antibody.

2.6. Quantification and statistical analysis

Schematic drawings of the representative sections used for counting Fos-positive cells are shown in Fig. 1. The counted areas are indicated by grey-filled squares. Fos-positive cells were counted manually within a 400 × 400 μm grid at a magnification of 125 in the dorsomedial, the ventromedial and the dorsolateral striatum, the lateral septum, the hypothalamic paraventricular nucleus and the central amygdala. In the prefrontal cortex an area of 320 × 480 μm was counted, while in the nucleus accumbens core and shell regions of 320 × 320 μm were quantified. Fos-positive cells were counted bilaterally and averaged per animal. Per experimental group, the mean number (±S.E.M.) of Fos-positive cells and the average levels (±S.E.M.) of plasma corticosterone were determined. The data of the different groups were compared using a one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls or the Dunn's Method for multiple comparison procedures. The differences were considered significant if $P < 0.05$.

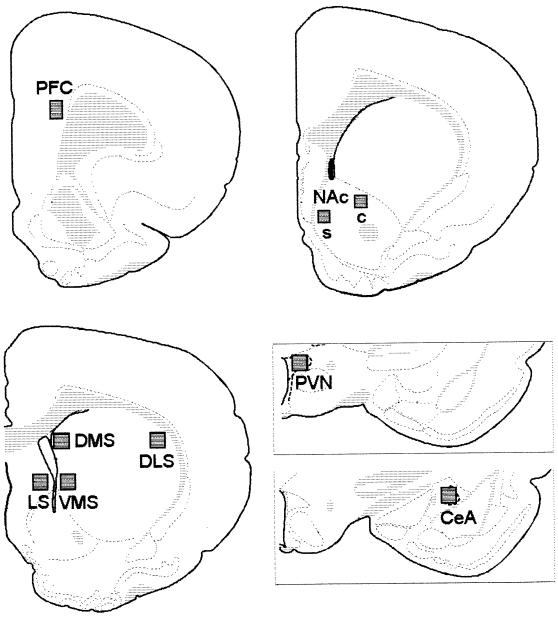

Fig. 1. Schematic representations of the levels used for the counting of Fos-positive cells. Grey-filled squares indicate the counted regions. PFC, prefrontal cortex; NAc c, nucleus accumbens core; NAc s, nucleus accumbens shell; DMS, dorsomedial striatum; VMS, ventromedial striatum; DLS, dorsolateral striatum; LS, lateral septum; PVN, paraventricular nucleus of the hypothalamus; CeA, central amygdala. Fos-positive cells were counted manually at a magnification of 125× within areas of 400×400 μm. In the PFC, this area was 320×480 μm and in the NAc c and NAc s 320×320 μm.

3. Results

3.1. Acute experiments

In general, all experimental groups induced a lower number of Fos-positive nuclei than observed in our previous studies (Sebens et al., 1995, 1996), possibly due to the fact that in the present study a cryostat microtome was used instead of a vibratome apparatus. Simultaneous administration of DOI and 8-OH-DPAT increased the number of Fos-positive nuclei in the prefrontal cortex, the nucleus accumbens core and shell, the dorsomedial, the ventromedial and the dorsolateral striatum, the lateral septum, the central amygdala and particularly in the hypothalamic paraventricular nucleus, where the response was

conspicuous. Less prominent were the increases in Fos expression induced by a single dose of clozapine in the dorsomedial striatum and the paraventricular nucleus of the hypothalamus, whereas in the prefrontal cortex, the nucleus accumbens core and the ventromedial striatum the Fos responses were not significantly altered compared to controls (Fig. 2).

