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Haloperidol and clozapine differentially regulate signals upstream of glycogen synthase kinase 3 in the rat frontal cortex

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Abbreviations: 5HT2 receptor, serotonin 5HT2 subtype receptor; D2 receptor, dopamine D2 subtype receptor; Dvl, dishevelled; GSK3, glycogen synthase kinase 3; PI-3K, phosphatidylinositol 3-kinase

Abstract

Glycogen synthase kinase 3 (GSK3) was recently suggested to be a potential target of psychotropics used in psychiatric illnesses such as schizophrenia and bipolar disorder. Relevant studies have found that antipsychotic drugs regulate GSK3 activity via an increase in either inhibitory serine phosphorylation or amount of GSK3 after acute or subchronic treatment. Recent evidence shows that GSK3 is regulated by dopaminergic or serotonergic systems implicated in the pathophysiology and treatment mechanisms of schizophrenia and bipolar disorder. Therefore, antipsychotics may regulate GSK3 via antagonizing dopaminergic or serotonergic activity. However, the signaling pathway that is involved in GSK3 regulation by dopaminergic or serotonergic systems has not been well established. Haloperidol is a typical antipsychotic with potent dopamine D_2 receptor antagonism. Clozapine is an atypical antipsychotic with potent serotonin $5HT_2$ receptor antagonism. We injected rats with haloperidol or clozapine and examined the phosphorylation and amount of GSK3α/β and its well-known upstream regulators Akt and Dvl in the rat frontal cortex by Western blotting. Both haloperidol and clozapine induced Ser21/9 phosphorylation of GSK3α/β. Haloperidol increased the Ser473 phosphorylation of Akt transiently, whereas clozapine maintained the increase for 1 h. Haloperidol did not affect the phosphorylation and amount of Dvl, whereas clozapine increased both phosphorylation and the amount of Dvl. Our results suggest that GSK3 activity may be regulated by both typical and atypical antipsychotics and that Akt or Dvl, depending on the D_2- or $5HT_2$-receptor antagonism properties of typical and atypical antipsychotics, mediate the regulation differently.

Keywords: antipsychotic agents; clozapine; dishevelled protein; glycogen synthase kinase-3; haloperidol; proto-oncogene proteins c-akt

Introduction

Glycogen synthase kinase 3 (GSK3) is an important protein kinase that controls multiple cellular functions in the brain. Its enzymatic activity is negatively regulated by the phosphorylation of N-terminal serine residues (Ser21 of GSK3α and Ser9 of GSK3β; Sutherland *et al*., 1993). Several protein kinases can phosphorylate these serine residues, including Akt (protein kinase B), protein kinase A, protein kinase C, phospholipase Cγ1 and others (Shin *et al*., 2002; Jope and Johnson, 2004). Among these, Akt is the most well-known upstream kinase of GSK3. In the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway, activated PI3-K leads to the subsequent phosphorylation-induced activation of Akt (reviewed in Gould, 2006). Activated Akt phosphorylates the inhibitory serine residue of GSK3. GSK3 activity can also be controlled via an entirely different regulatory mechanism. In the Wnt signaling pathway, the adaptor protein dishevelled (Dvl) is phosphorylated and activated when Wnt ligand binds to the Frizzled receptor. Activated Dvl interferes with the formation of the Axin/GSK3/β-catenin complex, which ultimately leads to the inhibition of GSK3 (reviewed in Kikuchi *et al*., 2006).

GSK3 has been linked to the pathophysiology and treatment mechanisms of psychiatric illnesses, including schizophrenia and bipolar disorder (Jope and Roh, 2006). Recent evidence indicates that the expression and phosphorylation of GSK3 can be regulated

by some neurotransmitter receptors such as dopamine D2 and serotonin 5HT1/5HT2 receptors. Interestingly, these receptors have long been proposed to be involved in the pathophysiology of schizophrenia and bipolar disorder. An acute stimulation of dopamine D_2 receptors elicits dephosphorylation-induced activation of GSK3 and vice versa (Beaulieu *et al*., 2004, 2005). D2 blockade-mediated phosphorylation of GSK3 was accompanied with the activation of Akt, suggesting that Akt may be an upstream regulator of GSK3 in D2 receptor-mediated signaling (Beaulieu *et al*., 2007). On the other hand, the acute stimulation of serotonin $5HT_1$ receptors or blockade of $5HT_2$ receptors produces phosphorylation-induced inhibition of GSK3 in the rodent brain (Li *et al*., 2004). However, the mediators of the 5HT receptor-coupled regulation of GSK3 have not been well established; a recent report stated that it was not mediated by Akt (Li *et al*., 2007).

