

Clozapine Modulates the Glycogen Synthase Kinase-3 Signaling partly via GABAB Receptors

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ABSTRACT

Schizophrenia is a severe and chronic mental illness and affects a large portion of the world population. Clozapine is currently used in the treatment of schizophrenia patients, but it causes serious side effects, such as extrapyramidal symptoms (EPS). Thus, understanding the details of signaling pathways in pathology of schizophrenia is critical for the identification of novel therapeutic targets to develop more effective and specific antipsychotic drugs with fewer side effects. Glycogen synthase kinase (GSK)-3 is a protein kinase that has been shown to involve in schizophrenia, and clozapine has been reported to increase phosphorylation of glycogen synthase kinase (GSK)-3 α/β (Ser21/9) in brain regions of rats related to schizophrenia. However, the pathways that mediate the effect of clozapine on GSK-3 were not clearly discovered. GABAB receptor has been reported to be associated with schizophrenia and activation of GABAB receptors may exert antipsychotic effects. Meanwhile, accumulated evidence suggests that the antipsychotic effect of clozapine may be partly mediated by modulating GABAB receptors. In addition, in our previous study, we have reported that GABAB receptors modulate GSK-3 signaling via the β -arrestin-dependent pathway. Activation of GABAB receptors will increase the phosphorylation of GSK-3 α/β (Ser21/9) via activation of Akt and this effect depends on the existence of β -arrestin. These data indicate that clozapine may modulate GSK-3 activity via GABAB receptors. Here we report that clozapine increases phosphorylation of GSK-3 α/β (Ser21/9) partly via GABAB receptors, which provides more specific target for treatment of schizophrenia, and avoidance of side effects of current antipsychotic drugs.

Keywords: Clozapine modulate, Glycogen synthase, GABAB receptors

INTRODUCTION

Schizophrenia, characterized by episodes of psychotic symptoms, is a severe and chronic mental illness affecting 0.5-1% of the world population, and it has been the 5th leading cause of disability in industrialized countries. Clozapine is currently used in treatment of schizophrenia patients; however, it causes serious side effects, including extrapyramidal symptoms (EPS), tardive dyskinesia, sexual dysfunctions, weight gain, and diabetes. Thus, understanding the details of signaling pathways in pathology of schizophrenia is critical for the identification of novel therapeutic targets to develop more effective and specific antipsychotic drugs with fewer side effects.

Glycogen synthase kinase (GSK)-3 is a protein kinase that has been shown to involve in schizophrenia, and decreased phosphorylation of GSK-3 α/β has been observed in post-mortem brain tissue of schizophrenia patients as well as animal models of schizophrenia [1-3]. Furthermore, inhibition of GSK-3 decreases hyperlocomotor activity in animal models of schizophrenia [4]. Clozapine, a currently used antipsychotic drug for the treatment of schizophrenia, has been reported to increase phosphorylation of glycogen synthase kinase (GSK)-3 α/β (Ser21/9) in brain regions of rats related to schizophrenia, such as the prefrontal cortex (PFC) and striatum, after repeated administration [5,6]. But the pathways that mediate the effect of clozapine on GSK-3 were not clearly discovered.

GABAergic neurotransmission has been more and more suggested as a potential target for the treatment of schizophrenia. The gene of human GABAB receptors locates in 6q21.3, a region associated with schizophrenia [7].

Human studies have shown reduced expression levels of GABAB receptors in brains of schizophrenia patients.

Furthermore, baclofen, a GABAB receptor agonist, have some efficacy in treatment of schizophrenia patients, and it was also shown to rescue [8,9].

Cognitive deficits in methamphetamine-induced animal model of schizophrenia [10] as well as rescuing prepulse inhibition of the startle response in a rat model of schizophrenia [11]. These data indicates that GABAB receptor is associated with schizophrenia and activation of GABAB receptors may exert antipsychotic effects.

