

Homer 1a Gene Expression Modulation by Antipsychotic Drugs: Involvement of the Glutamate Metabotropic System and Effects of D-Cycloserine

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*N-methyl-D-aspartate receptor hypofunction has been suggested to play a role in the pathophysiology of schizophrenia. New glutamatergic mechanisms involving metabotropic receptors have been recently proposed to further expand this hypothesis. "Homer" is a family of postsynaptic density proteins functionally and physically attached to glutamate metabotropic receptors. We investigated the activation of the early gene form of Homer after acute treatment with typical or atypical antipsychotic drugs alone or with the adjunction of D-cycloserine. This activation was compared with that of c-fos, considered a putative molecular marker of brain regions activated by antipsychotics. Male Sprague-Dawley rats were treated intraperitoneally with haloperidol (0.8 mg/Kg) or clozapine (15 mg/Kg) alone or with the adjunction of D-cycloserine (20 mg/Kg). Rats were sacrificed ninety minutes after injection and the brains were processed for quantitative *in situ* hybridization histochemistry. Haloperidol induced a statistically significant increase of Homer both in caudate-*

putamen and nucleus accumbens compared with controls; clozapine induced Homer significantly only in the accumbens. The adjunction of D-cycloserine attenuated the haloperidol-induced increase of Homer expression in caudate-putamen and nucleus accumbens and attenuated the clozapine-induced increase in the accumbens. The c-fos gene expression was potently induced by haloperidol in caudate-putamen and nucleus accumbens, and by clozapine only in the accumbens. The adjunction of D-cycloserine enhanced c-fos expression only for clozapine in both regions of the forebrain. These results demonstrate a differential involvement of glutamatergic metabotropic system in gene expression modulation induced by typical or atypical antipsychotic drugs and may suggest new molecular basis for the augmentation strategy by a glycine site partial agonist.

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The glutamatergic hypothesis of psychotic disorders postulates a cortical and subcortical N-methyl-D-aspartate (NMDA) Receptor Hypofunction (NRH) and a subcortical hyperdopaminergic (Breier et al. 1998; Olney et al. 1999). New glutamatergic mechanisms should be taken into account in order to investigate the molecular basis of dopamine-glutamate interactions in the pathophysiology of psychosis. Indeed, the study of the glutamatergic transmission has focused on the role of metabotropic receptors (Moghaddam and Adams 1998), post synaptic density proteins (de Bartolomeis et al. 2002) and glycine modulation of NMDA receptors (Heresco-Levy 2000). These molecular mechanisms have suggested new pharmacological strategies such as the adjunction of D-cycloserine to antipsychotic drugs (Goff et al. 1999a,b).

D-cycloserine is a partial agonist at the strychnine-insensitive glycine site of the NMDA receptor complex with approximately 40% to 60% activity compared with the endogenous ligand (Goff et al. 1999b). D-cycloserine at the dose of 50 mg/day has been demonstrated to improve primary negative symptoms in patients with schizophrenia when added to conventional neuroleptics (Goff et al. 1999b) but not to clozapine (Goff et al. 1996, 1999a). D-cycloserine's therapeutic effects probably result from a relative agonist rather than antagonist action at the glycine modulatory site (Goff et al. 1999b) since the full agonist glycine has been shown to improve negative symptoms in a similar manner (Heresco-Levy et al. 1996; Javitt et al. 1994). The findings of therapeutic effects on negative symptoms of schizophrenia obtained by NMDA receptor potentiation are consistent with the NRH hypothesis of psychosis. The drug-induced NRH model has been considered the most specific psychotomimetic drug model of schizophrenia for its ability to reproduce psychotic-like negative symptoms in healthy humans (Malhotra et al. 1996). In contrast, it has been shown that at doses of 100 mg/day or 250 mg/day D-cycloserine had no effect on negative symptoms and worsened positive symptoms of psychosis. This may be explained by the evidence that at higher concentrations D-cycloserine acts as an antagonist rather than a partial agonist at the glycine site (Casella et al. 1994; van Berckel et al. 1999). Finally, it has been suggested that glycine may be less effective when combined to clozapine than when combined to conventional neuroleptics (Evins et al. 2000).

