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Clozapine-induced Fos-protein expression in rat forebrain regions: differential effects of adrenalectomy and corticosterone supplement

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Abstract

Unlike classical antipsychotic drugs, clozapine activates the hypothalamo-pituitary–adrenal axis and induces a specific regional pattern of Fos-protein expression in the rat forebrain. Whether corticosterone plays a role in the clozapine-induced Fos response is the subject of this study. Some rats were adrenalectomized and in a number, including intact animals, a corticosterone pellet (100 mg s.c.) was implanted; after 1 week, a single dose of clozapine (20 mg kg^{-1} i.p.) was administered. The clozapine-induced Fos response was not affected by adrenalectomy, apart from the nucleus accumbens shell, the subfornical organ and the supraoptic nucleus; there was an increased response in the nucleus accumbens shell, while other regions showed less Fos immunoreactivity. Implantation of the corticosterone pellet in both sham-operated and adrenalectomized animals, reduced the clozapine-induced Fos responses strongly in the hypothalamic paraventricular nucleus, the subfornical organ and possibly in the prefrontal cortex; in the supraoptic nucleus, this effect was seen only in intact animals. The effect of clozapine on plasma corticosterone levels was also diminished by supplemental corticosterone treatment. These results imply that the effects of clozapine are partially dependent upon hypothalamo-pituitary–adrenal axis integrity and activation. The efficacy of clozapine in the treatment of polydipsia and hyponatremia in chronic psychiatric patients may involve clozapine-mediated activation of the cellular activity in the subfornical organ. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Clozapine; Fos expression; Hypothalamic paraventricular nucleus; Supraoptic nucleus; Subfornical organ; Nucleus accumbens; Adrenalectomy; Corticosterone

1. Introduction

The patterns of regional Fos-protein expression in the rat forebrain induced by acute and long-term antipsychotic treatment are specific for the various classes of neuroleptic drugs (e.g. Sebens et al., 1995, 1996). Thus, the atypical antipsychotic drug clozapine exerts little effect in the striatum, while its greatest response is found in the hypothalamic paraventricular nucleus, the supraoptic nucleus, the nucleus accumbens, the lateral septum and the central amygdala (Deutch et al., 1992; Dragunow et al., 1990; Fink-Jensen and Kristensen, 1994; Robertson et al., 1994; Robertson and Fibiger, 1992; Sebens et al., 1995, 1996). In contrast to typical antipsychotics, several atypical antipsychotic drugs enhance the plasma corticosterone levels in rats (Albinsson and Andersson, 1992; Gudelsky et al., 1989; Meltzer et al., 1989), presumably by activation of the hypothalamo-pituitary–adrenal axis. Indeed, our previous studies show that the hypothalamic paraventricular nucleus exhibits a most conspicuous increase in the expression of the immediate early gene–protein Fos after administration of a single dose of clozapine to rats (Sebens et al., 1995, 1996). In addition to its use as an antipsychotic drug, clozapine has also been used for treatment of polydipsia that occurs in about 20% of the chronic psychiatric inpatients (Fuller et al., 1996; Spears et al., 1996). In this respect, we considered the subfornical organ, an area surrounding the anteroventral part of the third ventricle, as a possible target for clozapine. This circumventricular organ, which is controlled by adrenal hormones, contains angiotensin- and 5-HT receptors (Verghese et al., 1993; Scrogin et al., 1998) and informs the brain about changes

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in body fluid homeostasis (McKinley et al., 1996). Supersensitive dopamine receptors and/or an overactive angiotensin II system have been suggested as possible causes of polydipsia (Verghese et al., 1993).

The aim of the present report is to study whether the status of the hypothalamo-pituitary–adrenal axis affects the clozapine-induced Fos responses in the rat brain. Accordingly, we compared the number of Fos-positive cells in several regions of the rat forebrain after administering a single dose of clozapine to rats with intact adrenals and to adrenalectomized animals, either with or without corticosterone supplement.

