Toxoplasma Gondii Dense Granule Protein 3 Promotes Endoplasmic Reticulum Stress-Induced Apoptosis by Activating the PERK Pathway

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Research Article

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Abstract

Background

Toxoplasma gondii is a neurotropic single-celled parasite that can infect mammals and birds. Central nervous system infection with *T. gondii* infection can lead to *Toxoplasma* encephalitis. Toxoplasma infection can cause endoplasmic reticulum (ER) stress and unfolded protein response (UPR) activation, which ultimately can lead to apoptosis of host cells. The dense granule protein GRA3 has been identified as one of the secretory proteins that contribute to the virulence of *T. gondii*; however, the mechanism remains enigmatic.

Methods

The expression of GRA3 gene in RH, ME49, Wh3 and Wh6 strains were determined using quantitative real-time PCR (qRT–PCR). pEGFP-GRA3$_{Wh6}$ was created by inserting Chinese 1 Wh6 GRA3 (GRA3$_{Wh6}$) cDNA into a plasmid encoding the enhanced GFP. Mouse neuro2a (N2a) cells were transfected with either pEGFP or pEGFP-GRA3$_{Wh6}$ and incubated for 24-36 hr. N2a cell apoptosis- and ER stress-associated proteins were determined using flow cytometry and immunoblotting. Furthermore, N2a cells were pretreated with GSK2656157 and Z-ATAD-FMK before pEGFP-GRA3$_{Wh6}$ transfection, and the effect of the inhibitors on GRA3$_{Wh6}$-induced ER stress and apoptosis was investigated.

Results

GRA3 gene expression was higher in the avirulent strains of type II ME49 and type Chinese 1 Wh6 strains compared to the virulent strains of type I RH strain and type Chinese 1 Wh3 strain. Transfection with pEGFP-GRA3$_{Wh6}$ induced neuronal apoptosis and increased the expression of GRP78, p-PERK, cleaved caspase-12, cleaved caspase-3, and CHOP compared to pEGFP. Pretreatment with GSK2656157 and Z-ATAD-FMK decreased apoptosis in N2a cells, and similarly, ER stress- and apoptosis-associated protein levels were significantly decreased.

Conclusion

GRA3 induces neural cell apoptosis via the ER stress signaling pathway, which could play a role in *Toxoplasmic* encephalitis.

Background

Toxoplasma gondii is a common intracellular coccidian parasite that infects human beings and animals [1–3]. Most *T. gondii* infections are usually asymptomatic and result in a self-limiting disease in

immunocompetent hosts [4]; however, in chronic immunocompromised individuals, in particular HIV patients, *T. gondii* can cause severe and fatal tissue damage [4, 5]. In pregnant women, *T. gondii* can cause miscarriages or deleterious effects to the infants or newborns [6–8]. Due to its preference for neural cells, *Toxoplasma* infection is responsible for neurological manifestations, including encephalitis, intracranial calcifications, and hydrocephalus [9–11].

Several conditions, including infectious and neurodegenerative diseases, are known to cause a build-up of misfolded proteins within the endoplasmic reticulum that interfere with normal functioning of the ER. This leads to ER stress [12, 13]. To alleviate the effect of the stress, ER-localized transmembrane signaling proteins activate the unfolded protein response (UPR) to restore protein homeostasis [14, 15]. However, an unremitted UPR can activate UPR-mediated inflammatory and apoptotic pathways, resulting in cell death [16, 17].

Previous studies demonstrated that the Chinese 1 Wh3 and type I RH strains can induce apoptotic neural stem cell apoptosis via ER stress-mediated apoptosis signaling pathways [18, 19]. Additionally, secretory proteins such as ROP 18 and GRA 15 have been demonstrated to induce apoptosis of neural cells [10, 19] and carcinoma JEG-3 cells [20]. An and collaborators in a recent study to unravel the host proteins targeted by ROP18 and its molecular mechanisms showed that ROP18 kinase induced neural cell apoptosis by phosphorylating reticulon 1-C, which in turn promotes GRP78 acetylation [11].

Among the extensively studied dense granule proteins (GRAs) in *T. gondii*, GRA3 is believed to interact with the host cell endoplasmic reticulum (ER) via calcium modulating cyclophilin ligand (CAMLG) [21, 22]. GRA3 is a 29 kDa dense granule protein localized to the parasitophorous vacuole membrane and intravacuolar network. Aside from its physiological role in the uptake of nutrients from host cells, GRA3 in type II strains has also been identified to contribute to its virulence [21, 23]. However, previous studies postulated that the interaction between *T. gondii* GRA3 and CAMLG of the host ER inhibits host cell apoptosis [24, 25]. We demonstrate for the first time that the interaction between GRA3 and CAML of the host endoplasmic reticulum contributes to *T. gondii*-induced cell death. We suggest that *Toxoplasma* GRA3 induces apoptosis in infected N2a cells by activating the PERK pathway to initiate the apoptotic cascade.

Methods

Parasite and cell Culture

T. gondii RH, ME49, Wh6, and Wh3 tachyzoites were cultured in human foreskin fibroblast (HFF) cells in Dulbecco's modified Eagle's medium (DMEM) which contained 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin amphotericin B (Biological Industries, Israel). N2a cells were cultured and maintained in DMEM which contained 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin amphotericin B (Biological Industries, Israel) at 37°C in a 5% CO_2 humified atmosphere. Cells were serially

passaged when they reached 80-90% confluency. HFF and N2a cells were regularly inspected for mycoplasma contamination.

Extraction of RNA and cDNA synthesis

Total RNA from *T. gondii* tachyzoites (RH, ME49, Wh6 and Wh3 strains) was obtained using TRIzol reagent (Invitrogen, SF, USA) following the manufacturer's protocols. Extracts with A260/A280 and A260/A230 absorbance ratios between 1.92 and 2.20 were considered pure contaminating reverse transcriptase or DNA polymerase inhibitors and were examined on 1% agarose gels. The concentration of purified RNA was measured using a NanoDrop™ One (Thermo Scientific CA, USA). RNA extracts were stored in -80°C refrigerator for subsequent use. Complementary DNA (cDNA) was synthesized from total RNA samples to using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA) following the manufacturer's recommendations. A 1:10 dilution of the cDNA reaction was prepared, measured, and stored at -20°C for use in subsequent steps.

Plasmid construction

The *ORF* encoding *T. gondii* GRA3 (http://toxodb.org) cDNA was amplified from Wh6 tachyzoite RNA using real-time (RT)-PCR. The oligonucleotides used included ME49-GRA3-EcorI-GFP (5'-CGGAATTCATGGACCGTACCATATG 3'; the EcoR1 site is underlined) and the reverse primer ME49-GRA3-SalI -GFP (5'- GTCGACTTATTTCTTGG AGGCTTTG 3'; the SalI site is underlined). GRA3 primer synthesis and gene sequencing were performed by General Biosystems Co., Ltd. (Anhui, China). A pEGFP-C2 vector (BD Biosciences Franklin Lakes, NJ, USA) was used to construct the pEGFP-GRA3$_{Wh6}$ plasmid by inserting digested TgGRA3$_{Wh6}$ cDNA into the digested pEGFP-C2 vector. The resulting pEGFP-GRA3$_{wh6}$ plasmid was transformed into *E. coli* TOP10 (Invitrogen Corp., U.S.A.) and screened.

Transfection of N2a cells with pEGFP and pEGFP-GRA3$_{Wh6}$ cDNA

Plasmid transfection was performed using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, China) in 6-well plates following the manufacturer's instructions. In brief, cells were seeded at a density of 1 × 10^5 cells/ml in 6-well plates and cultured to reach between 70-90% confluency at the time of transfection. DNA plasmid (2.5 μg) was diluted in 125 μl Opti-MEM containing 5 μl P3000 reagent, and 5 μl Lipofectamine 3000 transfection reagent was diluted in 125 μl Opti-MEM. The diluted Lipofectamine 3000 transfection reagent and diluted DNA were mixed gently and incubated at room temperature for 15 min. Following incubation, a total of 250 μl DNA-lipid complex was gently pipetted into each well. Control wells contained only cultured N2a medium. Cells were incubated between 18-48 hr, and the expression levels of pEGFP and pEGFP-GRA3$_{Wh6}$ were visualized using an Olympus IX51 fluorescence microscope

(Japan). After transfection, the cells were treated and analyzed by immunoblotting unless otherwise indicated.

Induction of ER stress

ER stress and apoptosis in N2a cells were induced using 4 μg/ml tunicamycin (TM) and 0.5 μM staurosporine (STS), respectively (MedChemExpress LLC, Shanghai, China), following the manufacturer's protocols.

