

## CHRONIC TREATMENTS WITH HALOPERIDOL AND CLOZAPINE ALTER THE LEVEL OF NMDA-R1 mRNA IN THE RAT BRAIN: AN *IN SITU* HYBRIDIZATION STUDY

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The aim of the present study was to examine the influence of 3-month administration of the typical neuroleptic haloperidol (1 mg/kg/day) and the atypical one clozapine (30 mg/kg/day) on the expression of the NMDA-R1 mRNA in different brain structures using *in situ* hybridization in rats. A long-term treatment with haloperidol decreased the NMDA-R1 mRNA level in intermediate and caudal parts of the caudate-putamen and in more caudally localized regions of parietal and frontal cortices, but increased it in the CA1 region of the hippocampus. No significant changes in the nucleus accumbens, insular cortex, CA3 and dentate gyrus of the hippocampus were found after haloperidol administration. Clozapine did not influence the NMDA-R1 mRNA expression in the hippocampus, as well as in the intermediate and caudal regions of the caudate-putamen, but significantly increased it in the rostral region of the latter structure, in the nucleus accumbens and insular cortex. The present study suggests that both these neuroleptics influence the expression of the mRNA of the NMDA-R1 subunit in brain structures which are thought to be important for development of psychotic symptoms.

**Key words:** *chronic treatment, haloperidol, clozapine, NMDA receptors, NMDA-R1 mRNA, in situ hybridization*

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*Abbreviations:* [ $^3\text{H}$ ]CGP 39653 – [ $^3\text{H}$ ]D,L-(E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid, [ $^3\text{H}$ ]MK-801 – dizocilpine – [ $^3\text{H}$ ](5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine, NMDA – N-methyl-D-aspartate

## INTRODUCTION

Neuronal mechanisms underlying psychotic symptoms in schizophrenia are still unknown. Although dopamine is suggested to play a very important role in this disease [18, 36], contribution of other neurotransmitter systems also seems to be essential.

Recently, a hypofunction of glutamatergic neurotransmission in the cerebral cortex and hippocampus has been suggested to contribute to the pathophysiology of schizophrenia [cf. 3]. A reduced level or release of glutamate, accompanied with an increase in the level of NMDA (N-methyl-D-aspartate) receptors, have been found in different cortical regions and the hippocampus in schizophrenic patients [7, 17, 37, 38, 40–42]. On this basis, it has been suggested that the presynaptic hypofunction leads to a compensatory up-regulation of glutamate postsynaptic receptors [17, 40]. However, most schizophrenics are treated with neuroleptics for many years, hence it is not possible to exclude a possibility that the observed receptor changes result not from the illness itself, but from a neuroleptic therapy. Moreover, some animal studies indicate that chronic neuroleptic administration influences NMDA receptors. An increase in the [ $^3\text{H}$ ]MK-801 binding to the ion channel in the prefrontal cortex [12], and elevation of the [ $^3\text{H}$ ]L-glutamate binding [43] to a recognition site of the NMDA receptor complex in the parietal cortex as a result of 3-week haloperidol administration have been reported. In contrast, McCoy and Richfield [27] and Tarazi et al. [39] showed that a 3–4-week treatment with typical (haloperidol and pimozide) or atypical (clozapine and risperidone) neuroleptics decreased the binding of [ $^3\text{H}$ ]MK-801 in medial prefrontal or frontal cortices. Moreover, according to Tarazi et al. [39], an 8-month treatment with either haloperidol or clozapine did not change the binding of [ $^3\text{H}$ ]MK-801. The above-cited data seem to suggest that neuroleptics may differently influence various binding sites of the NMDA receptor complex, and that their effect may depend on the duration of

treatment. Our recent study confirms the above conclusion. We found that haloperidol or clozapine administered to rats in drinking water for 3 months increased the number of NMDA receptors labeled with the competitive antagonist [ $^3\text{H}$ ]CGP 39653, but did not change the binding of [ $^3\text{H}$ ]MK-801 to those receptors in different cortical areas [31].

