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Chronic exposure to the atypical antipsychotic olanzapine differently up-regulates ERK 1/2 phosphorylation in subcellular compartments of rat prefrontal cortex.

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Running Title

Antipsychotic regulation of ERKs.

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List of non-standard abbreviations:

MEK 1/2= extracellular signal-regulated kinase kinase 1/2

GSK3β= glycogen synthase kinase-3

ERK 1/2= extracellular signal-regulated kinase 1/2

MAPK= mitogen activated protein kinase

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ABSTRACT

Antipsychotics are the drugs of choice for the treatment of schizophrenia. Besides blocking monoamine receptors, these molecules affect intracellular signaling mechanisms resulting in long-term synaptic alterations. Western blot analysis was employed to investigate the effect of chronic administration (14 days) with the typical antipsychotic haloperidol and the atypical olanzapine on the expression and phosphorylation state of ERK1 and ERK2, proteins involved in the regulation of multiple intracellular signaling cascades. A single injection of both drugs produced an overall decrease in ERK 1/2 phosphorylation, in different subcellular compartments. Conversely chronic treatment with olanzapine, but not haloperidol, increased ERK 1/2 phosphorylation in the prefrontal cortex, in a compartment-specific and time-dependent fashion. In fact, ERK 1/2 phosphorylation was elevated in the nuclear and cytosolic fractions 2 h after the last drug administration whereas it was enhanced only in the membrane fraction when the animals were sacrificed 24 h following the last injection. This effect might be the result of an activation of the MAP kinase pathway, since the phosphorylation of MEK 1/2 was also increased by chronic olanzapine. Our data demonstrate that chronic olanzapine exposure dynamically regulates ERK1/2 phosphorylation in different subcellular compartments, revealing a novel mechanism of action for this atypical agent and pointing to temporally-separated locations of signaling events mediated by these kinases following chronic olanzapine administration.

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INTRODUCTION

Antipsychotic drugs represent the mainstay of pharmacotherapy for schizophrenia. Whereas first generation (typical) drugs are mainly effective for positive symptoms, atypical agents improve also negative symptoms and cognitive deterioration (Keefe et al., 2005). Although these drugs act through the acute blockade of several neurotransmitter receptors, functional recovering of schizophrenic patients develops over time, implying neuroadaptive changes to take place in selected brain regions (Meltzer, 1991). One possible target of the action of antipsychotic drugs might be represented by intracellular signaling pathways governing information shuffling from the synatic cleft to the nucleus.

It has been shown that schizophrenics have altered brain expression and/or phosphorylation of β-catenin and AKT-GSK3β (Cotter et al., 1998; Kozlovsky et al., 2000; Beasley et al., 2001; Emamian et al., 2004). The relevance of these pathways to schizophrenia is corroborated by the evidence that antipsychotics increase levels and/or activation state of these proteins in the brain of experimental animals (Alimohamad et al., 2004, Kang et al., 2004; Emamian et al., 2004), regardless of the therapeutic class. In addition, antipsychotics regulate cAMP-dependent protein kinase (PKA)-mediated signaling in different rat cerebral regions (Turalba et al., 2004).

Recent data have shown that the MAP kinase pathway might also be modulated by acute administration of antipsychotics (Pozzi et al., 2003, Valjent et al., 2004, Browning et al., 2005). Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) represent a critical crossroad of multiple signaling cascades involved in the regulation of different cellular processes spanning from cell proliferation, differentiation and survival (Schaeffer and Weber, 1999; Colucci-

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D'Amato et al., 2003; Sweatt, 2004) to synaptic plasticity and cognition (Valjent et al., 2001; Adams and Sweatt, 2002; Thomas and Huganir, 2004).

Since these proteins operate as multifunctional signaling integrators involved in the regulation of gene transcription (Sweatt, 2004), we investigated whether chronic administration of antipsychotics could alter their expression and phosphorylation state in the prefrontal cortex, a region that contributes most to the cognitive impairments observed in schizophrenic patients (Weinberger et al., 2001).

Our results show that long-term treatment with the atypical antipsychotic olanzapine has specific modulatory effects on ERK 1/2 phosphorylation that might account for the improvements in cognitive symptoms of schizophrenia produced by the drug.

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METHODS AND MATERIALS

Materials

General reagents were purchased from Sigma-Aldrich (Milano, Italy), and molecular biology reagents were obtained from Ambion (Austin, Texas), New England Biolabs (Beverly, Massachusetts) and Promega (Milan, Italy). Olanzapine was obtained from Eli Lilly (Sesto Fiorentino, Italy); haloperidol was purchased from Sigma-Aldrich (Milano, Italy).

Animal treatment and drug paradigms

Male Sprague-Dawley rats (Charles River, Calco, Italy) weighing 225-250 g were used throughout the experiments. Animals were housed for 2 weeks before any treatment and maintained under a 12 hours light/12 hours dark cycle with food and water available ad libitum.

For the acute treatment, animals received a single injection only of either vehicle (saline), haloperidol (1 mg/kg) or olanzapine (2 mg/kg) and were sacrificed by decapitation 30 minutes or 2 hr later. For the chronic treatment rats were injected daily with the drugs for 14 days and were sacrificed 2 or 24 hrs after the last drug injection. Vehicle, haloperidol and olanzapine were administered by subcutaneous injection. Although appropriate drug dosing in rats is controversial, in our experiments drug doses were chosen in accordance with published protocols (Bubser and Deutsch, 2002, Schotte et al., 1996; Kapur et al., 2003). All animal handling and experimental procedures were performed in accordance with the EC guidelines (EC Council Directive 86/609 1987) and with the Italian legislation on animal experimentation (Decreto Legislativo 116/92).