Treatment with inhibitors

After seeding N2a cells for 24 hr, cells were pretreated with 4 μM GSK2656157 (MedChemExpress LLC, Shanghai, China) and 5 μM Z-ATAD-FMK (BioVision Inc., Milpitas, California) for 1.5 hr and 6 hr, respectively. Following pretreatment with inhibitors, Lipofectamine 3000 reagent was used to transfect cells with plasmids as previously described.

Apoptosis detection

Apoptosis of N2a cells was determined using the PE-Annexin V/7-AAD (BD Biosciences, USA) staining method following the manufacturer's instructions. In brief, cells in each well were washed twice with cold PBS and harvested using 0.25% trypsin solution. Growth medium was added to inactivate trypsin. Supernatants from each well were transferred to Eppendorf tubes and centrifuged at 800 RPM for 3 min. Pellets were then resuspended in 100 μL 1X Annexin V binding buffer. Five μL of PE-Annexin V and 5 μL of 7-AAD were added to the cell suspensions and mixed gently. Cells were incubated in the dark at room temperature for 15 min. After incubation, 300 μL of Annexin V binding buffer was added to each test tube. Apoptosis of N2a cells was detected using a FACSCalibur flow cytometer (BD Biosciences, USA) within 1 hr, and the data were analyzed using FlowJo/CytExpert software. Annexin V+/7-AAD represented cells in the early-stage apoptosis, while annexin V+/7-AAD+ represented cells in the late-stage apoptosis. Mock-transfected cells represented negative control, whereas STS and TM treated cells served as positive controls.

Cell viability assay

Following transfection and/or pretreatment with inhibitors, 10 μl of CCK-8 reagent was added to cultured N2a cells in a 96-well plate and incubated for 1 hr. The absorbance of each well was determined by an absorbance microplate reader (Tecan Infinite F50, Switzerland) at a wavelength of 450 nm.

Immunoblotting

N2a cells were harvested 24 hr after plasmid transfection, and the expression levels of GRA3, protein kinase R (PRK)-like ER kinase (PERK), glucose regulated protein (GRP)-78, cleaved caspase 12, cleaved caspase 3 and C/EBP-homologous protein (CHOP) were determined by immunoblotting. In brief, cells were washed with cold PBS and lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China). The cell lysate was centrifuged at 16,000 g for 10 min at 4°C, and the supernatant was collected. Proteins (40 μg) were separated on 10 - 12% SDS–PAGE and transferred onto 0.45 μM nitrocellulose membranes (Millipore, Billerica, MA, USA). The blotting membranes were then blocked with 5% skimmed milk in 1X TBST for an hour and incubated with primary antibodies (1:1000 dilution) overnight at 4 ℃. Blots were subsequently incubated for 1 hr with respective secondary antibodies (1:4000 dilution) at room temperature. Blots were washed and probed with an ECL kit (Affinity Bioscience Ltd., Jiangsu, China). Images from blots were viewed using a Bio–Rad ChemiDoc XRS+ imaging system, and ImageJ software (Rawak Software, Inc., Stuttgart, Germany) was used to calculate the relative optical densities of each band. The relative protein expression levels were normalized to that of β-actin. Mock-transfected cells served as a negative control. Rabbit anti-caspase 12, rabbit anti-caspase 3, mouse anti-CHOP, mouse anti-β-actin, goat anti-rabbit IgG, and goat anti-mouse IgM were all purchased from Proteintech (Wuhan, Hubei, China). Rabbit anti-phospho-PERK (p-PERK) was purchased from Affinity Bioscience Ltd. (Jiangsu, China), and rabbit anti-PERK was purchased from Cell Signaling Technology Inc. (Danvers, Massachusetts, United States), rabbit anti-GRP78 was purchased from Abcam (Cambridge, United Kingdom), and rabbit anti-GFP was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA).

Real Time-PCR

Total RNA was obtained from the cells by the TRIzol method as previously described. cDNA was synthesized from purified RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Reverse transcriptase quantitative polymerase chain reactions (RT–qPCR) were performed using SYBR Green ProTaq (Takara, Tokyo, Japan) following the manufacturer's protocols. The forward and reverse primers listed in Table 1 were synthesized by General Biosystems Co., Ltd. (Anhui, China). Briefly, 1 μg of RNA was reverse transcribed to a 10 μL final volume master mix reaction. Two microliters of cDNA, 0.4 μL of forward and reverse primers, 5 μL of SYBR Green ProTaq and 2.2 μL of ddH_2O were added to make a final reaction volume of 10 μL. PCR was carried out for 45 cycles of initial denaturation for 5 sec at 94°C, annealing for 15 sec at 55°C, and extension for 1 min at 72°C using a LightCycler 96 (Roche, Basel, Switzerland). All RT-qPCR reactions were carried out in triplicate. Gene expression levels were normalized to actin levels, and data were quantified with the delta-delta CT (ΔΔCT) method.

Table 1
Oligonucleotide sequences for Mus musculus genes and *Tg*GRA3 (RT-qPCR)

Primer Name	Sequence (5' to 3')
Caspase 12-F	ACAAAGGGATAGCCACTGCT
Caspase 12-R	ACCAGTCTTGCCTACCTTCC
Caspase 3-F	AAGGAGCAGCTTTGTGTGTG
Caspase 3-R	GGCAGGCCTGAATGATGAAG
PERK - F	CGGCAGGTCCTTGGTAATCA
PERK – R	CGTCCAAATCCCACTGCTTT
CHOP (C/EBP) - F	TCGCTCTCCAGATTCCAGTC
CHOP (C/EBP) – R	ACTGACCACTCTGTTTCCGT
GRP78 – F	GGTGGGCAAACCAAGACATT
GRP78 – R	TCAGTCCAGCAATAGTGCCA
Actin - F	AACTAGGCTGCTCCCTGAAG
Actin - R	TGCAAAGGATCCCGCTTAGA
GRA3 - F	TTCTCGCCGCCTACTACATT
GRA3 - R	TGTGTCCAATCTGCGTCAAC

Abbreviations: PERK Protein Kinase R (PRK)-like ER Kinase, GRP78 78-kDa glucose-regulated protein, CHOP C/EBP homologous protein, GRA3 Dense Granule Proteins.

Statistical analysis

Data are presented as mean ± SD of three or more independent experiments. A two-tailed independent Student's *t* test was used to determine the differences between pEGFP- and pEGFP-GRA3$_{Wh6}$-transfected N2a cells. One-way ANOVA was used to compare the GRA3 expression levels among RH, ME49, and Wh3 and Wh6 strains. A *P* value < 0.05 was considered statistically significant. Analysis was performed using GraphPad Prism 8 Version 8.02

Results

High expression of GRA3 in type II ME49 and Chinese 1 Wh6 strains

To understand the role of GRA3 in the virulence of *T. gondii*, first, GRA3 primers were used to amplify the GRA3 sequence in cDNA obtained from the wild-type (RH), type II strain (ME49) and the Chinese 1 Wh3

(virulent) and Wh6 (avirulent) parasite strains, and GRA3 mRNA expression levels were determined in each strain using RT–qPCR. Significant increases in GRA3 expression were observed in the ME49 ($P<0.05$) and Wh6 ($P<0.0001$) strains compared to the RH and Wh3 strains, respectively. However, the expression level of GRA3 in Wh6 strain was higher than in the ME49 strain ($P<0.05$) (Fig. 1). As such, we sought to compare the DNA sequences between $GRA3_{Wh6}$ and $GRA3_{ME49}$, the sequence alignment results revealed that $GRA3_{Wh6}$ was as same as $GRA3_{ME49}$, which is consistent with our previous study [26]. Due to the highest expression of GRA3 in Wh6 strain among the RH, ME49 and Wh3 strains, we adopted $GRA3_{Wh6}$ for subsequent experiments.

The expression of $GRA3_{Wh6}$ in N2a cells

To determine whether pEGFP-$GRA3_{Wh6}$ plasmid could be efficiently expressed in neural cells, we transfected both pEGFP and pEGFP-$GRA3_{Wh6}$ plasmids into the mouse neuroblastoma line N2a cells and determined the expression of $GRA3_{Wh6}$ protein. Twenty-four hours after transfection, GFP fluorescence was observed in both pEGFP- and pEGFP-$GRA3_{Wh6}$-transfected N2a cells. The fluorescence signal in pEGFP-$GRA3_{Wh6}$-transfected N2a cells was higher than that in pEGFP-transfected cells (Fig. 2a). To further confirm the presence of pEGFP and pEGFP-$GRA3_{Wh6}$ plasmids in N2a cells, proteins were extracted from the transfected cells 24 hr after transfection, and the expression of GFP and GFP-$GRA3_{Wh6}$ fusion protein was determined by immunoblotting. GFP and GFP-$GRA3_{Wh6}$ fusion proteins were blotted at 28 kDa and 58 kDa, respectively (Fig. 2b). We noted that the observed molecular weight of GRA3 was ~30 kD, which was similar to previous studies [21, 23].

