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항 정신성 약물 클로자핀의 아르기닌/N-말단 경로를 매개하는 RGS4 의 다중 유비퀴틴화 및 프로테아좀에 의한 분해 억제 기전 규명

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전 준 형

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The antipsychotic drug clozapine suppresses the RGS4 polyubiquitylation and proteasomal degradation mediated by the Arg/N-degron pathway

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Abstract

The antipsychotic drug clozapine suppresses the RGS4 polyubiquitylation and proteasomal degradation mediated by the Arg/N-degron pathway

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Although diverse antipsychotic drugs have been developed for the treatment of schizophrenia, most of their mechanisms of action remain elusive. Regulator of G-protein signaling 4 (RGS4) has been reported to be linked, both genetically and functionally, with schizophrenia and is a physiological substrate of the arginylation branch of the N-degron pathway (Arg/N-degron pathway). Here, I show that the atypical antipsychotic drug clozapine significantly inhibits proteasomal degradation of RGS4 proteins without affecting their transcriptional expression. In addition, the

levels of Arg- and Phe-GFP (artificial substrates of the Arg/N-degron pathway) were significantly elevated by clozapine treatment. Accordingly, treatment with clozapine resulted in reduced polyubiquitylation of Arg-GFP and RGS4 in the test tube and in cultured cells. Clozapine attenuated the activation of downstream effectors of G protein–coupled receptor signaling, such as MEK1 and ERK1, in neuronal cells. Overall, these results reveal an additional therapeutic mechanism of action of clozapine: this drug post-translationally inhibits the degradation of Arg/N-degron substrates, including RGS4. These findings imply that modulation of protein post-translational modifications, in particular the Arg/N-degron pathway, may be a novel molecular therapeutic strategy against schizophrenia.

* The study is further being explored for publication.

Keywords: schizophrenia, clozapine, RGS4, ubiquitination, N-degron pathway, ubiquitin-proteasome system(UPS)

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List of abbreviations

ATP : Adenosine triphosphate

APD: Anti psychotic drug

BSA : Bovine serum albumin

CBB : Coomassie brilliant blue

CP : Core particle

Degron : Degradation signal

DMEM : Dulbecco's modified eagle medium

DMSO : Dimethyl sulfoxide

DTT : 1,4-dithiothreitol

EDTA : Ethylenediaminetetraacetic acid

ERK : Extracellular signal-regulated kinaseu

FBS : Fetal bovine serum

GFP: Green Fluorescent Protein

GPCR: G protein-coupled receptors

IB : Immunoblotting

kDa : Kilo Dalton

MAPK : Mitogen-activated protein kinase

MEK : MAPK/ERK kinase

PBS : Phosphate-buffered saline

PVDF : Polyvinylidene fluoride

RGS4 : Regulator of G protein signaling 4

RGS5 : Regulator of G protein signaling 5

RT-PCR : Real-time polymerase chain reaction

SDS : Sodium dodecyl sulfate

SDS-PAGE : Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Ub : Ubiquitin

UPS : Ubiquitin-proteasome-system

WCL : Whole cell lysate

Introduction

Schizophrenia is a common psychiatric disorder that affects more than 1% of the global population [1]. Symptoms of schizophrenia include delusions, hallucinations, and paranoia (positive symptoms); blunted affect, anhedonia, and apathy (negative symptoms); and attention deficits, visual processing aberrations, and memory deficits (cognitive symptoms) [2-5]. The etiology and pathogenesis of schizophrenia remain largely unknown, but genetic variants, environmental factors affecting neurodevelopmental processes, and impaired neurotransmitter pathways in dopaminergic, glutamatergic, and serotonergic neurons probably collectively constitute risk factors of schizophrenia [6-8]. Despite high heritability of this disease (~80% [9]), identifying schizophrenia-associated genes is difficult. Individual candidate variants show only small effects; combined effects of common and rare variants as well as epigenetic mechanisms are involved too [10-12]. Nevertheless, convergent evidence from genetic and functional studies has yielded several putative schizophrenia-associated genes, such as *NRG1*, *DTNBP1*, *DISC1*, *G72*, *DAAO*, *RGS4*, *COMT*, and *PRODH* [13-16]. Further studies on these plausible genes at the molecular level are necessary to understand the pathogenesis of schizophrenia and relevant therapeutic mechanisms of action.

RGS4 is a member of the small regulator of G protein signaling (RGS) family, which inactivates G protein subunits including G_i/G_o and G_q by accelerating GTP hydrolysis on the G_α subunit and reassociation of $G_{\alpha\beta\gamma}$ proteins [17-19]. RGS4 is abundantly expressed in the prefrontal cortex and is strategically positioned not

only for postsynaptic but also presynaptic signaling in response to GPCR activation [20] and takes part in prefrontal cognitive functions [21, 22]. A significant reduction in *RGS4* mRNA levels in the prefrontal cortex was initially discovered by Mirnics et al. in six matched pairs of patients with schizophrenia and unaffected controls [23]. Many subsequent studies support the role of *RGS4* as a major candidate gene for susceptibility to schizophrenia [14, 24-26], while some studies have failed to confirm the positive association [27-29]. On the other hand, postmortem studies have provided more consistent findings that RGS4 mRNA and protein levels are lower in prefrontal cortical areas: a major dysfunctional region in schizophrenia [30-36]].

It was previously reported that RGS4 proteins are degraded by the arginylation branch of the N-degron pathway (Arg/N-degron pathway) *in vitro* and *in vivo* [37]. The Arg/N-degron pathway is the first identified degradation mechanism underlying ubiquitin–proteasome system (UPS)-mediated proteolysis [38, 39]. The sequential modifications at the N-terminus of RGS4 act as a licensing step for ubiquitylation mediated by UBR proteins (N-recognin E3 ubiquitin [Ub] ligases) and for proteasomal degradation [40]. Mice lacking UBR proteins die with various defects in neurogenesis as midgestation embryos [41, 42]]. RGS4 is one of the molecular hubs orchestrating neurotransmitter receptors and intracellular signaling and has been suggested as an important modulator of antipsychotic drug (APD) action [43]; however, the association between effects of APDs and RGS4 remains unclear.

APDs mostly act by binding to neurotransmitter receptors, and one of the main targets is the dopamine D2 receptor (D2R) [44-46]. Clozapine is an atypical APD for patients with severe and refractory psychotic disorders. It has relatively low

binding affinity for D2R as compared to other APDs and has rather pleomorphic receptor pharmacology, with activity toward D1 and D4 dopaminergic, 5-HT_{2A} and 5-HT_{2C} serotonergic, α1 and α2 adrenergic, M1 and M4 muscarinic, and H1 histaminergic receptors [47, 48]. Beyond the action on the neurotransmitter receptors, APDs directly and/or indirectly modulate multiple intracellular signaling pathways in the brain, resulting in neuronal and behavioral changes [49, 50]. Also, it was previously reported that a downstream crosstalk between various intracellular signaling pathways and autophagy is also involved in clozapine's action [51, 52].

Here, I show that the APD clozapine inhibits the Arg/N-degron pathway, thus stabilizing RGS4 not only in the test tube but also in cultured mammalian cells. Consistent with the results of biochemical degradation assay, downstream activation of GPCR signaling attenuated by clozapine treatment in cultured neuronal cells. Collectively, these data constitute the first evidence that Arg/N-degron pathway–dependent degradation is inhibited by clozapine both *in vitro* and mammalian cells. Therefore, pharmacological modulation of RGS4 homeostasis and of subsequent changes in downstream GPCR signaling may be a novel and improved strategy for the treatment of schizophrenia and other psychotic disorders.

