Switching antipsychotics: how and where a prolonged treatment with a typical antipsychotic affects the topography of postsynaptic density gene expression induced by an atypical antipsychotic.

Relevance for psychosis treatment.

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## INDEX

**SUMMARY** 3
**BACKGROUND** 4
**AIM OF THE STUDY** 7
**MATERIALS AND METHODS** 8
  - Acute paradigm 8
  - Switching paradigm 8
  - Animals 10
  - In Situ Hybridization 10
  - Data analysis 12
**PRELIMINAR DATA** 14
**RESULTS** 18
  - Homer1a 18
  - Arc 22
  - Homer1b 26
  - Psd-95 29
**TOPOGRAPHIC ANALYSIS** 32
  - Homer1a/Homer1b/c relative ratio 35
**DISCUSSION** 39
**BIBLIOGRAPHY** 47
SUMMARY

Typical and atypical antipsychotics can be characterized at preclinical levels by differential induction of several *Immediate Early Genes* localized at cortical and subcortical regions related to pathogenesis of psychiatric disorders and antipsychotics action. So, genes expression. Molecular imaging under different treatment paradigms could be considered a sensitive molecular tool to investigate where drugs act, predicting, with a translational approach a clinical outcome. In clinical practice psychiatrists frequently switch from one antipsychotic to another with different receptorial profile when patients show poor or no treatment response or intolerable adverse effect, but the post-switch antipsychotics response is different compared to drug naïve patients. Neuroimaging studies highlighted morphological and molecular changes after long-term administration in brain regions affected by antipsychotics, resulting in unexpected clinical outcome. In our study we analyze *Immediate Early Genes* expression of proteins localized ad Postsynaptic Density (PSD), where dopaminergic and glutamatergic transmission converging, in an animal model of switching. Thus we highlight that first chronic treatment with strong D₂ blocker affects genes expression induced by second chronic treatment with antipsychotic characterized by a different receptorial profile, confirming, at molecular level, what happens in clinical practice and giving new challenges towards antipsychotic mechanism of action.
BACKGROUND

Antipsychotics represent the cornerstone of schizophrenia pharmacological treatment. The efficacy of each antipsychotics is different among schizophrenic patients, but approximately 10%-30% of patients show little or no response or intolerable adverse effects, so necessitating a change in their medication regimen (Lieberman et al., 2005; Elkis and Meltzer, 2007).

Switching from one antipsychotic to another when facing poor response is common clinical practice, but it could be a potentially destabilizing event because it could lead to rebound and withdrawal phenomena (Correll, 2010; Correll, 2011). Rebound effects involve exposure of previously blocked receptors to the endogenous ligand or to the different receptor profile of the post-switch antipsychotic (Correll, 2010). Several evidence have established that chronic antipsychotic administration causes biological adaptation to counteract the postsynaptic dopamine D2 receptor blockade. SPECT studies show an increased number of DAT binding sites in the schizophrenic patients treated with D2 receptor blocking neuroleptics (Sjoholm et al., 2004). Furthermore preclinical evidence have shown recently that antipsychotics may affect directly brain structure determining morphological changes after long-term administration (Vernon et al., 2011).

MRI studies in schizophrenic patients, even if are not always consistent, show morphological changes induced by chronic antipsychotic administration in caudate, putamen and globus pallidus, regions related with schizophrenia symptoms, antipsychotic efficacy and motor side effect (Corson et al., 1999); switch to atypical antipsychotic as olanzapine or clozapine reverses the anatomical modification following typical antipsychotic long-term treatment (Scheepers et al., 2001; Lang et al., 2004). Indeed chronic antipsychotic administration causes changes across the synapse resulting in unexpected clinical outcome of patients after switching from one antipsychotic to another with different D2 affinity (Buckley et al., 2007), but studies in animal model are lacking.
Despite being a common clinical practice, switching antipsychotics has been surprisingly studied very little in preclinical paradigm, specifically with regard to the effects on brain activation and gene expression induction, leaving some question unexplored.

Mapping temporal and spatial changes in gene expression is a powerful tool for studying where antipsychotics switching. The Postsynaptic Density (PSD) is a specialized structure of postsynaptic membrane of glutamatergic synapses, where glutamatergic and dopaminergic system interact and which mediate transductional signalling and synaptic plasticity (Sheng et al., 2007).

To test changes in gene expression, among other components of PSD, we focus on Homer, a family of scaffold proteins localized at PSD, including both constitutive (Homer 1b/c, Homer 2 a/b, Homer 3) and inducible (Homer 1a and ania3) isoforms, which have a key role in regulating the synaptic architecture (Fagni et al., 2002; Hayashi et al., 2009) and the molecular rearrangement of PSD after challenging dopaminergic neurotransmission (Iasevoli et al., 2009). Moreover Homer 1-KO mice exhibit behavioural and neurochemical abnormalities that are consistent with the animal models of schizophrenia and the regulation of extracellular levels of glutamate within limbo-cortico-striatal structures by Homer 1 may be involved in the pathogenesis of schizophrenia (Szumlinski et al., 2005).

Homer constitutive isoform interact with other PSD proteins included glutamate receptors, kinases and cytoskeleton proteins so exerting a structural role in dendritic spines architecture, regulating trafficking and redistribution of metabotropic glutamate receptor (mGluRs), crosstalking between NMDARs and mGluRs and bridging of inositol 1,4,5-triphosphate receptors (IP3Rs) to mGluRs, providing a link between glutamate receptors and intracellular signal transduction system (de Bartolomeis et al., 2003). Homer 1a induction disrupts the cluster of proteins formed by constitutive isoforms leading to a changes in postsynaptic density morphology. Several stimuli have been demonstrated to induce Homer 1a expression, including antipsychotic administration. Homer-regulated PSD remodelling may represent a mechanism of
synaptic plasticity, and it could be involved in molecular changes associated to pharmacotherapy (Tomasetti et al., 2007). It has been shown that haloperidol, a strongly D₂ blocker, robustly induce the expression of Homer1a in all subregions of the caudate-putamen, whereas atypical antipsychotics, having a lesser D₂ blockade rate, does not (Iasevoli et al., 2010; Iasevoli et al., 2010; Iasevoli et al., 2011). Moreover, differently from other immediate early genes, expression of which is modulated by antipsychotics (i.e., cfos, arc) (Fumagalli et al., 2009), the up-regulation of Homer1a induced by acute antipsychotics administration hold steady in chronic paradigms (Ambesi-Impiombato et al., 2007; Tomasetti et al., 2011). From a clinical point of view, the lack of sensitization of Homer1a after long-term treatment makes it a good candidate as molecular marker of treatment response.

Arc is a PSD protein involved in rapid cytoskeletal rearrangements in response to several synaptic stimuli (Bramham et al., 2008), and its gene expression may mark fast structural changes in the PSD. Moreover, Arc expression has been found affected by antipsychotics (de Bartolomeis et al., 2013; Iasevoli et al., 2010; Fumagalli et al., 2009; Luoni et al., 2014). Psd-95 is a scaffolding protein, whose functions include the control of glutamatergic, dopaminergic, and serotonergic receptors trafficking throughout the PSD and positioning onto membrane surface (Iasevoli et al., 2013), thereby deeply influencing receptor signaling and PSD plasticity. All together these molecules are considered relevant for dendritic architecture and related functional changes as well as for synaptic plasticity disorders in neurobehavioral diseases (Kirov et al., 2012; Cohen et al., 2011).
AIM OF THE STUDY

The aim of the study is to investigate the effect on gene expression of chronic treatment with an atypical antipsychotic, after prolonged chronic treatment with a strong $D_2$ blocker, such as haloperidol, in order to analyze, at molecular level, a clinical practice of switching. We focus on Immediate Early Genes Homer1a and Arc and constitutive genes Homer1b and Psd-95, localized at PSD level, which likely could be involved in molecular changes related to antipsychotic response.

“Switching” is a change in medication regimen from a chronic treatment with an antipsychotic to a chronic administration with another antipsychotic characterized by a different receptorial profile. Clinical evidence showing an unexpected outcome in schizophrenic patients long lasting treated when switch antipsychotic, compared to patient drug-naive. Indeed several neuroimaging studies demonstrated a morphological changes induced by chronic antipsychotic administration in caudate, putamen and globus pallidus, regions related to schizophrenic symptoms, antipsychotic efficacy and motor side effect (Corson et al., 1999). Nevertheless, molecular finding that investigate in a preclinical paradigm switching are lacking.

We have chosen amisulpride as post-switch antipsychotic because, despite its high affinity to $D_2$ receptor, comparable to haloperidol, it has the clinical profile of atypical antipsychotics (eg a low potential to cause extrapyramidal symptoms (EPS)) (La Fougere et al., 2005).