Antipsychotics have therapeutic efficacy against schizophrenia or bipolar disorder by blocking neurotransmitter receptors. Typical antipsychotics such as haloperidol possess strong antagonism to D_2 receptors, but atypical antipsychotics such as clozapine possess strong $5HT_2$ antagonistic and weak D_2 antagonistic properties. Therefore, typical and atypical antipsychotics may regulate GSK3 activity via different signaling pathways. Previously, Kang *et al*. (2004) demonstrated that although both Dvl and Akt are activated by clozapine, only Dvl is responsible for Ser9-phosphorylation of GSK3β in SH-SY5Y cells. We compared the phosphorylation at Ser21/9 residues of GSK3α/β *in vivo* after acute treatment with haloperidol or clozapine. In addition, we examined the phosphorylation and amounts of Akt and Dvl to examine possible differences between haloperidol and clozapine in the regulation of GSK3 phosphorylation.

Materials and Methods

Experiments were carried out using male Sprague-Dawley rats (200-250 g) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23). Haloperidol (Sigma-Aldrich, Steinheim, Germany; 0.5, 1, 2, or 4 mg/kg) or clozapine (Tocris, St. Louis, MO; 5, 10, or 20 mg/kg) was dissolved in the vehicle (0.3% tartaric acid in saline). The solutions were then adjusted to pH 5.5 and administered intraperitoneally to the animals. Three independent sets of animals were used for each experiment. The doses of the two drugs were selected as equivalent doses based on clinical potency. Sham (S) animals were treated in precisely the same way, but were not

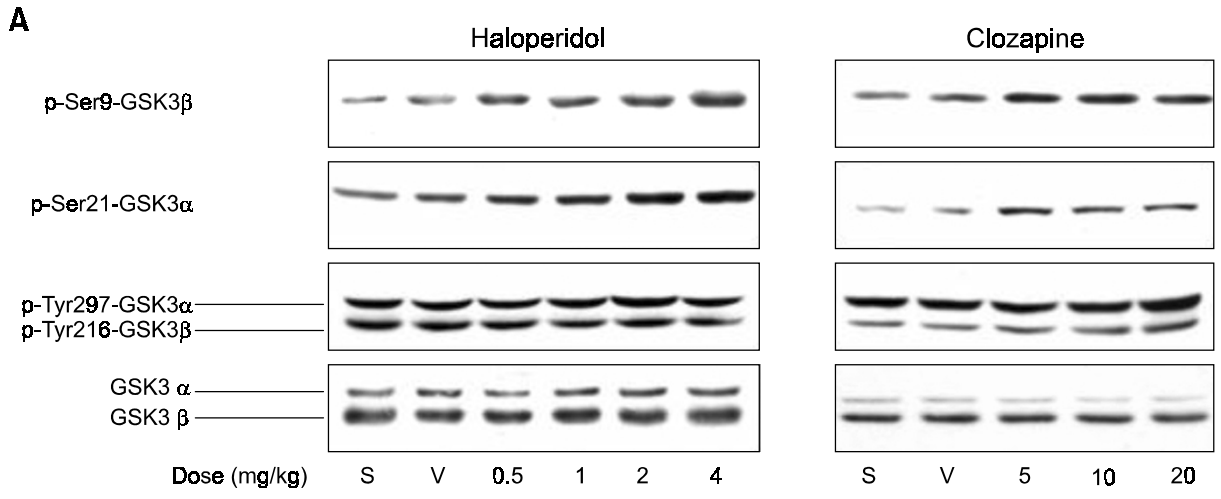

Figure 1. The Ser21/9 phosphorylation of GSK3α/β in the rat frontal cortex with acute haloperidol and clozapine treatment. (A) Representative immunoblots of the indicated proteins in the rat frontal cortex 1 h after injection with haloperidol (0.5, 1, 2, or 4 mg/kg i.p.) or clozapine (5, 10, or 20 mg/kg i.p.). S and V indicate the sham and vehicle-treated controls, respectively. (B) Quantification of immunoblot data for pSer9- GSK3β with acute haloperidol or clozapine treatment after 15, 30, or 60 min. (C) Quantification of immunoblot data for pSer21-GSK3α with acute haloperidol or clozapine treatment after 15, 30, or 60 min. The data are expressed as the mean and standard error of the relative OD from three independent experiments. The total numbers of animals used in the haloperidol and clozapine treatments were 54 (3 animals × 3 time points × 6 treatments) and 45 (3 animals × 3 time points × 5 treatments), respectively. The relative OD is the percentage OD compared to that of V. Data were analyzed statistically using two-way ANOVA to examine time and dose effects on the phosphorylation and amounts of proteins with haloperidol or clozapine treatment. The data were further analyzed using *post hoc* Tukey tests because a significant dose effect was found. Asterisks (*) indicate statistically significant differences in the OD of each dose from that of V (Tukey test, $P < 0.05$).

