The involvement of GABA\textsubscript{A} receptor in the molecular mechanisms of combined selective serotonin reuptake inhibitor-antipsychotic treatment

Lena Danovich\textsuperscript{1,2}, Orly Weinreb\textsuperscript{1,2}, Moussa B. H. Youdim\textsuperscript{2} and Henry Silver\textsuperscript{1}

\textsuperscript{1}Molecular Neuropsychiatry Unit, Shaar Menashe Brain Behavior Laboratory, Shaar Menashe MHC and Technion-Faculty of Medicine, Haifa, Israel
\textsuperscript{2}Eve Topf and National Parkinson Foundation Centers of Excellence for Neurodegenerative Diseases Research, Department of Pharmacology, Technion – Faculty of Medicine, Haifa, Israel

Abstract

There is evidence that combining selective serotonin reuptake inhibitor (SSRI) antidepressant and antipsychotic drugs may improve negative symptoms in schizophrenia and resistant symptoms in obsessive–compulsive and affective disorders. To examine the mechanism of action of this treatment we investigated the molecular modulation of \(\gamma\)-aminobutyric acid-A (GABA\textsubscript{A}) receptor components and biochemical pathways associated with GABA\textsubscript{A} receptor function following administration of the SSRI fluvoxamine (Flu) combined with the first-generation antipsychotic haloperidol (Hal) and compared it to the individual drugs and the atypical antipsychotic clozapine (Clz). We analysed prefrontal cortices of Sprague–Dawley rats injected intraperitoneally (i.p.) with the combination of Flu (10 mg/kg) and Hal (1 mg/kg), each drug alone, or Clz (10 mg/kg) after 30 min and 1 h. We found that haloperidol plus fluvoxamine (Hal-Flu) co-administration, and Clz, decreased the level of GABA\textsubscript{A}\(\beta\)/3 receptor subunit in the cytosolic fraction, and increased it in the membrane compartment in rat PFC. Flu or Hal alone did not produce changes in GABA\textsubscript{A}\(\beta\)/3 receptor protein expression. Additionally, Hal-Flu and Clz regulated molecular signalling pathways that modulate GABA\textsubscript{A} receptor function, including protein kinase C (PKC) and extracellular signal-regulated kinase-2 (ERK2). In primary cortical culture, short-term treatment (15 min) with Hal-Flu combination and Clz increased GABA\textsubscript{A} subunit phosphorylation levels. Pretreatment of the cells with PKC inhibitor abolished the effect of the combined treatment, or Clz on phosphorylation of GABA\textsubscript{A} receptor. Inhibition of ERK2 did not alter the effect of drugs on GABA\textsubscript{A} receptor phosphorylation levels. Our findings provide evidence that the combined treatment regulates GABA\textsubscript{A} receptor function and does so via a PKC-dependent pathway.

Received 7 October 2009; Reviewed 30 October 2009; Revised 3 January 2010; Accepted 13 January 2010; First published online 25 February 2010

Key words: Clozapine, fluvoxamine, GABA\textsubscript{A} receptor, haloperidol, schizophrenia, signalling pathways.

Introduction

Negative symptoms of schizophrenia such as apathy, loss of emotional response, and social withdrawal respond poorly to conventional antipsychotic treatments and are associated with poor psychosocial function and outcome (Lewis et al. 2005). One of the approaches to improve response of negative symptoms is augmentation of antipsychotic treatment with a selective serotonin reuptake inhibitor (SSRI) antidepressant. Several recent reviews and meta-analyses (Falkai et al. 2005, 2006; Möller, 2004; Rummel et al. 2005, 2006; Sepehry et al. 2007) found that the weight of available evidence supports the view that SSRI augmentation can improve negative symptoms in chronic schizophrenia patients, but emphasized the need for further research in a broad spectrum of patients particularly as the study of negative symptoms presents considerable difficulties (Murphy et al. 2006; Sepehry et al. 2007).
2007; Silver, 2008). These include methodological issues such as distinguishing primary and secondary negative symptoms, and uncertainty whether equally effective antidepressants of the SSRI class have similar effectiveness as augmenting agents for negative symptoms. The combination of SSRI and antipsychotic has also been reported useful in patients with resistant obsessive-compulsive and affective disorders (Bloch et al. 2006; Bobo & Shelton, 2009; Shelton, 2006).