Homer is a postsynaptic density protein localized at excitatory synapses that interacts with the C-terminal intracellular tail of group 1 metabotropic glutamate receptors (mGluRs) through a PDZ-like domain (Brakeman et al. 1997; Kato et al. 1998). Some PDZ domains (present in PSD-95, Dlg, and ZO-1/2 proteins) have been

shown to bind C-terminal polypeptides, whereas others appear to bind internal (non-C-terminal) polypeptides. Homer is thought to have a direct role in neuronal plasticity since it regulates the cytoskeletal attachment and clustering of group 1 mGluRs (Brakeman et al. 1997) coupling them physically and functionally to the intracellular IP3 receptors, thus modulating the specific phosphoinositide pathway. In detail, Homer 1a protein functions as a "dominant negative" in that its increase displaces the constitutively expressed Homers and disrupts group 1 mGluRs clustering (Tu et al. 1998). We previously described a differential effect of typical (haloperidol) or atypical (olanzapine) antipsychotics on Homer gene expression (de Bartolomeis et al. 2002).

Several immediate early genes (IEGs), such as *c-fos* (Hiroi and Graybiel 1996; Semba et al. 1999) have been demonstrated to be induced differentially by typical and atypical antipsychotics. Also, they are a potential molecular tool for the screening of the pharmacological profile of antipsychotic compounds (MacGibbon et al. 1994). While these effects may be relevant as a metabolic marker of antipsychotic action it needs to be emphasized that the role of *fos* family proteins in the mechanism of action of antipsychotics is probably more complex, specially with regards to chronic treatments. Inferences regarding the final target genes activated by IEGs and their direct impact on neuronal plasticity may be difficult to draw.

In the present study, we investigated the effects of the acute treatment with typical (haloperidol) or atypical (clozapine) antipsychotic drugs on the gene expression of Homer 1a and *c-fos* in the striatum and nucleus accumbens by means of quantitative *in situ* hybridization histochemistry (ISHH). Furthermore, we studied the impact of D-cycloserine adjunction to haloperidol or clozapine on the pattern of gene expression, in the attempt to better characterize the putative molecular substrate of antipsychotics augmentation strategy in an animal model.

MATERIALS AND METHODS

Animals and Treatments

Male Sprague Dawley rats weighing ~ 250 g were housed and adapted to human handling in a temperature and humidity controlled colony room with 12/12 h light/dark cycle (lights on from 6 a.m. to 6 p.m.) with *ad libitum* access to lab chow and water. On the day of the experiment rats were randomly assigned to one of the following six treatment groups: (1) saline (0.9% NaCl) plus saline (SAL); (2) saline plus haloperidol 0.8 mg/kg (HAL); (3) saline plus clozapine 15 mg/kg (CLO); (4) saline plus D-cycloserine 20 mg/kg (DCS); (5) haloperidol 0.8 mg/kg plus D-cycloserine 20 mg/kg (HAL+DCS); and (6) clozapine 15 mg/kg plus D-cycloserine 20 mg/kg (CLO+DCS). Each animal received

two 250 μ l injections intraperitoneally. The animals were sacrificed by decapitation 90 min after the second injection. The brains were rapidly removed, frozen on dry ice, and stored at -70°C prior to sectioning. All procedures were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the local Animal Care and Use Committee.

Synthetic Oligodeoxyribonucleotides

The Homer 1a probe was a 48-base oligodeoxyribonucleotide complementary to bases 1073–1120 of the rat Homer 1a mRNA (GenBank Accession U92079). The c-fos probe was a 48-base pair oligodeoxyribonucleotide complementary to bases 270–319 of the rat c-fos mRNA (Curran et al. 1987; Merchant and Dorsa 1993). The specificity of the antisense probe signal was assessed using the corresponding sense oligodeoxyribonucleotides in the same hybridization and stringency conditions. No autoradiographic signal was detected in any of the regions in which the antisense probe yielded positive results. Moreover, the same neuroanatomical signal distribution was observed for both probes in previous ISHH experiments (de Bartolomeis et al. 2002; Merchant and Dorsa 1993).