Material and Methods

Immunoblotting and co-immunoprecipitation

Whole cell extracts (WCEs) were prepared as following: cells were lysed in RIPA buffer (25 mM Tris-HCl [pH 8.0], 0.5% sodium deoxycholate, 150 mM NaCl, 0.1% NP-40, 0.1% SDS) containing protease inhibitors and centrifuged to remove insoluble matter. Protein concentration of the supernatant was measured using Coomassie protein assay reagent (Pierce). Supernatant fractions of an equal protein cell number, generally corresponding to 10-20 mg per lane, or 1/10 of the sample recovered from on well of a six-well plate, were separated by SDS-PAGE. After transfer onto PVDF membranes, they were analyzed by conventional western blot analysis. The primary antibodies and dilution factors used in this study included the following: anti-RGS4 (ABT17, 1:2000, Millipore), anti-RGS5 (HPA001821, 1:2000, Sigma), anti-His (MA1-21315, 1:2000,Thermo Fisher Scientific), anti-V5 (r96025, 1:2000, Thermo Fisher Scientific), anti-ubiquitin (sc-8107, 1:5000, Santa Cruz Biotechnology), anti-PSMD1 (sc-514809, 1:3000, Santa Cruz Biotechnology), anti-PSMC2 (sc-166972, 1:3000, Santa Cruz Biotechnology), anti-myc (ab9106, 1:3000, Abcam), anti-HA (11 867 423 001, 1:5000, Roche), anti-PSMA4 (BML-PW8115, 1:3000, Enzo Life Science), anti-GFP (E1T510, 1:3000, Enogene), anti-Flag (F7425, 1:3000, Sigma), anti-MEK1 (GTX102391, 1:1000, Genetex), anti-ERK1 (#9102, 1:2000, Cell signaling technology), phospho-MEK1/2 (sc-7995-R, 1:400, Santa Cruz Biotechnology), phospho-ERK1/2 (#4370, 1:1000, Cell signaling technology) and β-actin (A1978, 1:5000, Sigma). Secondary antibodies (horseradish peroxidase-

conjugated anti-mouse IgG (AP124P) and anti-rabbit IgG (AP132P) antibodies) were purchased from Millipore. For co-immunoprecipitation assay, HEK293 cells were transfected with 1.5-3ug of plasmids expressing HA-tagged Ub and/or His-tagged RGS4. WCEs were prepared in the co-IP buffer (25 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, and protease inhibitors) and then incubated with anti-His antibody for 1 h. After mixing with 30 μL of 50% slurry of protein G agarose (Santa Cruz Biotechnology) resin overnight, the beads were washed three times with the same buffer and the immunoprecipitated proteins were analyzed with SDS-PAGE/IB. The EasyBlot secondary antibodies (GTX221666 and GTX-221667; Genetex) were used for immunoprecipitation/immunoblotting experiments.

Cell cultures and transient expression

In this study, mammalian cells used including HEK293, HEK293-pre1-HTBH, SH-SY5Y cells were grown in DMEM with supplement of 10% FBS, 2 mM glutamine and 100 units/mL penicillin/streptomycin. Cells were sustained in humidified incubator with 5% CO_2 at 37°C. Cells were transfected with 1.5 – 3 μg of total plasmid DNA in 12-well or 6-well culture plate (> 95% confluent or at a density of 10^6 cells/well) for 24 h using Lipofectamine 3000 (Invitrogen) according to manufacturer's instruction.

Quantitative RT-PCR

Total RNA from cultured cells was prepared using TRIzol reagent (Invitrogen),

followed by additional purification through RNeasy mini-columns (Qiagen) with on-column DNase I treatment. cDNA samples were prepared by reverse transcription using Accupower RT-pre mix (Bioneer). Real-time PCR reactions were then conducted using StepOne Real-time PCR machine (ABI) with a diluted cDNA, SYBR Green qPCR master mixture (Enzynomics) and 10 pmol of gene-specific primers. Thermal cycling conditions comprised 95°C for 10 min for enzyme activation, 40 cycles at 95 °C for 15 sec, 60°C for 1min. After 40 cycles, conduct the other 95 °C for 15 sec, 60°C for 1 min and keep 95°C for 15 sec. Each mRNA level was normalized to that of GAPDH and quantified relative to DMSO (vehicle) groups. The values were plotted as means ± SD of triplicate experiments. Primer sequences were as follows: for human RGS4, forward (5'-ACATCGGCTAGGTTTCCTGC-3') and reverse (5'-GTTGTGGGAAGAATTGTGTTCAC-3'); for human RGS5, forward (5'-GACATGGCCCAGAAAAGAATTC-3') and reverse (5'-CACAAAGCGAGGCAGAGAATC-3'); for human GAPDH, forward 5'-CTGACTTCAACAGCGACACC-3') and reverse (5'-CTGACTTCAACAGCGACACC-3') primers.

Purification of the human 26S proteasome

Human proteasomes were purified by affinity chromatography from a HEK293 cell line stably expressing biotin-tagged human β4, as previously described [53]. Briefly, the cells were cultured in 15 cm dishes, harvested in lysis buffer (50 mM NaH_2PO_4 pH 7.5, 100 mM NaCl, 10% of glycerol, 5 mM $MgCl_2$, 0.5% of NP-40, 5 mM ATP, and 1 mM dithiothreitol [DTT]) containing a protease inhibitor cocktail and were

homogenized using a Dounce homogenizer. After centrifugation, supernatants were incubated with Biomag streptavidin beads (Bangslab) overnight at 4 °C. The beads were washed with lysis buffer and TEV buffer (50 mM Tris-HCl pH 7.5, 1 mM ATP, and 10% of glycerol). The 26S proteasome was eluted from the beads by incubation with TEV protease (Invitrogen) in TEV buffer for 1 h at 30 °C and was concentrated on an Amicon ultra-spin column (Millipore). Proteasomes were analyzed by SDS-PAGE in a 12% polyacrylamide gel followed by Coomassie Brilliant Blue (CBB) staining or immunoblotting.

Measurement of proteasome activity using fluorogenic peptide substrates

Hydrolysis of fluorogenic peptide substrates succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (suc-LLVY-AMC for a chymotrypsinlike-activity assay), Boc-Leu-Arg-Arg-AMC (Boc-LRR-AMC for a trypsinlike-activity assay), and Z-Leu-Leu-Glu-AMC (Z-LLE-AMC for a caspaselike-activity assay) was quantitated to determine proteolytic activity of proteasomes. Each proteasome activity assay involving a fluorogenic substrate was carried out with a purified proteasome or WCLs and 10 μM fluorogenic substrate (Enzo Life Sciences) in assay buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mg/mL BSA, 1 mM ATP, and 1 mM DTT). The activity in relative fluorogenic units was measured after a 60 min incubation at 30 °C. The WCLs were prepared in lysis buffer (100 mM NaCl, 50 mM NaH_2PO_4 pH 7.5, 10% of glycerol, 5 mM $MgCl_2$, 0.5% of NP40, 1 mM ATP, and 1 mM DTT) containing protease inhibitors and were homogenized by 15 strokes in a 1 mL syringe (needle: 26G × 1/2″) and centrifuged to remove insoluble matter. The proteasome

activity in the purified lysates and WCLs was measured by monitoring the fluorescence intensity of the liberated AMC in a black 96-well plate by means of a TECAN Infinite m200 fluorometer.

Nondenaturing gel electrophoresis

Native gel analysis of the purified proteasome was performed as described previously [54]. Briefly, proteins in samples were separated by native PAGE using NuPAGE 3-8% Tris-Acetate Protein Gels (Thermo Fisher Scientific) at 150 V for 3–4 h, followed by an in-gel suc-LLVY-AMC hydrolysis assay or were transferred to a PVDF membrane for immunoblot analysis with antibodies against proteasome subunits.

In vitro degradation assays of ubiquitylated recombinant Sic1

Polyubiquitylated Sic1 with a PY motif (Ub-sic1) was prepared as described elsewhere [53]. In brief, 20 nM Ub-sic1 was incubated with 5 nM purified human proteasome at room temperature for various periods in proteasome assay buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10% of glycerol, 2 mM ATP, 10 mM $MgCl_2$, and 1 mM DTT). The reaction was terminated by adding SDS-PAGE sample buffer and was analyzed by SDS-PAGE. Ub-sic1 degradation by the proteasome was determined by immunoblotting with an antibody against T7 (Novagen).

Ubiquitylation of recombinant Arg-GFP proteins *in vitro*

In a test tube, the ubiquitylation reaction mixture consisted of 450 nmol Arg-GFP,

70 nmol UBA1 (UBP Bio), 890 nmol HR6A (Boston Biochem), 3 μmol yeast UBR1, and 1.2 nmol Ub (Boston Biochem) in 50 μL of ubiquitylation buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, and 0.2 mM DTT). The ubiquitylation was performed at 37 °C for various periods.

Analysis of GPCR pathways

For the serum starvation assay of neuronal cells, neuroblastoma SH-SY5Y cells were pretreated with 25 μM clozapine for 16 h and then starved in the serum-free medium (containing clozapine) for 2, 4, or 6 h, and cell samples were harvested for immunoblotting to analyze the MAPK pathways.

Results

Clozapine delays degradation of RGS4 and RGS5 in mammalian cells

To investigate whether APDs affect RGS4 stability, I tested commonly prescribed atypical antipsychotic medications for schizophrenia: risperidone, olanzapine, and clozapine. First, I transiently overexpressed RGS4 and its subfamily member RGS5 [40, 55] in HEK293 cells and treated them with the APDs (10 μM for 24 h). A stable LacZ protein was cotransfected and used as a reference protein for changes in RGS4 levels. Although risperidone decreased steady-state levels of RGS4, and olanzapine only mildly affected them, clozapine significantly increased both RGS4 and RGS5 levels (Figure 1). Accordingly, RGS4 and RGS5 levels in HEK293 cells turned out to be gradually elevated by clozapine treatment in a dose-dependent manner (0, 10, and 25 μM; Figure 2)

On the basis of these results, I hypothesized that clozapine blocks the degradation of RGS4 and RGS5 as a novel pharmacological regulator of these proteins. To test this idea, I treated RGS4- or RGS5-overexpressing cells with cycloheximide to block *de novo* protein synthesis and monitored their levels by immunoblotting analysis. Consistent with the steady-state analysis, the half-life ($t_{1/2}$) of RGS4 in the cell significantly increased in the presence of clozapine ($t_{1/2}$ = 4.4 h with clozapine vs. 3.1 h with DMSO; Figure 3). RGS5 showed milder responses to clozapine and was mainly affected only after 6 h of treatment. The effect of clozapine on RGS protein stabilization was post-translational because mRNA levels of these proteins were either unchanged or reduced (Figure 4). These results strongly

indicated that clozapine potently inhibits posttranslational proteolysis of RGS proteins in the cell.