Reduced cell viability and apoptosis of neuronal cells transfected with $GRA3_{Wh6}$ In Vitro

To investigate whether GRA3 could impact the survival of neural cells, the cell viability and rate of apoptosis in N2a cells transfected with either pEGFP or pEGFP-$GRA3_{Wh6}$ were analyzed. Mock-transfected N2a cells showed no loss of viability (100%), whereas STS, which represented a positive control, decreased viability of N2a cells to 27.9%. On the other hand, pEGFP-$GRA3_{Wh6}$-transfected N2a cells significantly decreased cell viability ($t_{(2)}$ =6.099, P =0.0009, 46.2% vs 78.4%) compared to pEGFP-transfected N2a cells (Fig. 3a). To further determine the rate of apoptosis, flow cytometry analysis using Annexin V-PE/7-AAD staining assay was performed. The results showed that pEGFP-$GRA3_{Wh6}$-transfected N2a cells significantly promoted apoptosis ($t_{(2)}$ =54.09, P =0.0001, 44.8% *vs* 6.1%) compared to pEGFP-transfected N2a cells. The apoptotic rate in the mock-transfected cells was 5.2%, which was similar to that in pEGFP-transfected N2a cells (Fig. 3b).

ER stress-mediated apoptosis induced by $GRA3_{Wh6}$ in N2a cells

Our previous studies have identified certain effector proteins that can induce programmed cell death in mouse N2a and human choriocarcinoma JEG-3 cells via ER stress signaling pathways [10, 11, 20]. Here, we sought to determine whether the dense granule effector protein GRA3 activated ER stress-induced cell death. N2a cells were transfected with either pEGFP or pEGFP-$GRA3_{Wh6}$ vectors, and the expression levels

of ER stress-related proteins and apoptosis-associated proteins were assessed. Immunoblotting showed significantly increased ER stress-associated proteins, such as GRP78, an ER associated molecular chaperone ($t_{(2)}$ =7.937, P = 0.0014), and p-PERK ($t_{(2)}$ =5.422, P = 0.0056), in pEGFP-GRA3$_{Wh6}$-transfected cells compared with pEGFP-transfected cells. Consistent with the results obtained from ER stress-associated proteins, cells transfected with pEGFP-GRA3$_{Wh6}$ showed elevated levels of apoptosis-mediated proteins such as C/EBP homologous protein (CHOP) ($t_{(2)}$ = 3.514, P = 0.0246), cleaved caspase 12, ($t_{(2)}$ = 7.542, P = 0.0017) and cleaved caspase 3 ($t_{(2)}$ = 3.154, P = 0.0344) compared to pEGFP-transfected N2a cells (Fig. 4). These results suggested that GRA3 of Wh6 strain might induce ER stress-mediated apoptosis in neural cells.

Toxoplasma **GRA3 elevated the expression of ER stress- and apoptosis-associated genes**

Following immunoblotting, we performed RT–qPCR to further compare the mRNA expression levels of the associated apoptosis and ER stress genes between pEGFP- and pEGFP-GRA3$_{Wh6}$-transfected N2a cells. The results showed that pEGFP-GRA3$_{Wh6}$-transfected cells had elevated mRNA levels of GRP78 ($t_{(2)}$ = 2.800, P = 0.0488), PERK ($t_{(2)}$ = 7.186, P = 0.0020), CHOP ($t_{(2)}$ = 7.052, P = 0.0021), caspase-12 ($t_{(2)}$ = 21.090, P = 0.0003) and caspase-3 ($t_{(2)}$ = 4.066, P = 0.0153) compared to pEGFP-transfected N2a cells (Fig. 5).

Attenuation of GRA3-induced N2a cell apoptosis following pretreatment with GSK2656157 and Z-ATAD-FMK

Cell viability and apoptosis were analyzed in pEGFP-GRA3$_{Wh6}$-transfected N2a cells pretreated with GSK2656157 (PERK inhibitor, 4 μM, 1.5 hr) and Z-ATAD-FMK (Caspase-12 inhibitor, 5 μM, 6 hr). Mock-transfected N2a cells showed 100% viability and 8.4% apoptosis. pEGFP-transfected cells showed 89.6% viability and 12.3% apoptosis. treatment with TM decreased N2a cell viability to 48.6% and increased apoptosis to 61%. N2a cells transfected with pEGFP-GRA3$_{Wh6}$ significantly reduced cell viability ($t_{(2)}$ = 27.86, P <0.0001, 59.5% *vs* 89.6%) and increased cell apoptosis ($t_{(2)}$ = 40.74 P <0.0001, 37.7% *vs* 12.3%) when compared to pEGFP-transfected N2a cells. Our results showed that pretreatment with GSK2656157 and Z-ATAD-FMK significantly increased cell viability ($t_{(2)}$ = 10.22, P = 0.0005 and $t_{(2)}$ = 12.86, P = 0.0062) and significantly decreased apoptosis ($t_{(2)}$ = 29.56, P <0.0001 and $t_{(2)}$ = 12.86, P = 0.0002) in pEGFP-GRA3$_{Wh6}$-transfected cells (Fig. 6a, 6b). Furthermore, we analyzed the related proteins expressions of ER stress associated apoptosis in pEGFP-GRA3$_{Wh6}$-transfected cells following pretreatment with GSK2656157 and Z-ATAD-FMK. Immunoblotting results revealed that GRA3 increased the expression of cleaved caspase-12 ($t_{(2)}$ = 4.613, P = 0.0099), cleaved caspase-3 ($t_{(2)}$ = 8.118, P = 0.0013), p-PERK ($t_{(2)}$ = 5.989, P = 0.0039) and CHOP ($t_{(2)}$ = 8.373, P = 0.0011) when compared to pEGFP-transfected cells. Compared to pEGFP-GRA3$_{Wh6}$-transfected cells, the expression levels of p-PERK, cleaved caspase-12 ($t_{(2)}$ = 4.771, P = 0.0088), cleaved caspase-3 ($t_{(2)}$ = 10.110, P = 0.0005) and CHOP ($t_{(2)}$ = 5.668, P = 0.0048) were significantly decreased in pEGFP-GRA3$_{Wh6}$-transfected cells pretreated with GSK2656157. Similarly,

the protein expression levels of cleaved caspase-12 ($t_{(2)}$ = 3.471, P = 0.0256) and cleaved caspase-3 ($t_{(2)}$ = 6.980, P = 0.0022) were significantly decreased in Z-ATAD-FMK-pretreated pEGFP-GRA3$_{Wh6}$-transfected N2a cells (Fig. 6c). Collectively, these results demonstrated that GRA3$_{Wh6}$ induces ER stress-associated apoptosis *via* PERK pathway.

Discussion

Toxoplasmosis is known to be one of the most common parasitic infections that infects warm-blooded animals, including humans and pets. Nearly a third of the human population have been chronically infected with *T. gondii* [27]. *T. gondii* can infect neural cells, where it forms cysts that remain for a lifetime in the host, causing chronic subclinical neuroinflammation [3, 28].

During cell invasion, secretory proteins such as dense granule proteins and rhoptry proteins (ROPs) are released into the host nucleus and cause considerable harm [29]. Several studies have explored how parasite proteins such as ROP16, ROP18, GRA15 and GRA60 contribute to *T. gondii* virulence either by activating or subverting host defense mechanisms [10, 20, 30].

Although *Toxoplasmic* GRA3 participates in nutrient acquisition for the parasite, it has also been identified to be closely associated with strain virulence [23]. However, the mechanism is enigmatic. This study aimed to determine the mechanism by which GRA3 contributes to *T. gondii* virulence.

An important finding from the present study was that the GRA3 expression levels in avirulent type II ME49 and Chinese 1 Wh6 strains were significantly higher (P< 0.05 and P< 0.001, respectively) compared with those in the virulent type I RH and Chinese I Wh3 strains (Fig. 1). This was consistent with our previous study, which revealed that the expression level of GRA3 in the Wh6 strain was significantly higher than that in the RH and Wh3 virulent strains [31, 32].

The ER is responsible for the production of cellular organic molecules, including proteins, sterols, carbohydrates, and lipids [33–36]. Its role in protein folding is critical for cell survival. Cellular disturbances such as infections and reactive oxygen species can interfere with the normal functions of the ER. These cellular disturbances cause ER stress [37]. To alleviate this stress, the ER-localized transmembrane signaling proteins, i.e., inositol-requiring protein 1 (IRE1)-α, protein kinase R (PRK)-like ER kinase (PERK) and activating transcription factor 6 (ATF6), activate UPR to restore cellular homeostasis [14, 15]. However, an unremitted UPR can activate UPR-mediated inflammatory and apoptotic pathways, resulting in cell death [16, 17]. We, for the first time in this study, demonstrate that the ER-*Toxoplasma* GRA3 interaction activates downstream apoptotic cascades in *T. gondii*-infected mouse N2a cells via the ER stress pathway.

