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# Long-Term Exposure to the Atypical Antipsychotic Olanzapine Differently Up-Regulates Extracellular Signal-Regulated Kinases 1 and 2 Phosphorylation in Subcellular Compartments of Rat Prefrontal Cortex

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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 used to investigate the effect of long-term administration (14 days) with the typical antipsychotic haloperidol and the atypical olanzapine on the expression and phosphorylation state of extracellular signal-related kinases (ERKs) 1 and 2 (ERK1/2), proteins involved in the regulation of multiple intracellular signaling cascades. A single injection of both drugs produced an overall decrease in ERK1/2 phosphorylation in different subcellular compartments. Conversely, long-term treatment with olanzapine, but not haloperidol, increased ERK1/2 phosphorylation in the prefrontal cortex in a compartment-specific and time-dependent fashion. In fact, ERK1/2 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 killed 24 h after the last injection. This effect might be the result of an activation of the mitogen-activated protein kinase pathway, because the phosphorylation of extracellular signal-regulated kinase kinase 1/2 was also increased by long-term olanzapine administration. Our data demonstrate that long-term exposure to olanzapine 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 after long-term olanzapine administration.

Antipsychotic drugs represent the mainstay of pharmacotherapy for schizophrenia. Whereas first-generation (typical) drugs are mainly effective for positive symptoms, atypical agents also improve negative symptoms and cognitive deterioration (Keefe et al., 2006). Although these drugs act through the short-term blockade of several neurotransmitter receptors, functional recovering of schizophrenic patients develops over time, implying that neuroadaptive changes 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 synaptic cleft to the nucleus.

It has been shown that patients with schizophrenia have altered brain expression and/or phosphorylation of β-catenin and AKT-glycogen synthase kinase-3 (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 the activation state of these proteins in the brain of experimental animals (Emamian et al., 2004; Kang et al., 2004; Alimohamad et al., 2005) regardless of the therapeutic class. In addition, antipsychotics regulate cAMP-dependent protein kinase-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 short-term administration of

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**ABBREVIATIONS:** ERK1/2, extracellular signal-regulated kinase 1/2; MEK1/2, extracellular signal-regulated kinase kinase 1/2; MAP, mitogen-activated protein; ANOVA, analysis of variance.

antipsychotics (Pozzi et al., 2003; Valjent et al., 2004; Browning et al., 2005). Mitogen-activated protein kinase and 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-D'Amato et al., 2003; Sweatt, 2004) to synaptic plasticity and cognition (Valjent et al., 2001; Adams and Sweatt, 2002; Thomas and Huganir, 2004).

Because these proteins operate as multifunctional signaling integrators involved in the regulation of gene transcription (Sweatt, 2004), we investigated whether long-term 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 patients with schizophrenia (Weinberger et al., 2001).

Our results show that long-term treatment with the atypical antipsychotic olanzapine has specific modulatory effects on ERK1/2 phosphorylation that might account for the improvements in cognitive symptoms of schizophrenia produced by the drug.

## Materials and Methods

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

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

For the short-term treatment, animals received a single injection only of either vehicle (saline), haloperidol (1 mg/kg), or olanzapine (2 mg/kg) and were killed by decapitation 30 min or 2 h later. For the long-term treatment, rats were injected daily with the drugs for 14 days and were killed 2 or 24 h 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 (Schotte et al., 1996; Bubser and Deutch, 2002; 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).

**Preparation of Protein Extracts.** Prefrontal cortex (approximately weight, 8 mg) was dissected from 2-mm 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 described previously (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, and 0.1 mM phenylmethylsulfonyl fluoride, 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 dithiothreitol, and 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 with the Bio-Rad Protein Assay (Bio-Rad, Milano, Italy).