Clozapine blocked the Arg/N-degron pathway-dependent proteolysis

RGS4 is the *bona-fide* physiological substrate of the Arg/N-degron pathway. Under oxidative stress conditions, it becomes arginylated and ubiquitinated and then is degraded by proteasomes [40]. The penultimate Cys residue of wild-type RGS4 (RGS4-WT) is exposed after the cleavage of N-terminal Met and functions as a degradation signal or "N-degron" with several additional modifications by N-recognins (e.g., ATE1 and UBR1/UBR2 [55]). To determine whether the inhibitory effect of clozapine on RGS4 degradation is mediated by the Arg/N-degron pathway, I utilized Cys2Val-mutant RGS4 (RGS4-C2V), a long-lived version of RGS4 (control). I found that in contrast to RGS4-WT, steady-state levels of transiently overexpressed RGS4-C2V were virtually unaffected by clozapine treatment (Figure 5A). These results supported our principal conclusion that the decay of RGS4 is mainly regulated by UPS-mediated proteolysis through the Arg/N-degron pathway, which is potently inhibited by clozapine.

I further investigated the inhibitory actions of clozapine on the Arg/N-degron pathway by using artificial substrates. They contain N-terminal destabilizing residues, such as positively ("type 1") charged Arg or bulky hydrophobic ("type 2") Phe. Ub-X-GFP fusion proteins were expressed in HEK293 cells, and deubiquitylating enzymes cotranslationally cleaved the junction between Ub and X-GFP, yielding rapidly degrading Arg-GFP and Phe-GFP, in contrast to Met-GFP, a long-lived version of the model substrates (control; Figure 5B). After treatment with

clozapine, similarly to the results on RGS4-WT, the levels of Arg-GFP and Phe-GFP, but not Met-GFP, significantly increased largely in a dose-dependent manner (Figure 5B). Under the same conditions, however, *GFP* mRNA levels were comparable (Figure 5C), indicating that the attenuation of Arg- and Phe-GFP degradation during clozapine treatment occurred at the post-translational stage.

Clozapine does not affect levels, integrity, or activity of cellular proteasomes

A possible explanation for the clozapine-driven delay of degradation of RGS and X-GFP proteins is that this APD directly or indirectly antagonizes proteasome activity. To investigate the influence of clozapine on proteasome structure and activity, I isolated the 26S proteasome holoenzyme from HEK293-derived cells stably expressing PSMB2-biotin. After treatment with clozapine (25 μM for 24 h), human proteasomes were affinity-purified on streptavidin magnetic beads and compared with normal 26S proteasomes. I found that the overall abundance of proteasome subunits, both from the core particle (CP, also known as 20S) and regulatory particle (RP/19S), was virtually identical between the proteasomes regardless of clozapine treatment (Figure 6).

To assess the possibility that clozapine enhances proteasome activity directly or allosterically, I examined proteasome activity using suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (suc-LLVY-AMC), a fluorogenic reporter substrate for the chymotrypsinlike activity of the 26S proteasome. The fluorescence intensity resulting from suc-LLVY-AMC hydrolysis gradually increased over time as the

human proteasome was incubated with the substrates but did not change with clozapine treatment (Figure 7A). Similarly, trypsinlike PSMB7 and caspaselike PSMB6 activities were unaffected by clozapine (Figure 7B). The structural integrity of the clozapine-treated 26S proteasome was analyzed by nondenaturing (native) polyacrylamide gel electrophoresis (PAGE) and by subsequent in-gel activity analysis with the suc-LLVY-AMC. Purified human proteasomes from HEK293 cells treated with DMSO or clozapine showed comparable integrity of the complex (as evidenced by gel migration patterns of CP [20S], singly capped CP-RP [26S], and doubly capped RP-CP-RP [30S]) and substrate hydrolysis activity (judging by fluorescence intensities; Figure 8).

Furthermore, I examined degradation rates of a more physiologically relevant substrate, the polyubiquitinated Sic1 (Ub-sic1) protein [56], instead of the fluorogenic reporter peptides. The 26S proteasome purified after treatment with clozapine showed virtually no difference from the untreated proteasome in the degradation of Ub-sic1 (Figure 9). Collectively, these data indicated that the scenario where clozapine affects proteasome integrity, level, or activity is not likely and that clozapine probably targets the proteostasis of Arg/N-end rule substrates more specifically.

Clozapine inhibits poly-ubiquitination of Arg/N-degron substrate and decade of ubiquitination dependent substrate of proteasome

The results described above mean that the stabilization of RGS proteins by clozapine is mediated by Arg/N-degron pathway–dependent proteasomal degradation. To

examine the impact of clozapine on substrates ubiquitylation, I carried out the complete *in vitro* reconstitution in the presence of clozapine and found that clozapine effectively inhibited the time-dependent polyubiquitylation of Arg-GFP (Figure 10). In the presence of clozapine, Arg-GFP retained a much lower number of Ub moieties than poly-Ub chains under normal conditions. These results suggested that the inhibitory effect of clozapine on the Arg/N-degron pathway may be direct and specific to UBR N-recognins rather than mediated by the auxiliary components of the pathway. To further characterize the direct inhibitory influence of clozapine on polyubiquitylation, we transiently overexpressed RGS4-His and HA-Ub in HEK293 cells and treated the cells with 25 μM clozapine for 24 h. When we enriched overexpressed RGS4 from whole-cell lysates (WCLs) via immunoprecipitation with an anti-His antibody, we observed that clozapine significantly reduced the extent of RGS4 polyubiquitination (Figure 11). This result probably accounts for the delayed degradation of RGS4 (Figures 1, 2 and 3) in the cell. Future work on obtaining cocrystal structures of UBR proteins with clozapine will enable structure-guided optimization of Arg/N-end rule inhibitors.

Clozapine delays the downstream activation of GPCR signaling pathway in neuronal cells

RGS proteins, whose levels are controlled by the Arg/N-degron pathway, are GTPase-activating enzymes that negatively regulate G_q-mediated downstream signaling [57]. RGS4 is expected to activate GTP-G_α hydrolysis and induces reassociation of $G_{\alpha\beta\gamma}$ proteins to end G_q signaling pathways. I examined the impact

of clozapine on the downstream effectors of the G_q signaling pathway in SH-SY5Y neuroblastoma cells. In line with the physiological role of RGS4 in G_q protein signaling, time-course immunoblotting of SH-SY5Y cell lysates revealed that the phosphorylation of MEK1/2 and ERK1/2 were significantly attenuated by clozapine treatment during serum starvation (Figure12). These results implied that the regulatory mechanism of RGS4 proteostasis and of the Arg/N-degron pathway is an important mediator of atypical APDs' action on GPCR signaling, which is closely linked to their therapeutic action.

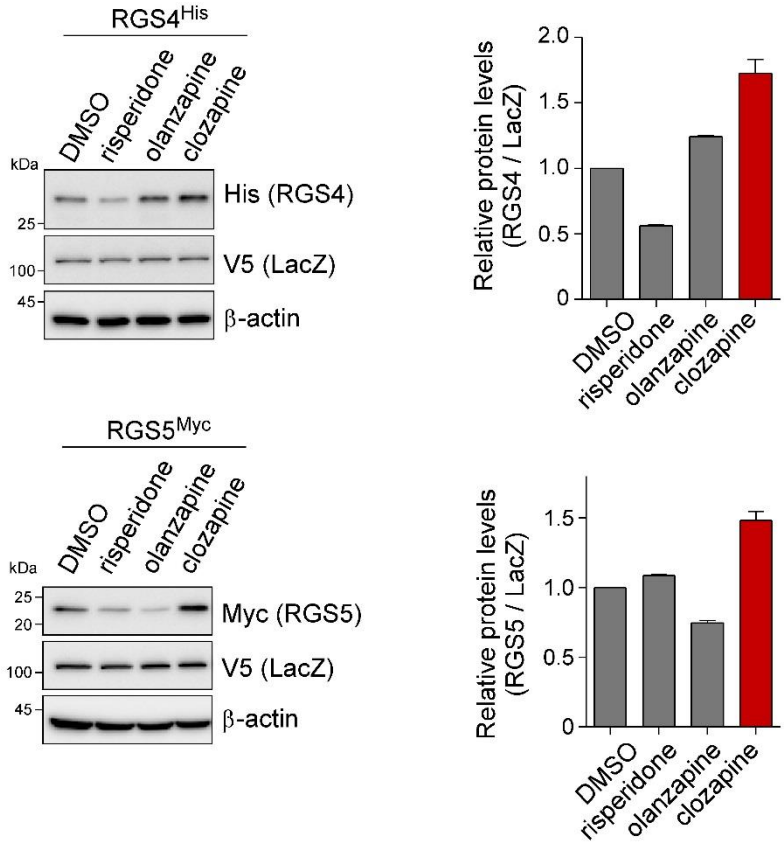

Figure 1. Effects of prescribed anti-psychotic drugs on RGS proteins in cultured cells.

