Regulation of mouse brain glycogen synthase kinase-3 by atypical antipsychotics

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Abstract

Glycogen synthase kinase-3 (GSK3) has been recognized as an important enzyme that modulates many aspects of neuronal function. Accumulating evidence implicates abnormal activity of GSK3 in mood disorders and schizophrenia, and GSK3 is a potential protein kinase target for psychotropics used in these disorders. We previously reported that serotonin, a major neurotransmitter involved in mood disorders, regulates GSK3 by acutely increasing its N-terminal serine phosphorylation. The present study was undertaken to further determine if atypical antipsychotics, which have therapeutic effects in both mood disorders and schizophrenia, can regulate phospho-Ser-GSK3 and inhibit its activity. The results showed that acute treatment of mice with risperidone rapidly increased the level of brain phospho-Ser-GSK3 in the cortex, hippocampus, striatum, and cerebellum in a dose-dependent manner. Regulation of phospho-Ser-GSK3 was a shared effect among several atypical antipsychotics, including olanzapine, clozapine, quetiapine, and ziprasidone. In addition, combination treatment of mice with risperidone and a monoamine reuptake inhibitor antidepressant imipramine or fluoxetine elicited larger increases in brain phospho-Ser-GSK3 than each agent alone. Taken together, these results provide new information suggesting that atypical antipsychotics, in addition to mood stabilizers and antidepressants, can inhibit the activity of GSK3. These findings may support the pharmacological mechanisms of atypical antipsychotics in the treatment of mood disorders.

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Introduction

Glycogen synthase kinase-3 (GSK3) has been increasingly recognized as a versatile enzyme that exerts profound influences on neuronal function, including gene expression, architecture, plasticity, and survival (Frame and Cohen, 2001; Grimes and Jope, 2001). These critical actions of GSK3 are mediated by more than 40 cytosolic and nuclear substrates of the enzyme (Jope and Johnson, 2004). With these numerous substrates and functions, the activity of GSK3 must be tightly controlled for normal function. The two isoforms of GSK3, GSK3α and GSK3β, are constitutively active, and their activities are primarily regulated by the phosphorylation of an N-terminal serine, Ser21 of GSK3α and Ser9 of GSK3β (Stambolic and Woodgett, 1994; Sutherland and Cohen, 1994; Sutherland et al., 1993). This N-terminal phosphorylation of GSK3 results in inhibition of its activity. Several different kinases are capable of phosphorylating these regulatory serines on GSK3, including Akt (also known as protein kinase B), protein kinase C, protein kinase A, and others (Jope and Johnson, 2004). Thus, many signalling systems converge on GSK3 to control its activity via serine phosphorylation, contributing to the regulation of its specific cellular functions.

GSK3 has been implicated as a contributory factor in some prevalent psychiatric diseases, such as mood disorders and schizophrenia (Emamian et al., 2004; Gould and Manji, 2005). The kinase was linked to mood disorders by the discovery that the mood stabilizer lithium directly inhibits GSK3 (Klein and Melton, 1996), raising the possibility that it may be inadequately controlled in mood disorders. This connection gained further support in the recent findings that administration of lithium increased levels of serine-phosphorylated GSK3 in animal brain (Beaulieu et al., 2004; De Sarno et al., 2002). GSK3
haploinsufficient mice demonstrated similar behavioural as that induced by lithium treatment (O’Brien et al., 2004). Certain GSK3 inhibitors produced antidepressant-like effects, as well as potently suppressed dopamine-induced hyperactivity in animals (Beaulieu et al., 2004; Gould et al., 2004; Kaidanovich-Beilin et al., 2004). In addition to the relevance to mood disorders, the Akt/GSK3 pathway can also be regulated by dopamine, a neurotransmitter that is suspected of playing a role in schizophrenia (Beaulieu et al., 2005; Emamian et al., 2004).

The neuronal actions of GSK3 and its possible links to mood disorders recently led us to examine the regulation of GSK3 by serotonergic activity, since serotonin is a major neurotransmitter that plays a critical role in mood disorders (Sobczak et al., 2002; Stockmeier, 2003) and is modulated by psychotropic drugs (Li et al., 2004). We found that several serotonergic modulators regulated GSK3 activity in the mouse brain, in which phospho-Ser9-GSK3\(\beta\) was increased by endogenous serotonin release, 5-HT\(\text{1A}\) receptor activation, and 5-HT\(\text{2}\) receptor blockade. The increased phospho-Ser9-GSK3\(\beta\) by endogenous serotonin or 5-HT\(\text{1A}\) receptor activation can be further enhanced by 5-HT\(\text{2}\) receptor blockade. Furthermore, inhibition of monoamine reuptake by the antidepressants imipramine and fluoxetine increased brain phospho-Ser9-GSK3\(\beta\), further suggesting a potential role of GSK3 as a molecular target in the treatment of mood disorders.

