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DOTTORATO DI RICERCA IN NEUROSCIENZE

AREA DI PSICHIATRIA MOLECOLARE

Modulation of *Homer1a* expression and Homer-mediated signaling in the post-synaptic density by antipsychotics: role of the cortical-subcortical dopamine-glutamate interaction

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Chapter 1.

INTRODUCTION.

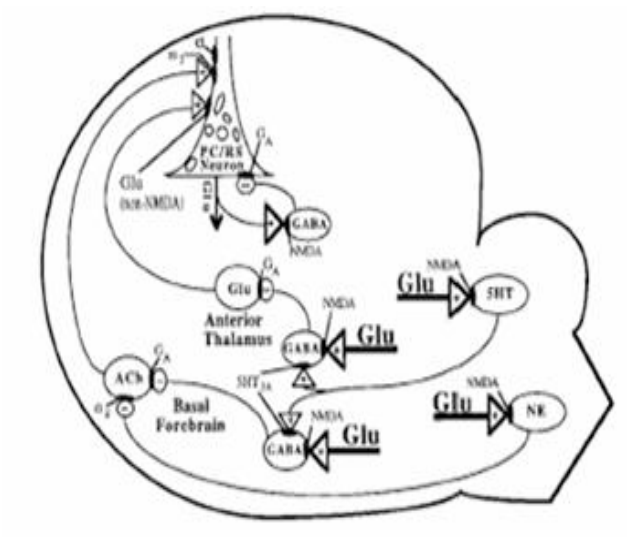
Cortical-subcortical dopamine-glutamate interplay in the pathophysiology of schizophrenia.

The dopaminergic hypothesis of schizophrenia states that subcortical hyperdopaminergy may result in the occurrence of psychotic symptoms, such as delusions and hallucinations (Kapur, 2004). Subcortical hyperdopaminergy may stem from the overactivation of the meso-striatal pathways. The meso-striatal tract originates from the ventro-tegmental area of the mesencephalon, where a great number of dopaminergic neuron bodies are located, and projects to the striatum, mainly the lateral aspects of the caudate-putamen (Van den Heuvel and Pasterkamp, 2008).

Several lines of evidence seem to corroborate this hypothesis (Meisenzahl et al., 2007): 1) the psychotomimetic action of dopamine-agonists compounds, as amphetamine; 2) therapeutic efficacy of antipsychotics, which is tightly correlated to the degree of affinity and blockade of the dopamine D2 receptors (enriched in the striatum); 3) the striatal supersensitvity to amphetamine in schizophrenic individuals compared to healthy volunteers (Breier et al., 1997).

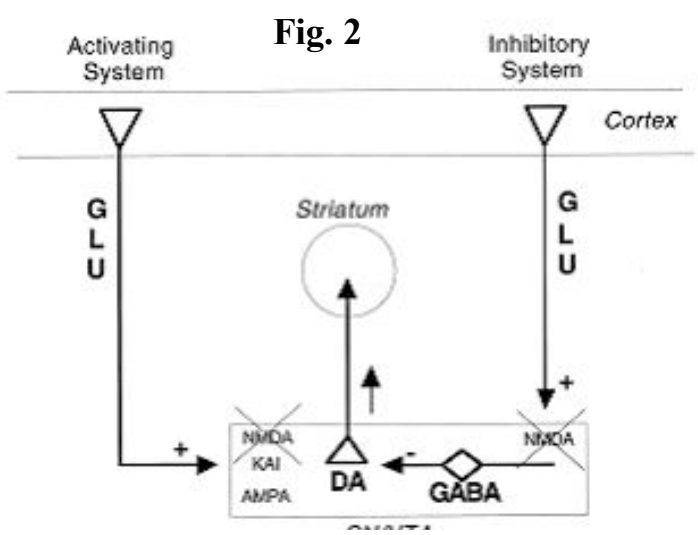
Recently, the so-called “cortical hypodopaminergy hypothesis” has also been proposed (Kapur, 2004). According to this view, a decreased function of the dopaminergic neurons belonging to the meso-cortico-limbic tract may explain the cognitive dysfunctions that are observed in schizophrenia. The meso-cortico-limbic tract originates from the ventro-tegmental area and projects to subcortical limbic regions (e.g.: the nucleus accumbens) and to the cortex, mainly the dorso-medial prefrontal cortex (Van den Heuvel and Pasterkamp, 2008).

Recent evidence let hypothesize a role for glutamate also in the pathophysiology of schizophrenic disease. Olney and Farber (Olney et al., 1999) proposed a model known as the NMDA receptor hypofunction (NRH) hypothesis. NRH may result in a decreased activation of GABAergic interneurons by collateral fibers of glutamatergic pyramidal neurons. GABAergic interneurons prevent from overactivation of glutamatergic fibers which project to other cortical regions and to the striatum and the mesencephalon. Decreased activation of GABAergic interneurons result in a lack of inhibition of glutamatergic pyramidal neurons which increase their firing onto post-synaptic neurons. Overactivation of glutamatergic pyramidal neurons determines a condition of paradoxical hyperglutamatergy (and presumably neurotoxicity) upon neurons connecting with glutamatergic pyramidal fibers: other cortical pyramidal neurons and subcortical dopaminergic fibers, among others. Hyperglutamatergy upon dopaminergic neurons may in turn overactivate these neurons and increase dopamine release in the striatum (Fig. 1).



It thus appears that the pathophysiology of schizophrenia may be more complex than that postulated by the hyperdopaminergy hypothesis. Schizophrenia may be provoked by a deep imbalance of the interconnections between

Fig. 2



dopaminergic and glutamatergic systems at cortical (i.e.: prefrontal cortex) and subcortical (i.e.: the caudate-putamen, the nucleus accumbens, the ventro-tegmental area) levels (Fig. 2).

The medium-sized spiny neurons and the post-synaptic density (PSD).

Striatal regions are classically considered to be relevant for both the pathophysiology and the therapy of schizophrenia (Laruelle et al., 2003). In the striatum, the interplay between the dopaminergic and the glutamatergic systems takes place at the level of GABAergic interneurons, called the medium-sized spiny neurons (MSNs, Fig. 3).

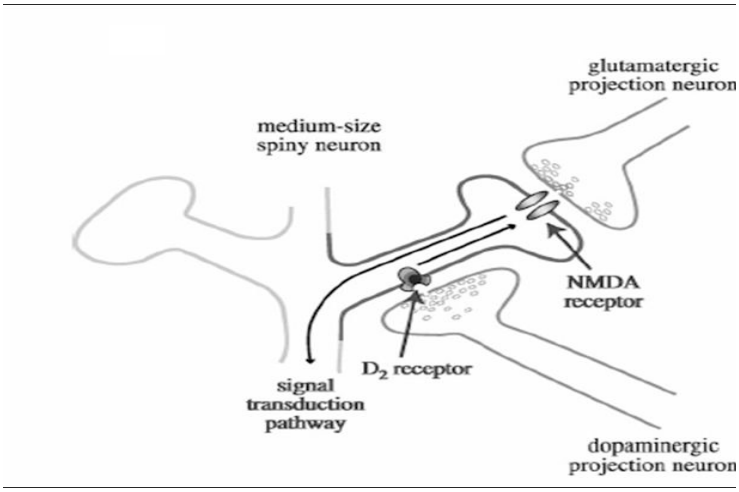


Fig. 3

Synaptic inputs from the two systems appear to regulate the direction, intensity, and type of output signal starting from MSNs (Kotter, 1994).

MSNs take part to a complex neuronal loop that projects to the cortex via glutamatergic thalamic neurons. It seems that the role of GABAergic interneurons may be to provide a filter to the flow of ascending inputs to the cortex. According to the "thalamic filter" hypothesis, postulated by Carlsson et al. (Carlsson, 1995), dopamine-glutamate cortical-subcortical imbalance may impair this subcortical filter, thus causing an overload of inputs to the cortex, which may result in psychotic symptoms.

The post-synaptic density (PSD) is an electrondense thickening located at the post-synaptic sites of glutamatergic synapes (Okabe, 2007; Sheng and Hoogenraad, 2007). In the striatum, the PSD is particularly enriched in the dendritic spines of MSNs. The PSD is a protein machinery that integrates synaptic signals from presynaptic neurons. Therefore, the PSD is a site of molecular interplay between dopaminergic and glutamatergic systems (de Bartolomeis et al., 2005). Within the PSD, a protein scaffold bridges receptors with: other surface receptors; cytoskeletric factors; or effectors of the second messengers pathway (Fig. 4).

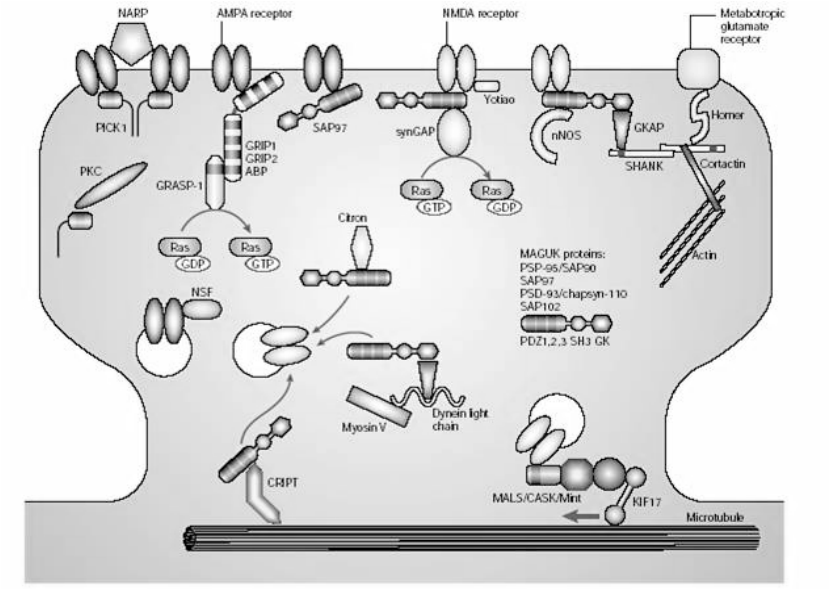


Fig. 4

As growing evidence is focusing on the dysfunction of synaptic plasticity processes at the glutamatergic synapses in the schizophrenia pathogenesis (Konradi and Heckers, 2003), the PSD may represent a key site in the pathophysiology of the disease and PSD factors may be considered as potential candidate genes for schizophrenia (Hashimoto et al., 2007b; Toro and Deakin, 2005).

Among all PSD factors, Homer proteins have been demonstrated to have a role in the regulation of post-synaptic architecture and in the tuning of post-synaptic glutamatergic

signaling (Sala et al., 2001; Sala et al., 2003; Sala et al., 2005). The inducible isoform Homer1a has been associated to schizophrenia and the gene coding for this isoform has been demonstrated to strongly increase when antipsychotics are administered.

The immediate-early gene *Homer1a*.

Homer1 is a gene coding for constitutively expressed isoforms (Homer1b/c) and for an activity-inducible isoform (Homer1a), which derives from an alternative splicing of the original gene (Fig. 5).

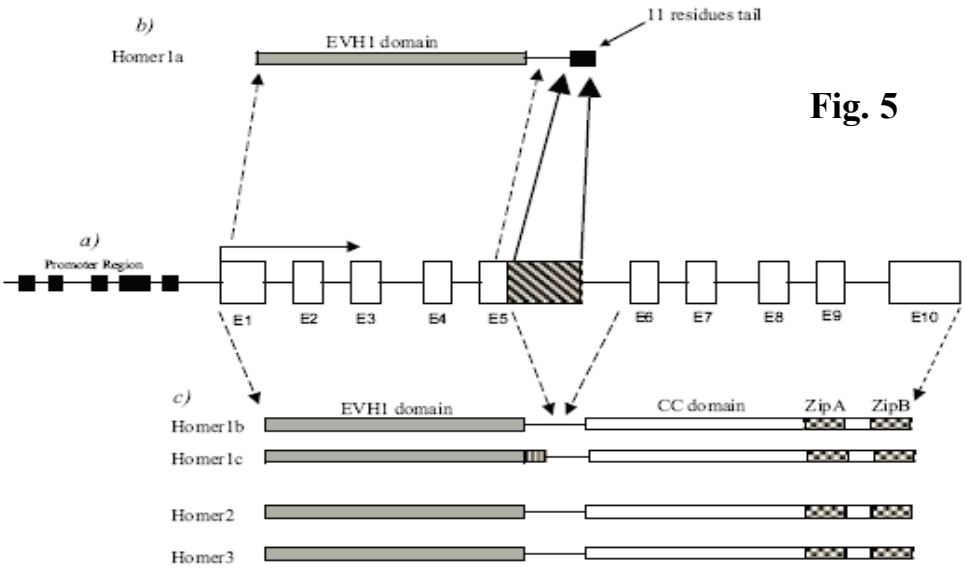


Fig. 5

Homer is a scaffolding protein. Constitutive isoforms (Homer1b/c/d) are bimodal proteins which include a domain of interaction with PSD substrates and a domain by which they self-holigomerize (de Bartolomeis and Iasevoli, 2003). Homer1b/c, by virtue of their molecular conformation, form a protein mesh with other PSD factors, which is principally devoted to approximate metabotropic glutamatergic receptors to their intracellular effectors and to preserve synaptic architecture (de Bartolomeis and Iasevoli, 2003; Shiraishi-Yamaguchi and Furuichi, 2007). The truncated isoform Homer1a (which lacks the CC domain allowing self-

holigomerization) competes with constitutive isoforms for binding to PSD substrates and disassemblies constitutive Homers-mediated clusters (Fig. 6).

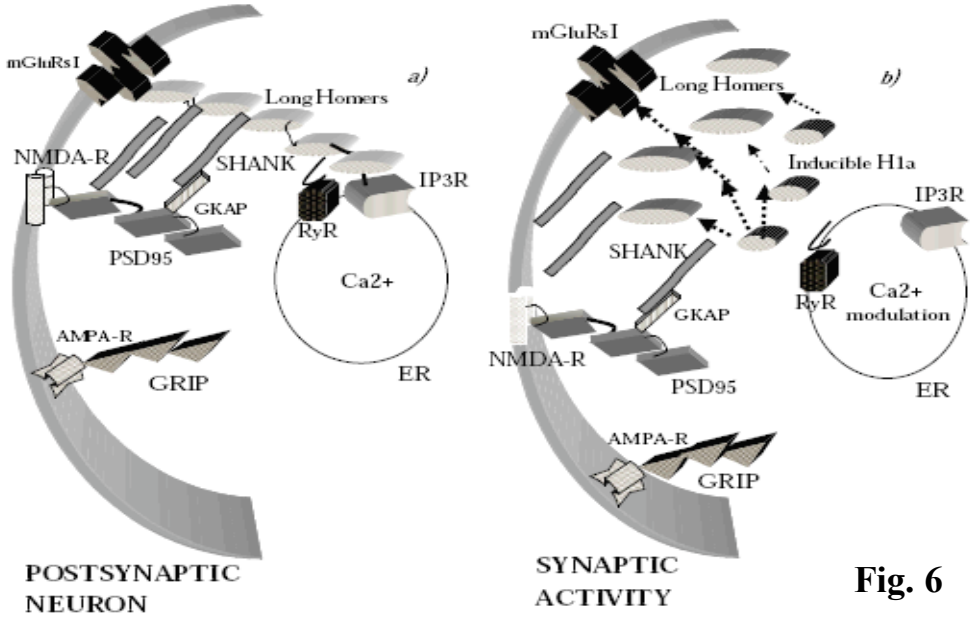


Fig. 6

As a result, the glutamatergic IEG *Homer1a* has been described to rapidly and transiently regulate intracellular Ca^{++} levels and glutamatergic signaling by type I metabotropic receptors (de Bartolomeis and Iasevoli, 2003; Shiraishi-Yamaguchi and Furuichi, 2007), when expressed. The Homer family of proteins has been shown to play a role in animal behavior, as locomotor activity (Diagana et al., 2002), and in behavioral plasticity related to memory processes (Klugmann et al., 2005). Overexpression of the inducible isoform Homer1a (the product of the IEG *Homer1a*) in the striatum of transgenic mice produces the impairment of motor performance and coordination and stereotyped response to administration of the psychostimulant amphetamine (Tappe and Kuner, 2006). A role for Homer proteins in behavioral disease, including drug addiction and mental retardation (Govek et al., 2004; Giuffrida et al., 2005; Szumlinski et al., 2006), has also been claimed. Behavioral abnormalities recalling those observed in animal models of schizophrenia have been recently described in *Homer1* knockout mice (Szumlinski et al., 2005). Intriguingly,

alterations in glutamatergic signaling have been hypothesized in the physiopathology of schizophrenia (Olney et al., 1999). Hence, *Homer1* may be a candidate gene for schizophrenia, given its function at glutamatergic post-synapses. According with this view, a borderline association with schizophrenia has been shown in a genetic analysis conduced on affected subjects (Norton et al., 2003). In our experiments, we have seen that the IEG *Homer1a* is significantly expressed in response to antipsychotics, in a region- and compound-specific manner.

Homer1a expression by discrete compounds and procedures: glutamatergic and dopaminergic mechanisms.

Homer1a expression appears to be triggered by several distinct experimental procedures that can be grouped in three major classes: paradigms that boost synaptic activity; psychostimulants and dopaminergic compounds; and drugs employed in the therapy of psychiatric diseases.

The interest in these drugs and procedures arises from the growing evidence that Homers are key factors in synaptic plasticity processes, as the fine-tuning of glutamatergic signaling and its postsynaptic integration with other neurotransmitters signaling cascades, above all the dopaminergic. Moreover, interest is further enhanced from the view that Homers may be implicated in the pathophysiology of behavioral and neurological disorders.

Homer1a is an activity-induced gene, which is rapidly upregulated in neurons in response to synaptic activity. As the main excitatory system in the brain is the glutamatergic, several direct and indirect reports, either *in vivo* or *in vitro*, suggest a direct regulation of *Homer1a* expression by glutamate, via the NMDA or the AMPA receptors. Glutamate agonists as kainate or NMDA elicit *Homer1a* expression (Kato et al., 1997; Ango et al., 2000; Sato et al., 2001). As well, conditions as seizure, kindling, long-term potentiation, or evidence-based

learning are all characterized by glutamate mediated synaptic activity and are all described to induce *Homer1a* expression (Brakeman et al., 1997; French et al., 2001; Matsuo et al., 2000; Morioka et al., 2001; Potschka et al., 2002; Vazdarjanova et al., 2002). It has been observed that Homer1a may directly reduce neuronal excitability. The induction of *Homer1a* may thus relieve neurons from excessive glutamate-induced depolarization and may have a protective role. The likelihood of this assumption appears to be confirmed by the strong induction of *Homer1a* in conditions of glutamate neurotoxicity as inflammations or stroke (Miyabe et al., 2006). Moreover, rats constitutively overexpressing *Homer1a* showed a reduced susceptibility to seizure (Potschka et al., 2002).

Homer1a overexpression may be involved in neuronal protection, by hyperpolarization and downscaling of neuronal excitation, after sleep deprivation-induced glutamate overstimulation.

Phencyclidine (PCP) is a non-competitive antagonist of NMDA receptors, which is also known as a drug of abuse. PCP has been demonstrated to induce *Homer1a* expression within the prelimbic region of the prefrontal cortex and the primary auditory cortex at 2 hours from administration and to reduce it in the granular retrosplenial cortex at 24 hours posttreatment (Cochran et al., 2002).

Overall, the data above confirm that glutamate may be a main modulator of *Homer1a* expression and suggest a strong involvement of *Homer1a* in behavioral-related processes. Glutamate-mediated modulation seems to occur mainly in the cortex and the hippocampus and appears to be negligible in the basal ganglia and the mesencephalon.

A strong increase of *Homer1a* expression has been described by direct or indirect activation of dopamine transmission. In opposition to environmental or electrical stimuli, which trigger gene expression mainly in the cortex and the hippocampus, dopaminergic compounds elicit

Homer1a expression in the striatum, which is highly enriched of dopaminergic terminals and receptors.

L-DOPA is a precursor in the metabolic pathway leading to dopamine formation and thus could be assumed as a direct agonist at both D_1-like and D_2-like receptors. Chronic L-DOPA has been described to induce *Homer1a* expression in the denervated striatum of dyskinetic rats (Sgambato-Faure et al., 2005).

A better discrimination of the dopaminergic mechanisms involved in *Homer1a e*xpression is provided by experiments exploring the induction by a subset of dopamine receptors. In a recent work, a significant increase of *Homer1a* mRNA levels in the striatum and the nucleus accumbens by the D_1 receptor direct agonist SKF38393 (20 mg/kg) has been observed (Yamada et al., 2007). The same compound was unable to trigger gene expression in the medial prefrontal cortex, the hippocampus, and the substantia nigra. Yamada and collaborators (Yamada et al., 2007) also demonstrated that the D_2 receptor direct agonist quinpirole had no significant effect on *Homer1a* expression. Thus, D_1 receptors may dose-dependently mediate the induction of *Homer1a* in the basal ganglia, while their role in cortical induction is questioned.

Studies employing indirect dopamine agonists appear to confirm the observations by direct agonists and antagonists at dopamine receptors.

Methylphenidate is a psychostimulant drug that behaves as an indirect dopamine agonist and produces striatal dopamine overflow, similar to that measured for cocaine (Swanson and Volkow, 2002). Acute administration of methylphenidate to adult rats increased *Homer1a* expression in cortical and striatal regions in a dose-dependent and regionally selective manner. These changes in gene expression were highly correlated in corticostriatal sectors and were prominent in regions involved in motor behavior (Yano and Steiner, 2005). Chronic methylphenidate in adolescent rats elicited a persistent increase of *Homer1a*

expression in adulthood (Adriani et al., 2006), presumably as a result of neuroplastic rearrangements occurring in the brain areas targeted by methylphenidate.

In a recent work, *Homer1a* induction in cortical and striatal areas has been evaluated after chronic exposure to vehicle or methylphenidate followed by an acute challenge with the same drug. While no significant changes were seen in the cortex as a consequence of acute methylphenidate in vehicle chronically treated rats, significant gene expression by acute exposure to methylphenidate was observed in several aspects of the caudate-putamen. These changes occurred mostly in sensorimotor and central parts of the striatum, and were more limited in the ventral sectors and negligible in the nucleus accumbens (Cotterly et al., 2007). Chronic treatment by methylphenidate significantly induced *Homer1a* expression in both the cortex and the striatum (Cotterly et al., 2007).

Acute administration of a neurotoxic dose of methamphetamine (40 mg/kg) significantly increased *Homer1a* mRNA levels in the striatum and the nucleus accumbens (Hashimoto et al., 2007a). The same treatment did not affect Homer1b and Homer1c striatal expression in any brain regions. Unfortunately, gene expression was measured in the striatum and the nucleus accumbens as a whole and no characterization was made of subregions in which changes in gene expression were more prominent.

An apparent exception to the framework depicted is represented from the psychostimulant drug lysergic acid diethylamide (LSD). LSD is a direct/indirect agonist at 5-HT_{1A} and 5-HT_{2A} receptors, which has been shown to induce acutely *Ania3* (a splice variant of *Homer1a*, which also behaves as an activity-induced gene) and *Homer1a* expression in the prefrontal cortex. This effect appears to be counteracted by antagonism at 5-HT_{2A} but not at 5-HT_{1A} receptors (Nichols et al., 2003). However, it is very difficult drawing an ultimate picture of LSD mechanism of action, since it holds partial agonism at 5-HT_{1A} receptors. Thus, *in vivo* action of this drug may change greatly according with dosages and cortical serotoninergic

tone, passing from agonism to functional antagonism. It has been described that partial agonists at 5-HT_{1A} receptors may facilitate dopamine outflow in the cortex and transmission via the D_1 receptor (Diaz-Mataix et al., 2005). Thus, LSD may trigger *Ania3* expression through dopamine release and D_1 receptor activation.

Aims of the research.

Here, we present a group of studies whose aim was to investigate the impact on *Homer1a* expression and Homer-mediated signalling by psychotropic compounds which elicit a perturbation of dopamine transmission.

The first goal of these studies was to clarify the modulation of *Homer1a* expression by psychotropic compounds, in terms of specificity for antipsychotics and of sensitivity to the degree of dopamine perturbation elicited. For these reasons, we studied the pattern of *Homer1a* expression by several different antipsychotics and other compounds which impacted differentially dopamine transmission.

In our opinion, *Homer1a* may represent a preclinical tool to characterize and differentiate compounds putatively provided of antipsychotic potential. The studies described herein were intended to confirm and strengthen this view.

However, *Homer1a* may be implied in antipsychotic mechanism of action. For this reason, we also evaluated the functional implications at the protein level of antipsychotic modulation upon the gene.

As a second goal, we also wanted to investigate whether other PSD factors may respond to acute and chronic dopamine perturbation by psychotropic compounds.

Moreover, the studies described may shed a light on the reciprocal interplay between dopamine and glutamate at both cortical and subcortical levels.

Chapter 2.

Experimental procedures.

Animals.

Male Sprague-Dawley rats of approx. 250g (Charles River Laboratories, Lecco, Italy) were housed and let to adapt to human handling in a temperature and humidity controlled colony room with 12/12h light/dark cycle (lights on from 6:00 a.m. to 6:00 p.m.) with *ad libitum* access to lab chow and water. All procedures were conducted in accordance with the NIH *Guide for Care and Use of Laboratory Animals* (NIH Publication N0.85-23, revised, 1996) and were approved by local Animal Care and Use Committee. Care was taken to minimize animal number and sufferance.

Drug treatment.

Ketamine paradigm.

On the day of the experiment rats (n=5) were randomly assigned to one of the following treatment groups: A) 12 mg/kg ketamine (Ket12); B) 50 mg/kg ketamine (Ket50); C) saline (0.9% NaCl) (Sal). All drugs were injected intraperitoneally at a volume of 1 ml/kg. The animals were sacrificed by decapitation 90 minutes after the treatment.

Aripiprazole paradigm.

Aripiprazole powder (Bristol-Myers Squibb, Italy), haloperidol injectable solution (Lusofarmaco, Italy), clozapine powder (Sigma-Aldrich, Italy), GBR12909 powder (Sigma-Aldrich, Italy) were used in this study. Aripiprazole, Clozapine and GBR12909 were dissolved in physiological saline solution (NaCl 0.9%) with the adjunction of a few drops of acetic acid. Physiological saline solution, added with a few drops of acetic acid, was used as

a control. All solutions were suited to physiological pH value and injected intraperitoneally (i.p.) at a volume of 1 ml/kg.

Acute experiment protocol. On the day of the experiment, rats (n=7 animals for each experimental group) were randomly assigned to one of the following treatment groups: A) control saline solution (VEH); B) aripiprazole 12 mg/kg (ARI12); C) aripiprazole 30 mg/kg (ARI30); D) haloperidol 0.8 mg/kg (HAL); E) GBR 12909 30 mg/kg (GBR); F) clozapine 15 mg/kg (CLO). The animals were sacrificed by decapitation 90 minutes after the treatment.

Chronic experiment protocol. Rats (n=7 animals for each experimental group) were randomly assigned to one of the following group: A) control saline solution (VEH); B) aripiprazole 12 mg/kg (ARI); C) clozapine 15 mg/kg (CLO); D) haloperidol 0.8 mg/kg (HAL) and treated daily for 21 days. The animals were sacrificed by decapitation 90 minutes after the last injection.

Dopamine Receptors Antagonists Paradigm.

SCH-23390 (R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride) (Tocris Cookson, Bristol, UK), L-741,626 (3-(4-(4-chlorophenyl-4-hydroxypiperidino)methyl)indole) (Tocris Cookson, Bristol, UK), U-99194 (5,6-dimethoxy-2-(dipropylamino)indan hydrochloride) (Tocris Cookson, Bristol, UK), L-745,870 (3-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-1H-pyrrolo(2,3-b)pyridine) (Tocris Cookson, Bristol, UK), S(+)-terguride (Sigma-Aldrich, Milano, Italy) were all purchased as a powder. Haloperidol was provided as the injectable solution (Lusofarmaco, Milano, Italy) and then diluted at the experimental dosages. All drugs were dissolved in a vehicle (VEH) of physiological saline solution (0.9% NaCl), which was used as a control. All solutions were suited to physiological pH value and injected intraperitoneally (i.p.) at a volume of 1 ml/kg.

Rats (n=5 for each experimental group) were randomly assigned to one of the following treatment groups: 1) vehicle (VEH); 2) SCH-23390 (D_1 receptor selective antagonist) 0.5 mg/kg; 3) L-741,626 (D_2 receptor selective antagonist) 2 mg/kg; 4) U-99194 (D_3 receptor selective antagonist) 5 mg/kg; 5) L-745,870 (D_4 receptor selective antagonist) 3 mg/kg; 6) terguride (D_2/D_3 receptor partial agonist) 0.5 mg/kg (TER); 7) haloperidol 0.8 mg/kg (HAL).

Atypical antipsychotics (risperidone, olanzapine, and sulpiride) paradigm.

Risperidone powder (Sigma-Aldrich, Milano Italy), olanzapine powder (Sequoia Research Products Ltd, London, UK), (-)-sulpiride powder (Sigma-Aldrich, Milano, Italy), haloperidol injectable solution (Lusofarmaco, Milano, Italy) were used in this study. All drugs were dissolved in a vehicle (VEH) of physiological saline solution (0.9% NaCl), with a few drops of 0.1M HCl, which was used as a control. All solutions were suited to physiological pH value and injected intraperitoneally (i.p.) at a volume of 1 ml/kg.

Rats (n=5 for each experimental group) were randomly assigned to one of the following treatment groups: 1) vehicle (VEH); 2) haloperidol 0.8 mg/kg (HAL); 3) risperidone 3 mg/kg (RISP); 4) olanzapine 2.5 mg/kg (OLA); 5) (-)-sulpiride 50 mg/kg (SULP).

Ziprasidone Paradigm.

Ziprasidone was provided by Pfizer as an HCl salt and was dissolved by few drops of DMSO and 0.9% NaCl (vehicle). Haloperidol (Serenase® 2mg/ml. Lusofarmaco; Milan, Italy), prepared from commercially available ampoules, and Clozapine powder (Sigma-RBI chemicals, Milan, Italy) were both diluted in vehicle. Vehicle was used as the control group. All injections were performed intraperitoneally (i.p.) using an injection volume of 1ml/kg. Animals (n=14) were randomly assigned to one of the following treatment groups: A) VEH; B) haloperidol 0.8mg/kg; C) ziprasidone 4mg/kg. Treatments were administered once per

day for 21 days. As *Homer1a* is acutely induced by antipsychotics in an IEG-like fashion, we asked whether this property was still conserved after a chronic treatment and/or whether chronic treatment may sustain *Homer1a* expression besides its acute expression. Therefore, after the last injection animals were split in two subgroups (n=7 each): those sacrificed acutely (90 minutes from the last injection: VEH90, HAL90, ZIP90, according to the treatment received) and those sacrificed after a 24-hour withdrawal (VEH24, HAL24, ZIP24). Then, we confronted expression of the genes at these two time-points.