Probe Labeling

The probes were labeled at the 3' end with $0.4\text{--}0.6 \times 10^6$ cpm [$\alpha^{35}\text{S}$] dATP (>1000 Ci/mmol, Amersham Pharmacia Biotech), terminal deoxynucleotidyl transferase (15 units/ml) (Roche), and tailing buffer. The unincorporated nucleotides were separated from radiolabeled DNA using Sephadex G-50 chromatographic columns (Amersham Pharmacia Biotech). The procedure for in situ hybridization histochemistry was taken from several standard published protocols (Austin et al. 1992; de Bartolomeis et al. 1994; Young et al. 1986).

In Situ Hybridization Histochemistry

Frozen 12 μ m sections were cut on a cryostat at -19°C at approximately bregma 1.60 mm forebrain level, using plate 12 of the rat brain atlas of Paxinos and Watson (1986) as an anatomical reference; sections were mounted onto gelatin-coated slides, and stored at -80°C for subsequent processing. Prior to hybridization, the sections were fixed in 1.5% formaldehyde in 0.12 M sodium-phosphate buffered saline (PBS, pH 7.4), quickly rinsed three times with $1 \times$ PBS, and placed in 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl, pH 8.0, for 10 min. Next, the sections were dehydrated in 70%, 80%, 90%, and 100% ethanol, delipidated in chloroform for 5 min, rinsed again in 100% and 95% ethanol and air dried. Sections were hybridized with $0.4\text{--}0.6 \times 10^6$ cpm of radiolabeled oligonucleotide in buffer containing 50% formamide, 600 mM NaCl, 80 mM Tris-HCl (pH 7.5), 4 mM

EDTA, 0.1% pyrophosphate, 0.2 mg/ml heparin sulfate, and 10% dextran sulfate. Incubations were carried out at 37°C in a humid chamber for 20 h. After hybridization the sections were washed 4×15 min in $2 \times$ SSC/50% formamide at 40°C , followed by two 30-min washes with $1 \times$ SSC at 40°C . The slides were rapidly rinsed in distilled water and then in 70% ethanol. The sections were dried and exposed to Biomax MR X-ray film (Kodak Scientific Imaging Film) for 14–30 days. The optimal time of exposure was chosen to maximize signal to noise ratio while preventing optical density from approaching the limits of saturation. ^{14}C standard sections with several known amounts of radioactivity cross-calibrated with ^{35}S standards were co-exposed with the samples.

Image Analysis

The quantitation of the autoradiographic signal was performed using a computerized image analysis system including: a light box (Northern Light) and a CCD camera, an Apple PowerPC, and NIH Image 1.62 software (W. Rasband, NIMH, Bethesda, MD). Sections were quantitated blind to the treatment conditions. In order to test for inter-observer reliability an independent quantitation was performed by a second investigator. Only quantitatively comparable results, in terms of consistency of any statistically significant effect, obtained by the two investigators were considered reliable. For each experimental group three to five slides were analyzed. Each slide contained 2–4 adjacent sections of a single animal. Care was taken to select identical anatomical levels of treated and control sections using thionin-stained reference slides. Quantitative comparisons among different experimental groups were performed using images from hybridized sections exposed on the same sheet of X-ray film. Regions of interest (ROI) were outlined on digitized autoradiograms and the transmittance was measured using NIH image software. Forebrain sections were analyzed in the caudate-putamen and nucleus accumbens regions. The caudate-putamen dorsolateral subregion was outlined and measured using an oval template of $0.70 \text{ mm} \times 1.17 \text{ mm}$ (Figure 1). A region of the nucleus accumbens was measured using an oval template of $0.60 \text{ mm} \times 0.72 \text{ mm}$ (Figure 1).

Data Analysis

Measurements of optical density within ROIs were then converted into "relative dpm" by a calibration curve based on the standard scale co-exposed to the sections. For this purpose a "best fit" third-degree polynomial was used. For each animal measurements from the 2–4 adjacent sections were averaged. The data were analyzed for treatment effects by a 1-way Analysis of Variance (ANOVA). Fisher's Protected LSD post hoc test was used to determine the locus of effects in any significant ANOVA.