Changes of steady-state levels of RGS proteins. Plasmids pcDNA3.1-RGS4^{His} (*top panels*) and RGS5^{myc} (*bottom*) were transiently transfected into HEK293 cells (for overexpression of respective proteins) along with plasmid pcDNA3.1-LacZ^{V5} as a transfection efficiency control. At 24 h post-transfection, risperidone, olanzapine, or clozapine (each 10 μM) were incubated with the cells for additional 24 h. (*left*) Whole-cell lysates (WCLs) were analyzed by SDS-PAGE/immunoblotting (IB) with the indicated antibodies. (*right*) Quantitation of IB signals of RGS proteins, normalized to those of LacZ.

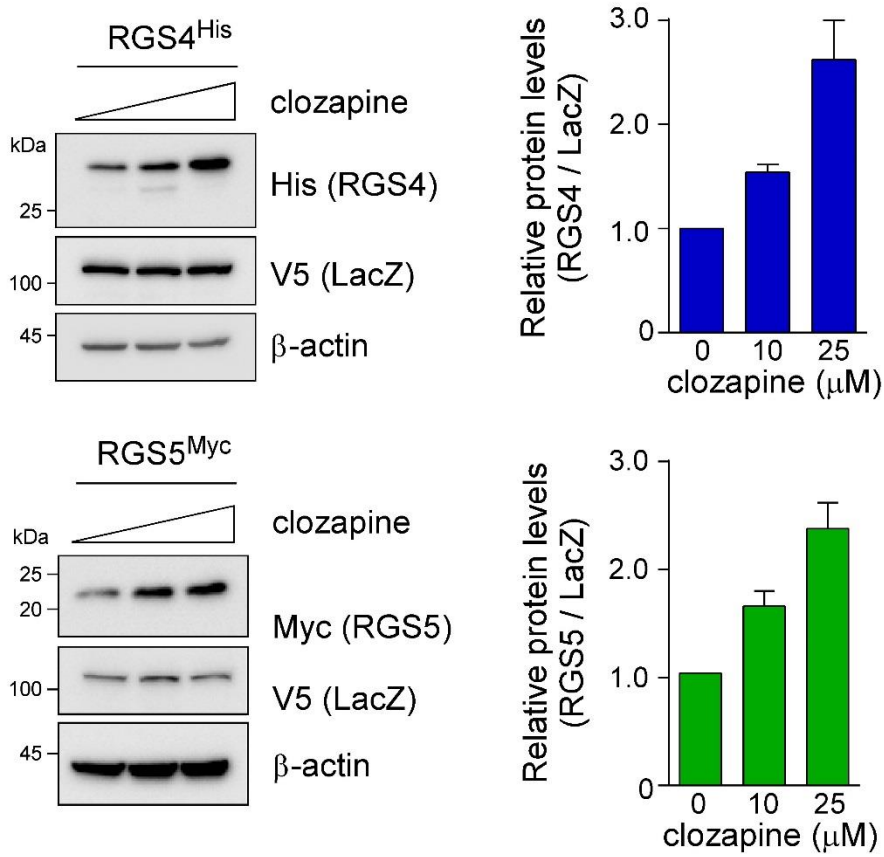

Figure 2. RGS4 and RGS5 were elevated in various concentrations of clozapine.

Similar to Figure1, different concentrations of clozapine (0, 10, or 25 μM for 24h) were added to examine their effect on steady-state levels of RGS4 and RGS5. (*left*) Whole-cell lysates (WCLs) were analyzed by SDS-PAGE/immunoblotting (IB) with the indicated antibodies. (*right*) Quantitation of IB signals of RGS proteins, normalized to those of LacZ.

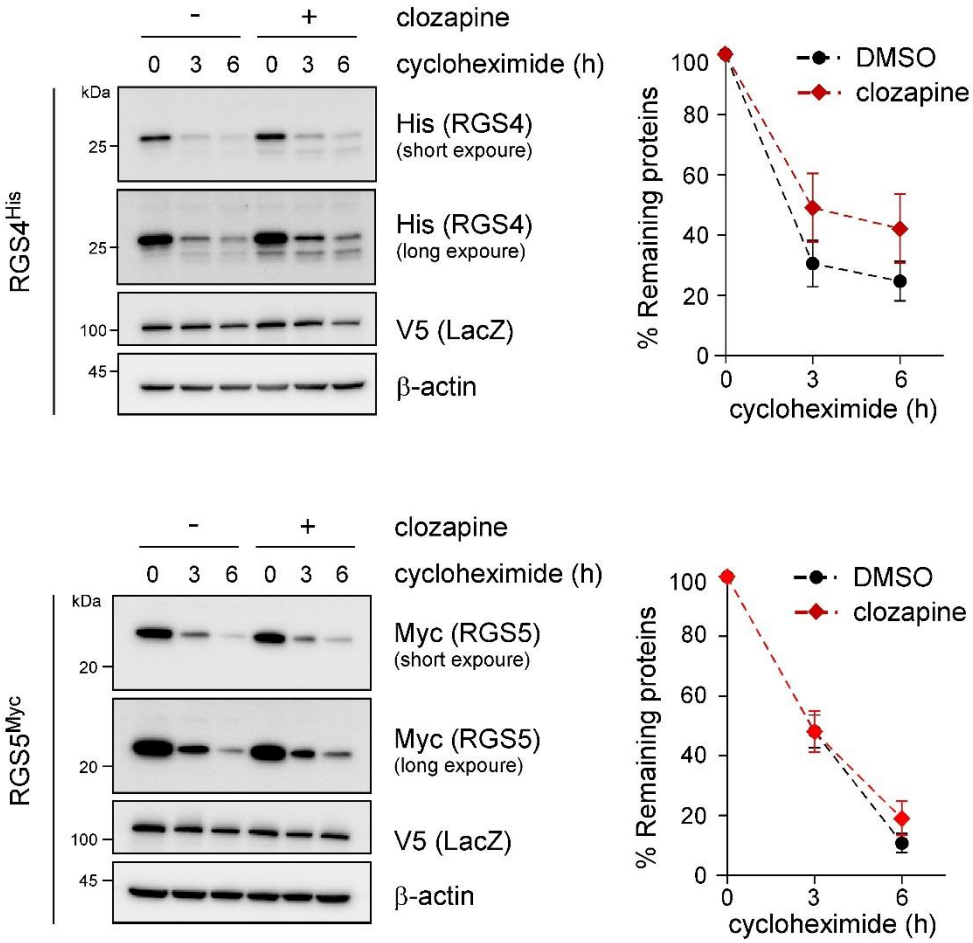

Figure 3. Clozapine delays degradation of RGS proteins in cycloheximide chase.

Cycloheximide chase analysis of the influence of clozapine on degradation of RGS4 (*top*) and RGS5 (*bottom*). HEK293 cells were cotransfected with LacZ and either RGS4 or RGS5 for 24 h. The cells were then treated with 80 μg/mL cycloheximide and either 50 μM clozapine or DMSO for 3 or 6 h. The decay of RGS4 and RGS5 was quantified by IB with normalization to LacZ levels. All the values represent means ± SD of three independent experiments.