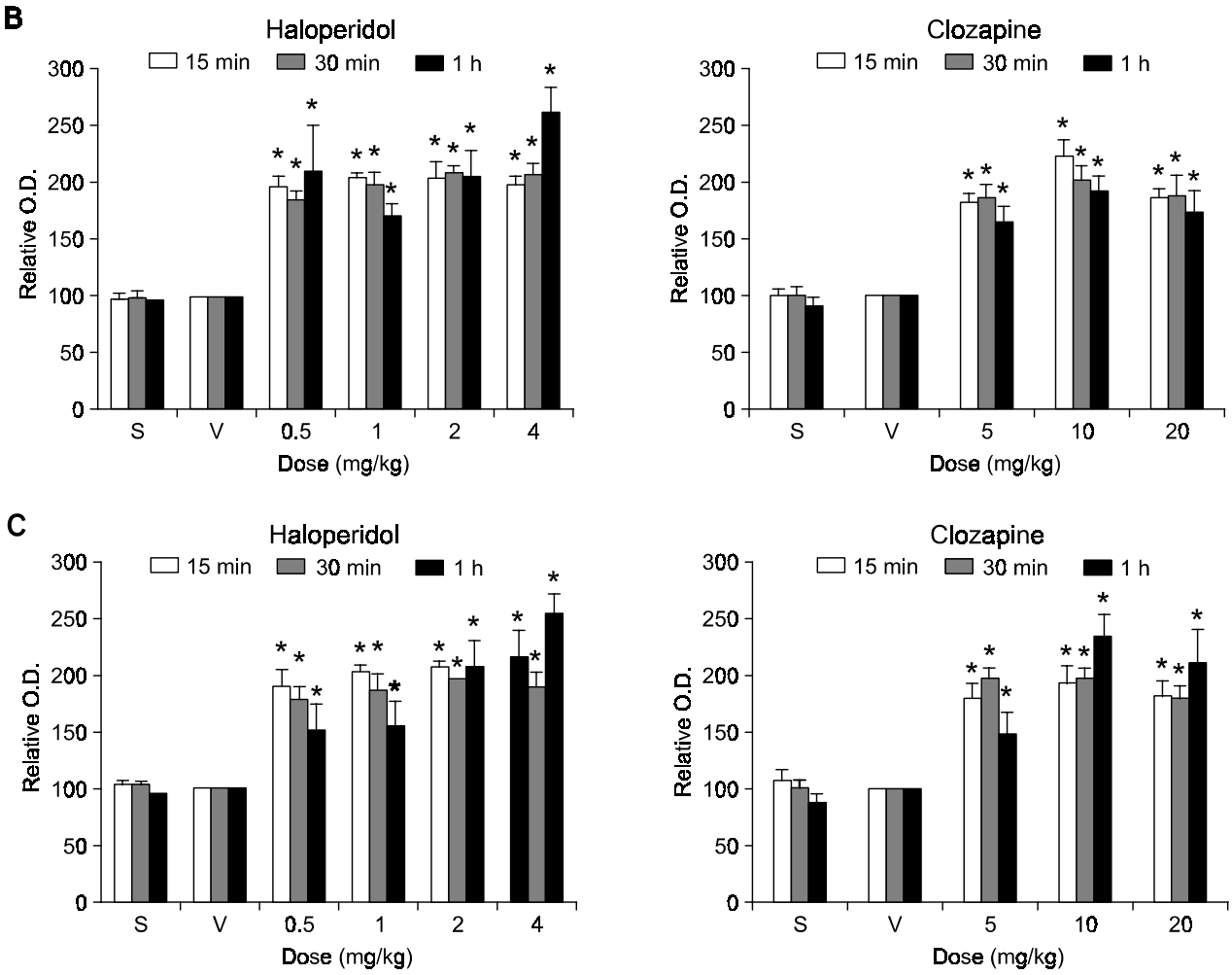

Figure 1. Continued.

injected. The rats were decapitated 15, 30, or 60 min after injection, and the frontal cortex was dissected on ice and immediately frozen in liquid nitrogen.