2. Materials and methods

2.1. Animals

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

2.2. Design of the study

Rats were adrenalectomized ($n = 20$) or sham-operated ($n = 17$) under anesthesia using 0.9% halothane in 30% O_2 and 70% N_2O. During the surgery, a 100-mg corticosterone pellet was implanted subcutaneously in 5 sham-operated and in 11 adrenalectomized rats, while 12 of the sham-operated and 9 adrenalectomized animals received a 100-mg cholesterol pellet to control the effects of pellet implantation. Adrenalectomized rats received 0.9% NaCl as their drinking water. To mitigate the effects of stress-induced Fos expression (Cullinan et al., 1995) by handling and injections, saline was given intraperitoneally for the last 5 days prior to the clozapine (or saline) challenge. Such control procedures revealed baseline levels of plasma corticosterone below 4 μg/dl. One week after surgery, a clozapine challenge (20 mg kg^{-1} i.p.) was given to sham-operated ($n = 6$), sham-operated/corticosterone ($n = 5$), adrenalectomized ($n = 4$) and adrenalectomized/corticosterone ($n = 6$) pretreated animals. All rats were perfused under pentobarbital anesthesia 2 h after the drug injection. Before perfusion, a blood sample was taken from the left heart ventricle to determine plasma corticosterone levels. To control the effects of the various treatments on the basal levels of Fos-protein expression and plasma corticosterone, six sham-operated, five adrenalectomized and five adrenalectomized/corticosterone-treated rats were challenged with saline (1 ml kg^{-1} i.p.). All experimental procedures were approved by the Committee on Animal Bio-ethics of the University of Groningen.

2.3. Drugs

Clozapine (generously supplied by Sandoz Basel, Switzerland) was dissolved in slightly acidified saline and pH adjusted to 5.8 with NaOH. Corticosterone (Sigma, St. Louis, MO, USA) was obtained commercially. Neither saline nor clozapine solutions were buffered. The injections did not produce any apparent discomfort.

2.4. Corticosterone assay and Fos immunohistochemistry

Quantification of levels of plasma corticosterone in the collected heparinised blood samples was performed as described by Dawson et al. (1984). The absolute detection threshold for corticosterone in plasma was 0.8 μg/dl.

For Fos immunohistochemistry, animals were perfused under deep anesthesia 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 postfixed overnight at 4°C in 4% paraformaldehyde solution and stored in 50 mM Tris-buffered saline pH 7.4 containing 0.1% Na-azide. Following cryoprotection by overnight immersion in 30% sucrose (in 50 mM Tris/HCl buffer pH 7.4) at room temperature, brains were sliced into 30-μm coronal sections using a cryostat microtome. The immunohistochemical procedure was performed on free floating sections with a Fos primary antiserum, diluted 1:2000

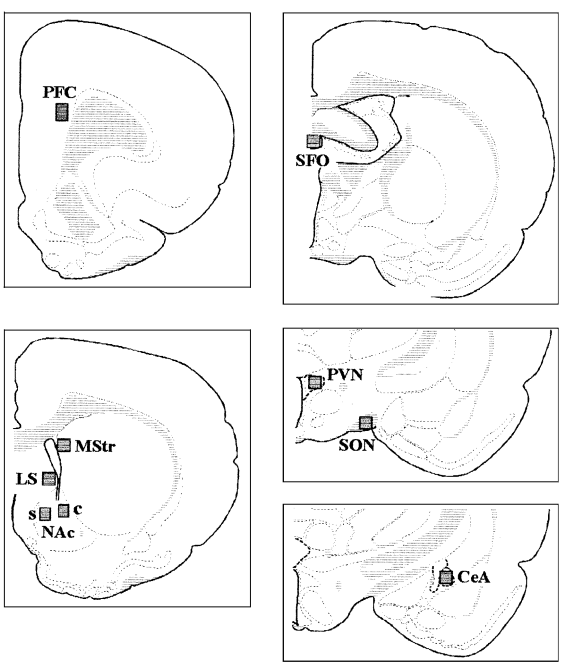

Fig. 1. Schematic representation of the levels used for quantification of Fos-positive cells. Grey-filled squares indicate the counted regions. PFC, prefrontal cortex; NAc s, nucleus accumbens shell; NAc c, nucleus accumbens core; MStr, medial striatum; LS, lateral septum; SFO, subfornical organ; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus; CeA, central amygdala. Fos-positive cells were quantified at a magnification of 125× in areas of 400×400 μm; PFC: 320×480 μm and NAc s and c: 320×400 μm.