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Preparation of Protein Extracts

Prefrontal cortex (approximately weight 8 mg) was dissected from 2-mm thick slices (prefrontal cortex defined as Cg1, Cg3, and IL subregions, corresponding to the plates 6–9 of the atlas of Paxinos and Watson (1996), immediately frozen on dry ice and stored at -80°C. Different subcellular fractions were prepared as previously described (Maragnoli et al., 2004). Tissues were homogenized in a glass-glass potter in cold 0.32 M sucrose containing 1 mM Hepes solution, 0.1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4, in presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail. The homogenized tissue was centrifuged at 5000 g for 10 min. The resulting pellet (P1), corresponding to the nuclear fraction, was resuspended in a buffer (20 mM HEPES, 0.1 mM DTT, 0.1 mM EGTA) with protease and phosphatase inhibitors; the supernatant (S1) was centrifuged at 9000 g for 15 min to obtain a clarified fraction of cytosolic proteins (S2**)** and the pellet, corresponding to the membrane fraction (P2), was resuspended in the same buffer used for P1.

Total protein content was measured in the P1, S2 and P2 fractions by the Bio-Rad Protein Assay (Bio-Rad, Milano, Italy).

Western Blot Analysis

ERK 1/2 protein analysis was performed on P1, S2 and P2 fractions as previously described (Fumagalli et al., 2005). Total protein concentrations were adjusted to the same amount for all samples (10 μg per lane). All the samples were run on a sodium dodecyl sulfate (SDS)-8% polyacrilamide gel under reducing conditions, and proteins were then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad). Blots were blocked with 10% nonfat dry milk, then incubated with primary antibody. The blots were first probed with antibodies against the

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phosphorylated forms of the protein and then stripped and re-probed with antibodies against total proteins of same type. ERK 1 and ERK 2 native forms were detected by evaluating the band density at 44 and 42 kDa, respectively, after probing with a polyclonal antibody (1:10000, 2 h, room temperature) (Santa Cruz Biotechnology). Membranes were incubated for 1 h at room temperature with a 1:10000 dilution of peroxidase-conjugated anti-rabbit IgG (Sigma). ERK 1 and ERK 2 phosphorylated forms were detected by evaluating the band density at 44 and 42 kDa, respectively, after probing with a monoclonal antibody (1:10000, 4°C, overnight) (Santa Cruz Biotechnology). Membranes were incubated 1 h with a 1:10000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma).

MEK 1/2 native form was detected by evaluating the band density at 45 kDa after probing with a polyclonal antibody (1:5000, 2 h, room temperature) (Cell Signalling). Membranes were incubated for 1 h at room temperature with a 1:5000 dilution of peroxidase-conjugated anti-rabbit IgG (Cell Signalling). MEK 1/2 phosphorylated form was detected by evaluating the band density at 45 kDa after probing with a polyclonal antibody (1:1000, 4°C, overnight) (Cell Signalling). Membranes were incubated 1 h at room temperature with a 1:1000 dilution of peroxidase-conjugated anti-rabbit IgG (Cell Signalling).

ERK 1 and ERK 2 phosphorylated immunocomplexes were visualized by chemiluminescence utilizing the SuperSignal West Femto (Pierce), whereas MEK 1/2 phosphorylated immunocomplexes were detected using the ECL Western Blotting kit (Amersham Life Science, Milano, Italy). ERK 1 and ERK 2 as well as MEK 1/2 native immunocomplexes were visualized by chemiluminescence utilizing the ECL Western Blotting kit (Amersham Life Science, Milano, Italy) according to the manufacturer's instructions.

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Results were standardized to a β-actin control protein which was detected by evaluating the band density at 43 kDa after probing with a polyclonal antibody with a 1:10000 dilution (Sigma). Membranes were incubated for 1 h at room temperature with a 1:10000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma).

Statistical Analysis

Expression and phosphorylation state of ERK 1/2 were measured using the Quantity One software from Biorad. The mean value of the control group within a single experiment was set at 100 and the data of animals injected with olanzapine or haloperidol were expressed as 'percentages' of saline-treated animals. The phosphorylation of ERK 1/2 was expressed as a ratio between phosphorylated ERKs and total ERKs (pERKs/tERKs). The total levels of ERKs were normalized with β-actin (ERKs/β-actin). The same analysis was carried out for the expression and phosphorylation state of MEK 1/2.

Statistical evaluation of the changes produced by antipsychotic treatment on the phosphorylation state or expression of targets proteins was performed using a one-way analysis of variance (ANOVA) followed by Scheffe F-test. Significance for all tests was assumed at $p < 0.05$.

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RESULTS

The major aim of the present study was to examine the subcellular expression and phosphorylation state of ERK 1/2 in rat prefrontal cortex following treatment with the typical antipsychotic haloperidol and the atypical olanzapine**.** The purity of cellular compartment preparation was previously demonstrated (Fumagalli et al., 2005). Both ERK isoforms were revealed by Western blot analysis, with a more intense immunoreactivity for ERK2.