However unlike other atypical antipsychotic which bind to multiple receptors, amisulpride is devoid of any significant affinity to other receptors (Schoemaker et al., 1997). Indeed, its activity at presynaptic $D_2/D_3$ receptors is responsible for enhancing dopamine transmission (Moller et al., 2003; Lecrubier et al., 2000).

Then for its unique receptorial profile, amisulpride allows to better understand molecular changes and then the downstream effects after manipulating dopaminergic neurotransmission system in subcortical regions involved in pathogenesis of schizophrenia, antipsychotic treatment response and motor side effects.
MATERIALS AND METHODS

Acute paradigm.
In the acute paradigm male Sprague-Dawley rats were randomly assigned to one of the following experimental treatment group: vehicle (VEH); haloperidol 0.8 mg/kg (HAL); amisulpride 10 mg/kg (AMS10); Amisulpride 35 mg/kg (AMS35) and received a unique administration. Animals were injected intraperitoneally (i.p.), mildly anaesthetized by chloral hydrate, and killed 90 min after administration. We had chosen two different doses to mimic clinical practice, where amisulpride is effective at low dose on dystimia and negative symptoms of schizophrenia (Boyer et al., 1995), at high dose on positive symptoms (Lecrubier et al., 2000; Moller et al., 2003). On the molecular point of view, low dose believed to block preferentially presynaptic dopamine autoreceptors, thereby regulating dopamine release (Schoemaker et al., 1997); high dose predicted to block both pre- and postsynaptic dopamine D2 receptors (Perrault et al., 1997).

Switching paradigm.
Experimental paradigm resembling, with a translational approach, a common clinical practice of switching was designed. Initially rats were randomly assigned to one of the following treatment groups (n=18 animals for each treatment group): vehicle (veh) or haloperidol 0.8 mg/kg (hal) and received daily treatments for 15 days; from on day sixteen rats of each group were switched to vehicle, haloperidol or amisulpride and received daily treatment for 15 days more; so they were randomly assigned to the following treatment groups (n=6 animals for each treatment group, pretreatment condition/switch condition): vehicle (veh/veh); haloperidol 0.8 mg/kg (veh/hal); amisulpride 35 mg/kg (veh/ams), which had receiving first vehicle treatment and
vehicle (hal/veh), haloperidol (hal/hal) and amisulpride (hal/ams), which had receiving first haloperidol treatment. We have used amisulpride at 35mg/kg dose because at this dosage it has been shown to block pre- and postsynaptic dopamine D₂ receptor (Perrault et al., 1997) and to induce a peculiar striatal pattern of Immediate Early Gene expression (de Bartolomeis et al., 2013).

**Picture 1.** Experimental design (above). Regions of Interest (below).
Animals
Male Sprague-Dawley rats (mean weight 250g) were obtained from Charles-River Labs. (Lecco, Italy). The animals were housed and let to adapt to human handling in a temperature and humidity controlled colony room with 12/12 h light–dark cycle (lights on from 6:00 a.m. to 6:00 p.m.) with ad libitum access to laboratory chow and water. All procedures were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and were approved by local Animal Care and Use Committee. All efforts were made to minimize animal number and suffering.

Amisulpiride powder (Sanofi-Aventis, Italy) and Haloperidol injectable solution (Lusofarmaco, Italy) were dissolved in saline solution (NaCl 0.9%). All solutions were adjusted to physiological pH value and injected i.p. at a final volume of 1 ml/kg. Animals were injected intraperitoneally (i.p.), and sacrificed by decapitation 90 minutes after the last administration.

In Situ Hybridization.
After sacrifice, the brains were rapidly removed, quickly frozen on powdered dry ice and stored at -70°C prior to sectioning. Serial coronal sections of 12 μm were cut on a cryostat at -18°C through the forebrain at the level of the middle-rostral striatum (approx. from Bregma 1.20mm to 1.00mm), using the rat brain atlas by Paxinos and Watson (Paxinos and Watson 1997) as an anatomical reference. Care was taken to select identical anatomical levels of treated and control sections. Sections were thaw-mounted onto gelatin-coated slides, and stored at -50°C for subsequent analysis.

Homer 1a, Arc, PSD-95 and Homer1b/c probes were oligodeoxyribonucleotides derived from identical probes used in previous hybridization studies (Ambesi -Impiombato et al., 2007; Tomasetti et al., 2007; Iasevoli et al. 2010; de Bartolomeis et a., 2011, 2013). All the oligodeoxyribonucleotides were purchased from MWG Biotech (Firenze, Italy).

For each probe a 50μl labeling reaction mix was prepared on ice using DEPC treated water, 1X tailing buffer, 7.5pmol/μl of oligo, 125 Units of TdT and 100mCi $^{35}$S-dATP.
The mix was incubated 20 min at 37°C. The unincorporated nucleotides were separated from radiolabeled DNA using ProbeQuant G-50 Micro Columns (Amersham-GE Healthcare Biosciences; Milano, Italy). As an assessment of the probe specificity, the autoradiographic signal distribution was compared and found to be consistent for topography and with previous in situ hybridization studies (Brakeman et al., 1997; Polese et al., 2002). The specificity of each oligonucleotide probe was also tested by pilot control experiment using the corresponding sense oligodeoxyribonucleotide.

Sections were processed for radioactive in situ hybridization according to previously published protocols (Ambesi-Impiombato et al., 2003; Iasevoli et al 2012, de Bartolomeis et al 2013). All solutions were prepared with sterile double-distilled water. The sections were fixed in 4% formaldehyde in 0.12 M phosphate buffered saline (PBS, pH 7.4), quickly rinsed three times with 1X PBS, and placed in 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl, pH 8.0, for 10 minutes. Next, the sections were dehydrated in 70%, 80%, 95% and 100% ethanol, delipidated in chloroform for 5 minutes, rinsed again in 100% and 95% ethanol and air dried.

Sections were hybridized with 0.4-0.6x10^6 cpm of radiolabeled oligonucleotide in buffer containing 50% formamide, 600mM NaCl, 80mM Tris-HCl (pH 7.5), 4mM EDTA, 0.1% pyrophosphate, 0.2mg/ml heparin sulfate, and 10% dextran sulfate. Slides were covered with coverslips and incubated at 37°C in a humid chamber for 22-24 hours. After hybridization the coverslips were removed in 1X SSC (saline sodium citrate solution) and the sections were washed 4x15 minutes in 2X SSC/50% formamide at 43-44°C, followed by two 30 min washes with 1X SSC at room temperature. The slides were rapidly rinsed in distilled water and then in 70% ethanol.

The sections were dried and exposed to Kodak-Biomax MR Autoradiographic film (Sigma-Aldrich, Milano, Italy). A slide containing a reference scale of 16 known amounts of ^14C standards (ARC-146C, American Radiolabeled Chemical, Inc., St. Louis, MO, USA) was co-exposed with the samples. The autoradiographic films were exposed in a time range of 14-30 days depending from the probe and the specific brain region to be analyzed. The optimal time of exposure was chosen to maximize signal-to-noise ratio but to prevent optical density from approaching the limits of saturation. Film
development protocol included a 1.5 min dip in the developer solution and 3 min in the fixer.

Data analysis.

The quantitation of the autoradiographic signal was performed using a computerized image analysis system including: a transparency film scanner (Microtek Europe B. V., Rotterdam, The Netherlands), an Apple iMac Intel Core 2 Duo 3.06GHz 3MB cache 4GB SDRAM DDR3 a 1066MHz, and ImageJ software (v. 1.40, Rasband, W.S., http://rsb.info.nih.gov/ij/). Sections on film were captured individually. The original characteristics of the scanned images (i.e. contrast, brightness, resolution) were preserved. Each experimental group contained 4-6 animals. Each slide contained 3 adjacent sections of a single animal. All hybridized sections were exposed on the same sheet of X-ray film. Signal intensity analysis was carried out on digitized autoradiograms measuring mean optical density (transmittance) within outlined Regions of Interest (ROIs) in correspondence of the cortex, caudate-putamen and nucleus accumbens. These cortical and striatal subregions are structurally and functionally interconnected through projections from the cortex, which targets specific striatal sectors. ROIs in the cortex were selected based on recent data describing functional and anatomical correlation between cortical and striatal subregions (Willuhn et al., 2003). ROIs in the striatum have been chosen according to classical subdivision of this region (Steiner and Gerfen 1993).

An oval template, proportional to the dimensions of the anatomical subregion, was used for computerized quantitations in each one of the ROIs depicted.

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. Results obtained by the first investigator were considered reliable, and then reported, only when they were quantitatively comparable, in terms of consistency of the statistically significant effects found, to that obtained by the second investigator. Measurements of mean optical density within ROIs were converted using a calibration curve based on the standard scale co-exposed to the sections. $^{14}$C standard values from 4
through 12 were previously cross-calibrated to $^{35}$S brain paste standards, in order to assign a dpm/mg tissue wet weight value to each optical density measurement through a calibration curve. For this purpose a “best fit” 3rd degree polynomial was used. For each animal, measurements from the 3 adjacent sections were averaged and the final data were reported in relative dpm as mean ± S.E.M.