(Cambridge Research Biochemicals, CRB, OA-11-824, UK) and a biotinylated anti-sheep secondary antibody (1:800, Pierce Chemical, Rockford, IL, USA), as described by Sebens et al. (1998).

2.5. Quantification and statistical analysis

Representative sections with the areas used for quantification of the number of Fos-positive cells (magnification of 125 ×) are shown in Fig. 1. In the nucleus accumbens shell and core, the counted areas were 320 × 400 μm; in the prefrontal cortex, 320 × 480 μm and in the medial striatum, the lateral septum, the subfornical organ, the hypothalamic paraventricular nucleus, the supraoptic nucleus and the central amygdala, these dimensions were 400 × 400 μm. Fos-positive cells were counted bilaterally and averaged per animal. Per experimental group, both the mean (± S.E.M.) number of Fos-positive cells and levels

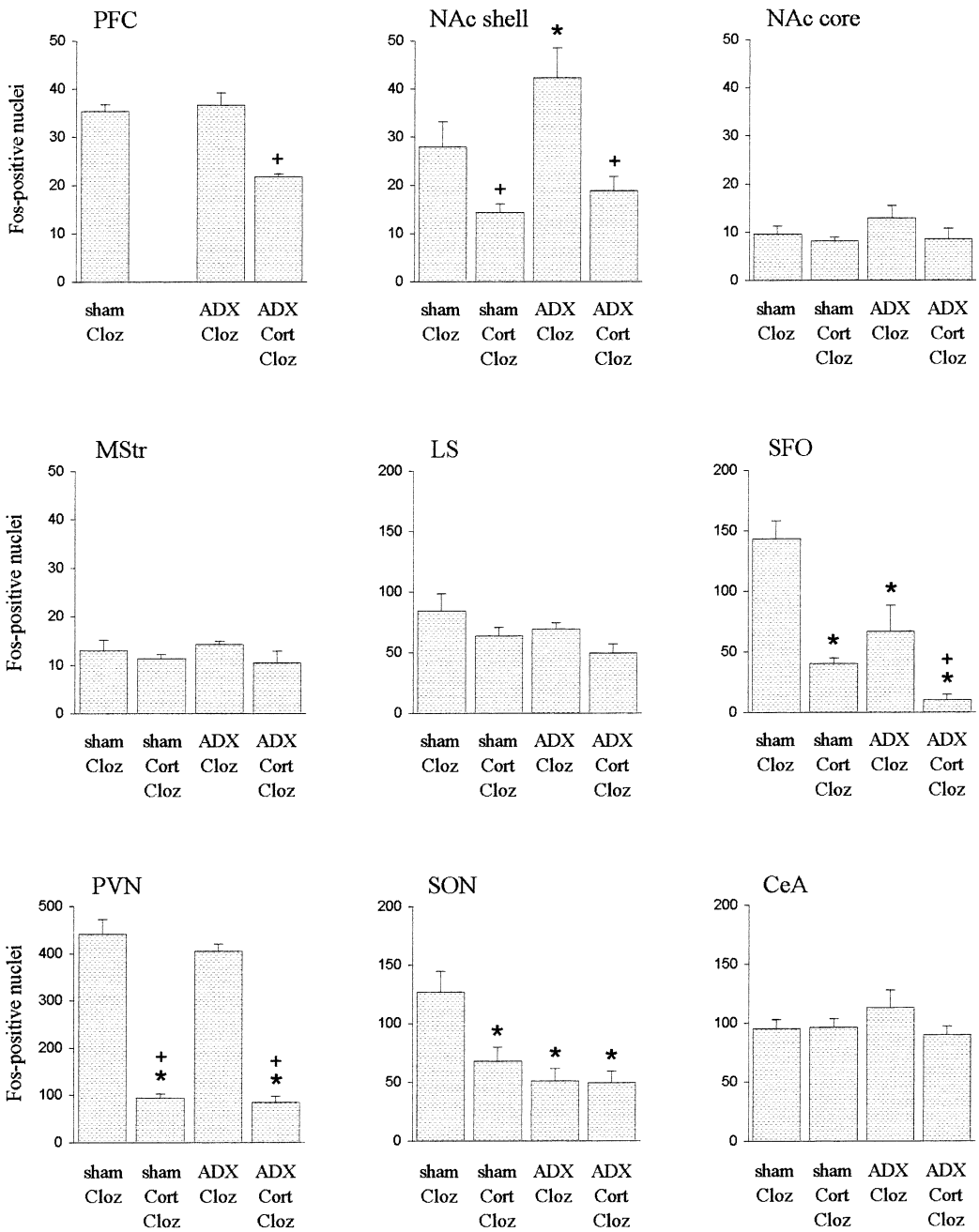

Fig. 2. Histogram illustrating the mean number (± S.E.M.) of clozapine-induced Fos-positive cells in the forebrain of sham-operated and adrenalectomized rats that had a subcutaneously implanted cholesterol pellet (100 mg, sham-Cloz and ADX-Cloz, respectively) or an implanted corticosterone pellet (100 mg, sham-Cort–Cloz and ADX-Cort–Cloz, respectively). Brain regions include: PFC, prefrontal cortex; NAc shell, nucleus accumbens shell; NAc core, nucleus accumbens core; MStr, medial striatum; LS, lateral septum; SFO, subfornical organ; PVN, hypothalamic paraventricular nucleus; SON, supraoptic nucleus; CeA, central amygdala. * Significantly different from the sham-Cloz group; + significantly different from the ADX-Cloz group ($P < 0.05$).

of plasma corticosterone were determined. In intact and adrenalectomized animals, and in adrenalectomized rats that had a corticosterone implant, the baseline levels of Fos expression were less than 10% of those induced by clozapine administration, with exception of the prefrontal cortex (data not shown). Fos data of the