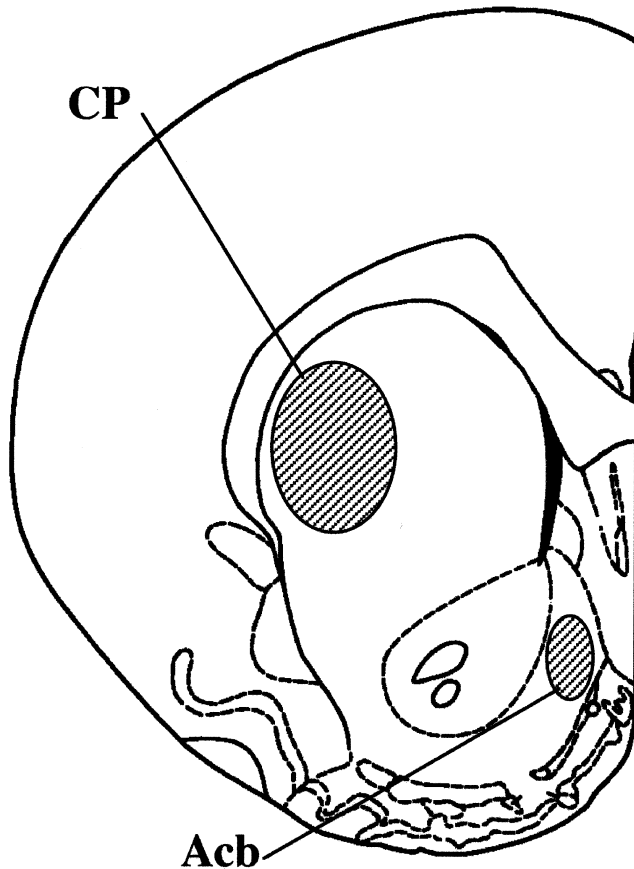


Figure 1. Diagram of rat forebrain section with regions of interest (ROIs) quantitated on film autoradiograms. CP = caudate-putamen; Acb = nucleus accumbens (modified from Paxinos and Watson 1986).

RESULTS

Homer 1a

Homer 1a gene expression was detected in several forebrain regions of the control animals. The signal was homogeneously distributed throughout the cerebral cortex, and was present in caudate-putamen, nucleus accumbens, and the islands of Calleja, consistently with previous reports (de Bartolomeis et al. 2002). Statistically significant differences in Homer 1a gene expression (Figure 2) have been found among experimental groups in the dorsolateral subregion of caudate-putamen (Figure 3) and in the nucleus accumbens (Figure 4).

In caudate-putamen the following statistically significant differences were found: HAL greater than SAL, DCS and CLO+DCS. In the same region, CLO was significantly greater than DCS. Moreover, HAL+DCS showed a statistically significant increase vs. DCS (ANOVA, $p = .0072$).

In the nucleus accumbens a statistically significant Homer 1a mRNA increase was found in HAL group

compared with SAL, HAL+DCS, CLO+DCS and in CLO compared with SAL (ANOVA, $p = .0143$).

c-fos

In the saline group the c-fos signal was homogeneously distributed throughout the cerebral cortex and the islands of Calleja, and a low signal was detected in the caudate-putamen and the nucleus accumbens.

In the dorsolateral subregion of caudate-putamen, a statistically significant increase of c-fos gene expression (Figure 5 and Figure 6) was detected under both haloperidol treatments, i.e. HAL and HAL+DCS vs. SAL, CLO, DCS and CLO+DCS; moreover, CLO+DCS was significantly greater than baseline (ANOVA, $p < .0001$).

In the nucleus accumbens, the densitometric analysis of autoradiographic images showed a statistically significant increase of c-fos mRNA expression (Figure 5 and Figure 7) after all the antipsychotic treatments, both with and without D-cycloserine adjunction. In this region, the following differences were statistically significant: HAL greater than SAL and than DCS; CLO greater than SAL; CLO+DCS greater than SAL, CLO, DCS; and finally HAL+DCS greater than SAL, HAL, CLO, DCS and CLO+DCS (ANOVA, $p < .0001$).

