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ORIGINAL ARTICLE

The Antipsychotic Drug Clozapine Suppresses the RGS4 Polyubiquitylation and Proteasomal Degradation Mediated by the Arg/N-Degron Pathway

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Abstract

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, we 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. In silico computational model suggested that clozapine may interact with active sites of N-recognin E3 ubiquitin ligases. Accordingly, treatment with clozapine resulted in reduced polyubiquitylation of RGS4 and Arg-GFP 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 HEK293 and SH-SY5Y cells. Furthermore, intraperitoneal injection of clozapine into rats significantly stabilized the endogenous RGS4 protein in the prefrontal cortex. Overall, these results reveal an additional therapeutic mechanism of action of clozapine: this drug posttranslationally 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.

Keywords Schizophrenia · Clozapine · RGS4 · Ubiquitination · N-degron pathway · Ubiquitin–proteasome system

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]. 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 [2, 3]. Despite high heritability of this disease (~ 80% [1]), 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

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are involved too [4, 5]. 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* [6, 7]. 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 [8]. 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 [9] and takes part in prefrontal cognitive functions [10, 11]. 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 [12]. Many subsequent studies support the role of *RGS4* as a major candidate gene for susceptibility to schizophrenia [13–16], while some studies have failed to confirm the positive association [17]. 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 [18–20].

We and other groups have previously reported that RGS4 and RGS5 proteins are degraded by the arginylation branch of the N-degron pathway (Arg/N-degron pathway) *in vitro* and *in vivo* [21–24]. The Arg/N-degron pathway is the first identified degradation mechanism underlying ubiquitin–proteasome system (UPS)-mediated proteolysis [25–27]. 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 [28, 29]. RGS2, another G_q-specific GTPase-activating protein implicated in cardiovascular function, is degraded by the acetylation branch of the similar system, termed the Ac/N-degron pathway [30–32]. Mice lacking UBR proteins die with various defects in neurogenesis as midgestation embryos [33, 34]. 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 [35]; however, the association between effects of APDs and RGS4 remains to be determined.

APDs mostly act by binding to neurotransmitter receptors, and one of the main targets is the dopamine D2 receptor (D2R) [36, 37]. 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 [38, 39]. 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 [40, 41]. We have previously reported that a downstream crosstalk between various intracellular signaling pathways and autophagy is also involved in clozapine's action [42, 43].

Here, we show that the APD clozapine inhibits the Arg/N-degron pathway, thus stabilizing RGS4 not only in the test tube but also in cultured neurons and in the rat prefrontal cortex. Consistent with the results of our biochemical degradation assay, in silico modeling results suggested that clozapine may be strongly bound to the active sites of Arg/N-degron E3 Ub ligases. Clozapine injection into rats resulted in significantly elevated levels of endogenous RGS4 in the frontal cortex. Collectively, these data constitute the first evidence that Arg/N-degron pathway–dependent degradation is inhibited by clozapine both *in vitro* and *in vivo*. 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.

Materials and Methods

Immunoblotting and Co-immunoprecipitation

Whole cell extracts (WCEs) were prepared as follows: 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), anti-LC3 (L7543, Sigma, 1/2,000), 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 1.5–3 μg 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 anti-mouse IgG secondary antibodies (GTX-221667; Genetex) were used for the detection of immunoprecipitated proteins.

Plasmids and Chemicals

The constructs encoding wild-type mouse RGS4 and RGS5 (RGS4-WT and RGS5-WT, respectively) were previously described [44, 45]. Their mutants having stabilizing Cys-2 residues (RGS4-C2V and RGS5-C2V) were generated by PCR-based site-directed mutagenesis. Open reading frames of the untagged RGS4 and RGS5 without stop codons were amplified to have C-terminal His- (CAT CAT CAC CAT CAC CAC) and myc (GAG CAG AAA CTC ATC TCA GAA GAG GAT CTG)-tags by using PCR and were subcloned into pcDNA3.1 plasmids (Invitrogen). DNA sequences of all plasmids were validated, and their expressions were confirmed using the rabbit reticulocyte lysate TnT system (Promega). Clozapine and prostaglandin E2 were purchased from Cayman Chemicals (#12,059 and #14,010, respecitvely). CCG-50014 was purchased from MedChemExpress (#HY-13509).

Cell Cultures and Transient Expression

Mammalian cells, including HEK293 and SH-SY5Y, were purchased from the Korea Cell Line Bank (catalog# 21573 and 22266, respectively) and authenticated by genetic profiling using polymorphic short tandem repeats. The HEK293-pre1-HTBH cell lines were generated as previously described [46]. SH-SY5Y cells with only less than passage number five were used in this study. All 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. The expression level of each protein was controlled by co-transfecting V5-tagged LacZ and by keeping the total amount of plasmids constant with addition of the empty vector if necessary.

Measurement of Cell Viability

Cell viability was assessed by the CellTiter-Glo luminescent cell viability assay kit (Promega) according to the manufacturer's instructions. Briefly, cells were grown in black-wall/clear-bottom 96-well plates, treated with either vehicle (0.1% DMSO) or clozapine at various concentrations (0.1 to 1000 μM) for 1 day and followed by the addition of luminescence substrates to the media and incubation for 10 min at room temperature on a shaker. The luminescence of the solution was measured using Tecan Infinite m200 luminometer.

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 s, 60 °C for 1 min. After 40 cycles, conduct the other 95 °C for 15 s, 60 °C for 1 min, and keep 95 °C for 15 s. 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′-CTGACTTCAACAGCG ACACC-3′) and reverse (5′-CTGACTTCAACAGCGACA CC-3′) primers.