prefrontal cortex of intact animals with a corticosterone implant were not available. The various groups were compared using a one way analysis of variance (ANOVA), followed by the Student–Newman–Keuls method for multiple comparison procedures; a difference was considered significant at $P < 0.05$.

3. Results

3.1. Clozapine in (sham-operated) controls

A single dose of clozapine induced small but significant rises in Fos-positive nuclei in the medial part of the striatum and the nucleus accumbens core, a moderate increase in the prefrontal cortex and the nucleus accumbens shell, and marked responses in the lateral septum and the central amygdala. In the subfornical organ, the supraoptic nucleus and, in particular, in the magno- and parvocellular division of the hypothalamic paraventricular nucleus, the Fos responses were substantial (Fig. 2). Clozapine also enhanced the basal levels (4–6 μg/dl) of plasma corticosterone to 58 ± 3 μg/dl (Fig. 3).

3.2. Clozapine in adrenalectomized rats

The clozapine-induced number of Fos-positive cells found in sham-operated rats was elevated by 50% in the nucleus accumbens shell following adrenalectomy, whereas a reduction was seen in the subfornical organ and the supraoptic nucleus by 53% and 60%, respectively. None of the other investigated brain areas showed a significant effect of adrenalectomy on clozapine-induced Fos expression (Fig. 2). Plasma corticosterone levels remained below 1 μg/dl after a challenge of clozapine in adrenalectomized animals (Fig. 3).

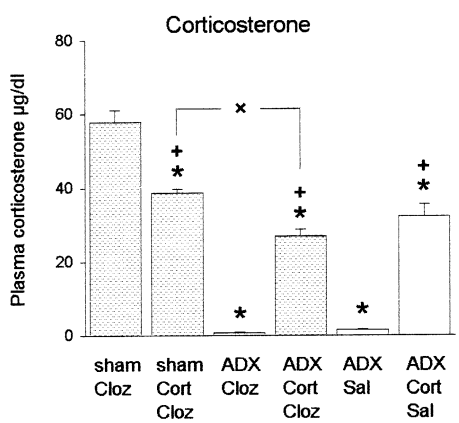

Fig. 3. Plasma corticosterone levels (mean ± S.E.M.) after a challenge dose of clozapine (Cloz) or saline (Sal) in sham-operated (sham) and adrenalectomized (ADX) animals, with an implanted cholesterol- or corticosterone (Cort) pellet. * Significantly different from the sham-Cloz group; + significantly different from the ADX-Cloz group; × significant difference between the indicated groups ($P < 0.05$).