The frontal cortex was homogenized in 10 v/w of ice-cold RIPA (+) buffer as described previously (Kang *et al*., 2006). After centrifugation at 20,000 × *g* for 10 min, the supernatants were boiled with Laemmli's sample buffer. Equal amounts of protein were separated by SDS-PAGE and immunoblotted with antibodies specific for Akt, pSer473-Akt, pThr308-Akt, pSer21- GSK3α, pSer9-GSK3β (Cell Signaling Technology, Beverly, MA), GSK3α/β (Santa Cruz Biotechnology, Santa Cruz, CA), pTyr297/216-GSK3α/β (Upstate Biotechnology, Lake Placid, NY), Dvl (Santa Cruz Biotechnology), or actin (Sigma-Aldrich). The membranes were developed using the enhanced chemiluminescence system (Pierce, Rockford, IL).

The results were quantified by densitometry. Data were statistically analyzed using two-way ANOVA to examine time and dose effects on the phosphorylation and amounts of protein with haloperidol or clozapine treatment. The data were further analyzed using Tukey *post hoc* tests when a significant time or dose effect was found. Statistical significance was set at 0.05.

Results

Two-way ANOVA indicated that the dose effect on pSer21-GSK3α and pSer9-GSK3β was significant ($P < 0.05$), but the time effect was not significant with haloperidol or clozapine treatment. Haloperidol and clozapine increased pSer21-GSK3α and pSer9-GSK3β significantly for all doses tested (Tukey test, $P < 0.05$ compared to the vehicle treatment; Figure 1). Haloperidol induced a greater increase in pSer21-