In Silico Docking Model of UBR Box–Clozapine Interactions

Crystal structures of the UBR2 box and ClpS box were retrieved from the PDB (IDs: 3NY3 and 3DNJ, respectively). The structure of clozapine was optimized at the B3LYP/6-31G(d,p) level of theory using the Gaussian 16 package [47]. AutoDockTools [48] was employed to add

polar hydrogens and assign charges to all structures. For the UBR box and ClpS box, affinity grids were created in the AutoGrid software. The grids were set to be large enough to cover all active sites. AutoDock2 with the Lamarckian genetic algorithm was applied to simulate ligand–receptor docking. Docking parameters were as follows: 200 trials of docking, population size of 300, random starting position and conformation, translation step range of 2.0 Å, rotation step range of 50°, elitism of 1.0, mutation rate of 0.02, crossover rate of 0.8, local search rate of 0.06, and 50 million energy evaluations. Docked conformations were clustered using a tolerance of 2.0 Å root mean square deviation. Experimental binding free energies were calculated from dissociation constants via the equation $\Delta G = R \cdot T \cdot \ln K_d$, where R is the gas constant (1.987 cal·K^{-1}·mol^{-1}) and T is temperature (298.15 K). Electrostatic potentials were calculated by means of the APBS package. All structural figures were prepared in the Chimera software.

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 [49]. 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 chymotrypsin-like activity assay), Boc-Leu-Arg-Arg-AMC (Boc-LRR-AMC for a trypsin-like activity assay), and Z-Leu-Leu-Glu-AMC (Z-LLE-AMC for a caspase-like 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 [50]. 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 [49]. 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).

Purification of Recombinant Usp2 and X-GFP Proteins

The recombinant X-GFP (Met-GFP, Arg-GFP, and Phe-GFP) proteins were prepared by *in vitro* cleavage of Ub from Ub-X-GFP proteins by means of DUB Usp2, containing a minimal catalytically active deubiquitylating domain, as described elsewhere [51] with some modifications. Briefly, to construct the vectors for bacterial expression of Ub-X-GFP, X-GFP sequences were obtained by PCR amplification of the Ub-X-GFP plasmid [52]. The amplicons were digested with SacII and NotI and cloned into a histidine-tagged-Ub expression vector, pHUE [51]. All the constructs were verified by sequencing. We transfected pHUE-X-GFPs into *Escherichia coli* strain BL21 (DE3), cultured the cells

at room temperature overnight, added 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) to each culture, and incubated them for 4 h. The cells were harvested in buffer A (50 mM Na_2HPO_4/NaH_2PO_4 pH 7.4, 300 mM NaCl, 20 mM β-mercaptoethanol, and 30% of glycerol) containing protease inhibitors, were incubated in 0.2% Triton X-100 on ice for 7 min, and then were lysed by sonication. After the lysates were centrifuged, the supernatants were incubated with Ni^{2+}-NTA resin (MCLAB) at 4 °C for 3 h. After a wash with buffer A containing 0.2% of Triton X-100, His-Ub-X-GFP proteins were eluted with 250 mM imidazole in buffer A. The recombinant Usp2 protein was purified using plasmid pHUsp2-cc, expressing a His-tagged Usp2 catalytic core (Usp2), as described before [51]. Ub cleavage from the resulting Ub-X-GFP was achieved by incubating both Usp2-cc and Ub-X-GFP proteins at room temperature for 1 h in buffer A. The purified Usp2-cc, Ub-X-GFPs, and X-GFPs were separated by SDS-PAGE, and the gels were stained with CBB to determine the size and purity of the proteins.

Ubiquitylation of Recombinant X-GFP Proteins *In vitro*

In a test tube, the ubiquitylation reaction mixture consisted of 37 nmol X-GFP, 4.2 nmol UBA1 (UBP Bio), 40 nmol HR6A (Boston Biochem), 9 μmol yeast UBR1, and 1.2 nmol Ub (Boston Biochem) in 25 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. For ubiquitylation using reticulocyte lysates, 2 μg of recombinant X-GFP proteins were incubated with T_NT rabbit reticulocyte lysates (12. 5 μL; Promega), which contain UBR proteins and other components for the Arg/N-degron pathway [53], in the T_NT® reaction buffer supplemented with 10 μg of additional Ub and 20 μM MG132 (total 25 μL reaction). The reaction was performed at 30 °C, and aliquots were taken at the indicated time periods for subsequent immunoblotting analysis.

Analysis of GPCR Pathways

For the serum starvation assay, HEK293 cells were incubated in a serum-free medium. For serum stimulation, the cells were kept in the serum-free medium for 16 h and then activated for the indicated time periods (5, 30, 60 min) by the addition of DMEM containing 10% of fetal bovine serum. Clozapine was present at 10 μM final concentration during the serum starvation and activation, and cell samples were harvested for immunoblotting to analyze the MAPK pathways. For the serum starvation assay of neuroblastoma SH-SY5Y cells, 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. For MAPK/ERK pathway stimulation, HEK293 cells were treated with prostaglandin E2 (PDE2) (5 or 10 μg/mL) for less than 30 min with or without CCG-50014 [4-(4-fluorobenzyl)-2-p-tolyl-1,2,4-thiadiazolidine-3,5-dione], a highly selective RGS4 inhibitor [54].

The Animal Experiment

Rats were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Formal approval of this experiment was obtained from the Animal Subjects Review Board of Seoul University Hospital. Male Sprague–Dawley rats (150–200 g) were acclimated for 1 week before the experiments and were maintained on a strict 12/12-h light/dark cycle (lights on at 08:00 h) with food and water available ad libitum. Clozapine (Tocris) was dissolved in vehicle (0.3% tartaric acid in distilled water), and pH was adjusted to 6.0–6.5 with NaOH [55]. A clozapine dose of 10 mg/kg was used in this study. This dose was selected based on our previous studies and on clinical relevance determined by the relations among drug dose, plasma levels, and *in vivo* dopamine D2 receptor occupancy [42, 43, 55, 56]. Control animals received an equivalent volume of 0.3% tartaric acid at the same pH. Each rat received an intraperitoneal injection of clozapine or vehicle in a volume of 1 mL for 14 days at the same time during the day (12:00–13:00 pm). The rats were decapitated 2 h after the last treatment. Four animals were assigned to each treatment group. Prefrontal-cortex tissues were harvested as previously described to prepare protein or mRNA samples and were subjected to SDS-PAGE/immunoblotting or quantitative reverse-transcription PCR (RT-PCR) analysis as described previously [42, 43, 55].