3.3. Clozapine in rats (sham-operated and adrenalectomized) with a corticosterone pellet

A substantial decline in clozapine-induced Fos expression was observed in the hypothalamic paraventricular nucleus and the subfornical organ after implantation of a corticosterone pellet in both sham-operated and adrenalectomized rats. In the hypothalamic paraventricular nucleus, these reductions were 79% in both sham-operated and adrenalectomized rats, whereas in the subfornical organ, the decrease was 72% in intact rats and more than 80% in adrenalectomized animals. The supraoptic nucleus showed a decline of 46% following corticosterone application, but only in intact animals, while the decrease of 55% in the nucleus accumbens shell was limited to adrenalectomized rats (Fig. 2). Not significantly altered were the Fos responses to clozapine in the nucleus accumbens core, the medial striatum, the lateral septum and the central amygdala, as compared to the appropriate controls (Fig. 2). In the prefrontal cortex, the clozapine-induced Fos results of adrenalectomy and corticosterone replacement in the adrenalectomized rats were comparable with those obtained in the hypothalamic paraventricular nucleus, albeit less pronounced (Fig. 2). The levels of plasma corticosterone in intact animals receiving clozapine were lowered by 33% following corticosterone implantation. No significant difference in levels of plasma corticosterone was seen between clozapine-administered corticosterone-treated animals, both intact and adrenalectomized, and a saline injection given to corticosterone/adrenalectomized rats (Fig. 3). These findings indicate that clozapine hardly affects hypothalamo-pituitary–adrenal axis activity in rats provided with an implanted corticosterone pellet.

4. Discussion

We tested whether Fos-protein induction following clozapine administration can, at least in part, be attributed to the involvement of corticosterone and/or the glucocorticoid receptor. This receptor is localized in most of the limbic brain regions and is sensitive to variations in circulating corticosterone concentrations (Aronsson et al., 1988; Cintra et al., 1994a,b; Joëls and De Kloet, 1994). Our experiments demonstrate that both excess and absence of corticosterone can modify the acute Fos effects of clozapine in some brain regions, such as the prefrontal cortex, the hypothalamic paraventricular nucleus, the supraoptic nucleus and the subfornical organ, but not in others, including the nucleus accumbens core, the medial striatum, the

lateral septum and the central amygdala. The latter observations also indicate that neither adrenalectomy nor the implantation of a corticosterone pellet does affect baseline levels of Fos immunoreactivity. The clozapine-induced Fos-protein expression in the nucleus accumbens shell was slightly increased following adrenalectomy, and this effect disappeared by supplemental corticosterone treatment.

Our observations of a strong decrease in the clozapine-induced Fos responses in the paraventricular nucleus of the hypothalamus, following continuous exposure to corticosterone, are in line with the findings of Helmreich et al. (1996) who reported no alterations in the c-*fos* mRNA responses in the paraventricular nucleus after stress in adrenalectomy-treated animals, while corticosterone replacement did cause a slight blunting of this response. Thus, in contrast to the clozapine-enhanced levels of corticosterone, the constantly high levels of plasma corticosterone caused by pellet implantation appear to disrupt the normal regulation and consequently diurnal variation in the activity of the paraventricular nucleus, as was demonstrated by the reduced Fos responses. The lack of response to elimination of the clozapine-enhanced levels of corticosterone by adrenalectomy in the paraventricular nucleus and in the prefrontal cortex as well, confirms the corticosterone independence of clozapine-induced Fos expression. In addition, in the prefrontal cortex, the clozapine effects on Fos induction are susceptible only to high levels of plasma corticosterone. Both the hypothalamic paraventricular nucleus and the supraoptic nucleus are similarly sensitive to exposure of corticosterone, presumably as the result of downregulation of the glucocorticoid receptor. However, the sensitivities of these nuclei to adrenalectomy are different, and in this respect, the supraoptic nucleus behaves more like the subfornical organ. Whether the latter effects are due to similar mechanisms is not yet clear.