Haloperidol-SSRI coadministration paradigm.

On the day of the experiment were randomly assigned to one of the six following groups: 1) Saline solution 0.9% NaCl (SAL); 2) Escitalopram 12mg/kg (ESC) (Lundbeck Italia S.p.A., Milan, Italy); 3) Citalopram 14mg/kg (CIT) (Lundbeck Italia S.p.A., Milan, Italy); 4) Haloperidol 0.8 mg/kg (HAL) (Lusofarmaco, Milan, Italy); 5) HAL + ESC; 6) HAL + CIT. All drugs were dissolved in a vehicle of physiological saline solution (0.9% NaCl, VEH), which was used as a control. All solutions were suited to physiological pH value and injected intraperitoneally (i.p.) at a volume of 1 ml/kg.

Sertindole paradigm.

Haloperidol (2mg/ml, Lusofarmaco, Italy) was used as commercially available ampoules and diluted in saline solution. Sertindole was gently gifted from Lundbeck H/S and dissolved in a vehicle (VEH) composed by saline solutions plus few drops of acetic acid. The vehicle was injected in the control group. All injections were performed subcoutaneously in a volume of 1ml/kg.

Rats were randomly assigned to one of the following treatment groups (n=7): 1) 0.9% NaCl plus acetic acid (VEH); 2) Haloperidol 0.8 mg/kg (HAL); 3) Sertindole 2 mg/kg (SERT).

Drug doses.

The doses of antipsychotics used were all chosen as to fit within the dose range that produces effects in animal behavioral models that are predictive of antipsychotic efficacy in humans, such as the conditioned avoidance suppression test (Seeger et al., 1995). A similar range was used in a recent paper assessing IEGs expression in rat brain (Jennings et al., 2006). Haloperidol dose is consistent with a dose range (0.25–1 mg/kg) used to elicit gene expression in several protocols (Merchant and Dorsa, 1993; Robertson and Fibiger, 1992; Rushlow et al., 2005).

Aripiprazole shows a dose-dependent dopamine D_2 receptors occupancy of 15-90% into a dose range between 0.3 and 30 mg/kg. Specifically, aripiprazole 12 mg/kg induces a dopamine D_2 receptors occupancy near 80% and is twice the ED_{50} to inhibit the stereotyped behavior evoked by apomorphine administration (Semba et al., 1995). Aripiprazole 30 mg/kg does not induce catalepsy, even if it gives rise to a D_2 receptors occupancy of >85% (Natesan et al., 2006; Semba et al., 1995).

The dosages of the selective dopamine receptors antagonists were chosen based on previous published observations, both in vitro and in vivo. The doses are at the lower limit of the dose-range used in behavioral studies, in order to preserve receptor selectivity (Bristow et al., 1997; Bristow et al., 1998; Chaperon et al., 2003; Costanza and Terry, 1998; Matsubara et al., 2006; Millan et al., 2000; Millan et al., 2004). As a further validation, neurochemical findings assessing dose-related IEGs expression by the compounds have been taken into account (Carr et al., 2002; LaHoste et al., 2000; Wirtshafter, 2007).

Risperidone, olanzapine, and sulpiride doses were chosen based on previous animal studies in which molecular and behavioural effects predictive of antipsychotic activity were elicited (Didriksen et al., 2006; Fujimura et al., 2000; Kapur et al., 2003; Marchese et al., 2004; Sams-Dodd, 1998; Wan et al., 1995).

The doses of ziprasidone were chosen as to fit within the dose range that produces effects in animal behavioral models that are predictive of antipsychotic efficacy in humans, such as the conditioned avoidance suppression test (Seeger et al., 1995). Ziprasidone was administered at two divergent doses to assess whether *Homer1a* expression might depend in a dose-dependant fashion on increasing D_2R blockade. The higher dose of ziprasidone (10 mg/kg) is also compatible with the dosage that produces minimal catalepsy, 12.1 mg/kg (9.7-15.1, 95% C.I.) (Seeger et al., 1995).

Citalopram and escitalopram doses were chosen based on previous animal studies in which molecular and behavioural effects predictive of either antidepressive or antipsychotic activity were elicited (Izumi et al., 2006; Kuipers et al., 2006; Tomasetti et al., 2007; Calcagno et al., 2007; Bondi et al., 2008). Citalopram and escitalopram were chosen because of their most selective effect on 5-HT reuptake blockade among all the currently available SSRIs, with escitalopram being approximately twice more potent than citalopram (Owens et al., 2001; Sanchez et al., 2003). Moreover, they significantly increase extracellular 5-HT, but not dopamine and noradrenaline levels in rat prefrontal cortex (Invernizzi et al., 1997; Bymaster et al., 2002; Bundgaard et al., 2006).

Sertindole was given in a dose-range known to be behaviorally active and to elicit gene expression (Andersen and Pouzet, 2001; de Bartolomeis et al., 2002; Depoortere et al., 1997; Gao et al., 1998; Sams-Dodd, 1997).

In situ hybridization histochemistry.

The procedure for *in situ* hybridization histochemistry was taken from standard published protocols (Ambesi-Impiombato et al., 2003).

Tissue preparation and sectioning

The brains were rapidly removed, quickly frozen on powdered dry ice and stored at -70ºC prior to sectioning.

Serial coronal sections of 12µm were cut on a cryostat at-18°C, through the forebrain using the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1997) as an anatomical reference. Care was taken to select identical anatomical levels of treated and control sections using thionin-stained reference slides. Sections were thaw-mounted onto gelatin-coated slides, and stored at -70°C for subsequent analysis.

Radiolabeling and purification of oligonucleotide probes

The *Homer1a* probe in the ketamine paradigm was specifically designed to label the *Homer1a* isoform of the *Homer1* gene and it was a 48-bases oligodeoxyribonucleotide complementary to bases 1073-1120 of the rat *Homer1a* mRNA (GenBank Accesion No U92079). The *αCaMKII* probe was a 45-bases oligodeoxyribonucleotide complementary to bases 937-981 of the rat *αCaMKII* mRNA (GenBank Accession No NM_012920). The *βCaMKII* probe was a 48-bases oligodeoxyribonucleotide complementary to bases 1143-1191 of the rat *βCaMKII* mRNA (GenBank Accesion No NM_021739).

In all other paradigms, the *Homer1a* probe was a specifically designed 48-base oligodeoxyribonucleotide complementary to bases 2527-2574 of the rat *Homer1* mRNA (GenBank # U92079) (MWG Biotech; Firenze, Italy).

The *ania3* probe was a 48-base oligodeoxyribonucleotide complementary to bases 1847-1894 of the rat *ania3* mRNA (GenBank # AF030088) (MWG Biotech; Firenze, Italy). The *Homer1b/c* probe was a 48-base oligodeoxyribonucleotide complementary to bases 1306-1353 of the rat *Homer1* mRNA (GenBank # AF093268) (MWG Biotech; Firenze, Italy). The *Homer2* probe was a 48-base oligodeoxyribonucleotide complementary to bases 710-757 of

the rat *Homer2* mRNA (GenBank # AB007689) (MWG Biotech; Firenze, Italy). The *D2R (dopamine D2 receptor)* probe was a 48-base oligodeoxyribonucleotide complementary to bases 374-421 of the rat *Drd2* mRNA (GenBank # NM012547) (MWG Biotech; Firenze, Italy). The *mGluR5* probe was a 45-base oligodeoxyribonucleotide complementary to bases 637-682 of the rat *mGluR5* mRNA (GenBank Accession #D10891). The *Homer1b* was a 48-base oligodeoxyribonucleotide complementary to bases 1306-1354 of the rat *Homer1b* mRNA (GenBank Accession #AF093267). The *shank* probe was a 48-base oligodeoxyribonucleotide complementary to bases 2757-2804 of the rat *shank1* mRNA (GenBank # NM_0317751) (MWG Biotech; Firenze, Italy). The *PSD-95* probe was a 45-base oligodeoxyribonucleotide complementary to bases 225–269 of the rat *PSD-95* mRNA (GenBank # M96853) (MWG Biotech; Firenze, Italy). The *Homer1b* was a 48-base oligodeoxyribonucleotide complementary to bases 1306-1354 of the rat *Homer1b* mRNA (GenBank Accession AF093267) (MWG Biotech; Firenze, Italy). The *P11* probe was a 48-bases oligodeoxyribonucleotide complementary to bases 313-361 of the rat *P11* mRNA (GenBank Accession # NM_134395). All the oligodeoxyribonucleotides were purchased from MWG Biotech (Firenze, Italy). These sequences were checked with blastn algorithm against GenBank, to avoid cross-hybridization. For each probe a 50µl labeling reaction mix was prepared on ice using DEPC-treated water, 1X tailing buffer, 1.5mM $CoCl_2$, 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 Biosciences; Milano, Italy). The autoradiographic signal distribution of *Homer* matched that of previous ISH studies (Berke et al., 1998; Brakeman et al., 1997; de Bartolomeis et al., 2002; Polese et al., 2002). Also, the specificity of each probe was tested by a control experiment using the corresponding sense probe.

In situ hybridization

All solutions were prepared with sterile double distilled water. The sections were fixed in 4% formaldehyde in 0.12 M sodium-phosphate buffered saline (PBS, pH 7.4), quickly rinsed three times with 1xPBS, 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%, 90% 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 37ºC in a humid chamber for 20 hours. After hybridization the coverslips were removed in 1X SSC and the sections were washed 4x15 minutes in 2xSSC/50% formamide at 40ºC, followed by two 30 min washes with 1xSSC at room temperature. The slides were rapidly rinsed in distilled water and then in 70% ethanol.

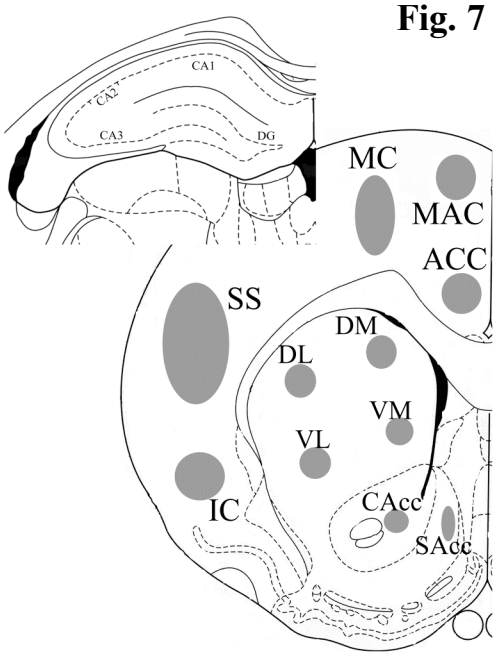
Autoradiography

The sections were dried and exposed to Kodak-Biomax MR Autoradiographic film (Sigma-Aldrich, Milano, Italy). A slide containing a scale of 16 known amounts of ^{14}C standards was co-exposed with the samples. The optimal time of exposure was chosen to maximize signal-to-noise ratio but to prevent optical density from approaching the limits of saturation. The film development protocol included a 1.5 min dip in the developer solution and 3 min in the fixer.

Image analysis

The quantitation of the autoradiographic signal was performed using a computerized image analysis system including: a transparency film scanner ScanMaker 9800XL (Microtek Europe B. V., Rotterdam, The Netherlands), an Apple PowerPC G4, and ImageJ software (v. 1.36, Rasband, W.S., http://rsb.info.nih.gov/ij/). Sections on film were captured individually. All hybridized sections were exposed on the same sheet of X-ray film. Analyses were carried out on digitized autoradiograms measuring mean optical density within outlined Regions of Interest (ROIs) in forebrain sections in correspondence of the cortex, caudate-putamen, and nucleus accumbens regions (Figure 7). ROIs in the cortex were selected based on recent acquisitions describing functional and anatomical correlation between cortical and striatal subregions (Cotterly et al., 2007; Willuhn et al., 2003; Yano and Steiner, 2005). ROIs in the striatum have been chosen according to classical subdivision of this region (Steiner and Gerfen, 1993). 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 statistically significant effects obtained by the two investigators, were considered reliable. Quantitative comparisons among different experimental groups were performed using images from hybridized sections exposed on the same sheet of X-ray film.

Fig. 7



Data processing

Measurements of mean optical density (OD) within ROIs were converted by a calibration curve based on the standard scale co-exposed to the sections. Standard values from 4 through 12 have been previously cross-calibrated to ^{35}S brain paste standards, in order to assign a dpm/mg tissue wet weight value to each OD measurement through a calibration curve. For this purpose a "best fit" 3rd degree polynomial was used. Experimental groups consisted of 3 to 5 animals. Data for each region was obtained by averaging measurements from 2-4 adjacent sections of single animals. The data were analyzed for treatment effects by a one-way analysis of variance (ANOVA).

Student-Newmann-Keuls was used as the *post hoc* test in the locus of any significant ANOVA. In the ketamine paradigm, Dunnett's *post hoc* test was used. Tukey's *post hoc* test was used in aripirazole and sertindole paradigms. Data in the ziprasidone paradigm were analyzed for treatment, time, and treatment-x-time effects by a two-way ANOVA.

Topographical distribution.

Topographical distribution of *Homer1a* expression has been evaluated by analyzing mean OD values for their frequency distribution (Distribution tool of ImageJ) among ROIs as induced by antipsychotics and vehicle. Frequency distribution tool provides a rate of the regional distribution of signal labeling according to measurement values in each ROI. Sum of measurements in a single ROI is compared to the total sum of measurement values in the striatum or the cortex, thus providing a relative weight of subregional signal labeling. Regional distribution of signal labeling for each treatment has been graphically expressed by means of a chart where calibrated (relative dpm) measurements are shown in relation to each ROI. As a further evaluation, averaged measurements from each treatment have been

analyzed for subregion effect by means of a one-way ANOVA. Student-Newmann-Keuls was used as the *post hoc* test in the locus of any significant ANOVA.

Western Blot.

After treatments, brains were lysed in a buffer containing 10 mmol/L KCl, 1.5 mmol/L MgCl2, 20 mmol/L HEPES, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and a mixture of protease inhibitors (Roche, Mannheim, Germany). Then, the lysates were centrifuged at 13000 rpm for 20 min to obtain the supernatants and the pellets. Protein concentration of supernatants and pellets was determined by Bradford method. Next, proteins were visualized on the filter by reversible staining with Ponceau-solution and de-stained in a Tris-buffered saline solution with 0.1% Tween 5%. Membranes were first blocked in milk buffer [Tris-buffered saline solution with 0.1% Tween 5% plus fat dry milk] and then incubated overnight at 4°C with 1:500 monoclonal anti-Homer1a antibody and 1:1000 polyclonal anti goat antibody (Santa Cruz Biotecnology, Santa Cruz, CA, USA). The resulting complexes were detected using chemiluminescent western blotting detection reagents (ECL, Amersham). Filter was exposed to Autoradiographic film and then visualized by a transparency film scanner (Microtek Europe B. V., Rotterdam, The Netherlands). ImageJ software was used to measure the optical density of the bands. Normalization of results was ensured by running parallel western blots with the appropriate actin antibody. The data were analyzed for treatment effects by a One Way Analysis of Variance (ANOVA). Tukey-Kramer *post-hoc* test was used to determine the locus of effects in any significant ANOVA.

Chapter 3.

Ketamine-related expression of glutamatergic postsynaptic density genes: possible implications in psychosis.

Rationale.

The non-competitive antagonist of glutamatergic N-methyl-D-aspartate (NMDA) receptor ketamine is known to induce psychotomimetic effects in healthy volunteers and to re-exacerbate psychotic symptoms in chronic schizophrenic patients (Krystal et al., 1994). Due to the non-competitive blockade of the channel site of NMDA receptor, ketamine is predicted to induce a state of NMDA receptor hypofunction (NRH) (Olney et al., 1999). NRH is considered one of the putative molecular mechanisms involved in psychosis and has been extensively used in humans and animals for mimicking some aspects of schizophrenia pathophysiology (Lipska and Weinberger, 2000). Beyond impairing NMDA-mediated glutamate transmission, non-competitive NMDA receptor antagonists are demonstrated to affect dopamine transmission, in terms of both dopamine synthesis and release and in dopamine transporter (DAT) availability (Schiffer et al., 2003; Tsukada et al., 2000). According to preclinical and clinical data, an imbalance of dopamine-glutamate transmission in striatum or prefrontal cortex appears crucial in schizophrenia (de Bartolomeis et al., 2005). The NMDA receptor is the core of the postsynaptic density (PSD), a multiproteic mesh providing physical interconnection between surface receptors and intracellular effectors (Boeckers, 2006). Moreover, PSD is a site of integration of glutamate and dopamine signalling by mechanisms of trans-activation (Hakansson et al., 2006) or convergence (Valjent et al., 2005) of intracellular pathways.

Two major components of PSD involved in dopamine-glutamate interplay are Homer and CaMKII. CaMKII is a dodecameric enzyme activated by Ca^{++} entry through the NMDA

receptor. CaMKII is composed by alpha subunits, the catalytic component, and beta subunits, mediating interaction with cytoskeleton. NMDA receptor stimulation leads to the disconnection from actin filaments and translocation in the PSD, where the enzyme exerts its kinase activity (Shen and Meyer, 1999). CaMKII has been involved in both glutamate signalling via the NMDA receptor and dopamine transduction (Pan et al., 2006). Homer1 and CaMKII may be functionally linked to modulate dopamine-glutamate interplay in PSD. CaMKII phosphorylates Homer1 in striatal synaptosomes (Yoshimura et al., 2002) and NMDA receptors in response to the stimulation of D_1 or D_2 receptors in striatal slices (Oh et al., 1999). Since Homer1a and alphaCaMKII represent reliable markers of glutamatergic function and are related to dopaminergic activity in the striatum, we employed *in situ* hybridization histochemistry to perform a quantitative analysis of topographic anatomical pattern of *Homer1a*, *alpha* and *betaCaMKII* gene expression in the ketamine-related model of glutamatergic and dopaminergic imbalance. We have chosen a low (12 mg/kg) and a high (50 mg/kg) subanaestethic dose of ketamine to observe gene expression in a condition of low and high dopamine-glutamate perturbation in the regions of interest (Moghaddam et al., 1997).

Results.

Animals treated with 12 mg/kg ketamine showed hyperlocomotion and stereotypy, which appeared rapidly after injection and subsided few minutes later. For the 50 mg/kg dose, we observed a moderate level of sedation, flat body posture, ataxia, head weaving. Rats were, however, full responsive to sound stimulation and were not anaesthetized. These effects lasted more than those observed with the 12 mg/kg dose.

Low levels of *Homer1a* and *αCaMKII* gene expression were detected in the forebrain of control animals in both cortical and subcortical regions (Fig 8).

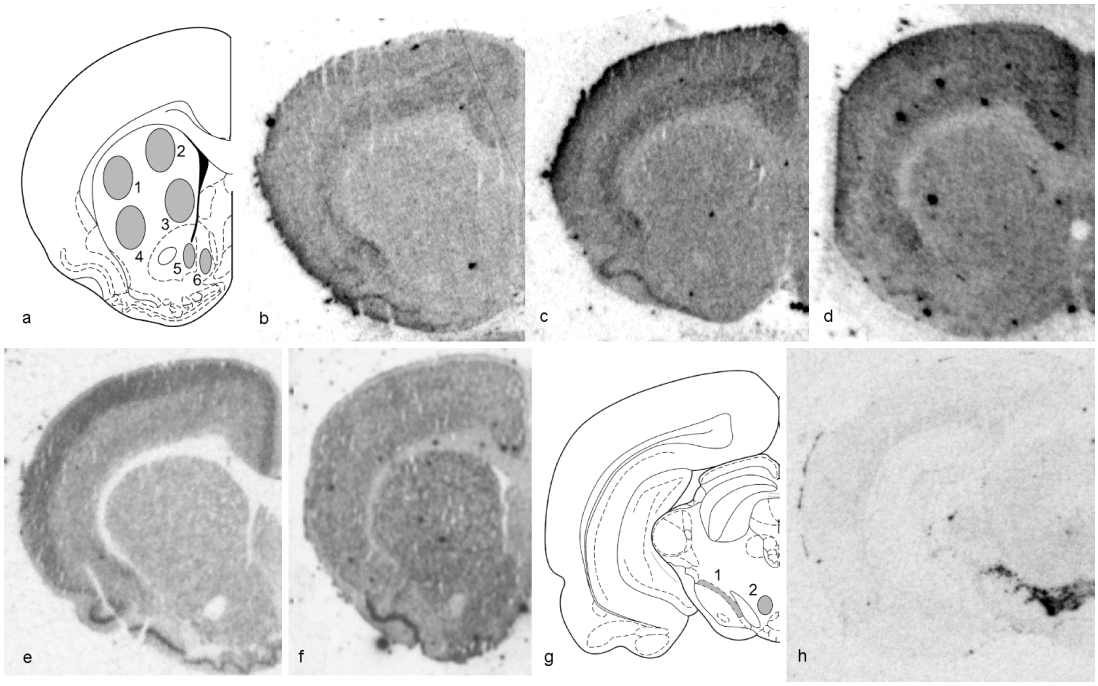


Figure 8. Upper row: ***a)*** Reference forebrain section highlighting Regions of Interest, Autoradiograms of *Homer1a* mRNA expression in: saline (***b***), ketamine 12mg/kg (***c***), ketamine 50mg/kg (***d***) treated-rats. Lower row: ***g)*** Reference midbrain section highlighting Regions of Interest, modified from *Paxinos and Watson (1997)*: substania nigra pars compacta (1), ventro-tegmental area (2). Autoradiograms of *αCaMKII* (***e***), *βCaMKII* (***f***), and *DAT* (***h***) mRNA expression from saline-treated control rats.

Homer1a autoradiographic signal was widely distributed in the brain, mainly in the frontal and the parietal cortex, the caudate-putamen and the nucleus accumbens (Fig. 9). We found a significant difference in the ventral striatum, whereas no significant changes in mRNA expression were detected in the dorsal striatum (dorsomedial, DM, and dorsolateral, DL: ANOVA, $p > 0.05$). In both the ventrolateral (VL) and the ventromedial (VM) putamen *Homer1a* was increased by Ket50 over the Sal group (VL: ANOVA, $p = 0.0379$, $F_{2,9} = 5.93$;

VM: ANOVA, p= 0.0273, $F_{2,9}$= 10.98). Moreover, Dunnett's post hoc test showed a significant induction by Ket12 over the control group in VM. In the shell (ANOVA, p= 0.0015, $F_{2,9}$= 18.93) and the core (ANOVA, p= 0.0008, $F_{2,9}$= 22.47) of the nucleus accumbens Ket50 significantly induced *Homer1a* expression compared to both Sal and Ket12, whereas Ket12 was significant against Sal (Fig. 9).

Fig. 9

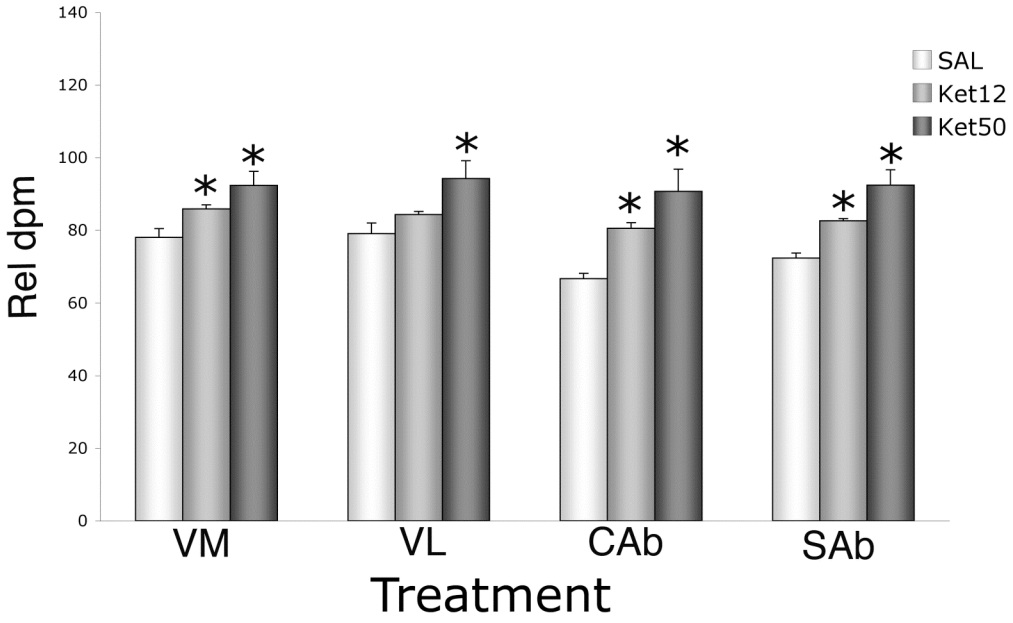


Figure 9. *Homer1a* mRNA expression. *Homer1a* mRNA levels measured after acute treatment in striatal subregions quantitated by densitometry of in situ hybridization histochemistry autoradiograms. VL= ventrolateral putamen. VM= ventromedial putamen. CAb= core of the accumbens. SAb= shell of the accumbens. Post-hoc: * $p<0.05$ vs saline. Data are expressed as relative dpm ± S.E.M.

The highest density of the *αCaMKII* autoradiographic signal was detected in frontal and parietal cortex, caudate-putamen and nucleus accumbens. In either DL, DM, and VM subregions of caudate-putamen and in the shell of nucleus accumbens (SAcb) a statistically significant increase of *αCaMKII* gene expression was observed in Ket12 group as compared with Sal and Ket50 groups (DL: ANOVA, p= 0.0067, $F_{2,9}$= 7.81; DM: ANOVA, p= 0.0060, $F_{2,9}$= 8.05; VM: ANOVA, p= 0.0063, $F_{2,9}$= 7.93; SAcb: ANOVA, p= 0.0333; $F_{2,9}$= 4.57) (Fig. 10). No significant changes were detected in both VL and core of accumbens (ANOVA, p>0.05). *βCaMKII* mRNA expression was detected throughout rat forebrain. Nonetheless, no significant differences among groups were detected in any of the region assessed (ANOVA, p>0.05).

Fig. 10

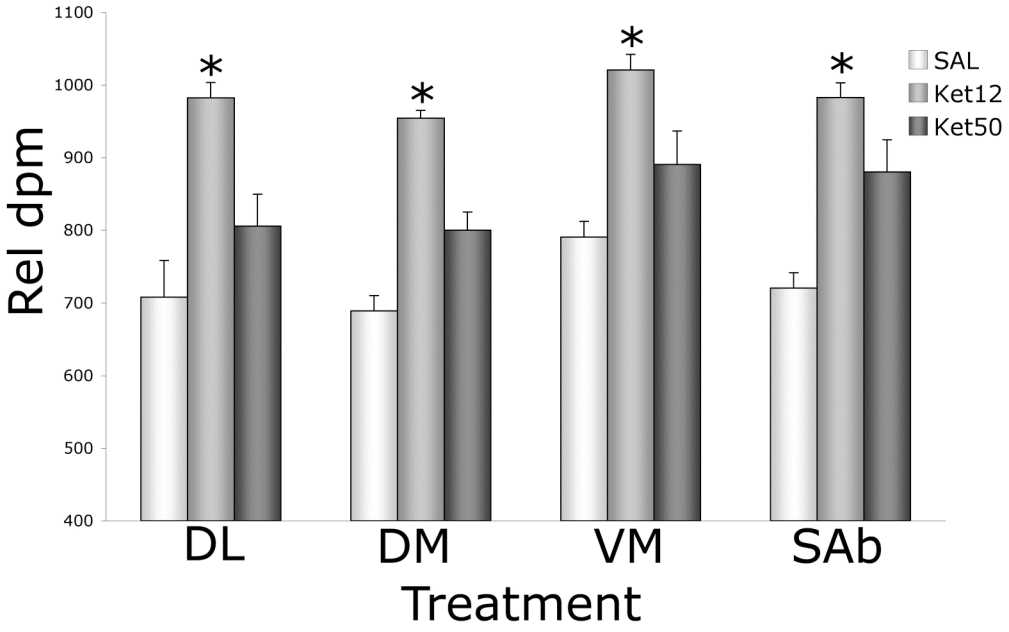


Figure 10. *AlphaCaMKII* mRNA expression. *AlphaCaMKII* mRNA levels measured after acute treatment in striatal subregions quantitated by densitometry of in situ hybridization histochemistry autoradiograms. DL= dorsolateral putamen. DM= dorsomedial putamen. Post-hoc: * p<0.05 vs saline. Data are expressed as relative dpm ± S.E.M.

Chapter 4.

Homer splicing variants modulation in cortico-subcortical regions by dopamine D2 antagonists, partial agonists, and indirect agonist: implication for glutamate in antipsychotics action.

Rationale.

Several lines of evidence support the hypothesis that the dysregulation of dopamine systems in schizophrenia may be associated with a persistent dysfunction of glutamate system (Olney et al., 1999; Goff et al., 2001). Moreover, genes that have been reported to be altered in schizophrenia play essential roles in glutamatergic neurotransmission (Harrison and Weinberger, 2005). In addition, recent studies reported that the inhibition of dopamine D_2 receptors by typical and atypical antipsychotics may influence glutamate neurotransmission (Leveque et al., 2000).

Thus, the analysis of the expression patterns of genes linked both to dopamine and glutamate neurotransmission may provide an interesting tool to understand the mechanism of action of antipsychotic drugs.

The present study aimed to give a more complete picture of *Homer* genes expression by investigating, in both acute and chronic paradigms, its modulation by compounds differently impacting the dopaminergic function: the typical antipsychotic haloperidol, the atypical antipsychotic clozapine, the dopamine transporter (DAT) inhibitor GBR12909, and the novel dopamine partial agonist aripiprazole.