Atypical antipsychotics are a group of newer and widely used psychotropics originally developed to improve the treatment of schizophrenia, which have recently increased use in the treatment of mood disorders, such as bipolar disorder and depression (Hirschfeld, 2003; Papakostas, 2005; Yatham, 2003). In addition to their binding affinity for the dopamine D\(\text{2}\) receptors, one of the major pharmacological differences between these agents and the conventional antipsychotics is their prevalent binding to serotonin 5-HT\(\text{2}\)\(\text{A}\) receptors (Meltzer et al., 1989). Although not conclusive, it has been hypothesized that the 5-HT\(\text{2}\)\(\text{A}\) receptor-blocking property of the atypical antipsychotics may play a role in their improved and extended therapeutic applications in the treatment of schizophrenia and mood disorders (Meltzer et al., 2003). Recently, emerging evidence has shown that chronic treatment of animals with antipsychotics may regulate GSK3 in the brain by increasing the level of total GSK3 (Alimohamad et al., 2005a, b) or by Akt-induced serine phosphorylation of GSK3 (Alimohamad et al., 2005a; Emamian et al., 2004). These chronic effects of antipsychotics appear to be mediated by D\(\text{2}\) dopamine receptor blockade and are shared by classical and atypical antipsychotics (Alimohamad et al., 2005a).

Based on our finding that 5-HT\(\text{1A}\) receptor blockade can increase phospho-Ser-GSK3 and thus inhibit GSK3 activity, we hypothesized that atypical antipsychotics may have an additional regulatory effect on GSK3 that mimics the acute effect of serotonin modulators. In the present study, we examined the acute regulatory effects of several atypical antipsychotics on serine phosphorylation of GSK3, aimed at improving our understanding of the mechanisms of these atypical antipsychotics in the treatment of mood disorders.

**Methods**

**Animals and treatments**

The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved the experimental protocol used in this study.

Adult male C57BL/6 mice (Frederick Cancer Research, Frederick, MD, USA), 8–12 wk old, were used for all experiments. Mice were injected intra-peritoneally (i.p.) with the indicated drugs. Risperidone, haloperidol (Sigma, St. Louis, MO, USA), clozapine (NIMH Chemical and Drug Supply Program), olanzapine (Eli Lilly and Company, Indianapolis, IN, USA), quetiapine (AstraZeneca, Macclesfield, Cheshire, UK), and ziprasidone (Pfizer Inc., Groton, CT, USA) were dissolved in 5% acetic acid in saline and adjusted to pH 5.5 for injection with 5% acetic acid as control vehicle for these drugs. Dosages of these drugs, indicated in the Results section, were chosen from previously published effective dose ranges in animal studies (Kapur et al., 2003; Weiner et al., 2001). Fluoxetine (NIMH Chemical Synthesis and Drug Supply Program) and imipramine (Sigma) were dissolved in saline with saline as vehicle for these drugs. All drugs were dissolved to a concentration (mg/ml) that, when injected at 5 \(\mu\)l/g, yielded the desired final dosage (mg/kg). At the end of each treatment, the mice were euthanized in a CO\(\text{2}\) chamber for 10 s, followed by rapid decapitation. Brain regions (cortex, hippocampus, striatum, and cerebellum) were immediately dissected in ice-cold saline.