The protein folding function of the ER requires the presence of Ca^{2+}-dependent ER molecular chaperone proteins. GRP78, commonly known as BiP, is one of the most-studied ER chaperone proteins [38]. Aside from being critical for protein quality control and thus sensing and targeting misfolded and/or unfolded proteins for degradation, GRP-78 controls the activation of ER-stress transducers and acts as an ER

stress sensor [39]. Within the cell, GRP78 levels are kept relatively low; however, they are upregulated in response to stressors that alter ER and Ca^{2+} homeostasis [40]. In our study, we demonstrated that *T. gondii* $GRA3_{Wh6}$ induced ER stress in N2a, which significantly increased GRP78 mRNA and protein levels after a 24 hr transfection with $GRA3_{Wh6}$ (Fig. 4, 5). This observation is similar to our previous study, which involved the transfection of carcinoma JEG-3 cells with *Toxoplasma* $GRA15_{II}$. In that study, pEGFP-$GRA15_{II}$ increased the expression levels of GRP78. This suggests that the ER-GRA3 interaction induces ER stress and that the levels of GRP78 are upregulated in an attempt to restore homeostasis.

Caspase-12 plays a crucial role in ER stress-mediated cell death. Under ER stress conditions, procaspase-12 is cleaved, and the activated forms accumulate (Nakagawa *et al*., 2000). Here, $GRA3_{Wh6}$ was found to cleave pro-caspase-12 into active caspase-12, accelerating apoptosis. Having demonstrated that GRA3-induced ER stress activates caspase 12, we next examined the downstream targets of GRA3-induced apoptosis following caspase 12 activation. We observed that the activation of caspase 12 resulted in the activation of caspase 3, as demonstrated by the increased cleaved caspase-3 expression in $GRA3_{Wh6}$-transfected mouse N2a cells. Our results showed that GRA3 induced the activation of caspase-12 which contributes to the pathogenesis of encephalitis during *T. gondii* infection. Moreover, pretreatment of N2a cells with the caspase-12 inhibitor Z-ATAD-FMK significantly decreased cleaved caspase-12 and cleaved caspase-3 protein expression levels; consequently, Z-ATAD-FMK downregulated apoptosis in $GRA3_{Wh6}$ N2a cells.

As an important initiator of the unfolded protein response (UPR), PERK dimerizes and autophosphorylates upon dissociation from GRP78. The kinase domain is then activated by phosphorylation of PERK, which then targets substrates such as eIF2a to activate the cascade [41]. The PERK signaling pathway is activated in response to excessive amounts of misfolded proteins in the ER and temporarily blocks protein translation, which results in neuronal cell death [42, 43]. Our results showed that GRA3 significantly activated ER stress and UPR, as observed by the significantly increased levels of phosphorylated PERK proteins following immunoblotting. Similarly, qPCR results showed that the mRNA levels of PERK were elevated in GRA3-infected N2a cells. Consistent with previous findings, our immunoblotting results showed that pretreatment of N2a cells with GSK2656157, a PERK inhibitor, significantly suppressed phosphorylated PERK expression. CCK8 and Annexin V-PE/7-AAD apoptosis assays revealed that N2a cells that were pretreated with GSK26561157 also suppressed neuronal cell death 24 hr after GRA3 transfection. Furthermore, inhibition of PERK downregulated CHOP, cleaved caspase-12, and cleaved caspase-3 expression. GSK2606414 was shown to have neuroprotective effects by rescuing the loss of dendritic development and number of synapses in neurons following traumatic brain injury and decreasing the expression of downstream targets such phospho-eIF2a, ATF4, and CHOP [44, 45]. Therefore, initiation of UPR by signaling through the PERK pathway appears to play a crucial role in GRA3-mediated ER stress apoptosis.

CHOP plays a pathologic role in ER stress-related diseases. During unremitted UPR, activation of PERK results in phosphorylation of the eukaryotic translation initiation factor (eIF2), resulting in general

translational block. However, ATF4 (activating transcription factor 4) is translated, activating downstream targets such as C/EBP homologous protein (CHOP) [46]. PERK-ATF4-CHOP pathway activation during prolonged UPR induces apoptosis [47]. Our results showed that the mRNA and protein expression levels of CHOP in N2a cells were significantly increased 24 hr after *T. gondii* $GRA3_{Wh6}$ transfection, which translated into increased N2a cell death, as observed in cell viability and cell apoptosis flow cytometry assays. This further indicates that $GRA3_{Wh6}$ induces ER stress and activates the PERK-ATF4-CHOP signaling pathway to induce apoptosis in neuronal cells.

Conclusion

In conclusion, we have come a long way in our understanding of this protozoan parasite and its interaction with host cells. Our study highlights the mechanism by which dense granule protein (GRA3) increases the virulence of *T. gondii*. $GRA3_{Wh6}$ induces neuronal apoptosis via the endoplasmic reticulum stress-mediated apoptosis pathway. This study provides further understanding of the mechanisms by which *T. gondii* causes neuropathology.

Abbreviations

GRA 3: Dense granule protein 3

CAML: Calcium modulating cyclophilin ligand

ER: Endoplasmic reticulum

UPR: Unfolded protein response

HIV*:* Human immunodeficiency virus

N2a: Neuro2a

ROP: Rhoptry protein

cDNA: complementary DNA

mRNA: messenger RNA

DNA: deoxyribonucleic acid

RNA: Ribonucleic acid

GRP78: glucose regulated protein-78

PERK: protein kinase R (PRK)-like ER kinase

CHOP: C/EBP-homologous protein

SDS–PAGE: sodium dodecyl sulfate-polyacrylamide gels

Declarations

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Availability of data and materials

Data are available from the corresponding authors upon reasonable request.

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Author Contributions

Jian Du, Jilong Shen: Conceived and designed the experiments. Cudjoe Obed: Performed experiments, Data analysis, Writing - review & editing. Minmin Wu, Jie Wang and Fang Liu performed the experiments and data analysis. Ying Chen: Review & editing. Ran An, Haijian Cai, Qingli Luo, Li Yu: Formal analysis. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

There are no competing interests with regard to the work reported in this manuscript.

References

1. Meng M, Zhou A, Lu G, Wang L, Zhao G, Han Y, Zhou H, Cong H, Zhao Q, Zhu XQ *et al*: **DNA prime and peptide boost immunization protocol encoding the Toxoplasma gondii GRA4 induces strong protective immunity in BALB/c mice**. *BMC infectious diseases* 2013, **13**:494.
2. Jones JL, Parise ME, Fiore AE: **Neglected parasitic infections in the United States: toxoplasmosis**. *Am J Trop Med Hyg* 2014, **90**(5):794-799.
3. Parlog A, Schluter D, Dunay IR: **Toxoplasma gondii-induced neuronal alterations**. *Parasite immunology* 2015, **37**(3):159-170.
4. Robben J, Hertveldt K, Bosmans E, Volckaert G: **Selection and identification of dense granule antigen GRA3 by Toxoplasma gondii whole genome phage display**. *The Journal of biological chemistry* 2002, **277**(20):17544-17547.
5. Niedelman W, Gold DA, Rosowski EE, Sprokholt JK, Lim D, Farid Arenas A, Melo MB, Spooner E, Yaffe MB, Saeij JP: **The rhoptry proteins ROP18 and ROP5 mediate Toxoplasma gondii evasion of the murine, but not the human, interferon-gamma response**. *PLoS pathogens* 2012, **8**(6):e1002784.