The biochemical mechanisms mediating the favourable clinical response to antipsychotic and SSRI co-administration remain to be determined. A recent in-vivo microarray study (Chertkow et al. 2006) showed that gene expression profile after haloperidol + fluvoxamine (Hal-Flu) administration was different from that of fluvoxamine (Flu) or haloperidol (Hal) given alone. Clozapine (Clz), the prototype ‘atypical’ antipsychotic that was shown to have some efficacy for obsessive–compulsive and affective disorders (Bloch et al. 2006; Bobo & Shelton, 2009; Shelton, 2006).

The biochemical mechanisms mediating the favourable clinical response to antipsychotic and SSRI co-administration remain to be determined. A recent in-vivo microarray study (Chertkow et al. 2006) showed that gene expression profile after haloperidol + fluvoxamine (Hal-Flu) administration was different from that of fluvoxamine (Flu) or haloperidol (Hal) given alone. Clozapine (Clz), the prototype ‘atypical’ antipsychotic that was shown to have some efficacy for obsessive–compulsive and affective disorders (Bloch et al. 2006; Bobo & Shelton, 2009; Shelton, 2006). These findings supported the hypothesis that chronic treatment with SSRI-antipsychotic combination, results in biochemical changes which differ substantially from those of the individual drugs. In particular that study indicated that Hal-Flu administered together regulated γ-aminobutyric acid-A (GABA\(_{A}\)β2/3 receptor subunit and glutamic acid decarboxylase (GAD67) gene and protein expressions differently than Hal or Flu administered alone.

Findings that the GABAergic system in rat frontal cortex might be distinctively influenced by chronic Hal-Flu treatment is interesting in light of evidence that abnormal GABAergic transmission might play an important role in the pathophysiology of schizophrenia (Benes & Berretta, 2001; Tamminga, 2006; Wassef et al. 2003), and evidence of altertions in several essential elements of the GABAergic system, primarily in frontal cortex and hippocampus in post-mortem brains of schizophrenia patients (Deng & Huang, 2006; Hashimoto et al. 2008; Ishikawa et al. 2004; Lewis et al. 2008; Reynolds & Beasley, 2001). It has been proposed that dysregulation of pyramidal neurons in the dorsolateral prefrontal cortex by GABA interneurons may be implicated in cognitive dysfunction, particularly in working memory, in schizophrenia (Lewis et al. 2005). Published data has indicated that GABA\(_{A}\) receptor modulating drugs, such as benzodiazepines, may be useful in the treatment of positive and negative symptoms and cognitive deficits associated with schizophrenia (Carpenter et al. 1999; Menzies et al. 2007). These observations predicted that modulation of inhibitory transmission via GABA\(_{A}\) receptors could be relevant in schizophrenia treatment, particularly of negative symptoms and cognitive impairments (Guidotti et al. 2005).

Despite the potential relevance of GABAergic function to antipsychotic drug action, little is known about how these compounds modulate GABA\(_{A}\) receptors. In previous studies we have shown that chronic Hal-Flu treatment alters PKCβ2 mRNA levels and protein expression (Chertkow et al. 2006). Published evidence indicates that phosphorylation is an important mechanism of GABA\(_{A}\) receptor regulation, particularly serine phosphorylation of β subunit by PKCβ2 (Brandon et al. 2000).

In the present study we aimed to examine the molecular effects produced by acute administration of Hal-Flu in rat frontal cortex and in cultured primary cortical cells. We focused on regulation of the GABA\(_{A}\)β2/3 receptor, and the involvement of signaling pathways, particularly protein kinase C (PKC), and mitogen-activated protein kinase (MAPK).

Methods

Animal experimental procedures

Adult male Sprague–Dawley rats, weighing 180–220 g, were purchased from Harlan (Israel), and housed for an acclimatization period of 1 wk before starting the experiment. Animals were maintained 3–4 rats per cage under standard conditions of lighting and room temperature. Food and water were available ad libitum. All procedures were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Technion Animal Ethics Committee.