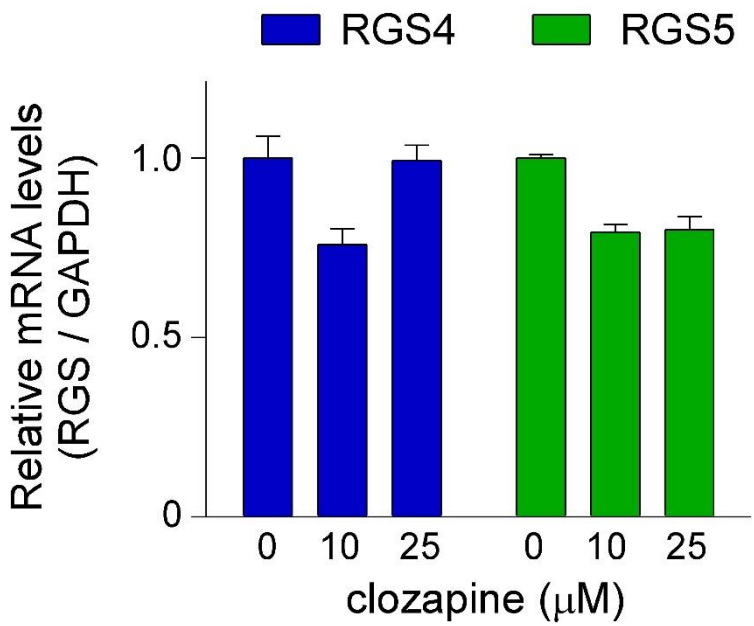

Figure 4. Transcriptional levels of RGS proteins in clozapine treated mammalian cells.

Post-translational modulation of RGS protein levels by clozapine. Total RNA was isolated from HEK293 cells after the treatment with clozapine (10 or 25 μM for 24 h) and used for quantitative RT-PCR of *RGS4* and *RGS5* transcripts with normalization to *GAPDH*. The values plotted are means ± SD from three independent experiments.

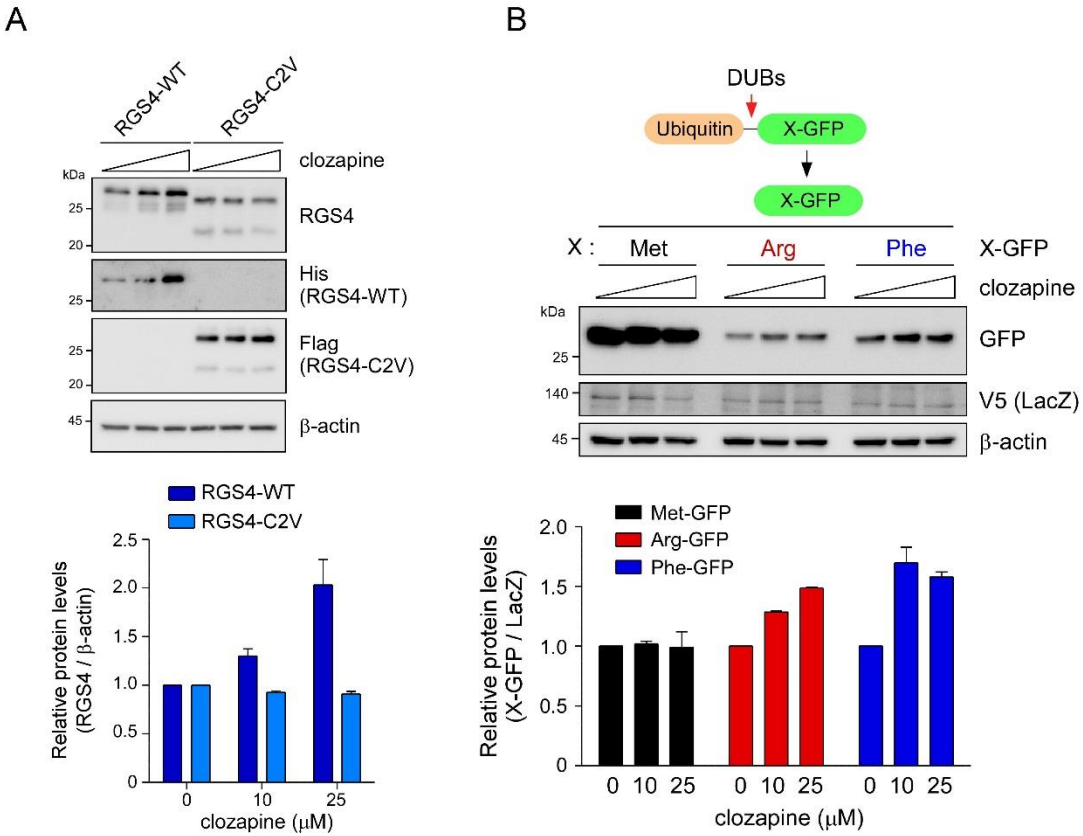
A
RGS4-WT
RGS4-C2V
clozapine
kDa
25
20
RGS4
His
(RGS4-WT)
Flag
(RGS4-C2V)
45
β-actin
RGS4-WT
RGS4-C2V
Relative protein levels
(RGS4 / β-actin)
2.5
2.0
1.5
1.0
0.5
0
0
10
25
clozapine (μM)
B
DUBs
Ubiquitin
X-GFP
X-GFP
X :
Met
Arg
Phe
X-GFP
clozapine
kDa
25
GFP
140
V5 (LacZ)
45
β-actin
Met-GFP
Arg-GFP
Phe-GFP
Relative protein levels
(X-GFP / LacZ)
2.0
1.5
1.0
0.5
0
0 10 25 0 10 25 0 10 25
clozapine (μM)
C
X-GFP
Arg
Met
X-GFP
- + - +
clozapine
RT-PCR
GFP
GAPDH
Met-GFP
Arg-GFP
Relative mRNA levels
(X-GFP / GAPDH)
1.5
1.0
0.5
0
0 25 0 25
clozapine (μM)

Figure 5. Inhibitory effects of clozapine on degradation of model N-degron substrates.

(A) The N-terminus–dependent regulatory effect of clozapine on RGS protein degradation. (*top*) RGS proteins, either wild-type (WT) with N-terminal Met-Cys residues or a Cys2>Val (C2V) mutant, were overexpressed in HEK293 cells in the presence of clozapine (25 μM for 12 h). (*bottom*) Quantification of RGS4-WT and RGS4-C2V. RGS4 IB signals were normalized to those of endogenous β-actin. Quantification was conducted by ImageJ densitometric analysis. (B) Artificial Arg/N-degron substrates, X-GFP, were generated from ubiquitin (Ub)-X-GFP fusion constructs (X represents a variable amino acid residue). When cotransfected into HEK293 cells with LacZ-V5 control constructs, cotranslational cleavage by deubiquitylating enzymes at the Ub-X junction yielded either short-lived substrate Arg-GFP (type 1) or short-lived substrate Phe-GFP (type 2). Met-GFP is a long-lived (stable) control protein. (*top*) At 24 h post-transfection, clozapine (0, 10, or 25 μM) was added to the cells for further 24 h incubation, and then WCLs were prepared and subjected to SDS-PAGE/IB. (*bottom*) Relative amounts of remaining X-GFP proteins were quantified in the ImageJ software, and the data were normalized to the levels of LacZ.

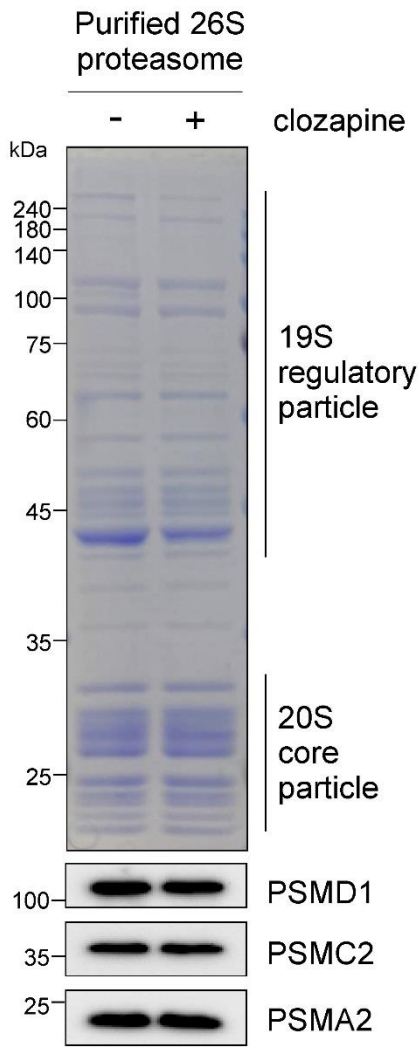

Figure 6. Comparison of assembly or levels of purified proteasomes with and without clozapine treatment.

Comparison of purified proteasomes with and without clozapine treatment. Affinity-purified 26S human proteasomes were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining or IB using various antibodies against 19S (PSMD1 and PSMC2) or 20S subunits (PSMA4).