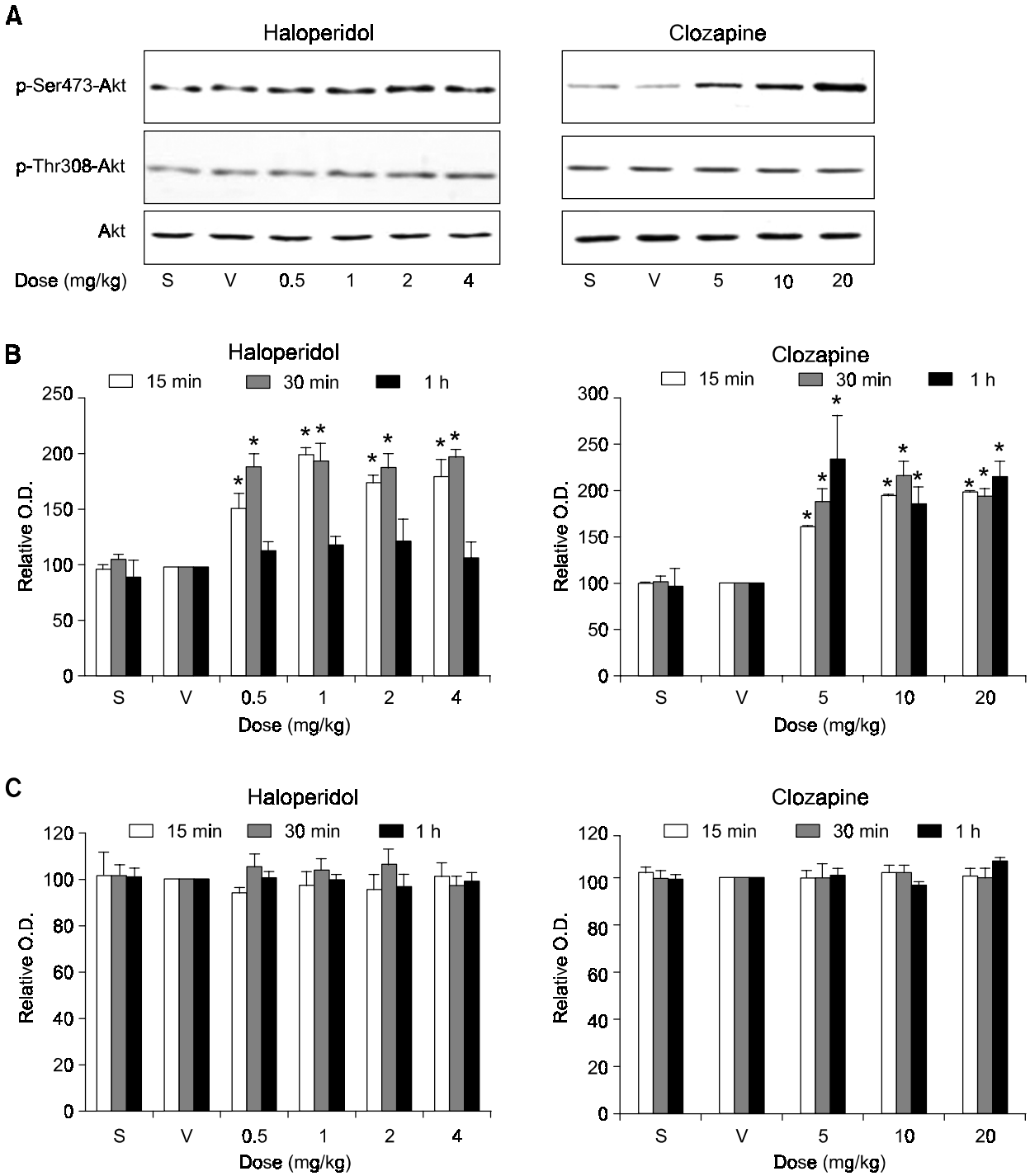

Figure 2. The Ser473 and Thr308 phosphorylation of Akt in the rat frontal cortex with acute haloperidol and clozapine treatment. (A) Representative immunoblots of the indicated proteins in the rat frontal cortex 1 h after injection with haloperidol (0.5, 1, 2, or 4 mg/kg i.p.) or clozapine (5, 10, or 20 mg/kg i.p.). S and V indicate the sham and vehicle-treated controls, respectively. (B) Quantification of the immunoblot data for pSer473-Akt with acute haloperidol or clozapine treatment after 15, 30, or 60 min. (C) Quantification of the immunoblot data for pThr308-Akt with acute haloperidol or clozapine treatment after 15, 30, or 60 min. The data are expressed as the mean and standard error of the relative OD from three independent experiments. The total numbers of animals used in the haloperidol and clozapine treatments were 54 (3 animals $\times$ 3 time points $\times$ 6 treatments) and 45 (3 animals $\times$ 3 time points $\times$ 5 treatments), respectively. The relative OD is the percentage OD compared to that of V. Data were analyzed statistically using two-way ANOVA to examine time and dose effects on the phosphorylation and amounts of proteins with haloperidol or clozapine treatment. The data were further analyzed using *post hoc* Tukey tests because significant time and dose effects were found. Asterisks (*) indicate statistically significant differences in the OD of each dose from that of V (Tukey test, $P < 0.05$). Statistically significant differences among time points are not marked in the graph.