**Protein preparation**

To prepare protein lysate from brain homogenate, brain regions were homogenized in ice-cold lysis buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 10 \(\mu\)g/ml
leupeptin, 10 \text{mg/ml} aprotinin, 5 \text{mg/ml} pepstatin, 0.1 \text{mM} \beta\text{-glycerophosphate}, 1 \text{mM} phenylmethanesulphonyl fluoride, 1 \text{mM} sodium vanadate, and 100 \text{nM} okadaic acid. The lysate was collected after the homogenate was centrifuged at 20,800 \text{g} for 10 \text{min} to remove insoluble debris. To obtain cytosolic and nuclear fractions, the brain cortex was suspended in a cavitation buffer containing 5 \text{mM} HEPES (pH 7.4), 3 \text{mM} MgCl2, 1 \text{mM} EGTA, 250 \text{mM} sucrose, 10 \text{mg/ml} leupeptin, 10 \text{mg/ml} aprotinin, 5 \text{mg/ml} pepstatin, 0.1 \text{mM} phenylmethanesulphonyl fluoride, 1 \text{mM} sodium vanadate, 1 \text{mM} okadaic acid, and 50 \text{mM} sodium fluoride (Bijur and Jope, 2001). Cells were disrupted by nitrogen cavitation using a Cell Disruption Bomb (Parr Instrument Company, Moline, IL, USA) at 200 psi, followed by centrifugation at 700 \text{g} for 10 \text{min} at 4\C. The supernatant was centrifuged at 100,000 \text{g} for 30 \text{min} at 4\C and the resultant supernatant was used as the soluble cytosolic fraction. The nuclear-containing pellet from the 700 \text{g} spin was washed twice with cavitation buffer and passed through 10 \text{times} volume of 1 \text{m} sucrose by centrifugation at 700 \text{g} for 10 \text{min}. The pellet was washed once with the cavitation buffer and the nuclear proteins were extracted from the pellet with nuclear extraction buffer containing 20 \text{mM} HEPES (pH 7.9), 300 \text{mM} NaCl, 1.5 \text{mM} MgCl2, 0.2 \text{mM} EDTA, 10 \text{mg/ml} leupeptin, 10 \text{mg/ml} aprotinin, 5 \text{mg/ml} pepstatin, 0.1 \text{mM} phenylmethanesulphonyl fluoride, 1 \text{mM} sodium vanadate, 1 \text{mM} okadaic acid, and 0.1 \text{mM} \beta\text{-glycerophosphate. Protein concentrations of lysate, cytosol and nuclei were determined using the Bradford protein assay (Bradford, 1976).}

**Immunoblotting**

Proteins from lysate, cytosol or nuclei were mixed with Laemmli sample buffer (2% SDS) and placed in a boiling water bath for 5 \text{min}. Proteins (20 \text{mg} of lysate or 5 \text{mg} of cytosol or nuclei) were resolved in 10% SDS–polyacrylamide gels, and transferred to nitrocellulose. Blots were probed with antibodies to phospho-Ser9-GSK3β, phospho-Ser21-GSK3α, total GSK3β, total GSK3α, α-tubulin, or CREB (Cell Signalling Technology, Beverly, MA, USA). Immunoblots were developed using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG, followed by detection with enhanced chemiluminescence. Protein bands were quantitated with a densitometer. Statistical significance was determined using analysis of variance (ANOVA).

**Immunohistochemistry**

The immunohistochemistry method was derived from the immersion-fixation and tyramide signal amplification (TSA) method (Roth et al., 1999). When treatments were completed, mice were euthanized in a CO2 chamber for 10 \text{sec} followed by rapid decapitation. Brains were immediately immersion-fixed in Bouin’s fixative overnight at 4\C. The fixed brains were processed in paraffin, and 4 \text{mm} brain sections were prepared on a microtome. The sections were deparaffinized in serial solutions of Citrisolv (Fisher Scientific, Pittsburgh, PA, USA), isopropanol, and water, followed by antigen retrieval by steaming in 10 \text{mM} citric acid (pH 6.0) for 20 \text{min}. Endogenous peroxidase activity was inhibited by incubation in 3% H2O2 in PBS for 5 \text{min}, followed by three PBS washes. Sections were incubated for 30 \text{min} in blocking buffer (1% bovine serum albumin, 0.2% skim milk, 0.3% Triton X-100 in PBS) to inhibit non-specific antibody binding, and then incubated overnight with anti-phospho-Ser9-GSK3β or anti-total GSK3β diluted in blocking buffer. After PBS washes, sections were labelled with horseradish peroxidase-conjugated anti-rabbit (for anti-phospho-Ser9-GSK3β labelled sections) or anti-mouse (for anti-total GSK3β labelled sections) secondary antibodies for 1 \text{hr} at room temperature, and then washed in PBS again. Cyanine-3-conjugated tyramide was deposited according to the manufacturer’s protocol to localize sites of antibody binding (TSA Plus, PerkinElmer Life Science Products, Boston, MA, USA). Sections were then washed in PBS, counter-stained with Hoechst 33,258, and coverslipped with PBS-glycerol (1:1). Fluorescence was viewed with a Zeiss-Axioskop microscope equipped with epifluorescence. Brain sections were examined under the microscope using 10× and 20× objectives. Digital images were captured with the Zeiss Axioscam and Axiovision software. All images were collected using identical camera settings and post-collection image-processing parameters. No immunoreactivity was observed if primary antibodies were omitted from the immunoreaction protocol (data not shown).

**Data analysis**

Data are presented as means±S.E. Statistical comparisons were performed using one-way ANOVA or unpaired Student’s t test. In all cases, p<0.05 is considered statistically significant.