Statistical Analysis

Statistical significance of differences between various groups was determined by Student's *t* test or one-way ANOVA followed by the Bonferroni post hoc test, mostly using GraphPad Prism 5 (GraphPad Inc.). Data are reported as mean ± SD of three or four independent experiments. Differences were considered significant at $p < 0.05$. The half-lives ($t_{1/2}$) of RGS proteins were obtained by fitting the experimental data to a nonlinear regression model in Prism 5.

Results

Clozapine Delays Degradation of RGS4 in HEK293 Cells

To investigate whether APDs affect RGS4 stability, we tested commonly prescribed atypical antipsychotic medications for schizophrenia: risperidone, olanzapine, and

clozapine. First, we transiently overexpressed RGS4 and its subfamily member RGS5 [23, 28] 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 ($p < 0.01$ from one-way ANOVA; Fig. 1a). Accordingly, RGS4 and RGS5 levels in HEK293 cells turned out to be gradually elevated by clozapine treatment

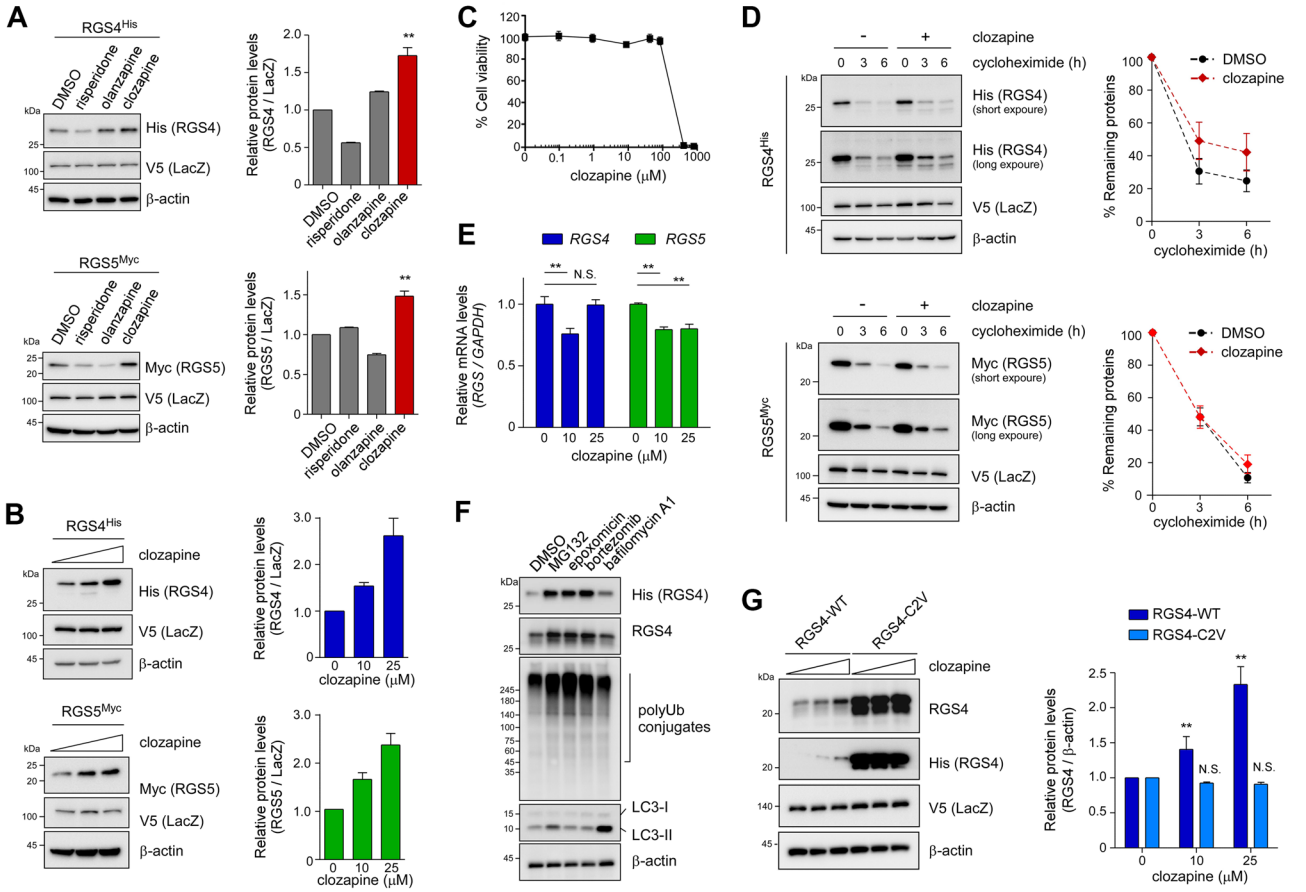


Fig. 1 Antipsychotic drug (APD) clozapine post-translationally stabilizes RGS4 and RGS5 in cultured cells. **a** Changes of steady-state levels of RGS proteins. Plasmids pcDNA3.1-RGS4^{His} (top panels) and RGS5^{myc} (bottom) were transiently transfected into HEK293 cells (for overexpression of respective proteins) along with plasmid pcDNA3.1-LacZ^{V5} as a transfection efficiency control. All tags were C-terminally labeled. At 24 h posttransfection, 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 anti-RGS4 and anti-His antibodies. (Right) Quantitation of IB signals of RGS proteins, normalized to those of LacZ. **b** As in **a**, except that different concentrations of clozapine (0, 10, or 25 μM) were added to examine their effect on steady-state levels of RGS4 and RGS5. **c** Assessment of clozapine cytotoxicity. HEK293 cells were treated with clozapine at the indicated concentrations. After 24 h, the CellTiter-Glo bioluminometric cell viability assay was performed. Each data point is a mean ± SD of three independent experiments. **d** 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. **e** Posttranslational 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. **f** Inhibition of proteasomes, not of autophagy, increased the cellular RGS4 levels. HEK293 cells were transfected with His-tagged RGS4 for 36 h and treated with proteasome inhibitors [MG132 (10 μM), epoxomicin (1 μM), or bortezomib (1 μM)] or autophagy inhibitor bafilomycin A1 (100 nM), for 6 h. Protein samples were then collected and analyzed by SDS-PAGE/IB. **g** The N-terminus-dependent regulatory effect of clozapine on RGS protein degradation. (Left) 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). (Right) 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. N.S., not significant. $^{**}p < 0.01$ from one-way ANOVA

in a dose-dependent manner (0, 10, and 25 μM; Fig. 1b). The cytotoxicity of clozapine was observed only at a concentration above 200 μM after 24-h incubation (Fig. 1c).