The NMDA receptor is a heterooligomeric protein complex composed of NMDA-R1 (8 splice variants) and NMDA-R2 (A-D) subunits. Different subunit combinations constitute various subpopulations of NMDA receptors, which also differ with respect to their affinity for competitive antagonists, ion channel blockers or agonists [20, 21]. The NMDA-R1 subunit is required for the normal function of the NMDA ionophore [29, 33]. NMDA-R2 subunits appear to serve a modulatory role in NMDA channel function [16]. Recently, it has been reported that knock-out mice expressing only 5% of normal levels of the NMDA-R1 subunit display schizophrenic-like behaviors (increased motor activity, stereotypy and deficits in social and sexual interactions) which can be ameliorated by neuroleptics [28]. These findings support the importance of the NMDA-R1 subunit for neuronal mechanisms underlying psychotic symptoms of schizophrenia and the antipsychotic therapy.

The aim of the present study was to examine the influence of a long-term (3 months) treatment of rats with the typical neuroleptic haloperidol and the atypical one clozapine on the expression of the mRNA of the NMDA-R1 subunit in different cortical regions and in some subcortical structures.

## MATERIALS and METHODS

### Animals

The experiment was performed on male Wistar rats weighing 160–245 g at the beginning of the investigation. The animals were kept under an artificial light/dark cycle (12/12 h, the light on from 7 a.m. to 7 p.m.). The experiments were carried out in compliance with the Animal Protection Bill of August 21, 1997; (published in Dziennik Ustaw no. 111/1997 poz. 724), and according to the NIH Guide for the Care and Use of Laboratory Animals.

### Drug administration

Haloperidol base (RBI, USA) and clozapine (Polfa, Starogard Gdański, Poland) were dissolved

in a small amount of 1% lactic acid and were then diluted with tap water. Haloperidol (1 mg/kg/day) and clozapine (30 mg/kg/day) were given to rats in drinking water for 3 months and were withdrawn afterwards. The weight of the rats rose throughout the treatment (up to 365–505 g after 3 months). The rats were weighed twice a week to adjust the amount of drugs administered to their actual body weight. The volume of a consumed liquid was estimated for a few days before the start of treatment with neuroleptics. Afterwards, drug solutions were prepared in the volume to be consumed as a whole during one day. The solution consumption was checked every day, and was either slightly diminished or augmented on the following day, depending on the intake. Parallel to drug-drinking rats, control groups of animals received tap water *ad libitum*. Experimental groups consisted of 10 animals each.

### Tissue preparation

On day 5 after the drug withdrawal, the rats were sacrificed by decapitation, their brains were rapidly removed, frozen on dry ice cold heptane ( $-70^{\circ}\text{C}$ ) and stored at  $-70^{\circ}\text{C}$ . Coronal 10  $\mu\text{m}$  sections were cut up using a cryostat microtome ( $-20^{\circ}\text{C}$ ). The sections were thaw-mounted on gelatine-coated microscopic slides, and stored for 1–4 days at  $-20^{\circ}\text{C}$  before the assay.

### In situ hybridization according to [44]

A mixture of two 46-mer synthetic deoxyoligonucleotide probes (New England Nuclear), complementary to bases 375–420 and 1011–1056 of mRNA of the NMDA-R1 subunit, were labeled using [ $^{35}\text{S}$ ]dATP (1200 Ci/mmol, New England Nuclear) and terminal deoxynucleotidyl transferase (Boehringer, Germany). Tissue sections were thawed and fixed for 10 min in 4% paraformaldehyde in phosphate-buffered saline (PBS). Afterwards, the sections were rinsed in PBS for 5 min, acetylated in 0.1 M triethanolamine (pH 8.0)/acetic anhydride (0.25%) for 10 min, dehydrated through graded alcohols and allowed to air-dry. After fixing and prehybridization, the sections were treated with [ $^{35}\text{S}$ ]dATP-labeled probes. The probes were diluted in a hybridization buffer [50% (v/v) formamide, 10% (w/v) dextran sulphate,  $4 \times \text{SSC}$  (pH = 7.0),  $1 \times \text{Denhardt's}$  solution (= 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), yeast tRNA (0.25 mg/ml), herring sperm DNA (0.2