6. Dubey JP: **Toxoplasmosis of animals and humans**: CRC press; 2016.
7. Shwab EK, Saraf P, Zhu X-Q, Zhou D-H, McFerrin BM, Ajzenberg D, Schares G, Hammond-Aryee K, van Helden P, Higgins SA *et al*: **Human impact on the diversity and virulence of the ubiquitous zoonotic parasite *Toxoplasma gondii***. *Proceedings of the National Academy of Sciences* 2018, **115**(29):E6956-E6963.
8. Martin S: **Congenital toxoplasmosis**. *Neonatal network : NN* 2001, **20**(4):23-30.
9. Petersen E: **Toxoplasmosis**. *Seminars in fetal & neonatal medicine* 2007, **12**(3):214-223.
10. Wan L, Gong L, Wang W, An R, Zheng M, Jiang Z, Tang Y, Zhang Y, Chen H, Yu L *et al*: **T. gondii rhoptry protein ROP18 induces apoptosis of neural cells via endoplasmic reticulum stress pathway**. *Parasites & vectors* 2015, **8**:554.
11. An R, Tang Y, Chen L, Cai H, Lai D-H, Liu K, Wan L, Gong L, Yu L, Luo Q *et al*: **Encephalitis is mediated by ROP18 of Toxoplasma gondii, a severe pathogen in AIDS patients**. *Proceedings of the National Academy of Sciences of the United States of America* 2018, **115**(23):E5344-E5352.
12. Inácio P, Zuzarte-Luís V, Ruivo MT, Falkard B, Nagaraj N, Rooijers K, Mann M, Mair G, Fidock DA, Mota MM: **Parasite-induced ER stress response in hepatocytes facilitates Plasmodium liver stage infection**. *EMBO reports* 2015, **16**(8):955-964.
13. Hetz C, Mollereau B: **Disturbance of endoplasmic reticulum proteostasis in neurodegenerative diseases**. *Nature reviews Neuroscience* 2014, **15**(4):233-249.
14. Liu CY, Kaufman RJ: **The unfolded protein response**. *Journal of cell science* 2003, **116**(10):1861-1862.
15. Bravo R, Parra V, Gatica D, Rodriguez AE, Torrealba N, Paredes F, Wang ZV, Zorzano A, Hill JA, Jaimovich E *et al*: **Endoplasmic reticulum and the unfolded protein response: dynamics and metabolic integration**. *International review of cell and molecular biology* 2013, **301**:215-290.
16. Sprenkle NT, Sims SG, Sánchez CL, Meares GP: **Endoplasmic reticulum stress and inflammation in the central nervous system**. *Molecular neurodegeneration* 2017, **12**(1):42.
17. Bellezza I, Grottelli S, Mierla AL, Cacciatore I, Fornasari E, Roscini L, Cardinali G, Minelli A: **Neuroinflammation and endoplasmic reticulum stress are coregulated by cyclo(His-Pro) to prevent LPS neurotoxicity**. *The international journal of biochemistry & cell biology* 2014, **51**:159-169.
18. Zhou J, Gan X, Wang Y, Zhang X, Ding X, Chen L, Du J, Luo Q, Wang T, Shen J *et al*: **Toxoplasma gondii prevalent in China induce weaker apoptosis of neural stem cells C17.2 via endoplasmic reticulum stress (ERS) signaling pathways**. *Parasites & vectors* 2015, **8**:73.
19. Wang T, Zhou J, Gan X, Wang H, Ding X, Chen L, Wang Y, Du J, Shen J, Yu L: **Toxoplasma gondii induce apoptosis of neural stem cells via endoplasmic reticulum stress pathway**. *Parasitology* 2014, **141**(7):988-995.
20. Wei W, Zhang F, Chen H, Tang Y, Xing T, Luo Q, Yu L, Du J, Shen J, Zhang L: **Toxoplasma gondii dense granule protein 15 induces apoptosis in choriocarcinoma JEG-3 cells through endoplasmic reticulum stress**. *Parasites & vectors* 2018, **11**(1):251.

21. Kim JY, Ahn HJ, Ryu KJ, Nam HW: **Interaction between parasitophorous vacuolar membrane-associated GRA3 and calcium modulating ligand of host cell endoplasmic reticulum in the parasitism of Toxoplasma gondii**. *The Korean journal of parasitology* 2008, **46**(4):209-216.
22. Holloway MP, Bram RJ: **Co-localization of Calcium-modulating Cyclophilin Ligand with Intracellular Calcium Pools**. *Journal of Biological Chemistry* 1998, **273**(26):16346-16350.
23. Craver MP, Knoll LJ: **Increased efficiency of homologous recombination in Toxoplasma gondii dense granule protein 3 demonstrates that GRA3 is not necessary in cell culture but does contribute to virulence**. *Mol Biochem Parasitol* 2007, **153**(2):149-157.
24. Nam HW: **GRA proteins of Toxoplasma gondii: maintenance of host-parasite interactions across the parasitophorous vacuolar membrane**. *The Korean journal of parasitology* 2009, **47 Suppl**(Suppl):S29-37.
25. Feng P, Park J, Lee BS, Lee SH, Bram RJ, Jung JU: **Kaposi's sarcoma-associated herpesvirus mitochondrial K7 protein targets a cellular calcium-modulating cyclophilin ligand to modulate intracellular calcium concentration and inhibit apoptosis**. *Journal of virology* 2002, **76**(22):11491-11504.
26. Cheng W, Wang C, Xu T, Liu F, Pappoe F, Luo Q, Xu Y, Lu F, Shen J: **Genotyping of polymorphic effectors of Toxoplasma gondii isolates from China**. *Parasit Vectors* 2017, **10**(1):580.
27. Halonen SK, Weiss LM: **Toxoplasmosis**. *Handbook of clinical neurology* 2013, **114**:125-145.
28. Weiss LM, Dubey JP: **Toxoplasmosis: A history of clinical observations**. *International journal for parasitology* 2009, **39**(8):895-901.
29. Laliberté J, Carruthers VB: **Host cell manipulation by the human pathogen Toxoplasma gondii**. *Cellular and molecular life sciences : CMLS* 2008, **65**(12):1900-1915.
30. Nyonda MA, Hammoudi PM, Ye S, Maire J, Marq JB, Yamamoto M, Soldati-Favre D: **Toxoplasma gondii GRA60 is an effector protein that modulates host cell autonomous immunity and contributes to virulence**. *Cellular microbiology* 2021, **23**(2):e13278.
31. Cheng W, Liu F, Li M, Hu X, Chen H, Pappoe F, Luo Q, Wen H, Xing T, Xu Y *et al*: **Variation detection based on next-generation sequencing of type Chinese 1 strains of Toxoplasma gondii with different virulence from China**. *BMC Genomics* 2015, **16**:888.
32. Li M, Mo X-W, Wang L, Chen H, Luo Q-L, Wen H-Q, Wei W, Zhang A-M, Du J, Lu F-L *et al*: **Phylogeny and virulence divergency analyses of Toxoplasma gondii isolates from China**. *Parasites & vectors* 2014, **7**(1):133.
33. Reid DW, Nicchitta CV: **Diversity and selectivity in mRNA translation on the endoplasmic reticulum**. *Nature reviews Molecular cell biology* 2015, **16**(4):221-231.
34. Rapoport TA: **Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes**. *Nature* 2007, **450**(7170):663-669.
35. Braakman I, Hebert DN: **Protein folding in the endoplasmic reticulum**. *Cold Spring Harbor perspectives in biology* 2013, **5**(5):a013201.

36. Westrate LM, Lee JE, Prinz WA, Voeltz GK: **Form follows function: the importance of endoplasmic reticulum shape**. *Annual review of biochemistry* 2015, **84**:791-811.
37. Zhang K, Kaufman RJ: **From endoplasmic-reticulum stress to the inflammatory response**. *Nature* 2008, **454**(7203):455-462.
38. Adams CJ, Kopp MC, Larburu N, Nowak PR, Ali MMU: **Structure and Molecular Mechanism of ER Stress Signaling by the Unfolded Protein Response Signal Activator IRE1**. *Frontiers in Molecular Biosciences* 2019, **6**(11).
39. Wang M, Wey S, Zhang Y, Ye R, Lee AS: **Role of the unfolded protein response regulator GRP78/BiP in development, cancer, and neurological disorders**. *Antioxid Redox Signal* 2009, **11**(9):2307-2316.
40. Casas C: **GRP78 at the Centre of the Stage in Cancer and Neuroprotection**. *Frontiers in Neuroscience* 2017, **11**(177).
41. Bell MC, Meier SE, Ingram AL, Abisambra JF: **PERK-opathies: An Endoplasmic Reticulum Stress Mechanism Underlying Neurodegeneration**. *Current Alzheimer research* 2016, **13**(2):150-163.
42. Radford H, Moreno JA, Verity N, Halliday M, Mallucci GR: **PERK inhibition prevents tau-mediated neurodegeneration in a mouse model of frontotemporal dementia**. *Acta Neuropathologica* 2015, **130**(5):633-642.
43. Meng C, Zhang J, Dang B, Li H, Shen H, Li X, Wang Z: **PERK Pathway Activation Promotes Intracerebral Hemorrhage Induced Secondary Brain Injury by Inducing Neuronal Apoptosis Both in Vivo and in Vitro**. *Frontiers in Neuroscience* 2018, **12**(111).
44. Sen T, Gupta R, Kaiser H, Sen N: **Activation of PERK Elicits Memory Impairment through Inactivation of CREB and Downregulation of PSD95 After Traumatic Brain Injury**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2017, **37**(24):5900-5911.
45. Atkins C, Liu Q, Minthorn E, Zhang SY, Figueroa DJ, Moss K, Stanley TB, Sanders B, Goetz A, Gaul N *et al*: **Characterization of a novel PERK kinase inhibitor with antitumor and antiangiogenic activity**. *Cancer research* 2013, **73**(6):1993-2002.
46. Ron D, Walter P: **Signal integration in the endoplasmic reticulum unfolded protein response**. *Nature Reviews Molecular Cell Biology* 2007, **8**(7):519-529.
47. Walter F, Schmid J, Düssmann H, Concannon CG, Prehn JHM: **Imaging of single cell responses to ER stress indicates that the relative dynamics of IRE1/XBP1 and PERK/ATF4 signalling rather than a switch between signalling branches determine cell survival**. *Cell Death & Differentiation* 2015, **22**(9):1502-1516.