Accumulated evidence suggests that the antipsychotic effect of clozapine may be partly mediated by modulating GABAB receptors. Using transcranial magnetic stimulation (TMS) indices of GABAergic neurotransmission, previous studies have shown lower inhibitory neurotransmission mediated by both GABAB receptors and GABAA receptors [12], while clozapine treatment significantly change the GABAB receptor-mediated inhibitory neurotransmission, but not GABAA receptor [13] suggesting that clozapine may potentiate the GABAB receptors during treatment of schizophrenia. In addition, in vivo studies reported that clozapine increased the binding affinity of GABAB receptor with its antagonist [3H]-CGP54626 [14], further highlighting GABAB receptor as a therapeutic target for clozapine.

In our previous study, we have found that GABAB receptors modulate GSK-3 signaling via the β -arrestin-dependent pathway. Activation of GABAB receptors will increase the phosphorylation of GSK-3 α/β (Ser21/9) via activation of Akt and this effect depends on the existence of β -arrestin [13]. Thus, we hypothesized that clozapine may modulate GSK-3 signaling via activation of GABAB receptors in the treatment of schizophrenia. Here we report that clozapine increases phosphorylation of GSK-3 α/β (Ser21/9) partly via activation of

GABAB receptors, which provide more specific target for treatment of schizophrenia, and avoidance of side effects of current antipsychotic drugs.

MATERIALS AND METHODS

cDNA constructs and DNA subcloning

Human GABAB receptor R1a and R2 cDNA were purchased from Origene. cDNA encoding full-length GABAB receptor R1a and R2 were amplified by PCR and subcloned into pcDNA3.1 vector. All constructs were sequenced to confirm appropriate splice fusion and absence of spurious PCR-generated nucleotide errors.

Cell culture and DNA transfection

HEK293 cells were maintained at 37°C in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Gibco). Cells were grown to 90% confluency before being transiently transfected with DNA constructs using Lipofectamine 2000 (Invitrogen), following the manufacturer's instruction. Cells were used for various experiments after 24-48 h of transfection

Drugs and treatments

CGP52432 (Tocris Bioscience) was dissolved in dimethyl sulfoxide (DMSO) to a stock solution of 10 mM. Clozapine (Selleck Chemicals) were dissolved in ethanol for a stock solution of 10 mM. Haloperidol and Ketanserin were purchased from Sigma-Aldrich, and both were dissolved in DMSO to a stock solution of 10 mM (Haloperidol) and 1 mM (Ketanserin).

Animals

Male C57BL/6 mice were obtained from Charles River Farms (St. Constant, QC, Canada), with each experiment performed on separate groups. The 12:12 h light–dark cycle was maintained with artificial light. All procedures were approved by the Centre for Addiction and Mental Health Animal Care Committee.

Protein extraction and western blot

Protein extraction and Western Blot analyses were performed as previously described [3,15]. Brain slices or primary cultured neurons were homogenized in ice-cold lysis buffer containing (in mM): 50 Tris-Cl, pH 7.4, 150 NaCl, 2 EDTA, 1 PMSF plus 1% Igepal CA-630, 0.5-1% sodium deoxycholate, 1% Triton X-100 and protease inhibitor

mixture (5 μ L/100 mg of tissue; Sigma-Aldrich, Okaville, ON, Canada) on ice and shaken at 4°C for 1 h. Striatal tissues dissolved in the lysis buffer was then centrifuged at 12,000 g for 10 min at 4°C to yield the total protein extract in the supernatant. Protein concentration was measured with BCA protein assay kit (Pierce Protein Biology, ON, Canada). Equal amounts of samples (50~100 μ g) were denatured and subjected to 10% SDS-PAGE and Western blot analyses.

The antibodies used include Anti-phospho-GSK-3 α/β (Ser21/9) (Cell Signaling Technology, rabbit); Anti-GSK-3 α (Cell Signaling Technology, rabbit); Anti- GSK-3 β (Cell Signaling Technology, rabbit).

Acute striatal slices

Acute striatal slices (350 μ m thick) were dissected and prepared from C57BL/6 mice with a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, United Kingdom) and remained in ice-cold artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1.25 mM KH₂PO₄, 26 mM NaHCO₃ and 20 mM glucose, which was bubbled continuously with 95% O₂/5% CO₂ for 5 min. Freshly cut slices were then placed in an incubating chamber with carbogenated aCSF and recovered from stress at 35°C for 1 h. They were treated with Haloperidol (10 μ M, 30 min)/Ketanserin (1 μ M, 30 min) with/without CGP52432 (10 μ M, 30 min) prior to treatment with clozapine (10 μ M, 30 min) and harvested for Western blot analysis.