Dopamine partial agonists, such as aripiprazole, represent a novel therapeutic approach to schizophrenia. As other partial agonists, aripiprazole binds to D_2 dopamine receptors with high affinity and low intrinsic activity (Kikuchi et al., 1995) and exerts antagonistic or agonistic effects at D_2 receptors depending on their level of basal activation (Burris et al.,

2002). Aripiprazole, indeed, has been demonstrated to display D_2 receptors antagonist activity in conditions of dopaminergic hyperactivity, e.g. blocking apomorphine-induced stereotypies (Semba et al., 1995), and D_2 receptors agonist effects in conditions of dopamine hypoactivity, e.g. reducing increased dopamine synthesis in reserpinized rats (Kikuchi et al., 1995). However, the molecular mechanisms involved in the dopamine partial-agonist activity of aripiprazole are not completely known at present. Recent theorizations suggest that the peculiar functional characteristics of aripiprazole at dopamine D_2 receptors could be ascribed, rather than a classical partial agonist activity, to a mechanism of "functional selectivity" (Lawler et al., 1999). Specifically, the agonistic or antagonistic properties of aripiprazole at D_2 receptors would depend upon the cellular location and the signalling pathways (i.e. G-proteins and downstream effectors) of the targeted D_2 receptors (Shapiro et al., 2003; Urban et al., 2007). These features may explain why aripiprazole could act as an antagonist in subcortical regions of schizophrenic patients, where a hyperdopaminergy is postulated (Breier et al., 1997), and as an agonist in the cortex, where it could counterbalance the putative hypodopaminergy (Laruelle et al., 2003; Guillin et al., 2007).
In this paradigm, we also evaluated whether a differential induction of *Homer1a* and *ania-3* splice variants may be elicited by the different compounds administered.
Moreover, the chronic treatment protocol, resembling the timing to obtain pharmacological effects with antipsychotics in clinical practice, set out to assess the impact on *Homer* expression of prolonged drug administration. Indeed, the chronic paradigm may help to discriminate tolerance effects potentially displayed by IEGs after repeated drug administration (Persico et al., 1993).

Results.

Acute administration experiment

Homer 1a and ania-3

Caudate-putamen

ANOVA revealed a statistically significant induction of both *Homer1a* and *ania-3* following aripiprazole at the lower dose of 12 mg/kg in all the subregions of the caudate-putamen: dorsomedial (DM), dorsolateral (DL), ventrolateral (VL), and ventromedial (VM). The *post hoc* test displayed a significant signal increase in DM and VM by ARI12 compared to VEH and in DL and VL by ARI12 compared to VEH and CLO (Figure 12b, e). Differently, there was no statistically significant induction of *Homer1a* and *ania-3* in rats treated with aripiprazole at the higher dose (30 mg/kg) in any subregion (Figure 12b, e).

Fig. 11

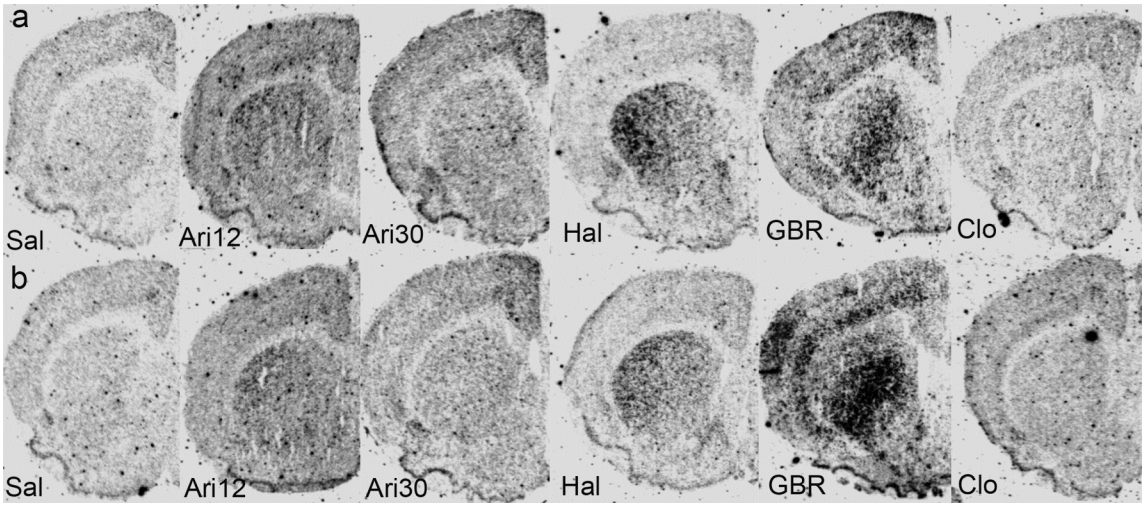


Figure 11. Autoradiographic film images of *Homer1a* (panel a) and *ania-3* (panel b) mRNA detected by means of *in situ* hybridization histochemistry (ISHH) in coronal brain sections after acute treatment with vehicle (VEH), aripiprazole 12 mg/kg (ARI12), aripiprazole 30 mg/kg (ARI30), haloperidol (HAL), GBR 12909 (GBR) or clozapine (CLO).

As reported in previous works (de Bartolomeis et al., 2002; Polese et al., 2002; Ambesi-Impiombato et al., 2007), haloperidol significantly induced *Homer1a* expression in all the subregions of caudate-putamen, where the *post hoc* test showed significant signal changes in DM and VM by HAL compared to VEH and CLO; in DL and VL by HAL compared to VEH, CLO and ARI30 (Figure 12b). Within the caudate-putamen, the pattern of *ania-3* expression induced by haloperidol resembled that of *Homer1a* in all the subregions (Figure 12e).

GBR12909 treated group displayed a statistically significant induction of *Homer1a* gene expression in all the subregions of caudate-putamen (Figure 12b) where the *post hoc* test indicated a significant signal increase in DM and VL by GBR compared to VEH, CLO and ARI30; in DL and VM by GBR compared to VEH and CLO. The pattern of *ania-3* expression in the caudate-putamen following GBR administration was similar to that of *Homer1a* (Figure 12e).

Clozapine administration induced no significant expression in the caudate-putamen of both splice variants of *Homer1* gene (Figure 12b, e).

Nucleus accumbens

Aripiprazole 30 mg/kg significantly increased *Homer1a*, but not *ania-3*, expression in the shell of nucleus accumbens compared to VEH (Figure 12c, f). Also, clozapine acute administration significantly increased *Homer1a* expression, but not *ania-3*, in the shell compared to VEH, as described in previous works (Polese et al., 2002) (Figure 12c, f). *Homer1a* and *ania-3* were significantly induced by haloperidol in both core and shell subregions (Figure 12c, f). At the *post hoc* test the HAL treated group showed a significant *Homer1a* signal increase compared to VEH and CLO in the core and compared to VEH in

the shell. *Ania-3* was significantly induced by HAL compared to VEH and CLO in both core and shell.

GBR12909 significantly induced *Homer1a* in core and shell compared to control (Figure 12c). As opposed to *Homer1a*, no significant induction of *ania-3* was detected in the nucleus accumbens by GBR12909 (Figure 12f).

Cortex

There was a significant induction of *Homer1a* in both the outer (FCo) and the inner layers (FCi) of the frontal cortex by GBR compared to VEH and HAL and in the cingulate cortex (Cg) by GBR compared to VEH (Figure 12a). *Ania-3* was significantly increased in the FCo by GBR compared to VEH, CLO and HAL and in the FCi by GBR compared to all the other treatments (Figure 12d).

Aripiprazole 30 mg/kg significantly induced *Homer1a* in the cortex (Figure 12a) and the *post hoc* test showed a significant signal increase in FCo by ARI30 compared to VEH and HAL, in FCi by ARI30 compared to VEH, and in Cg by ARI30 compared to VEH, HAL, ARI12 and CLO.

No significant induction of *Homer1a* was detected in the parietal cortex (PC), whereas *ania-3* was strongly upregulated by GBR in both the outer (PCo) and the inner layers (PCi) of the parietal cortex compared to all the other treatments (Figure 12a, d).

There was no statistically significant expression in the cortex for both splicing variants of *Homer1* gene following clozapine and haloperidol administration (Figure 12a, d).

Fig. 12

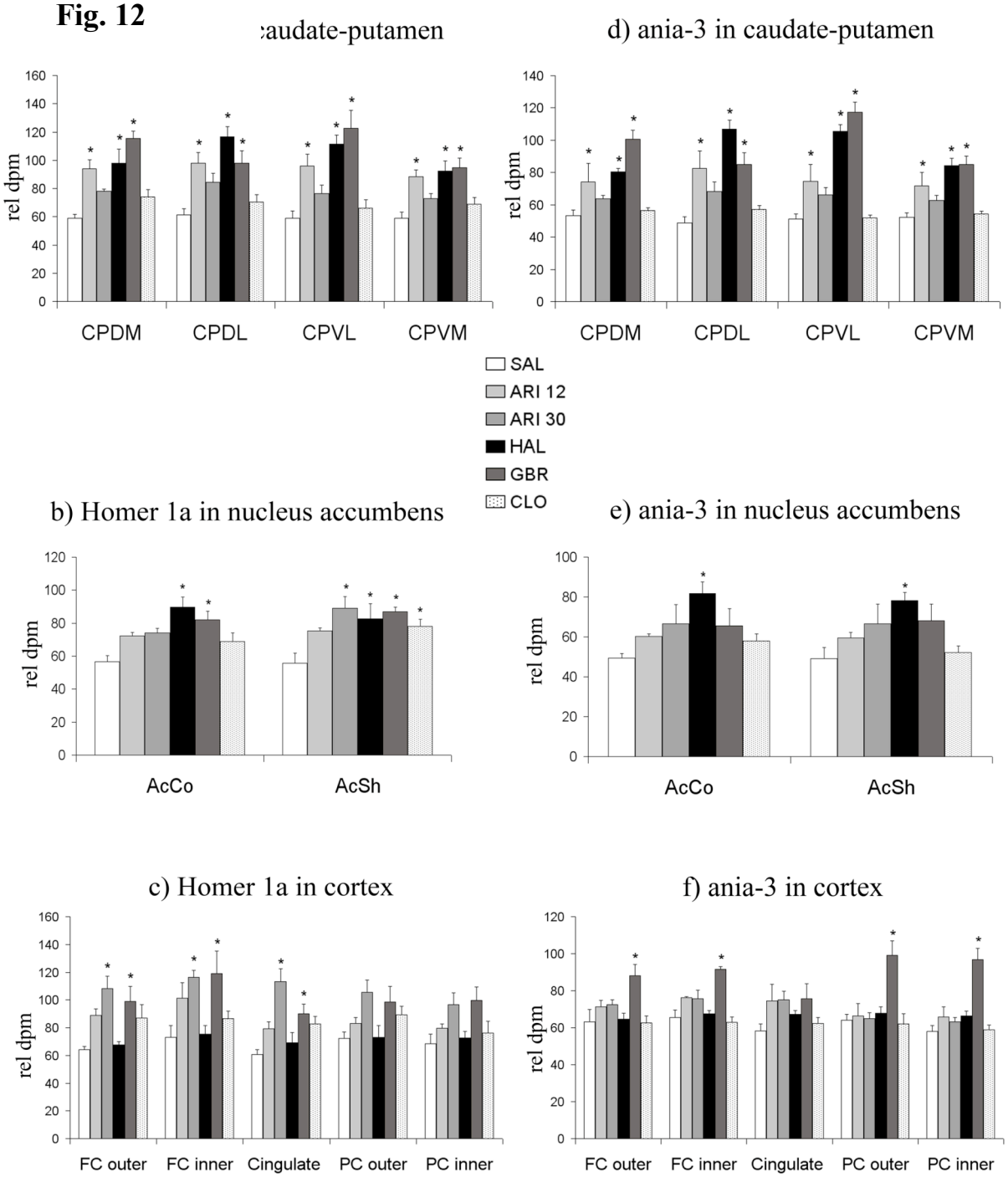


Figure 12. *Homer1a* and *ania-3* mRNA levels after acute treatment. Panels a, b and c: *Homer1a* mRNA levels in cortex, caudate-putamen and nucleus accumbens. Panels d, e and f: *ania-3* mRNA levels in cortex, caudate-putamen and nucleus accumbens. Data are reported in relative dpm as mean ± S.E.M. Tukey's *post hoc* test: * *vs.* VEH (ANOVA, $p<0.05$).

ACUTE TREATMENT

Probes	ARI 12	ARI 30	HAL	GBR	CLO	P-Value (ANOVA)	$F_{(df)}$-Value (ANOVA)
Homer1a							
Cortex							
FC outer	138.5±7.05	168.4±14.1*	105.4±3.41	153.9±16.82*	135.3±14.76	0.0029	$5.543_{(5,18)}$
FC inner	138.2±15.42	156.1±6.79*	103.01±8.18	162.49±22.53*	118.05±7.89	0.01	$4.077_{(5,18)}$
Cingulate ctx	131.2±8.03	186.9±15.73*	114.6±12.02	148.6±11.74*	136.9±8.51	0.0005	$7.74_{(5,18)}$
PC outer	115±6.11	145.8±12.5	100.8±11.77	136.5±15.1	123.6±8.41	0.0336	$3.118_{(5,18)}$
PC inner	116.5±9.97	141±12.26	106.3±6.35	145.3±14.46	111±12.36	0.0316	$3.171_{(5,18)}$
Caudate-putamen							
DM	159.4±10.5*	132.7±2.74	115.3±17.05*	195.7±8.82*	126±8.63	0.0001	$12.742_{(5,17)}$
DL	160±11.88*	137.9±10.03	190.3±11.92*	159.6±14.34*	114.8±8.68	0.0002	$9.598_{(5,17)}$
VL	163.3±14.07*	130±10.02	189.5±10.57*	208.1±22.01*	112.5±9.85	0.0001	$11.358_{(5,17)}$
VM	150.1±7.95*	123.8±5.66	156.9±12.01*	160.6±11.39*	116.5±8.71	0.0006	$7.687_{(5,17)}$
Nucleus accumbens							
Core	127.4±4.12	130.6±4.91	158.3±10.82*	145±8.93*	121.4±9.10	0.0019	$6.212_{(5,17)}$
Shell	134.8±3.24	159.5±12.48*	148±16.24*	155.7±5.12*	139.8±7.93*	0.0041	$5.306_{(5,17)}$
Ania-3							
Cortex							
FC outer	112.9±5.75	114.7±4.11	102.2±5.33	139.7±9.6*	99.3±5.88	0.0103	$4.012_{(5,21)}$
FC inner	116.5±0.90	115.5±7.47	103.1±2.88	140.1±2.10*	96±4.55	0.0001	$9.153_{(5,21)}$
Cingulate ctx	127.9±15.6	129±7.94	115.6±3.24	130.3±13.6	107.2±5.51	0.1218	$1.991_{(5,21)}$
PC outer	103.7±10.33	101.7±4.80	106±5.67	155±12.36*	96.8±8.87	0.0006	$6.932_{(5,21)}$
PC inner	113.4±9.62	109±3.95	114.3±4.78	166.9±10.27*	101.4±4.67	0.0001	$13.564_{(5,21)}$
Caudate-putamen							
DM	139.3±21.3*	119.7±4	150.7±3.92*	188.7±10.14*	105.5±3.55	0.0001	$12.160_{(5,21)}$
DL	169.9±21.68*	140.5±11.94	219.8±11.57*	174.6±15.26*	117.4±5.18	0.0001	$11.941_{(5,21)}$
VL	145.3±20.46*	129.1±8.95	205.9±7.98*	228.7±12.67*	101.1±3.77	0.0001	$26.147_{(5,21)}$
VM	137.3±16.28*	120.1±5.95	161.6±8.39*	162.8±10.39*	104±3.27	0.0001	$10.378_{(5,21)}$
Nucleus accumbens							
Core	121.8±2.42	134.6±19.33	165.1±11.84*	132.6±17.23	117.2±7.25	0.0122	$3.866_{(5,21)}$
Shell	121.3±5.73	135.7±20.36	159.3±8.71*	138.6±17.4	106.3±6.74	0.0228	$3.326_{(5,21)}$

Table 1. mRNA levels of *Homer 1a* and *ania-3* after acute treatments, expressed as percent of VEH relative dpm mean value ± SEM and presented for each brain region analyzed with the relative ANOVA values. FC = frontal cortex; PC = parietal cortex; DM = dorsomedial; DL = dorsolateral; VL = ventrolateral; VM = ventromedial. * = statistical significance *vs.* VEH at the Tukey's *post hoc* test

Chronic administration experiment

Homer 1a and ania-3

Caudate-putamen

ANOVA revealed a significant induction of *Homer1a* expression by aripiprazole in the lateral subregions of the caudate-putamen (Figure 14b), where the *post hoc* test showed a significant signal increase in DL and VL by ARI12 compared to VEH and CLO.

Fig. 13

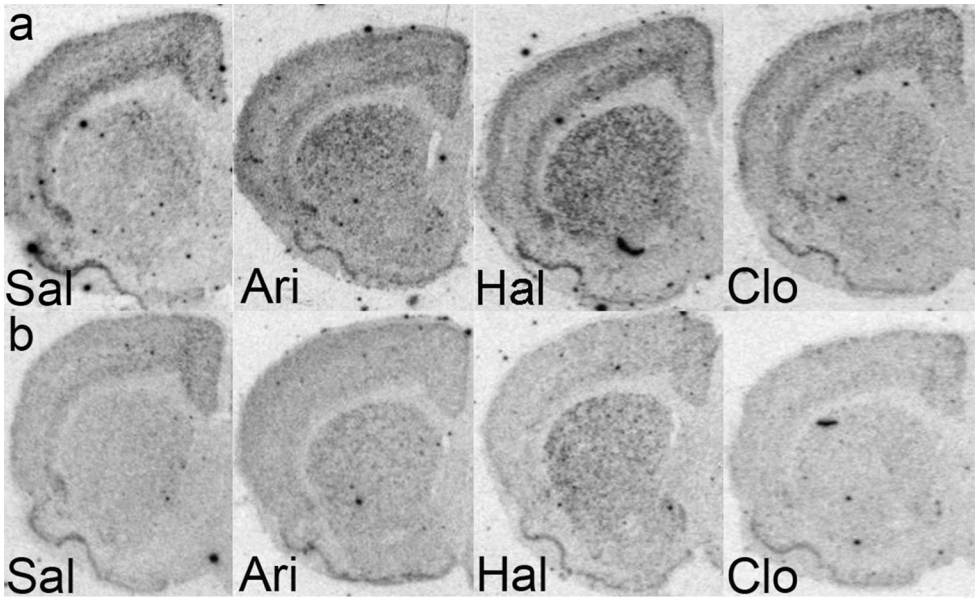


Figure 13. Autoradiographic film images of *Homer1a* and *ania-3*. Autoradiographic film images of *Homer1a* (panel a) and *ania-3* (panel b) mRNA detected by means of *in situ* hybridization histochemistry (ISHH) in coronal brain sections after chronic treatment with saline (VEH), aripiprazole (ARI), clozapine (CLO) or haloperidol (HAL).

Homer1a was significantly upregulated in the caudate-putamen following haloperidol chronic treatment (Figure 14b). The *post hoc* test displayed a significant signal increase in all caudate-putamen subregions by HAL compared to all the other treatments.

No significant changes in *Homer1a* signal were detected in the caudate-putamen following chronic clozapine administration.

As described for the acute paradigm, *ania-3* followed, in the chronic protocol, the same pattern of signal induction as *Homer1a* in the caudate-putamen (Figure 14e).

Nucleus accumbens

There was a statistically significant upregulation of *Homer1a* in the nucleus accumbens core by HAL compared to VEH and CLO and of *ania-3* by HAL compared to all the other treatments (Figure 14c, f). No significant changes were observed in both *Homer1a* and *ania-3* expression in the nucleus accumbens following all the other treatments.

Cortex

Homer1a and *ania-3* expression was differently modulated in the subregions of the cortex. *Homer1a* was significantly downregulated in FCi and Cg by aripiprazole 12 mg/kg and clozapine compared to control, and in PCi by CLO compared to VEH (Figure 14a). Differently, *ania-3* gene expression did not show any significant change in the same regions, although it followed a similar trend as *Homer1a* (Figure 14d).

Haloperidol did not give rise to any significant signal change of both *Homer1a* and *ania-3* in the cortex (Figure 14a, d).

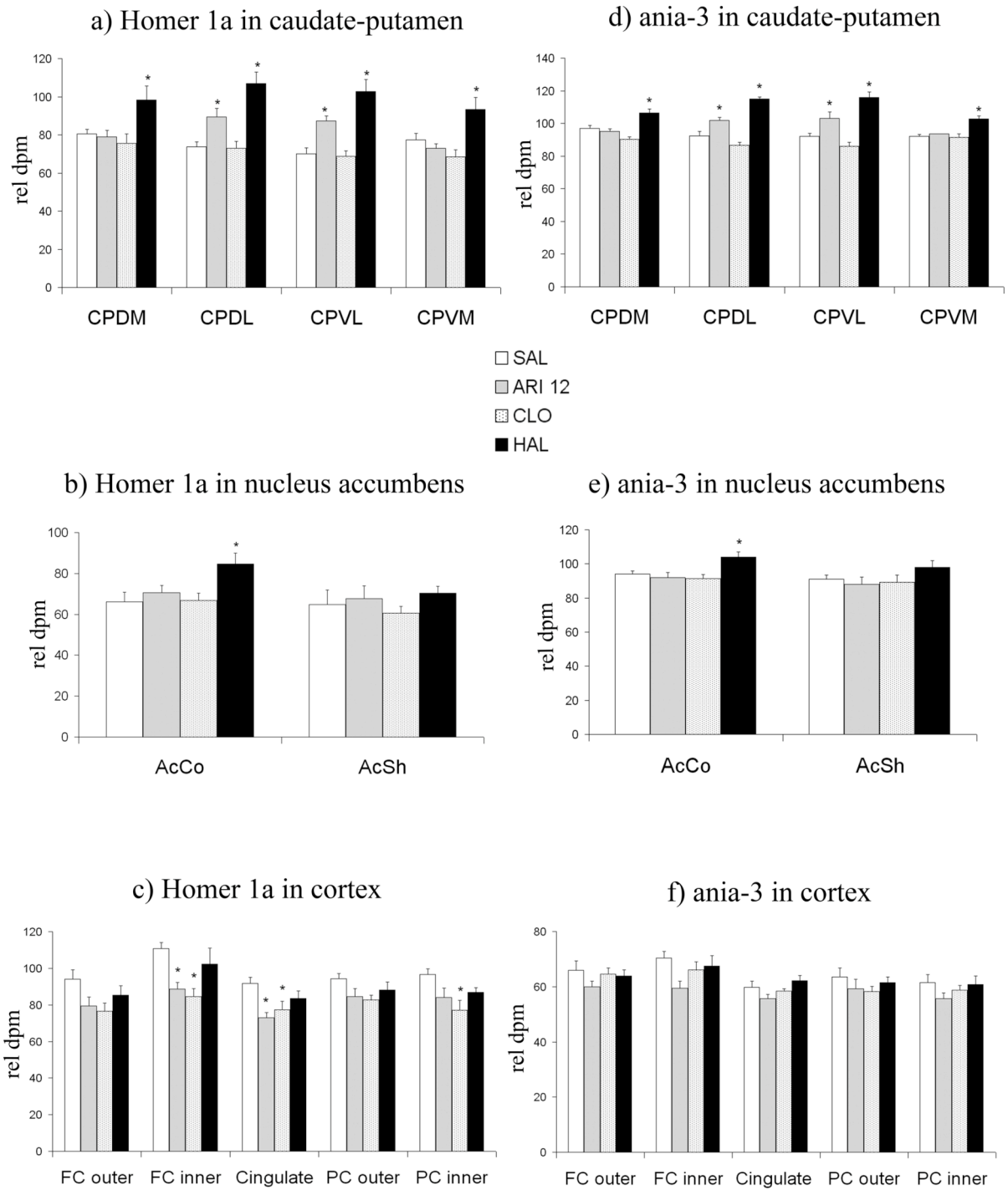


Figure 14. *Homer1a* and *ania-3* mRNA levels after chronic treatment. Panels a, b and c: *Homer1a* mRNA levels in cortex, caudate-putamen and nucleus accumbens. Panels d, e, and f: *ania-3* mRNA levels in cortex, caudate-putamen and nucleus accumbens. Data are reported in relative dpm as mean ± S.E.M. Tukey's *post hoc* test: * *vs*. VEH (ANOVA, $p<0.05$).

Homer 1b/c

The *Homer1b/c* gene expression showed no statistically significant variations in the caudate-putamen and in the nucleus accumbens, as well as in the cortex, following the treatments administered (data not shown).

Dopamine D2 receptors

There was a trend toward the upregulation of the *dopamine D2 receptor* gene expression in all the striatal regions following chronic haloperidol and aripiprazole treatments, even if there were no statistically significant differences among experimental groups (data not shown). These results are consistent with previous reports (Hurley et al., 1996).

CHRONIC TREATMENT

Probes	ARI	HAL	CLO	P-Value ANOVA	$F_{(df)}$-Value ANOVA
Homer1a					
Cortex					
FC outer	84.50±5.12	90.77±5.38	81.41±4.66	0.1076	$2.409_{(3,15)}$
FC inner	80.1±3.23*	92.42±7.84	76.4±3.95*	0.0183	$4.568_{(3,15)}$
Cingulate ctx	79.5±3.10*	91.1±4.29	84.31±5*	0.0119	$5.171_{(3,15)}$
PC outer	89.64±4.71	93.64±4.43	87.82±2.68	0.1842	$1.835_{(3,15)}$
PC inner	86.98±5.28	89.9±2.84	79.98±5.48*	0.0366	$3.668_{(3,15)}$
Caudate-putamen					
DM	97.9±4.32	122.1±8.88*	93.6±6.17	0.0189	$4.526_{(3,15)}$
DL	121.1±5.89*	144.8±8*	98.7±4.8	0.0001	$13.710_{(3,15)}$
VL	124.9±3.5*	146.8±8.99*	98.2±4.16	0.0001	$15.305_{(3,15)}$
VM	94.3±3.03	120.4±8.28*	88.3±4.69	0.0059	$6.212_{(3,15)}$
Nucleus accumbens					
Core	106.8±5.37	128.1±8*	101.1±5.3	0.0316	$3.852_{(3,15)}$
Shell	104.7±9.65	108.8±4.92	93.7±4.98	0.6536	$0.553_{(3,15)}$
Ania-3					
Cortex					
FC outer	90.9±3.21	96.8±3.40	97.8±3.59	0.4238	$0.987_{(3,16)}$
FC inner	84.4±3.54	95.9±5.41	94±3.97	0.0968	$2.487_{(3,16)}$
Cingulate ctx	93.2±2.64	103.9±3.38	97.6±1.63	0.0989	$2.502_{(3,16)}$
PC outer	93.3±5.44	96.7±3.18	91.5±2.97	0.5305	$0.764_{(3,16)}$
PC inner	90.6±3.27	99±4.87	95.49±2.94	0.3814	$1.091_{(3,16)}$
Caudate-putamen					
DM	98.1±1.58	109.8±2.42*	92.9±1.78	0.0001	$13.746_{(3,15)}$
DL	110.1±1.94*	124.4±1.52*	93.8±2.12	0.0001	$38.038_{(3,15)}$
VL	111.7±4.18*	125.7±3.54*	93.31±2.56	0.0001	$22.555_{(3,15)}$
VM	101.7±0.18	111.8±1.84*	99.4±2.24	0.0003	$11.946_{(3,15)}$
Nucleus accumbens					
Core	97.8±3.20	110.5±3.42*	97±2.73	0.0128	$5.063_{(3,15)}$
Shell	96.8±4.41	107.55±4.47	97.8±4.63	0.2773	$1.415_{(3,15)}$

Table 2. mRNA levels of *Homer1a and ania-3* after chronic treatments, expressed as percent of VEH relative dpm mean value ± SEM and presented for each brain region analyzed with the relative ANOVA values. FC = frontal cortex; PC = parietal cortex; DM = dorsomedial; DL = dorsolateral; VL = ventrolateral; VM = ventromedial. * = statistical significance *vs.* VEH at the Tukey's *post hoc* test.

Topography of *Homer1a* expression.

Acute experiment. According to other observations (see following chapters), vehicle showed a homogeneous signal distribution (Fig. 15). No significant differences among subregions were detected at the ANOVA ($p > 0.05$). Clozapine also exhibited a homogeneous signal distribution with no significant differences at the ANOVA, although a slight prominence of signal expression could be noted in the shell of the accumbens (Fig. 15). This pattern may correlate with the putative limbic-selectivity of clozapine and agrees with previous studies describing a preferential IEGs expression in the shell of the accumbens by this compound (REF). *Homer1a* distribution by aripiprazole was obviously different at the two doses used. The lower dose (12 mg/kg) gave raise to a signal distribution which highly resembled those by haloperidol, while the 30 mg/kg dose showed a trend toward the attenuation of signal peaks and a stronger expression in the shell of the accumbens (Fig. 15). *Homer1a* induction by 12mg/kg aripiprazole was significant ($p<0.0001$) in the lateral and medial putamen compared with the nucleus accumbens. However, no significant differences were detected between lateral and medial subregions. *Homer1a* induction by 30mg/kg aripiprazole showed a weak trend to significance ($p=0.0417$), with expression in the dorsolateral putamen and the shell of the accumbens being significantly higher than that in the dorsomedial putamen and the core of the accumbens.

Haloperidol exhibited its characteristic “three-steps” pattern of signal distribution, with significant higher levels of *Homer1a* expression in the lateral subregions and with lower levels in the accumbens. Intermediate values of expression were observed in the medial subregions (Fig. 15).

Taken together, these observations let hypothesize that the profile of *Homer1a* signal distribution may be strictly related to dopaminergic perturbation exerted by a compound. In

particular, the higher dopamine perturbation seems to be associated with higher levels of gene expression in the lateral subregions and lower levels in the accumbens. Thus, profiling of *Homer1a* expression may be a tool to characterize the actual impact on dopamine transmission by a compound.

GBR12909 showed a peculiar pattern of signal distribution, with significant higher values of expression in the dorso-medial and the ventro-lateral striatum among all other subregions (Fig. 15).

Chronic experiment. Signal distribution resembled those observed in the acute experiment. Vehicle distribution was homogeneous although a slight induction could be observed in medial regions. Clozapine distribution, as well, was homogeneous. Aripiprazole and haloperidol exhibited the same distribution pattern described in the acute experiment (Fig. 15).