The drugs were dissolved in a sterile saline solution containing 2% DMSO and 70 mM acetic acid. The animals were randomly divided into five groups and injected intraperitoneally (i.p.) with Hal (1 mg/kg), Flu (10 mg/kg), a combination of both (1 and 10 mg/kg, respectively), Clz (10 mg/kg) or vehicle (control group). Previous studies in our laboratory have demonstrated that these dosages of drugs produce levels in rat serum similar to the ranges which are considered to be therapeutic in humans, and that brain and serum concentrations of Hal or Flu, given individually, were not significantly changed when they were co-administered (Chertkow et al. 2006). Thus, at this dosage range, the distinctive pharmacological profile of Hal-Flu could not be attributed to the pharmacokinetic interaction. The animals were sacrificed by decapitation 30 min or 1 h after injection. The prefrontal cortices
(PFC) and the striatum tissues were dissected, frozen immediately in liquid nitrogen, and stored at \(-80\,^{\circ}\mathrm{C}\) for further analyses, including high-performance liquid chromatography (HPLC) and Western blotting analyses.

Brain tissue lysate was homogenized in Tris-sucrose buffer (pH 7.4) (containing a mixture of protease and phosphatase inhibitors) and centrifuged at 1000 \(g\) for 10 min. The supernatant, consisting of the cytosolic fraction, was centrifuged at 100 000 \(g\) for 1 h at \(4\,^{\circ}\mathrm{C}\). The pellet, consisting of the membrane fraction, was resuspended in Tris-sucrose buffer, containing a mixture of protease inhibitors and 0.5% Triton X-100, placed on ice for 30 min and vortexed thoroughly. All the fractions were analysed for protein concentration by Bradford assay (Bradford, 1976).

**HPLC analysis**

Striatum and PFC tissues were homogenized in ice-cold 0.1 M perchloric acid with mini-homogenizer (Pellet Pestle + Motor, Kontes, USA), followed by centrifugation at 14 000 \(g\) for 5 min. Samples were analysed as previously described (Chertkow et al. 2007). The content was calculated by comparison to dopamine (DA) standard (Sigma, USA) in known concentration and normalized relative to the weight of the tissue.

**Primary neuronal cortical culture and cell lysates**

Cerebral cortices were isolated from Sprague–Dawley rat embryonic (E18) pups, incubated in 0.25% trypsin EDTA solution at 37 \(^{\circ}\mathrm{C}\) for 10 min, trypsin inhibitor-DNase solution was added, and mechanical dissociation was performed by intensive trituration. The cells were maintained in the neurobasal medium supplemented with B27, 2 mM l-glutamine, 5 mM Hepes, and 100 U/ml penicillin/streptomycin solution 4 h after seeding. The maintenance of the cells in the neurobasal medium supplemented with B27 yields almost pure neuronal cultures, as judged by immunocytochemistry for glial fibrillary acidic protein (GFAP) and neuron-specific enolase (NSE) (Brewer et al. 1993; Brewer, 1995). The cells were incubated for 7–8 d before the start of the experiments.

The cells were harvested in cold lysis buffer [200 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 mM sodium vanadate, 1% Triton X-100, provided with protease and phosphatase inhibitor cocktails], centrifuged at 14 000 \(g\) for 10 min at 4 \(^{\circ}\mathrm{C}\) and protein concentration was determined by Bradford assay.

**Western blotting analysis**

Samples were separated by electrophoresis and proteins were quantified as previously described (Chertkow et al. 2006). The following antibodies were used: affinity-purified goat anti-GABA\(_{\alpha}\beta2/3\) (Upstate, USA), mouse anti-PKC\(_b\)2 (Transduction Laboratories, USA), rabbit anti-extracellular signal-regulated kinase (ERK)1/2 and anti-phospho-ERK1/2 (Cell Signaling, USA), mouse anti-\(\beta\)-actin (Sigma).