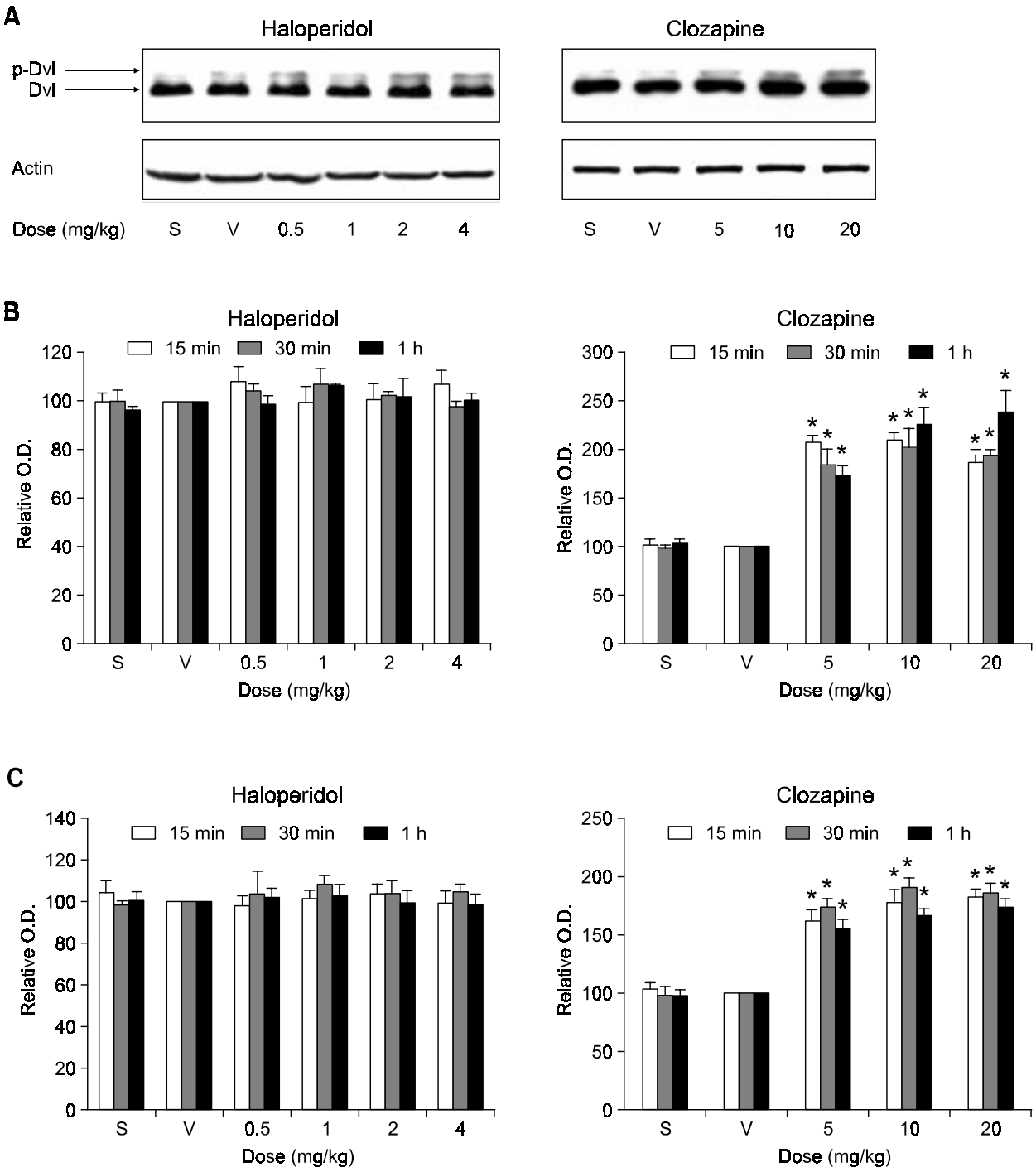

Figure 3. The phosphorylation and total amount of Dvl in the rat frontal cortex with acute haloperidol and clozapine treatment. (A) Representative immunoblots of the indicated proteins in the rat frontal cortex 1 h after injection with haloperidol (0.5, 1, 2, or 4 mg/kg i.p.) or clozapine (5, 10, or 20 mg/kg i.p.). S and V indicate the sham and vehicle-treated controls, respectively. (B) Quantification of the immunoblot data for phosphorylated Dvl with acute haloperidol or clozapine treatment after 15, 30, or 60 min. (C) Quantification of the immunoblot data for total Dvl with acute haloperidol or clozapine treatment after 15, 30, or 60 min. The data are expressed as the mean and standard error of the relative OD from three independent experiments. The total numbers of animals used in the haloperidol and clozapine treatments were 54 (3 animals × 3 time points × 6 treatments) and 45 (3 animals × 3 time points × 5 treatments), respectively. The relative OD is the percentage OD compared to that of V. Data were analyzed statistically using two-way ANOVA to examine time and dose effects on the phosphorylation and amounts of proteins with haloperidol or clozapine treatment. The data were further analyzed using *post hoc* Tukey tests because a significant dose effect was found. Asterisks (*) indicate statistically significant differences in the OD of each dose from that of V (Tukey test, $P < 0.05$).

GSK3α at 4 mg/kg than at 0.5 or 1 mg/kg (Tukey test, $P < 0.05$), and clozapine induced a greater increase in pSer9-GSK3β at 10 mg/kg than at 5 mg/kg (Tukey test, $P < 0.05$). The total amounts of GSK3α/β and pTyr297/Tyr216-GSK3α/β, which is known to enhance GSK3 activity (Hughes *et al*., 1993), were not affected by any dose of haloperidol or clozapine at any time point.

The dose and time effects on pSer473-Akt were both significant with haloperidol treatment (two-way ANOVA, $P < 0.05$). Haloperidol increased pSer473-Akt significantly compared to the vehicle for all doses tested (Tukey test, $P < 0.05$; Figure 2). The induced pSer473-Akt returned to the basal level at 1 h compared to that at 15 and 30 min, regardless of the dosage (Tukey test, $P < 0.05$). The dose effect, but not that of time, was significant with clozapine treatment (two-way ANOVA, $P < 0.05$). Clozapine increased pSer473-Akt significantly compared to the vehicle for all doses tested (Tukey test, $P < 0.05$; Figure 2). The clozapine-induced pSer473-Akt was maintained until 1 h after the treatment. The total amounts of Akt and pThr308-Akt were not affected by any dose of haloperidol or clozapine at any time point.