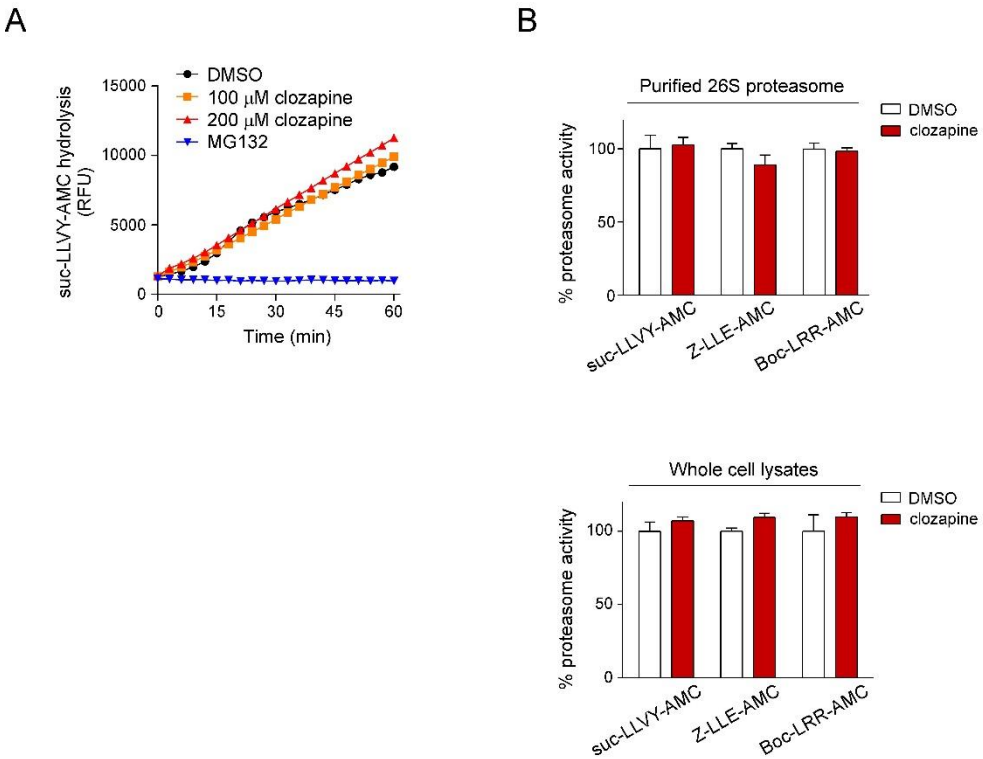

Figure 7. Measurement of catalytic activity of cellular proteasomes using fluorogenic substrates

(A) Purified proteasome (0.5 nM) activity was kinetically monitored by hydrolysis of the fluorogenic substrate suc-LLVY-AMC (10 μM) in the presence of 100 μM or 200 μM clozapine. MG132 was used as a control for baseline proteasome activity. RFU: relative fluorescence unit. **(B)** The three different catalytic activities of purified proteasomes (*upper panel*) and whole cell lysates (*lower*) after DMSO or clozapine treatment were measured by using fluorogenic substrates suc-LLVY-AMC (for chymotrypsin-like activity), Z-LLE-AMC (for caspase-like activity), and Boc-LRR-AMC (for trypsin-like activity). The substrate hydrolysis was stopped after 60

min of reactions and the proteasome activities were compared. Hydrolysis values normalized to vehicles and the error bars represent means ± SD (n=3).

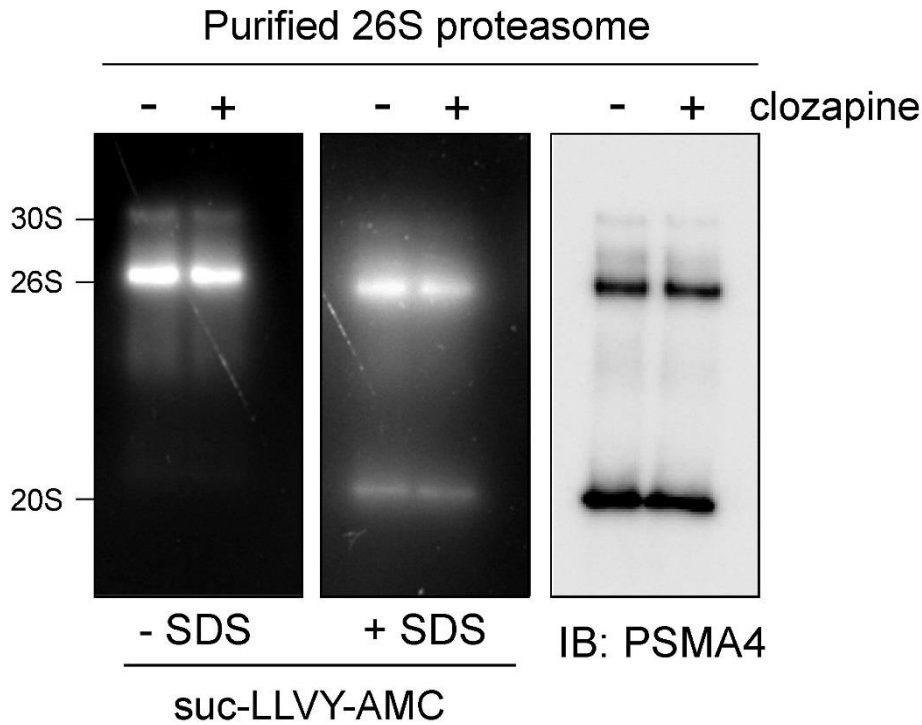

Figure 8. Native PAGE and in gel activity assays with immunoblotting.

The structure and activity of purified proteasome from untreated or clozapine (25 μM, 24 h)-treated HEK293 cells were determined by nondenaturing (native) PAGE, followed with suc-LLVY-AMC in-gel hydrolysis and IB analysis with anti-PSMA4 antibody. The addition of sodium dodecyl sulfate (SDS) opens the substrate-translocating gate of the CP and subsequently activates the CP [58]. This, together with Figure 6 and Figure 7, indicates that there is essentially no change in proteasome integrity and activity upon clozapine treatment.

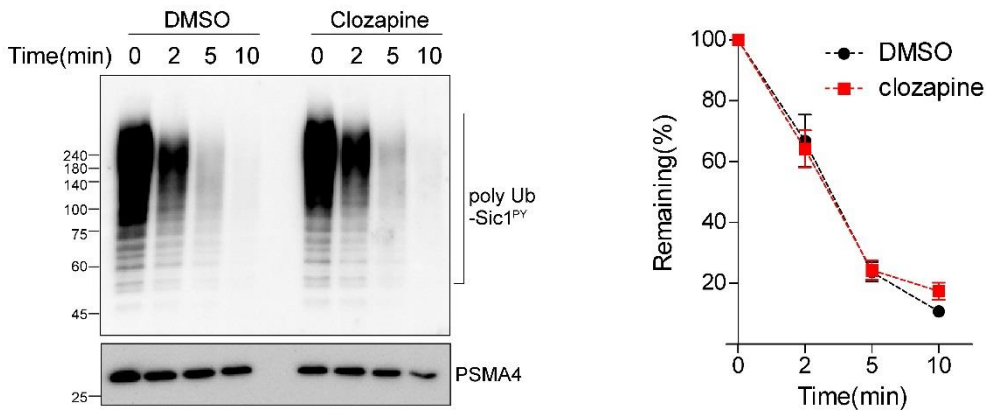

Figure 9. Time dependent *in vitro* degradation of Ub-Sic1 by purified proteasomes.

Time-dependent *in vitro* proteolysis of Ub-Sic1 by purified proteasomes treated with clozapine, No significant change in the abundance of Ub-sic1 was detected by immunoblotting analysis (*left*) and quantification of Ub-sic1 levels (*right*). Values normalized to the starting levels of Ub-sic1 present with the error bars (SD, n=3).

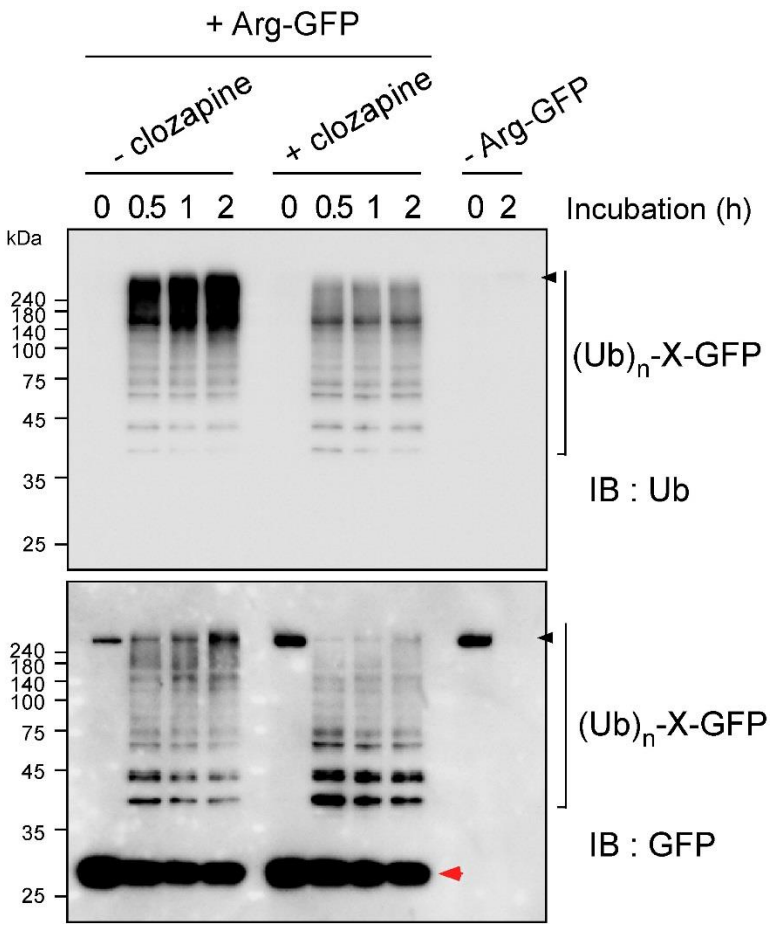

Figure 10. Inhibitory effects of clozapine on *in vitro* poly-ubiquitylation of Arg-GFP

In vitro ubiquitylation of Arg-GFP was performed in the presence or absence of clozapine for 0.5, 1, or 2 h. The red arrow indicates monomeric forms of Arg-GFP and black arrow indicates nonspecific bands.