DISCUSSION

Based on previous animal studies, our dosage of D-cycloserine of 20 mg/kg can be predicted to exert agonistic effects on the glycine site of the NMDA receptor. This dosage has been shown to facilitate performance on learning tasks in rodents, a property attributed to NMDA receptor potentiation (Andersen et al. 2002; Baran et al. 1995; Depoortere et al. 1999; Monahan et al. 1989). Antagonistic effects of D-cycloserine such as its anticonvulsive properties are associated to much higher doses (160–320 mg/kg) in rodents (Baran et al. 1995; Loscher et al. 1994; Wlaz et al. 1994). It is conceivable that the D-cycloserine dose of 20 mg/kg in rats exerts an agonistic effect similarly to the dosage proven to be effective in adjunction to haloperidol in humans. Interestingly, we previously found that quantitatively comparable results of Homer1a expression in caudate-putamen are obtained using D-cycloserine at a dose of either of 20 mg/kg or 100 mg/kg in adjunction to haloperidol (unpublished data). This may suggest that even higher doses (at least up to 100 mg/kg) may exert agonistic effects in our paradigm. However, caution must be used when interpreting our findings in terms of reproduction of pharmacological effects reported in clinical trials, as effects on psychotic symptoms occur over a period of weeks, whereas our study is based on acute treatments. While further studies using chronic treatments will be

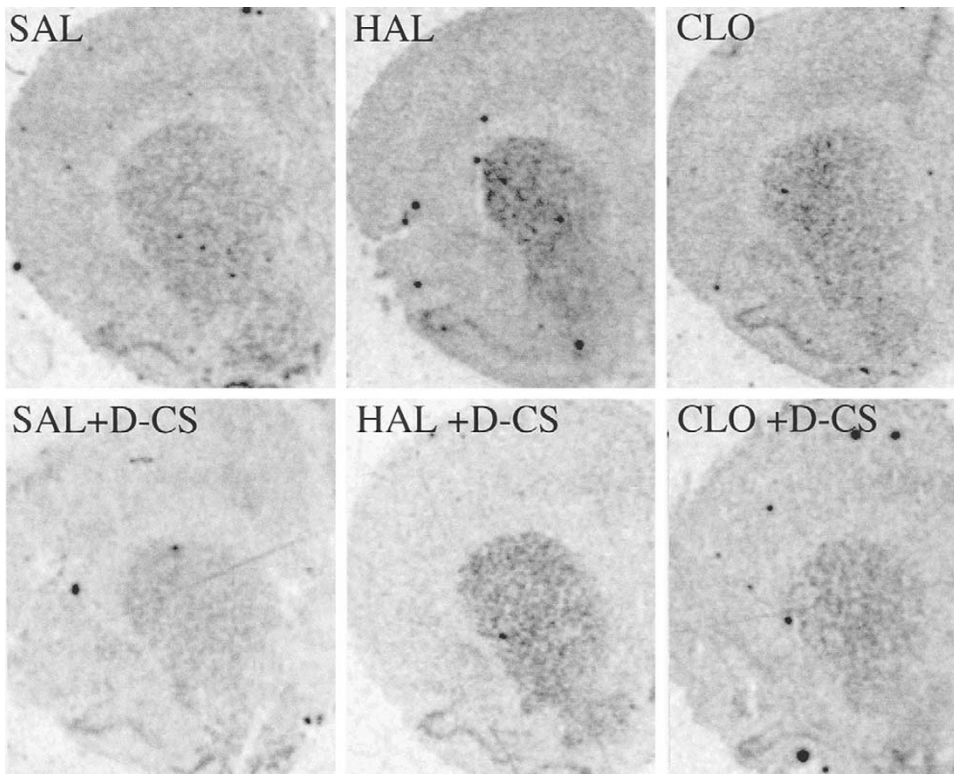


Figure 2. Autoradiograms of rat forebrain sections hybridized with Homer 1a probe. The comparison of treated groups vs. saline shows a remarkable signal increase of haloperidol and clozapine groups in caudate-putamen and nucleus accumbens. Moreover, the signal appears less intense when D-cycloserine is added to haloperidol and clozapine, in the same regions.

of great interest, our findings may represent an important first step in the understanding of the molecular mechanisms of D-cycloserine augmentation strategy. The acute treatment used in the present study is further justified by our specific focus on the modulation of immediate early genes as Homer 1a and c-fos.