**Immunoprecipitation analysis of GABA\(_{\alpha}\) phosphoserine**

Cell lysates (1 \(\mu\)g/ml) were taken for immunoprecipitation. Mouse anti-GABA\(_{\alpha}\beta3/\beta2\) (Upstate) antibody was added to the lysate, followed by overnight incubation at 4 \(^{\circ}\mathrm{C}\). Then protein G sepharose (50 \(\mu\)l) was added to the mixture, incubated for 2 h and centrifuged at 12 000 \(g\) for 20 s at 4 \(^{\circ}\mathrm{C}\). The pellet containing receptor-antibody-bead complex was washed and the receptor-antibody-bead complex was re-suspended in loading buffer. The beads were separated from the immunoprecipitate, which was subjected to SDS–PAGE and Western blotting with rabbit anti-phosphoserine antibodies (Chemicon, USA). GABA\(_{\alpha}\beta2/3\) receptor levels were used to normalize the values of GABA\(_{\alpha}\beta2/3\) serine phosphorylation.

**Statistical analysis**

Statistical significance was analysed by one-way analysis of variance (ANOVA) followed by unpaired Student’s \(t\) test (two-tailed) to determine whether compared groups are distinct. Results are expressed as mean ± S.E.M.

**Results**

Our previous study demonstrated that in rat PFC chronic (28 d) administration of Hal-Flu altered GAD67 and PKC\(_b\)2 genes, associated with GABAergic transmission, differently than each drug alone (Chertkow et al. 2006). In the present study we investigated the effects of acute administration in rat PFC and in primary cortical culture.

**Hal-Flu acute treatment induced changes in GABA\(_{\alpha}\beta2/3\) subunit expression and cellular regulatory molecules in rat PFC**

Figure 1 demonstrates that 30 min after treatment with Hal-Flu, GABA\(_{\alpha}\beta2/3\) protein level was significantly decreased in the cytosolic fraction, and concomitantly...
increased in the membrane compartment of rat PFC (27 ± 8.7% and 26 ± 8.2%, vs. control, respectively, p < 0.05). After 1 h of Hal-Flu treatment GABA\(_{\beta}^{2/3}\) subunit level remained reduced in the cytosolic fraction (37 ± 8.0% vs. control, p < 0.05). Similarly, Clz decreased GABA\(_{\beta}^{2/3}\) subunit level in the cytosol after 30 min and 1 h of treatment (35 ± 8.3% and 40 ± 14.5% vs. control, p < 0.05, respectively), and increased GABA\(_{\beta}^{2/3}\) level in the membrane fraction after 30 min of treatment (34 ± 18.2% vs. control, p < 0.05). Hal or Flu alone increased GABA\(_{\beta}^{2/3}\) protein level in the membrane fraction after 1 h and 30 min of treatment (27 ± 8.9% and 31 ± 13.7% vs. control, p < 0.05) (Fig. 1b, d) with no change in the cytosol.

Our previous study showed that chronic Hal-Flu decreased PKC\(_{\beta}\) protein expression (Chertkow et al. 2006). In this study Hal-Flu decreased PKC\(_{\beta}\) protein expression in the cytosol and increased it in the membrane compartment after 30 min of treatment (22 ± 10.3% and 73 ± 38.3%, vs. control, respectively, p < 0.05) (Fig. 2). These changes persisted for at least 1 h after the administration of the combined treatment. Clz produced changes in PKC\(_{\beta}\) protein levels 30 min after the treatment, similarly to those found with the drug combination (decrease of 34 ± 6.55%, p < 0.05 in PFC cytosol, increase of 133 ± 36.5%, p < 0.01, in the PFC membrane compartment) (Fig. 2a, b). At 1 h cytosolic PKC\(_{\beta}\) levels were decreased in Clz- and Flu-treated rats (31 ± 6.9% and 22 ± 9.4% vs. control, respectively, p < 0.05) (Fig. 2c).