mg/ml), 10 mM dithiothreitol] to yield a final concentration of  $2 \times 10^6$  dpm/30  $\mu\text{l}$ . All the sections were made up with autoclaved 0.1% diethylpyrocarbonate-treated water. The diluted probes (30  $\mu\text{l}$ ) were applied to sections which were then covered with Parafilm. The same probe was used for sections of control and neuroleptic-treated animals. Hybridization occurred overnight in a humidified chamber at  $37^{\circ}\text{C}$ . Following hybridization, the sections were washed in  $1 \times \text{SSC}$  for 10 min, then 4 times for 15 min each in  $1 \times \text{SSC}/50\%$  formamide at  $42^{\circ}\text{C}$ , rinsed briefly in  $1 \times \text{SSC}$  and water at a room temperature, and air-dried. The sections were exposed to a  $^3\text{H}$ -Hyperfilm (Amersham) for 4 weeks at  $4^{\circ}\text{C}$ .

### Autoradiographic analysis

After the exposure, the films were developed with a Dectol developer (Kodak), fixed with a GBX Fixer and Replenisher (Kodak), and dried. The optical density of the resultant film images was determined using a computer-assisted image analysing system (MCID, St. Catharines, Ontario, Canada).

The mRNA level of NMDA-R1 subunit was estimated at 4 levels: level I – A = 1.60 to 1.20 mm from the bregma [frontal, parietal and insular (agranular and disgranular regions) cortices, rostral part of caudate-putamen – ventrolateral (VL) and dorsolateral region (DL), nucleus accumbens – shell and core]; level II – A = 0.20 to  $-0.26$  mm from the bregma [frontal, parietal and insular (agranular and disgranular) cortices, intermediate part of caudate-putamen – VL and DL]; level III – A =  $-0.40$  to  $-0.92$  mm from the bregma [parietal and insular (agranular and disgranular) cortices, caudal part of caudate-putamen – VL and DL], and level IV – A =  $-3.30$  to  $-3.80$  mm from the bregma [the CA1, CA3 regions and dentate gyrus of the hippocampus], according to the stereotaxic atlas of Paxinos and Watson [32] (Fig. 1). The frontal and parietal cortices were arbitrarily divided into outer and inner layers. The outer and inner layers corresponded approximately to 1–4, and 5–6 cortical layers, respectively, according to the atlas of Paxinos and Watson [32]. A single slice which represented each brain level for each rat was evaluated. A mean value of both sides of the brain in each region was calculated and accepted as a value/animal. Slices of control and neuroleptic-treated animals were exposed at the same film. The data were

## NMDA-R1 *in situ* hybridization

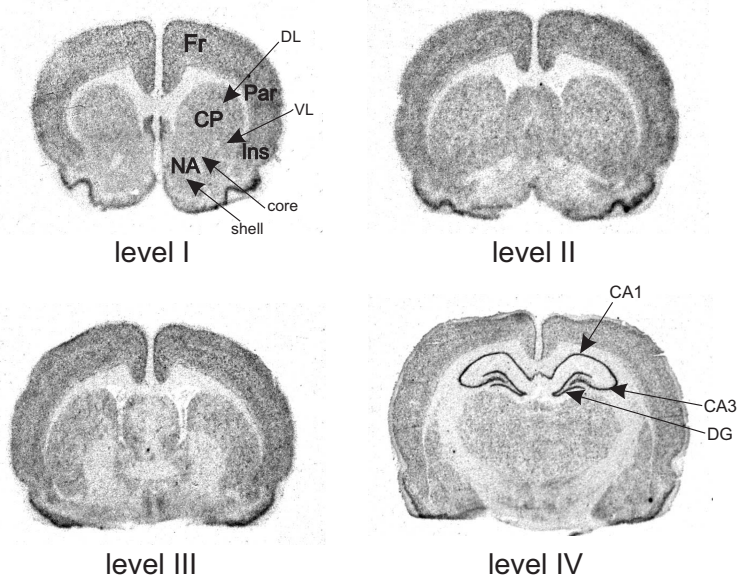


Fig. 1. Distribution of mRNA of the NMDA-R1 subunit in 4 frontal sections of the brain. Level I: A = 1.60 to 1.20; level II: A = 0.20 to -0.26; level III: A = -0.40 to -0.92; level IV: A = -3.30 to -3.80 from the bregma (according to the stereotaxic atlas of Paxinos and Watson [32]). CA1 – the CA1 region of the hippocampus, CA3 – the CA3 region of the hippocampus, core – the core region of the nucleus accumbens, CP – the caudate-putamen, DG – the dentate gyrus of the hippocampus, DL – the dorsolateral region of the caudate-putamen, Fr – the frontal cortex, Ins – the insular cortex, NA – the nucleus accumbens, Par – the parietal cortex, shell – the shell region of the nucleus accumbens, VL – the ventrolateral region of the caudate-putamen. The left side of a rat's brain is shown on the right side of the figure