We first analyzed the acute modulation of ERK expression and phosphorylation by both antipsychotics, sacrificing the animals 30 minutes and 2 hours after drug treatment. Overall, acute treatment with antipsychotics showed a generalized decrease of ERK 1/2 phosphorylation, although subtle differences can be detected. In the nuclear fraction, haloperidol and olanzapine significantly reduced ERK 1/2 phosphorylation 30 minutes (haloperidol: ERK1= -30%, $p<0.05$ and ERK2= - 29%, $p<0.05$; olanzapine: ERK1= -25%, $p<0.05$, ERK2= -25%, $p<0.05$) as well as 2 hours (haloperidol: ERK1= -44%, $p<0.05$ and ERK2= - 34%, $p<0.05$; olanzapine: ERK1= -51%, $p<0.05$, ERK2= -35%, $p<0.05$) after injection (Fig 1a and 1c). In the cytosolic compartment, the two antipsychotics did not produce any change in ERK 1/2 phosphorylation 30 minutes post-injection whereas both drugs significantly decreased ERK 2 phosphorylation (haloperidol= -32%, $p<0.05$; olanzapine= -33%, $p<0.05$), with a tendency toward a decrease for ERK1, 2 hours after drug treatment (Fig. 1c). A similar trend was also detected in the membrane fraction, at both the time points investigated, although a statistically significant reduction was only found for ERK 2 phosphorylation after olanzapine injection (-25%, $p<0.05$) when examined 2 hours after injection.

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While 30 minutes after administration of both drugs total levels of ERK 1/2 were not changed (Fig. 1b), single injection of olanzapine increased total ERK 1/2 levels in the nucleus, with a concomitant decrease of their levels in the cytosol 2 hours later (Fig. 1d). Conversely, at this time point, the acute administration of haloperidol did not produce any significant change of the levels for both ERK isoforms in any cellular compartments.

We then performed two different long-term treatments (14 days) with haloperidol and olanzapine that could be distinguished on the basis of the time of sacrifice from the last injection. We reasoned that these distinct experimental paradigms could allow us to dissect between mechanisms directly related to (2h), and those independent from (24h), the last drug administration.

Fig. 2a shows a representative immunoblotting demonstrating the increased ERK 1/2 phosphorylation 2h after the last injection of a two week treatment with olanzapine in rat prefrontal cortex. Quantitative analysis demonstrated that chronic treatment with olanzapine significantly enhanced ERK1 (+59%, $p<0.01$) and ERK2 (+35%, $p<0.01$) phosphorylation in the nuclear fraction of this brain region (Fig. 2b), whereas in the cytosolic fraction, the increase was restricted to ERK2 (+25%, $p<0.05$) (Fig. 2b). Conversely, chronic haloperidol treatment did not elicit any significant change of ERK1/2 phosphorylation in the nuclear as well as in the cytosolic fractions (Fig. 2b). Furthermore, at this time point, phosphorylation of ERK 1/2 isoforms was not changed following chronic haloperidol or olanzapine in the membrane fraction (Fig. 2b). No changes were measured in the total levels of ERK isoforms with either drugs in the different subcellular fractions (Fig. 2c).

When the animals were sacrificed 24 h after last drug injection, olanzapine, but not haloperidol, up-regulated ERK 1 and ERK 2 phosphorylation (+58%, $p<0.05$

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and +68%, $p<0.05$ respectively) only in the membrane fraction, whereas in the nuclear or cytosolic compartments the levels of ERK 1/2 phosphorylation decayed back to control level (Fig. 3b). No changes were measured, in these experimental conditions, in the total levels of ERK isoforms with either drugs in the different fractions examined (Fig. 3c).

In order to dissect out the molecular mechanisms underlying increased ERK 1/2 phosphorylation produced by chronic olanzapine, we analyzed the expression and phosphorylation state of MEK 1/2, a kinase upstream of ERK 1/2. The analysis of MEK 1/2 phosphorylation in the cytosol, the subcellular compartment where MEK 1/2 is primarily located and enriched, revealed that olanzapine significantly up-regulated MEK 1/2 phosphorylation 24 hours after last drug treatment, whereas only a trend was observed 2 hours after the last drug injection. No significant changes were produced by chronic olanzapine treatment on MEK 1/2 total expression (Fig. 4c).

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DISCUSSION

Our results demonstrate that, in rat prefrontal cortex, ERK 1/2 phosphorylation is selectively increased by chronic treatment with olanzapine, but not haloperidol, according to a finely tuned, compartment- and temporal-specific profile. The subcellular localization of such effect is strictly correlated to the time of sacrifice from the last injection: in fact, such enhancement is specifically confined to the nuclear and cytosolic fraction 2 hours after the last injection but it is restricted to the membrane fraction when the animals are sacrificed 24 hours later.

A single injection of haloperidol or olanzapine produced an overall reduction of ERK 1/2 phosphorylation in the nuclear and cytosolic compartments, an effect that might be the consequence of the blockade of dopaminergic and serotonergic receptors. In the case of olanzapine, however, while the antipsychotic directly reduces ERK 1/2 phosphorylation 30 minutes after drug injection, the effect observed 2 hours later may be the result of increased protein expression: based on these data, we suggest that olanzapine might promote protein translocation from the cytosol to the nucleus 2 hours post-treatment, presumably in an attempt to counteract the decreased ERK 1/2 phosphorylation.