Fig. 15

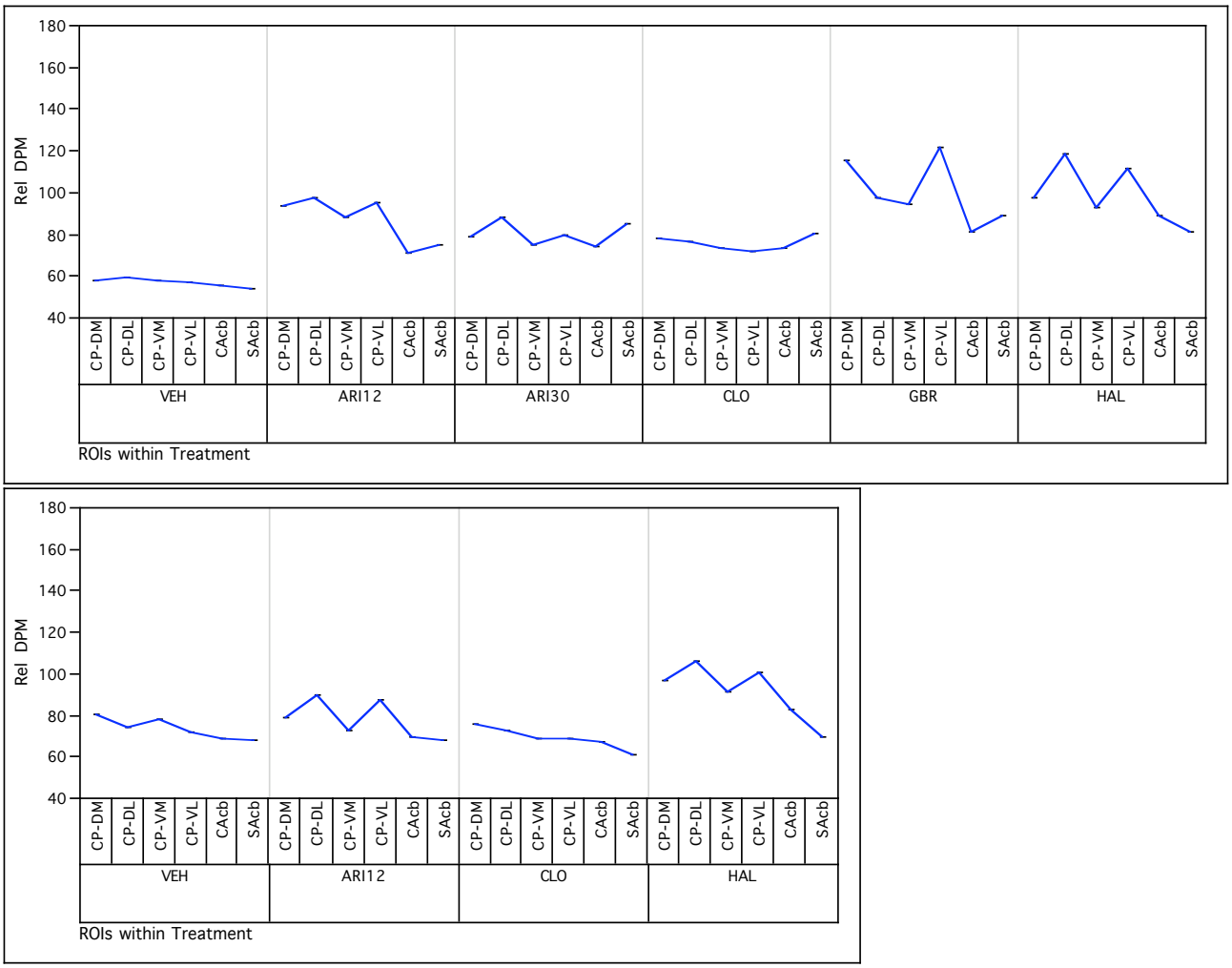


Figure 15. Topographical distribution of *Homer1a*. *Homer1a* expression has been evaluated related to signal distribution among ROIs within each treatment in both the acute (upper panel) and the chronic (lower panel) paradigm Data are reported as averaged relative d.p.m. (Rel DPM). For clarity matter, S.E.M. bars have not been traced.

Chapter 5.

Dopamine receptor subtype dynamic contribution to the induction of Homer: insights into antipsychotics molecular mechanism of action.

Rationale.

Both glutamate and dopamine have been observed to induce *Homer1a* expression. Glutamate agonists as kainate or NMDA elicit *Homer1a* expression (Ango et al., 2000; Kato et al., 1997; Sato et al., 2001). The antagonist of NMDA receptors, ketamine, induces *Homer1a* (Iasevoli et al., 2007), most likely as a result of secondary hyperglutamatergy on ionotropic receptors. On the other hand, *Homer1a* is induced by indirect dopamine agonists, such as cocaine (Brakeman et al., 1997; Swanson et al., 2001; Zhang et al., 2007), amphetamines (Yano et al., 2006), and cocaine-like psychostimulants (Ambesi-Impiombato et al., 2007). *Homer1a* induction has also been described by SKF38393, a selective and direct dopamine D_1 receptors agonist (Yamada et al., 2007).

However, little is known about changes in gene expression as a consequence of antagonism at dopamine receptors. We demonstrated that *Homer1a* is consistently induced by antipsychotic drugs. The prototype typical antipsychotic haloperidol induced the gene in all subregions of the caudate-putamen (de Bartolomeis et al., 2002), while two atypical antipsychotics, clozapine and quetiapine, produced little or no expression of the gene (Ambesi-Impiombato et al., 2007). Moreover, the dopamine D_2 receptor partial agonist, aripiprazole, induced the gene significantly more when given at a lower (12 mg/kg) than a higher (30 mg/kg) dosages (Tomasetti et al., 2007). Since all effective antipsychotics share the property to block dopamine D_2 receptors, we hypothesized that antagonism at dopamine D_2 receptors may be responsible for *Homer1a* induction by antipsychotics. However, most antipsychotics interact with several dopaminergic receptors (Arnt and Skarsfeldt, 1998),

raising the issue of whether *Homer1a* induction may be triggered by non-D_2 dopaminergic receptors.

In this study, we aimed to confirm that *Homer1a* may be induced by dopamine receptors blockade and to characterize which subtype of dopamine receptors may trigger the expression of the gene. Hence, we tested *Homer1a* induction by selective antagonists at dopamine receptors (D_1, D_2, D_3, D_4 subtypes) and compared with that by haloperidol and the putative antipsychotic terguride, which behaves as a partial agonist at dopamine D_2 receptors. The constitutive isoform, Homer1b, and the metabotropic glutamatergic receptor, mGluR5, interact with Homer1a and are involved in Homer-mediated signaling (Kammermeier and Worley, 2007; Mao et al., 2005; Ronesi and Huber, 2008) and in synaptic plasticity processes (Sala et al., 2005; Vanderklish and Edelman, 2002). In the present study we evaluated also the expression of these genes by haloperidol, terguride, and dopamine receptors selective antagonists.

Results.

Caudate-putamen

The *post hoc* test showed a significant *Homer1a* induction by HAL in all subregions compared both to controls and selective antagonists of dopamine receptors (Fig. 16, Table 3). L-741,626 showed a pattern of *Homer1a* induction closely similar to that of haloperidol, increasing *Homer1a* signal in all caudate-putamen subregions as compared to controls and to all other dopamine receptors antagonists (Fig. 16). A significant increase of *Homer1a* expression as compared to VEH was observed by the D_2/D_3 receptors partial agonist TER in the lateral caudate-putamen and by SCH-23390 in the VL caudate-putamen (Fig. 16).

Nucleus Accumbens

The *post hoc* test indicated that HAL significantly induced *Homer1a* expression compared to all the other treatments in both CAcb and SAcb (Fig. 16, Table 3). *Homer1a* was significantly induced by SCH-23390 and L-741,626 in both accumbal subregions compared to VEH (Fig. 16).

Cortex

A significant *Homer1a* induction by SCH-23390 was observed in all cortical subregions, with the exception of the ACC (Fig. 16, Table 3). A significant induction was also elicited by L-741,626 and U-99194 in the MAC and the MC (Fig. 16). On the contrary, terguride and haloperidol decreased *Homer1a* expression compared to basal levels in the MC. It is worth noting that SCH-23390 and L-741,626 induced *Homer1a* expression significantly more than haloperidol and terguride in the ACC, the MAC, and the MC (Fig. 16).

Hippocampus

In the CA1, SCH-23390 induced *Homer1a* expression significantly higher than L-745,870. In the CA3, VEH group induced significantly the gene compared to L-745,870 (Fig. 19, Table 4). No difference is found in both the CA2 and DG subregions among groups.

Fig. 16

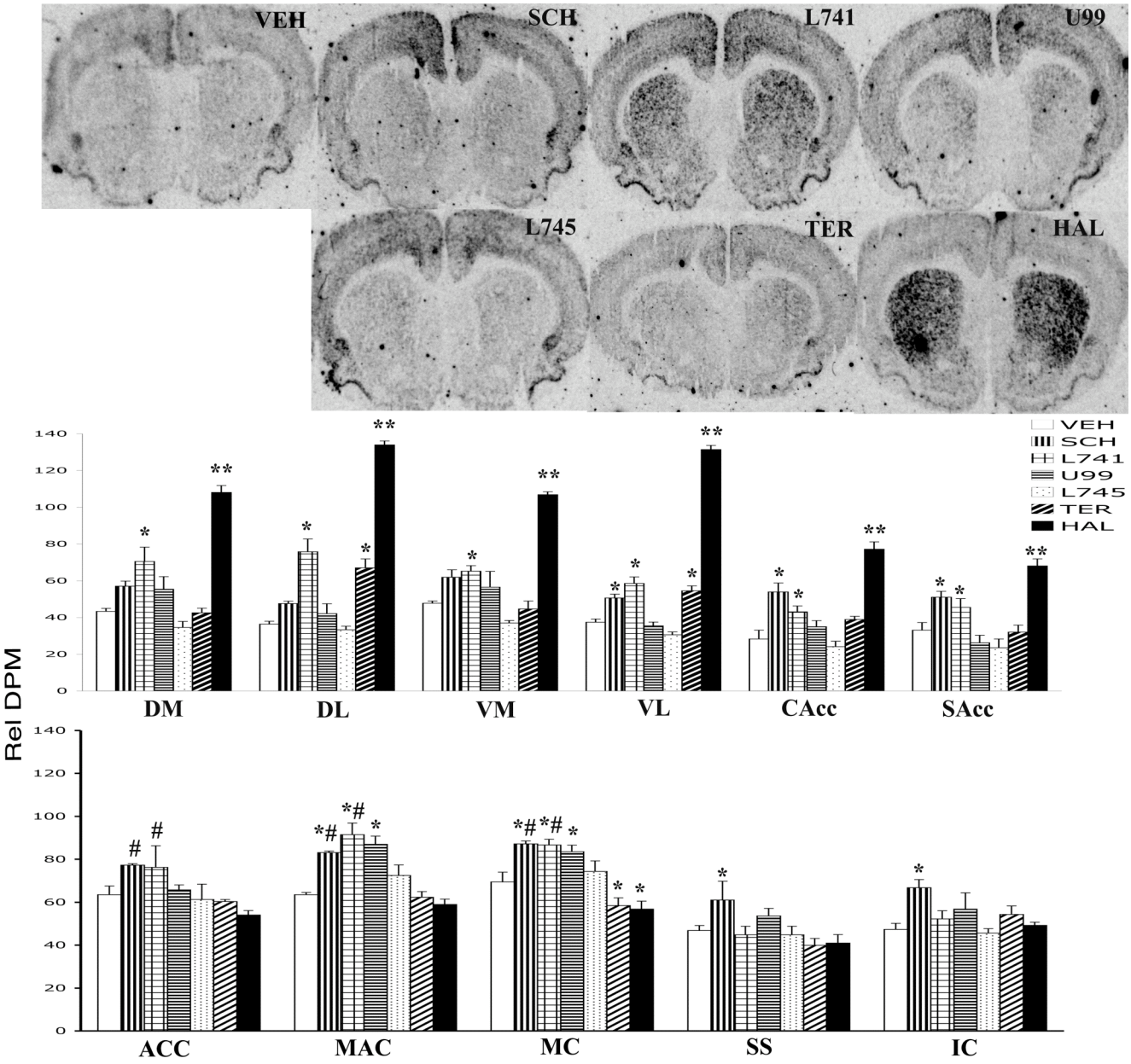


Figure 16. *Homer 1a* mRNA expression by dopamine receptors antagonists. Upper panel: autoradiographic film image of *Homer 1a* detected by means of ISHH in rat coronal brain sections after treatment with control (VEH), SCH-23390 (SCH), L-741,626 (L741), U-99194 (U99), L-745,870 (L745), terguride (TER), and haloperidol (HAL). Treatments are outlined in the upper right corner of each section. Intermediate panel: *Homer1a* levels in caudate-putamen and nucleus accumbens. Values are expressed in relative dpm as mean ± SEM. Student-Neumann-Keuls post hoc test: ** = *vs.* all treatments; * = *vs.* controls. Lower panel: *Homer 1a* levels in the cortex. Student-Neumann-Keuls post hoc test: * = *vs.* controls; #= *vs.* TER and HAL.

	SCH-23390	L-741,626	U-99194	L-745,870	Terguride	Haloperidol	ANOVA
Striatum							
DM		121.15±3.7				165.33±3.1	$p < 0.0001$, $F_{6,22}=31.613$
DL		130.61±2.07			121.71±1.36	224.44±1.65	$p < 0.0001$, $F_{6,22}=111.731$
VM		111.07±0.97				156.29±0.86	$p < 0.0001$, $F_{6,22}=48.936$
VL	109.12±1.21	115.83±2.13			112.37±1.61	221.51±4.06	$p < 0.0001$, $F_{6,22}=234.308$
CAcb	120.32±1.61	110.96±0.97				143.61±1.65	$p < 0.0001$, $F_{6,22}=28.618$
SAcb	116.02±0.95	111.16±1.35				131.52±1.37	$p < 0.0001$, $F_{6,22}=17.056$
Hippocampus							
CA1							$p = 0.0394$, $F_{6,20}=2.777$
CA2							$p > 0.05$, $F_{6,20}=2.939$
CA3				*80.05±1.89*			$p = 0.0161$, $F_{6,20}=3.477$
DG							$p > 0.05$, $F_{6,20}=2.310$
Cortex							
ACC							$p = 0.0297$, $F_{6,22}=3.187$
MAC	131.14±0.59	144.11±5.02	137.21±3.56				$p < 0.0001$, $F_{6,22}=15.947$
MC	125.55±0.31	124.88±0.57	120.38±0.65		*84.19±0.78*	*81.86±0.79*	$p < 0.0001$, $F_{6,22}=13.145$
SS	130.38±3.83						$p < 0.0275$, $F_{6,23}=3.194$
IC	141.25±1.33						$p < 0.0265$, $F_{6,23}=3.227$

Table 3. Summary table of results from experimental *Homer1a* profiles. The table summarizes significant changes *vs.* controls of *Homer1a* expression in striatum, hippocampus, and cortex after acute treatment by selective antagonists of dopamine receptors, terguride and haloperidol. Data are expressed as a percentage of vehicle relative d.p.m. mean value ±S.E.M. and listed by brain region analyzed, along with the relative ANOVA p and F(df) values. Increases in gene expression are shaded in dark grey and decreases in light grey.

Homer1b

Caudate-putamen

At the *post hoc* test both L-741,626 and HAL significantly decreased *Homer1b* expression as compared to VEH in the VL caudate-putamen (Fig. 17, Table 4). L-745,870 and terguride significantly decreased *Homer1b* expression with respect to VEH in the VM and the VL caudate-putamen (Fig. 17). No significant differences among groups were observed in the dorsal subregions.

Nucleus Accumbens

In the CAcb, *Homer1b* was significantly induced by SCH-23390 as compared to all other treatments (Fig. 17, Table 4). In the SAcb, *Homer1b* expression was significantly decreased by L-741,626, L-745,870, terguride and HAL compared to SCH-23390 and VEH (Fig. 17).

Cortex

HAL and TER decreased *Homer1b* expression compared to the VEH in almost all subregions, with the exception of the ACC where only a borderline trend toward significance was observed (Fig. 17, Table 4). In the MC, the SS, and the IC, L-741,626 decreased *Homer1b* expression compared to the VEH (Fig. 17). Gene expression was reduced in comparison to basal amounts by L-745,870 in the MC and the SS and by SCH-23390 in the MC only (Fig. 17).

Hippocampus

No significant differences among groups in *Homer1b* expression were found at the ANOVA test in any hippocampal subregion (data not shown).

Fig. 17

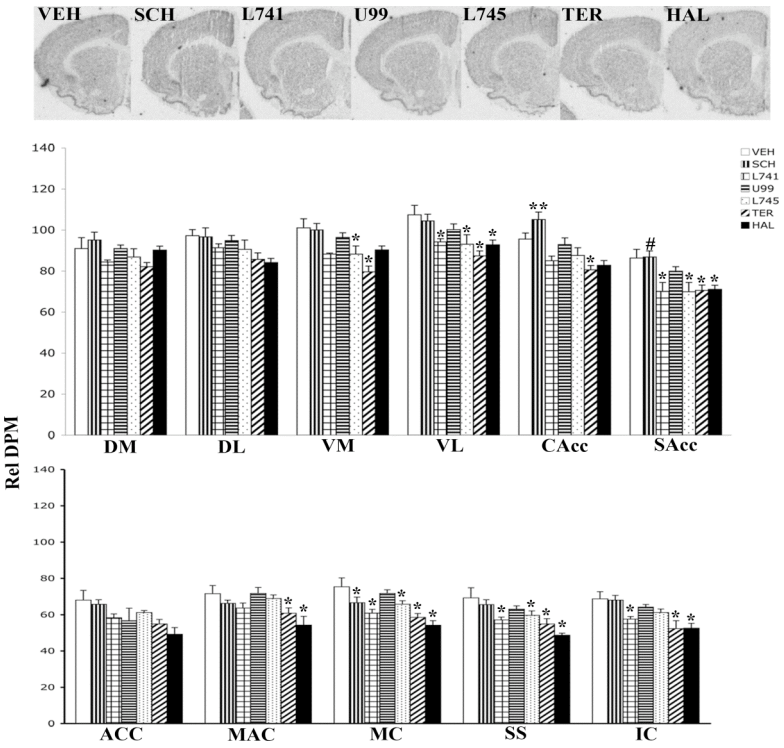


Figure 17. ***Homer1b*** **mRNA expression by dopamine receptors antagonists.** Upper panel: autoradiographic film image of *Homer 1b* detected by means of ISHH in rat coronal brain sections after treatment with control (VEH), SCH-23390 (SCH), L-741,626 (L741), U-99194 (U99), L-745,870 (L745), terguride (TER), and haloperidol (HAL). Treatments are outlined in the upper right corner of each section. Intermediate panel: *Homer1b* levels in caudate-putamen and nucleus accumbens. Values are expressed in relative dpm as mean ± SEM. Student-Neumann-Keuls post hoc test: ** = *vs*. all treatments; * = *vs*. controls; # = *vs*. L,741,626, L-745,870, TER, and HAL. Lower panel: *Homer 1b* mRNA levels in the cortex. Student-Neumann-Keuls post hoc test: * = *vs*. controls.

mGluR5

Caudate-putamen

Expression of *mGluR5* was increased by SCH-23390 and L745,870 compared to VEH in the DM putamen (Fig. 18, Table 4). In the same subregion, TER decreased significantly gene expression compared to VEH (Fig. 18). SCH-23390 gave raise to *mGluR5* expression levels significantly higher than that elicited by HAL, TER, and L-741,626 in all subregions (Fig. 18), with the only exception of the DL putamen where only a trend toward significance *vs*. L-741,626 was recognized. L-745,870 induced gene levels significantly more than HAL and TER in all subregions and L-741,626 in the DM and the DL putamen (Fig. 18). In the DL putamen, U-99194 induced significantly *mGluR5* expression compared to HAL and TER (Fig. 18).

Nucleus Accumbens

In the core of the accumbens SCH-23390 induced significantly *mGluR5* expression compared to VEH (Fig. 18, Table 4). In both core and shell of the nucleus accumbens, SCH-23390 induced significantly *mGluR5* expression compared to L-741626, HAL, and TER (Fig. 4). In the CAcb, also L-745,870 induced significantly gene expression compared to L-741626, HAL, and TER (Fig. 18).

Cortex

Levels of *mGluR5* expression in the cortex were negligible in all treatment groups and were not quantitable.

Hippocampus

SCH-23390 significantly induced *mGluR5* expression compared to VEH and all other treatments (with the exception of U-99194 in the CA1 subregion) in the CA1, the CA3, and the DG (Fig. 19, Table 4). In the CA2 region, a significant *mGluR5* induction by SCH-23390 compared to L-741,626 and HAL was observed.

Fig. 18

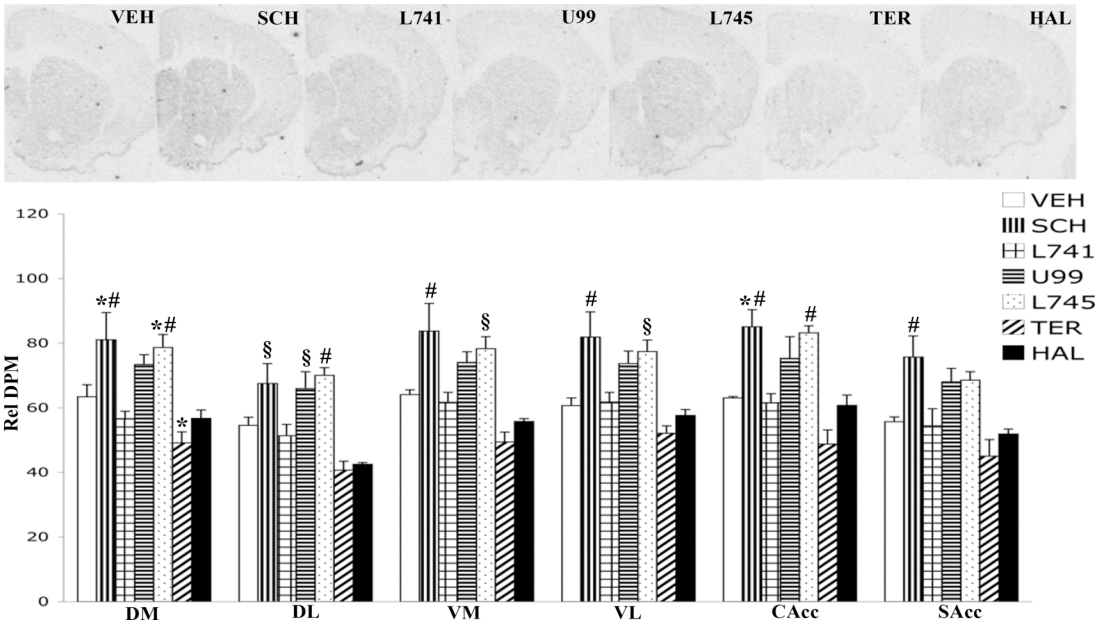


Figure 18. ***mGluR5* mRNA expression by dopamine receptors antagonists.** Upper panel: autoradiographic film image of *mGluR5* mRNA detected by means of ISHH in rat coronal brain sections after treatment with control (VEH), SCH-23390 (SCH), L-741,626 (L741), U-99194 (U99), L-745,870 (L745), terguride (TER), and haloperidol (HAL). Treatments are outlined in the upper right corner of each section. Lower panel: *mGluR5* mRNA levels in the caudate-putamen and nucleus accumbens. Values are expressed in relative dpm as mean ± SEM. Student-Neumann-Keuls post hoc test: * = *vs*. controls; # = *vs*. L-741,626, HAL, and TER; § = *vs*. HAL and TER.

Fig. 19

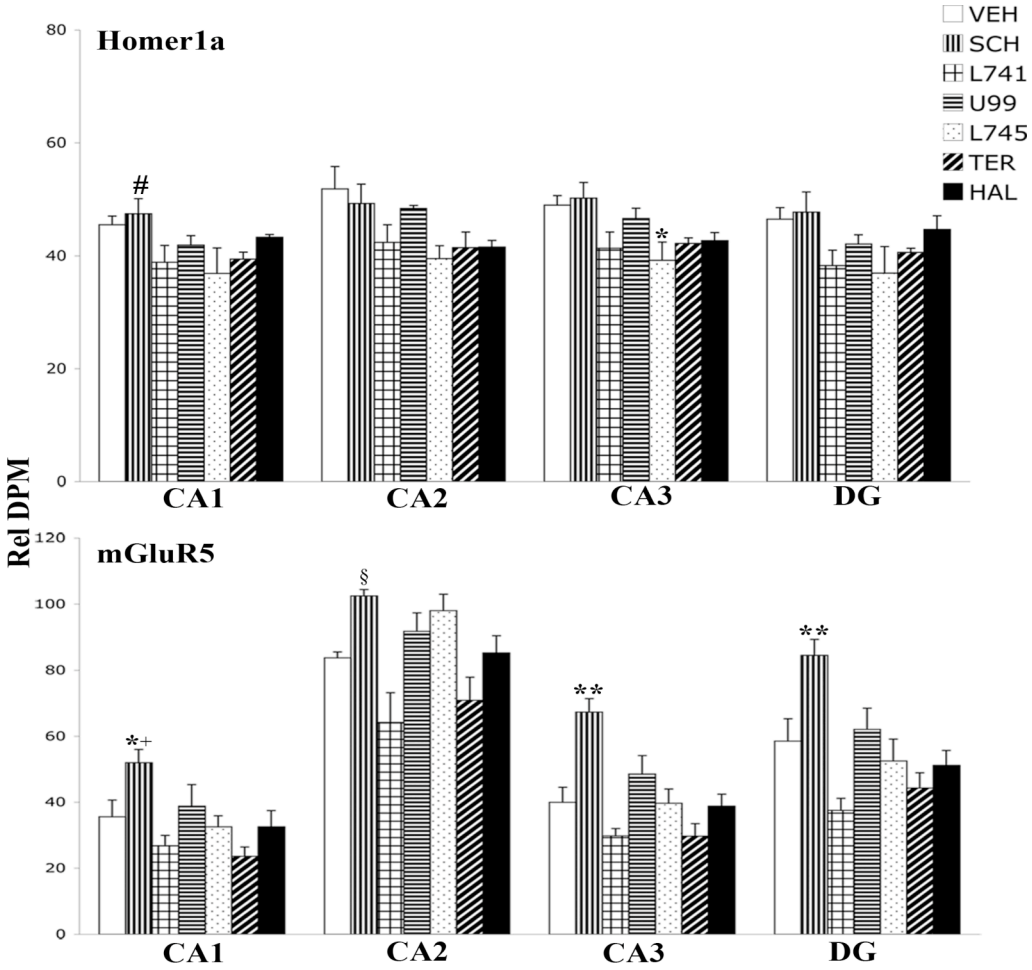


Figure 19. Hipppocampal expression of *Homer1a* and *mGluR5* by dopamine receptors antagonists. Upper panel: Homer1a mRNA levels in the hippocampus. Values are expressed in relative dpm as mean ± SEM. Student-Neumann-Keuls post hoc test: # = *vs.* L-745,870; * = *vs.* controls. Lower panel: *mGluR5* mRNA levels in the hippocampus. Student-Neumann-Keuls post hoc test: ** = *vs.* all treatments; * = *vs.* controls; + = *vs.* L-741,626, L-745,870, HAL and TER; § = *vs.* L-741,626 and HAL.

	SCH-23390	L-741,626	U-99194	L-745,870	Terguride	Haloperidol	ANOVA
Homer1b							
Striatum							
DM							$p > 0.05$, $F_{6,20}=2.074$
DL							$p > 0.05$, $F_{6,20}=2.962$
VM				*87.37±0.91*	*78.85±0.61*		$p = 0.0004$, $F_{6,20}=7.078$
VL		*87.84±0.32*		*86.75±0.99*	*81.48±0.51*	*86.46±0.48*	$p = 0.0022$, $F_{6,20}=5.223$
CAcb	110.07±1.21				*84.41±0.68*		$p = 0.0002$, $F_{6,18}=8.604$
SAcb		*81.22±1.02*		*81.03±1.06*	*81.74±0.62*	*82.32±0.49*	$p = 0.0025$, $F_{6,18}= 5.367$
Cortex							
ACC							$p = 0.052$, $F_{6,25}=2.598$
MAC					*84.95±0.66*	*75.81±1.09*	$p = 0.0142$, $F_{6,25}=3.635$
MC	*88.46±0.61*	*80.89±0.42*		*87.39±0.38*	*77.69±0.43*	*71.93±0.51*	$p = 0.0003$, $F_{6,25}=7.429$
SS		*82.53±0.27*		*86.11±0.45*	*79.36±0.51*	*70.37±0.21*	$p = 0.0006$, $F_{6,25}=6.691$
IC		*83.81±0.36*			*76.09±1.13*	*76.48±0.68*	$p = 0.0014$, $F_{6,25}=5.841$
mGluR5							
Striatum							
DM	127.83±2.24			124.09±1.07	*77.46±0.92*		$p = 0.0001$, $F_{6,17}=9.291$
DL							$p = 0.0001$, $F_{6,17}=9.735$
VM							$p < 0.0001$, $F_{6,17}=9.976$
VL							$p = 0.0003$, $F_{6,17}=8.192$
CAcb	134.86±12.02						$p = 0.0003$, $F_{6,16}=8.727$
SAcb							$p = 0.0021$, $F_{6,16}=5.909$
Hippocampus							
CA1	146.29±0.81						$p = 0.0051$, $F_{6,19}=4.544$
CA2							$p = 0.0016$, $F_{6,19}=5.680$
CA3	168.51±0.91						$p = 0.001$, $F_{6,19}=10.573$
DG	144.55±0.71						$p = 0.0001$, $F_{6,19}=8.670$

Table 4. Summary table of results from experimental *Homer1b* and *mGluR5* profiles. The table summarizes significant changes *vs.* controls of *Homer1b* (upper section) and *mGluR5* (lower section) expression in striatum, hippocampus, and cortex after acute treatment by selective antagonists of dopamine receptors, terguride and haloperidol. Data are expressed as a percentage of vehicle relative d.p.m. mean value ±S.E.M. and listed by brain region analyzed, along with the relative ANOVA p and F(df) values. Increases in gene expression are shaded in dark grey and decreases in light grey.

Topography of gene expression.

Profiling of *Homer1a* expression in striatum by selective antagonists at dopamine receptors, terguride, and haloperidol allows a clear-cut distinction among compounds. Gene expression by vehicle and the D_4 receptor antagonist L-745,870 was homogeneous, although a non-significant peak in VM putamen could be observed for vehicle. D_1 and D_3 receptors antagonists, SCH-23390 and U-99190, exhibited a similar distribution of *Homer1a* expression, with prominent expression in the medial subregions of the caudate-putamen (Fig. 20). However, *Homer1a* expression in the accumbens was significantly induced by SCH-23390 but not by U-99190. Taken together, these data may suggest that a partial involvement of D_1 and D_3 receptors in the medial putamen and of D_1 receptors only in the accumbens may take place in the modulation of *Homer1a* expression. Thus, compounds provided of D_1 or D_3 blocking potential may induce the gene in the medial putamen and in the accumbens. These findings may shed a light on the subregional profile of gene expression by compounds as clozapine (which is also a D_1 receptor antagonist) or amisulpride (which is a D_2/D_3 receptor antagonist). Nonetheless, it must be noted that D_1 and D_3 receptor antagonists profile of gene distribution partially resembled those by vehicle. It may well be that SCH-23390 and U-99190 induced the gene over basal levels without a particular distribution. More studies are needed to clarify this point.