ERK2, a key molecule in MAPK cell signalling pathway, has been reported to be regulated by antipsychotic and SSRI drugs (Fumagalli et al. 2005; Kim et al. 2008; Qi et al. 2008). Here we found that in rat PFC the relative phosphorylation levels of ERK2 were significantly decreased by Hal-Flu both in cytosolic and membrane compartments at 30 min and remained low after 1 h (40 ± 10.8% and 52 ± 8.7% vs. control, respectively).
and 40 ± 10.3% and 38 ± 10.0% vs. control, respectively, p < 0.01 (Fig. 3a, b). Clz-treated animals also showed decreased ERK2 phosphorylation at 30 min (51 ± 5.4% vs. control in PFC cytosol and 48 ± 5.9% vs. control in PFC membrane fraction, p < 0.05) and at 1 h (40 ± 11.2% vs. control in PFC cytosol and 32 ± 8.6% vs. control in PFC membrane fraction, p < 0.01) (Fig. 3). We also investigated glycogen synthase-3β (GSK-3β) which is a cell-signalling pathway component considered to be a possible target molecule for psychiatric drugs (Jope & Roh, 2006). Hal-Flu significantly increased GSK-3β phosphorylation levels in PFC cytosolic fraction after 30 min and this effect persisted for at least 1 h after the treatment (144 ± 31% and 98 ± 52% vs. control, p < 0.01 and p < 0.05, respectively) (Fig. 4). In addition, in Clz-treated rats, a significant increase in GSK-3β phosphorylation levels was also observed (150 ± 30% vs. control, p < 0.01, at 30 min after the treatment, and 84 ± 44% vs. control, p < 0.01, at 1 h after the treatment).

**DA content in rat frontal cortex following Hal-Flu administration**

DA content in PFC was reduced (51 ± 9.82% vs. control, p < 0.01) following Hal-Flu treatment in animals that were sacrificed 1 h after drug administration (Table 1). No statistically significant alterations in PFC of DA content were found in animals sacrificed 30 min after drug administration. Striatal DA content was not altered by any of the drug treatments (data not shown), indicating regional specificity.

**Hal-Flu effect on GABA_A receptor, ERK, and PKC phosphorylation in primary cortical culture**

To investigate the regulation of PKC and MAPK by the drug treatments, we measured phosphorylation levels of total PKC and ERK2 in primary cortical neurons after 15 min of drug treatment. PKC phosphorylation was significantly increased by Hal and Flu (126 ± 40% and 153 ± 51% vs. control, p < 0.05). Neither the Hal-Flu combination nor Clz produced any significant
Fig. 3. The effect of acute drug treatments on ERK2 phosphorylation in rats. Male Sprague–Dawley rats were treated as described in Fig. 1. Protein samples from individual frontal cortices were subjected to subcellular fractionation and consequent Western blotting analysis of the cytosolic (a, c) or membrane compartments (b, d) using primary antibodies against phospho-ERK2. Immunoreactive bands were analysed by densitometry and normalized against total ERK2 levels. Percent of control values are given as mean ± S.E.M. from 8–12 rats, controls are expressed as 1 (t test: * p<0.05, ** p<0.01, *** p<0.001 vs. control).

Fig. 4. The effect of acute drug treatments on GSK-3β phosphorylation in rats. Male Sprague–Dawley rats were treated as described in Fig. 1. Protein samples from individual frontal cortices were subjected to subcellular fractionation and consequent Western blotting analysis of the cytosolic compartment using primary antibodies against phospho-GSK-3β. Immunoreactive bands were analysed by densitometry and normalized against total GSK-3β levels. Percent of control values are given as mean ± S.E.M. from 8–12 rats, controls are expressed as 1 (t test: * p<0.05).
changes in PKC phosphorylation (Fig. 5b). ERK2 phosphorylation was significantly decreased by Hal-Flu (49 ± 14.1% vs. control, \( p < 0.01 \)). Treatment with Flu or Clz also reduced phosphorylation of ERK2 (46 ± 7.3% and 67 ± 6.8% vs. control, \( p < 0.05 \), respectively) (Fig. 5a).

Phosphorylation of serine residues on GABA_A beta subunits by PKC was reported to be an important regulatory mechanism of GABA_A receptor activity (Brandon et al. 2000; McDonald et al. 1998). In order to investigate the molecular mechanism responsible for changes in GABA_A receptor cellular distribution induced by the drugs, we applied Hal, Flu, Hal-Flu, or Clz to primary cortical cells culture for 15 min. Negative controls, including inhibitor plus vehicle were performed for both inhibitors (GF109203X, PD98059), and showed similar results to vehicle alone (Fig. 6). Hal-Flu significantly increased GABA_A beta2/3 subunit phosphorylation (52 ± 18% vs. control, \( p < 0.05 \)) (Fig. 6). Hal or Flu applied alone did not alter GABA_A beta2/3 subunit phosphorylation, while Clz treatment increased the phosphorylation (169 ± 59% vs. control, \( p < 0.001 \)) (Fig. 6), similarly to the combined treatment.