## NMDA-R1 *in situ* hybridization

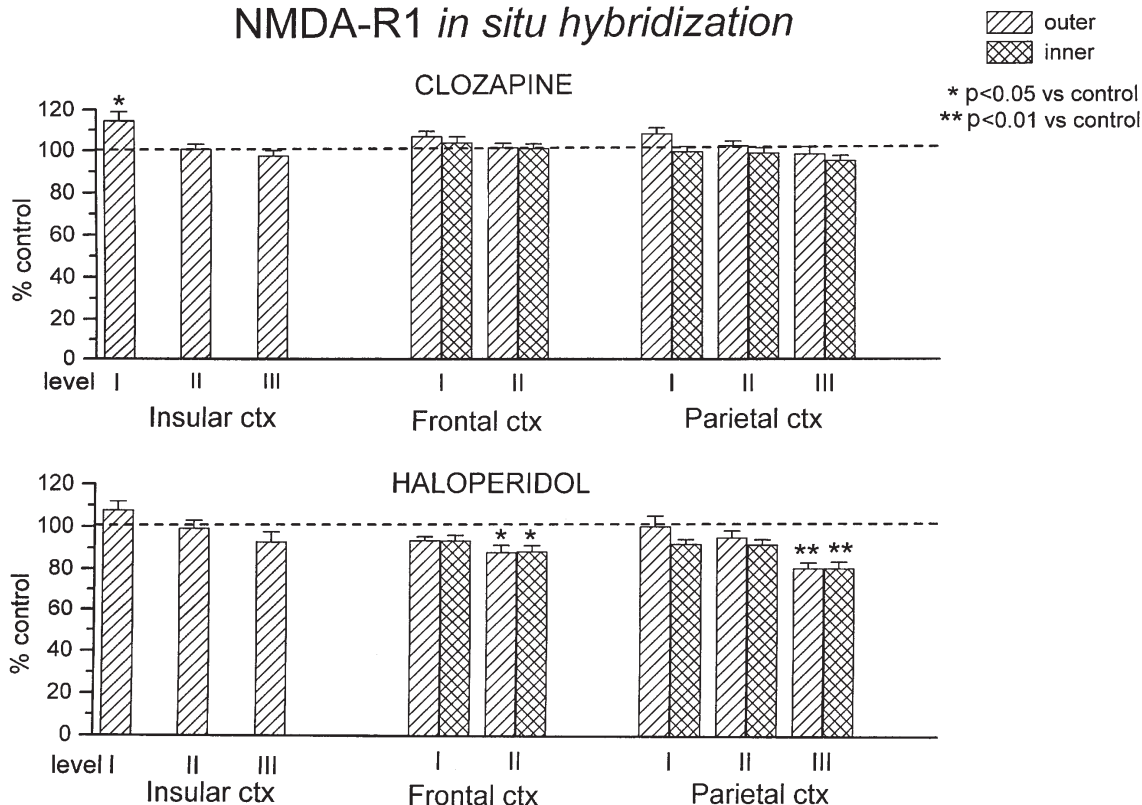


Fig. 2. The influence of long-term (3-month) clozapine or haloperidol administration on the level of mRNA of the NMDA-R1 subunit in insular, frontal and parietal cortices. Level I: A = 1.60 to 1.20; level II: A = 0.20 to -0.26; level III: A = -0.40 to -0.92 (according to the stereotaxic atlas of Paxinos and Watson [32]). Outer – outer layers; inner – inner layers. Each group consisted of 10 rats. ctx – the cortex. The results are presented as a percentage of control values (mean  $\pm$  SEM)

presented as a percentage (mean ± SEM) of the respective control values (Figs. 2 and 3).

**Statistics**

A statistical analysis of the autoradiographic data was carried out using the Kruskal-Wallis and Mann-Whitney U-tests.