**Results**

The atypical antipsychotic risperidone increased serine phosphorylation of GSK3 in mouse brain

To examine if the inhibitory serine phosphorylation of GSK3 is regulated by acute antipsychotic treatment,
we first tested the effect of an atypical antipsychotic, risperidone, at doses of 0.1 and 1 mg/kg. These two doses of risperidone were chosen based on the available animal studies showing that the efficient dopamine D$_2$ receptor occupancy for risperidone is 0.5–1 mg/kg, whereas its binding affinity to 5-HT$_2$A receptors is at least three times higher than its binding to D$_2$ receptors (Kapur et al., 2003; Weiner et al., 2001). Mice were treated with a single injection of risperidone (0.1 mg/kg or 1 mg/kg) and brains were dissected 1 h after the injection. Relative to control, the low dose of risperidone (0.1 mg/kg) increased phospho-Ser9-GSK3$\beta$ in the cortex and hippocampus, and, to a lesser degree, in the striatum and cerebellum (Table 1). To compare the effect of the atypical antipsychotic to the conventional antipsychotic, phospho-Ser9-GSK3$\beta$ was measured after mice were treated with the conventional antipsychotic haloperidol. Haloperidol at 0.2 mg/kg, a dose that is sufficient for clinically comparable D$_2$ receptor occupancy (Kapur et al., 2003) and is clinically equivalent to 0.1 mg/kg risperidone, had almost no effect on the level of phospho-Ser9-GSK3$\beta$ (92±38%, 97±33%, 124±45%, and 88±35% of controls (n=3) in the cortex, hippocampus, striatum, and cerebellum respectively) as shown in the representative immunoblot in Figure 1a.

Since GSK3$\beta$ distributes widely throughout the cells, including cytosol and nucleus (Bijur and Jope, 2001), we examined the subcellular distribution of GSK3$\beta$ after treatment with risperidone (Figure 1b). Although there was a substantial level of GSK3$\beta$ in both cytosol and nuclei, risperidone (0.1 mg/kg, 1 h) increased phospho-Ser9-GSK3$\beta$ only in the cytosol. The purity of the cytosolic and nuclear preparations was examined using $\alpha$-tubulin (a protein marker for cytosol) and CREB (a protein marker for nuclei). There

Figure 1. Risperidone increased the level of phospho-Ser-GSK3 in the mouse brain. Mice were treated with vehicle (Ctl), risperidone (Ris; 0.1 or 1 mg/kg i.p.), or haloperidol (Hal; 0.2 mg/kg i.p.), for 1 h. (a) Phospho-Ser9-GSK3$\beta$ and total GSK3$\beta$ in brain homogenate from the cortex, hippocampus, striatum, and cerebellum were detected by immunoblot. (b) Cytosolic and nuclear phospho-Ser9-GSK3$\beta$, total GSK3$\beta$, $\alpha$-tubulin, and CREB in the cortex were detected by immunoblots. (c) Phospho-Ser21-GSK3$\alpha$ and total GSK3$\alpha$ in brain homogenate from the cortex, hippocampus, striatum, and cerebellum were detected by immunoblots after mice were treated with risperidone (0.1 mg/kg).
was an abundant level of α-tubulin in the cytosolic fraction and a trace amount identified in the nuclear fraction. CREB was only detected in the nuclear fraction, indicating relative purity of cytosolic/nuclear preparation.

In addition to increasing phospho-Ser9-GSK3β, risperidone (0.1 mg/kg) also increased the level of phospho-Ser21-GSK3α in all four brain regions (Figure 1c). This effect of risperidone was statistically significant in the cortex, hippocampus, and cerebellum, and had a p value of 0.058 in the striatum (Table 1). Conversely, the levels of brain phospho-Ser473-Akt or phospho-Thr308-Akt did not change after risperidone treatment (data not shown), suggesting that the effect of risperidone on GSK3 phosphorylation is specific to GSK3 and independent of the Akt signalling pathway.

**Risperidone-induced increase of phospho-Ser9-GSK3β was rapid and dose-dependent**

The increase of phospho-Ser9-GSK3β in the cortex and hippocampus following a single injection of risperidone (0.1 mg/kg) was rapid, but transient (Figure 2a, b). There was a tendency for it to increase within 0.5 h and to reach a peak 1 h after treatment, followed by a gradual (cortex) or rapid (hippocampus) decline to near control levels between 2 and 6 h of treatment. Thus, we chose to measure phospho-Ser-GSK3 levels after 1 h treatment for most of the experiments in this study.

To characterize the dose range of risperidone that increased the level of phospho-Ser9-GSK3β, mice were treated with 0, 0.01, 0.03, 0.1, 0.3, and 1 mg/kg risperidone for 1 h (Figure 2c, d). In both cortex and hippocampus, risperidone increased the level of phospho-Ser9-GSK3β at a dose as low as 0.03 mg/kg. The peak effect of risperidone in both brain regions occurred at 0.1 mg/kg, and the effect started to decline at 0.3 mg/kg. Risperidone at 1 mg/kg had much less effect on the level of phospho-Ser9-GSK3β.