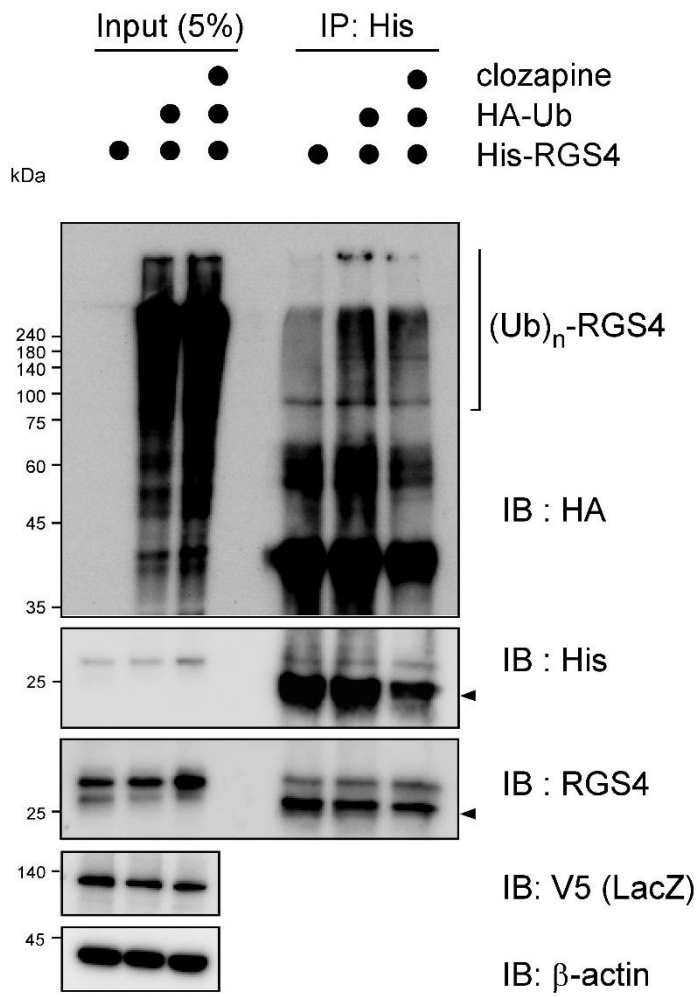

Figure 11. Inhibited poly-ubiquitylation of RGS4 by clozapine in HEK293 cells

Inhibition of RGS4 polyubiquitylation by clozapine in HEK293 cells. HA-tagged Ub and/or His-tagged RGS4 were cotransfected into HEK293 cells for 24 h with $LacZ^{V5}$ as a transfection control. After treatment with clozapine (25 μM) for 24 h, WCLs were prepared and subjected to immunoprecipitation with the anti-His antibody. Poly-Ub chains of RGS4 were monitored by IB using anti-HA and anti-His antibodies. The black arrow indicates IgG light chain.

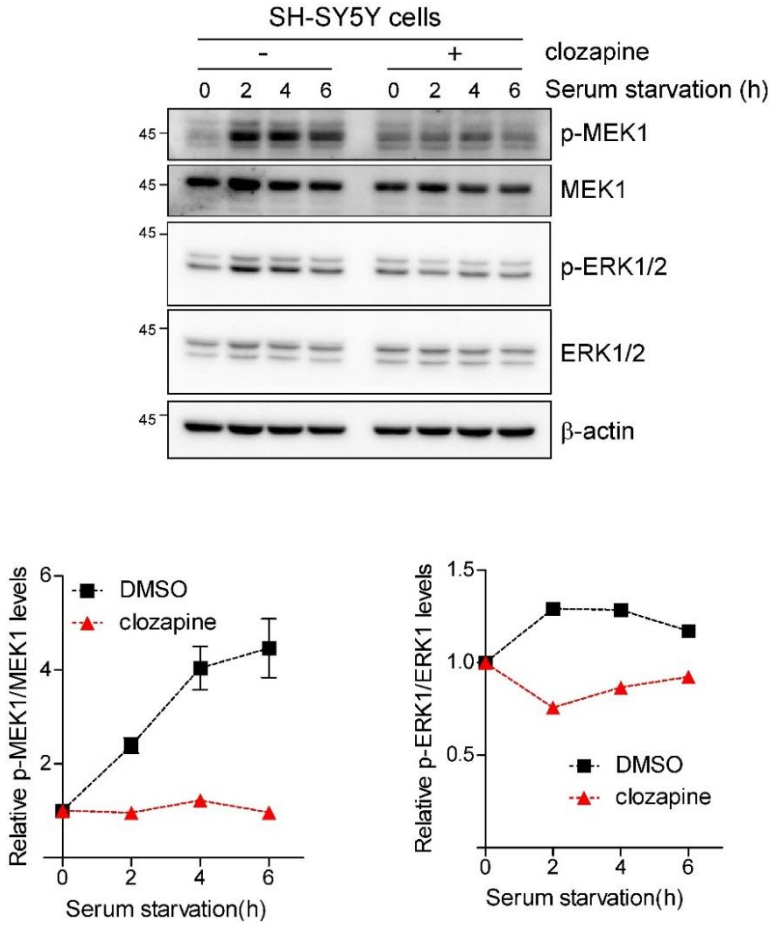

Figure 12. Clozapine delays the downstream activation of GPCR signaling pathway in neuroblastoma cells.

After treatment of 25 μM clozapine, neuroblastoma SH-SY5Y cells were incubated with a serum-free medium (stravation) for the indicated periods. WCLs were collected at the indicated time points and analyzed by SDS-PAGE/IB (*top pannel*). Using multiple IB images, levels of phospho-MEK1 (*left bottom*) and phospho-ERK1/2 (*right bottom*) were quantified with normalization to total MEK1 and ERK1/2, respectively. Data represent mean ± SD (n = 3)

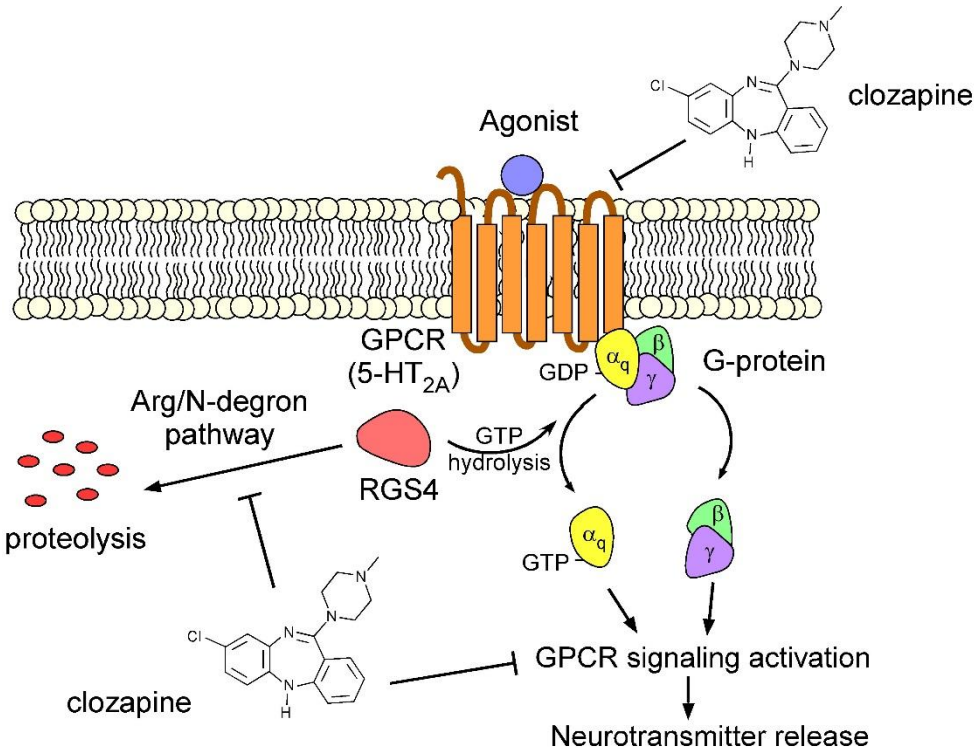

Figure 13. Proposed model of clozapine mechanism in neuronal cells.