The results show that typical or atypical antipsychotics may modulate differentially the gene expression of the marker of the glutamatergic system Homer 1a, and

of the IEG c-fos. The adjunction of D-cycloserine produced relevant effects on Homer and c-fos gene expression. Homer 1a mRNA levels in caudate-putamen are significantly increased by haloperidol treatment compared with DCS and CLO+DCS but not to treatment with clozapine alone (Figure 3). This finding suggests that D-cycloserine, when added to clozapine, attenuates clozapine-induced Homer 1a increase vs. DCS. More-

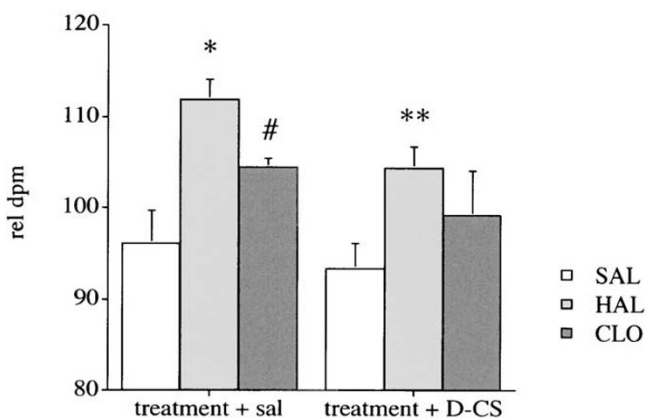


Figure 3. Homer mRNA levels in caudate-putamen quantitated by densitometry of in situ hybridization histochemistry autoradiograms and expressed in "relative dpm". ANOVA: $p = .0072$. Fischer's Protected LSD post hoc test: *HAL vs. SAL, DCS and CLO+DCS; **HAL+DCS vs. DCS; #CLO vs. DCS.

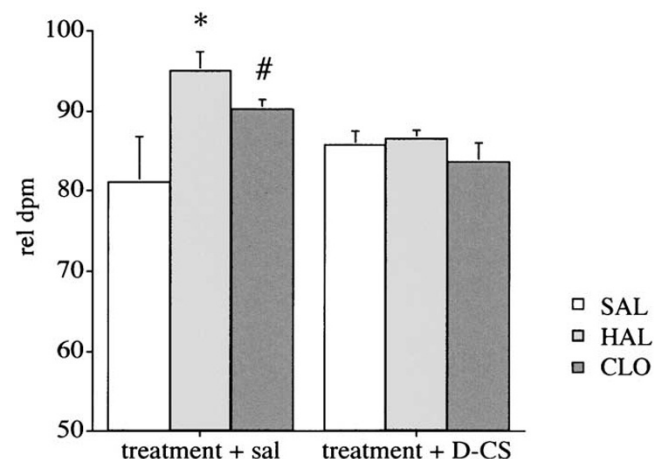


Figure 4. Homer mRNA levels in nucleus accumbens quantitated by densitometry of In Situ Hybridization Histochemistry autoradiograms and expressed in "relative dpm". ANOVA: $p = .0143$. Fischer's Protected LSD post hoc test: *HAL vs. SAL, HAL+DCS and CLO+DCS; #CLO vs. SAL.