3.2. Absence of tolerance in prefrontal cortex, nucleus accumbens shell, dorsolateral striatum, hypothalamic paraventricular nucleus and central amygdala of DOI / 8-OH-DPAT challenged, clozapine pretreated animals

The acutely induced Fos expression of a clozapine challenge was attenuated by clozapine pretreatment in the

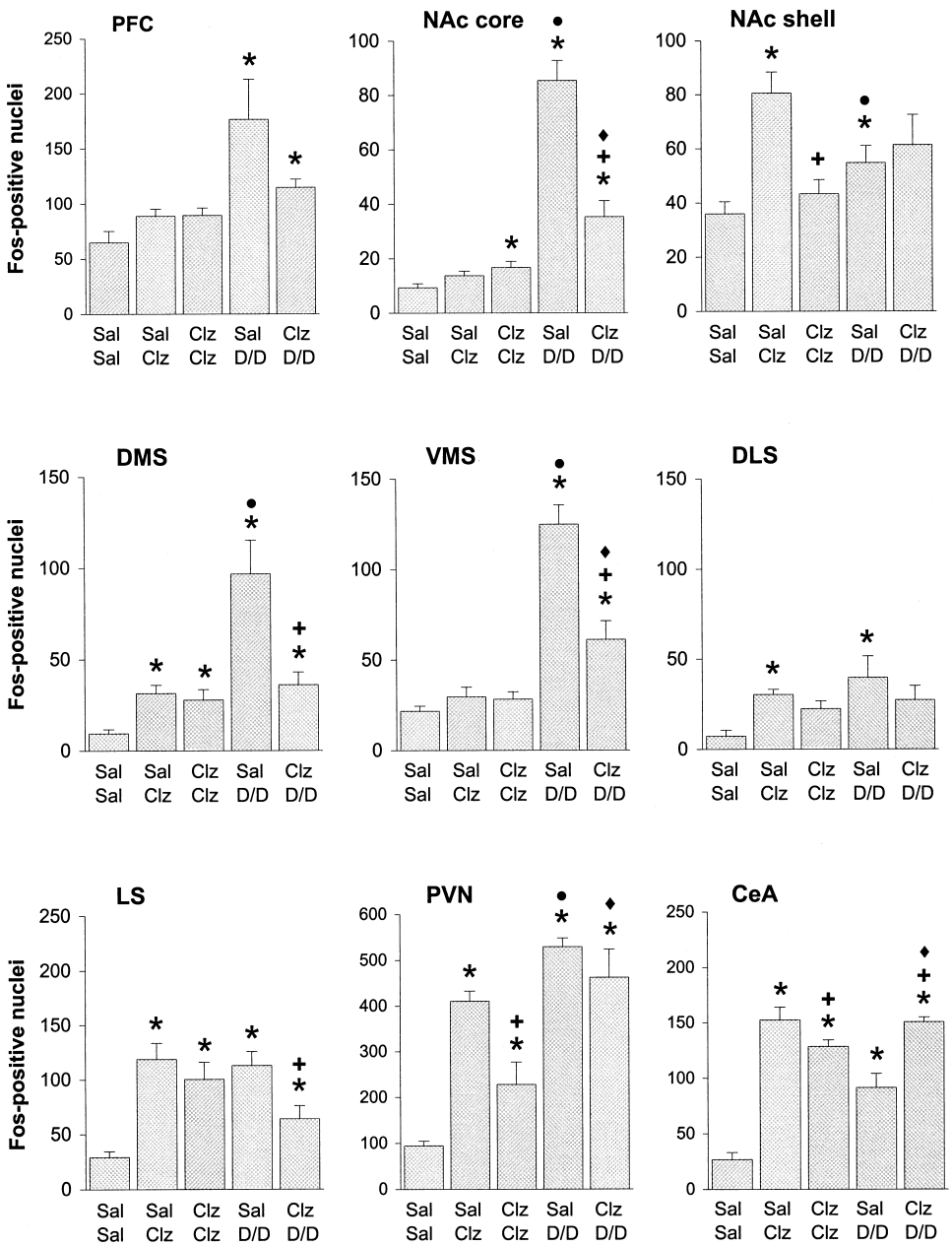

Fig. 2. Distribution patterns of Fos-positive cells (mean number ± S.E.M.) induced by a challenge dose of saline, clozapine or a combination of the 5-HT receptor agonists DOI and 8-OH-DPAT, following 3-week pretreatment with saline (Sal–Sal, Sal–Clz, Sal–D/D) or clozapine (Clz–Clz, Clz–D/D). Brain regions include: PFC, prefrontal cortex; NAc core, nucleus accumbens core; NAc shell, nucleus accumbens shell; DMS, dorsomedial striatum; VMS, ventromedial striatum; DLS, dorsolateral striatum; LS, lateral septum; PVN, paraventricular nucleus of the hypothalamus; CeA, central amygdala. Counted areas are 400 × 400 μm; in the PFC 320 × 480 μm and in the NAc core and NAc shell 320 × 320 μm. (*) Significantly different from Sal–Sal; (+) significantly different from acute treatment; (●) significant difference between Sal–Clz and Sal–D/D; (♦) significant difference between Clz–Clz and Clz–D/D.

nucleus accumbens shell, the hypothalamic paraventricular nucleus and the central amygdala by 47%, 45% and 16%, respectively. Following a challenge with the 5-HT receptor agonists DOI/8-OH-DPAT in the long-term clozapine pretreated rats, no significant reduction in Fos response was found in the nucleus accumbens shell and the hypothalamic paraventricular nucleus and there was only a tendency towards decrease in the prefrontal cortex and the dorsolateral striatum, while a significant rise in gene expression was seen in the central amygdala (Fig. 2). The absence of a significant decrease in the acutely induced Fos effects of the agonists by clozapine pretreatment in the nucleus accumbens shell, the hypothalamic paraventricular nucleus and the central amygdala suggests that 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors do not interfere in the clozapine-induced Fos expression in these areas.