On the basis of these results, we hypothesized that clozapine blocks the degradation of RGS4 and RGS5 as a novel pharmacological regulator of these proteins. To test this idea, we 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 mildly increased in the presence of clozapine ($t_{1/2}$ = 4.4 h with clozapine vs. 3.1 h with DMSO; Fig. 1d). RGS5 showed weaker responses to clozapine and was mainly affected only after 6 h of treatment. The effect of clozapine on RGS protein stabilization was posttranslational because mRNA levels of these proteins were either unchanged or reduced (Fig. 1e). Moreover, the protein homeostasis (proteostasis) of the RGS proteins appeared to be mainly mediated by the UPS rather than by the autophagy–lysosome system; chemical inhibition of proteasomes (by MG132, epoxomicin, or bortezomib) increased RGS4 and total Ub conjugate levels in the cell, whereas autophagy inhibitor bafilomycin A1 had little effect (Fig. 1f). The levels of LC3-II, a key autophagic marker protein [57], were robustly elevated after cells were treated with bafilomycin A1, but not with proteasome inhibitors. These results strongly indicated that clozapine potently inhibits UPS-mediated proteolysis of RGS proteins in the cell.

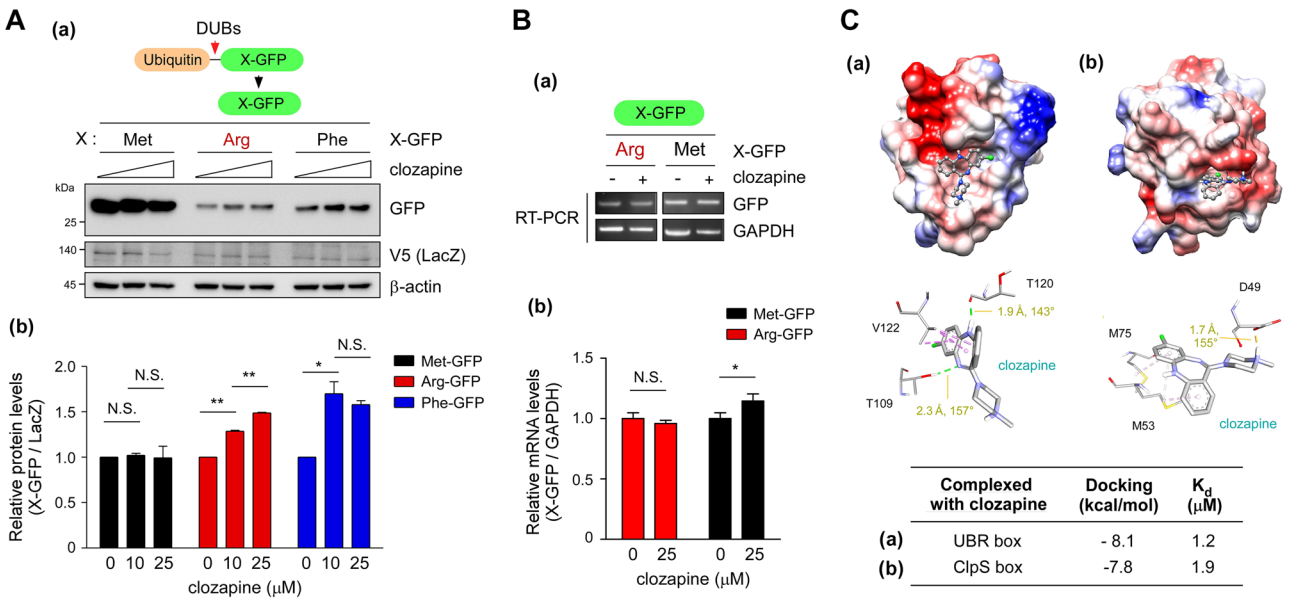
Biochemical and Computational Analysis Reveals that Clozapine Efficiently Blocks the Arg/N-degron Pathway

Studies by the Levitt group and others established RGS4 is the only gene among other RGS family, whose expression was significantly reduced in the prefrontal cortex of the postmortem brains of patients with schizophrenia [12, 17]. In addition, 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 [28]. 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). To determine whether the inhibitory effect of clozapine on RGS4 degradation is mediated by the Arg/N-degron pathway, we utilized Cys2Val-mutant RGS4 (RGS4-C2V), a long-lived version of RGS4 (control). We found that in contrast to RGS4-WT, steady-state levels of transiently overexpressed RGS4-C2V were virtually unaffected by clozapine treatment (Fig. 1g). 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.

We 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; Fig. 2a). 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 ($p < 0.05$ from one-way ANOVA; Fig. 2a). Under the same conditions, however, *GFP* mRNA levels were only comparable ($p > 0.05$ from Student's *t* test; Fig. 2b), indicating that the attenuation of Arg- and Phe-GFP degradation during clozapine treatment occurred at the posttranslational stage.

In silico docking analysis suggested strong interactions between clozapine and the two substrate-binding sites of N-recognins. Binding affinities between clozapine and the UBR box (which binds to a "type 1" Arg/N-degron) or the ClpS domain (to a "type 2" Arg/N-degron) were calculated by a ligand-receptor docking computation using AutoDock with the Lamarckian genetic algorithm [47] (Fig. 2c; see the "Materials and Methods" section for detailed docking parameters and methodology). Crystal structures of the UBR box and N-domain were retrieved from the Protein Data Bank (PDB; ID 3NY3 for mouse UBR2 and PDB ID 3DNJ for yeast ClpS, a sequelog of the mammalian N-domain). Substantiating the biochemical data, the computational modeling proposed that clozapine may significantly interact with both the UBR box (−8.1 kcal/mol binding affinity, meaning a 1.2 μM dissociation constant) and the ClpS box (−7.8 kcal/mol binding affinity, 1.9 μM dissociation constant).