One-way Analysis of Variance (ANOVA) was used to analyze treatment effects. The Tukey’s post hoc test was used to determine the locus of effects in any significant ANOVA.

A comparison between groups with same treatment, but different first treatment, in order to analyze the effect of the last one, were carried out by Student’s t test. In all tests, significance was set at p>0.05 (two-tailed).

To calculate the Homer1a/Homer1b/c ratio we first divided the mean value of Homer1a and Homer1b/c mRNA expression to the correspondent value of vehicle mRNA expression in each region. The normalized Homer1a value of expression was then divided for normalized Homer1b/c value of expression. A result >1 indicated prominent Homer1a over Homer1b/c expression; a result <1 indicated prominent Homer1b/c over Homer1a expression (de Bartolomeis et al., 2013).

To determine whether gene expression differed between the ROIs, measurements from each treatment group were taken as the dependent variable, and the ROIs in which expression was measured as the independent variable (i.e.: measurements were analyzed per region effect). As distribution of measurements values did not follow a normal distribution (as assessed by the Shapiro-Wilk test), the non-parametric Kruskal-Wallis test was used to investigate the null hypothesis that no significant differences could be found in mRNA expression throughout cortical or striatal subregions. Significant differences among groups were analyzed by the Wilcoxon test for multiple comparisons (Iasevoli et al., 2014). In a subsequent step we used Two Way Analysis of Variance (ANOVA) in order to evaluate first treatment effect, second treatment effect and the interaction between first treatment and second treatment effect on gene expression. So we investigated whether the observed gene expression was mainly induced by first
treatment effect or by second treatment effect or by first treatment*second treatment interaction.

PRELIMINAR DATA

In the acute paradigm Homer1a is induced by amisulpride at both doses compared to control in ventral and medial striatal subregions, even though in a no statistically significant manner (AMS35 vs VEH and AMS10 vs VEH). Haloperidol induced Homer1a in dorsolateral subregion of caudate-putamen compared to all other treatment (hal vs ams35, ams10, veh), in ventrolateral subregions compared to veh and ams35 (hal vs veh, ams35), in dorsomedial subregions compared to vehicle (hal vs veh). No significant Homer1a induction by haloperidol or amisulpride was detected in the cortex (ANOVA, p<0.05). In the acute paradigm we analyzed Immediate Early Genes expression induced by haloperidol and amisulpride at two different doses: 10mg/kg and 35 mg/kg. So, we identified in the striatum, a peculiar pattern of expression induced by haloperidol, mainly impacting lateral subregions and by amisulpride at high doses, mainly impacting medial subregions. Specifically we observed that, after acute administration, in the lateral subregions of the striatum Immediate Early Genes expression was prevalently induced by haloperidol as compared to vehicle and to amisulpride, both at low doses (10 mg/kg) and at high doses (35 mg/kg). Specifically, we analyzed the expression of Immediate Early Genes Homer1a, Ania3, its splicing variant, Arc, Zif-268 and c-fos that had been demonstrated to be involved in synaptic plasticity and to be affected by antipsychotic treatment. In agreement with our previous work (Ambesi-Impiombato et al.,2007; Tomasetti et al.,2007), we showed that haloperidol acute administration significantly induced the expression of Immediate Early Genes Homer1a, Ania-3, its splicing variant, Arc, Zif-268, compared to vehicle, in
lateral striatal subregions. However, the extent of *Immediate Early Genes* induction by haloperidol in medial regions was lesser than in lateral regions, never reaching statistical significance. In the opposite of haloperidol, amisulpride at high dose (35mg/kg) induced a significant expression of the *Immediate Early Genes Homer1a, Ania-3, Arc* and *Zif-268* that was restricted to medial regions of caudate-putamen, compared to vehicle. Indeed, a statistically significant induction of *c-fos* and *Zif-268* transcripts expression, but not of the other genes, was highlighted by amisulpride 10mg/kg in medial striatal regions, compared to vehicle. Altogether, results suggest that haloperidol and amisulpride trigger different patterns of *Immediate Early Genes* expression in the striatum, with a prevailing effect of haloperidol in the lateral subregions and a more definite dose-dependent impact of amisulpride in the medial subregions. In the cortex, the expression of *Immediate Early Genes* was poorly affected by haloperidol or amisulpride administration. Indeed, *Homer1a* expression was induced by haloperidol compared to vehicle in the ACC, whereas amisulpride at low doses reduced the expression of the gene in the MC. *Arc* expression was significantly induced by haloperidol compared to vehicle in the I. These results confirm previous findings in which haloperidol acute administration demonstrated a weak impact on cortical gene expression. Moreover, amisulpride, like haloperidol, showed a low effect on *Immediate Early Genes* expression in the cortex, probably due to its poor impact on serotonergic neurotransmission. Topographic analysis confirmed the impact of antipsychotic treatment on *Immediate Early Genes* distribution along cortical and subcortical regions. Resembling the results of quantitative analysis of gene expression, haloperidol and amisulpride differently affected radioactive signal distribution in the cortex and in the striatum. Specifically, haloperidol administration induced a signal peak in the lateral subregions of the striatum, consistently with prominent action of this compound on motor performances. On the contrary, amisulpride administration induced a signal peak in the medial striatal subregions, that was highly pronounced for the 35 mg/kg dose. Prominent medial induction of *Immediate Early Genes* expression by amisulpride may be consistent with its proposed action on limbic structures and with its lower propensity to induce motor side effects, compared to haloperidol. In the cortex, antipsychotic
treatment elicited poor changes in the topographic distribution curves of *Immediate Early Genes* analyzed. Given the distinct and specific impact on *Immediate Early Genes* expression by amisulpride and haloperidol in striatal regions, as observed by both quantitative and topographic analyses, we hypothesized that the two antipsychotics may exert prevailing action in two macro-regions of interest, corresponding to discrete medial and lateral regions of striatum with peculiar anatomic and functional connections (Steiner and Gerfen, 1993; Willuhn et al., 2003) that were different from the ROIs we originally used. Therefore, we carried out adjunctive quantitative analysis of gene expression by haloperidol and the two doses of amisulpride in these macro-areas of the striatum. So, we observed that 1) haloperidol induced *Immediate Early Genes* expression prominently in lateral striatal regions, which are targeted by cortical areas that control motor and visuo-spatial behaviors (Featherstone and McDonald, 2004); 2) amisulpride, at high dose, mainly triggered *Immediate Early Genes* expression in medial aspects of striatum, which have been implicated in reward, motivation, and goal-directed learning (Graybiel, 2008).
PICTURE 2. Model of the peculiar pattern of gene induction in striatum by amisulpride, at both 10 and 35 mg/kg doses, compared to haloperidol. The selected area in each autoradiographic image has been magnified on the right. No other adjustments have been carried out on the images. On the right side of the picture we have provided a diagram with the adjunctive ROIs (Lst, lateral striatum, and Mst, medial striatum) that have been considered for the further quantitative evaluation.
RESULTS

In order to better recapitulate the results of the study, in a first step I’ll present, for each gene: 1) quantitative analysis of genes expression, first in the striatum and then in the cortex. For each gene, I’ll consider separately groups that had received vehicle as first treatment or haloperidol as first treatment, focusing on differences between groups (One-Way ANOVA); 2) a comparison between gene expression induced by treatment preceded by vehicle as first treatment, with gene expression induced by the same treatment preceded by haloperidol as first treatment (Student’s t); 3) treatment effect, first treatment effect or interaction between first treatment and second treatment effect, assessed by Two-Way ANOVA. In a second step, I’ll present 4) topographic analysis in the cortex and in the striatum showing the differences in genes expression throughout cortical and subcortical regions among groups; 5) Homer1a/Homer1b ratio.

HOMER1A

STRIATUM. In the experimental groups treated by vehicle as first treatment, a statistically significant Homer1a induction were found in all striatal subregions, with the exception of Sab (ANOVA: DM, p<.0001, df=2,9, F=36.3; DL, p=.0008, df=2,9, F=17.6; VM, p=.0008, df=2,9, F=17.4; VL, p=.022, df=2,9, F=5.9; CAb, p=.016, df=2,9, F=6.6; SAb, p>.05, df=2,9).

Veh/ams significantly induced Homer1a compared to veh/veh in DM, DL, VM e Cab (Tukey’s: p<.0001; p=.003; p=.0006; p=.013, respectively). Homer1a mRNA signal was enhanced in a statistically significant manner by veh/hal compared to veh/veh in DM, DL, VM, VL (Tukey’s: p=.0002; p=.0009; p=.029; p=.023, respectively). No significant differences were found between veh/ams and veh/hal in any striatal subregions (Tukey’s: p>.05).