3.3. Tolerance in nucleus accumbens core, dorsomedial and ventromedial striatum and lateral septum of DOI/8-OH-DPAT challenged animals following clozapine pretreatment

Following long-term clozapine treatment, the acute Fos effects of a combination of the 5-HT receptor agonists DOI and 8-OH-DPAT were decreased in the nucleus accumbens core, the dorsomedial and ventromedial striatum and the lateral septum. In the dorsomedial striatum, this reduction was 63%. The remaining number of Fos-positive cells was similar to that elicited by a clozapine challenge in long-term treated rats, indicating the existence of a complete interaction between the agents (Fig. 2). In the lateral septum, acute administration of clozapine and DOI/8-OH-DPAT resulted in similar patterns of Fos distribution. However, whereas no tolerance developed following an injection of clozapine in long-term clozapine-treated animals, a decrease of 43% was seen after a challenge with the agonists in these rats (Fig. 2). These findings suggest that in the lateral septum, 5-HT_{1A} and/or 5-$HT_{2A/2C}$ receptor subtypes interfere in the processes of clozapine-induced Fos-protein production. In the nucleus accumbens core and the ventromedial striatum, where a single dose of clozapine had hardly any effect, administration of DOI/8-OH-DPAT caused a considerable increase in Fos immunoreactivity. Long-term clozapine pretreatment reduced these acute agonistic effects by 59% and 51%, respectively (Fig. 2). Thus, despite its lack of Fos response, clozapine seems to be able to inhibit the actions of the 5-$HT_{2A/2C}$ receptor agonists in the nucleus accumbens core and the ventromedial striatum.

3.4. Plasma corticosterone levels

Both an injection with clozapine and combined DOI/8-OH-DPAT administration enhanced the levels of plasma corticosterone significantly. These increases disappeared following clozapine pretreatment; the remaining levels of corticosterone were no longer significantly above baseline (Fig. 3). These results strongly support the assumption that the clozapine-induced elevation in plasma corticosterone levels is mediated by 5-HT receptors.