**Increase of phospho-Ser9-GSK3β in mouse brain is a common effect among atypical antipsychotics**

Since all clinically applied atypical antipsychotics, including risperidone, olanzapine, clozapine, quetiapine, and ziprasidone, share a dual-acting effect on dopamine and serotonin receptors (Schotte et al., 1996), we next examined if these atypical antipsychotics also share a regulatory effect on GSK3β in the mouse brain (Figure 3). Olanzapine (5 mg/kg, 1 h) caused a large increase in phospho-Ser9-GSK3β to about 300–400% of control levels in the cortex, hippocampus, striatum, and cerebellum. A parallel comparison of the effects of olanzapine and clozapine indicated that they had a similar intensity in increasing phospho-Ser9-GSK3β in all tested brain regions, and the increase was statistically significant in the cortex and hippocampus (Figure 3a–c). A lower dose of quetiapine (10 mg/kg, 1 h) had a moderate effect, increasing phospho-Ser9-GSK3β to 208%, 194%, 365%, and 134% in the cortex, hippocampus, striatum, and cerebellum respectively, whereas the higher dose of quetiapine (50 mg/kg, 1 h) paradoxically had a smaller effect (Figure 3d). Noticeably, this dose effect mirrored that of risperidone, where the higher dose of risperidone (1 mg/kg) had less effect. Ziprasidone (2.5 mg/kg, 1 h) also caused an increase in phospho-Ser9-GSK3β in the cortex, hippocampus, and striatum (172%, 182% and 179% respectively), but the effect was smaller than those caused by clozapine, olanzapine, and quetiapine. None of these atypical antipsychotics changed the total amount of GSK3β in the tested brain regions (data not shown).

### Table 1. Risperidone increased phospho-Ser-GSK3 in the mouse brain

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>Phospho-Ser9-GSK3β</th>
<th>Phospho-Ser21-GSK3α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ris % Ctl (Av. ± s.e.)</td>
<td>n</td>
</tr>
<tr>
<td>Cortex</td>
<td>246.69 ± 45.40</td>
<td>15</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>237.66 ± 42.63</td>
<td>15</td>
</tr>
<tr>
<td>Striatum</td>
<td>189.84 ± 28.35</td>
<td>12</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>168.60 ± 26.90</td>
<td>12</td>
</tr>
</tbody>
</table>

Mice were treated with risperidone (Ris; 0.1 mg/kg) or vehicle (Ctl) for 1 h. Phospho-Ser9-GSK3β and phospho-Ser21-GSK3α in homogenates of indicated brain regions were detected by immunoblot.

*p < 0.05 when risperidone was compared to control using unpaired Student’s t test.
Combined risperidone and antidepressant treatment caused larger increases in mouse brain phospho-Ser9-GSK3β

We previously reported that the monoamine reuptake inhibitor antidepressants imipramine and fluoxetine increased phospho-Ser9-GSK3β in the mouse brain (Li et al., 2004). In this study, we further examined whether a combination of risperidone and a monoamine reuptake inhibitor antidepressant could cause a larger increase of phospho-Ser-GSK3 than the effect of either agent alone. In one group of experiments, mice received an injection of risperidone (0.1 mg/kg) alone, imipramine (30 mg/kg) alone, or risperidone + imipramine for 1 h. Figure 4(a,b) shows that risperidone and imipramine each increased phospho-Ser9-GSK3β. The combined treatment caused a significantly larger increase of phospho-Ser9-GSK3β in the cortex, hippocampus, striatum and cerebellum when compared to each individual drug treatment. In this group of experiments, risperidone and imipramine each caused an ~4-fold increase in phospho-Ser9-GSK3β in the cortex, whereas the combined treatment caused an ~11-fold increase in the same region.
In another group of experiments, mice were treated with risperidone (0.1 mg/kg) alone, a serotonin-selective antidepressant fluoxetine (20 mg/kg) alone, or risperidone + fluoxetine for 1 h. The risperidone and fluoxetine combination caused a statistically significant larger increase of phospho-Ser9-GSK3β in the cortex, hippocampus, and striatum than each individual drug treatment, and the effect appeared to be the sum of each individual treatment (Figure 5a, b). In addition, the presence of phospho-Ser9-GSK3β and total GSK3β in the hippocampus was visualized by immunohistochemistry (Figure 5c). Phospho-Ser9-GSK3β immunoreactivity was visible in the hippocampus of mice treated with risperidone or fluoxetine, and it was more prominent in the hippocampus of mice receiving combined treatment with risperidone and fluoxetine. A strong signal of total GSK3β immunoreactivity was visible in the hippocampus but there was no apparent intensity difference between control and treated mice. The phospho-Ser9-GSK3β and total GSK3β immunoreactivity was most pronounced in hippocampal pyramidal neurons located in the CA3 and hilar regions.