The different effect of a single versus repeated injections of olanzapine is suggestive of the possibility that the chronic treatment with the atypical antipsychotic might determine adaptive mechanisms that lead to the up-regulation of ERK 1/2 phosphorylation in selected cell compartments.

The specific, compartimentalized increase in ERK 1/2 phosphorylation poses an interesting question regarding the role of ERKs in the different subcellular fractions with respect to the mechanism of action of antipsychotic drugs. Evidence exists that activated ERK 1/2 play distinct roles in the nucleus [where they activate

transcription factors or immediate early gene such as Elk-1 or c-fos, (Sgambato et al., 1998a, 1998b)], in the cytoplasm [where they appear to be implicated in the regulation of cytoplasmic proteins involved in synaptic rearrangements such as MAP2 (Bhat et al., 1998) or Cdk5 (Veeranna et al., 2000)] or in the membrane fraction [where, postsynaptically, they participate in the regulation of plasticity and learning (Komiyama et al., 2002) whereas, presynaptically, are involved in regulating neurotransmitter release (Schenk et al., 2005)]. To this regard, Harding and associates (2005) recently demonstrated that a different threshold of activation exists for ERK 1/2 in different subcellular compartments that might explain the temporal redistribution of the activated kinase following chronic olanzapine administration.

Olanzapine, similarly to other novel antipsychotics, has a complex pharmacodynamic profile, thus posing an important question as to how it enhances ERK 1/2 phosphorylation in prefrontal cortex. Direct blockade of dopamine D2 receptors is not likely to contribute to ERK 1/2 changes since haloperidol, a preferential antagonist of these receptors, is devoid of any effect. While we can not exclude a role for a specific receptor subtype, it could be hypothesized that the activation of ERKs in prefrontal cortex might represent the consequence of complex events involving direct receptor antagonism and enhancement of neurotransmitter release produced by olanzapine. In fact, it has been shown that olanzapine increases dopamine and norepinephrine release in rat prefrontal cortex **(**Bymaster et al., 1999), an effect that may contribute to the increase in ERK 1/2 phosphorylation (Zhong and Minneman, 1999; Runyan and Dash, 2004). In addition, atypical drugs enhance glutamate transmission thus facilitating NMDA responses in pyramidal cells of medial prefrontal cortex (Ninan et al., 2003, Heresco-Levy et al., 2003).

Increased ERK 1/2 phosphorylation may be the result of a direct activation of the MAP kinase pathway or, otherwise, it might depend on indirect modulation by other pathways involved in the phosphorylation of these proteins. Based on our data, we propose that increased phosphorylation of MEK 1/2, the kinase upstream of ERK 1/2, is responsible of the up-regulation of ERK 1/2 phopshorylation. However, we can not rule out the possibility that other mechanisms participate to such enhancement. Among the intracellular cascades relevant for the action of olanzapine, the pathway of PKA can increase ERK 1/2 phosphorylation (Yao et al., 1998; York et al., 1998; Grewal et al., 1999, Roberson et al., 1999). Alternatively, enhanced phosphorylation of ERK 1/2 after chronic olanzapine treatment might also result from the inhibition of protein phosphatases. Indeed, chronic administration of atypical antipsychotics down-regulates the expression of serine-threonine phosphatases, the enzymatic system responsible for ERK 1/2 dephosphorylation (MacDonald et al., 2005).

Post-mortem studies in schizophrenic patients (Kyosseva et al., 1999) as well as preclinical investigations in animal models of the disease using glutamate NMDA receptor antagonists (Kyosseva et al., 2001, Ahn et al., 2005) have shown dysregulation of ERK pathway suggesting that it may represent a target for therapeutic interventions. In support of these molecular observations, clinical studies have demonstrated that patients with schizophrenia display abnormalities in prefrontal information processing (for a review see Weinberger et al., 2001) which could contribute to the cognitive impairments associated with the disease. Clinical data indicate that olanzapine, and more in general second generation antipsychotics, successfully improve specific cognitive domains in schizophrenic patients, whereas haloperidol and classical neuroleptics are not effective (Purdon et al., 2000; Woodward et al, 2005). Given that ERK pathway modulates cognition in the

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prefrontal cortex (Runyan and Dash, 2004), our data raise the possibility that olanzapine might improve cognitive processes via the up-regulation of ERK 1/2 phosphorylation in prefrontal cortex.

To sum up, our results point to ERK as a dynamic target of antipsychotic administration unraveling a previously unappreciated degree of subcellular regulation promoted by olanzapine. Although the findings need to be confirmed and extended to other antipsychotic drugs, these results could explain, at least in part, the ability of atypical antipsychotics in alleviating some of the schizophrenic negative symptoms and improving cognitive dysfunctions.

The potential implication of increased ERK 1/2 phosphorylation following chronic antipsychotic administration might extend from schizophrenia to other psychiatric conditions. To this end olanzapine, but not haloperidol, delays or prevents relapse during long-term maintenance therapy of bipolar disorder (Bowden, 2005; McCormack and Wiseman, 2004). It should be emphasized that lithium and valproate, which are widely used for the treatment of bipolar disorder, increase ERK 1/2 phosphorylation in rat prefrontal cortex following chronic administration (Einat et al., 2003) pointing to enhanced function of ERK pathway in this brain structure as a common event of the long-term therapeutic action of psychotropic drugs.