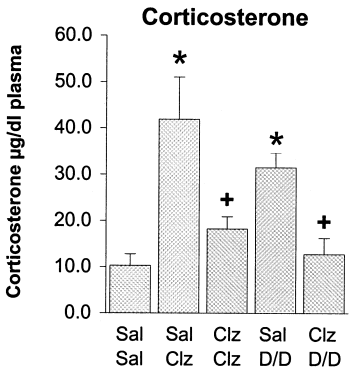

Fig. 3. Plasma corticosterone levels (mean ± S.E.M.) after a challenge dose of saline, clozapine or a combination of DOI and 8-OH-DPAT administered in either saline (Sal–Sal, Sal–Clz, Sal–D/D) or clozapine (Clz–Clz, Clz–D/D) pretreated animals. (*) Significantly different from Sal–Sal; (+) significantly different from acute treatment.

4. Discussion

The involvement of 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors in the clozapine-induced pattern of Fos expression appeared to be different in the various rat forebrain regions. We observed attenuated responses in the nucleus accumbens core, the dorsomedial and ventromedial striatum and the lateral septum, indicating a contribution of the aforementioned 5-HT receptors to the clozapine-induced Fos effects. In other brain areas (nucleus accumbens shell, dorsolateral striatum, hypothalamic paraventricular nucleus and central amygdala), the results suggest, however, that clozapine also affects Fos induction — and presumably other functions as well — independent of 5-$HT_{1A/2A/2C}$ receptors. The involvement of 5-HT receptors (presumably of the 1A subtype) in the clozapine-enhanced release of corticosterone was confirmed here, indicating that clozapine may exhibit agonistic activity at the 5-HT_{1A} receptor in vivo. In the prefrontal cortex, the effects of both acute and long-term clozapine treatment were only marginal and there was no apparent interaction with the 5-HT receptor agonists. The general conclusion that emerges from the present study is that the 5-HT receptor agonists DOI/8-OH-DPAT and clozapine share common sites of action only in a few brain regions.

The brain regional distribution of Fos-protein induced by 5-HT_{1A} receptor agonists has been well documented. Increased Fos immunoreactivity was seen in the central

amygdala, the dorsolateral part of the bed nucleus of the stria terminalis, the corticotropin-releasing hormone-containing neurons of the hypothalamic paraventricular nucleus and the prefrontal cortex (Compaan et al., 1996, 1997; Rouillard et al., 1996). In contrast, 5-$HT_{2A/2C}$ receptor agonists have, thus far, revealed that activation of these receptor subtypes generates the Fos-protein, particularly in the cerebral cortex, the olfactory tubercle, the dorsomedial striatum, the nucleus accumbens and the amygdala (Leslie et al., 1993; Moorman and Leslie, 1998). Combined administration of these agonists induces Fos expression in the medial part of the striatum, the nucleus accumbens and the prefrontal cortex (Tremblay et al., 1998). In the present study, similar results of Fos distribution were seen in these brain areas, following acute administration of the 5-$HT_{1A/2A/2C}$ receptor agonists. The acute and long-term effects of clozapine on the pattern of Fos expression were, except in the prefrontal cortex, comparable with those of our previous studies and with several other reports (e.g. Deutch and Duman, 1996; Dragunow et al., 1990; Robertson and Fibiger, 1992; Robertson et al., 1994). In our experimental set-up, we took particular care to avoid nonspecific stress responses, due to handling and injections of the drugs. Accordingly, all animals were injected daily for 3 weeks with clozapine for the long-term effects and during 1 week with saline for the acute effects of the agents. The necessity of these precautions to reduce baseline levels of Fos has been emphasized in previous studies (see Deutch et al., 1991; Sharp et al., 1991).