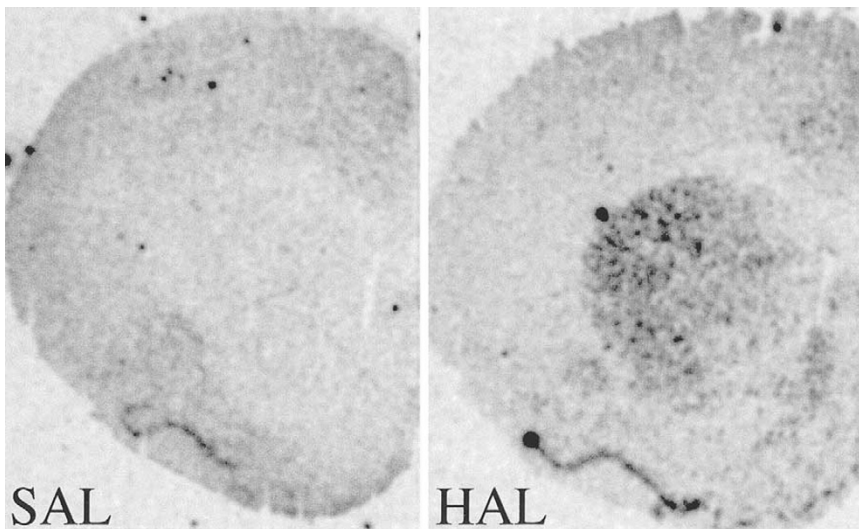


Figure 5. Autoradiographic images of rat forebrain sections hybridized with c-fos probe in saline and haloperidol treated groups. In saline control group, the signal is homogeneously distributed throughout the cerebral cortex and the islands of Calleja, but not in caudate-putamen and nucleus accumbens. The comparison of the two images clearly shows the c-fos induction in caudate-putamen and nucleus accumbens by haloperidol treatment.

over, the clozapine treated group showed a statistically significant increase vs. DCS but not vs. SAL, and the HAL+DCS group also showed an increased signal compared with DCS group but not to SAL. Both these observations may be explained by the negative trend of the D-cycloserine group. It can also be observed that the adjunction of D-cycloserine to haloperidol attenuates the haloperidol induced Homer 1a increase, as HAL is significantly greater than SAL whereas HAL+DCS is not. In fact, HAL+DCS is reduced as compared with HAL, although this difference is not statistically significant. These effects seem to be neuroanatomically specific to caudate-putamen. At the molecular level, considering that Homer 1a functions as a “dominant negative” it can be predicted that the Homer 1a decrease by D-cycloserine

augmentation determines a relative increase of mGluR clustering. This could be interpreted as a mechanism of metabotropic glutamatergic enhancement by a partial agonism at glycine site of NMDA receptor in subcortical neurons receiving cortical glutamatergic inputs.

In the nucleus accumbens, both antipsychotics significantly increased Homer 1a gene expression as compared with the saline group (Figure 4). This antipsychotic induced effect is abolished when the three D-cycloserine treated groups (DCS, HAL+DCS and CLO+DCS) are compared. Remarkably, HAL+DCS is significantly reduced as compared with HAL. So it

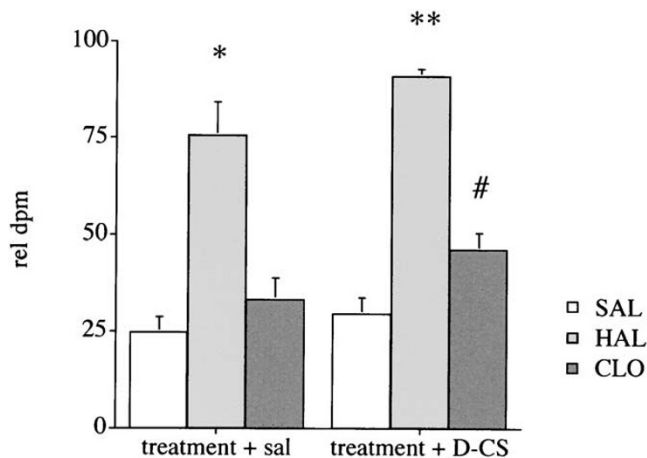


Figure 6. c-fos mRNA levels in caudate-putamen quantitated by densitometry of in situ hybridization histochemistry autoradiograms and expressed in “relative dpm”. ANOVA: $p < .0001$. Fischer’s Protected LSD post hoc test: *HAL vs. SAL, DCS, CLO and CLO+DCS; **HAL+DCS vs. SAL, DCS, CLO and CLO+DCS; #CLO+DCS vs. SAL.