A proposed model for the consequences of clozapine treatment. Under normal conditions, RGS4 levels in the brain are maintained below the critical concentrations in neuronal cells by the Arg/N-degron pathway. In contrast, when clozapine inhibits N-recognins, the RGS4 protein is stabilized and subsequently attenuates downstream G_q signaling pathways mediated by GPCRs. Although pharmacological significance of this phenomenon is yet to be determined, it can be hypothesized that dysregulation of the Arg/N-degron pathway is associated with the abnormal intraneuronal signaling in schizophrenia.

Discussion

Here I report that the atypical APD clozapine is capable of inhibiting the Arg/N-degron pathway. Clozapine potently and specifically stabilized the physiological Arg/N-degron substrate RGS4, and this phenomenon was found to be linked to the attenuation of downstream GPCR signaling in cultured neuronal cells. Clozapine increased the RGS4 protein amount without affecting its mRNA expression in the mammalian cells. *In vitro* ubiquitination and immunoprecipation results indicated the clozapine directly inhibits the poly-ubiquitination of Arg/N-degron substrates, which suggest potential inhibitory mechanism of clozapine. Genetic linkage, association, and postmortem brain studies have revealed that RGS4 is a strong candidate gene of schizophrenia susceptibility [59]. These data collectively suggest that clozapine may alleviate the genetic or functional defect of RGS4 in schizophrenia through post-translational protein stabilization (Figure 13). Thus, this study points to a novel mechanism of action of clozapine having superior pharmacological efficacy, possibly through modulating the Arg/N-degron pathway.

This pathway was the first identified UPS-mediated proteolytic mechanism that degrades proteins bearing N-terminal destabilizing residues or N-degrons [38, 39]. It is noteworthy that the Arg/N-end rule pathway evolutionarily developed prior to the UPS and is present even in prokaryotes, which lack Ub [60, 61]. Dysfunctions of protein homeostasis and of related intraneuronal signaling in schizophrenia have also been repeatedly reported [62], pointing to the UPS as a molecular target of APDs [62, 63]. Upregulation of polyubiquitinated proteins in erythrocytes, in postmortem

orbitofrontal cortex tissue [64], and in the superior temporal gyrus [65] of individuals with schizophrenia has been reported. Nonetheless, the underlying molecular mechanism is unclear, and further evidence is still needed. These data suggest that clozapine specifically blocks the polyubiquitylation of RGS4 instead of globally altering the proteome. In addition, considering that clozapine stabilizes Arg-GFP and Phe-GFP (artificial model substrates of Arg/N-degron pathway), it is expected that global Arg/N-degron substrates also stabilized by clozapine. Thus, Arg/N degron specific E3 ligases (N-recognins) or arginyl transferase-1(ATE1) are possibly targeted by clozapine directly or indirectly, but it will be need to further elucidated. The present findings provide a rationale for analyzing the ubiquitination of Arg/N-degrons in schizophrenia, as a novel therapeutic target in psychosis.

Availability of chemical structures contributes to the therapeutic profiles of APDs [66]. Clozapine is a synthetic dibenzodiazepine derivative, and its bulky planar structure is believed to contribute to its characteristic receptor-binding profile, which underlies the atypical features of this APD [67]. On the other hand, the action of APDs is not necessarily limited to their characteristics of binding to neurotransmitter receptors: direct intracellular action of APDs (and their metabolites as well) along with plasma membrane penetration may be a part of the mechanisms of action of APDs [68-70]. By stabilizing RGS4, and subsequently attenuating GPCR signaling, clozapine may compensate for the dysfunctional neurotransmission dynamics in psychosis. It is also notable that a sympathomimetic amine, *para*-chloroamphetamine, which functions as a serotonin releaser and was originally developed as an antidepressant [71], is another small-molecule inhibitor of the Arg/N-degron pathway with weaker binding affinity [72]. Further research on CNS

drugs should provide novel compounds acting on the Arg/N-degron pathway in the brain.

Exposure to APDs, including haloperidol and olanzapine, does not change the *RGS4* mRNA level in the monkey frontal cortex [23, 31], but in the postmortem prefrontal cortex of patients with schizophrenia, immunoreactivity of the RGS4 protein has been found to be significantly elevated in the APD-treated subgroup [33]. Likewise, here I report that clozapine stabilizes the RGS4 protein without affecting its mRNA levels *in vitro*. RGS4 is a key regulator of GPCR signaling via negative regulation of G_q subunits of neurotransmitter receptors [43]. Therefore, the stabilization of RGS4 by clozapine can have diverse downstream effects and related clinical manifestations. In addition to dopaminergic and serotonergic receptor signaling [73, 74], RGS4 is deeply involved in glutamatergic-neurotransmission regulation [74]. Recent studies show interactions of RGS4 with metabotropic glutamate receptor 1α (mGluR1α) and mGluR5 signaling in the prefrontal cortex of individuals with schizophrenia [31, 75]. These observations suggest a role of RGS4 not only in positive and negative symptoms but also in the cognitive dysfunction in schizophrenia. Stabilization of the RGS4 protein by clozapine may contribute to the superior efficacy of clozapine in a wide array of symptom domains, including positive, negative, depressive, and cognitive [76-78].

Accumulating evidence indicates that the UPS and related molecular mechanisms have critical roles in neuronal signaling, neural development, survival and death of neural cells, neurogenesis, and neuroprotection by determining functions and structures of the brain [79]. The Arg/N-degron pathway also participates in various neural functions [41, 80-82], yet to be explored further. In this

study, clozapine was demonstrated to stabilize RGS4 through inhibition of the Arg/N-degron pathway. The changes in RGS4 homeostasis by clozapine can have diverse downstream signaling consequences and relevant clinical manifestations, which may be associated with the superior and characteristic therapeutic efficacy of clozapine. Assessing the therapeutic effects of clozapine on the basis of diverse genetic variants with a focus on GPCR signaling may give more definitive clues to its mechanism of action. The clinical implications of the effect of clozapine and its derivatives on RGS4 will be investigated in further translational studies.

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국문초록

항 정신성 약물 클로자핀의 아르기닌/N-말단 경로를 매개하는 RGS4 의 다중 유비퀴틴화 및 프로테아좀에 의한 분해 억제 기전 규명

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전 준 형

조현병 (schizophrenia)의 치료를 위해 다양한 항 정신성 약물이 개발되었음에도 불구하고, 작용 기전의 대부분은 아직 완벽히 파악되지 않았다. G-단백질 신호전달 조절자 (Regulator of G-protein signaling 4, RGS4)는 유전적 혹은 기능적으로 조현병과 연관되어 있고, 아르기닌/ N-말단 경로(Arg/N-degron pathway)의 생리학적 기질로 보고되어 있다. 본 논문에서는 비전형적인 (atypical) 항 정신성 약물 클로자핀이 전사 발현에는 영향을 주지 않고 RGS4 단백질의 프로테아좀에 의한 분해를 상당히 억제함을 밝혔다. 게다가 아르기닌/ N-말단 경로의 인공 기질인

아르기닌 (Arg) 및 페닐알라닌 (Phe) 이 부착된 녹색형광단백질 (Arg or Phe-GFP)의 단백질 수준은 클로자핀 처리에 의해 유의미하게 증가되었다. 또한, 클로자핀 처리는 시험관 및 배양된 동물세포에서 아르기닌-녹생형광단백질(Arg-GFP) 및 RGS4 의 다중 유비퀴틴화를 감소시켰다. 클로자핀은 뉴런 세포에서 MEK1 및 ERK1 과 같은 G 단백질 결합 수용체 (G-protein coupled receptor, GPCR) 신호전달 체계 하위 반응기의 활성화를 약화시켰다. 종합적으로 이러한 결과들은 번역(translation) 후 단계에서 RGS4 를 포함한 아르기닌/N-말단 경로 기질들의 분해 억제라는 클로자핀의 추가적인 치료상의 기전을 밝혀냈다. 이러한 발견은 아르기닌/N-말단 경로와 같은 단백질 번역 후 변형(post-translational modification, PTM)들이 조현병에 대한 새로운 분자치료전략이 될 것을 암시한다.

* 본 논문의 내용은 출판을 위해 더 연구 중에 있다.

주요어 : 조현병, 클로자핀, RGS4, 유비퀴틴화, N-말단 경로, 유비퀴틴-프로테아좀 시스템

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