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Footnotes section

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Figure legend

Fig.1. Effect of acute treatment with haloperidol or olanzapine on ERK 1/2 in rat prefrontal cortex. The phosphorylation (panels a and c) and expression (panels b and d) of ERK 1/2 were investigated in animals that received a single injection of haloperidol (1 mg/kg) or olanzapine (2 mg/kg) and that were killed 30 minutes (panels a and b) or 2 hours (panels c and d) later. The mean value of the control group was set at 100 and the data of animals injected with haloperidol or olanzapine were expressed as 'percentages' of saline-treated animals. Data are the mean ± SEM from 6-8 independent determinations. *p< 0.05 vs. saline-injected rats (one way ANOVA with Scheffe F-test).

Fig. 2 Effect of chronic antipsychotic treatment on ERK 1/2 expression and phosphorylation in rat prefrontal cortex as measured 2 hours after last drug injection. The photographs are representative immunoblots of native (t-ERK) and phosphorylated (p-ERK) forms of ERK 1 and ERK 2 in the nuclear, cytosolic and membrane fractions from prefrontal cortex of saline (S), haloperidol (H) or olanzapine (O) treated rats (panel a).

Quantitative analysis of the effects of chronic haloperidol or olanzapine treatment are shown in panel b (ERK 1/2 phosphorylation) and panel c (ERK 1/2 expression). Animals were treated for 14 consecutive days with haloperidol (1 mg/kg) or olanzapine (2 mg/kg, twice a day) and killed 2 hours after the last injection. The mean value of the control group was set at 100 and the data of animals injected with haloperidol or olanzapine were expressed as 'percentages' of saline-treated animals. Data are the mean ± SEM from 8 independent determinations. *p< 0.05 and **p< 0.01 vs. saline-injected rats (one way ANOVA with Scheffe F-test).

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Fig. 3 Effect of chronic antipsychotic treatment on ERK 1/2 expression and activity in rat prefrontal cortex as measured 24 hours after last drug injection. The photographs are representative immunoblots of native (t-ERK) and phosphorylated (p-ERK) forms of ERK 1 and ERK 2 in the nuclear, cytosolic and membrane fractions from prefrontal cortex of saline (S), haloperidol (H) or olanzapine (O) treated rats (panel a).

Quantitative analysis of the effects of chronic haloperidol or olanzapine treatment are shown in panel b (ERK 1/2 phosphorylation) and panel c (ERK 1/2 expression). Animals were treated for 14 consecutive days with haloperidol (1 mg/kg) or olanzapine (2 mg/kg, twice a day) and killed 24 hours after the last injection. The mean value of the control group was set at 100 and the data of animals injected with haloperidol or olanzapine were expressed as 'percentages' of saline-treated animals. Data are the mean ± SEM from 8 independent determinations. *$p < 0.05$ vs. saline-injected rats (one way ANOVA with Scheffe F-test).

Fig. 4 Effect of chronic antipsychotic treatment on MEK 1/2 expression and activity in rat prefrontal cortex 2 and 24 hours after last drug injection. (panel a) The photographs are representative immunoblots of native (t-MEK) and phosphorylated (p-MEK) forms of MEK 1/2 in the cytosolic fraction from prefrontal cortex of saline (S) or olanzapine (O) treated rats. Quantitative analysis of the effects of chronic olanzapine treatment are shown in panel b (MEK 1/2 phosphorylation) and panel c (MEK 1/2 expression). Animals were treated for 14 consecutive days with olanzapine (2 mg/kg, twice a day) and sacrificed 2 or 24 hours after the last injection. The mean value of the control group was set at 100 and the data of animals injected with

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olanzapine were expressed as 'percentages' of saline-treated animals. Data are the mean ± SEM from **8** independent determinations. *$p<$ 0.05 vs. saline-injected rats (one way ANOVA with Scheffe F-test).