Figures

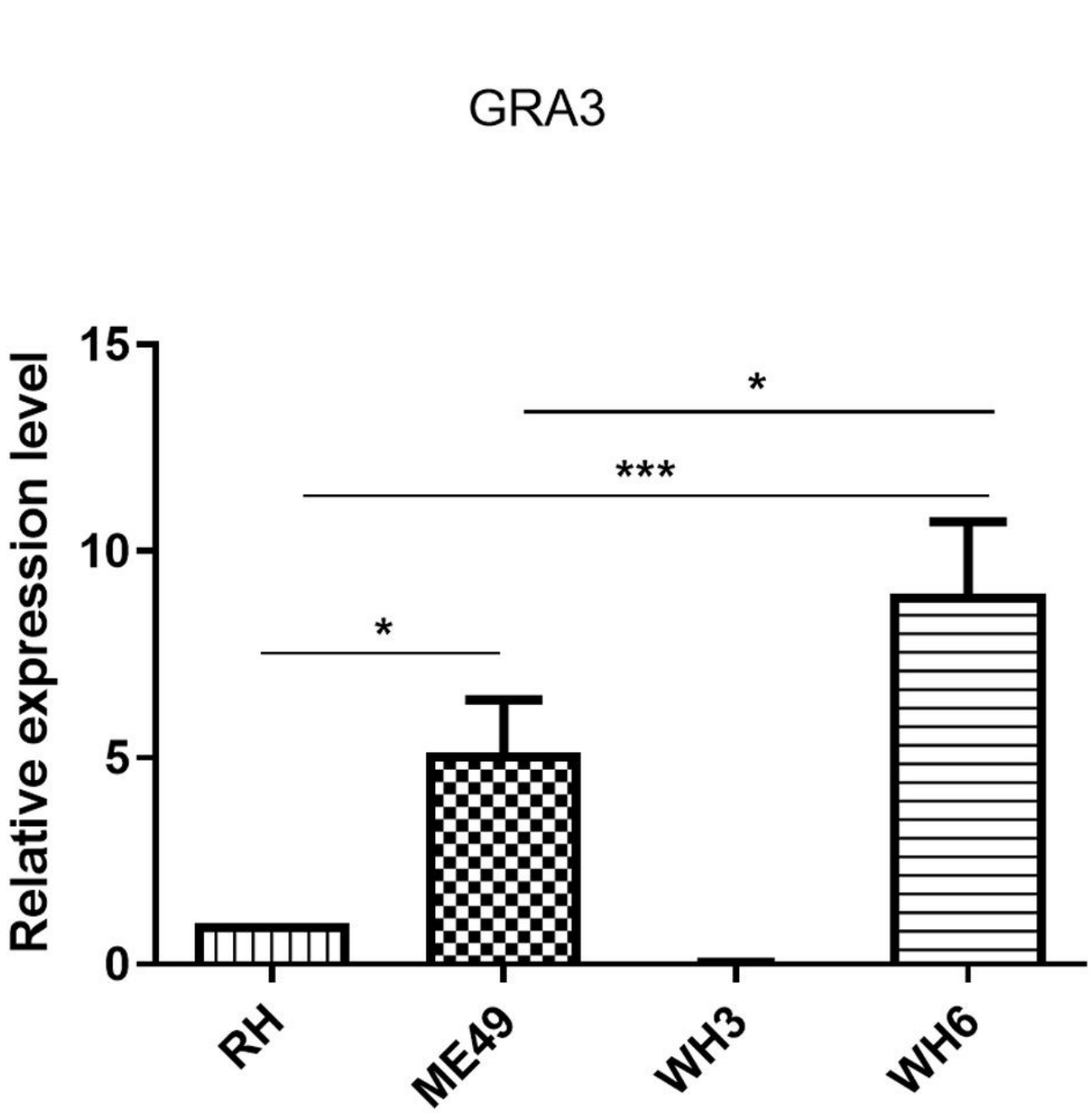

Figure 1

Dense Granule Protein 3 (GRA3) Gene Expression Among Different Isolates. GRA3 expression levels were compared between virulent RH and Wh3 strains and less virulent ME49 and Wh6 strains. The RT-qPCR was performed in triplicate and values were expressed as mean ± SD. *P<0.05 ***P < 0.001.

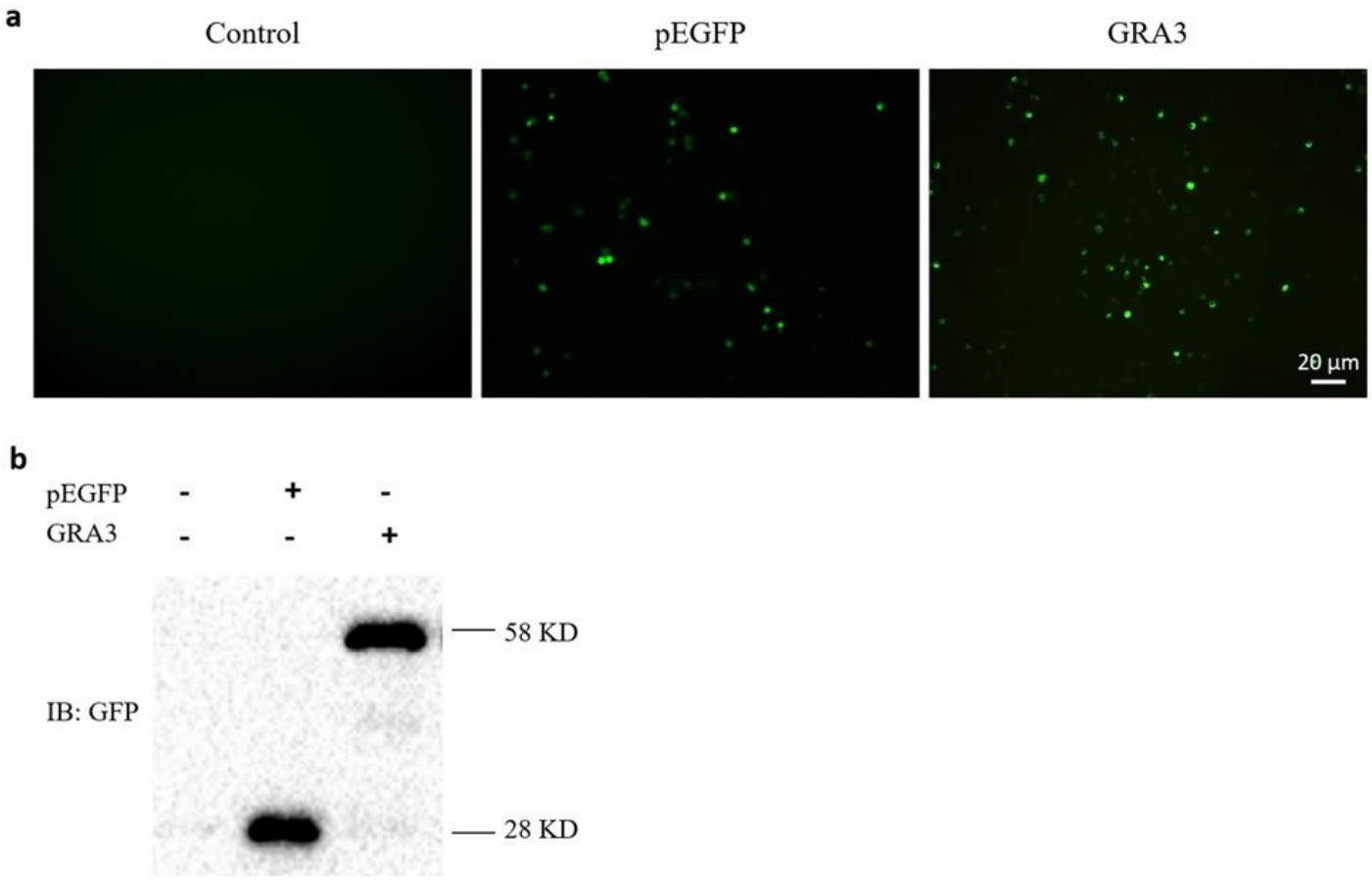

Figure 2

The Expression of Dense Granule Protein 3 ($GRA3_{Wh6}$). Mouse neuroblastoma (N2a) cells were transfected with either a control pEGFP vector or pEGFP-$GRA3_{Wh6}$ vector for a period of 24h. Mock transfected N2a cells served as a negative control. **a** The expression of green fluorescent protein (GFP) was observed using fluorescent microscope. Scale-bar: 20μm. **b** The expression of $GRA3_{Wh6}$ was confirmed by immunoblotting.