Haloperidol showed its classical "three-steps" pattern of *Homer1a* distribution, which was strongly approximated by terguride (a partial agonist at D_2 receptors) but, surprisingly, not by the selective D_2 receptor antagonist L-741,626. This latter compound showed the typical prominent expression in the DL putamen, which appears to be common to all compounds provided of substantial D_2 receptors blocking liability. However, L-741,626 induced *Homer1a* more in the medial than in the VL putamen. This discrepancy with haloperidol (and terguride) may suggest that selective blockade of D_2 receptors may be not sufficient to

produce a haloperidol-like distribution of *Homer1a* expression. It could be hypothesized that other receptors (presumably not dopaminergic) may influence *Homer1a* distribution by haloperidol. However, terguride does not share with haloperidol interaction with not-D_2 receptors and it exhibited a *Homer1a* profile of distribution which highly resembled those by haloperidol. Moreover, other antipsychotics, which shared with haloperidol almost exclusively their antagonism at D_2 receptors, also exhibited a profile of *Homer1a* distribution that was similar to haloperidol. One suggestive explanation of the discrepancy between haloperidol and L-741,626 may be that *Homer1a* distribution is influenced by compound affinity to D_2 receptors. According to this hypothesis, compounds provided of significant affinity at D_2 receptors, besides their selectivity for this receptor, may produce a profile of *Homer1a* distribution resembling the "three-steps" pattern exhibited by haloperidol. Thus, *Homer1a* expression profiling may be a measure of a compound affinity, rather than selectivity, at striatal D_2 receptors.

Distribution slope in the cortex by vehicle, dopamine receptors antagonists, terguride, and haloperidol followed a highly similar shape. The similarity of the distribution pattern may perhaps imply that the neuronal type expressing *Homer1a* are not homogeneously distributed in the cortex. According to this view, cells expressing *Homer1a* would be more concentrated in the medial agranular cortex and in the motor cortex, while they would be scarcely represented in the somatosensory and infralimbic cortices. Intriguingly, the subregions preferentially expressing *Homer1a* are involved in motor-related tasks, which could lend some support to recent findings of a role for *Homer* in motor behavior.

Quantitatively, *Homer1a* expression appears to be heightened in these subregions by antagonism at D_1, D_2, and D_3 receptors. However, both terguride and haloperidol seem to

reduce *Homer1a* expression in the same regions. Thus, high affinity to D_2 receptors may reduce, rather than increase, gene expression in the cortex. Otherwise, some not-dopaminergic receptors may concur to modulate cortical *Homer1a* expression by terguride and haloperidol.

Fig. 20

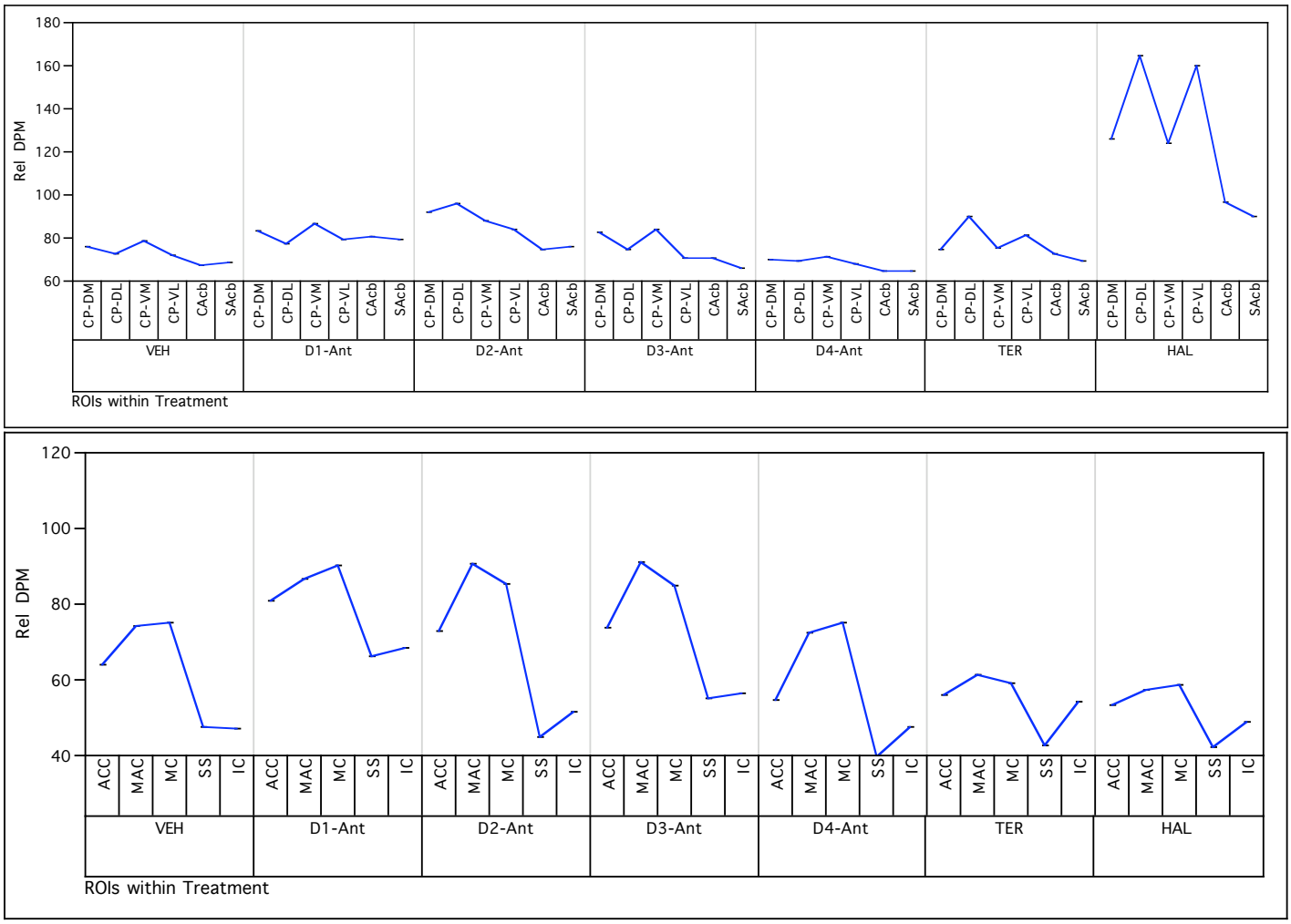


Figure 20. Topographical distribution of *Homer1a*. *Homer1a* expression has been evaluated related to signal distribution among ROIs within each treatment in both the striatum (upper panel) and the cortex (lower panel). Data are reported as averaged relative d.p.m. (Rel DPM). For clarity matter, S.E.M. bars have not been traced.

Chapter 6.

Post-synaptic density genes differential expression by antipsychotics with different dopamine D_2 receptor affinity: dissecting post-receptorial mechanisms of antipsychotic action.

Rationale.

Antipsychotic drugs are the mainstay treatment for schizophrenia. According to clinical and preclinical evidence dopamine D_2 receptors (D_2Rs) antagonism is a crucial mechanism for antipsychotics therapeutic effects (Laruelle et al., 2005). Indeed, typical antipsychotics show high rates of D_2Rs affinity and induce extrapyramidal side-effects (EPSEs) (Seeman and Tallerico, 1998), whereas atypical antipsychotics exhibit lower rates of D_2Rs affinity and interact with multiple receptors, with a lack or low liability for EPSEs (Richtand et al., 2007). However, little is known about post-receptorial mechanisms of antipsychotic action. Moreover, a clear molecular distinction among typical and atypical antipsychotics is still lacking.

Recently, preliminary evidence has been provided that antipsychotics may modulate the levels of post-synaptic density (PSD) factors (Fumagalli et al., 2008; O'Connor et al., 2007). PSD has been regarded as a putative site of dopamine-glutamate interaction, which can be involved in synaptic plasticity remodeling triggered by antipsychotics (Konradi and Heckers, 2003). It has been observed that antagonism at D_2Rs by typical or atypical antipsychotics might influence glutamate system (Leveque et al., 2000) and increasing evidence supports the hypothesis that the dysregulation of dopamine neurotransmission in schizophrenia may be associated with a persistent dysfunction of glutamate system (Olney et al., 1999). Alterations of PSD factors have been widely described in schizophrenia (Kristiansen et al., 2006; Toro and Deakin, 2005). Thus, the study of antipsychotics-induced changes in PSD

factors may increase the knowledge of post-receptorial mechanisms of action of these drugs and may shed a light on the pathophysiology of the disease.

Homers are a family of PSD proteins that are involved in intracellular glutamatergic signaling and in synaptic architecture (de Bartolomeis and Iasevoli, 2003). Constitutively expressed Homer isoforms (namely Homer1b/c) form a scaffold between metabotropic glutamatergic receptors and either intracellular effectors or other glutamatergic receptors (Shiraishi-Yamaguchi and Furuichi, 2007). The inducible isoform Homer1a disrupts these connections, modifying glutamatergic signaling (de Bartolomeis and Iasevoli, 2003; Shiraishi-Yamaguchi and Furuichi, 2007).

Recently, we have demonstrated that *Homer1a*, the gene coding for the Homer1a isoform, is induced by typical and atypical antipsychotics (de Bartolomeis et al., 2002; Polese et al., 2002). Thus, Homer may represent a putative site of intracellular action of antipsychotics. Moreover, our studies suggest that *Homer1a* may be differentially modulated by antipsychotics with different dopaminergic profile (Ambesi-Impiombato et al., 2007). To test these hypotheses we investigated *Homer1a* expression by antipsychotic drugs with different D_2Rs affinity profiles: haloperidol, risperidone, olanzapine and sulpiride.

Results.

Caudate-putamen

ANOVA revealed statistically significant differences among groups in all subregions of the caudate-putamen (Table 5). The *post hoc* test showed that HAL significantly induced *Homer1a* compared to VEH, according to our previous observations (de Bartolomeis et al., 2002; Polese et al., 2002). Moreover, HAL-treated group showed significant *Homer1a* signal increase also compared to all the other antipsychotics. RISP and OLA significantly induced

Homer1a compared to VEH in the lateral caudate-putamen (Fig. 21) and SULP significantly elicited the induction of *Homer1a* as compared to VEH in the VL caudate-putamen (Fig. 21).

Nucleus Accumbens

In the core of the nucleus accumbens (CAcb) the densitometric analysis of autoradiographic images showed a statistically significant signal increase of *Homer1a* by HAL, OLA, and SULP as compared to VEH (Table 5). No changes among groups were detected in the shell (SAcb) subregion (Fig. 21).

	RISP	OLA	SULP	HAL	ANOVA (*df*)
DM				159.85±0.83	$p = 0.0018$, $F_{4,12}=9.66$
DL	145.89±1.21	135.45±0.54		177.96±1.09	$p = 0.0025$, $F_{4,12}=7.76$
VM				151.66±1.24	$p = 0.0093$, $F_{4,12}=5.78$
VL	144.04±2.21	136.59±1.12	128.51±0.81	185.31±0.77	$p = 0.0014$, $F_{4,12}=8.98$
CAcb		113.18±0.86	111.33±0.41	115.97±0.44	$p = 0.0008$, $F_{4,12}=11.01$
SAcb					$p > 0.05$, $F_{4,14}=0.74$

Table 5. Summary table of results from experimental Homer1a profiles.

The table summarizes significant changes vs. controls of *Homer1a* expression in striatum after acute treatment by risperidone (RISP), olanzapine (OLA), sulpiride (SULP), and haloperidol (HAL). Data are expressed as a percentage of vehicle relative d.p.m. mean value ±S.E.M. and listed by brain region analyzed, along with the relative ANOVA p and F(df) values.

Fig. 21

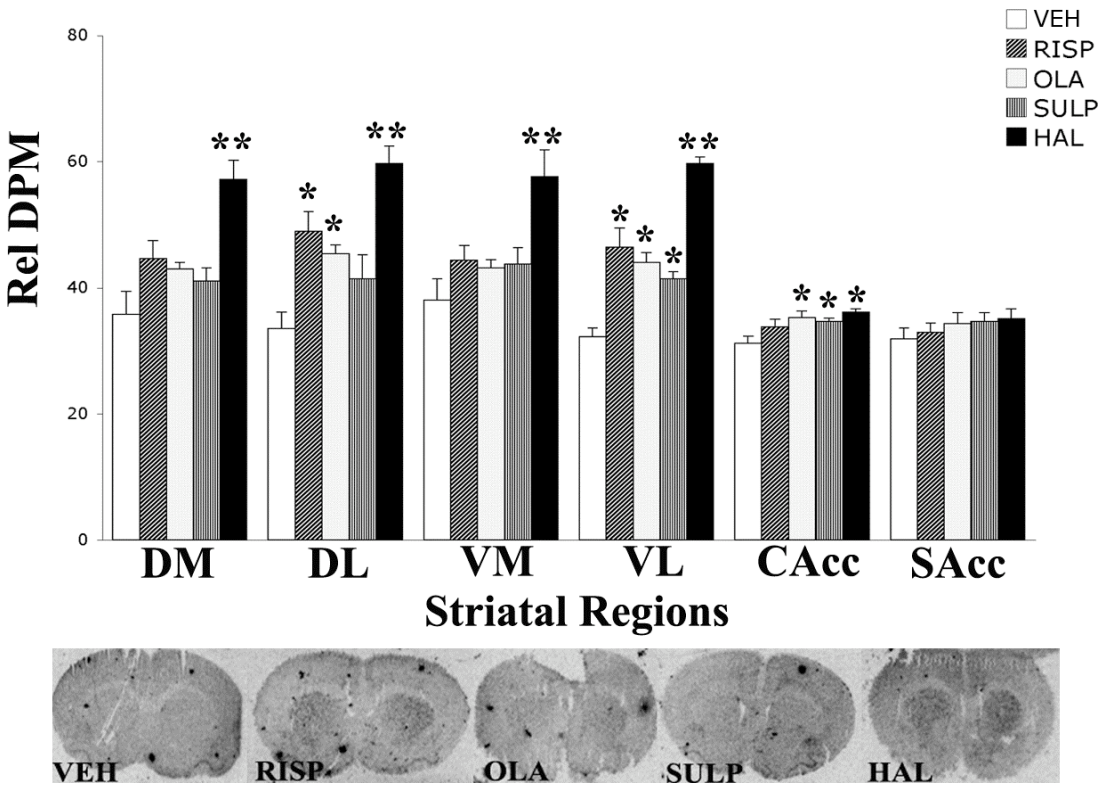


Figure 21. ***Homer1a*** **signal distribution in striatal areas by antipsychotics.**

Homer1a expression has been evaluated related to signal distribution among ROIs within each treatment. Data are reported as averaged relative d.p.m. (cal(rel dpm) in the figure). For clarity matter, S.E.M. bars have not been traced. Student-Neumann-Keuls post hoc test: *p<0.05 vs. core and shell of the accumbens.

Topography of *Homer1a* expression.

Homer1a profile of expression by vehicle was not homogeneous, showing a peak in the VM putamen and another slight peak in the DM putamen (Fig. 22). Although this pattern was already observed in other studies, it has never gained significance. Gene distribution in the vehicle group may be biased by high standard error, perhaps due to low signal-to-noise ratio. Haloperidol-induced distribution of *Homer1a* expression resembled the classical "three-steps" pattern, although with an attenuated gradient between lateral and medial caudate-putamen subregions and an enhanced gradient between caudate-putamen and nucleus accumbens (Fig. 22). The same distribution pattern was also induced by risperidone and olanzapine, which have substantial action at D_2 receptors. On the contrary, sulpiride showed a prominent expression in the VM putamen, an intermediate expression in DM and lateral putamen, and the lowest expression in the nucleus accumbens (Fig. 22). This pattern by sulpiride may be influenced by its action at D_3 receptors.

Fig. 22

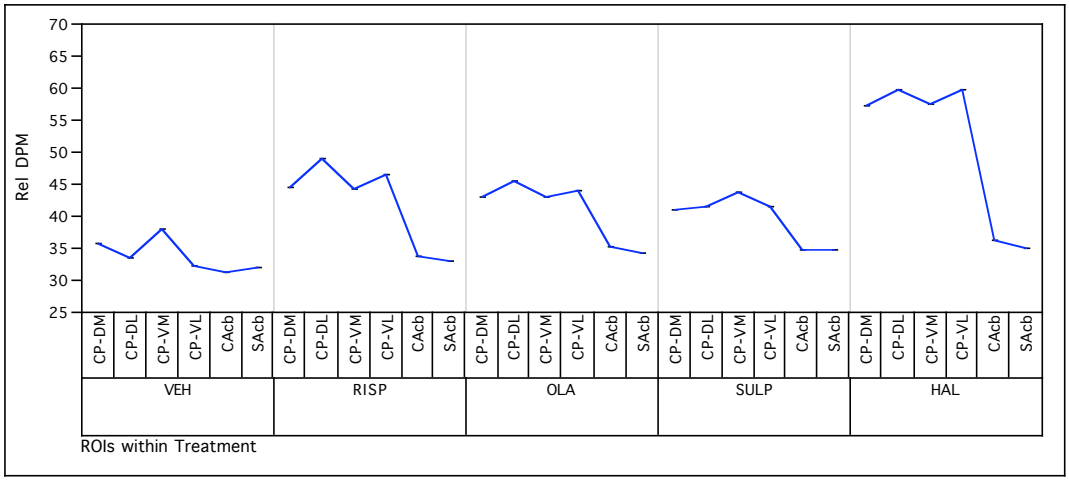


Figure 22. Topographical distribution of *Homer1a*. *Homer1a* expression has been evaluated related to signal distribution among ROIs within each treatment in the striatum. Data are reported as averaged relative d.p.m. (Rel DPM). For clarity matter, S.E.M. bars have not been traced.

Chapter 7.

Topographical and temporal distribution of *Homer1a* expression in corticostriatal regions is influenced by antipsychotics dopaminergic profile: new clues for the preclinical distinction among typical and atypical antipsychotics.

Rationale.

We have demonstrated that *Homer1a* is strongly induced by haloperidol, while being scarcely or not induced by several atypical compounds: clozapine, quetiapine, and olanzapine at a low dose (Ambesi-Impiombato et al., 2007; de Bartolomeis et al., 2002; Polese et al., 2002). The results led us to hypothesize that measuring the increase of *Homer1a* in rat striatum may be helpful to discriminate typical from atypical antipsychotics. These data suggest that the extent of *Homer1a* expression may be, at least partially, influenced by antipsychotics affinity to D_2Rs and it might be predictive of motor side effects. To address these issues we compared the expression of *Homer1a* by haloperidol, clozapine, and ziprasidone. The choice of ziprasidone was based on its low liability to EPS despite its relatively high affinity for D_2Rs (Nemeroff et al., 2005). Thus, ziprasidone appears a compelling candidate to discern whether *Homer1a* expression may be affected by D_2Rs blockade and whether it may be a marker of antipsychotics EPS liability.

In the present study, we also provided a subregional analysis of *Homer1a* signal distribution to evaluate whether regional distribution of *Homer1a* expression is influenced by antipsychotic treatment and whether distinct patterns of gene distribution in striatal and cortical areas could be detected for each antipsychotic. Indeed, antipsychotics efficacy has been related to *c-fos* induction in the nucleus accumbens, whereas *c-fos* induction in the dorsolateral striatum has been considered predictive of EPS (Robertson et al., 1994).

To investigate the dynamics of *Homer1a* response to antipsychotics, in a way that more closely resembles the clinical administration of these compounds, we explored *Homer1a* expression after a chronic (21 days) treatment by haloperidol and ziprasidone. We evaluated whether IEG-like properties of *Homer1a* are preserved after a chronic antipsychotic treatment by assessing gene expression either acutely (90 minutes) or after a 24 hours withdrawal from the last chronic administration.

Results.

Topographical distribution of *Homer1a* expression in the acute administration paradigm

Striatum. The most prominent expression was observed in the DL putamen, while variable degrees of signal labelling were detected in other striatal subregions. VEH group did not show any peculiar pattern of signal distribution, although prominent gene expression in DL putamen was still observed (Fig. 23). On the contrary, the general trend of distribution by antipsychotics was along a dorsal-to-ventral and lateral-to-medial gradient (Fig. 23). As this pattern was induced by all antipsychotics employed in this work, it may represent a common mark of acute *Homer1a* expression by antipsychotic compounds. Nonetheless, some difference in the relative distribution among groups has to be outlined. HAL, ZIP4, and ZIP10 exhibited a "three-levels" pattern of distribution (Fig. 23) with higher frequencies in the DL and VL putamen, intermediate frequencies in the medial putamen, and lower frequencies in the accumbens. CLO showed an attenuated gradient, with higher frequencies in the accumbens compared to other antipsychotics. At the ANOVA test, no significant differences among striatal subregions were detected for VEH and CLO groups (*VEH*: $F_{(5;18)}$=2.37, p=0.08; *CLO*: $F_{(5;16)}$=0.29; p=0.91). In the HAL, ZIP4, and ZIP10 groups,

significant gene expression was detected in the DL putamen compared with VM putamen and accumbens (*HAL*: $F_{(5;23)}$=17.01, p<0.0001; *ZIP10*: $F_{(5;22)}$=7.16, p=0.0004; *ZIP4*: $F_{(5;22)}$=15.36, p<0.0001). Moreover, significant expression was observed in the VL putamen compared with VM putamen and accumbens in the HAL group; with the accumbens in the ZIP4 group; and with the SAcb only in the ZIP10 group.

Cortex. Differences in regional distribution of signal labelling were less pronounced in the cortex than in the striatum. Distribution of *Homer1a* expression by antipsychotics was not as similar as seen in the striatum. Further, gene distribution among subregions in the control group was not homogeneous and revealed a preferential basal expression in the motor-related subregions MAC and MC (Fig. 23). This pattern of distribution was almost overlapped from distribution by HAL (Fig. 23), which suffered of lower levels of standard error compared to controls (data not shown). CLO and ZIP10 showed higher levels of gene expression than controls. CLO-induced gene expression distributed preferentially in the MAC, while differences among other subregions were attenuated in comparison to VEH (Fig. 23). Signal distribution by ZIP10 peaked in the MC, reached intermediate levels in the ACC and the IC, and exhibited the lowest levels in the MC and the SS (Fig. 23). Signal induction by ZIP4 was lower than controls and resembled those by ZIP10, with the exception of the IC. Statistical analysis showed that significant differences among subregions were detectable in the HAL group only (*HAL*: $F_{(4;10)}$=8.86, p=0.0025; *ZIP10*: $F_{(4;15)}$=1.38, p=0.28; *ZIP4*: $F_{(4;10)}$=2.65, p=0.09; *CLO*: $F_{(4;10)}$=1.67, p=0.23; *VEH*: $F_{(4;10)}$=2.31, p=0.14). Post hoc test revealed that gene expression by HAL was significantly higher in the MAC and MC compared to all other subregions (Fig. 23).

Fig. 23

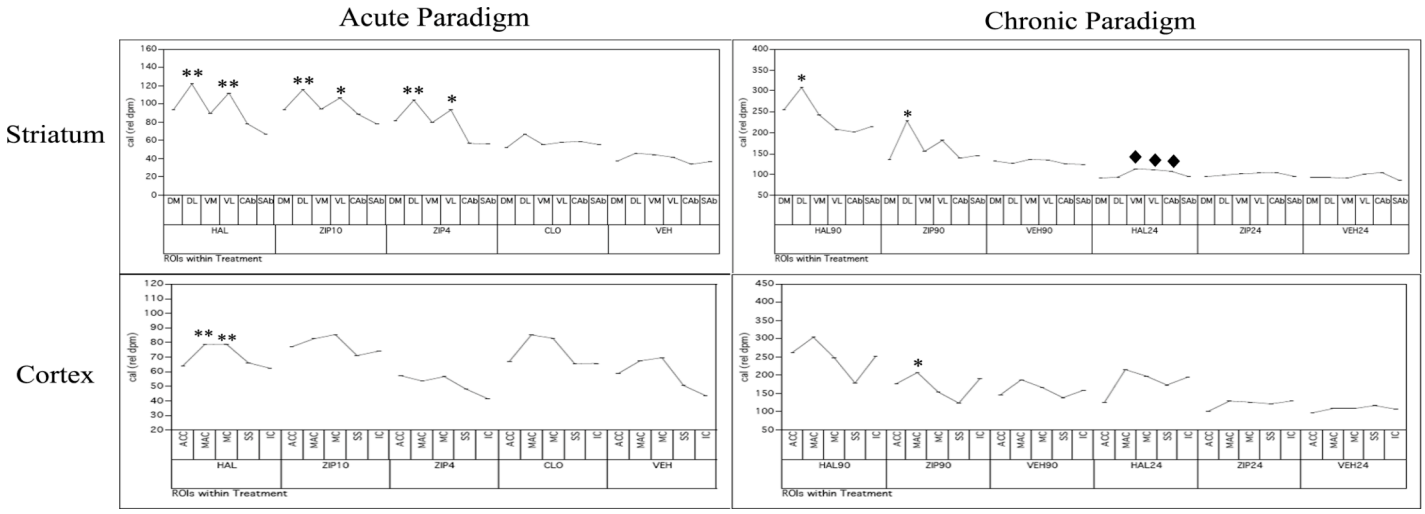


Figure 23. Topographical analysis of *Homer1a* expression in the striatum and the cortex. *Homer1a* expression has been evaluated related to signal distribution among ROIs within each treatment in both the striatum and the cortex and graphically compared between the acute and the chronic paradigm. ZIP90 and ZIP24 in the chronic paradigm correspond to ZIP4 in the acute one. Data are reported as averaged relative d.p.m. (cal(rel dpm) in the figure). For clarity matter, S.E.M. bars have not been traced. Student-Newman-Keuls *post hoc* test: **P<0.05 vs. all ROIs. *P<0.05 vs. discrete ROIs (*ZIP4, striatum*: vs. CAcb and SAcb; *ZIP10, striatum*: vs. SAcb; *HAL90 and ZIP90, striatum*: vs. VM, CAcb, and SAcb; *ZIP90, cortex*: vs. ACC, MC, SS cortex). ♦P<0.05 vs. DM, DL, SAcb.

Chronic administration paradigm

Striatum

Two-way ANOVA revealed that significant differences among experimental groups were attributable in all subregions to either treatment or time effect, and to the interaction between these factors (Table 6). Post-hoc analysis showed that HAL90 induced significantly the gene in all subregions compared to both ZIP90 and VEH90. Gene induction by ZIP90 was significantly higher compared to VEH90 in the lateral putamen and the core of the accumbens, whereas in the medial putamen and the shell of accumbens a trend toward significance was observed (Fig. 24).

In all subregions, HAL90-induced expression was significantly higher compared to HAL24. ZIP90 induced significantly gene expression compared to ZIP24 in all subregions, while no significant difference was observed between VEH90 and VEH24 (Fig. 24). Thus, basal *Homer1a* expression was similar at both time-points. No significant differences were observed among groups sacrificed after the 24-hour withdrawal (Fig. 24).

Cortex

Significant differences among experimental groups were mostly attributable to treatment effect in all cortical subregions. Time effect was significant in all subregions with the exception of SS. No treatment-x-time effect was observed in any cortical subregion (Table 6). Post-hoc analysis showed that HAL90 induced significantly *Homer1a* expression compared to either VEH90 in all subregions and ZIP90 in all subregions excluding the ACC. ZIP90 did not induce significantly the gene compared to VEH90 in any cortical subregion (Fig. 24).

HAL90 induced significantly *Homer1a* compared to HAL24 in all subregions with the exception of the SS. Expression by ZIP90 was significantly higher than that by ZIP24 in the MAC and IC, while expression by VEH90 was significantly higher than VEH24 expression in the MAC, MC, and IC (Fig. 24). As no treatment-x-time interaction has been observed at the 2-way ANOVA test, differences between 90min and 24h groups may presumably reflect the decay of gene expression with time. Thus, AP treatments are not able to sustain *Homer1a* expression in the cortex in the time window chosen by us, with the exception of the SS where antipsychotics appear to trigger an enduring gene expression. However, delayed expression of the gene was still observable. In fact, HAL24 induced significantly *Homer1a* expression compared to VEH24 and ZIP24 in all subregions, with the exclusion of the ACC (Fig. 24).

Table 6. mRNA levels of *Homer1a, Homer1b, PSD95, and Shank* after chronic treatment. Data are expressed as percentage of VEH relative d.p.m. mean value ±S.E.M. and listed by brain region analyzed, along with the relative two-way ANOVA values for each of the significant changes ($p < 0.05$). Only significant changes *vs*. controls were cited. Groups belonging at the 90min time point are compared to VEH90 and groups belonging to the 24h time point are compared to VEH24. *= significant differences from VEH90 or VEH24 at the Student-Newmann-Keuls post hoc test. §= significant differences from the corresponding group at the 24h time point.