Figure 1

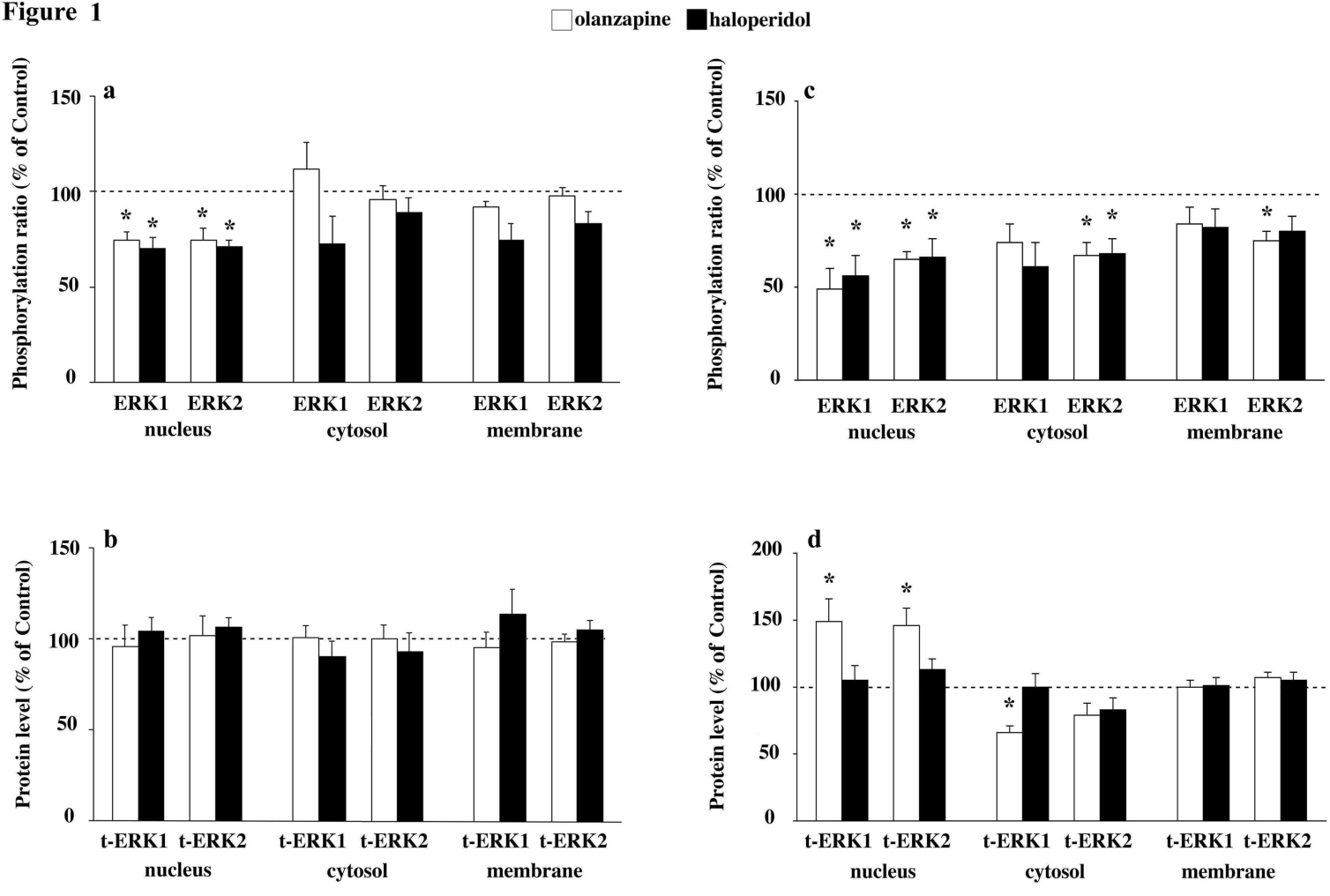
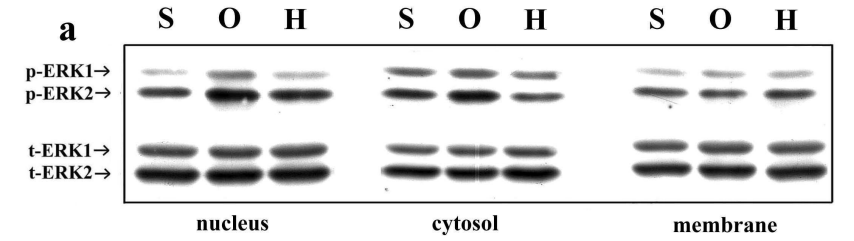
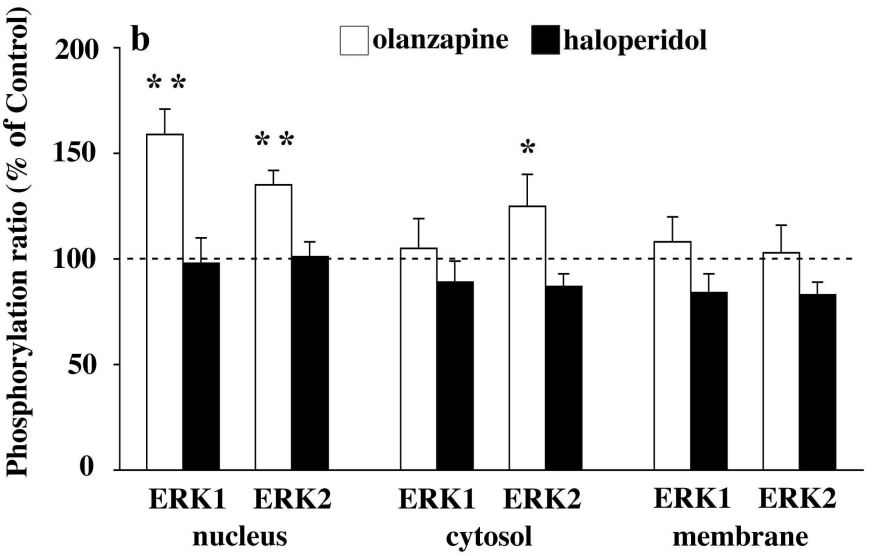


Figure 2


a
S O H S O H S O H
p-ERK1→
p-ERK2→
t-ERK1→
t-ERK2→
nucleus
cytosol
membrane


b
olanzapine
haloperidol
Phosphorylation ratio (% of Control)
200
150
100
50
0
* *
* *
*
ERK1 ERK2 ERK1 ERK2 ERK1 ERK2
nucleus
cytosol
membrane