To examine the involvement of PKC in the effect of the drug treatments on GABA_A phosphorylation, we pretreated the cultured cells with the unselective inhibitor of PKC pathway, GF109203X. This inhibitor abolished the effects of Hal-Flu on GABA_A beta2/3 subunit phosphorylation (Fig. 5). The same effect was observed in Clz-treated cells. Thus, the PKC pathway appears to have a role in GABA_A beta2/3 phosphorylation.
induced by Hal-Flu treatment, although it is possible that other kinase pathways, such as MAPK, are also involved (Bell-Horner et al. 2006; Obrietan et al. 2002). Therefore we tested for MAPK pathway involvement in GABA_A phosphorylation induced by the combined treatment and Clz by pretreatment of the cells with the unselective inhibitor of ERK1/2 activity, PD98059. This did not prevent increase of GABA_Aβ2/3 subunit phosphorylation following treatments with Hal-Flu or Clz (70 ± 12% and 130 ± 44% vs. control, p < 0.01) (Fig. 6).

Discussion
In agreement with findings in our previous chronic in-vivo study, acute Hal-Flu treatment produced changes in various cellular regulators and pathways in rat PFC and primary cortical cultures, which were distinct from the effects of individual treatments. The unique effects included changes in protein and phosphorylation levels of GABA_A receptor subunit, regulation of the cell-signalling proteins PKCβ2, ERK2, GSK-3β, and in content of the catecholamine DA.

The effects of Hal-Flu on GABA_A receptor protein and phosphorylation levels
The present study demonstrates that Hal-Flu affected the relative expression levels of GABA_A receptor in cellular compartments. In rat PFC, GABA_A receptor level was decreased in the cytosolic fraction and increased in membrane. In cultured cortical neurons, Hal-Flu caused a significant increase in serine phosphorylation on serine. (Fig. 6)
phosphorylation of GABA<sub>αβ</sub>/2/3 receptor subunit. This is consistent with our previous findings showing that the combined treatment affects GABAergic system components differently than each drug given alone (Chertkow et al. 2006). Previous studies have shown that Clz and Hal differentially regulate GABA<sub>α</sub> receptor binding in rat brain (Farnbach-Pralong et al. 1998; Skilbeck et al. 2007; Zink et al. 2004) and it has been postulated that differences in regulation of GABA transmission may mediate the ‘atypical’ effects of second-generation antipsychotics. Indeed, our findings in vivo and in vitro demonstrated that Hal or Flu alone had no effect on GABA<sub>α</sub> receptor, but that Hal-Flu led to alterations in GABA<sub>α</sub> levels and phosphorylation, effects similar to those following Clz.

The mechanism by which Hal-Flu increases serine phosphorylation of GABA<sub>αβ</sub> subunit in cultured primary cortical neurons is of particular interest. There is no evidence of synthesis of DA or 5-HT in primary cortical cultures, thus, Hal-Flu or Clz are unlikely to mediate GABA<sub>α</sub> phosphorylation increase through DA antagonism or 5-HT reuptake inhibition and this mechanism is unlikely to be explained by the classic receptor/transporter action of the drugs. Several in-vitro studies suggested that antipsychotic and antidepressant compounds may act via mechanisms that include effects on cell-signalling regulators, such as ERK, PKC, and GSK-3β (Li et al. 2007; Pakaski et al. 2005; Pereira et al. 2009) and our findings support such potential mechanisms.