In the experimental groups treated by haloperidol as first treatment, statistically significant differences were found in the DM, DL, and VL (ANOVA: DM, p=.041, df=2,10, F=4.4; DL, p=.002, df=2,10, F=11.5; VM, p>.05, df=2,10; VL, p=.012,
Hal/ams did not induce Homer1a expression compared to hal/veh in all caudate-putamen subregions and nucleus accumbens (Tukey’s: p > .05). On the contrary, Homer1a expression was significantly induced by hal/hal compared to hal/veh in DM, DL, VL (Tukey’s: p = .043; p = .002; p = .011, respectively), and compared to hal/ams in the DL and VL (Tukey’s: p = .007; p = .038).

Comparing groups with same treatment, but different first treatment, we observed that Homer1a expression was statistically significant induced by veh/ams compared to hal/ams in the DL, VM, and VL (Fig. 4B; Student’s t; DM, p > .05, df = 1.7; DL < .0001, df = 1.7, F = 80.3; VM, p = .031, df = 1.7, F = 7.2; VL, p = .044, df = 1.7, F = 5.9; CAb, p > .05, df = 1.7; SAb, p > .05, df = 1.7). No significant differences were found between hal/veh and veh/veh, and between hal/hal and veh/hal in the striatum (Student’s t: p > .05, df = 1.7 in all regions). Thus, in the striatum haloperidol first treatment prevented amisulpride to induce Homer1a, whereas expression of Homer1a by haloperidol was not significantly affected by first treatment.

Two-way ANOVA revealed that Homer1a expression was not significantly affected by first treatment in the striatum (first treatment effect: DM: p > 0.05, F = 1.05, df = 1.19; DL: p > 0.05, F = 1.65, df = 1.19; VL: p > 0.05, F = 0.01, df = 1.19; VM p > 0.05, F = 0.33, df = 1.19; CAb: p > 0.05, F = 0.67, df = 1.19; SAb: p > 0.05, F = 0.04, df = 1.19). Gene expression was significantly affected by treatment in all caudate putamen subregions and in CAb (second treatment effect: DM: p < 0.0001, F = 16.64, df = 2.19; DL: p < 0.0001, F = 24.81, df = 2.19; VL: p < 0.001, F = 11.96, df = 2.19; VM: p < 0.01, F = 9.73, df = 2.19; CAb: p < 0.01, F = 3.53, df = 2.19; SAb: p > 0.05, F = 3.09, df = 2.19). In DL, Homer1a expression was also affected by the combination of first and second treatment (first treatment*second treatment effect: DM: p > 0.05, F = 1.17, df = 2.19; DL: p < 0.0001, F = 24.81, df = 2.19; VL: p < 0.001, F = 11.96, df = 2.19; CAb: p < 0.05, F = 3.53, df = 2.19; SAb: p > 0.05, F = 3.01, df = 2.19).

CORTEX. In the experimental groups treated by vehicle as first treatment, statistically significant differences in Homer1a expression were observed in all cortical regions (ANOVA, ACC: p = .001, df = 2.9, F = 15.9; MAC: p = .002, df = 2.9, F = 11.9; MC: p = .003,
df=2,9, F=11.3; SS: p=.005, df=2,9, F=9.5; IC: p<.0001, df=2,9, F=39.5). In all cortical subregions considered, veh/ams induced Homer1a expression compared to veh/veh (Tukey’s: ACC, p=.0008; MAC, p=.002; MC, p=.002; SS: p=.005; IC: p<.0001), and by veh/hal compared to veh/veh in the MC (Tukey’s, p=.032). Homer1a expression was also significantly induced by veh/ams compared to veh/hal in the ACC (Tukey’s, p=.035), SS (Tukey’s, p=.041), and IC (Tukey’s, p=.0001). In the experimental groups treated by haloperidol as first treatment, no significant differences among groups were found (ANOVA, ACC: p>.05, df=2,10; MAC: p>.05, df=2,10; MC: p>.05, df=2,10; SS: p>.05, df=2,10; IC: p>.05, df=2,10).

Comparing groups with same treatment, but different first treatment, had revealed that veh/ams significantly induced Homer1a expression compared to hal/ams in all cortical regions (Student’s t: ACC, p=.0007, df=1,7, F=32.8; MAC, p=.0002, df=1,7, F=46.9; MC: p=.004, df=1,7, F=16.7; SS, p=.007, df=1,7, F=13.5; IC, p=.004, df=1,7, F=17.6).

No significant differences were found between hal/veh and veh/veh, and between hal/hal and veh/hal in the striatum (Student’s t: p>.05, df=1,7 in all regions).

Thus, in the cortex, such as in the striatum, haloperidol first treatment prevented amisulpride to induce Homer1a; on the contrary Homer1a expression was limitedly affected by haloperidol.

In the cortex we highlighted a significant interaction effect between first treatment and second treatment. Specifically amisulpride treatment affected Homer1a expression following vehicle first treatment, whereas did not when preceded by haloperidol treatment (first treatment*second treatment effect: ACC: p<0.05, F=5.38, df=1,19; MAC: p<0.05, F=8.91, df=1,19; MC: p<0.05, F=8.59, df=1,19; SS: p<0.05, F=6.24, df,19; IC: p<0.05, F=5.66, df=1,19). No first or second treatment effect were detected in all subregions considered (first treatment effect: ACC: p>0.05, F=0.65, df=1,19; MAC: p>0.05, F=2.17, df=1,19; MC: p>0.05, F=2.47, df=1,19; SS: p>0.05, F=0.84, df=1,19; IC: p>0.05, F=0.17, df=1,19; second treatment effect: ACC: p>0.05, F=3.22, df=1,19; MAC: p>0.05, F=0.91, df=1,19; MC: p>0.05, F=1.12, df=1,19; SS: p>0.05, F=2.11, df=1,19; IC=p>0.05, F=5.66, df=1,19).
PICTURE 3.  A. Homer1a mRNA expression (in relative dpm means ± standard error means) and in cortical (above) and striatal (below) regions. * = Tukey’s post-hoc test, p<.05, vs. vehicle. ** = Tukey’s post-hoc test, p<.05, vs. all treatments. B. Distribution of Homer1a expression throughout cortical (above) and striatal (below) in rats receiving vehicle, haloperidol, or amisulpride as second treatment. *= Student’s t test, p<.05.
**Arc**

**Striatum.** In the experimental groups treated by vehicle as first treatment, significant differences were found in the DM, DL, and VL (ANOVA: DM, p=.015, df=2,9, F=6.9; DL, p=.003, df=2,9, F=11.5; VM, p>.05, df=2,9; VL, p=.004, df=2,9, F=10.7; CAb, p>.05, df=2,9; SAb, p>.05, df=2,9). Arc expression was not significantly increased by veh/ams in all striatal subregions (Tukey’s: p>.05). In DM, DL e VL, veh/hal induced Arc expression compared to veh/veh (Tukey’s: DM, p=.012; DL, p=.002; VL, p=.003).

No statistically significant differences were detected in the striatum between veh/hal and veh/ams (Tukey’s: p>.05).

In the experimental groups treated by haloperidol as first treatment, statistically significant differences in Arc expression were found in all caudate-putamen regions (ANOVA: DM, p=.014, df=2,8, F=7.5; DL, p=.002, df=2,8, F=14.8; VM, p=.001, df=2,8, F=15.1; VL, p=.0007, df=2,8, F=20.7), but not in the nucleus accumbens (ANOVA: CAb, p>.05, df=2,8; SAb, p>.05, df=2,8).

Arc expression was significantly induced by hal/hal and hal/ams compared to hal/veh in the medial caudate-putamen (Tukey’s: DM, p=.025 and p=.024, respectively; VM, p=.011 and p=.002, respectively), and by hal/hal compared to both hal/veh (Tukey’s: DL, p=.001; VL, p=.0006) and hal/ams (Tukey’s: DL, p=.013; VL, p=.005) in the lateral caudate-putamen.

Comparing groups with the same treatment, but different first treatment, we had found that hal/ams significantly induced specifically Arc expression compared to veh/ams in the VM (Student’s t: p=.036, df=1.5, F=7.9). No other significant differences were observed between veh/ams and hal/ams (Student’s t: p>.05, df=1.5 in all subregions with the exclusion of VM), between veh/hal and hal/hal (Student’s t: p>.05, df=1.6 in all subregions), and between veh/veh and hal/veh (Student’s t: p>.05, df=1.6 in all subregions).