**RESULTS**

**The influence of long-term treatments with haloperidol and clozapine on the level of the NMDA-R1 mRNA in the cerebral cortex**

Haloperidol given in drinking water for 3 months significantly decreased the mRNA level of the NMDA-R1 subunit in outer and inner layers of the frontal (level II) and parietal (level III) cortices, but did not change it in insular cortex (Fig. 2). Clo-

zapine administered for 3 months did not change the mRNA level in the frontal, parietal and more caudal regions of the insular cortex, but it significantly increased the NMDA-R1 mRNA expression in the rostral (level I) insular cortex (Fig. 2).

**The influence of long-term treatments with haloperidol and clozapine on the level of the NMDA-R1 mRNA in the caudate-putamen and nucleus accumbens**

Haloperidol given in drinking water for 3 months significantly decreased the mRNA level of the NMDA-R1 subunit in a dorsolateral part of the intermediate (level II) and caudal (level III) regions of the caudate-putamen (Fig. 3).

Clozapine did not change the NMDA-R1 mRNA expression in the intermediate (level II) and caudal (level III) regions of the caudate-putamen, but increased it in the shell and core of the nucleus ac-

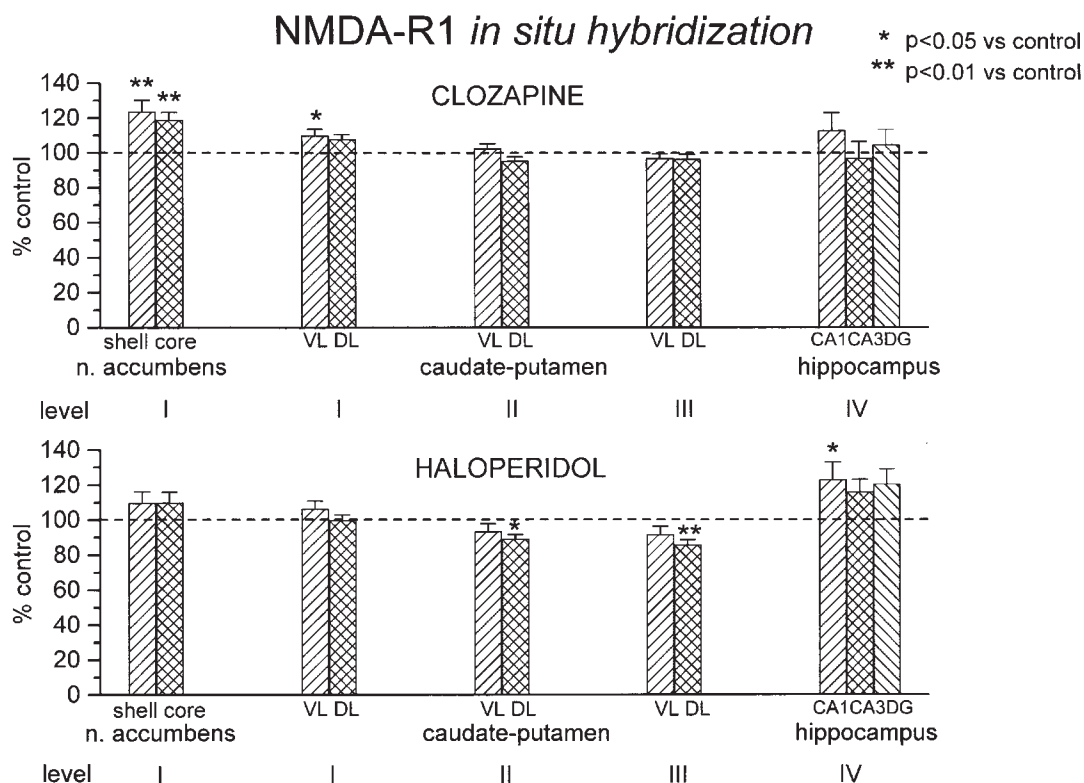


Fig. 3. The influence of long-term (3-month) clozapine or haloperidol administration on the level of mRNA of the NMDA-R1 subunit in rostral (level I), intermediate (level II) and caudal (level III) parts of the caudate-putamen, in the nucleus accumbens – the shell and core, and in the hippocampus – CA1, CA3 and the dentate gyrus. Level I: A = 1.60 to 1.20; level II: A = 0.20 to -0.26; level III: A = -0.40 to -0.92; level IV: A = -3.30 to -3.80 (according to the stereotaxic atlas of Paxinos and Watson [32]). Each group consisted of 10 rats. VL – the ventrolateral region, DL – the dorsolateral region. The results are presented as a percentage of control values (mean ± SEM)

cumbens and in the neighboring ventrolateral region (level I) of the caudate-putamen (Fig. 3).