The combined treatment of mice with risperidone + imipramine or fluoxetine not only increased phospho-Ser9-GSK3β, but also increased phospho-Ser21-GSK3α in the cortex and hippocampus (Figure 6). When the combined treatments were compared to the effect of each agent alone, risperidone + imipramine caused a 3-fold more increase of phospho-Ser21-GSK3α in the cortex and a 2-fold more increase in the hippocampus (Figure 6a, c). Similarly, risperidone + fluoxetine caused a 2.5- to 3-fold more increase of phospho-Ser21-GSK3α than did each agent alone (Figure 6b, d). Neither of these treatments caused a significant change in the total level of GSK3α.
Discussion

Based on the findings that the mood stabilizer lithium directly inhibits GSK3 (Klein and Melton, 1996), that reduced GSK3 activity in mice has antidepressant-like effects (Gould et al., 2004; Kaidanovich-Beilin et al., 2004; O’Brien et al., 2004), and that serotonin modulators robustly regulate GSK3 in the mouse brain (Li et al., 2004), this study sought to further identify the in-vivo regulation of brain GSK3 by psychotropic medications that have clinical implications in mood disorders. Our previous work has shown that either activating 5-HT\textsubscript{1A} receptors or blocking 5-HT\textsubscript{2A} receptors increased N-terminal serine-9 phosphorylation of GSK3\textsubscript{β}, causing inactivation of GSK3 (Li et al., 2004). Therefore, we hypothesized that atypical antipsychotics, which block both dopamine D\textsubscript{2} and serotonin 5-HT\textsubscript{2A} receptors (Meltzer et al., 1989), may regulate GSK3 in the mouse brain, and may enhance the GSK3-regulating effect of monoamine reuptake inhibitor antidepressants. In this study, we report several new findings that support this hypothesis.

Most noticeably, several clinically applicable atypical antipsychotics rapidly regulated mouse brain GSK3 by increasing its inhibitory N-terminal serine phosphorylation. Additionally, we found that combined treatment of mice with risperidone and a monoamine reuptake inhibitor antidepressant caused a significantly larger increase in the N-terminal serine phosphorylation of mouse brain GSK3. These new findings add additional support to the growing body of evidence that brain GSK3 may be involved in the development and treatment of mood disorders.

In our first group of experiments we tested the acute effect of risperidone, a drug with clinical indications in the treatment of both bipolar mania and schizophrenia. Risperidone has dual actions of blocking both dopamine D\textsubscript{2} and serotonin 5-HT\textsubscript{2A} receptors (Sumiyoshi et al., 1994). Due to its high affinity to the 5-HT\textsubscript{2A} receptors, it is thought that risperidone may have an additional pharmacological effect as a 5-HT\textsubscript{2A} receptor antagonist to increase phospho-Ser9-GSK3. Indeed, we found that a low dose of risperidone acutely increased phospho-Ser9-GSK3\textsubscript{β} and phospho-Ser21-GSK3\textsubscript{α} in...

![Figure 4. Risperidone and imipramine combination treatment robustly increased phospho-Ser9-GSK3β.](http://ijnp.oxfordjournals.org/)

(a) Representative immunoblots of phospho-Ser9-GSK3\textsubscript{β} and total GSK3\textsubscript{β}, in the cortex (CTX), hippocampus (HIP), striatum (STR), and cerebellum (CBL) after mice received i.p. injection of vehicle (Ctl), risperidone (R; 0.1 mg/kg), imipramine (I; 30 mg/kg), or risperidone + imipramine (R + I) for 1 h. (b) Quantitative analysis of phospho-Ser9-GSK3\textsubscript{β} immunoblots. Values are expressed as percent of control. Means ± s.e. (n = 6), **p < 0.05 when risperidone + imipramine treatment was compared with either risperidone or imipramine alone using one-way ANOVA.
all tested brain regions. Interestingly, risperidone appeared to have an effective window in which lower doses (0.03–0.3 mg/kg) increased phospho-Ser9-GSK3β, whereas the higher dose (1 mg/kg) had less effect. In contrast, haloperidol is a conventional antipsychotic with highly potent D2 receptor antagonism and minimal 5-HT2A receptor affinity (Leysen et al., 1988), making it a useful control to differentiate the effect of the dual actions of risperidone. As a comparison in our experiments, we chose a dose of haloperidol (0.2 mg/kg) at which it is less likely to block 5-HT2A receptors, and at this dose, haloperidol did not cause an acute change of phospho-Ser9-GSK3β.