Indeed, the pattern of Arc expression by antipsychotics in striatum was not substantially affected by the type of first treatment, vehicle or haloperidol.
In all subregions of striatum no significant intergroups differences in *Arc* expression were observed depending on first chronic treatment (first treatment effect: DM: p>0.05, F=1.05, df=1,17; DL: p>0.05, F=1.65, df=1,17; VL: p>0.05, F=11.96, df=1,17; CAb: p>0.05, F=0.67, df=1,17; SAb: p>0.05, F=0.04, df=1,17).

In the striatum second chronic treatment significantly impacted *Arc* expression. Specifically in lateral striatal subregions haloperidol second treatment induced *Arc* expression compared to amisulpride and control, whereas in medial subregions only compared to control (second treatment effect: DM: p<0.001, F=16.64, df=2,17; DL: p<0.0001, F=24.81, df=2,17; VL: p<0.001, F=11.95, df=2,17; VM: p<0.01, F=9.73, df=2,17; CAb: p<0.05, F=3.53, df=2,17; SAb: p>0.05, F=3.09, df=2,17).

No interaction effect between first and second treatment was observed impacting *Arc* expression (first treatment*second treatment effect: DM: p>0.05, F=1.04, df=2,17; DL: p>0.05, F=1.94, df=2,17; VL: p>0.05, F=1.49, df=2,17; VM: p>0.05, F=2.24, df=2,17; CAb: p>0.05, F=0.78, df=2,17; SAb: p>0.05, F=0.71, df=2,17).

**Cortex.** In both first treatment groups (haloperidol or vehicle), no significant intergroup differences in *Arc* expression were found in cortical regions (ANOVA: p>.05, df=2.9 in all cortical regions in groups treated by vehicle as the first treatment; p>.05, df=2.8 in all cortical regions in groups treated by haloperidol as the first treatment). No significant differences in *Arc* expression were observed comparing groups with same treatment, but different pretreatment, between veh/ams and hal/ams (Student’s t: p>.05, df=1.5 in all regions), between veh/hal and hal/hal (Student’s t: p>.05, df=1.6 in all regions), and between veh/veh and hal/veh (Student’s t: p>.05, df=1.6 in all regions).

Therefore, *Arc* expression in the cortex was not affected by antipsychotics after either paradigms of first treatment. No changes in *Arc* expression was observed when second treatment was preceded by haloperidol or vehicle first treatment (first treatment effect: ACC: p>0.05, F=0.08, df=1,17, MAC: p>0.05, F=0.74, df=1,17; MC: p>0.05, F=1.82, df=1,17; SS: p>0.05, F=3.62, df=1,17; IC: p>0.05, F=0.58, df=1,17; second treatment effect: ACC: p>0.05, F=0.41, df=2,17; MAC: p>0.05, F=0.84, df=2,17; MC: p>0.05, F=0.51, df=2,17; SS: p>0.05, F=1.15, df=2,17; IC: p>0.05, F=2.52, df=2,17). Indeed no
interaction effect was detected between first and second treatment in any cortical subregions considered (first treatment*second treatment: ACC: p>0.05, F=0.41, df=2,17; MAC: p>0.05, F=0.03, df=2,17; MC: p>0.05, F=0.51, df=2,17; SS: p>0.05, F=1.11, df=2,17; IC: p>0.05, F=0.81, df=2,17).
PICTURE 4. A. *Arc* mRNA expression (in relative dpm means ± standard error means) in cortical (above) and striatal (below) regions. *=Tukey’s post-hoc test, p<.05, vs. vehicle. **=Tukey’s post-hoc test, p<.05, vs. all treatments. B. Distribution of *Arc* expression throughout cortical (above) and striatal (below) regions in rats receiving vehicle, haloperidol, or amisulpride as second treatment. *= Student’s t test, p<.05.
**HOMER1B**

**STRIATUM.** In the experimental groups treated by vehicle as first treatment, significant inter-group differences in Homer1b/c expression were found in the VL only (ANOVA: DM, p>.05, df=2.8; DL, p>.05, df=2.8; VM, p>.05, df=2.8; VL, p=.004, df=2.8, F=14.9; CAb, p>.05, df=2.8; SAb, p>.05, df=2.8). In this subregion, Homer1b/c expression by both veh/veh and veh/hal was significantly higher than that by veh/ams (Fig. 6A; Tukey’s: p=.035 and p=.004, respectively).

In the groups treated by haloperidol as first treatment, significant inter-group differences in Homer1b/c expression were found in all striatal subregions, with the exception of the VL (ANOVA: DM, p=.003, df=2.7, F=5.3; DL, p=.016, df=2.7, F=7.7; VM, p=.021, df=2.7, F=7.1; VL, p>.05, df=2.7; CAb, p=.014, df=2.7, F=8.2; SAb, p=.019, df=2.7, F=7.3). Homer1b/c expression was significantly increased by hal/ams compared to hal/hal and hal/veh in DL (Tukey’s: p=.041 and p=.018, respectively), VM (Tukey’s: p=.033 and p=.031, respectively), and CAb (Tukey’s: p=.043 and p=.014, respectively), and compared to hal/veh only in the DM (Tukey’s: p=.037) and SAb (Tukey’s: p=.018). No significant differences between hal/hal and hal/veh were found.

Comparing groups with same treatment, but different first treatment, we observed that Homer1b/c expression was significantly higher in the hal/ams group compared to veh/ams in all striatal subregions (Student’s t: DM, p=.011, df=1.6, F=20.9; DL, p=.006, df=1.6, F=28.2; VM, p=.002, df=1.6, F=46.3; VL, p=.003, df=1.6, F=38.8; CAb, p=.004, df=1.6, F=32.7; SAb, p=.013, df=1.6, F=17.5). No statistically significant differences were detected between veh/hal and hal/hal (Student’s t: p>.05, df=1.6 in all subregions), and between veh/veh and hal/veh (Student’s t: p>.05, df=1.6 in all subregions).

Thus, Homer1b/c expression was mainly increased by amisulpride compared to haloperidol. Amisulpride-mediated increase was significantly higher after haloperidol first treatment than after vehicle first treatment.

Two-way ANOVA revealed a first treatment effect on the Homer1b expression in DL and VM (first treatment effect: DM: P>0.05, F=3.29, df=1.13; DL: p<0.05, F=5.19, df=1.13; VL: p<0.05, F=3.46, df=1.13; VM: p<0.05, F=7.11, df=1.13; CAb: p<0.05,
F=5.72, df=1,13; SAb: p>0.05, F=4.44, df=1,13). Specifically in DL and in VM
haloperidol first treatment significantly affected \textit{Homer1b} expression compared to
vehicle first treatment. No second treatment effect on \textit{Homer1b} expression was detected
in the striatum (second treatment effect: DM: P>0.05, F=1.04, df=2,13; DL: p>0.05,
F=3.11, df=2,13; VL: p>0.05, F=1.29, df=2,13; VM: p>0.05, F=1.91, df=2,13; CAb:
p<0.05, F=6.01, df=2,13; SAb: p>0.05, F=3.63, df=2,13). In DL, in ventral striatal
subregions and in nucleus accumbens Two-way ANOVA showed an interaction effect
between first and second chronic treatment: amisulpride enhanced \textit{Homer1b} expression
when preceded by haloperidol as first treatment, whereas following vehicle first
treatment did not (first treatment*second treatment effect: DM: P>0.05, F=1.04,
df=2,13; DL: p<0.05, F=9.54, df=2,13; VL: p<0.05, F=9.24, df=2,13; VM: p<0.05,
F=10.32, df=2,13; CAb: p<0.05, F=5.72, df=2,13; SAb: p<0.05, F=4.15, df=2,13).
**Homer1b/c**

**A**

A. Homer1b/c mRNA expression (in relative dpm means ± standard error means) and in cortical (above) and striatal (below) regions. * = Tukey’s post-hoc test, p<.05, vs. vehicle. ** = Tukey’s post-hoc test, p<.05, vs. all treatments.

**B**

B. Distribution of Homer1b/c expression throughout cortical (above) and striatal (below) regions in rats receiving vehicle, haloperidol or amisulpride as second treatment. *= Student’s t test, p<.05.

**PICTURE 5.**
**PSD-95**

Striatum. No significant inter-group differences in **PSD-95** expression were found in groups treated by vehicle as first treatment (ANOVA: p>.05, df=2,9 in all striatal regions) or haloperidol as first treatment (ANOVA: p>.05, df=2,9 in all cortical and in all striatal regions).

Comparing groups with same second treatment and different first treatment, we found that **PSD-95** expression was significantly higher by veh/veh compared to hal/veh in the DL (Student’s t: p=.038, df=1,6, F=7.8), VM (Student’s t: p=.036, df=1,6, F=7.9), and VL (Student’s t: p=.031, df=1,6, F=8.9). No other significant differences were found between these treatment groups. No significant differences between veh/ams and hal/ams were observed (Student’s t: p>.05, df=1,6 in all regions).