Primary culture of striatal neurons

Striatal tissues were dissected from E15 embryonic mouse brains and digested with 0.25% trypsin-EDTA (Invitrogen) at 37°C for 15 min, followed by Neurobasal Medium (Gibco)+10% fetal bovine serum (Invitrogen) to inactivate trypsin activity, and centrifuged at 100 g for 5 min. The supernatant solution was replaced with Neurobasal/B27 (Invitrogen) medium supplemented with Glutamax (Invitrogen). The pellet was then mechanically dissociated by gentle trituration through a fire-polished Pasteur pipette. The cell suspension was plated at a density of $1 \times 10^5/\text{cm}^2$ onto poly-D-lysine (Sigma Aldrich) coated coverslips (Fisher Scientific) in 24-well culture plates, poly-D-lysine-coated 6-well culture plates or 60 mm culture dishes (Falcon). Cultures were incubated at 37°C in a 5% CO₂ incubator in Neurobasal/B27+Glutamax medium. Half of the medium was changed every 3 days until DIV 10. Cytarabine (Ara-C) (Sigma Aldrich) was added to the medium from DIV 4 onwards to prevent glial cell differentiation.

RESULTS

Clozapine increases phosphorylation of GSK-3 α/β (Ser 21/9) in HEK293 transfected with GABAB receptors

To determine whether clozapine is able to modulate GABAB receptor-mediated GSK-3 signaling, we test phosphorylation of GSK-3 α/β (Ser 21/9), an important phosphorylation site for GSK-3 activity, in HEK293 cells transfected with GABAB receptor and pretreated with clozapine (10 μ M, 30 min). As shown in Figure 1A and Figure 1B, clozapine increases phosphorylation of GSK-3 α/β (Ser 21/9) in HEK293 cells transfected with GABAB receptors. To further confirm this effect is mediated by GABAB receptor, we pretreated GABAB receptor-expressing HEK293 cells with CGP52432 (10 μ M, 30 min), a specific antagonist of GABAB receptors, prior to clozapine treatment. As shown in Figure 1C and Figure 1D, clozapine-induced increase of GSK-3 α/β (Ser 21/9) phosphorylation was blocked by pretreatment with CGP52432, indicating that clozapine increases GSK-3 α/β (Ser 21/9) phosphorylation by activating GABAB receptors.

To exclude the nonspecific effect of clozapine on HEK293 cells, we test phosphorylation of GSK-3 α/β (Ser 21/9) in HEK293 cells without any transfection. As shown in Figure 1E and Figure 1F, there is no effect on phosphorylation of GSK-3 α/β (Ser 21/9) with treatment of clozapine; further confirming that clozapine increases GSK-3 α/β (Ser 21/9) phosphorylation via activation of GABAB receptor.

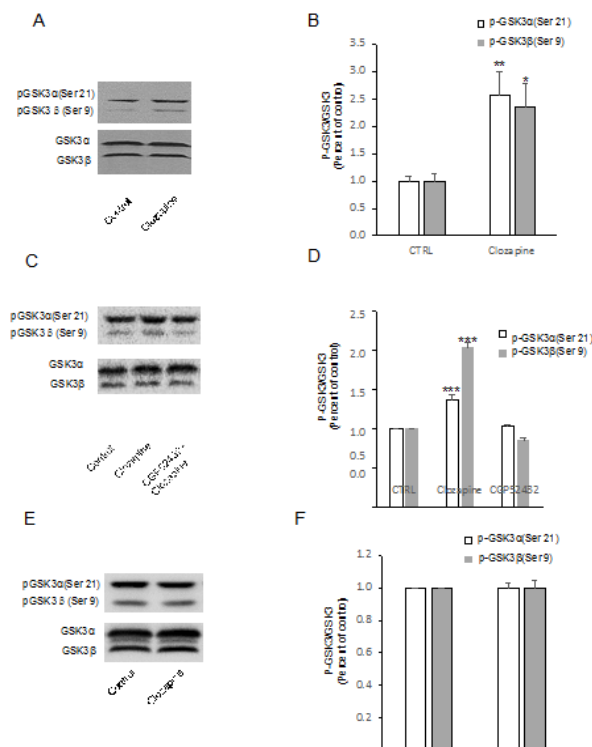


Figure 1: Clozapine increases phosphorylation of GSK-3α/β (Ser 21/9) in HEK293 transfected with GABAB receptors