**Western Blot Analysis.** ERK1/2 protein analysis was performed on P1, S2, and P2 fractions as described previously (Fumagalli et al., 2005). Total protein concentrations were adjusted to the same amount for all samples (10 μg per lane). All of the samples were run on an SDS-8% polyacrylamide gel under reducing conditions, and proteins were then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad). Blots were blocked with 10% nonfat dry milk and then incubated with primary antibody. The blots were first probed with antibodies against the phosphorylated forms of the protein and then stripped and reprobed with antibodies against total proteins of same type. ERK1 and ERK2 native forms were detected by evaluating the band density at 44 and 42 kDa, respectively, after probing with a polyclonal antibody (1:10,000, 2 h, room temperature) (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated for 1 h at room temperature with a 1:10,000 dilution of peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich). ERK1 and ERK2 phosphorylated forms were detected by evaluating the band density at 44 and 42 kDa, respectively, after probing with a monoclonal antibody (1:10,000, 4°C, overnight) (Santa Cruz Biotechnology). Membranes were incubated for 1 h with a 1:10,000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich).

MEK1/2 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 Signaling Technology). Membranes were incubated for 1 h at room temperature with a 1:5000 dilution of peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology). MEK1/2 phosphorylated form was detected by evaluating the band density at 45 kDa after probing with a polyclonal antibody (1:1000, 4°C, overnight) (Cell Signaling Technology). Membranes were incubated for 1 h at room temperature with a 1:1000 dilution of peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology).

ERK1 and ERK2 phosphorylated immunocomplexes were visualized by chemiluminescence using the SuperSignal West Femto (Pierce Chemical, Rockford, IL), whereas MEK1/2 phosphorylated immunocomplexes were detected using the ECL Western Blotting kit (Amersham Life Science, Milano, Italy). ERK1, ERK2, and MEK1/2 native immunocomplexes were visualized by chemiluminescence using the ECL Western Blotting kit (Amersham Life Science) according to the manufacturer's instructions.

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 1:10,000 dilution (Sigma-Aldrich). Membranes were incubated for 1 h at room temperature with a 1:10,000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich).

**Statistical Analysis.** Expression and phosphorylation state of ERK1/2 were measured using the Quantity One software from Bio-Rad. 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 ERK1/2 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 MEK1/2.

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 Scheffé's *F* test. Significance for all tests was assumed at $p < 0.05$.

## Results

The major aim of the present study was to examine the subcellular expression and phosphorylation state of ERK1/2 in rat prefrontal cortex after treatment with the typical antipsychotic haloperidol and the atypical olanzapine. The purity of cellular compartment preparation was demonstrated

previously (Fumagalli et al., 2005). Both ERK isoforms were revealed by Western blot analysis, with a more intense immunoreactivity for ERK2.

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

Although 30 min after administration of both drugs total levels of ERK1/2 were not changed (Fig. 1b), single injection of olanzapine increased total ERK1/2 levels in the nucleus, with a concomitant decrease of their levels in the cytosol 2 h later (Fig. 1d). Conversely, at this time point, the short-term 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 (2 h) and those independent from (24 h) the last drug administration.

Figure 2a shows a representative immunoblotting demonstrating the increased ERK1/2 phosphorylation 2 h after the last injection of a 2-week treatment with olanzapine in rat prefrontal cortex. Quantitative analysis demonstrated that long-term 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, long-term haloperidol treatment did not elicit any significant change of ERK1/2 phosphorylation in the nuclear or in the cytosolic fractions (Fig. 2b). Furthermore, at this time point, phosphorylation of ERK1/2 isoforms was not changed after long-term 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 killed 24 h after the last drug injection, olanzapine, but not haloperidol, up-regulated ERK1 and ERK2 phosphorylation (+58%, $p < 0.05$ and +68%, $p < 0.05$, respectively) only in the membrane fraction, whereas in the nuclear or cytosolic compartments, the levels of ERK1/2 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).

To dissect out the molecular mechanisms underlying increased ERK1/2 phosphorylation produced by long-term olanzapine treatment, we analyzed the expression and phosphorylation state of MEK1/2, a kinase upstream of ERK1/2. The analysis of MEK1/2 phosphorylation in the cytosol, the subcellular compartment in which MEK1/2 is primarily located and enriched, revealed that olanzapine significantly