On the basis of the docking results, we postulated that hydrogen bonds and CH–π interactions may play important roles in the binding of clozapine to the N-recognins (Fig. 2c). Our modeling showed that, in the clozapine–UBR box complex, clozapine may form two hydrogen bonds (with amino acid residues T109 and T120). Moreover, three CH–π interactions between clozapine and hydrophobic V122 were found, which could further stabilize this complex. These residues are key components of the β-sheet secondary structures of the Cys- and His-enriched UBR box [58]. Likewise, in the clozapine–ClpS complex, clozapine was found to form a strong hydrogen bond with D49 with a short O–H distance (1.7 Å); this amino acid residue is known to be indispensable for the degradation of "type 2" substrates [59]. In this model, clozapine also engages in CH–π interactions with residues M53 and M75. Even though effective inhibition



	Complexed with clozapine	Docking (kcal/mol)	K_d (μM)
(a)	UBR box	- 8.1	1.2
(b)	ClpS box	-7.8	1.9

Fig. 2 Inhibitory effects of clozapine on the degradation of model Arg/N-degron substrates. **a** 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. (**a**) At 24 h posttransfection, 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. (**b**) Relative amounts of remaining X-GFP proteins were quantified in the ImageJ software, and the data were normalized to the levels of LacZ. N.S., not significant. $*p<0.05$; $**p<0.01$ from one-way ANOVA. **b** Posttranslational regulation of Arg/N-degron substrates by clozapine (25 μM for 24 h). Conventional (**a**) and quantitative (**b**) RT-PCR was carried out using primers for *GFP* and *GAPDH* (for normalization). The values plotted are means (±SD) of three independent experiments $*p<0.05$ from two-tailed Student's *t* test. **c** In silico docking model of binding modes, binding affinities (docking, kcal/mol), and dissociation constants (K_d, μM) of clozapine (**a**) with the UBR box (PDB ID: 3NY3) or (**b**) with the ClpS domain (PDB ID: 3DNJ). Note that the structure of full-length UBR1 is not reported yet

was achieved only at millimolar concentrations, the binding of clozapine to UBR from our calculation was found to be significantly stronger (> 100-fold) than those of a previously reported *in vivo* Arg/N-degron pathway inhibitor, *para*-chloroamphetamine [60–62]. Thus, the atypical APD clozapine seems to exert a direct inhibitory activity toward the Arg/N-degron pathway. Using clozapine as a lead compound to determine the structural basis of its inhibitory effects may contribute to the development of more potent APDs with improved efficacy, tissue distribution, and safety.

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, we 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. We 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 (Supplementary Fig. 1A).

To assess the possibility that clozapine enhances proteasome activity directly or allosterically, we examined proteasome activity using suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (suc-LLVY-AMC), a fluorogenic reporter substrate for the chymotrypsin-like 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 (Supplementary Fig. 1B). Similarly, trypsin-like PSMB7 and caspase-like PSMB6 activities were unaffected by clozapine (Supplementary Fig. 1C). 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; Supplementary Fig. 1D).

Furthermore, we examined degradation rates of a more physiologically relevant substrate, the polyubiquitinated Sic1 (Ub-sic1) protein [63], instead of the fluorogenic reporter peptides. The 26S proteasome purified after treatment with clozapine showed no significant difference from the untreated proteasome in the degradation of Ub-sic1 (Supplementary Fig. 1E). 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 Polyubiquitylation of Arg/N-degron Substrates Including RGS4

The results described above mean that the stabilization of RGS proteins by clozapine is mediated by Arg/N-degron pathway–dependent proteasomal degradation. To identify the target of clozapine in the UPS, we set up a complete *in vitro* ubiquitylation system for the Arg/N-degron pathway. Recombinant fusion proteins (Ub-X-GFP) with His-tagged Ub were immobilized on Ni^{2+}-NTA beads and then on-column cleaved by recombinant USP2 to elute the model substrates Arg-GFP and Phe-GFP and their control substrate, Met-GFP (~27 kDa; Fig. 3a, b). These recombinant proteins were first incubated with rabbit reticulocyte lysates, which are enriched with components of the UPS. Arg-GFP and Phe-GFP proved to be efficiently ubiquitylated *in vitro* (Fig. 3c). In sharp contrast, Met-GFP was virtually