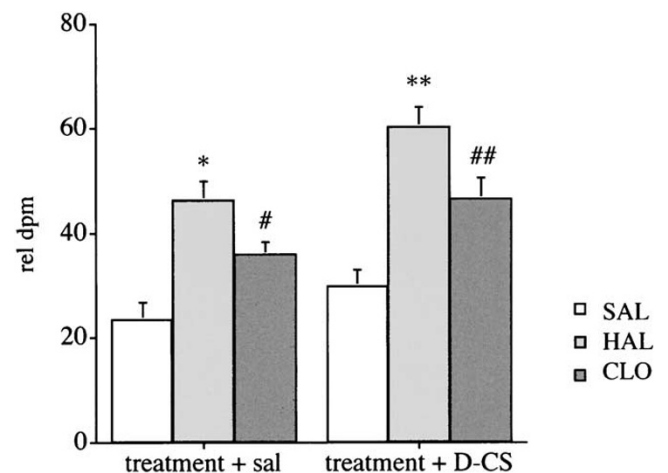


Figure 7. c-fos mRNA levels in nucleus accumbens quantitated by densitometry of In Situ Hybridization Histochemistry autoradiograms and expressed in “relative dpm”. ANOVA analysis of variance test detected significant variations among the experimental groups ($p < .0001$). Fischer’s Protected LSD post hoc test showed the following statistically significant differences: *HAL vs. SAL and DCS; **HAL+DCS vs. SAL, DCS, CLO and CLO+DCS; #CLO vs. SAL; ##CLO+DCS vs. SAL, CLO and DCS.

seems that D-cycloserine negatively modulates Homer 1a gene expression when added to haloperidol and in a non-significant manner, when added to clozapine.

The discrete effect of the adjunction of D-cycloserine to haloperidol on Homer gene expression could underlie a limbic-specific activity of this drug association. Indeed, the signal decrease (HAL > HAL+DCS) is statistically significant in the nucleus accumbens, which is largely associated with limbic function, but not in the caudate-putamen, which is characteristically involved in motor control.

At the molecular level, the relevant role of Homer in the regulation of glutamate receptor clustering, their physical proximity, as well as the recent demonstrations of their ability to interact at the synapse and to modulate NMDA receptor function (Naisbitt et al. 1999) must be considered. The results of the present study suggest that D-cycloserine, added to antipsychotic drugs, may be involved in the modulation of neuronal plasticity and may functionally affect the postsynaptic density, which could be involved in the antipsychotics mechanism of action.

Finally, the IEG c-fos also proved to be differentially modulated by the different acute treatments. A differential induction of c-fos gene expression by typical or atypical antipsychotics has been demonstrated in several acute studies by independent reports (Hiroi and Graybiel 1996; Semba et al. 1999). Previous studies show that c-fos immunoreactivity is downregulated in the caudate-putamen after chronic treatment by haloperidol and clozapine but not by metoclopramide. In contrast, FosB-like immunoreactivity is upregulated after chronic administration of haloperidol but not clozapine (Hiroi and Graybiel 1996). All together these data point out that acute and chronic administration of antipsychotics may exert relevant differential effects on IEG induction and this observation needs to be considered when interpreting our results. In our acute paradigm, both haloperidol treatments, either alone or with D-cycloserine, potentially induce its expression in the caudate-putamen (Figure 6). The atypical antipsychotic clozapine did not induce c-fos in this region unless DCS was added. The CLO+DCS induced c-fos expression was significantly higher than SAL, although it was at the same time significantly lower than both the HAL treatments. In the nucleus accumbens (Figure 7), c-fos mRNA was induced by both haloperidol and clozapine and the adjunction of D-cycloserine proportionally enhanced this induction. The c-fos mRNA induction pattern in both striatum and accumbens by haloperidol and clozapine that we observed is consistent with previous studies of c-fos mapping after treatment with typical or atypical antipsychotics (Guo et al. 1995; MacGibbon et al. 1994; Robertson and Fibiger 1992; Robertson et al. 1994). Those studies point out that both haloperidol and clozapine induce c-fos expression in nucleus accumbens, whereas haloperidol but not clozapine increases

c-fos in the striatum. The differential D-cycloserine effect in the striatum may prove to better characterize the index of "atypicality" based on antipsychotic-induced c-fos expression pattern in the striatum (Robertson et al. 1994). Our findings may prove useful in the preclinical characterization of new antipsychotics and possibly in predicting clinical efficacy of associations of new antipsychotics with D-cycloserine. However, in order to draw more precise correlations to the efficacy of antipsychotic augmentation therapy on the negative symptoms of psychotic disorders, new experiments using different doses of D-cycloserine and chronic treatments will be a useful means of further investigation.

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