(A. Clozapine treatment (10 μ M, 30 min) in HEK293 cells transfected with GABAB receptors increased the level of phosphorylation of GSK-3α/β (Ser 21/9), as compared to those in control group. Total GSK-3α/β was used as loading control

B. Densitometric analysis of phosphorylation of GSK-3α/β (Ser 21/9) in HEK293 cells transfected with GABAB receptors following treatment of clozapine. The levels of phosphorylation of GSK-3α/β (Ser 21/9) were normalized after dividing by the level of total GSK-3α/β. Results for each sample are presented as the percentage of the control sample (CTRL). * $p < 0.05$, ** $p < 0.01$, $n = 6$, t-test

C. GABAB receptor antagonist, CGP52432 (10 μ M, 30 min) pretreatment in HEK293 cells transfected with GABAB receptors blocked clozapine-induced increased level of phosphorylation of GSK-3α/β (Ser 21/9), as compared to those in control group. Total GSK-3α/β was used as loading control

D. Densitometric analysis of phosphorylation of GSK-3α/β (Ser 21/9) in HEK293 cells transfected with GABAB receptors following treatment of clozapine with/without pretreatment of CGP52432. The levels of phosphorylation of GSK-3α/β (Ser 21/9) were normalized after dividing by the level of total GSK-3α/β. Results for each sample are presented as the percentage of the control sample (CTRL). *** $p < 0.001$ as compared to control group, $n = 3$, one-way ANOVA analysis followed by Dunnett's test

E. Clozapine treatment (10 μ M, 30 min) in untransfected HEK293 cells did not change the level of phosphorylation of GSK-3α/β (Ser 21/9), as compared to those in control group. Total GSK-3α/β were used as loading control

F. Densitometric analysis of phosphorylation of GSK-3α/β (Ser 21/9) in HEK293 cells transfected with GABAB receptors following treatment of clozapine. The levels of phosphorylation of GSK-3α/β (Ser 21/9) were normalized after dividing by the level of total GSK-3α/β. Results for each sample are presented as the percentage of the control sample (CTRL). $n = 3$, t-test)

Clozapine increases GSK-3α/β (Ser 21/9) phosphorylation via activation of GABAB receptor in primary cultured neurons

As HEK293 cells are not neuronal cells, we further test if clozapine is able to increase GSK-3α/β (Ser 21/9) phosphorylation via activation of GABAB receptors in nervous system. Firstly, we detected GSK-3α/β (Ser 21/9)

phosphorylation in primarily cultured striatal neurons treated with clozapine (10 μ M, 30 min). As shown in Figure 2A-B, clozapine increases GSK-3 α/β (Ser 21/9) phosphorylation in primary cultured striatal neurons, consistent with the antipsychotic effect of clozapine by inhibiting GSK-3 activity. Furthermore, we tested if antagonism of GABAB receptors is able to block the effect of clozapine on GSK-3 α/β (Ser 21/9) phosphorylation in primary cultured neurons pretreated with CGP52432 (10 μ M, 30 min), the specific antagonist of GABAB receptors. As shown in Figure 2C and Figure 2D, clozapine-induced increased phosphorylation of GSK-3 α/β (Ser 21/9) was blocked with pretreatment of CGP52432, indicating the involvement of GABAB receptors in the effect of clozapine-induced inhibition of GSK-3 activity in nervous system.