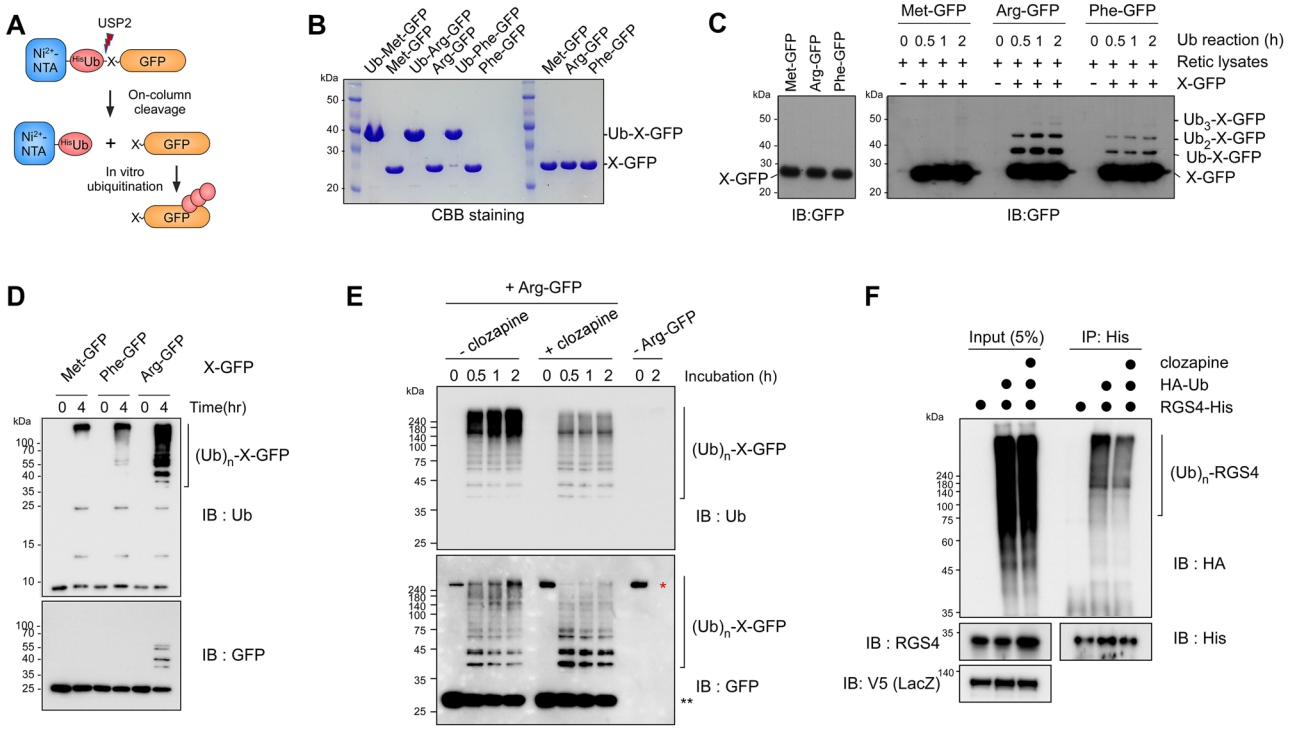


Fig. 3 Clozapine inhibits the formation of a polyubiquitination chain on Arg/N-degron substrates. **a** The process of purification of recombinant Arg/N-degron substrates. His-tagged Ub-X-GFP fusion proteins were expressed in *E. coli* and immobilized on a Ni^{2+}-NTA column. X-GFP proteins were eluted from the beads by on-column cleavage of the Ub-X junction using recombinant USP2. **b** Recombinant Met-GFP (not an Arg/N-degron substrate), Arg-GFP (type 1 substrate), and Phe-GFP (type 2) were confirmed by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. **c** Recombinant Arg- and Phe-GFP proteins, but not Met-GFP, were actively ubiquitylated in rabbit reticulocyte lysates, which are enriched in components of protein ubiquitylation machinery. **d** Complete *in vitro* reconstitution of Arg- and Phe-GFP ubiquitylation reactions, where these proteins were incubated with recombinant Ub, UBA1 (E1), HR6A (E2), and yeast UBR1 (E3) at 37 °C for 4 h. The modification of Arg- and Phe-GFP was monitored by SDS-PAGE/IB with anti-Ub and anti-GFP antibodies. **e** As in **d**, except that *in vitro* ubiquitylation of Arg-GFP was performed in the presence or absence of clozapine (10 μM) for 0.5, 1, or 2 h. The red arrow indicates monomeric forms of Arg-GFP. *Nonspecific bands. **IgG light chain. **f** Inhibition of RGS4 polyubiquitylation by clozapine in HEK293 cells. HA-tagged Ub and/or C-terminally 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 antibodies and EasyBlot anti-mouse IgG