Therefore, **PSD-95** expression may be reduced by chronic treatment by haloperidol, while it may be unaffected by amisulpride. Haloperidol or vehicle first chronic treatment did not impact **Psd-95** mRNA expression level in the striatum (first treatment effect: DM: P>0.05, F=1.21, df=1,18; DL: p>0.05, F=0.14, df=1,18; VL: p>0.05, F=2.21, df=1,18; VM: p>0.05, F=2.69, df=1,18; CAb: p>0.05, F=0.93, df=1.18; SAb: p>0.05, F=0.11, df=1,18; second treatment effect: DM: P>0.05, F=0.34, df=2,18; DL: p>0.05, F=0.53, df=2,18; VL: p>0.05, F=1.59, df=2,18; VM: p>0.05, F=0.63, df=2,18; CAb: p>0.05, F=0.04, df=2,18; SAb: p>0.05, F=0.29, df=2,18). No interaction effect on **PSD-95** expression was observed between first and second treatment (first treatment*second treatment effect: DM: P>0.05, F=2.03, df=2,18; DL: p>0.05, F=2.83, df=2,18; VL: p>0.05, F=2.84, df=2,18; VM: p>0.05, F=2.36, df=2,18; CAb: p>0.05, F=0.31, df=2,18; SAb: p>0.05, F=0.45, df=2,18).

Cortex. No significant differences among experimental groups in **PSD-95** expression were found both in groups treated by vehicle as first treatment (ANOVA: p>.05, df=2,9 in all cortical regions) and in those treated by haloperidol as first treatment (ANOVA: p>.05, df=2,9 in all cortical regions).

Comparing groups treated by same second treatment, but different first treatment, we found that **PSD-95** expression was significantly higher by veh/hal compared to hal/hal in the MAC (Student’s t: p=.006, df=1,7, F=15.1), MC (Student’s t: p=.028, df=1,7,
F=7.5), and IC (Student’s t: p=.025, df=1,7, F=7.9), and by veh/veh compared to hal/veh in the DL (Student’s t: p=.038, df=1,6, F=7.8), VM (Student’s t: p=.036, df=1,6, F=7.9), and VL (Student’s t: p=.031, df=1,6, F=8.9). No other significant differences were found between these treatment groups. No significant differences between veh/ams and hal/ams were observed (Student’s t: p>.05, df=1,6 in all regions).

Also in cortical subregions, PSD-95 expression may be affected by chronic treatment with haloperidol, while by amisulpride did not. In the cortex PSD-95 expression level did not change after haloperidol or amisulpride treatment. Haloperidol chronic first administration did not impact PSD-95 expression, compared to vehicle first treatment. No statistically significant difference was observed between veh/ams and hal/ams or between veh/hal and hal/hal in all cortical subregions (ACC: p>0.05, F=0.62, df=5,18; MAC: p>0.05, F=1.18, df=5,18; MC: p>0.05, F=0.81, df=5,18; SS: p>0.05, F=0.83, df=5,18; IC: p>0.05, F=2.08, df=5,18).

No first or second treatment effect on PSD-95 expression was found (first treatment effect: ACC: P>0.05, F=0.67, df=1,18; MAC: p>0.05, F=3.17, df=1,18; MC: p>0.05, F=1.53, df=1,18; SS: p>0.05, F=1.84, df=1,18; IC: p>0.05, F=1.46, df=1,18; second treatment effect: ACC: P>0.05, F=0.26, df=2,18; MAC: p>0.05, F=0.37, df=2,18; MC: p>0.05, F=0.64, df=2,18; SS: p>0.05, F=0.84, df=2,18; IC: p>0.05, F=2.46, df=2,18).

Moreover two-way ANOVA did not revealed any first and second treatment interaction effect on gene expression in the cortex (first treatment*second treatment effect: ACC: P>0.05, F=0.97, df=2,18; MAC: p>0.05, F=0.86, df=2,18; MC: p>0.05, F=0.65, df=2,18; SS: p>0.05, F=0.35, df=2,18; IC: p<0.05, F=2.43, df=2,18).
PICTURE 6. A. *Psd*-95 mRNA expression (in relative dpm means ± standard error means) and in cortical (above) and striatal (below) regions. B. Distribution of *Psd*-95 expression throughout cortical (above) and striatal (below) regions in rats receiving vehicle, haloperidol or amisulpride as second treatment. *= Student’s t test, p<.05.
Topographical analysis

Striatum. In topographical analysis we have described spatial distribution of gene expression along striatal and cortical subregions following different treatment paradigm. In each analysis we compared two experimental groups treated with the same second treatment but different first treatment, in order to individuate where haloperidol first treatment impacts topographic distribution of genes induced by typical and atypical antipsychotic.

In antipsychotic-naïve groups, *Homer1a* mRNA signal distribution by vehicle and amisulpride was homogeneous (Kruskal-Wallis: *p*>.05, df=5), while treatment with haloperidol increased *Homer1a* expression in the caudate-putamen regions (Kruskal-Wallis: *p*<.0001, df=5, $\chi^2$: 30.5) compared to the nucleus accumbens. In the groups treated with haloperidol as first treatment, signal distribution by vehicle and haloperidol was homogeneous throughout striatal regions (Kruskal-Wallis: *p*>.05, df=5). On the contrary, treatment by amisulpride increased *Homer1a* expression in the medial striatum (Kruskal-Wallis, *p*=.0004, df=5, $\chi^2$: 22.8) compared to the lateral striatum and the nucleus accumbens.

In antipsychotic-naïve rats, *Arc* mRNA signal was higher in the caudate-putamen regions compared to the nucleus accumbens regions by vehicle and haloperidol (Kruskal-Wallis: *p*<.0001, df=5, $\chi^2$: 35.6 and *p*<.0001, df=5, $\chi^2$: 39.7, respectively), while signal was homogeneously distributed throughout striatal regions in rats treated by amisulpride (Kruskal-Wallis: *p*>.05, df=5, $\chi^2$: 6.7). In antipsychotic-treated rats, *Arc* mRNA signal distribution throughout striatal regions was homogeneous in rats treated by vehicle (Kruskal-Wallis: *p*>.05, df=5, $\chi^2$: 9.1). *Arc* signal was significantly higher in the caudate-putamen compared to the nucleus accumbens regions in haloperidol and amisulpride-treated rats (Kruskal-Wallis: *p*=.0005, df=5, $\chi^2$: 22.1 and *p*<.0001, df=5, $\chi^2$: 32.7, respectively).

In antipsychotic-naïve rats, *Homer1b/c* mRNA signal was significantly higher in the lateral caudate-putamen compared to the nucleus accumbens regions after vehicle treatment (Kruskal-Wallis: *p*=.01, df=5, $\chi^2$: 14.1) and in the VL compared to the nucleus
accumbens regions after haloperidol treatment (Kruskal-Wallis: $p=.02$, $df=5$, $\chi^2$: 12.6). 

*Homer1b/c* signal was homogeneously distributed throughout striatal regions after amisulpride treatment (Kruskal-Wallis: $p>.05$, $df=5$, $\chi^2$: 4.4). In rats treated by haloperidol as the first treatment, *Homer1b/c* mRNA signal was significantly higher in DL, VM, and VL compared to the nucleus accumbens regions in the rats treated by vehicle (Kruskal-Wallis: $p=.001$, $df=5$, $\chi^2$: 20.1). However, signal distribution was homogeneous throughout striatal regions in haloperidol and amisulpride-treated rats (Kruskal-Wallis: $p>.05$, $df=5$, $\chi^2$: 6.2, and $p>.05$, $df=5$, $\chi^2$: 5.9).

*PSD-95* mRNA signal distribution was homogeneous throughout striatal regions in all treatment groups (Kruskal-Wallis, veh/veh: $p>.05$, $df=5$, $\chi^2$: 4.1; veh/ams: $p>.05$, $df=5$, $\chi^2$: 4.3; hal/veh: $p>.05$, $df=5$, $\chi^2$: 0.9; hal/ams: $p>.05$, $df=5$, $\chi^2$: 0.3).

**CORTEX**. In the groups treated by vehicle as first treatment, *Homer1a* mRNA signal distribution by vehicle and amisulpride was homogeneous (Kruskal-Wallis: $p>.05$, $df=4$, $\chi^2$: 2.5, and $p>.05$, $df=4$, $\chi^2$: 4.4, respectively), while treatment by haloperidol increased *Homer1a* expression in the MAC and MC compared to the ACC (Kruskal-Wallis: $p=.041$, $df=4$, $\chi^2$: 9.9). In the groups treated by haloperidol as the first treatment, signal distribution by all treatments was homogeneous throughout cortical regions (Kruskal-Wallis: $p>.05$, $df=4$).