We detected phosphorylated Dvl using the mobility shift in gradient gels (6-12%; Ruel *et al*., 1999; Kang *et al*., 2004). The OD of phosphorylated Dvl (pDvl) was obtained from the upper band, and that of total Dvl was obtained from the upper and lower bands. The dose effects on pDvl and total Dvl were significant (two-way ANOVA, $P < 0.05$), but the time effect was not significant with clozapine treatment. Both pDvl and total Dvl increased significantly with all doses of clozapine treatment compared to the vehicle treatment (Tukey test, $P < 0.05$; Figure 3). Clozapine induced a greater increase in total Dvl at 20 mg/kg than at 5 mg/kg (Tukey test, $P < 0.05$). In contrast to the effects of clozapine, there was no change in pDvl or total Dvl after haloperidol treatment, regardless of the time or dosage.

Discussion

Acute treatments with haloperidol or clozapine increased pSer21/9-GSK3α/β *in vivo*, but they affected the upstream regulators of GSK3 differently. Haloperidol transiently increased the phosphorylation of Akt, but did not affect either the phosphorylation or amount of Dvl, whereas clozapine increased the phosphorylation of Akt, as well as the phosphorylation and total levels of Dvl (Figure 4).

The phosphorylation of GSK3 and Akt occurred simultaneously after haloperidol treatment. Although GSK phosphorylation can be regulated by kinases other than Akt, this coactivation may suggest a causal relationship. Other researchers have also suggested that D_2 receptor-mediated signaling regulates GSK3 via the Akt pathway (Beaulieu *et al*., 2007); however, Li *et al*. (2007) used a lower dosage of haloperidol (0.2 mg/kg), and their results contrasted with our findings. The results of research using long-term haloperidol treatment generally correspond with our results using acute treatment. Haloperidol is reported to increase the level of pSer9-GSK3β, accompanied by either an increase or no change in total GSK3 in subchronic treatment (Emamian *et al*., 2004; Alimohamad *et al*., 2005a), but Kozlovsky *et al*. (2006) reported that chronic haloperidol treatment decreased pSer9-GSK3β. The variable states of GSK3 phosphorylation may depend on the different treatment dosages and periods of time. The lingering effect of long-term haloperidol treatment on GSK3 further supports the possibility that D2 receptor-mediated inhibition of GSK3 may be related to the therapeutic mechanisms of haloperidol.

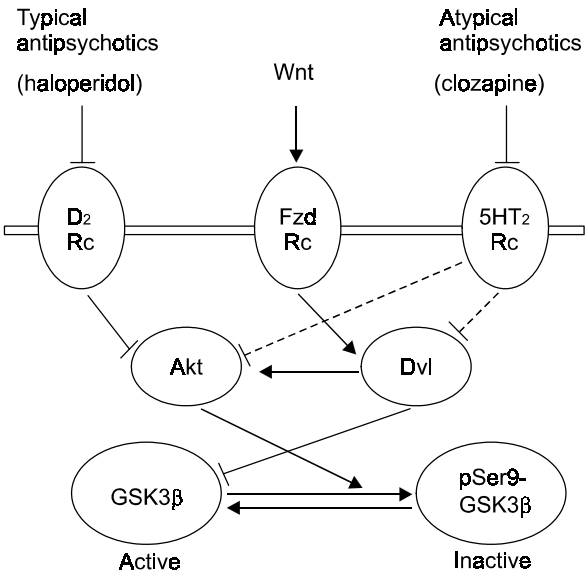

Figure 4. Schematic diagram of signaling pathways mediating the regulation of GSK3 by neurotransmitter receptor activity. D_2 Rc, dopamine D_2 subtype receptors; Dvl, dishevelled; Fzd Rc, frizzled receptor; GSK3, glycogen synthase kinase 3; $5HT_2$ Rc, serotonin $5HT_2$ subtype receptors; arrow, activating signal; blunt arrow, inhibitory signal.

The results of the clozapine treatment confirmed previous findings that acute clozapine treatment increases pSer9-GSK3β (Li *et al*., 2007). Clozapine also increased the phosphorylation of Akt. In contrast, Li *et al*. (2007) reported that risperidone, another 5HT2 and D2 blocker, did not increase the phosphorylation of Akt. The $5HT_2$ receptor antagonism of clozapine is much greater than that of risperidone, whereas the D2 antagonism is the reve-

rse. This difference in the $5HT_2/D_2$ affinity ratio may contribute to the difference in signal transduction.