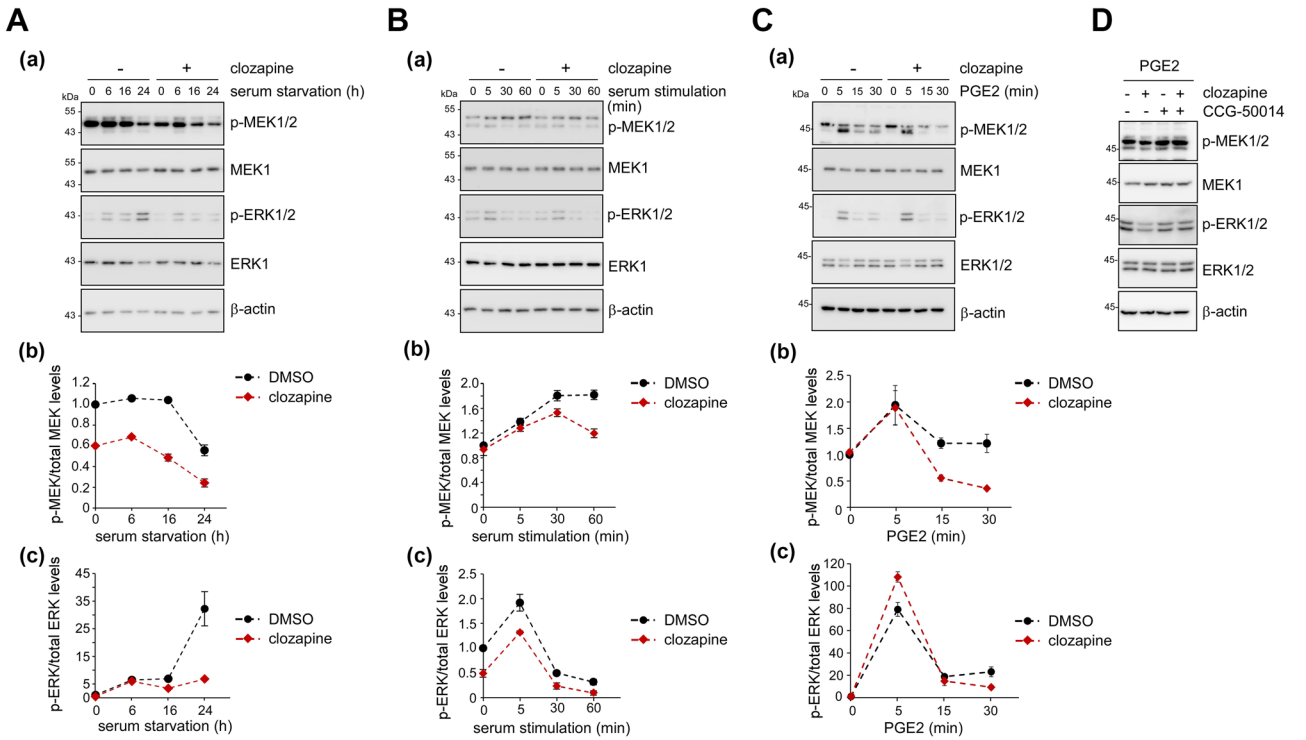


Fig. 4 Clozapine delays the downstream activation of GPCR signaling in cultured cells. **a** Profiles of MEK1/2 and ERK1/2 in HEK293 cells in the absence or presence of 10 μM clozapine after incubation with a serum-free medium (starvation) for the indicated periods. In multiple IB images (**a**), levels of phospho-MEK1 (**b**) and phospho-ERK1/2 (**c**) were quantified with normalization to total MEK1 and ERK1/2, respectively. **b** After serum starvation (16 h) with 10 μM clozapine, the cells were stimulated with a serum-containing medium (DMEM supplemented with 10% of fetal bovine serum) in the absence or presence of clozapine. WCLs were collected at the indicated time points and analyzed with SDS-PAGE/IB. **c** As in **b**, except that prostaglandin E2 (PGE2; 10 μg/mL) was used to stimulate GPCRs for indicated time periods. To exclude the input of receptor tyrosine kinases on MAPK phosphorylation, cells were serum-deprived for 2 h prior to PGE2 treatment. Data represent mean ± SD from independent triplicate experiments. **d** Inhibitory effects of clozapine on PGE2-induced ERK phosphorylation was abolished by RGS4 inhibitors. HEK293 cells were pre-treated with clozapine (10 μM) in the presence and absence of the irreversible RGS4 inhibitor CCG-50014 (30 nM) for 24 h. Cells were serum-starved for 2 h and then treated with PGE2 (5 μg/mL) for 10 min

nonubiquitylated. Next, we carried out biochemical reactions involving X-GFPs, purified UBA1 (as E1), HR6A (E2), yeast UBR1 (E3), and Ub, which yielded identical results (strong polyubiquitylation of Arg-GFP and Phe-GFP but not Met-GFP), as evidenced by the partial reconstitution in reticulocyte lysates (Fig. 3d).

To examine the impact of clozapine on X-GFP ubiquitylation, we 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 (Fig. 3e). 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 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 (Fig. 3f). This result is in agreement with the strong binding of clozapine to UBR proteins identified by our in silico computational analysis (Fig. 2c) and probably accounts for the delayed degradation of RGS4 (Fig. 1a, b) 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 Treatment Impairs the Activation of the GPCR Signaling Pathway in Mammalian Nonneuronal and Neuronal Cells

RGS proteins, whose levels are controlled by the Arg/N-degron pathway, are GTPase-activating enzymes that

negatively regulate G_q and $G_{i/o}$-mediated downstream signaling. RGS4 is expected to activate GTP-G_α hydrolysis and induces reassociation of $G_{\alpha\beta\gamma}$ proteins to end G protein signaling pathways. We then examined the impact of clozapine on the downstream effectors of the G_q signaling pathway. In line with the physiological role of RGS4 in G protein signaling, time-course immunoblotting of HEK293 cell lysates revealed that the activities of MEK1/2 and ERK1/2 were significantly attenuated by clozapine treatment, and this phenomenon was more prominent during serum starvation (Fig. 4a and Supplementary Fig. 2A).

When GPCR was reactivated with subsequent serum stimulation, the activation of MEK1/2 and ERK1/2 was also significantly delayed in the presence of clozapine (Fig. 4b). In addition, we treated the HEK293 cells with prostaglandin E2 (PGE2), which exerts its multiple effects through GPCR without directly involving receptor tyrosine kinases [64]. After 2 h of serum deprivation, stimulation with PGE2 rapidly induced the phosphorylation of MEK1/2 and ERK1/2 (Fig. 4c). However, this activation of the MAPK pathway was delayed in the presence of clozapine. This effect of clozapine was effectively abrogated when RGS4 was chemically inhibited (Fig. 4d). These data collectively reflect clozapine-mediated inhibition of RGS4 degradation, which in turn potentially blocks the downstream G_q and $G_{i/o}$ signaling. The influence of clozapine on the GPCR signaling pathway was examined in immortalized SH-SY5Y neuroblastoma cells as well. Just as in the non-neuronal cells, phosphorylation of MEK1/2 and ERK1/2 in SH-SY5Y cells was significantly delayed by clozapine treatment (Supplementary Fig. 2B). These results imply 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.

Clozapine Inhibits Endogenous RGS4 Degradation in the Rat Prefrontal Cortex

To test whether clozapine affects the stability of RGS4 *in vivo*, we intraperitoneally injected clozapine into rats at 10 mg/kg for 14 days and euthanized them 2 h after the