Although we did not find an acute effect by the D2 antagonist haloperidol in this study, we do not rule out the possibility that the dopaminergic action of atypical antipsychotics may contribute to the regulation of GSK3 as reported by other groups of researchers. Noticeably, chronic treatments of animals with a higher dose of risperidone (0.9–2 mg/kg) or haloperidol (1 mg/kg) increased the level of either total GSK3 or phospho-Ser9-GSK3β in rats and mice respectively (Alimohamad et al., 2005a,b; Emamian et al., 2004). The acute increase of phospho-Ser-GSK3β was noticed only after dopamine transporter knock-out mice were treated with a D2/D3 receptor antagonist raclopride (Beaulieu et al., 2004). However, the acute effect of risperidone and haloperidol on serine phosphorylation of GSK3 was not reported in these studies. As the average dose of risperidone used to attain a 50% in-vivo blockade of D2 receptors in rats is ~0.3 mg/kg (Kapur et al., 2003), and the binding affinity of risperidone to 5-HT2A receptors is at least three times higher than to D2 receptors (Weiner et al., 2001), we suspect that the increase of phospho-Ser-GSK3 by a lower dose range of risperidone (0.03–0.3 mg/kg) may be mediated by a mechanism other than blockade of dopamine D2 receptors, including the potential involvement of serotonin regulation. However, to substantiate the above speculation will require extensive studies in the future.

In this study, we focused on acute treatment with antipsychotics. This may call into question the therapeutic relevance of the observed effects on serine phosphorylation of GSK3. It is important to emphasize that regulation of protein phosphorylation is a necessary post-translational regulatory step during receptor-coupled signal transduction process, which

Figure 5. Risperidone and fluoxetine combination treatment largely increased phospho-Ser9-GSK3β. (a) Representative immunoblots of phospho-Ser9-GSK3β and total GSK3β, in the cortex (CTX), hippocampus (HIP), striatum (STR), and cerebellum (CBL) after mice received i.p. injection of vehicle (Ctl), risperidone (R; 0.1 mg/kg), fluoxetine (F; 20 mg/kg), or risperidone + fluoxetine (R + F) for 1 h. (b) Quantitative analysis of phospho-Ser9-GSK3β immunoblots. Values are expressed as percent of control. Means ± S.E. (n = 6), **p < 0.05 when risperidone + fluoxetine treatment was compared with either risperidone or fluoxetine alone using one-way ANOVA. (c) Immunohistochemical detection shows phospho-Ser9-GSK3β and total GSK3β immunoreactivity in hippocampal pyramidal neurons, particularly those in the CA3 and hilar region following indicated treatments.
further triggers the down-stream transcriptional activation and gene expression (Grimes and Jope, 2001). The observed acute effect of atypical antipsychotics in the increased level of phospho-Ser-GSK3 may serve as the first step in the long-term regulation of gene expression and thus, might well be compatible with the drugs’ therapeutic effects.

A time-course of risperidone revealed that after a single injection, the effect of risperidone was large but transient, peaking at 1 h and lasting no longer than 2–4 h. Several factors may have contributed to this observation. In order to meet the need for a selective receptor profile, the dose of risperidone used in this study was relatively low. Consequently, the well-characterized phenomenon of shorter half-lives (by 8–10 times) of most drugs in mice compared to humans (Urquhart et al., 1984) are likely to contribute to the observed transient time-course. Additionally, in the presence of several types of protein phosphatases in the brain, it is not surprising that receptor-mediated increase of protein phosphorylation might be rapidly returned to baseline by one or more active protein phosphatases. Nevertheless, an extended study applying repeated or continued treatment with serotonin modulators is warranted, aimed at further identification of the therapeutic relevance of the observed results.

Although we primarily focused on one antipsychotic, risperidone, in order to elucidate its effect on GSK3, we also sought to extend our results to a group of atypical antipsychotics because they all have indications for schizophrenia and bipolar disorder and share the dual-acting pharmacological property (Schotte et al., 1996). Indeed, a comparison of several atypical antipsychotics, including olanzapine, clozapine, quetiapine, and ziprasidone showed a common effect of acutely increasing phospho-Ser9-GSK3β. Clozapine was reported to increase phospho-Ser9-GSK3β in cultured neuroblastoma cells (Kang et al., 2004), but none of these antipsychotics have been reported to increase phospho-Ser-GSK3 in vivo. Among these atypical antipsychotics, olanzapine and clozapine appeared to have a more prominent effect, and quetiapine and ziprasidone a moderate effect. Interestingly, we found that, like risperidone, a lower dose of quetiapine appeared to have a stronger effect on GSK3 phosphorylation than did a higher dose. Due to its limited solubility, the maximum dose of