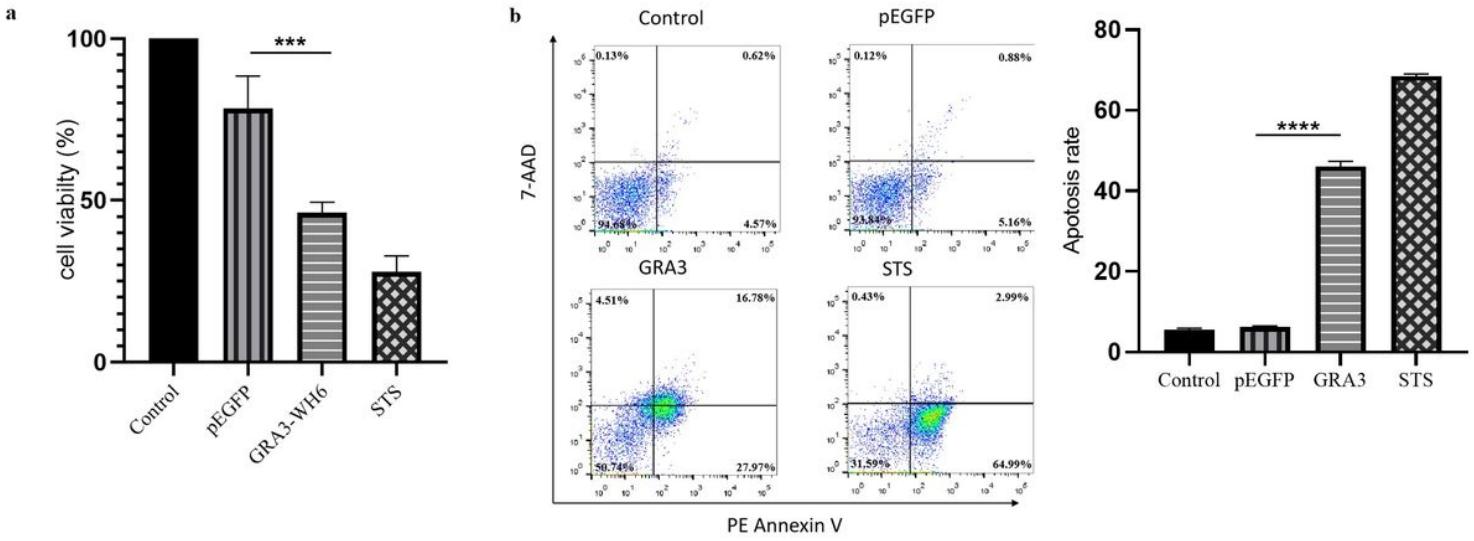

Figure 3

Dense granule protein 3 ($GRA3_{Wh6}$)-induced loss of cell viability and apoptosis. N2a cells were transfected with either a control vector (pEGFP) or pEGFP-$GRA3_{Wh6}$ vector for a 24h period. Mock transfected N2a cells served as the negative control, and N2a cells treated with staurosporine (STS) (1 μM, 12 h) served as the positive control. **a** Cell viability was measured using the CCK-8 (Cell Counting Kit-8) assay. **b** Apoptosis of cells were determined using flow cytometry after staining with Annexin V-PE/ /7-AAD.The plots are from a representative measurement and the data were expressed as mean ± SD on three different assays (n = 3). ***P < 0.001, ****P < 0.0001 vs. pEGFP transfected N2a cells.

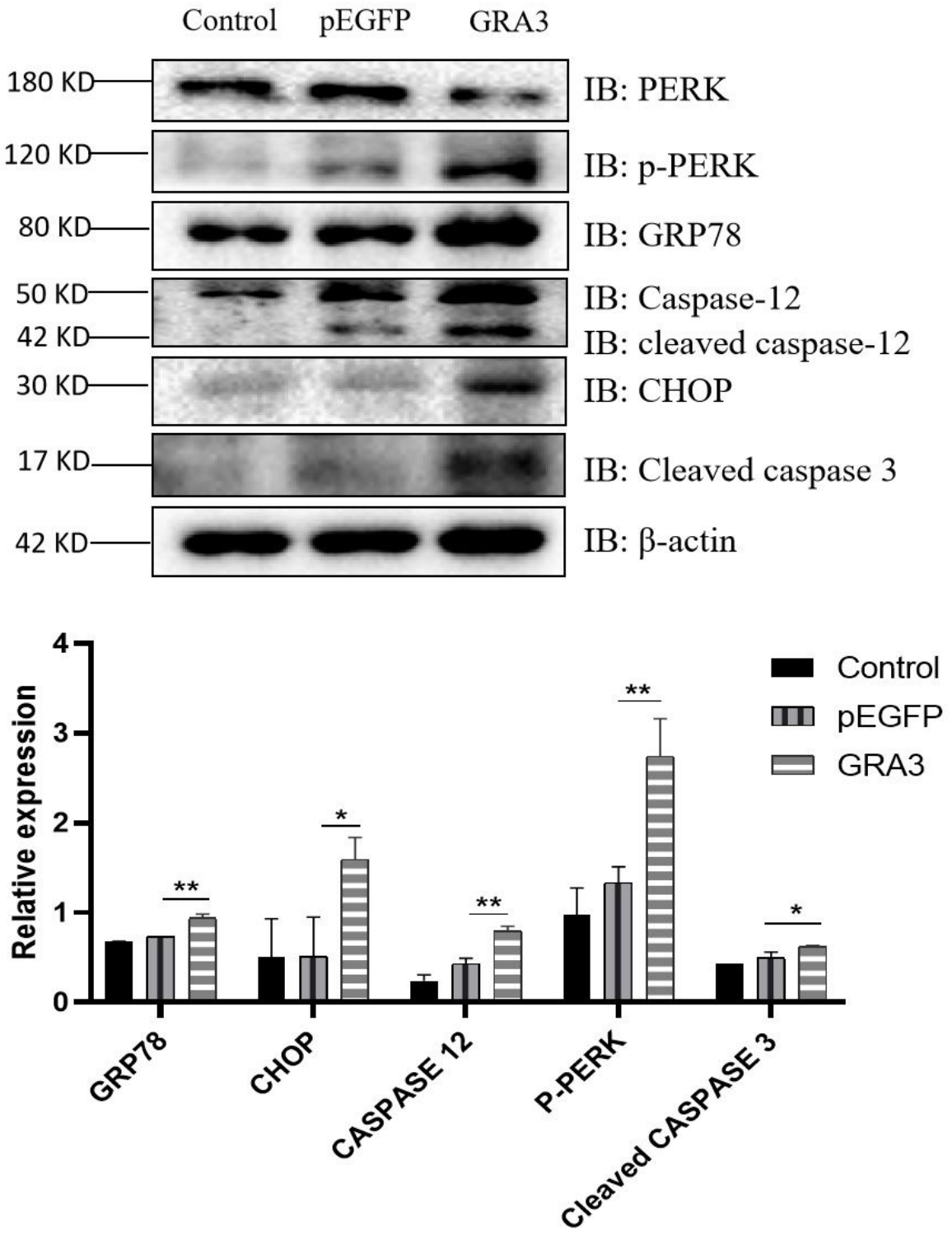

Figure 4

Expression of apoptosis-associated proteins and Endoplasmic reticulum stress (ERS) proteins. N2a cells were transfected with either pEGFP vector or pEGFP-GRA3$_{Wh6}$ vector for a 24 hr period. Then the expression levels of ER stress- and apoptosis-associated proteins were determined by immunoblotting. Mock transfected cells served as the negative control. Abbreviations: CHOP, C/EBP homologous protein; GRP78, 78-kDa glucose-regulated protein; PERK, PRK-like ER kinase; P-PERK, phosphorylated PERK. Data

were expressed as mean ± SD on three different assays (n = 3). *P < 0.05, **P < 0.01, when compared to the pEGFP-transfected N2a cells.