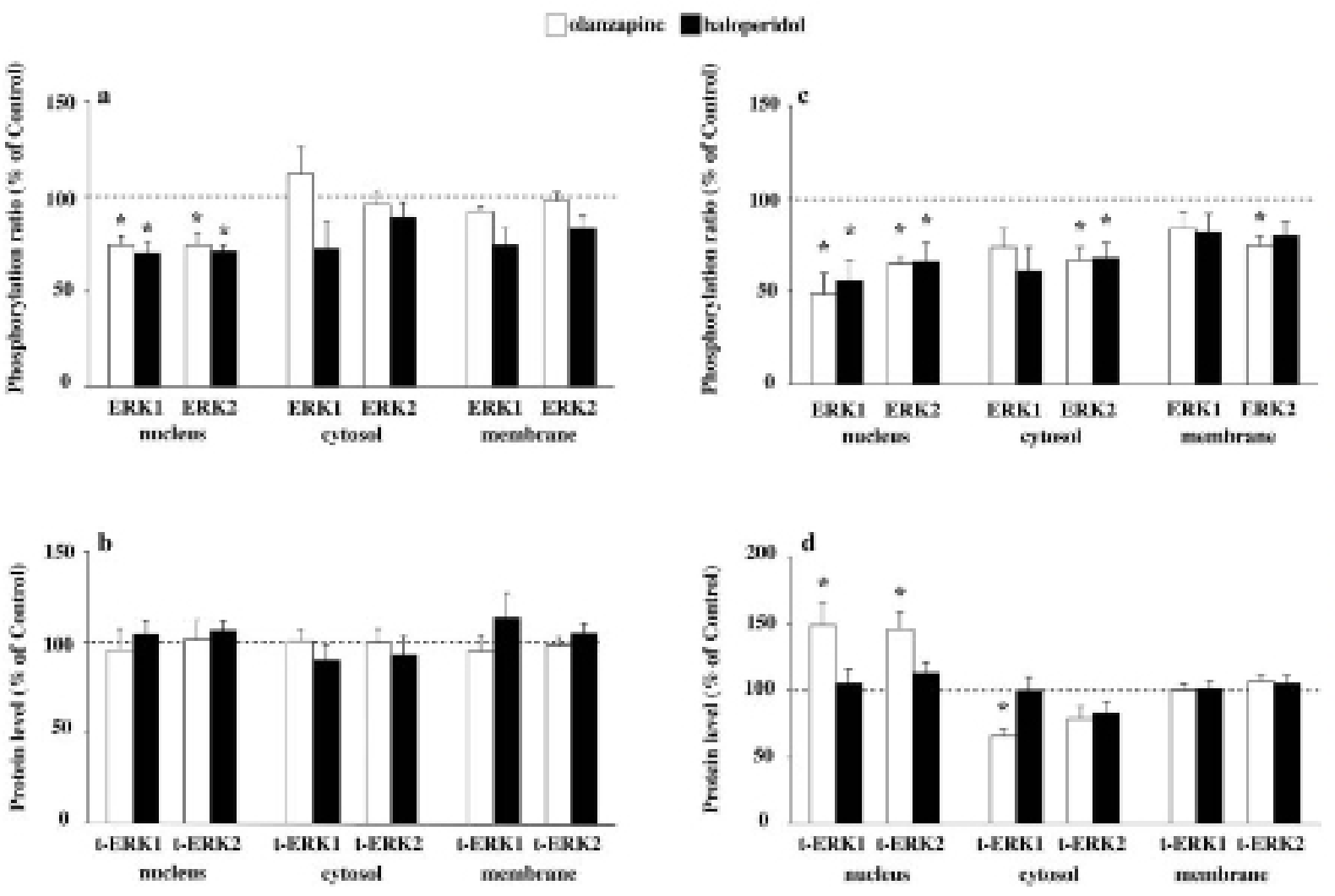


Fig. 1. Effect of short-term treatment with haloperidol or olanzapine on ERK1/2 in rat prefrontal cortex. The phosphorylation (a and c) and expression (b and d) of ERK1/2 were investigated in animals that received a single injection of haloperidol (1 mg/kg) or olanzapine (2 mg/kg) and that were killed 30 min (a and b) or 2 h (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 ± S.E.M. from six to eight independent determinations. *, $p < 0.05$ versus saline-injected rats (one-way ANOVA with Scheffé's $F$ test).