Probes/ Regions	HAL90	ZIP90	HAL24	ZIP24	P-value; $F_{(df)}$-value (2-way ANOVA) Treatment	P-value; $F_{(df)}$-value (2-way ANOVA) Time	P-value; $F_{(df)}$-value (2-way ANOVA) Treatment-x-Time
Homer1a							
Striatum							
DM	214.8±30.81*§	121.8±8.29§	100.4±2.92	105.6±9.62	0.0018; $11.25_{(2;12)}$	<0.0001; $32.46_{(1;12)}$	0.0024; $10.44_{(2;12)}$
DL	276.5±30.59*§	193.3±29.34*§	106.1±7.23	107.5±4.62	0.0005; $15.27_{(2;12)}$	<0.0001; $57.64_{(1;12)}$	0.0007; $14.35_{(2;12)}$
VM	200.1±30.25*§	134.3±6.05§	114.5±6,61	113.1±8.74	0.0021; $10.78_{(2;12)}$	<0.0001; $35.60_{(1;12)}$	0.0150; $6.08_{(2;12)}$
VL	176.2±6.21*§	150.1±12.79*§	115.3±7.17	105.5±7.49	<0.0001; $43.05_{(2;12)}$	<0.0001; $180.19_{(1;12)}$	<0.0001; $27.97_{(2;12)}$
CAcb	179.2±9.01*§	126.2±2.31*§	105.4±4.42	102.6±9.34	<0.0001; $22.40_{(2;12)}$	<0.0001; $59.15_{(1;12)}$	0.0002; $18.56_{(2;12)}$
SAcb	180.6±23.74*§	127.7±5.68§	111.2±1.69	114.4±10.44	0.0090; $7.16_{(2;12)}$	0.0001; $33.65_{(1;12)}$	0.0234; $5.22_{(2;12)}$
Cortex							
ACC	162.3±36.28*§	112.2±6.28	126.4±4.77*	102.6±8.66	0.0268; $4.97_{(2;12)}$	<0.0001; $36.95_{(1;12)}$	*n.s.*
MAC	157.8±21.19*§	114.7±6.97§	189.7±24.44*	118.1±6.41	0.0002; $18.06_{(2;12)}$	<0.0001; $38.22_{(1;12)}$	*n.s.*
MC	138.1±12.49*§	87.8±6.49	174.8±14.44*	114.3±3.31	0.0002; $18.61_{(2;12)}$	0.0011; $18.19_{(1;12)}$	*n.s.*
SS	129.3±9.71*	91.9±5.78	150.9±9.98*	109.5±8.27	0.0006; $14.44_{(2;12)}$	*n.s.*	*n.s.*
IC	155.8±7.52*§	123.6±8.88§	180.3±19.04*	122.1±14.78	0.0001; $21.37_{(2;12)}$	<0.0001; $36.04_{(1;12)}$	*n.s.*
Homer1b							
Striatum							
DM	102.3±2.86	106.5±5.22§	103.4±2.01	100.7±2.22	*n.s.*	0.0005; $18.30_{(1;18)}$	*n.s.*
DL	105.4±2.77§	110.6±2.27*§	111.5±1.09	109.6±1.97	0.0002; $14.58_{(2;18)}$	<0.0001; $112.08_{(1;18)}$	*n.s.*
VM	99.4±2.01§	102.4±3.41§	104.2±2.24	101.4±2.06	*n.s.*	<0.0001; $35.33_{(1;18)}$	*n.s.*
VL	99.9±3.51§	99.3±2.55§	108.1±1.17	89.1±2.93	*n.s.*	<0.0001; $28.43_{(1;18)}$	*n.s.*
CAcb	103.3±3.17	108.9±5.36§	111.4±1.09	102.8±2.81	*n.s.*	0.0066; $9.43_{(1;18)}$	*n.s.*
SAcb	103.6±2.57	99.4±5.41	114.4±2.17	110.4±1.32	*n.s.*	0.0039; $10.94_{(1;18)}$	*n.s.*
Cortex							
ACC	99.4±3.33§	104.4±5.01§	101.7±0.71	99.9±4.43	*n.s.*	<0.0001; $33.24_{(1;18)}$	*n.s.*
MAC	96.1±3.72	105.5±8.19§	104.5±2.81	96.7±2.78	*n.s.*	0.0003; $19.46_{(1;18)}$	*n.s.*
MC	96.2±1.79	110.2±14.04§	108.1±3.49	100.5±3.58	*n.s.*	0.0026; $12.19_{(1;18)}$	*n.s.*
SS	97.1±1.85	106.5±9.45§	103.6±1.99	103.5±2.49	*n.s.*	0.0028; $11.95_{(1;18)}$	*n.s.*
IC	104.9±3.03§	108.1±4.31§	111.8±1.98	113.4±0.97	*n.s.*	0.0012; $14.75_{(1;18)}$	*n.s.*
PSD95							
Striatum							
DM	144.8±11.06*§	125.4±6.27*	109.5±3.86	112.9±1.92	0.0040; $9.06_{(2;12)}$	*n.s.*	*n.s.*
DL	145.8±12.7*§	123.2±9.36*	108.2±2.39	106.9±1.64	0.0209; $5.43_{(2;12)}$	0.0286; $6.18_{(1;12)}$	*n.s.*
VM	139.6±7.94*§	123.3±4.11*	110.1±2.76	114.9±1.72*	0.0016; $11.60_{(2;12)}$	0.0280; $6.25_{(1;12)}$	0.0320; $4.65_{(2;12)}$
VL	142.5±11.6*§	120.3±6.29*	104.1±2.42	111.2±4.48	0.0220; $5.33_{(2;12)}$	0.0116; $8.85_{(1;12)}$	0.0413; $4.21_{(2;12)}$
CAcb	138.5±8.61*§	120.3±5.37*	106.2±3.96	111.5±5.49	0.0064; $7.92_{(2;12)}$	0.0050; $11.78_{(1;12)}$	0.0361; $4.43_{(2;12)}$
SAcb	135.9±8.21*§	123.6±4.07*	102.6±3.02	109.6±4.25	0.0085; $7.28_{(2;12)}$	0.0180; $7.49_{(1;12)}$	0.0327; $4.61_{(2;12)}$
Cortex							
ACC	109.3±3.26	114.1±8.92*	116.2±3.13*	122.1±0.17*	0.0045; $8.75_{(2;12)}$	*n.s.*	*n.s.*
MAC	117.9±2.82*	110.1±3.61	118.9±5.57*	123.3±0.22*	0.0003; $17.55_{(2;12)}$	*n.s.*	*n.s.*
MC	120.8±6.77*	107.7±8.03	115.8±2.13*	122.8±5.27*	0.0101; $6.90_{(2;12)}$	*n.s.*	*n.s.*
SS	136.7±14.91*	110.9±10.62	113.1±5.31	122.4±11.38	0.0322; $4.64_{(2;12)}$	*n.s.*	*n.s.*
IC	124.7±11.91	113.4±9.59	109.9±6.88	113.6±2.74	*n.s.*	*n.s.*	*n.s.*
Shank							
Striatum							
DM	105.9±2.94	84.7±4.91	114.1±2.95	113.7±3.39	*n.s.*	*n.s.*	*n.s.*
DL	109-6±2.08	82.3±4.35	111.9±3.39	116.2±5.14	*n.s.*	*n.s.*	*n.s.*
VM	103.8±1.88	88.1±3.81	107.3±2.03	111.2±3.53	*n.s.*	*n.s.*	*n.s.*
VL	105.3±2.67	83.7±6.01	110.1±3.57	114.2±5.05	*n.s.*	*n.s.*	*n.s.*
CAcb	107.9±3.36	90.4±2.91	104.6±2.67	113.6±7.41	*n.s.*	*n.s.*	*n.s.*
SAcb	101.9±5.01	95.9±3.12	102.1±2.69	104.9±5.33	*n.s.*	*n.s.*	*n.s.*
Cortex							
ACC	104.3±1.11§	102.5±1,51	97.9±0.66	99.1±2.75	*n.s.*	0.0014; $17.07_{(1;12)}$	*n.s.*
MAC	102.5±0.23	94.3±1.91	99.5±1.89	99.1±2.95	0.0081; $7.39_{(2;12)}$	0.0197; $7.23_{(1;12)}$	*n.s.*
MC	100.2±0.51	93.8±0.37	101.3±1.83	102.1±2.52	0.0496; $3.90_{(2;12)}$	0.0276; $6.28_{(1;12)}$	*n.s.*
SS	98.8±2.01	96.8±1.32	98.5±3.34	99.7±1.43	*n.s.*	0.0099; $9.36_{(1;12)}$	*n.s.*
IC	98.2±0.17§	95.1±0,97	98.1±1.19	101.3±2.24	*n.s.*	0.0216; $6.97_{(1;12)}$	*n.s.*

Fig. 24

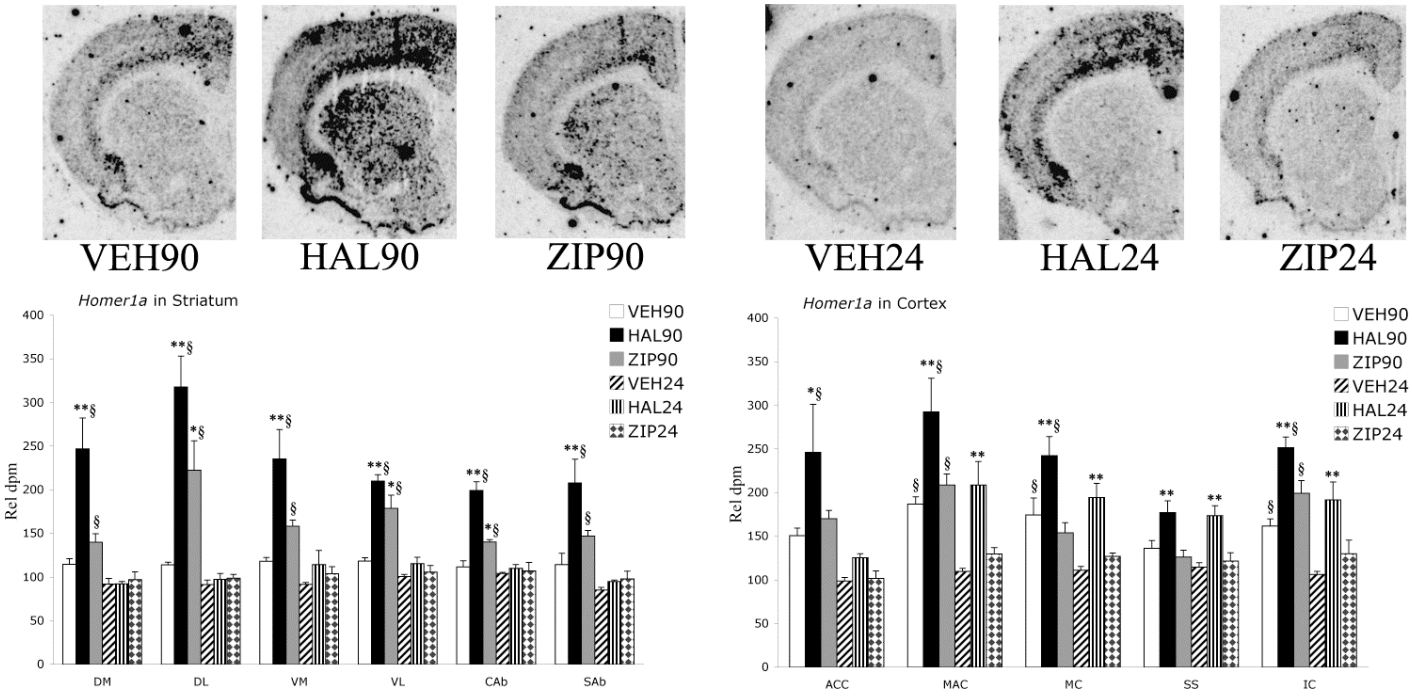


Figure 24. *Homer1a* expression in chronic treatment. *Upper panel*: autoradiographic film images of *Homer1a* mRNA detected by means of ISHH in coronal brain sections after chronic treatment with haloperidol, ziprasidone, or vehicle at both 90min time point ("90" suffix near each treatment) and 24h time point ("24" suffix near each treatment). *Lower panel*: *Homer1a* mRNA levels in striatum (left) and cortex (right). Data are reported in relative d.p.m. as mean ±S.E.M. Comparison at the post hoc test was performed within groups from the same time point and within corresponding treatments at the two time points. No comparison was made within not corresponding groups at the two time points. Student-Newmann-Keuls post hoc test: *= p <0.05 *vs*. VEH90. **= p <0.05 *vs*. all treatments belonging to the same time point. §= p <0.05 *vs*. the corresponding groups belonging to the other time point.

Topographical distribution of *Homer1a* expression

Striatum. Topography of gene expression by both HAL90 and ZIP90 resembled that observed by these compounds in the acute paradigm, although with some subtle discrepancy. A prevalent distribution of signal labeling in the DL putamen was recognized for both groups, with intermediate values for the medial subregions and the lowest values for the accumbens (Fig. 23). A remarkable exception to this pattern was represented from HAL90, which showed signal labeling rates in the VL putamen lower than the medial putamen subregions. It is not clear whether this result depends on individual variability or it underlies neuroplastic changes resulting in the desensitization of *Homer1a* expression in this subregion. Signal labeling in the VEH90 group was homogeneous, partially differentiating from the distribution observed in the acute paradigm, where a prevalent, although not significant, labeling in the DL putamen was detected. Signal labeling in the 24h groups was homogeneous and did not show a characteristic pattern. Nonetheless, HAL24 group exhibited a slightly prominent gene expression in the ventral subregions (Fig. 23), a feature that represents a divergence with what observed in both the acute paradigm and the HAL90 group of the chronic paradigm.

At the ANOVA test, no statistical differences among subregions were detected for VEH90 ($F_{(5;12)}$=0.13, p=0.98), VEH24 ($F_{(5;18)}$=2.21, p=0.31), and ZIP24 ($F_{(5;12)}$=0.28, p=0.91). HAL90 showed a trend toward significance ($F_{(5;12)}$=2.56, p=0.08), while significant differences were recognized for ZIP90 ($F_{(5;18)}$=3.89, p=0.0143) and HAL24 groups ($F_{(5;18)}$=3.91, p=0.0141). Gene expression in both HAL90 and ZIP90 was significantly higher in the DL putamen compared to that in VM putamen and accumbens. In the HAL24 group a significant expression in ventral putamen and the core of accumbens compared to the other subregions has been recognized (Fig. 23).

Cortex. In VEH90, HAL90, and ZIP90, a prevalent gene expression in the MAC was observed. Both HAL90 and ZIP90 showed high-to-intermediate signal labeling rates in the IC, MC, and ACC, and the lowest induction in the SS cortex (Fig. 23). This pattern partially corresponds to what observed by the same compounds in the acute paradigm. As compared to the acute paradigm, it appears that *Homer1a* expression may be reduced in the MC and enhanced in the IC, relatively to the other subregions.

At the ANOVA test, no significant differences among subregions were recognized in VEH90 ($F_{(4;10)}$=2.92, p=0.07) and HAL90 ($F_{(4;15)}$=1.61, p=0.22). Significant changes were detected in ZIP90 group ($F_{(4;15)}$=8.73, p=0.0008). At the post hoc test, gene expression in the MAC was significant compared with all other subregions, with the exclusion of the IC.

Signal labeling in VEH24 and ZIP24 groups was homogeneous, with slight peaks in the SS cortex and the MAC, respectively. HAL24 followed a pattern that was comparable to that of HAL90, with a marked reduction of expression in the ACC (Fig. 23). ANOVA showed that no significant differences among subregions were detectable for VEH24 ($F_{(4;15)}$=2.13, p=0.12) and ZIP24 ($F_{(4;10)}$=1.49, p=0.27), while a trend toward significance was observed for HAL24 ($F_{(4;10)}$=3.41, p=0.0523).

Homer1b

Striatum

The two-way ANOVA revealed that significant differences among experimental groups could be almost entirely attributed to time effect, with the exception of the DL putamen where also treatment effect was observed. The interaction between treatment and time was not significant in any subregion (Table 6).

At the post hoc test, no statistical differences among VEH90, HAL90, and ZIP90 were detected in any subregion, with the exception of the DL putamen where ZIP90 induced

significantly the gene compared with VEH90 (Fig. 25). No statistical differences were also detected between VEH24, HAL24, and ZIP24 groups. VEH90, HAL90 and ZIP90 were significant compared to VEH24, HAL24 and ZIP24 respectively in DL, VM, and VL putamen. In the DM putamen and core of the accumbens, only ZIP90 induced significantly the gene compared to ZIP24, while in the shell of the accumbens only VEH90 induced significantly compared to VEH24 (Fig. 25).

Cortex

Significant differences among experimental groups at the two-way ANOVA were exclusively attributable to time effect. No treatment effect or interaction effect was observed, although a borderline significance for interaction was seen in the MAC (Table 6).

No significant differences were observed between VEH90, HAL90, and ZIP90 and between VEH24, HAL24, and ZIP24 in any subregion assessed. In all subregions, VEH90 and ZIP90 induced significantly the gene compared to VEH24 and ZIP24, respectively. On the other hand, *Homer1b* expression was significantly induced by HAL90 compared to HAL24 in the ACC and the IC only (Fig. 25).

PSD95

Striatum

Treatment, time, and interaction effects were significant in all subregions, with the exception of the DM putamen for time effect and the DM and DL putamen for interaction effect (Table 6).

Gene expression was significantly enhanced by HAL90 compared to both VEH90 and ZIP90 and by ZIP90 compared to VEH90 in all subregions, with the exception of the shell of

accumbens where no significant difference was recognized between HAL90 and ZIP90 (Fig. 25).

ZIP24 induced significantly *PSD95* expression compared to VEH24 in the VM putamen. No other significant differences were detected among VEH24, HAL24, and ZIP24 groups. In all subregions, gene expression by HAL90 was also significantly higher than by HAL24, whereas neither ZIP90 nor VEH90 were significant compared to ZIP24 and VEH24, respectively, in any striatal subregion (Fig. 25).

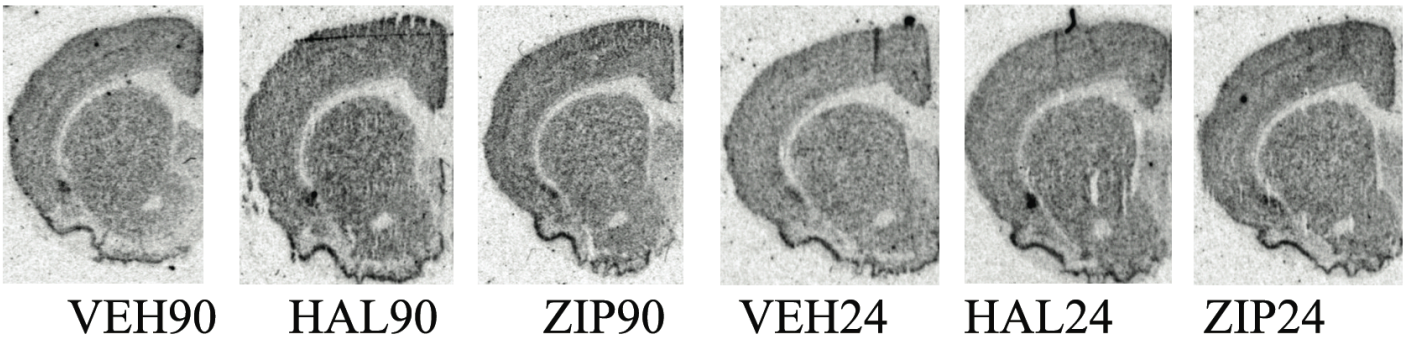
Cortex

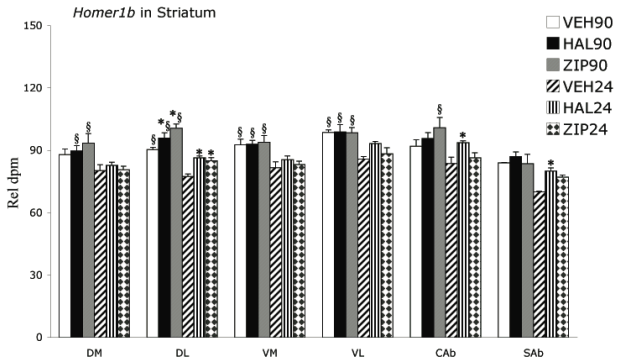
PSD95 expression in the cortex was affected by treatment in almost all subregions, with the exclusion of the IC where only a trend toward significance was observed. Gene expression was not affected by time. No treatment-x-time interaction was detected in any cortical subregion (Table 6). In the ACC, ZIP90 induced significantly the gene compared to VEH90 (Fig. 25). In the MAC, MC, and SS cortex HAL90, but not ZIP90, yielded significant *PSD95* induction compared to VEH90 (Fig. 25). In all subregions, no significant differences were observed between HAL90 and ZIP90.

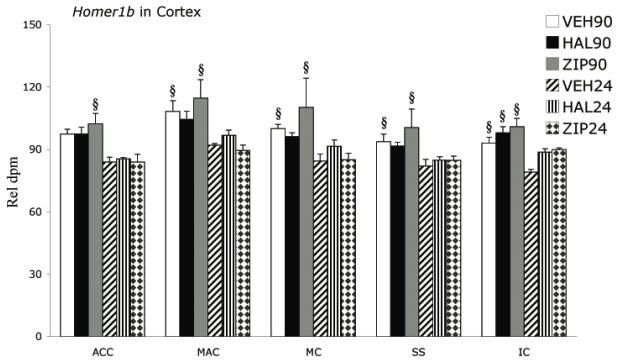
In the ACC, MAC, and MC, both HAL24 and ZIP24 were significant compared to VEH24 (Fig. 25). No significant differences were recognized in the SS cortex and the IC. No differences were observed between VEH90, HAL90, ZIP90 and VEH24, HAL24, ZIP24 groups, respectively (Fig. 25).

Fig. 25

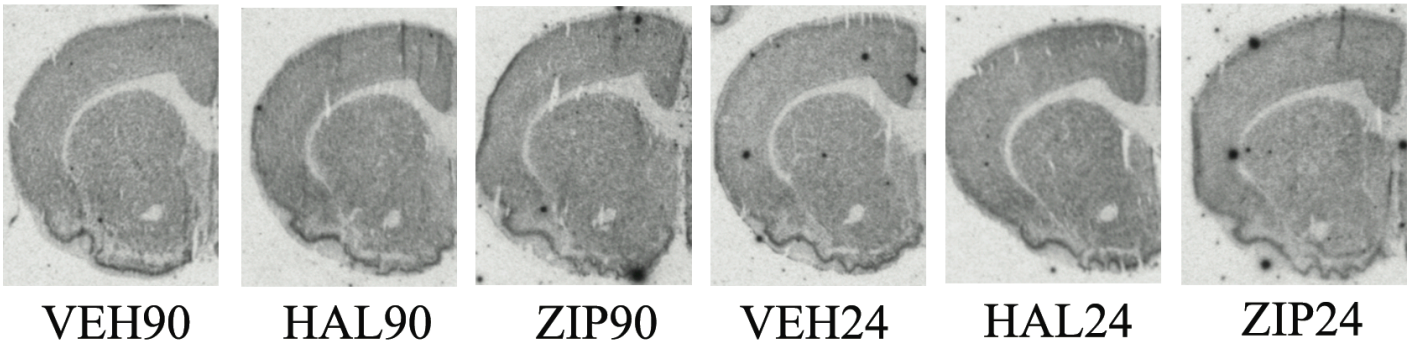
Homer1b autoradiograms

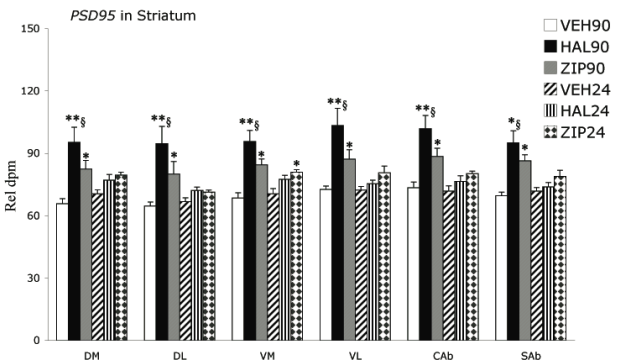






PSD95 autoradiograms





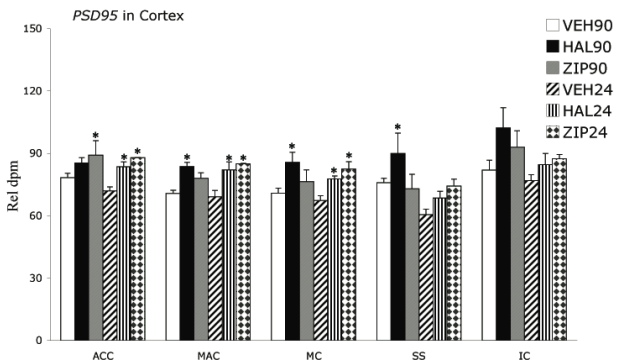


Figure 25. *Homer1b* and *PSD95* expression in chronic treatment. *Panel a,c*: autoradiographic film images of *Homer1b* (a) and *PSD95* (c) mRNA detected by means of ISHH in coronal brain sections after chronic treatment at both time points. *Panel b, d*: *Homer1b* (b) and *PSD95* (d) mRNA levels in striatum (left) and cortex (right). Data are reported in relative d.p.m. as mean ±S.E.M. Comparison at the post hoc test was performed within groups from the same time point and within corresponding treatments at the two time points. No comparison was made within not corresponding groups at the two time points. Student-Newmann-Keuls post hoc test: *= p <0.05 *vs*. VEH (90 or 24). **= p <0.05 *vs*. all treatments belonging to the same time point. §= p <0.05 *vs*. the corresponding groups belonging at the other time point.

Shank

Striatum

Shank expression in the striatum was affected neither by treatment nor by time. Interaction of the two factors was not significant (Table 6).

No significant differences were detected between experimental groups at both time points and between groups belonging to the 90min time point compared to the corresponding groups belonging to the 24h time point. Nonetheless, ZIP90 induced gene expression constantly less than HAL90 and VEH90, showing a trend toward significance in several subregions (data not shown). On the contrary, both HAL24 and ZIP24 showed a not significant trend to induce *Shank* expression more than VEH24 in many subregions (data not shown).

Cortex

Significant differences in cortical gene expression among experimental groups may be obviously attributed to time effect, while only a minimal treatment effect has been detected. No significant interaction of the two factors was seen (Table 6).

At the post hoc test, HAL90 showed signal increase compared with ZIP90 but not with VEH90 in the MAC only (Table 6). No other significant differences were observed between VEH90, HAL90, and ZIP90 groups, although ZIP90 showed a trend toward reduction of gene expression compared with VEH90. No significant differences were seen between VEH24, HAL24, and ZIP24 groups.

HAL90 induced significantly gene expression compared to HAL24 in the ACC and IC (Table 6). A trend toward significance was observed in the other subregions. VEH90 was significant compared with VEH24 in the IC only (Table 2), although it constantly yielded a not-significantly enhanced gene expression as related to VEH24 in the other subregions. No significant differences were recognized between ZIP90 and ZIP24.

Chapter 8.

Antipsychotic and antidepressant co-treatment: effect on transcripts of postsynaptic density genes possibly implicated in behavioural disorders.

Rationale.

Several studies demonstrated that the combination of antipsychotics and selective serotonin reuptake inhibitors (SSRIs) might be effective for treating both psychotic-like symptoms in depression and depressive symptoms of schizophrenia (for review, see Rummel et al., 2005). Despite the relatively frequent use of antipsychotics and antidepressants co-administration, there are few pre-clinical studies about the impact of this combination on brain molecular signalling. This prompted us to explore inducible genes that have been suggested to be implicated in the pathophysiology of schizophrenia and depression as well as in the putative therapeutic action of antipsychotics and antidepressants beyond receptor interaction (de Bartolomeis et al., 2002; Polese et al., 2002; de Bartolomeis & Iasevoli, 2003).

SSRIs inhibit the reuptake of 5-hydroxytriptamine (5-HT) by blocking the serotonin transporter (SERT), leading to increased extracellular levels of 5-HT (Bundgaard et al., 2006; Millan et al., 1999). Antipsychotics exert their effects through a prevalent dopamine D_2 receptor (D_2R) antagonism (Laruelle et al., 2005) and, when acutely administered, induce a significant increase in dopamine release (Moghaddam & Bunney, 1990; Laruelle et al., 2005). Recent studies showed that the combined administration of SSRIs and antipsychotics might result in biochemical and molecular changes different from their individual effects (Chertkow et al., 2006, 2007).

A growing body of evidence suggests that neuroplastic changes may be implicated in the therapeutic effects of either class of drugs and perhaps of their combination (Konradi and Heckers, 2001; Ohashi et al., 2002). Indeed, it has been observed that the expression of

neurotrophic factors is enhanced by the concomitant treatment with fluoxetine and olanzapine (Maragnoli et al., 2004), while the subchronic administration of these two compounds suppresses the induction of two immediate-early genes (i.e., CREB and Fos) associated with long-lasting changes in synaptic efficacy (Horowitz et al., 2003).
Thus, we hypothesized that the combination of these two classes of drugs may result in changes in the expression of genes involved in synaptic plasticity. To test this hypothesis, we evaluated the effects of two SSRIs, citalopram and escitalopram, alone or in combination with haloperidol, on the expression profile of *Homer1a*, *ania-3* and *p11*. Our previous studies (de Bartolomeis et al., 2002; Polese et al., 2002; Ambesi-Impiombato et al., 2007; Tomasetti et al., 2007), in fact, pointed out a tight correlation between the pattern of *Homer1a* and *ania-3* expression and the impact onto the dopaminergic function exerted by typical and atypical antipsychotics. *P11* expression, indeed, has been reported to be related to serotonergic neurotransmission and to be affected by antidepressant therapy (Svenningsson et al., 2006).

Results.

Homer1a.

ANOVA test revealed significant differences in *Homer1a* expression among treatments in the outer layer of the parietal cortex (PCo: $p = 0.002$; $F_{(5;17)} = 6.10$) but not in the inner one (PCi: $p > 0.05$, $F_{(5;17)} = 0.90$), in the frontal cortex (outer layer, FCo: $p > 0.05$; $F_{(5;17)} = 1.95$; inner layer, FCi: $p > 0.05$; $F_{(5;17)} = 0.92$), and in the cingulate cortex (Cg: $p > 0.05$; $F_{(5;17)} = 0.41$).The post hoc test showed that *Homer1a* expression was significantly reduced in PCo by all treatment groups compared to VEH (Fig. 26 panel A; Fig. 27C).
Significant differences in *Homer1a* expression were detected among treatment groups in all striatal subregions with the exception of the nucleus accumbens shell (dorsomedial caudate-

putamen, DM: $p = 0.012$; $F_{(5;15)} = 4.39$; dorsolateral, DL: $p = 0.0001$; $F_{(5;15)} = 12.76$; ventromedial, VM: $p = 0.005$; $F_{(5;15)} = 5.29$; ventrolateral, VL: $p = 0.0001$; $F_{(5;15)} = 20.31$; core of the nucleus accumbens, CAcb: $p = 0.002$; $F_{(5;15)} = 6.33$; shell of the nucleus accumbens, SAcb: $p > 0.05$; $F_{(5;15)} = 2.24$) (Fig. 27A). The post hoc test showed that HAL significantly increased *Homer1a* expression: 1) compared to VEH in all regions of the caudate-putamen; 2) compared to ESC and CIT in DL and VL; and 3) compared to VEH in CAcb. *Homer1a* signal was significantly increased by HAL+ESC: 1) in DL and VL compared to VEH, ESC and CIT; and 2) in CAcb compared to VEH. Finally, HAL+CIT significantly induced *Homer1a*: 1) in all regions of the caudate-putamen compared to VEH; 2) in DL compared to ESC, CIT, HAL+ESC; 3) in VL compared to all the other treatments, 4) in VM compared to ESC; and 5) in AcCo compared to VEH (Fig. 27 A-B).

Ania-3.

Ania-3 showed a pattern of expression similar to *Homer1a* (Fig. 26 panel B; Fig. 27D-E-F). The ANOVA test revealed significant differences among treatments in all striatal subregions with the exception of the nucleus accumbens shell (DM: $p = 0.001$; $F_{(5;24)} = 6.25$; DL: $p = 0.0001$; $F_{(5;24)} = 13.99$; VM: $p = 0.003$; $F_{(5;24)} = 4.885$; VL: $p = 0.0001$; $F_{(5;24)} = 16.32$; CAcb: $p = 0.033$; $F_{(5;24)} = 2.91$; SAcb: $p > 0.05$; $F_{(5;24)} = 1.31$). Only a few differences from *Homer1a* were detected at the post hoc test: 1) in DM and in VM, HAL-treated group significantly induced *ania-3* expression compared to all the other treatments; 2) in DL, a significant *ania-3* signal increase was induced by HAL compared to VEH, ESC, CIT and HAL+ESC; 3) in DL and VL, HAL+CIT significantly induced *ania-3* compared to VEH, CIT and ESC; 4) in CAcb, *ania-3* was significantly induced by HAL compared to VEH (Fig. 27 D-E).