Signal of *Arc, Homer1b/c*, and *PSD-95* mRNA was homogeneously distributed throughout cortical regions by all treatments groups.

All together, data on topographic distribution confirm the difference in molecular effects of antipsychotics when given in previously-treated subjects compared to antipsychotic-naïve ones.
PICTURE 7. Illustrative autoradiograms of *Homer1a*, *Arc*, *Homer1b/c*, and *PSD-95* mRNA expression in the cortex and the striatum.
Homer1a/Homer1b/c relative ratio

In the groups treated by vehicle as the first treatment the relative ratio was shifted toward Homer1a expression by haloperidol in the caudate-putamen, the MAC, and the MC. Amisulpride shifted the ratio toward Homer1a expression in all cortical and striatal regions.

In the groups treated by haloperidol as the first treatment, haloperidol shifted the ratio toward Homer1a expression in striatum, while the genes’ relative expression was about equal in the cortex. Amisulpride treatment resulted in approximately equivalent relative gene expression or in some cortical regions shifted the ratio toward Homer1b/c expression.
PICTURE 8. Homer1a/Homer1b ratio.
<table>
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<tr>
<th>Homer1a</th>
<th>DL</th>
<th>DM</th>
<th>VM</th>
<th>VL</th>
<th>CAb</th>
<th>SAb</th>
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<td>$F_{1,19}=1.651; P=0.2143$</td>
<td>$F_{1,19}=1.052; P=0.2288$</td>
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<td>SECOND TREATMENT</td>
<td>$F_{2,19}=24.806; P=0.0001$</td>
<td>$F_{2,19}=16.643; P=0.0001$</td>
<td>$F_{2,19}=9.732; P=0.0012$</td>
<td>$F_{2,19}=11.958; P=0.0004$</td>
<td>$F_{2,19}=3.530; P=0.0497$</td>
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<td>FIRST*SECOND TREATMENT</td>
<td>$F_{2,19}=4.237; P=0.0301$</td>
<td>$F_{2,19}=1.172; P=0.3310$</td>
<td>$F_{2,19}=2.181; P=0.1404$</td>
<td>$F_{2,19}=1.683; P=0.2123$</td>
<td>$F_{2,19}=0.450; P=0.2593$</td>
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<td>SECOND TREATMENT</td>
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<tr>
<td>FIRST*SECOND TREATMENT</td>
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</tbody>
</table>

**TABLE 1.** In this table two-way ANOVA results in the striatum are recapitulated. In red we have indicated statistically significant data.
**TABLE 2.** In this table two-way ANOVA results in the cortex are recapitulated. In red we have indicated statistically significant data.

<table>
<thead>
<tr>
<th>Homer1a</th>
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<th>MAC</th>
<th>MC</th>
<th>SS</th>
<th>IC</th>
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<td>FIRST TREATMENT</td>
<td>F₁₁ = 0.653; 6</td>
<td>P = 0.4289</td>
<td>F₁₁ = 2.168;</td>
<td>P = 0.1573</td>
<td>F₁₁ = 2.468;</td>
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<td>SECOND TREATMENT</td>
<td>F₁₁ = 3.216;</td>
<td>P = 0.0625</td>
<td>F₁₁ = 0.905;</td>
<td>P = 0.4213</td>
<td>F₁₁ = 1.122;</td>
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<td>FIRST*SECOND TREATMENT</td>
<td>F₁₁ = 5.578;</td>
<td>P = 0.0141</td>
<td>F₁₁ = 8.908;</td>
<td>P = 0.0019</td>
<td>F₁₁ = 8.593;</td>
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**Arc**

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<th>Homer1b</th>
<th>Psd.95</th>
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<td>F₁₁ = 0.403;</td>
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<td>F₁₁ = 2.446;</td>
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**Psd.95**

<table>
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<td>FIRST TREATMENT</td>
<td>F₁₁ = 0.667;</td>
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<td>SECOND TREATMENT</td>
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<tr>
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DISCUSSION

The present study is the first to explore at molecular level the common clinical treatment paradigm of “switching” between antipsychotics. Specifically, using gene expression as preclinical marker of antipsychotic action, we investigated the induction of Postsynaptic Density *Immediate Early Genes* and the topography of brain activation induced by an atypical antipsychotic treatment after prolonged treatment with a typical one. We focused on genes localized at crossroads of dopaminergic and glutamatergic system, related to psychosis, synaptic plasticity and demonstrated to be modulated by antipsychotics. We chose amisulpride as post-switch agent based on the evidence that, despite its high affinity for the D₂R, comparable to haloperidol, showed low liability for extrapyramidal side effects, a clinical features of atypical antipsychotics. The results of the study are reported below, expounded point by point:

1) In the acute paradigm, haloperidol induced a branded gene expression throughout the striatum, with a preferential impact on the lateral subregions; by contrast, the effect of amisulpride on gene expression was preferentially restricted to medial striatum (de Bartolomeis et al., 2013). So, modeling gene expression in animal model, haloperidol and amisulpride induced a peculiar and distinct pattern of *Immediate Early Gene* expression. It has been highlighted that the extent of Homer1a induction by antipsychotics may be related to the degree of D₂R blockade of each drug (Iasevoli et al., 2009). So, it could be deduced that the different pattern of Homer1a induction by haloperidol or amisulpride may be due to their distinct impact on dopamine receptors, with haloperidol inducing a stronger postsynaptic D₂R blockade than amisulpride, whereas amisulpride preferentially act on presynaptic D₂/D₃R at low doses and on both pre-and postsynaptic D₂/D₃R at higher doses. Moreover amisulpride seems to preferentially act on D₂R/D₃R located in limbic and associative striatum (Stone et al., 2005). Indeed, it has been observed that induction of Homer1a may depend on the activation of NMDA receptors.
and/or D$_2$R-mediated signaling (Yamada et al., 2007; Sato et al., 2001). So, amisulpride and haloperidol may indirectly trigger Homer1a expression by blockade of D$_2$Rs and concomitant activation of D$_1$Rs by residual synaptic dopamine, which may in turn modulate glutamatergic neurotransmission at PSD level, regulating NMDA receptor activation (Borroto-Escuela et al., 2011). Indeed, the effect of haloperidol and amisulpride on gene expression seems to be restricted on striatal regions, in agreement with previous studies highlighting 5-HT$_{2A}$ receptors high affinity for the antipsychotics that specifically impact gene expression in the cortex, such as aripiprazole, ziprasidone and clozapine (Iasevoli et al., 2011a; Mo et al., 2005; Tomasetti et al., 2007). The peculiar pattern of Immediate Early Genes expression of amisulpride suggest a selectivity of this compound for limbic forebrain regions. The “molecular profile” of amisulpride may explain its “atypical” atypicality and provide a useful tool to address the molecular processes underlying clinical efficacy of atypical antipsychotics.

2) The specific pattern of Immediate Early Genes expression induced by haloperidol and amisulpride held on after 15 days of treatment, in a chronic paradigm, when antipsychotic administration was preceded by vehicle first treatment. This finding are consistent with our previous observation that demonstrated a lacking sensitization of Homer1a after chronic treatment and suggests that both antipsychotics activate Homer-mediated postsynaptic glutamatergic signaling even after prolonged administration (Iasevoli et al., 2011). Notably, in a precedent study, we found no significant Homer1a induction by amisulpride in the cortex in the acute paradigm (de Bartolomeis et al., 2013). Therefore, amisulpride appears to trigger cortical Homer1a expression after a prolonged rather than an acute treatment. This feature may indicate the propensity of amisulpride to progressively activate glutamatergic signaling, and possibly neuronal depolarization, in the cortex during sustained treatments. It has been proposed that discrete glutamatergic activation may revert hypofrontality and decreased prefrontal activation, supposed in schizophrenia (Liemburg et al., 2012). Our results may partially explain the relative efficacy of amisulpride on negative symptoms (Arango et al., 2013). The impact of chronic amisulpride administration on cortical
glutamatergic signaling may depend on the action on D₃ receptors. It has been observed that selective antagonists at D₃ receptors increase extracellular levels of dopamine in the rat prefrontal cortex (Lacroix et al., 2003), most presumably by acting on inhibitory autoreceptors located on dopamine neurons of the ventral tegmental area. In turn, increased cortical dopamine may enhance NMDA receptor-mediated response via activation of D₁ receptors (Tseng et al., 2004).