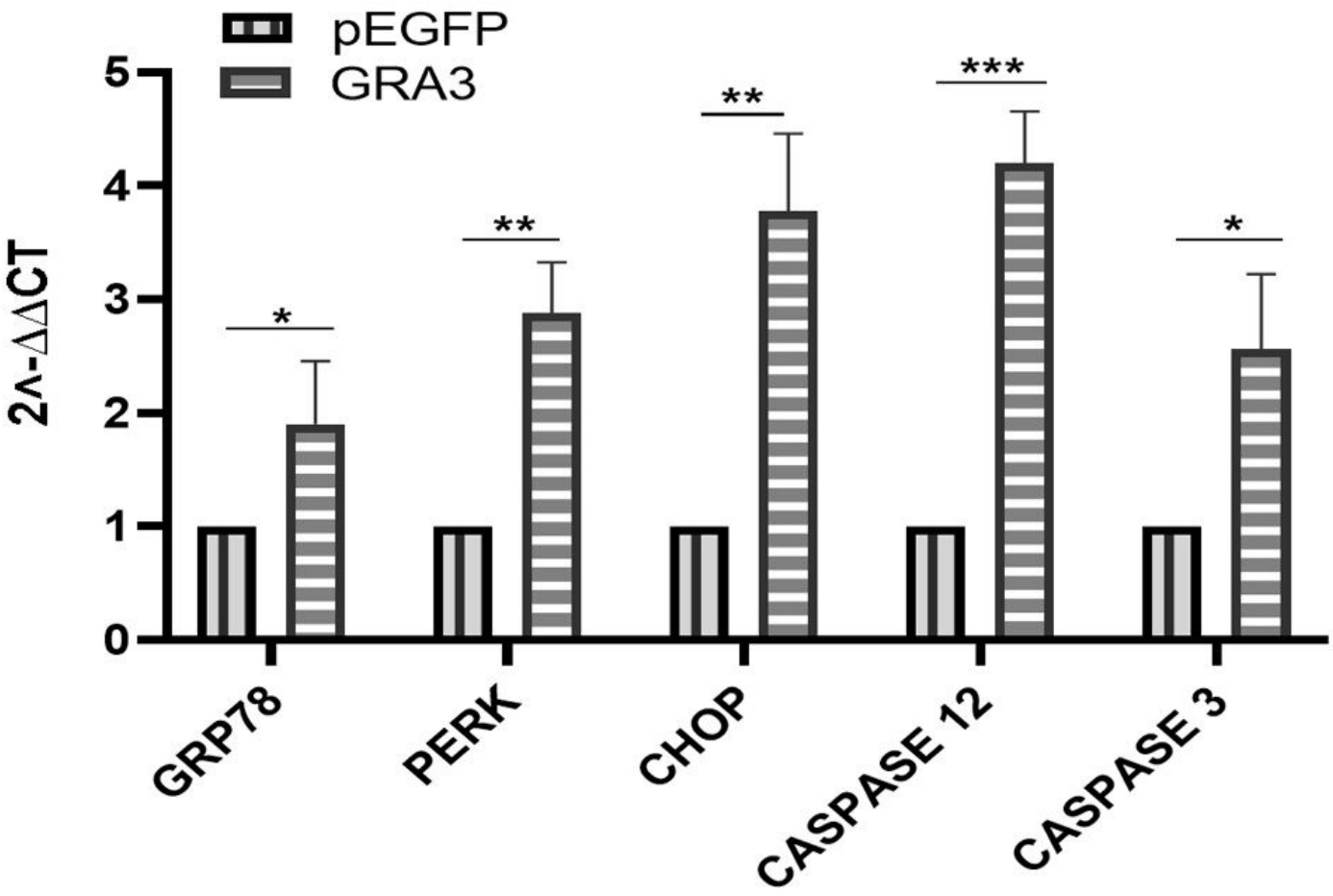

Figure 5

Transcription levels of apoptosis-associated genes. N2a cells were transfected with either a control vector (pEGFP) or pEGFP-GRA3$_{Wh6}$ vector for a 24h period. mRNA expressions of the associated apoptosis and ER stress genes were measured using RT-qPCR. Abbreviations: GRP78, 78- kDa glucose-regulated protein; CHOP, C/EBP homologous protein; PERK, PRK-like ER kinase. Data were expressed as mean ± SD on three different assays (n = 3). *P < 0.05, **P < 0.01, when compared to the pEGFP-transfected cells.

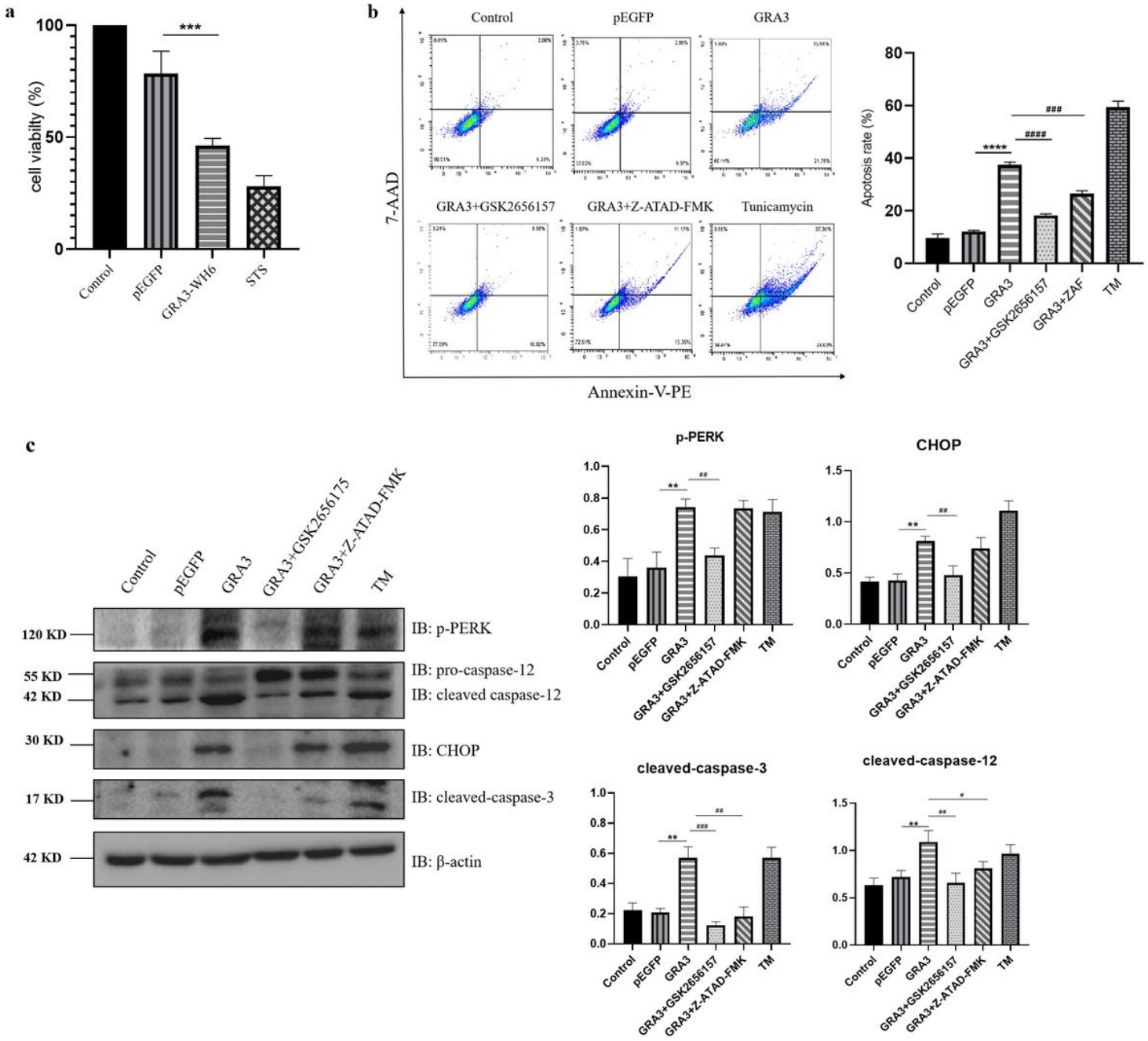

Figure 6

Effects of PERK and Caspase-12 inhibitors on loss of cell viability and apoptosis pEGFP-GRA3-WH6 transfected N2a cells. N2a cells were treated with or without GSK2656157 (4μM) and Z-ATAD-FMK (ZAF, 5μM) for 1.5 h and 6 h respectively, and transfected with either a control vector (pEGFP, plasmid encoding enhanced green fluorescent protein) or pEGFP-GRA3$_{Wh6}$ vector for 24h. **a** Cell viability was measured using the CCK-8 assay. **b** Apoptosis of cells were determined using flow cytometry after staining with Annexin V-PE/ /7-AAD. The plots are from a representative measurement and the data were expressed as mean ± SD on three different assays (n = 3). **c** The protein expression levels of ER stress- and apoptosis-related proteins were determined by immunoblotting. The data were expressed as mean ± SD on three different assays (n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs. pEGFP transfected N2a cells. $^{\#}$P <

0.05, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ vs. pEGFP-GRA3$_{Wh6}$ transfected N2a cells. pEGFP-GRA3$_{Wh6}$ + GSK2656257 represents N2a cells pretreated with GSK2656157, followed by transfection with pEGFP-GRA3$_{Wh6}$ plasmid. pEGFP-GRA3$_{Wh6}$ + ZAF represents N2a cells pretreated with ZAF, followed by transfection with pEGFP-GRA3$_{Wh6}$ plasmid.

Supplementary Files

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