In the cortex, significant differences in *ania-3* expression were observed at the ANOVA in the FCi ($p = 0.006$; $F_{(5;24)} = 4.39$) and in the PCi ($p = 0.024$; $F_{(5;24)} = 3.20$) but not in the other subregions assessed (FCo: $p > 0.05$; $F_{(5;24)} = 1.55$; PCo: $p > 0.05$; $F_{(5;24)} = 1.55$; Cg: $p > 0.05$; $F_{(5;24)} = 1.46$). At the post hoc test, in the FCi *ania3* expression was significantly reduced by HAL + ESC compared to VEH and CIT. In PCi, a significant reduction of a*nia-3* expression by HAL + ESC compared to CIT was observed (Fig. 26 panel B; Fig. 27F).

Fig. 26

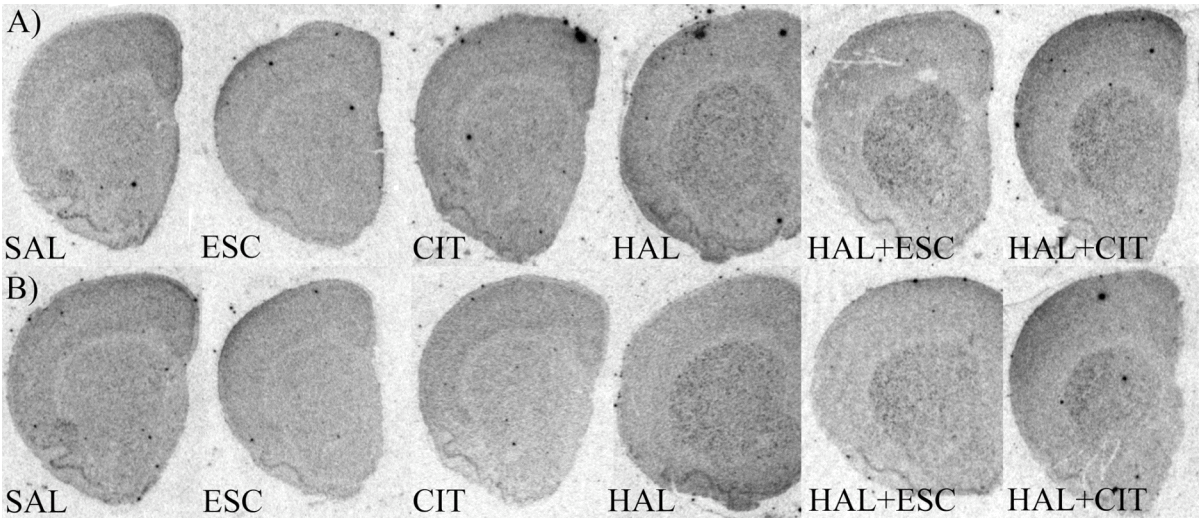
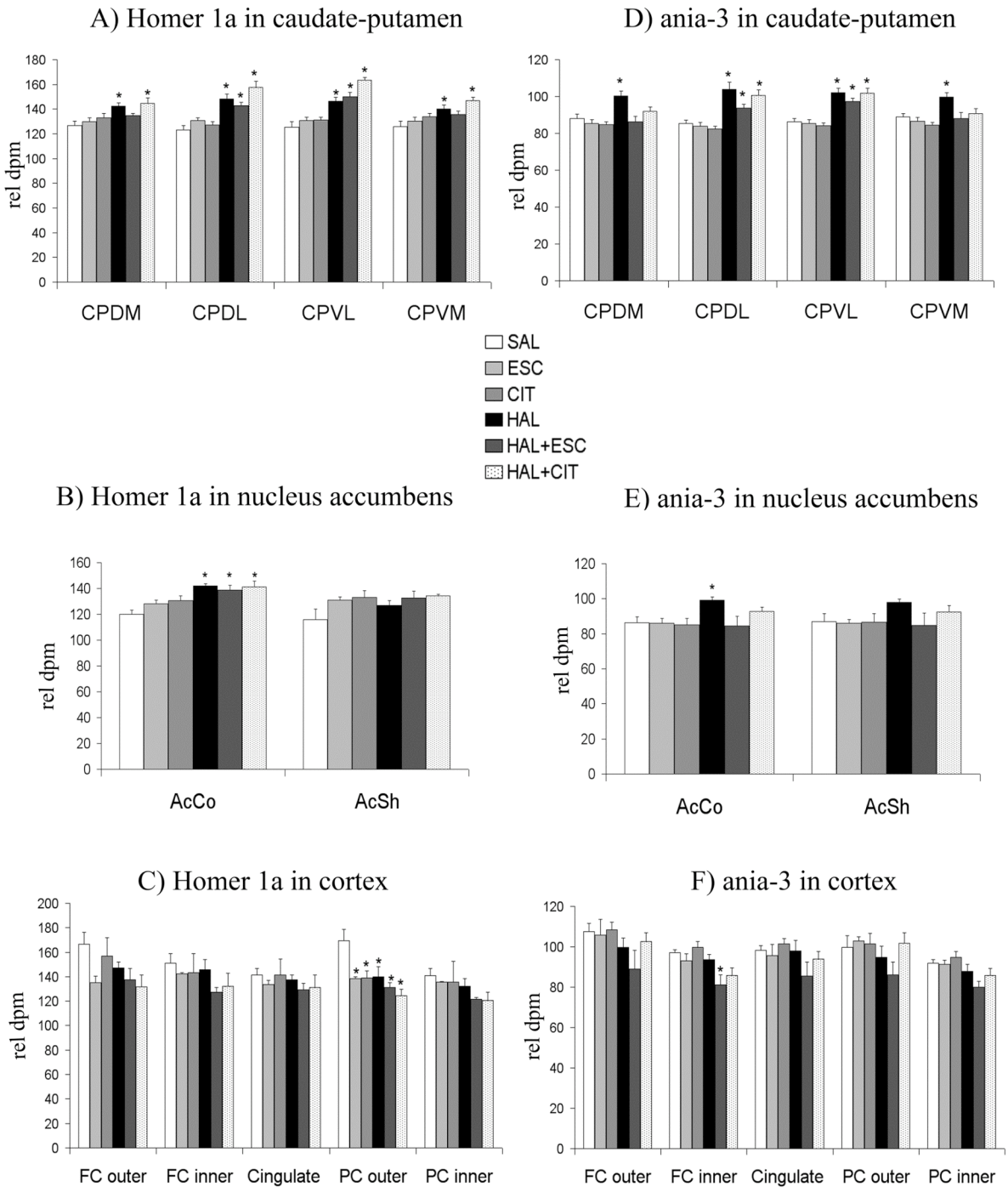


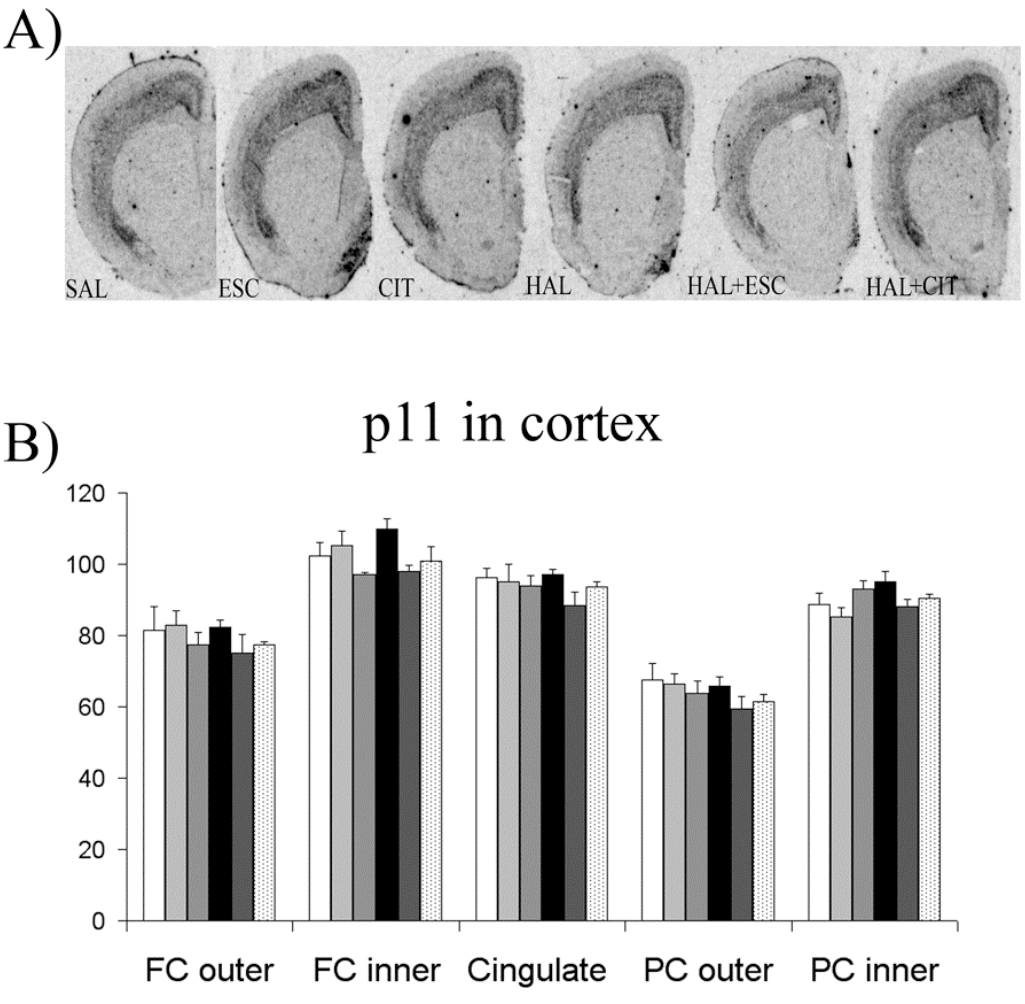
Fig. 27


A) Homer 1a in caudate-putamen
D) ania-3 in caudate-putamen
rel dpm
CPDM
CPDL
CPVL
CPVM
SAL
ESC
CIT
HAL
HAL+ESC
HAL+CIT
B) Homer 1a in nucleus accumbens
E) ania-3 in nucleus accumbens
AcCo
AcSh
C) Homer 1a in cortex
F) ania-3 in cortex
FC outer
FC inner
Cingulate
PC outer
PC inner

P11.

P11 expression was negligible in the striatum while it was detected in each subregion of the cortex (Fig. 28 A-B). Nonetheless, no significant differences among treatments were observed at the ANOVA test in any cortical subregion (FCo: $p > 0.05$; $F_{(5;23)} = 0.55$; FCi: $p > 0.05$; $F_{(5;23)} = 2.23$; PCo: $p > 0.05$; $F_{(5;23)} = 0.86$; PCi: $p > 0.05$; $F_{(5;23)} = 1.93$; Cg: $p > 0.05$; $F_{(5;23)} = 0.92$).

Fig. 28



Chapter 9.

Early genes expression by compounds with high and low dopamine D2 receptors affinity: insights into the preclinical classification of antipsychotics.

Rationale.

Sertindole is a limbic selective antipsychotic drug with a very low propensity to give extrapyramidal side effects (EPSE) that has been successfully employed against schizophrenia (Zimbroff et al., 1997). The compound shows affinity for the D_2 family of dopamine receptors, serotonin $5HT_{2A}$ and $5HT_{2C}$, and α_1 noradrenergic receptors (Arnt, 1992). Sertindole has demonstrated to be effective in animal models predictive of antipsychotic liability, antagonizing cocaine- and methamphetamine-induced place preference (Suzuki and Misawa, 1995), reversing impaired prepulse inhibition (PPI) (Andersen and Pouzet, 2001; Depoortere et al., 1997), and reducing the level of PCP-induced stereotyped behavior (Sams-Dodd, 1997). Assessments carried out by radioligand displacement in rat brain have shown that sertindole is characterized by low or minimum occupancy of D_2 receptors in vivo (Takahashi et al., 1998). Accordingly, sertindole seems not to induce oral chewing movements in rats (Gao et al., 1998) and catalepsy, even at very high doses (i.e., 40 mg/kg ip) (Ninan and Kulkarni, 1999). These preclinical observations correlate with the negligible motor side effects seen in humans.

Sertindole has been shown: i) to act preferentially upon mesolimbic over nigrostriatal dopaminergic neurons (Marcus et al., 2000); ii) to dissociate rapidly from D_2 receptors; iii) and to have a cholinergic profile, all features that can explain the low incidence of EPSE in patients. In similarity with clozapine and quetiapine, sertindole appears to not trigger EPSE at any dose used, whereas other atypical antipsychotics (namely, risperidone, olanzapine, and

ziprasidone) may induce motor impairment in a dose-dependent fashion, although far less than that observed with typical antipsychotics.

In our previous studies we have demonstrated that a clear-cut separation exists in the expression of the glutamatergic postsynaptic density (PSD) gene *Homer1a* by different class of antipsychotics. The prototypical neuroleptic haloperidol significantly induces the gene in all subregions of the caudate-putamen and nucleus accumbens (de Bartolomeis et al., 2002). Moreover, the expression of the gene is significantly higher than that triggered by any other atypical antipsychotic (Ambesi-Impiombato et al., 2007; Polese et al., 2002; Tomasetti et al., 2007). Indeed, *Homer1a* expression may be modulated by D_2 antagonism, as compounds with higher affinity for D_2 receptors (i.e.: haloperidol) induced the gene more than compounds with lower D_2 affinity (Iasevoli et al., 2007).

The aim of the study was to evaluate *Homer1a* expression by the atypical compound sertindole, which is almost completely devoid of EPSE propensity and is known to give low D_2 affinity, according to the hypothesis that sertindole may belong to the same class of quetiapine and clozapine in respect to *Homer1a* expression. Further, the study may expand our knowledge on the molecular discrimination among antipsychotic compounds. Further, we evaluated the impact of sertindole administration on the expression of genes relevant for PSD plasticity, namely the *Homer1a* splicing variant *Ania-3* and the early gene *Arc*, as to provide a more detailed picture of sertindole-induced perturbation in PSD.

Results.

Homer1a.

Sertindole did not induced *Homer1a* expression in any subregion of caudate-putamen and nucleus accumbens (Fig. 29a). Mean levels of mRNA expression by sertindole were lower than that elicited by vehicle. However, statistical significance was not reached, with the exception of a trend toward significance in the core of the accumbens. Consistently with our previous reports (de Bartolomeis et al., 2002), haloperidol induced *Homer1a* expression compared to vehicle in all striatal subregions (dorsomedial, DM, $p=0.0006$, $F_{2,11}=18.7841$; dorsolateral, DL, $p<0.0001$, $F_{2,11}=74.7199$; ventromedial, VM, $p=0.0064$, $F_{2,11}=9.3192$; ventrolateral, VL, $p<0.0001$, $F_{2,11}=40.8582$; core of accumbens, CAcb, $p=0.0016$, $F_{2,11}=14.2238$; shell of accumbens, SAcb, $p=0.0098$, $F_{2,10}=8.7056$). Moreover, haloperidol induced *Homer1a* expression significantly more than sertindole in all subregions (Fig. 29a).

Ania-3.

Ania-3 pattern of expression by haloperidol and sertindole highly resembled that of *Homer1a*, according with our previous preliminary observations (Ambesi-Impiombato et al., 2007). However, sertindole-induced mean levels of *Ania-3* expression were not lower than that by vehicle, as observed for *Homer1a*, being rather equivalent or slightly higher, though not reaching statistical significance (Fig. 29b). Hence, *Ania-3* was not induced by sertindole in any striatal subregions, while it was strongly induced by haloperidol in comparison to both vehicle and sertindole in all caudate putamen subregions (DM, $p=0.0001$, $F_{2,10}=32.7942$; DL, $p=0.0146$, $F_{2,10}=7.5026$; VM, $p=0.0024$, $F_{2,10}=14.0333$; VL, $p=0.0029$, $F_{2,10}=13.2941$). In the core of the accumbens no statistical significance was found at the ANOVA ($p=0.1113$, $F_{2,10}=2.9245$), whereas in the shell haloperidol induced significantly the gene compared to vehicle but not to sertindole ($p=0.0276$, $F_{2,10}=5.8136$) (Fig. 29b).

Arc.

Arc pattern of expression in the present paradigm was highly similar to that described for *Homer1a* and *Ania-3*. Arc was induced by haloperidol in comparison to both vehicle and sertindole in all striatal subregions, with the exception of the shell of the accumbens where haloperidol-induced expression was significant compared to sertindole- but not to vehicle-induced expression (DM, $p=0.0002$, $F_{2,10}=28.6929$; DL, $p=0.0048$, $F_{2,10}=11,2354$; VM, $p<0.0001$, $F_{2,10}=41.1899$; VL, $p=0.0036$, $F_{2,10}=12.2833$; CAcb, $p=0.0016$, $F_{2,10}=15.9017$; SAcb, $p=0.0269$, $F_{2,9}=6.3290$) (Fig. 29c). *Arc* was not induced by sertindole in any striatal subregions. Mean expression levels by sertindole were lower than that by vehicle, though not reaching statistical significance.

Topography of gene expression.

Homer1a signal distribution by vehicle and sertindole was homogeneous (Fig. 30). Haloperidol exhibited the typical distribution in three steps. As a control, distribution of two other early genes was assessed: the *Homer1a* transcript variant, *ania-3*, and *Arc*. Overall, signal distribution was highly similar to those observed for *Homer1a* (Fig. 30).

Western Blot.

Analysis was carried out on two independent sets of samples. In both sets, no significant differences among groups were detected (ANOVA: $p=0.986$; $F_{2,8}=0.014$; and ANOVA: $p=0.822$; $F_{2,8}=0.202$) (Fig. 29d).

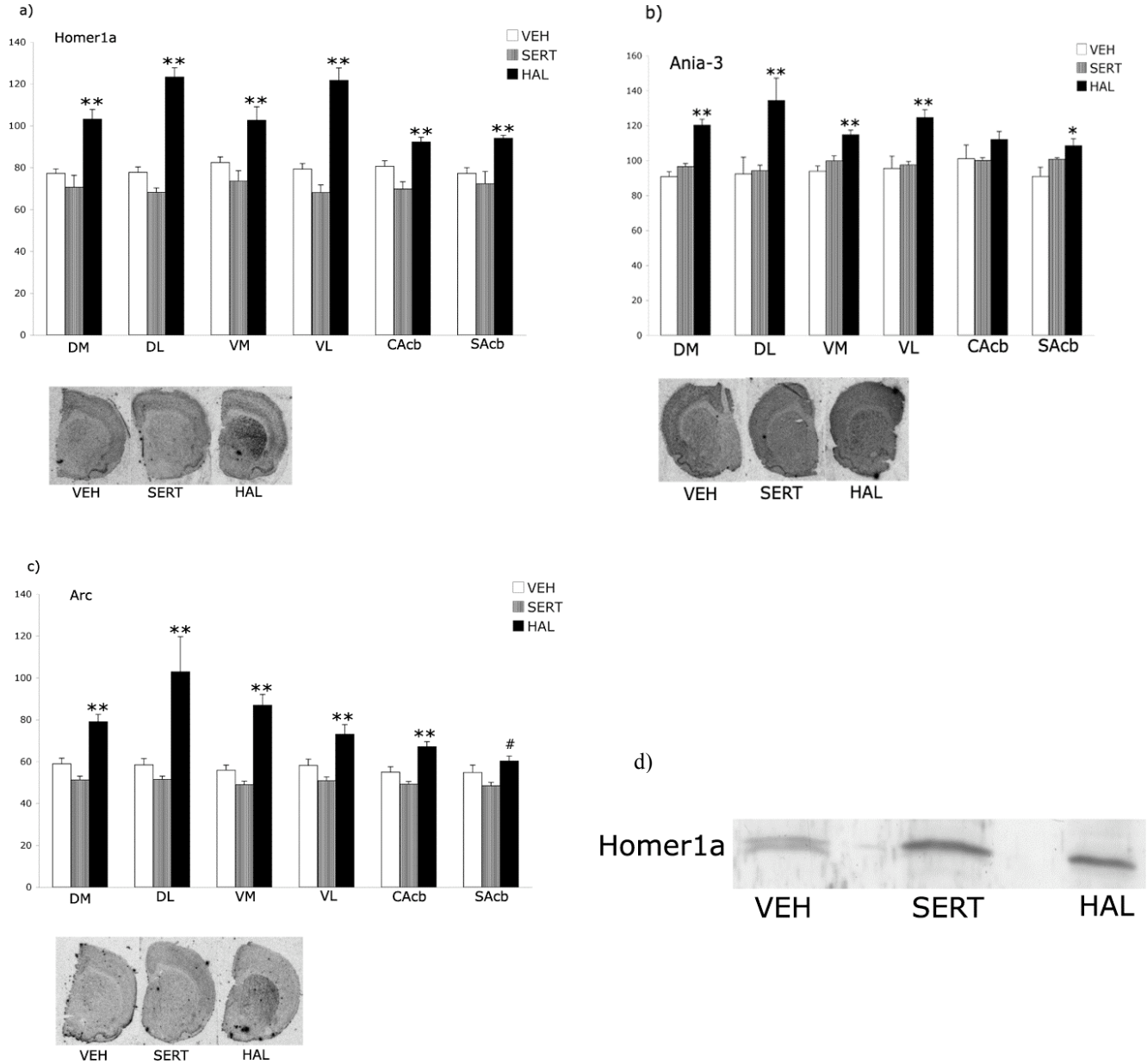


Figure 29. a) Upper panel: *Homer1a* mRNA levels in the caudate-putamen and nucleus accumbens. In all graphics, values are expressed in relative dpm as mean ± SEM. Post hoc test levels of significance: ** treatment vs VEH and SERT; * treatment vs VEH; # treatment vs SERT. Lower panel: autoradiographic film image of *Homer1a* mRNA after treatment with vehicle (VEH), sertindole (SERT), and haloperidol (HAL). **b)** Upper panel: *Ania-3* mRNA levels in the caudate-putamen and nucleus accumbens. Lower panel: autoradiographic film image of *Ania-3* mRNA after treatment with vehicle, sertindole, and haloperidol. **c)** Upper panel: *Arc* mRNA levels in the caudate-putamen and nucleus accumbens. Lower panel: autoradiographic film image of *Arc* mRNA after treatment with vehicle, sertindole, and haloperidol. **d)** Homer1a protein levels in the whole striatum as assessed by western blot.

Fig. 30

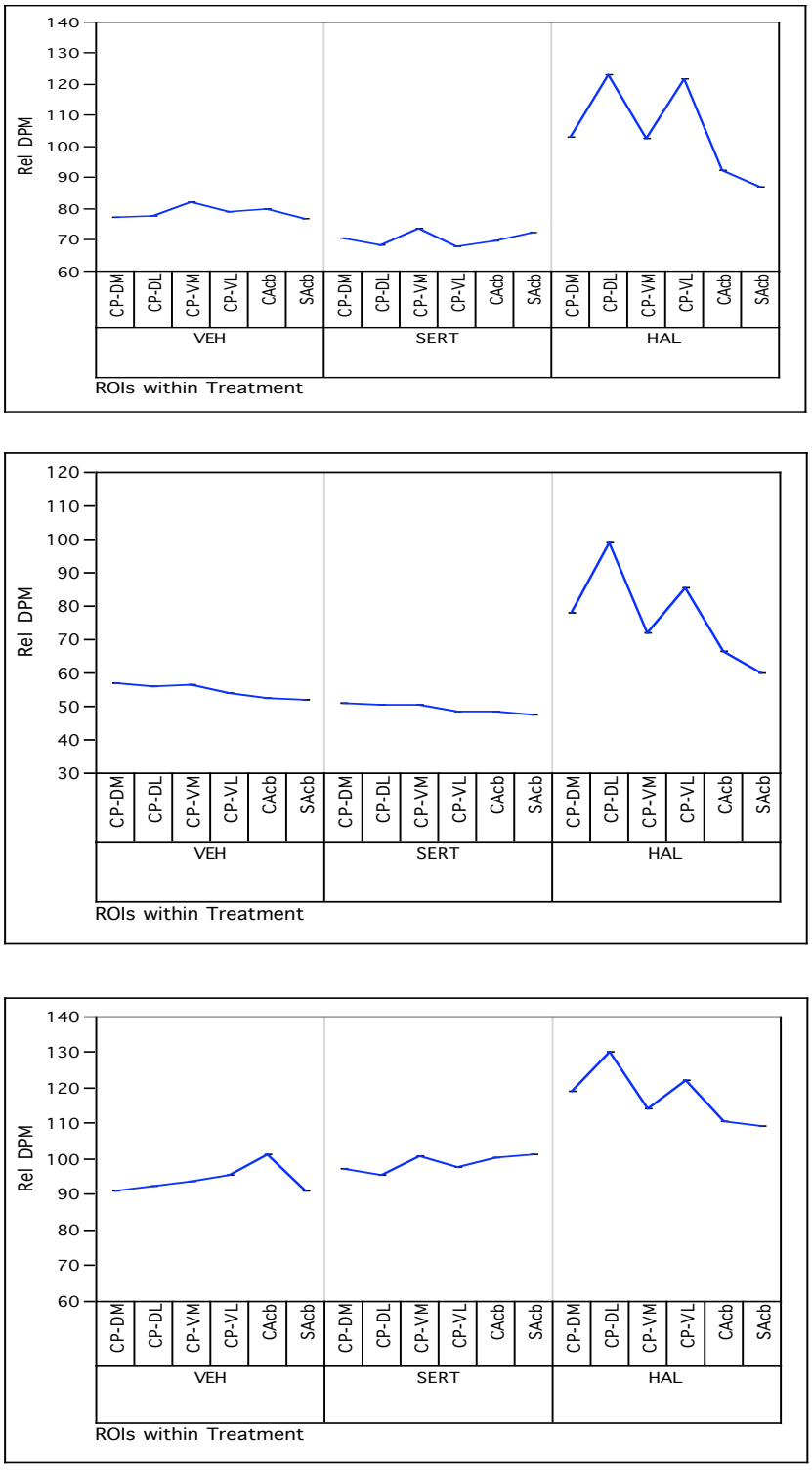


Figure 30. Topographical distribution of gene expression. *Homer1a* (upper panel), *ania-3* (intermediate panel), and *Arc* (lower panel) expression has been evaluated related to signal distribution among ROIs within each treatment in the striatum. Data are reported as averaged relative d.p.m. (Rel DPM). For clarity matter, S.E.M. bars have not been traced.

Chapter 10.

DISCUSSION.

Quantitative patterns of *Homer1a* expression by antipsychotic compounds.

Antipsychotic drugs share the property to block the dopamine D_2 receptor and increase acutely dopamine transmission in the striatum (Westerink et al., 2001). However, antipsychotics may be obviously distinct in typical and atypical ones according to their receptor profile. Typical antipsychotics exhibit high D_2 blocking potential, while atypical antipsychotics may have lower propensity to D_2 receptor antagonism and a broader profile of receptor interaction (Arnt and Skarsfeldt, 1998).

The quantitative pattern of *Homer1a* expression is sharply divergent according to the class of antipsychotic.

In several experiments conduced in our laboratory, we have seen that the prototype typical antipsychotic haloperidol triggered the expression of the gene in the whole striatum. Such induction was significantly higher than the expression observed in striata from control rats (Ambesi-Impiombato et al., 2007; de Bartolomeis and Iasevoli, 2003; Polese et al., 2002; Tomasetti et al., 2007). Moreover, induction by haloperidol was significantly higher than the induction obtained by any other antipsychotic compound. Thus, haloperidol shows the best propensity to induce the subcortical expression of *Homer1a* among compounds tested.

Atypical antipsychotics show different rates of *Homer1a* expression.

Risperidone and olanzapine when given at behaviorally active doses induced the expression of *Homer1a* significantly less than haloperidol and in the lateral caudate-putamen only. Olanzapine induced the gene also in the core of the nucleus accumbens, while risperidone did not. However, olanzapine when given at low doses (0.5 mg/kg) did not induced *Homer1a* expression (de Bartolomeis et al., 2002). Clozapine has been repeatedly shown to exhibit a trend toward the expression of *Homer1a* in the nucleus accumbens, but it did not trigger gene

expression in the caudate-putamen in any of the experimental paradigms in which it was tested (Polese et al., 2002; Tomasetti et al., 2007). Quetiapine did not induce gene expression at either a low (15 mg/kg) and an intermediate dose (30 mg/kg) in any striatal regions (Ambesi-Impiombato et al., 2007).

Ziprasidone appears to be an intriguing exception to this trend. In fact, the compound induced *Homer1a* expression in all striatal regions at a dose (4 mg/kg) known to exert antipsychotic action in animal models of psychosis. *Homer1a* induction was significantly less than induction by haloperidol in the lateral caudate-putamen, partially overlapping what observed with risperidone and olanzapine. However, the rate of *Homer1a* expression was virtually comparable to that by haloperidol when ziprasidone was given at a higher dose (10 mg/kg).

Another atypical antipsychotic showing a peculiar pattern of *Homer1a* expression was the D_2 receptor partial agonist aripiprazole. This compound exhibited an inverted biphasic action on *Homer1a*, as opposed to the other antipsychotics. In fact, aripiprazole triggered *Homer1a* expression at the lower (12 mg/kg) but not at higher dose (30 mg/kg) (Tomasetti et al., 2007).

Results from all antipsychotics tested so far have been pooled together by normalizing values of gene expression by haloperidol (which was assumed as the positive control in all the experiments) in each paradigm and then relating values of *Homer1a* expression by vehicle and other antipsychotics to normalized values of haloperidol-induced expression. The general picture that emerges from this pooling is that antipsychotics may be obviously differentiated in discrete subgroups as related to the quantitative pattern of *Homer1a* transcription: high, intermediate, low, and negligible, respectively.

The first group is represented from haloperidol, the compound that elicits the highest gene expression in all subregions. A second group comprises risperidone, ziprasidone, and

olanzapine, which elicit intermediate levels of *Homer1a* expression. However, in this group some exceptions need to be described. *Homer1a* induction by ziprasidone in the lateral caudate-putamen is significantly higher than that by risperidone and olanzapine, although not reaching levels by haloperidol. Moreover, in the dorsolateral striatum risperidone-induced *Homer1a* transcription is consistently, although not significantly, higher than that by olanzapine. Indeed, in the medial parts of the striatum the three antipsychotics induce comparable levels of *Homer1a* transcription. A third group is constituted by sulpiride and aripiprazole. These two antipsychotics induce low levels of *Homer1a* expression, although still differentiable from vehicle-induced levels. *Homer1a* induction by sulpiride and, less, by aripiprazole is near the amount by risperidone group in the medial striatum, while it separates obviously in the lateral striatum.