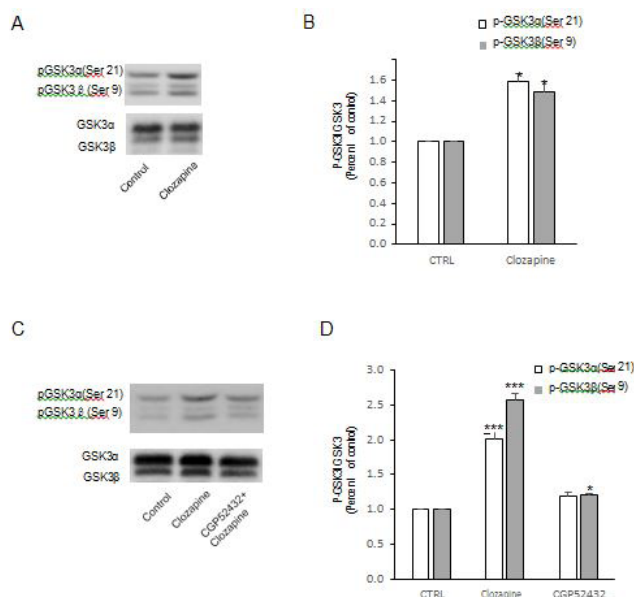


Figure 2: Clozapine increases GSK-3 α/β (Ser 21/9) phosphorylation via activation of GABAB receptor in primary cultured neurons

(A. Clozapine treatment (10 μ M, 30 min) in primary cultured neurons increased the level of phosphorylation of GSK-3 α/β (Ser 21/9), as compared to those in control group. Total GSK-3 α/β were used as loading control

B. Densitometric analysis of phosphorylation of GSK-3 α/β (Ser 21/9) in primary cultured neurons following treatment of clozapine. The levels of phosphorylation of GSK-3 α/β (Ser 21/9) were normalized after dividing by the level of total GSK-3 α/β . Results for each sample are presented as the percentage of the control sample (CTRL). * $p < 0.05$, $n = 3$, t-test

C. GABAB receptor antagonist, CGP52432 (10 μ M, 30 min) pretreatment in primary cultured neurons blocked clozapine-induced increased level of phosphorylation of GSK-3 α/β (Ser 21/9), as compared to those in control group. Total GSK-3 α/β was used as loading control

D. Densitometric analysis of phosphorylation of GSK-3 α/β (Ser 21/9) in primary cultured neurons following treatment of clozapine with/without pretreatment of CGP52432. The levels of phosphorylation of GSK-3 α/β (Ser 21/9) were normalized after dividing by the levels of total GSK-3 α/β . Results for each sample are presented as the percentage of the control sample (CTRL). * $p < 0.05$, *** $p < 0.001$ as compared to control group, $n = 3$, one-way ANOVA analysis followed by Dunnett's test)

Clozapine-induced increased GSK-3 α/β (Ser 21/9) phosphorylation is partly mediated by activation of GABAB receptor

To further investigate whether GABAB receptors are sufficient for the inhibiting effect of clozapine on GSK-3 activity, we tested GSK-3 α/β (Ser 21/9) phosphorylation in striatal slices pretreated with antagonists of dopamine D2 receptor (Haloperidol) and 5-HT_{2A} receptor (Ketanserin), as these two receptors have been reported as the main targets for clozapine. As shown in Figure 3A-B, clozapine still could increase GSK-3 phosphorylation despite the slices were pretreated with haloperidol (10 μ M, 30 min) and ketanserin (1 μ M, 30 min), indicating there are other mechanisms other than antagonism of D2R and 5-HT_{2A}R involved in the inhibiting effect of clozapine on GSK-3 activity. We further treated striatal slices with CGP52432 (10 μ M, 30 min) following antagonists of D2R and 5-HT_{2A}R, but prior

to clozapine treatment. As shown in Figure 3C-D, in the presence of antagonists of D2R and 5-HT_{2A}R, CGP52432 could further block clozapine-induced increased phosphorylation of GSK-3 α/β (Ser 21/9). These data suggest that the effect of clozapine on inhibition of GSK-3 activity is partly mediated by activation of GABAB receptors.