### **The influence of long-term treatments with haloperidol and clozapine on the level of the NMDA-R1 mRNA in the hippocampus**

Haloperidol elevated the level of mRNA of the NMDA-R1 subunit in CA1, CA3 and dentate gyrus regions of the hippocampus. However, only in the CA1 region that increase was significant (Fig. 3).

Clozapine did not significantly change the expression of the NMDA-R1 mRNA in the whole hippocampus (Fig. 3).

## **DISCUSSION**

The present study shows that a 3-month treatment with the typical neuroleptic haloperidol and with the atypical one clozapine affects differently the expression of the NMDA-R1 mRNA in various brain structures. Haloperidol decreased NMDA-R1 mRNA expression in intermediate and caudal parts of the caudate-putamen and in more caudally localized regions of the parietal and frontal cortices, but increased it in the CA1 region of the hippocampus. Clozapine did not influence the level of NMDA-R1 mRNA in intermediate and caudal regions of the caudate-putamen, but significantly increased its expression in the rostral region of that structure, in the nucleus accumbens and insular cortex.

A different influence of haloperidol and clozapine on the expression of the mRNA of NMDA-R1 subunit in intermediate and caudal regions of the caudate-putamen may contribute to mechanisms important for differentiation of typical and atypical neuroleptics. In contrast to clozapine, haloperidol affects several striatal processes. Haloperidol, but not clozapine, increases the number of dopamine D2 receptors in the caudate-putamen after chronic administration [cf. 45], and, when injected acutely, it activates c-fos expression in this structure [35]. The above-mentioned effects of haloperidol on striatal functions are presumably related to its ability to induce extrapyramidal side-effects, e.g. tardive dyskinesia and parkinsonism, of which clozapine is devoid.

Our earlier study showed that 6-week haloperidol administration produced subsensitivity of NMDA receptors in intermediate and caudal regions of the

caudate-putamen [30]. Such a treatment diminished the number of contralateral head turns induced by a unilateral intrastriatal injection of NMDA. The present study seems to indicate that a decreased expression of at least one subunit of the NMDA receptor complex (NMDA-R1) may be responsible for the above-mentioned behavioral effect of haloperidol. Similar data were obtained by Chih-Hui Chen et al. [4] who found a decline in mRNA level not only of the NMDA-R1 but also of the NMDA-R2A, B and C subunits in subcortical structures, including the caudate-putamen, after a 3-month treatment with *cis*- and *trans*-optical isoforms of flupenthixol, another typical neuroleptic. An opposite effect, i.e. an increase in the NMDA-R1 subunit immunoreactivity or in mRNA of that subunit in the caudate-putamen, was reported after a considerably shorter period (3–4 weeks) of haloperidol administration [9, 34]. Moreover, the lack of effect on mRNA in the striatum was reported by Brene et al. [2] after 3-week or 6-month administration of the latter neuroleptic. Such discrepancies may be due to some methodological differences [Northern blot [9] vs *in situ* hybridization (present study)], or to the rat's strain used [Sprague Dawley [2, 9, 34] vs Wistar (present study) rats]. However, they may also suggest that the striatal expression of the NMDA-R1 subunit varies during a neuroleptic therapy.