Since the effects of the 5HT receptor agonists were attenuated after long-term clozapine pretreatment in the nucleus accumbens core, the dorsomedial and the ventromedial striatum and the lateral septum, the question arises whether such apparent effects have to be attributed to tolerance in the target cells or to residual clozapine attached to the $5HT_{2A/2C}$ receptor. Most studies on biodistribution and pharmacokinetics suggest that hardly any clozapine remained 24 h following long-term treatment. Half-life values of 6 h based on tissue assay as well as biodistribution studies with radioactive clozapine were mentioned (Bender et al., 1994; Kuoppamäki et al., 1993). Although it seems unlikely, we can as yet not exclude the existence of some residual clozapine. Alternatively, downregulation of $5HT_{2C}$ receptors following long-term clozapine treatment has been described (Kuoppamäki et al., 1993). Anyhow, the present results emphasize that an interaction between clozapine and the explored 5-HT receptors can, only in part, explain the pattern of Fos distribution. Thus, the clinical effects of clozapine may not be exclusively attributed to an interaction with the here investigated 5-HT receptors, despite its high affinity for these receptors (Meltzer, 1999).

It should also be recognised that clozapine exhibits a high affinity for several other brain receptors as well, including the histamine H_1 receptor (Leysen et al., 1993), the α_1-adrenoceptor, the muscarinic receptor and, to a lesser extent, the different dopamine receptor subtypes (Bymaster et al., 1996, 1997). Recently, the possible role of some of these receptors in the clozapine-induced pattern of Fos expression has been investigated in acute experiments. Blocking of the adenosine A_{2A} receptors indeed caused an attenuation of the Fos response to clozapine in the nucleus accumbens and the striatum (Pinna et al., 1999), whereas the study of Fink-Jensen et al. (1995) suggested that neither adrenergic α_1- nor 5-HT_2 receptor blockade accounts for the unique pattern of Fos expression produced by clozapine. Involvement of the dopamine D_3 receptor can also be excluded, as the Fos results of clozapine in mice lacking the dopamine D_3 receptor appeared to be similar to those in the wild type (Carta and Gerfen, 1999). In a preliminary study, we emphasized that the regional pattern of Fos distribution induced by the nonspecific histamine H_1 receptor antagonist promethazine mimics that of clozapine (Korf et al., 1997). This histaminergic receptor appeared to be downregulated in the frontal cortex of patients with schizophrenia and may therefore be involved in the pathophysiology of this disorder (Nakai et al., 1991).

The general idea emerging from this and other reports is, that the pattern of regional Fos-protein expression following acute clozapine (and olanzapine) treatment, cannot be attributed solely to interactions with a few receptor types. More than likely it is determined by a relatively high degree of receptor-nonspecificity. Most of the current concepts in (psycho)pharmacology define receptor specificity as an interaction with a single receptor entity, usually with a single binding site at the receptor-protein. However, the specificity of drug actions may also be described in terms of cellular specificity. Irrespective of the molecularly defined interactions (e.g. partial or complete receptor blockade or activation), specificity of centrally active drugs may also be determined by their influence on a defined set of neurons in the brain. Consequently, nonspecific drugs interacting with a limited set of different receptor types, will influence the function (e.g. neurotransmission) only of those neurons, which express all these receptors. Accordingly, neurons expressing a lower number of these receptor types will only be marginally affected. Of course, it should be kept in mind that possible effects also depend on factors such as receptor reserve and micro-environment, although these parameters were left out of consideration here. Contributing to the clinical profile of the atypical drug clozapine, this reasoning implies that although its interactions in limbic brain areas are shared with most of the typical antipsychotics, both the involved number and the receptor types of the various drugs may differ. Our previous cross-tolerance studies with clozapine and haloperidol have indeed demonstrated that although both compounds induced Fos-protein in several limbic forebrain regions, the involved receptors and/or mechanisms must be different, as cross-tolerance was seen in the lateral septum only (Sebens et al., 1996). In retrospect, the concept of cellular speci-

ficity, rather than molecular specificity of the mechanism of action of (atypical) antipsychotics, may lead to the development of novel drugs, that have an apparent lack of receptor specificity. In this search for novel antipsychotics, cross-tolerance designs in combination with markers of nonspecific cell-activity (e.g. immediate early gene expression) may prove valuable tools.

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