The present report, to our knowledge, is to first describe the effects of clozapine on Fos expression in the subfornical organ, a brain region closely involved in the regulation of fluid and electrolyte balance (e.g. McKinley et al., 1996). Administration of clozapine causes an increase in number of Fos-positive cells that was suppressed by both adrenalectomy and long-term exposure to corticosterone. Although adrenalectomy affects the basal Fos response to clozapine, the relative effects of supplemental corticosterone treatment were alike in intact and adrenalectomized animals, and therefore independent of endogenous corticosterone. The decreased Fos response to clozapine following adrenalectomy may be explained by suppression of the activity of mineralocorticoid receptors and the consequently increased levels of angiotensin II. Apparently, the effects of clozapine depend on angiotensin II levels that are thought to be responsible for salt appetite and perhaps polydipsia (Verghese et al., 1993; Shelat et al., 1998). Additional corticosterone treatment demonstrates even less responsiveness to clozapine in expressing Fos, essentially in a way similar to that seen in the paraventricular nucleus of the hypothalamus. Sustained exposure to glucocorticoids is known to reduce the number of glucocorticoid receptors in the brain (Spencer et al., 1991), and may therefore lead to diminished Fos effects. Yet, other receptor types may be implicated as well. The effect of clozapine on the expression of Fos in various regions of the brain is mediated, at least in part, by 5-HT_{1A} and 5-$HT_{2A/C}$ receptors (Sebens et al., 2000). In the hypothalamic paraventricular nucleus, the 5-HT_{1A} receptors appear to be, though indirectly, most important to explain the Fos responses (e.g. Compaan et al., 1996, 1997). Both 5-HT_{1A} and 5-$HT_{2A/C}$ receptor types are present in the subfornical organ and involved in the regulation of nerve cell activity (Scrogin et al., 1998). So, it is possible that the actions of clozapine are also mediated directly through (partial) blockade of 5-HT_{1A} and 5-$HT_{2A/C}$ receptors. Clozapine has been used clinically to treat polydipsia and hyponatremia (Fuller et al., 1996; Spears et al., 1996). Hyponatremia has been associated with the use of selective 5-HT reuptake inhibitors (Liu et al., 1996), whereas increased 5-HT activity causes polydipsic behaviour in animals (Hubbard et al., 1989). Besides, the weak anti-dopamine D_2 receptor activity and the moderate dopamine D_1 receptor blocking properties of clozapine have been associated with drinking behaviour (Verghese et al., 1993; Ljunburg, 1989). Furthermore, increased monoamine turnover was observed in the subfornical organ area following body fluid depletion, implying that both dopaminergic and serotonergic systems in this region may be involved in controlling body fluid balance (Kariya et al., 1992). Taken together, these and our data suggest that clozapine does not necessarily affect the function through angiotensin-mediated processes only, but possibly by a direct action on 5-HT and/or other receptor types as well.

In the nucleus accumbens shell, the effect of clozapine on Fos expression was enhanced following adrenalectomy. One explanation could be that changes in the neuronal input from the hippocampus act (indirectly) upon Fos-protein inducing processes. This assumption is not inconceivable since the nucleus accumbens, in particular, the shell division, receives indirect hippocampal input via the subiculum, the main output structure of the hippocampal formation (Groenewegen et al., 1987). In a previous study (Jaarsma et al., 1992), we found that complete removal of the adrenals—providing undetectable levels of plasma corticosterone—and consequently loss of glucocorticoids initiates and continues the degeneration of granule neurons in the dentate gyrus, including mineralocorticoid receptor-containing neurons, within a few days after surgery. In addition, the observed augmentation was abolished when adrenalectomy was accompanied by replacement of corticosterone (pellet implantation), which prevents neuronal degeneration (Gould et al., 1990; Sloviter et al., 1989).

In conclusion, the pattern of clozapine-induced Fos-protein expression in several limbic rat forebrain regions is highly influenced by sustained corticosterone exposure and

the question is whether this response is also susceptible to changes in physiological levels of corticosterone. Elimination of the clozapine-enhanced plasma corticosterone levels (by adrenalectomy) affects only the subfornical organ and the supraoptic nucleus by suppression of the antipsychotic-induced neuronal activity, whereas in the nucleus accumbens shell, this activity increases, possibly as a consequence of neuronal degeneration in the dentate gyrus. The present results demonstrate that the effects of clozapine in the various brain regions are, in general, not mediated by the drug-evolved increase in levels of plasma corticosterone. On the other hand, our results suggest that the clinical antipsychotic effects may, in part, be dependent on the status of the hypothalamo-pituitary–adrenal axis.

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