The regulatory effects of Hal-Flu on cell-signaling pathways associated with GABA<sub>α</sub> receptor

The present study shows that Hal-Flu increased PKCβ2 protein expression levels in the membrane and decreased PKCβ2 levels in cytosolic fractions in PFC. Furthermore, in primary cortical culture, inhibition of PKC abolished the effect of Hal on GABA<sub>α</sub> receptor phosphorylation levels. PKCβ2 is one of the major modulators of GABA<sub>α</sub> receptor activity via phosphorylation of serine residues on the β subunits (Brandon et al. 2000), so our findings suggest that the unique effect of Hal-Flu on the GABA<sub>α</sub> receptor is mediated, at least in part, through the PKC signalling pathway. Interestingly, total PKC phosphorylation levels were not altered following Hal-Flu treatment in vivo or in vitro. One explanation for these results is that Hal-Flu does not affect PKC activation but that PKC enzyme activity in required for Hal-Flu induced GABA<sub>α</sub> phosphorylation. In such a case, another cell-signalling kinase, may be a rate-limiting step for GABA<sub>α</sub> phosphorylation activation induced by Hal-Flu.

Unlike each drug alone, Hal-Flu combination treatment significantly reduced the phosphorylation levels of ERK2, a key effector of the MAPK pathway, in PFC of acutely treated animals and in primary cortical neuronal culture. These findings support previous reports which implicated MAPK pathway in pharmacological actions of antipsychotic and antidepressant drugs (Fumagalli et al. 2005; Kim et al. 2008; Pereira et al. 2009). Studies of the effects of SSRIs and antipsychotic medications on ERK, describe a complex and unclear picture. Chronic fluoxetine administration decreased ERK2 phosphorylation levels in PFC of naïve rats (Fumagalli et al. 2005), but increased ERK2 phosphorylation levels in rats exposed to chronic forced swim stress (Qi et al. 2008). Reports on acutely administered Hal showed down-regulation of ERK2 phosphorylation in murine primary cortical neurons (Pereira et al. 2009) and in rat PFC (Fumagalli et al. 2006), while Kim et al. (2008) described a biphasic effect of Hal in rat PFC. One explanation for such inconsistencies is that ERK2 phosphorylation is a dynamic process, and that dosages and timing of examination in relation to drug administration can substantially affect results.

Our findings are consistent with previous reports that ERK2 is regulated differently by Hal and Clz in vivo (Ahmed et al. 2008; Pozzi et al. 2003). Pereira et al. (2009) also reported a differential effect of first-generation and second-generation antipsychotics on ERK2 phosphorylation in primary cortical culture. Our results in vivo and in vitro show, that Hal-Flu treatment produces similar effects to Clz in down-regulating ERK2 phosphorylation, while Hal or Flu alone does not affect ERK2 phosphorylation levels. In addition, we have found that ERK inhibition does not affect Hal-Flu-induced GABA<sub>αβ</sub> subunit phosphorylation. This is consistent with reports that the putative phosphorylation site for ERK is the α subunit of the GABA<sub>α</sub> receptor and suggest the MAPK pathway as a negative modulator of GABA<sub>α</sub> receptor function (Bell-Horner et al. 2006). Thus ERK activity, unlike PKC, may not be critical for the effect of Hal-Flu or Clz on GABA<sub>α</sub> phosphorylation.

Hal-Flu increased GSK-3β phosphorylation

Since GSK-3β has recently been suggested to be an intracellular target of many antipsychotic and mood-stabilizing agents (Jope & Roh, 2006), we examined the effects of the drug treatments on GSK-3β phosphorylation. Our finding that Hal-Flu significantly
increased GSK-3β phosphorylation in the cytosolic fraction of rat PFC is consistent with reports that acute treatment with risperidone combined with fluoxetine or second-generation antipsychotics given alone, but not Hal given alone, significantly increased GSK-3β phosphorylation in mouse PFC (Li et al. 2007).

Here, we did not find changes in GSK-3β phosphorylation levels in primary cortical culture. One explanation may be that the effect of the psychotropic treatments on GSK-3β phosphorylation levels requires intact serotonergic and dopaminergic neuronal pathways. GSK-3β has been reported to act downstream to dopaminergic receptors (Beaulieu et al. 2007) and specific serotonergic compounds can modulate GSK-3 phosphorylation; it is increased by 5-HT1A and inhibited by 5-HT2 receptor activation (Li et al. 2004).