**Figure 6.** Risperidone + imipramine or risperidone + fluoxetine combination treatment synergistically increased phospho-Ser21-GSK3α. (a, b) Representative immunoblots and (c, d) quantitative analysis of immunoblots showing phospho-Ser21-GSK3α in the cortex (CTX) and hippocampus (HIP) after mice were treated with risperidone (R), imipramine (I), risperidone + imipramine (R + I), fluoxetine (F), or risperidone + fluoxetine (R + F). Values are expressed as percent of risperidone alone, imipramine alone, or fluoxetine alone. Values shown were an average of four samples from each treatment. *p < 0.05 on unpaired Student’s t test when risperidone + imipramine treatment was compared to risperidone or imipramine alone, and when risperidone + fluoxetine treatment was compared to risperidone or fluoxetine alone.
ziprasidone tested in this study was 2.5 mg/kg, and no further ziprasidone dose–response experiment was conducted. With this promising observation, each of these atypical antipsychotics needs to be further investigated in detail before the similarities and differences, as well as the clinical implications of any such findings, can be further clarified.

In our previous study, we found that treatment with a 5-HT2 receptor antagonist allowed induction of a much greater increase in brain phospho-Ser9-GSK3β by agents that increase brain serotonergic activity, such as treatment with d-fenfluramine + clorglyline or with the 5-HT1A receptor agonist 8-OH-DPAT (Li et al., 2004). We, therefore, hypothesized that a combination of an atypical antipsychotic with a monoamine reuptake inhibitor antidepressant may produce a larger increase in brain phospho-Ser-GSK3 in a similar fashion. Our finding that risperidone + imipramine or fluoxetine caused significantly larger increases in both phosphorylated isoforms of GSK3 in the mouse brain strongly supports this hypothesis. In fact, the enhancing effects between atypical antipsychotics and monoamine reuptake inhibitor antidepressants have previously been observed in both clinical practice (Li et al., 2005; Ostroff and Nelson, 1999; Tohen et al., 2003) and in other lines of pharmacological studies (Marek et al., 2003). Although the pharmacological characteristics of the robust GSK3 regulation by this combined treatment, such as receptor profile, additive vs. synergistic effect, and dose range, etc, remain to be identified, our findings provide additional affirmation of the growing clinical impression that atypical antipsychotics may be used as an adjunct to antidepressants for the treatment of severe mood disorders, with a therapeutic target on brain GSK3.

In this study, neither risperidone nor risperidone and a monoamine reuptake inhibitor antidepressant changed the phosphorylation of Akt, a major protein kinase that regulates serine phosphorylation of GSK3 (data not shown). Thus, it appears that the regulation of GSK3 by risperidone is mediated by a signalling pathway different from the D2/D3 antagonist raclopride-induced acute increase of phospho-Ser-GSK3 – a response coupled to an increase of phospho-Thr308-Akt (Beaulieu et al., 2004). It is possible that the atypical antipsychotic-induced increase of phospho-Ser-GSK3 is mediated by other protein kinases, such as protein kinase C or protein kinase A (Cook et al., 1996; Fang et al., 2000; Goode et al., 1992). The risperidone-induced serine phosphorylation of GSK3β seems to be localized in the cytosol, since there was no increase of phospho-Ser-GSK3β in the nucleus. This suggests that the protein kinase regulating GSK3 in response to atypical antipsychotics localizes in the cytosol. However, the physiological consequence of this localized regulation of GSK3 remains to be studied.

Taken together, findings from this study suggest that atypical antipsychotics have an acute inhibitory effect on mouse brain GSK3, and that the effect is delivered through increased N-terminal phosphorylation of GSK3. Thus, GSK3 may play a role as a therapeutic target of atypical antipsychotics. Additionally, atypical antipsychotics and monoamine reuptake inhibitor antidepressant combination treatment elicited much stronger inhibition of GSK3 activity, providing a biological background for the increasingly favoured psychopharmacological practice in which atypical antipsychotics are used as an adjunct to enhance the efficacy of antidepressants. The effects of atypical antipsychotics on GSK3 are shared with the previously identified effects of lithium and other GSK3 inhibitors – all inhibit GSK3. Although the precise role of GSK3 in the pathophysiology and treatment of mood disorders remains to be identified, the shared effect of atypical antipsychotics with mood stabilizers and antidepressants may further support their increased clinical application in mood disorders.

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Statement of Interest

None.

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