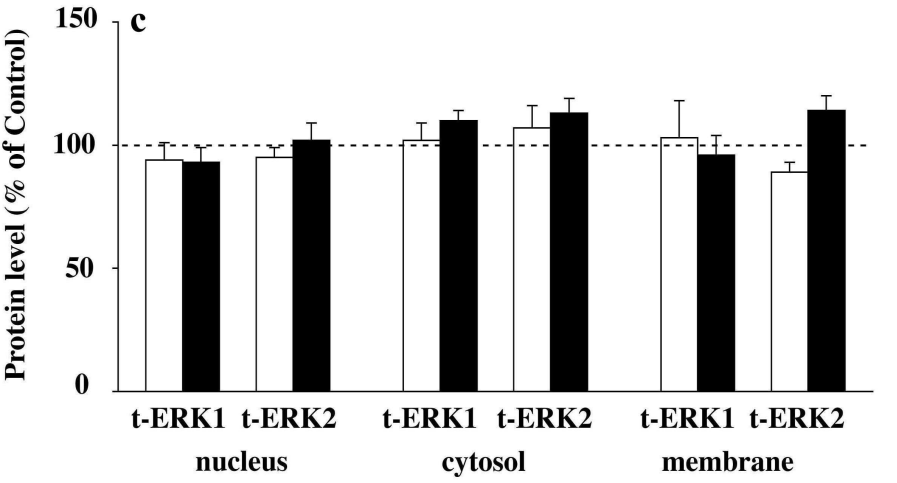
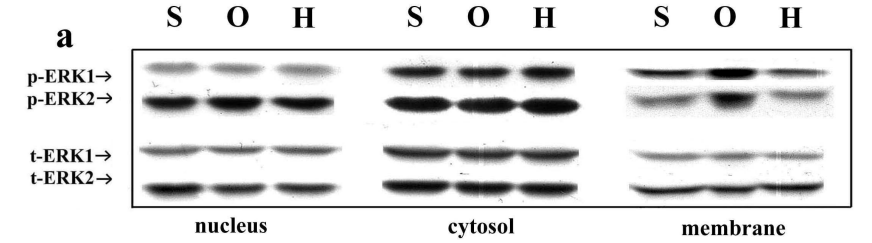
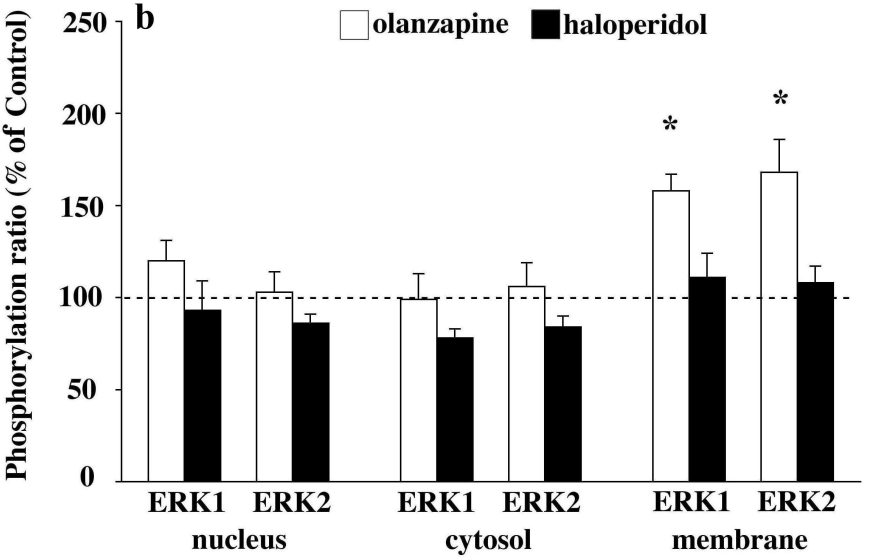

c
Protein level (% of Control)
150
100
50
0
t-ERK1 t-ERK2 t-ERK1 t-ERK2 t-ERK1 t-ERK2
nucleus
cytosol
membrane

Figure 3


a
S O H S O H S O H
p-ERK1→
p-ERK2→
t-ERK1→
t-ERK2→
nucleus
cytosol
membrane


b
olanzapine
haloperidol
Phosphorylation ratio (% of Control)
250
200
150
100
50
0
*
*
ERK1 ERK2 ERK1 ERK2 ERK1 ERK2
nucleus
cytosol
membrane