Our *in vivo* results that clozapine increased pSer9-GSK3β, pSer473-Akt, and phosphorylated and total Dvl are consistent with the findings of Kang *et al*. (2004) using SH-SY5Y cells. However, Kang *et al*. (2004) showed that clozapine-induced Akt phosphorylation is not related to GSK3 serine phosphorylation. Because SH-SY5Y cells lack D_2 or $5HT_2$ receptors, the initial event in clozapine-induced signaling would be different between the cell line and brain models. Further studies are needed to clarify whether Akt or Dvl is responsible for the clozapine-induced phosphorylation of GSK3 *in vivo*. A notable finding was that clozapine induced increases in the phosphorylation and total amount of Dvl, contrary to haloperidol. Acute clozapine treatment rapidly increased the amount of Dvl, as well as its phosphorylation status, within 15 min, but the mechanism behind the rapid increase in the expression level by clozapine is unknown. Dvl is an adaptor protein linked to Frizzled receptors. Because the relationship of Dvl with neurotransmitter receptors is still unknown, it is unclear whether clozapine induces the alteration of Dvl via $5HT_2$ receptors. More studies on the possible relation between Dvl and clozapine are needed.

Dvl regulates GSK3 activity via two different regulating mechanisms (Shin *et al*., 2006). In the canonical Wnt pathway, Dvl is activated, binds to the Axin/GSK3/β-catenin protein complex, and consequently inhibits GSK3. The other regulating mechanism is the interaction of Dvl with Akt. The activation of Dvl results in the activation of Akt and facilitates GSK3 phosphorylation by Akt (Fukumoto *et al*., 2001; Shin *et al*., 2006). Shin *et al*. (2006) showed that ectopic expression of Dvl induced Akt phosphorylation in U-87MG cells and inhibited H_2O_2-induced GSK3 dephosphorylation. Therefore, clozapine-induced Dvl activation can contribute to the increased phosphorylation of GSK3 via two separate mechanisms. However, as stated above, the findings from Kang *et al*. (2004) favored the mechanism independent of Akt.

Recent evidence regarding the functions of Dvl in the nervous system suggests that the Dvl-GSK3 pathway may play an important role in the therapeutic mechanisms of antipsychotics. Activated Dvl stabilizes the microtubules that are important for neural plasticity, neural remodeling, and vesicle transport (Ciani *et al*., 2004; Rosso *et al*., 2005; Ahmad-Annuar *et al*., 2006). Fan *et al*. (2004) reported that Dvl regulates microtubule stability through the inhibition of GSK3. Moreover, Alimohamad *et al*. (2005b) reported that subchronic treatment with haloperidol or clozapine increases the total amount of Dvl-3 and GSK3 in the rat brain.

Table 1. Comparison of clinical effects, receptor affinity profile, and signal transduction mechanisms between typical and atypical antipsychotic drugs. 0, None or not significantly different from placebo; +, mild; ++, moderate; +++, marked; D_2 receptor, dopamine D_2 subtype receptors; GSK3, glycogen synthase kinase 3; $5HT_2$ receptor, serotonin $5HT_2$ subtype receptors.

	Haloperidol	Clozapine
Clinical effects[ʃ]		
Therapeutic efficacy	++	+++
Extrapyramidal symptoms	+++	0
Receptor affinity profile[ʃ]		
D_2 receptor	+++	+
$5HT_2$ receptor	+	+++
Signal transduction pathway		
GSK3	Increase Ser9 and Ser21 phosphorylation	Increase Ser9 and Ser21 phosphorylation
Akt	Increase Ser473 phosphorylation transiently	Increase Ser473 phosphorylation
Dishevelled	No change	Increase phosphorylation and total amount

[ʃ]Adapted from Ananth *et al*. (2004).

Clinically, atypical antipsychotics can be distinguished from typical antipsychotics by their improved efficacy and reduced extrapyramidal side effects (Ananth *et al*., 2004). Different receptor affinity profiles between typical and atypical antipsychotics may account for the different effects on the signaling pathways and possibly lead to the different clinical effects (Table 1). We compared two antipsychotics to explore their different effects on the GSK3 signaling pathway according to their receptor binding profiles, although there is a limitation that haloperidol and clozapine are not receptor-specific drugs. The finding that GSK3 is inhibited by both haloperidol and clozapine supports the suggestion that GSK3 can be a common target of typical and atypical antipsychotics. Further studies regarding the different effects on the signals upstream of GSK3 between typical and atypical antipsychotics will clarify the individual molecular mechanisms contributing to the clinical efficacy and side effects of these drugs.

Acknowledgment

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