The last group is formed by quetiapine, sertindole, and clozapine, which exhibit low or negligible induction of the gene almost indistinguishable from that by vehicle.

The picture is more complex in the nucleus accumbens, where the highest levels of expression are reached by aripiprazole and the lowest by ziprasidone. Risperidone also induces poorly the gene in these subregions, while clozapine gives raise to intermediate amounts of *Homer1a* transcription. Olanzapine and sulpiride induces the gene at intermediate levels in the core and at low levels in the shell of the accumbens. Quetiapine triggers gene expression at intermediate levels in the shell of the accumbens.

The differences described above may be related to the impact on dopaminergic transmission exerted by each antipsychotic, and particularly to the ability of a compound to block D_2 receptors. D_2 receptor blockade may elicit *Homer1a* expression either postsynaptically by trans-activation of second messenger pathways (i.e.: the adenyl cyclase-cAMP mediated cascade) or presynaptically via the increase of dopamine and glutamate release and their subsequent action on D_1 and ionotropic receptors respectively (Surmeier et al., 2007).

According with this view, it has been described that antipsychotics increase striatal dopamine outflow when administered acutely (Wadenberg et al., 2001). However, antipsychotics vary greatly in their affinity to D_2 receptors and consequently in their ability to block these receptors (Kapur and Seeman, 2001). Acute dopamine release by antipsychotics appears consistent with their affinity to D_2 receptors (Westerink et al., 2001). The extent of *Homer1a* expression seems to overlap precisely the levels of dopamine released by antipsychotics and to be related to antipsychotic affinity to D_2 receptors, at least in the caudate-putamen. Further, it appears that higher D_2 receptors affinity could translate in higher levels of *Homer1a* expression in the lateral striatum. Among compounds tested, haloperidol exhibits the highest D_2 receptor affinity, followed from ziprasidone and risperidone, while quetiapine and clozapine holds very low D_2 receptor affinity (Kapur and Seeman, 2001). This subdivision appears to closely match the subdivision observed above for *Homer1a* expression. Thus, the extent of *Homer1a* expression may be a marker of drug affinity to D_2 receptors.

This assumption appears not to be valid in the nucleus accumbens, where other molecular mechanisms beyond mere D_2 blockade may concur at *Homer1a* induction. As described in the previous section, we have observed that D_1 receptor blockade may trigger *Homer1a* expression in the ventral striatum, including the nucleus accumbens. It could be hypothesized that a concomitant D_1/D_2 receptors blockade mechanism may facilitate *Homer1a* expression in these subregions. Indeed, aripiprazole and clozapine hold moderate antagonism at D_1 receptors and significantly induce the gene in the nucleus accumbens, while risperidone, which is virtually devoid of D_1 receptor action (Arnt and Skarsfeldt, 1998), did not.

The evaluation of haloperidol induction of *Homer1a* expression in cortical regions led to contrasting findings. However, the overall trend appears to delineate a low impact on *Homer1a* expression in this region. This result may depend on the lower abundance of D_2

receptors in the cortex, as compared to the striatum. More studies are needed to substantiate these speculations. The evaluation of *Homer1a* expression by antipsychotics in dopamine-depleted rats may help corroborate the view that *Homer1a* induction is dependent from dopamine transmission. This experiment may be complemented by a study combining the evaluation of dopamine release by microdyalisis and *Homer1a* expression by in situ hybridization or PCR to relate the extent of *Homer1a* expression to striatal dopamine outflow. The coadministation of antipsychotics and blockers of dopamine D_1 receptors may reveal whether dopamine action via postsynaptic D_1 receptors is the key mechanism to induce *Homer1a*. Further, a more stringent evidence of the putative relation between *Homer1a* expression and affinity to D_2 receptors must be traced by expanding the number of compounds tested.

The dose-issue.

A mighty confounding factor may be compound dosing since *Homer1a* levels are dose-dependently enhanced by antipsychotics. A clear-cut separation exists between *Homer1a* induction by the 4 mg/kg and the 10 mg/kg ziprasidone doses. The latter produces levels of *Homer1a* transcription which are virtually indistinguishable from that by haloperidol, while *Homer1a* induction by 4 mg/kg ziprasidone is significantly lower than that by haloperidol and 10 mg/kg ziprasidone in the lateral striatum. *Homer1a* expression by a low olanzapine dose (0.5 mg/kg) is not significantly different from vehicle (de Bartolomeis et al., 2002), while induction by a behaviorally active dose (2.5 mg/kg) is significantly higher than basal levels in the lateral striatum and in the core of the accumbens. Higher levels of *Homer1a* levels by the 30 mg/kg quetiapine dose in comparison with the 15 mg/kg dose were observed, although significance was not reached (Ambesi-Impiombato et al., 2007). A singular exception to this trend is represented from aripiprazole, which induces significantly

higher *Homer1a* mRNA levels at the lower dose used (12 mg/kg *vs.* 30 mg/kg) (Tomasetti et al., 2007). The apparent discrepancy from the general picture may be explained by the peculiar mechanism of action of the compound, which behaves as a partial agonist at D_2 receptors. Hence, in a condition of basal dopaminergic tone, a lower dose of the compound may impair dopaminergic transmission via D_2 receptors more than a higher dose (Miyamoto et al., 2005).

The considerations about dose-dependency of *Homer1a* expression may suggest that the rate of gene expression may be linked to D_2 receptor occupancy by an antipsychotic. It has been observed that striatal D_2 receptor occupancy increases with antipsychotic dose until reaching a plateau, which is different for each compound (Kapur and Seeman, 2001). Moreover, some antipsychotics (i.e.: clozapine and quetiapine) exhibit low D_2 receptor occupancy even at very high doses (Kapur and Seeman, 2001). However, a lengthy correlation between *Homer1a* expression by an antipsychotic and its D_2 receptor occupancy is still speculative and must be substantiated by further experiments evaluating *Homer1a* levels at different values of D_2 receptor occupancy. The lack of *Homer1a* expression by clozapine and quetiapine in the caudate-putamen appears to be consistent with the observed low D_2 receptor occupancy of these compounds. Nevertheless, their action on *Homer1a* might be also investigated at very high doses to confirm their negligible induction of the gene, similarly to their negligible D_2 receptor occupancy.

Correlation between *Homer1a* expression by antipsychotics and their extrapyramidal side-effects liability.

Another interesting issue is whether the extent of *Homer1a* expression may be related to the liability to extrapyramidal side effects of an antipsychotic. Typical antipsychotics are classically described to hold high D_2 receptor affinity and D_2 receptor occupancy and to

cause extrapyramidal side effects (Seeman and Tallerico, 1999). As haloperidol is a prototype typical antipsychotic, it is expected that typical antipsychotic drugs would show quantitative patterns similar to haloperidol once tested for *Homer1a* expression, according with the suggestion that *Homer1a* expression may be linked to D_2 receptor affinity and occupancy. However, recent evidence shows that some atypical antipsychotics (i.e.: risperidone and ziprasidone) may exhibit higher D_2 receptor affinity than most of the typical antipsychotics (Kapur and Seeman, 2001). Moreover, it has been proposed that low EPS liability of a compound may be more likely related to its action at serotoninergic receptors than to low D_2 receptor affinity (Meltzer, 1995). The considerations above may suggest that the amount of *Homer1a* induction by most typical antipsychotics may be less than that obtained by some atypical antipsychotics, irrespective of EPS liability of a compound. To date, no studies have explored the levels of *Homer1a* mRNA as induced by typical antipsychotics other than haloperidol: assessing *Homer1a* expression by typical antipsychotics may expand our knowledge in the field.

Ziprasidone is a suggestive example of an atypical antipsychotic exhibiting high D_2 receptor affinity and occupancy but low EPS liability, due to its favorable serotoninergic profile. In our study, a behaviorally active dose of ziprasidone (4 mg/kg, which has been observed to revert amphetamine-induced hyperlocomotion) induced *Homer1a* in all caudate-putamen subregions, although significantly less than haloperidol. The high ziprasidone dose of 10 mg/kg falls at the lower limit of the dose-range shown to be slightly cataleptic (Seeger et al., 1995). This dose elicited a strong induction of *Homer1a*, virtually indistinguishable from haloperidol. Thus, quantitative patterns of *Homer1a* induction may be related to antipsychotic dopaminergic profile, and possibly to D_2 receptor occupancy, but apparently not to motor impairment exerted by an antipsychotic compound.

Distinction between *Homer1a* and *c-fos.*

The analysis of quantitative patterns of expression allows tracing a first distinction between *Homer1a* and *c-fos*. The early gene *c-fos* is induced by all antipsychotics in the shell of the nucleus accumbens and by cataleptic antipsychotics only in the dorsolateral striatum (Robertson et al., 1994). These features may reflect the preferential site of action of antipsychotics, i.e.: the nigrostriatal over the mesolimbic neuronal pathway. Conversely, *Homer1a* is not induced by all antipsychotics in the shell of the accumbens. Moreover, induction in the dorsolateral striatum may not be seen as a thorough sign of the liability to motor side effects of an antipsychotic. The discrepancy likely reflects divergent mechanisms of induction of the two early genes. It has been observed that *c-fos* induction is obtained after several pharmacological stimuli, including opiodergic, adenosinergic, and cholinergic compounds. On the contrary, *Homer1a* induction appears to be under a definite dopaminergic and glutamatergic control. Thus, *c-fos* may be less specific than *Homer1a* in evaluating the impact on dopaminergic transmission of a putative antipsychotic compound. Furthermore, *Homer1a* is an effector IEG and it has been demonstrated to play a role in synaptic plasticity and behavioral tasks (Tappe and Kuner, 2006). Thus, *Homer1a* may be a key factor in the molecular action of antipsychotics, a feature that is not shared by *c-fos*.

Temporal pattern of *Homer1a* expression by antipsychotics: triggering neuroplastic changes at the PSD.

Homer1a expression has been evaluated also along prolonged time-course administration of antipsychotics in three independent sets of experiments. Sustained antipsychotic administration allows drawing experimental paradigms more closely related to real-world practice where therapies are often long lasting and life spanning.

Neuroplastic changes in the cytoarchitecture of synapses are believed to be the molecular correlate of behavioral effects of antipsychotics (Konradi and Heckers, 2003). Neuroplasticity may rely on changes in the pattern by which gene transcripts respond to chronic *vs.* acute administration of psychotropic compounds. Indeed, a large body of evidence confirms that sustained administration of psychotropic compounds may cause stable changes in gene expression, i.e.: sensitization or tolerance phenomena (Robinson and Kolb, 2004). Upregulation or downregulation of gene expression may thus represent a preliminary step of a complex cascade of molecular events leading to changes in neuronal architecture and/or neurophysiology (Nestler, 2004).

Homer proteins are known to bridge metabotropic glutamate receptors to either intracellular second messengers machinery or ionotropic glutamate receptors and may thus regulate postsynaptically the strength and the direction of glutamatergic signals. Moreover, our studies have demonstrated that *Homer1a* expression is profoundly affected by dopaminergic compounds, suggesting that Homers may represent crossroad factors in the interplay between dopaminergic and glutamatergic systems.

In our experiments, we administered antipsychotics for 21 days and then sacrificed rats at 90 minutes from the last injection, thus mimicking the condition of an acute challenge with the same compounds. We hypothesized that the sustained administration could modify the response to the acute antipsychotic challenge. To fully characterize the pattern of temporal response, we employed the typical and high D_2 receptors blocking antipsychotic haloperidol and a series of atypical antipsychotics that differ for their mechanism of action at D_2 receptors: the intermediate D_2 receptor antagonist ziprasidone, the low D_2 receptor antagonists quetiapine and clozapine, and the D_2 receptor partial agonist aripiprazole. In one paradigm we also explored *Homer1a* expression after a 24-hour withdrawal from antipsychotic administration, as to investigate whether prolonged antipsychotics may sustain

or decrease gene expression in a wider time window than that explored in the previous experiments.

As a first striking finding, we observed that sustained antipsychotic administration does not modify grossly the pattern of *Homer1a* acute expression (i.e. 90 minutes after antipsychotic injection). In the first chronic study we carried out, *Homer1a* induction was observed in lateral caudate-putamen subregions by haloperidol (0.8 mg/kg) while quetiapine (15 mg/kg) did not induce the gene. These results overlapped those obtained after acute administration of the same compounds, although in the acute paradigm haloperidol had induced the gene in all caudate-putamen subregions. Consistent with these findings are the results from two other studies employing haloperidol (0.8 mg/kg), aripiprazole (12 mg/kg), and clozapine (20 mg/kg) in one case and haloperidol (0.8 mg/kg) and ziprasidone (4 mg/kg) in another.

In the latter study, *Homer1a* expression was also assessed after a 24-hour withdrawal from the last injection scheduled. Indeed, there is some evidence that *Homer1a* expression may decrease significantly under control values at time-points delayed from administration of psychotropic compounds (Cochran et al., 2002). No significant induction by either haloperidol or ziprasidone was recognized at the 24-hour time-point. Two-way ANOVA showed that differences in antipsychotic induction of *Homer1a* expression at the two time-points were due to treatment effect, time effect, and to the interaction between these two variables. Indeed, haloperidol induction at the 90 minutes time-point was significantly higher than that observed at the 24-hour time-point, and the same attained for ziprasidone induction. Interestingly, no differences were observed in baseline *Homer1a* expression at the two time-points. These results suggest that *Homer1a* induction by antipsychotics does not undergo sensitization or tolerance phenomena: acute *Homer1a* expression preserves its features also after sustained antipsychotics treatments. Hence, profiling of *Homer1a* expression represents a specific and sensible marker of acute antipsychotic administration even after a prolonged

treatment. This feature could render the gene a precious tool to study the pathways of neuronal activation and perhaps *in vivo* blockade of D_2 recptors by antipsychotics in preclinical models aimed to mimic the time-course of therapy in clinical practice.
At a molecular level, the lack of modifications in *Homer1a* pattern of expression after a prolonged antipsychotic treatment may depend on its specific role in inhibiting neuronal hyperpolarization and damage.

Topography of *Homer1a* expression by antipsychotics.

Besides the quantitative evaluation of *Homer1a* expression by antipsychotics, a less studied field is whether the topographic (i.e.: subregional) distribution of gene expression signal may separate treated *vs.* untreated groups and whether signal distribution may relate to molecular mechanisms of action of antipsychotics.
In an acute administration paradigm *Homer1a* expression by haloperidol and ziprasidone distributed in the striatum along a dorsolateral-to-ventromedial gradient, following a "three-steps" model of distribution. The amount of gene expression was higher in the dorsolateral striatum and decreased progressively in ventral and medial striatal subregions, being the lowest in the shell of the nucleus accumbens. Differences in gene expression among subregions were significant at the ANOVA in several cases. On the other hand, gene expression in control striata was homogeneous (no significant differences at the ANOVA) and did not show any peculiar pattern of subregional distribution, although a prominent signal labeling was still recognizable in the dorsolateral putamen. Clozapine also showed no significant differences in the rate of mRNA expression among striatal subregions. However, *Homer1a* expression was prominent in the dorsolateral putamen, according with all other compounds, and it was relatively higher in the nucleus accumbens than other antipsychotics.

To strengthen these data, we replicated the topographical analysis on measurements from other paradigms, including those availed in some previously published papers.

Topography of *Homer1a* distribution by haloperidol was consistent in all paradigms, although some not-significant discrepancies could be detected in the distribution curve profile. These discrepancies are likely attributable to biological variability.

Homer1a distribution by 12 mg/kg aripiprazole resembled, although not overlapped, those of haloperidol, while 30 mg/kg aripiprazole showed a relative abundance of *Homer1a* expression in the shell of the accumbens. *Homer1a* induction by 12 mg/kg aripiprazole was significant ($p<0.0001$) in the lateral and medial putamen compared with the nucleus accumbens. However, no significant differences were detected among lateral and medial subregions. *Homer1a* induction by 30 mg/kg aripiprazole showed a weak trend to significance ($p=0.0417$), with expression in the dorsolateral putamen and the shell of the accumbens being significantly higher than that in the dorsomedial putamen and the core of the accumbens. Clozapine and vehicle distributions in this paradigm were homogeneous ($p>0.05$), consistent with what observed in the ziprasidone-paradigm.

Risperidone and olanzapine also induced *Homer1a* with a distribution profile that resembled that of haloperidol. As in the case of 12 mg/kg aripiprazole, both risperidone and olanzapine gave significant higher *Homer1a* expression in the lateral and medial putamen compared with the nucleus accumbens ($p<0.0001$), without significant differences between the lateral and medial subregions. Sulpiride-induced distribution profile of *Homer1a* signal evidenced a prominent expression in the ventral caudate-putamen, with significant higher *Homer1a* induction in the ventral and dorsolateral caudate putamen compared with nucleus accumbens ($p=0.0353$).

It could be concluded from these data that *Homer1a* appear to distribute not homogeneously in the striatum after antipsychotic administration as compared to basal gene distribution and

to display distinctive distribution profiles, according with the dopaminergic profile and the dose of the compound tested.

As a further verification, the pattern of topographic distribution of the dopamine reuptake inhibitor GBR12909 was evaluated to test whether the topographic analysis may discern antipsychotics from non-antipsychotic compounds.

Homer1a distribution by GBR12909 was strikingly divergent from the distribution by any antipsychotic. In fact, GBR12909 induced a prominent expression in the ventrolateral and the dorsomedial caudate putamen, which was significantly higher ($p<0.0001$) than in all other striatal subregions. We have already demonstrated that the preferential ventral distribution of *Homer1a* expression may be a distinctive feature of psychotomimetic drugs, in particular those provided of dopamine agonism liability (Ambesi-Impiombato et al., 2007). Thus, this additional evidence appears to confirm that a separation may be drawn on the basis of topographic distribution of *Homer1a* expression among antipsychotic and non-antipsychotic compounds.

These findings allow many suggestions. It has been recently theorized that, in opposition to the early-proposed dorsal-ventral subdivision of caudate-putamen organization, a more functional and reliable subdivision of the region along a dorsolateral-to-ventromedial gradient would need to be drawn (Voorn et al., 2004). This gradient may account for anatomical and histochemical observations and it may best fit with the course of neuronal projections from the cortex. Interestingly, this newly proposed organization of caudate-putamen subdivision appears to closely match the graded density of medium-sized spiny neurons, the GABAergic postsynaptic interneurons that constitute approximately 95% of all striatal cells and express the postsynaptic density machinery, including Homers, in their dendritic arborizations (Tappe and Kuner, 2006). Moreover, there is some evidence that also distribution of dopamine D_2 receptors may follow a similar dorsolateral-to-ventromedial

gradient in the striatum (Alcantara et al., 2003; Fisher et al., 1994; Hall et al., 1994; Meador-Woodruff et al., 1996; Russell et al., 1992; Szele et al., 1991).

Thus, the pattern of striatal distribution of *Homer1a* may be consistent with the distribution of medium-sized spiny neurons and perhaps of dopamine D_2 receptors. Indeed, significantly higher gene expression in the lateral striatal subregions compared to other striatal aspects is seen by antipsychotics that robustly affect dopamine D_2 receptors. This observation could be explained by a lower concentration of either GABAergic interneurons or dopamine D_2 receptors in ventromedial striatum and nucleus accumbens compared to the dorsolateral striatum. Although attractive, these hypotheses are preliminary and need to be substantiated by more studies. A double in situ immunohistochemistry study may allow localizing *Homer1a* and D_2 receptor mRNAs, confirming (or rejecting) the hypothesis of their matching distribution. However, since D_2 receptor involved in *Homer1a* modulation may be presynaptic, such experiment may also yield confounding results. A further study by double immunostaining for *Homer1a* and enkephalin (which is a marker for GABAergic neurons carrying D_2 recptors) may help to precisely correlate the distribution of *Homer1a* to the concentration of GABAergic interneurons in discrete subregions of the striatum. These experiments may allow shed some lights on the issues discussed above.

If confirmed by adjunctive experiments, the pattern of distribution of *Homer1a* may help discern antipsychotics provided of *in vivo* action at the dopamine D_2 receptor, as haloperidol and ziprasidone, from that, as clozapine, which are virtually devoid of this action *in vivo*. Thus, the profiling of *Homer1a* expression may be predictive of the antidopaminergic property of a compound. However, behavioral studies coupled with the examination of topographic distribution of the gene would be needed to corroborate this view.

Another issue which may come out from the assessment of topographic distribution of *Homer1a* expression is that antipsychotics prominent induction in specific subregions may

relate to their clinical and side-effects profile and may correspond to the activation of discrete neuronal networks, For example, the relative abundance of *Homer1a* expression in the nucleus accumbens by clozapine compared to the other antipsychotics evaluated may depend on the well-known preferential action of clozapine upon the mesolimbic over the nigrostriatal neuronal pathway and it may give a further molecular explanation of clozapine's lack of motor side effects. The potent and significant induction of *Homer1a* by haloperidol in the lateral striatum over other striatal subregions and the low relative expression in the ventral limbic areas of the striatum it represents a further and refined confirmation of the prevalent action of this compound on non-limbic tasks and on nigrostriatal projections. However, ziprasidone show a striatal distribution similar to that of haloperidol but at the doses tested is not cataleptic and is generally considered to hold low liability to motor side effects in humans. *Homer1a* distribution in the striatum may be obviously affected by the interaction with dopamine D_2 receptor. However, since tight interaction with dopamine D_2 receptor is a key condition to give motor disturbances, this is not sufficient. In the case of ziprasidone and of other atypical antipsychotics, intermediate action at the dopamine D_2 receptor is counteracted by their strong serotoninergic action. As demonstrated in the paradigm of SSRIs+haloperidol co-administration, *Homer1a* expression seems to be not affected by serotoninergic agonism/antagonism in the striatum, while it could be in the cortex. This may explain why ziprasidone, even exhibiting a striatal distribution profile of *Homer1a* expression similar to that of haloperidol, may not be accounted for a cataleptic compound.

Cortical distribution has been examined by delineating five regions of interest according with expert opinions in the field. All groups tested, including controls, showed a not-homogeneous distribution of *Homer1a* signal that resulted prominent in the motor-related

regions of the Medial Agranular Cortex and of the Motor Cortex. This feature may reflect a relative abundance in these regions of the cells expressing the gene.

While these statements appear true for acute antipsychotics, are they still suitable in chronic treatment paradigms? Chronic regimens do rise to complex pictures. When *Homer1a* profiling is observed in rats sacrificed acutely after the last injection, the general trend of distribution mimic, although not overlap, those observed after acute antipsychotics. Gene expression is prominent in the dorsolateral caudate-putamen and is lower in the nucleus accumbens. Distribution in control striata is homogenous, while both ziprasidone and haloperidol exhibit significant expression in the dorsolateral putamen compared to other ventral subregions. However, some subtle changes are particularly evident for haloperidol, which may imply the occurrence of neuroplastic changes.

The similarity of striatal distribution profile in acutely sacrificed chronic treated rats is however not paralleled by cortical distribution profile. In fact, the general trend of distribution resembled partially those observed in the acute paradigm, with prominent gene expression in the Medial Agranular Cortex, the Motor Cortex, the Anterior Cingulate cortex, and the Infralimbic Cortex. Significant differences among subregion expression were seen for ziprasidone but not for haloperidol.

It could be hypothesized that, although the overall feature of signal distribution may seem preserved after a prolonged antipsychotic treatment, the profiling of gene distribution may be not a valuable tool to discriminate antipsychotics after their sustained administration. A presumable explanation may be that some neuroplastic changes may have been occurred during sustained antipsychotic treatment. This view seems to be confirmed by the distribution profiling in rats sacrificed 24 hours after the last chronic injection. In fact, signal distribution is homogeneous in all groups in either the striatum or the cortex, with the

intriguing exception of haloperidol that shows significant induction in the ventral regions of striatum compared to the other striatal subregions.

Changes in the expression of Homer1a-related genes.

In the different experimental paradigms, several other genes, mainly constitutive, have been tested to investigate whether significant changes in their levels of expression may be observed by the psychotropic compounds administered. Genes tested were chosen among PSD factors which can functionally relate to Homer1a and can be modulated by perturbation of dopamine-glutamate interplay. As a general consideration, it could be stated that changes in non-inducible genes were low from a quantitative point of view and substantially less evident than that described for *Homer1a*.

Constitutive genes expression was assessed in either acute or chronic paradigms.

Acute administration of haloperidol or selective antagonists at dopamine receptors was observed to overall reduce the levels of both *Homer1b* and *mGluR5* expression in striatal and cortical areas. Reduction of gene expression was particularly evident for compounds provided of antagonism at D_2 receptors, namely L-741,626, terguride, and haloperidol. Further, a negative modulation by L-745,870 was also observed. On the contrary, a positive modulation by SCH-23390 was recognized in some subregions. These data may suggest an opposite control upon *Homer1b* and *mGluR5* expression by D_1 and D_2 receptors. Moreover, it appears that D_2 receptors may modulate the expression of *Homer1a* at one side and *Homer1b* and *mGluR5* at the other side in opposite directions. This contrary modulation may reflect the opposite physiological role of these factors: as Homer1a appears to break mGluRs-mediated transduction, Homer1b facilitates transduction by approximating mGluRs to intracellular effectors.

CaMKII expression was acutely increased in several striatal areas by a low ketamine dose. As CaMKII function is positively modulated by NMDA receptor activation (REF), the increase in gene expression by a non-competitive NMDA receptor blocker may reflect a feedback mechanism to preserve neuronal homeostasis or be sustained by hyperglutamatergy on non-NMDA glutamatergic receptors.

Evaluation of changes in constitutive genes expression reveals more attractive when conduced in chronic paradigms. No significant changes in *Homer1b* expression were observed in two independent chronic experiments. However, levels of expression were affected by time: basal, ziprasidone-, and haloperidol-induced gene expression was significantly higher at 90minutes than at 24hours from the last injection in a chronic (21 days) paradigm of compound administration.

Both time and treatment may instead affect the expression of the *PSD95* constitutive gene. In the striatum, gene expression was acutely increased by both haloperidol and ziprasidone after a prolonged treatment. After a 24 hours withdrawal from the last injection, however, only ziprasidone increased gene expression in the VM putamen only. In the cortex, both haloperidol and ziprasidone increased gene expression at either 90 minutes or 24hours from the last injection. These findings seem to confirm the view that antipsychotics may trigger neuroplastic changes at the PSD.

A trend toward the increase was also recognized for the gene coding for the dopamine D_2 receptor after a prolonged treatment with antipsychotics. This finding is in line with reports dcescribing an up-regulation of D_2 receptors following chronic antipsychotic treatments.

Ania-3 is a *Homer1a* transcript variant which is induced in an IEG-like fashion. Our studies corroborate the view that *ania-3* follows patterns of quantitative and topographic induction similar to *Homer1a*.

Arc is another early gene coding for a PSD factor which regulates cytoskeleton organization. Patterns of quantitative and topographic expression appear similar to *Homer1a*, although assessments are limited to one experimental paradigm only.

Concluding remarks.

The studies summarized here were intended to analyze the features of *Homer1a* expression and of some Homer1a-related genes in different conditions of dopamine (and sometime glutamate) transmission perturbation. The different paradigms employed allowed to draw a comprehensive picture on the issue and opened new interesting questions.

Homer1a seems to be obviously induced by compounds provided of antagonist action at dopamine D_2 receptors. This property renders *Homer1a* a valuable tool to study antipsychotics, which main mechanism of action is represented by blockade of D_2 receptors. Moreover, analysis of *Homer1a* expression may be also employed to study the action upon D_2 receptors of novel putative antipsychotics or procedures taken from clinical practice, as adjunction to antipsychotics of non-antipsychotic compounds to increase efficacy.

Another suggestion appears to be that *Homer1a* may be integral at the mechanism of antipsychotics action at the PSD. More studies, assessing protein level and functional changes after administration of these compounds, may clarify this intriguing issue.

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Come qualcuno avrà notato, non ho iniziato la tesi, come è oramai di moda, con dediche strappalacrime e/o melodrammatiche pagine di ringraziamenti a madripadrisuocerecanigattiamantifiglifratelliamicinemici.
Non amo l'ostentazione: le cose preziose vanno protette e tenute lontane da eccessivi sguardi.
Dunque, il mio primo ringraziamento va a chi avrà avuto la pazienza e la benevolenza di arrivare con la lettura fin quaggiù, a scoprire questa ghost-track.

Ho inserito in questa tesi esperimenti (ed esperienze) compiute nel corso dei tre anni di dottorato, ma in alcuni casi intraprese già durante la specializzazione. Inoltre ci sono i riferimenti anche ai lavori effettuati quando ero ancora al corso di laurea. Ciò per dire che questa tesi rappresenta una sorta di *summa* del lavoro svolto nel corso di questi anni di ricerca universitaria. Ho preso la decisione di non limitare la tesi ad un singolo studio o ad una parte dei lavori svolti, innanzitutto, per un motivo teorico: credo che la portata scientifica del nostro lavoro sia più facilmente apprezzabile se la si guarda nel suo complesso. Difatti nella discussione, piuttosto che indugiare sugli aspetti problematici e sulle criticità che inevitabilmente emergevano da ciascuno studio, ho cercato di tratteggiare un quadro d'insieme dei nostri risultati, rianalizzandoli nella loro globalità. Questo, a mio parere, permette anche di comprendere meglio il razionale generale e la direzione delle nostre ricerche e ci consente di avere più chiari i successivi passi che noi (o chi ci seguirà) dovremo intraprendere per proseguire il cammino teorico intrapreso.
Ciononostante, la decisione di mettere giù una tesi così ampia e complessa ha avuto anche una motivazione personale. Volevo che questa tesi fosse la celebrazione, il compendio, la festa (e forse anche il necrologio) di una esperienza personale importante e lunga. Credo che

sia difficile ripensare a noi stessi in un certo periodo temporale senza collocarci all'interno di un particolare sfondo spaziale. Detto meno astruso, il ricordo di un lungo periodo della mia vita (e che periodo!) sarà inscindibile dalla mia esperienza di ricerca e di lavoro in laboratorio e da quanti, anche al di fuori dell'ambito lavorativo, in quello stesso periodo hanno incrociato la mia via, scherzato parlato lavorato sofferto studiato discusso imparato litigato aspettato sperato e tanto altro ...ato con me. Proust aveva il talento per sugellare gli anni ed il loro ricordo in un'opera come la Recerche. Io mi devo accontentare dell'arido linguaggio della comunicazione scientifica. Ciononostante, dietro le formule, i grafici, le tabelle, ritrovo persone in carne e sangue, avvenimenti, emozioni, viaggi, dischi, quadri, in generale: ricordi.

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