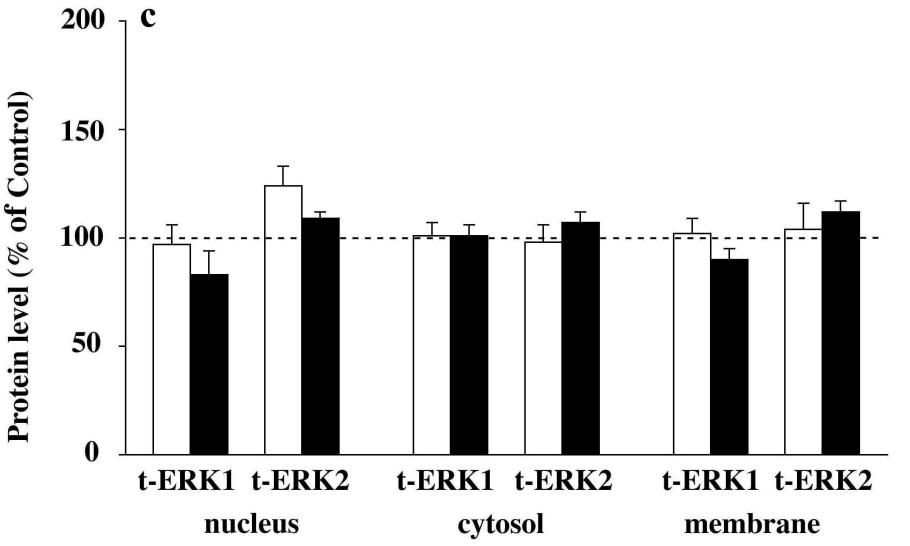
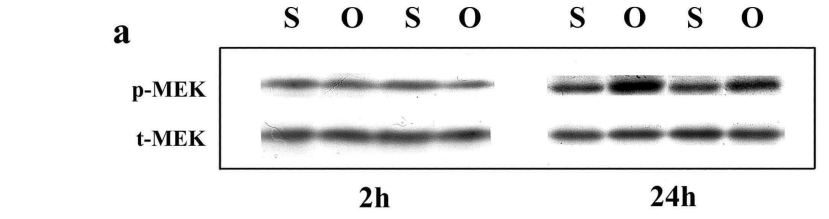
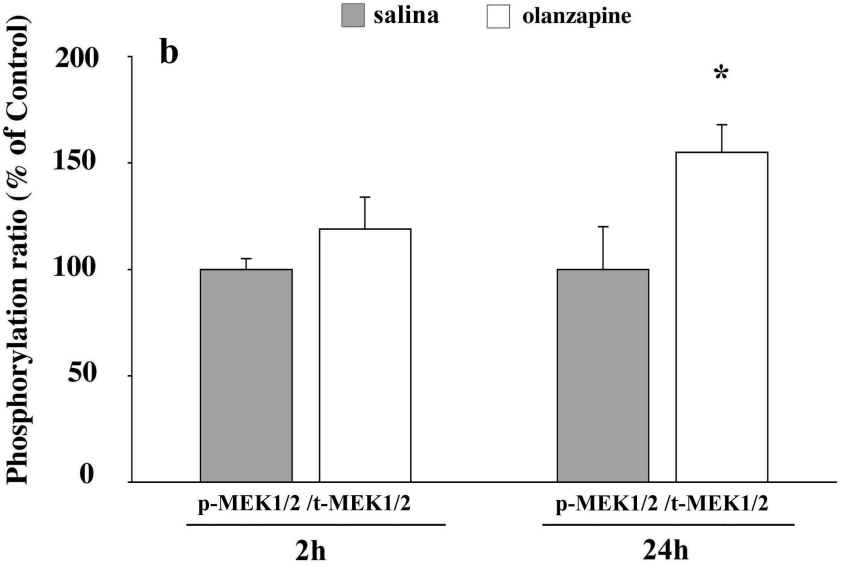
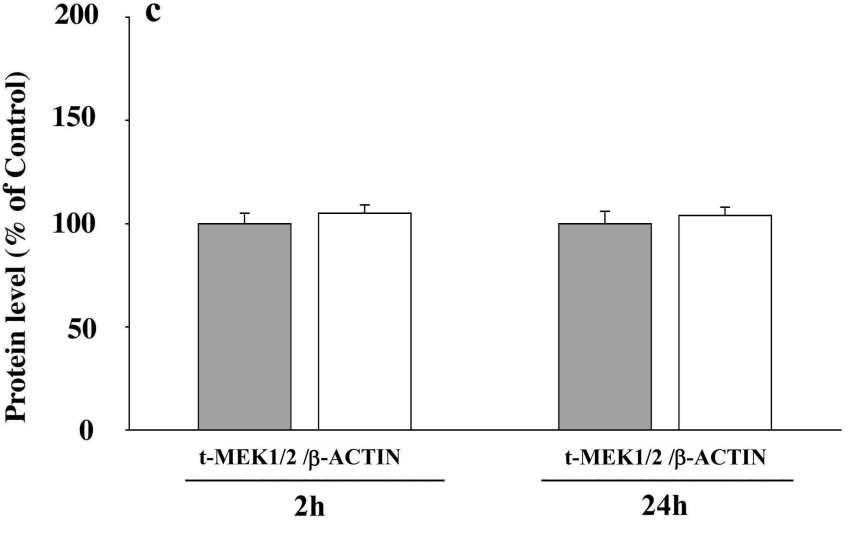

c
Protein level (% of Control)
200
150
100
50
0
t-ERK1 t-ERK2 t-ERK1 t-ERK2 t-ERK1 t-ERK2
nucleus
cytosol
membrane

Figure 4


a
S O S O S O S O
p-MEK
t-MEK
2h
24h


salina
olanzapine
b
*
Phosphorylation ratio (% of Control)
200
150
100
50
0
p-MEK1/2 /t-MEK1/2
p-MEK1/2 /t-MEK1/2
2h
24h


c
Protein level (% of Control)
200
150
100
50
0
t-MEK1/2 /β-ACTIN
t-MEK1/2 /β-ACTIN
2h
24h