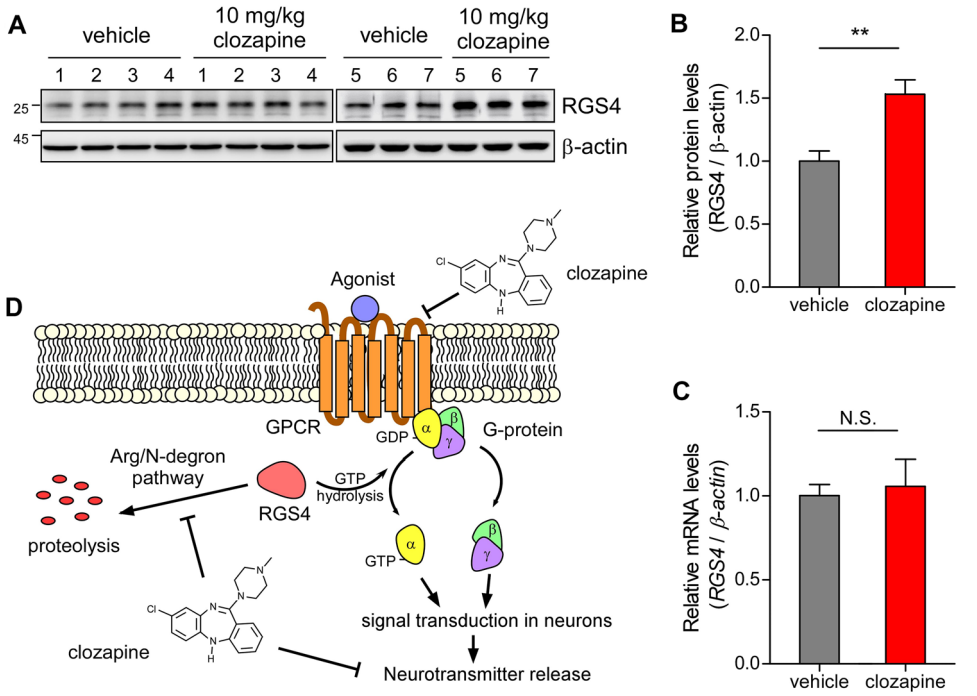


Fig. 5 Treatment with clozapine inhibits the degradation of endogenous RGS4 in the rat brain. **a** Endogenous RGS4 levels in the rat prefrontal cortex significantly increased after intraperitoneal injections of clozapine (10 mg/kg) for 14 days. WCLs were extracted from the right hemisphere and analyzed by SDS-PAGE/IB with RGS4 antibodies. β-Actin: loading control. **b** Quantification of relative RGS4 levels in the prefrontal cortex, with normalization to β-actin. Mean ± SD ($N = 7$/group from two independent experiments). $^{**}p < 0.001$ (two-tailed Student's t test). **c** Total RNA was isolated from the left hemisphere of the rats subjected to the experiments depicted in **a**. Quantitative RT-PCR analysis of *RGS4* and β-actin mRNA levels was performed, and the results are plotted as means ± SD of four independent experiments. N.S., not significant ($p > 0.05$ from two-tailed Student's t test). **d** 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 protein 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

final injection. In the rat prefrontal cortex, which is known to strongly express RGS4 [65], immunoreactivity of RGS4 in WCLs was significantly higher ($p < 0.001$, $t = 4.657$ from Student's t test, $N = 7$) in the clozapine-treated group compared to the control group (vehicle only; 0.3% tartaric acid in distilled water; Fig. 5a, b). No significant changes in *RGS4* mRNA levels were observed (Fig. 5c). These effects of clozapine on the brain are similar to those when components of the Arg/N-degron pathway are genetically disrupted [21], thus indicating that clozapine targets RGS4 proteolysis in the rat brain. Notably, the prefrontal cortex is the region most consistently implicated in schizophrenia and where RGS4 levels were initially found to be reduced [12, 65].

Discussion

Here, we report that the atypical APD clozapine is capable of inhibiting the Arg/N-degron pathway *in vitro* and *in vivo*. 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 rat prefrontal cortex. In silico computational analysis implies a direct interaction between clozapine and the UBR protein, which was found to potentially mediate the direct inhibition of RGS4 polyubiquitination. Genetic linkage, association, and postmortem brain studies have revealed that RGS4 is a strong candidate gene of schizophrenia susceptibility [17]. Our data collectively suggest that clozapine may alleviate the genetic or functional defect of RGS4 in schizophrenia through post-translational protein stabilization (Fig. 5d). Thus, this study points to a novel mechanism of action of clozapine, having superior pharmacological efficacy, 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 [25, 27]. 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 [66, 67]. We have reported gross developmental defects of neurogenesis, such as malformation of the brain and cerebral hemorrhages, that may originate from abnormal proliferation of the neuroepithelium [68]. Dysfunctions of protein homeostasis and of related intraneuronal signaling in schizophrenia have also been repeatedly reported [69], pointing to the UPS as a molecular target of APDs [69, 70]. Upregulation of polyubiquitinated proteins in erythrocytes, in postmortem orbitofrontal cortex tissue [71], and in the superior temporal gyrus [72] of individuals with schizophrenia has been reported. Nonetheless, the underlying molecular mechanism is unclear, and further evidence is still needed. Our data suggest that clozapine specifically blocks the polyubiquitylation of RGS4 instead of globally altering the proteome. 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 may contribute to the therapeutic profiles of APDs [73]. 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 [39]. 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 [74–76]. By direct binding to UBR, 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 [77], is another small-molecule inhibitor of the Arg/N-degron pathway with weaker binding affinity [61]. 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 [12, 19], 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 [20]. Likewise, here we report that clozapine stabilizes the RGS4 protein without affecting its mRNA levels *in vitro* and *in vivo*. RGS4 is a key regulator of GPCR signaling via negative regulation of G_q and $G_{i/o}$ subunits of neurotransmitter receptors [35]. Therefore, the stabilization of RGS4 by clozapine can have diverse downstream effects and related clinical manifestations. In addition to dopaminergic and serotonergic receptor signaling [78, 79], RGS4 is deeply involved in glutamatergic-neurotransmission regulation [80]. Recent studies show interactions of RGS4 with metabotropic glutamate receptor 1α (mGluR1α) and mGluR5 signaling in the prefrontal cortex of individuals with schizophrenia [19, 81]. 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 [82, 83]. Therefore, future research is required to further clarify the *in vivo* consequences of clozapine therapy on the intercellular signaling pathways in the brain as well as their behavioral effects. This will expand our knowledge of the mechanisms responsible for the superior antipsychotic efficacy of clozapine.

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 [84]. The Arg/N-degron pathway also participates in various neural functions [33, 68, 85, 86], yet to be explored further. In the present 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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Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

Author Contribution J.H.J. and T.R.O. carried out most *in vitro* studies and cell-based assays. J.H.L. analyzed the signaling pathway after clozapine treatment. S.P. and J.H.K. purified recombinant X-GFP proteins. B.K.M. performed in silico analysis. S.H. and S.H.K. contributed to the rat experiments. J.H.L. and M.J.L. were responsible for the overall design and oversight of the project. J.H.L., S.H.K., and M.J.L. contributed to the preparation of the paper.

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Declarations

Conflict of Interest The authors declare that they have no competing interests.

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