3) In the switching group, where amisulpride treatment is preceded by haloperidol first treatment, haloperidol first treatment prevented amisulpride to induce Homer1a. Moreover, a prolonged administration of D₂ strong blocker also affected the other Immediate Early Genes and constitutive gene expression. So, in animal model, the sequential antipsychotics administration affects brain transcripts in a significantly different manner compared to antipsychotic-naïve animals. One possible explanation for this finding is that chronic haloperidol treatment may lead to several molecular adaptations in dopaminergic and glutamatergic signaling: D₂R up-regulation (Varela et al., 2014); switch to D₂R high-affinity status and/or in a condition of dopamine supersensitivity (Samaha et al., 2007; Seeman et al., 2007); altered AMPA or NMDA receptor trafficking (Fumagalli et al., 2008). Adaptations to prolonged haloperidol administration may partially change the molecular substrate where antipsychotic given subsequently acts, so giving rise to an unexpected downstream signaling. These results may suggest that the timing and the order by which antipsychotics are administered to subjects may substantially change their molecular action and the clinical outcome, such as efficacy and side effect. Indeed, this effect is more sharp in the striatum than in the cortex. These results suggest a differential activation of postsynaptic glutamatergic signaling induced by antipsychotic administration, based on previous treatments. A relevant translational consequence of these results is that amisulpride behaves differentially, at the molecular level, when given after a previous antipsychotic treatment or in antipsychotic-naïve subjects. On the contrary, Homer1a induction by haloperidol didn’t depend on treatment length, so maintaining its molecular effect on postsynaptic glutamatergic signaling when given for a prolonged period of time.
The functional consequences of the *Homer1a* induction by antipsychotic in acute, chronic and switching paradigm are unknown. It has been demonstrated that *Homer1a* induction is involved in neuronal downscaling, thus preventing excessive postsynaptic depolarization, in neuronal population where it is expressed, i.e. GABAergic medium sized spiny neurons (Tappe et al., 2006; Hu et al., 2010; Siddoway et al., 2014; Li et al., 2013). Intriguingly, medium spiny neurons are both intrastriatal interneurons and extrastriatal projecting fibers belonging to the output pathways implicated in motivated motor behavior (Kreitzer et al., 2008). Moreover, it has been demonstrated that striatal *Homer1a* expression may be implicated in motor control (Tappe et al., 2006). So, it could be hypothesized that the functional result of *Homer1a* induction by antipsychotics may be a dopamine-induced glutamatergic regulation of GABAergic postsynaptic neurons and may indicate a putative effect on motor behaviours. The distinct preferential distribution of *Immediate Early Genes* induction by haloperidol and amisulpride seems to follow the regional functional specificity and, under the translational point of view, may account for the differences in the clinical profile of the two drugs. Several studies in animal model, by means of lesions or pharmacological blockade of dopamine signaling in specific regions, have demonstrated that dopaminergic projections to the ventro- and dorso- medial striatum are related to reward, motivation and goal-directed learning (Graybiel et al., 2008), whereas lateral striatum is involved in motor control, such as motor performances and visuo-spatial learning and memory (Featherstone and McDonald et al., 2004). Moreover, recent studies showed a direct connection of medial striatum with central and lateral thalamic nucleus, which is in turn linked to prefrontal cortex and implicated in cognition and learning, as well as in neuropsychiatric disorders where cognitive tasks are impaired, such as schizophrenia (Shirvalkar et al., 2006; Smith et al., 2011).

Indeed, a recent finding have reported that haloperidol or amisulpride induced a coordinated gene expression in anatomically and functionally related cortico-striatal regions, even though with distinctive patterns. Haloperidol induced a coordinated corticostriatal gene expression prominently in regions related to motor performances (de Bartolomeis et al., 2013). These results are in line with the well-known propensity of
haloperidol to impact motor behaviors and to induce catalepsy at the dose used in this study (Wadenberg et al., 2001). Both doses of amisulpride triggered coordinated cortico-striatal gene expression in both motor and limbic regions, thereby resulting in a more balanced gene induction, compared to haloperidol (de Bartolomeis et al., 2013). Taken together, these results account to the propensity of haloperidol to induce motor side effect in a dose-dependent manner (Kapur et al., 2001) and with a scarce or absent induction of motor impairment maybe triggered by the doses of amisulpride analyzed (Natesan et al., 2008).

4) The effect of interaction between first and second treatment is more prominent in the dorsolateral caudate-putamen, compared to all other cortical and subcortical regions.

These results suggest that haloperidol first treatment affects gene expression induced by amisulpride second treatment in a greater extent in dorsolateral subregion, compared to all other striatal subregion analyzed. These finding are in line with a previous observation that demonstrated a more sustained effect of haloperidol on gene expression in dorsolateral striatal subregion (Iasevoli, 2010).

In switching group the effect of antipsychotic administration on the other genes analyzed are different from which induced by Homer1a. Arc expression was mainly induced by haloperidol, while limitedly by amisulpride. Indeed, haloperidol first treatment enhanced in a statistically significant manner Arc expression induced by amisulpride in medial striatal regions. Arc protein is involved in rapid cytoskeletal rearrangements secondary to a wide range of stimuli including to both glutamate and dopamine modulation (Bramham et al., 2008). So, also for Arc, the timing and the order in which antipsychotics are administered affect its expression. Moreover, the putatively biological effects on synaptic plasticity may be more sensitive to haloperidol than to amisulpride.

Homer1b/c expression was significantly increased by chronic amisulpride compared to both vehicle and haloperidol-mediated levels, mainly in the striatum and when amisulpride was given in the subjects previously treated with haloperidol. Homer1b/c is the constitutive isoform of the Homer1 gene (de Bartolomeis et al., 2003), coding for a
scaffolding protein that bridge metabotropic glutamate receptors to their intracellular effectors and participates in the architecture of dendritic spines (Hayashi et al., 2009; de Bartolomeis et al., 2014). It has been observed that increased levels enrichment of Homer1b/c in the PSD is related to enlargement in dendritic spines shape and size (Sala et al., 2005; Sala et al., 2003; Sala et al., 2001). Therefore, it has been hypotized that the increase of Homer1b/c after a chronic antipsychotic treatment may reinforce mGluR-mediated glutamatergic signaling and may trigger synaptic plasticity processes. Intriguingly, increase of Homer1b/c expression by amisulpride occurred when the compound was given in subjects pretreated with haloperidol but not in antipsychotic-naïve ones, further reinforcing the view that the biological action of antipsychotics is crucially affected by prior antipsychotic treatment.

Indeed, among other Immediate Early Genes, Homer1a showed a characteristic features such as 1) the extent of Homer1a induction by antipsychotics may be related to the degree of D2R blockade of each drug (Iasevoli et al., 2009); Homer1a induction hold on following chronic treatment (Iasevoli et al., 2011); in switching paradigm, the effect of a strong D2R blockade first treatment on Homer1a expression induced by amisulpride is more pronounced in dorsolateral subregions, where haloperidol impact in a greater extent. Taken toghether, these results suggest the Homer1a is more sensitive as a molecular marker of antipsychotic treatment related to other Immediate Early Genes in acute, chronic and in switching paradigm.

Homer1a and Homer1b/c exert opposite molecular functions on postsynaptic density, so the relative ratio of Homer1a/Homer1b/c levels has been demonstrated to affect mGluR-mediated signaling (Kammermeier et al., 2008), with substantial impact on PSD neurobiology, including synaptic plasticity (Ronesi et al., 2012; Sala et al., 2003; Sala et al., 2005). Relative ratio was shifted toward Homer1a in both the cortex and the striatum by both antipsychotic compounds when they were administered after a previously treated by vehicle. This result implicates that chronic haloperidol and amisulpride in antipsychotic-naïve subjects may preferentially elicit the biological actions mediated by Homer1a. However, in subjects previously treated by haloperidol, haloperidol shifted the ratio toward Homer1a in the striatum; moreover, amisulpride did
not affect the relative ratio of expression or shifted it toward *Homer1b/c*. Therefore, a previous treatment by an antipsychotic has a relevant and antipsychotic-specific effect on the direction of *Homer1a/Homer1b/c*-mediated molecular effects triggered by these compounds.

Topographic analysis showed that haloperidol prominently induced gene expression in the medial agranular cortex, in the motor cortex, and in the lateral caudate-putamen, a regions related to motor side effect. This pattern of topographic expression was only observed when haloperidol was given in antipsychotic-naïve animals. This result may depend on molecular adaptations following prolonged treatments, leading to a more homogeneous *Homer1a* expression and neuronal activation throughout brain regions. Amisulpride prominently induced *Homer1* expression in the medial caudate-putamen when the compound was given in animals previously treated with haloperidol. The preferential activation of medial striatal regions had been reported in our previous study (de Bartolomeis et al., 2013) and is consistent with the supposed limbic selectivity of the compound (Moller et al., 2003), which has been accounted for its claimed superiority against negative symptoms (Nuss et al., 2010).

In conclusion, the results of this study add further insight to the hypothesis that the molecular actions exerted by antipsychotics are substantially different according to the order in which they are administered (i.e. antipsychotic administration in antipsychotic-naïve animals vs. previously antipsychotic-treated ones), confirming, at molecular level, what it had been observed in neuroimaging studies and in clinical practice.


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