**Time dependence of Hal-Flu effect on DA concentration and regional specificity**

GABAergic and dopaminergic transmissions in PFC are interrelated (Wassef et al. 2003), we therefore investigated the effects of the drug treatments on DA content in PFC. We found that after 1 h of treatment Hal-Flu reduced the total DA levels in rat PFC. Other studies reported that acute treatment with SSRI-antipsychotic combination, including Hal-Flu, increased extracellular DA release in rat PFC (Ago et al. 2005; Koch et al. 2004; Zhang et al. 2000). Clz has also been reported to increase DA release in PFC (Devoto et al. 2003; Jaskiw et al. 2005). Such apparent discrepancy with our findings can be explained by methodological differences between the studies. Unlike the studies mentioned above, which measured DA release (i.e. extracellular DA), we measured total DA content (intracellular and extracellular DA). It is our contention that the acute response to drugs involves initially an increase in DA release followed by depletion of total DA content. Chertkow et al. (2007), found that in chronically treated animals, total DA content was increased when measured 24 h after the last injection of Hal-Flu, but was similar to control levels when measured 1.5 h after the last injection in rat PFC. This indicates that in both acute and acute on chronic treatment conditions, an acute bolus of drug is followed by a phase of reduction in total DA content in the PFC. However, while DA content is reduced 1 h after acute treatment, it is elevated after chronic administration indicating that adaptive changes, e.g. increased DA synthesis, occur over time. Increase in PFC DA levels with chronic treatment is consistent with evidence that reduced DA in PFC is related to negative symptoms (Abi-Dargham & Moore, 2003).

We found no similar effects in the striatum (data not shown), suggesting that this adaptive process may be regionally specific.

**Final remarks**

This study provides further evidence that combination of SSRI with an antipsychotic has a unique pharmacological profile, distinct from the effects of the individual drugs.

Importantly, several of the changes unique to the SSRI-antipsychotic combination, particularly the effects on GABA<sub>A</sub> receptor and PKCβ2 cellular localization in rat PFC, increase in GABA<sub>A</sub>β receptor subunit phosphorylation and decrease of ERK2 phosphorylation in rat PFC and primary cortical neurons, are similar to those induced by the atypical antipsychotic Clz. The finding of such common effects in clinically convergent but pharmacologically distinct treatments raises the likelihood that they are relevant to the molecular mechanisms underlying the clinical improvement in negative symptoms and indicate that the SSRI-antipsychotic combination may serve as a useful paradigm for investigation of mechanisms of drug treatment in schizophrenia.

Several questions remain open regarding the generalizability of our finding. First, it is not clear whether the observed changes are class related or specific to the particular chemical compounds. For example, Flu has pharmacological properties, such as α<sub>2</sub> receptor agonism, not shared by other members of the SSRI class (Narita et al. 1996; Silver, 2001) and it is unknown whether these contribute to Hal-Flu induced molecular changes. Similarly, Clz has a unique pharmacological profile, and it is not clear whether its effects on GABA<sub>A</sub> system and signalling pathways are shared by other ‘second-generation’ antipsychotic compounds. The effect of adding a SSRI to Clz, or other second-generation antipsychotic, also remains to be studied.

While the limitations of the study and of extrapolating findings from the laboratory to the clinic must be borne in mind, this study provides important information of potential molecular mechanisms of SSRI-antipsychotic treatment. Since SSRI-antipsychotic combinations may be effective in refractory obsessive–compulsive disorder (Bloch et al. 2006), treatment-resistant depression (Bobo & Shelton, 2009), and bipolar depression (Shelton, 2006), in addition to schizophrenia, this knowledge may be relevant to a range of psychiatric conditions. More broadly, this study highlights the potential importance of intracellular signalling pathway molecules in the mechanism of action of SSRI-antipsychotic treatment.
Acknowledgements

The authors acknowledge the support of Rappaport Institute, Technion – Faculty of Medicine, Haifa, Israel.

Statement of Interest

None.

References


Guidotti A, Aua J, Davis JM, Dong E, *et al.* (2005). GABAergic dysfunction in schizophrenia: new treatment...


Tamminga CA (2006). The neurobiology of cognition in schizophrenia. *Journal of Clinical Psychiatry* 67 (Suppl. 9), 9–13; discussion 36–42.