Clozapine increased the mRNA level of the NMDA-R1 subunit in the nucleus accumbens, neighboring ventrolateral part of the rostral caudate-putamen and rostral region of agranular and disgranular insular cortices. Haloperidol significantly increased the expression of the NMDA-R1 mRNA in the CA1 of the hippocampus. All these structures belong to a corticosubcortical circuit which is related to the ventral striatopallidal system [14]. The hippocampus is a part of an allocortical area, and the agranular insular cortex of a periallocortical area, which send glutamatergic projections to the nucleus accumbens [5, 11, 13, 19] which, together with the adjacent ventral part of the caudate-putamen and tuberculum olfactorium, constitutes the ventral striatum [14]. From there neuronal impulses are conveyed, *via* a ventral extension of the pallidum and mediodorsal thalamic nucleus, to the prefrontal cortex [14]. The malfunctioning of that circuit is suggested to be involved in neuropsychiatric disorders, including psychotic symptoms of schizophrenia [8, cf. 12, cf. 14, 26]. Therefore, it

may be speculated that an increase in the expression of the NMDA-R1 mRNA within that neuronal circuit induced by both haloperidol and clozapine may be important for antipsychotic action of these drugs. However, differential contribution of individual brain structures to that effect may reflect differences in their pharmacological and clinical profile.

Our recent study showed that a 3-month treatment with haloperidol and clozapine increased the number of NMDA receptors labeled with [<sup>3</sup>H]CGP 39653 in insular and parietal cortices [31]. Haloperidol also augmented their number in the frontal cortex [31]. Surprisingly, neither clozapine nor haloperidol increased the NMDA-R1 mRNA expression in frontal and parietal cortices. Contrariwise, haloperidol decreased it in both those cortical regions. Furthermore, only clozapine significantly increased that mRNA expression in the insular cortex. Therefore, the present study suggests that changes in the mRNA level of the NMDA-R1 subunit may not account for the increased number of NMDA receptors in the above-mentioned cortical regions. Other authors did not find any increases in the expression of the NMDA-R1 subunit in those cortical regions, either. According to Fitzgerald et al. [9] and Riva et al. [34], neither clozapine nor haloperidol administered for a shorter period of time (3–4 weeks) influenced the level of mRNA or the protein of the NMDA-R1 subunit in the frontal or parietal cortex. Moreover, in line with the present study, Chih-Hui Chen et al. [4] reported its decrease in the frontal cortex as a result of 6-month administration of *cis*- or *trans*-flupenthixol.

It is difficult to speculate about the cause of elevation of the [<sup>3</sup>H]CGP 39653 binding in these structures. Alteration of subunit composition of the NMDA receptor by long-term treatment with neuroleptics may be a possible explanation. [<sup>3</sup>H]CGP 39653, a competitive antagonist, binds preferentially to the combination of NMDA-R1a/NMDAR-2A subunits [20, 21, 24]. However, there are no data available on changes in the expression of subunits belonging to the other family (NMDA-R2A, B and C) after 3-month administration of haloperidol or clozapine. Moreover, data obtained in experiments in which neuroleptics were administered for much shorter periods of time did not offer any additional explanation, either. According to Riva et al. [34], 3-week treatments with haloperidol or clozapine did not change the mRNA level of the

NMDA-2A and 2B subunits in the frontal cortex. However, the lack of effect of neuroleptics on the mRNA level does not exclude a possibility that they may alter the level of the above mentioned subunits by affecting posttranslational processes. Further studies using a Western blot analysis for direct estimation of NMDA-R subunit levels may help to solve this problem.

A question arises whether the influence of chronic haloperidol and clozapine on the expression of the mRNA of the NMDA-R1 subunit is a direct one, or rather results from the action of these neuroleptics on other neurotransmitter systems. In the light of the present knowledge, a direct influence of these drugs does not seem unlikely. It is known that acute administration of both haloperidol and clozapine, as well as of some other neuroleptics influences directly the NMDA receptor complex. It has been suggested that these compounds may act as partial agonists of a glycine or another modulatory site of the NMDA receptor complex [1, 6, 10, 15, 23, 25]. In *in vitro* experiments, haloperidol and clozapine given at micromolar concentrations have been found to displace [<sup>3</sup>H]MK-801, [<sup>125</sup>I]MK-801, [<sup>3</sup>H]ifenprodil or [<sup>3</sup>H]TCP from the binding to NMDA receptors [1, 6, 22, 25]. It has also been suggested that haloperidol binds with a high affinity to a combination of the NMDA-R1a/NMDA-R2B subunits [25].

Summing up, the present results give further support to the notion that a long-term treatment with neuroleptics influences NMDA receptors in brain structures that are thought to be important for the development of psychotic symptoms. However, the exact meaning of the above-described alterations is still obscure.

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