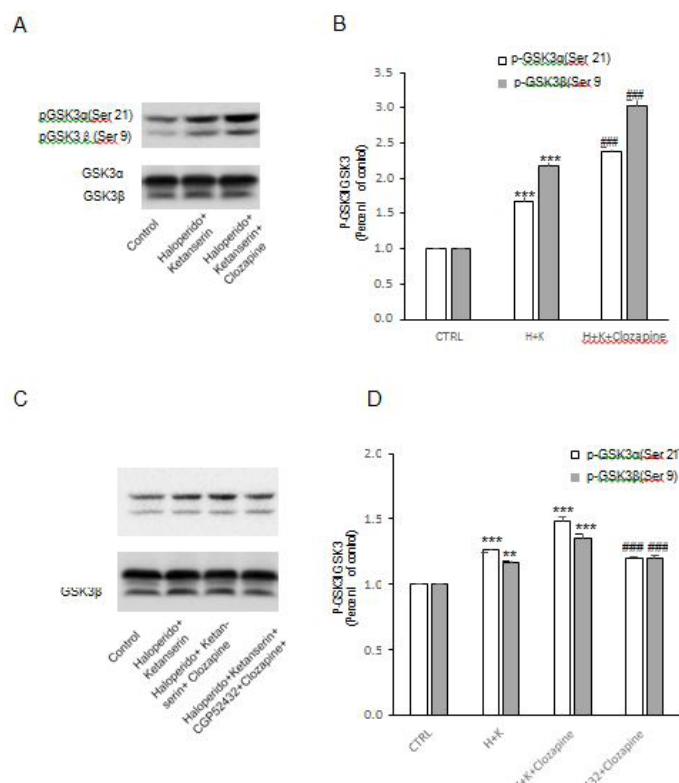


Figure 3: Clozapine-induced increased GSK-3 α/β (Ser 21/9) phosphorylation is partly mediated by GABAB receptor

(A. Clozapine treatment (10 μ M, 30 min) in striatal slices following pretreatment of haloperidol (10 μ M, 30 min) and ketanserin (1 μ M, 30 min) increased the level of phosphorylation of GSK-3 α/β (Ser 21/9), as compared to those only pretreated with haloperidol and ketanserin. Total GSK-3 α/β was used as loading control

B. Densitometric analysis of phosphorylation of GSK-3 α/β (Ser 21/9) in striatal slices following pretreatment of haloperidol and ketanserin in the presence or absence of clozapine. The levels of phosphorylation of GSK-3 α/β (Ser 21/9) were normalized after dividing by the level of total GSK-3 α/β . Results for each sample are presented as the percentage of those only pretreated with haloperidol and ketanserin (haloperidol+ketanserin). *** p <0.001 as compared to control group, ### p <0.001 as compared to H+K group, n =3, one-way ANOVA analysis followed by Bonferroni's test

C. GABAB receptor antagonist, CGP52432 (10 μ M, 30 min) pretreatment in striatal slices in the presence of haloperidol and ketanserin blocked clozapine-induced increased level of phosphorylation of GSK-3 α/β (Ser 21/9), as compared to those only pretreated with haloperidol and ketanserin (haloperidol+ketanserin). Total GSK-3 α/β was used as loading control

D. Densitometric analysis of phosphorylation of GSK-3 α/β (Ser 21/9) in striatal slices in the presence of haloperidol and ketanserin following treatment of clozapine with/without pretreatment of CGP52432. The levels of phosphorylation of GSK-3 α/β (Ser 21/9) were normalized after dividing by the levels of total GSK-3 α/β . Results for each sample are presented as the percentage of those only pretreated with haloperidol and ketanserin (haloperidol+ketanserin). *** p <0.001 as compared to control group, ### p <0.001 as compared to H+K+Clozapine group n =3, one-way ANOVA analysis followed by Bonferroni's test)

DISCUSSION

These results show that clozapine, a currently used antipsychotic drug in the treatment of schizophrenia, increases phosphorylation of GSK-3 partly via activation GABAB receptors.

These data is consistent with previous study showing that clozapine increases phosphorylation of GSK-3 during treatment of schizophrenia, and is an extension of our previous study determining that activation of GABAB receptors modulates Akt/GSK-3 signaling.

In addition, our finding provides a novel target for clozapine in the treatment of schizophrenia, besides dopamine D2 receptors and serotonin 2A receptors. Currently used antipsychotic drugs mostly targeted D2R and 5-HT2A receptors, but most of them cause severe side effects. In addition to D2R and 5-HT2AR, our finding supplies novel specific pathway for drug development and therapeutics of schizophrenia without inducing serious side effects.

CONFLICT OF INTEREST

The authors declare no conflict of financial interests.

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