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Research paper

Germinated brown rice protects against glutamate toxicity in HT22 hippocampal neurons through the jnk-mediated apoptotic pathway via the $GABA_A$ receptor

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ABSTRACT

The anti-apoptosis effect of germinated brown rice (GBR) focusing on differentiated HT22 cells results in improved nutritional values after the germination process of GBR which contains total phenolic compounds and γ-aminobutyric acid (GABA). Cell death induced by 5 mM glutamate was investigated for 24 h to determine whether GBR mediates cell death through GABA receptors by using antagonists. The results showed that GBR (100 μg/ml) suppressed glutamate-induced cytotoxicity and caused arrest at the G1/S phase of the cell cycle in differentiated HT22 cells. Furthermore, GBR significantly decreased the expression level of c-Jun, while its active form, p-c-Jun, is the downstream product of the JNK-mediated apoptotic pathway and causes subsequent cell death. In addition, bicuculline (12.5 nM), a $GABA_A$ antagonist, could eliminate GBR effects, but phaclofen (1 mM), a $GABA_B$ antagonist, could not. Surprisingly, GBR exhibited a better neuroprotective effect than a pure commercial GABA compound (0.115 μM). These results indicated that GBR possessed high anti-apoptotic activity and inhibited cell death in differentiated HT22 cells by perturbing re-entry of the cell cycle and apoptosis via the $GABA_A$ receptor. Hence, GBR could be further used as a valuable nutritional compound to prevent apoptosis-induced neurodegenerative diseases.

1. Introduction

Neurodegenerative disease is mostly associated with neuronal damage after oxidative stress, which may cause apoptosis initiated by DNA damage. Neurotransmitter imbalances in the brain, especially glutamate, play a principal role in pathological brain functions. Normally, glutamate is an important promoter of neural differentiation and migration during brain development. However, excessive glutamate concentrations can initially cause neuronal cytotoxicity. Glutamate cytotoxicity is mediated by nonreceptor-mediated oxidative stress and receptor-initiated excitotoxicity (Maher and Schubert, 2000; Mattson, 2000). In the oxidative toxicity pathway induced by glutamate, cystine uptake via the cystine/glutamate antiporter is decreased by glutamate, leading to a lack of cellular glutathione, while neuronal amino acid receptors are overactivated (excitotoxicity) (Mattson, 2000). Decreased glutathione levels accelerate various downstream signaling pathways, leading to cell death via abnormal calcium uptake in neurons. Likewise, glutamate cytotoxicity seems to cause mitochondrial dysfunction, thereby initiating cell death pathways by intracellular Ca^{2+} accumulation and oxidative stress-induced apoptosis (Maher and Schubert, 2000).

Depending on the neuron type, two pathways are associated with apoptosis-induced glutamate: the caspase-3-dependent (D'Amelio et al., 2010) and caspase-independent pathways. The latter employs apoptosis-inducing factor (AIF, a kind of mitochondrial-membrane

Abbreviations: GBR, germinated brown rice; GABA, gamma-aminobutyric acid; Glu, glutamate; Bic, bicuculline; Pha, phaclofen; GABRG2, $GABA_A$ receptor (γ2 subunit); NMDA, N-methyl-D-aspartate receptors; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; MAPKs, mitogen-activated protein kinase; ROS, reactive oxygen species; JNKs, c-Jun N-terminal kinases; HT22, mouse hippocampal neuronal cell line.

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flavoprotein with NADH oxidase properties) and calpain (Polster et al., 2005). Intracellular Ca^{2+} accumulation activates the truncation process of pro-apoptotic Bid to tBid mediated by calpain, and then activates the Bax apoptotic protein (Tobaben et al., 2011). Oxidative stress generation leads to mitochondrial dysfunction and activates translocation of AIF from mitochondria to the nucleus, which then induces apoptosis via a caspase-independent process (Daugas et al., 2000; Xu et al., 2007; Landshamer et al., 2008).

Glutamate-induced oxidative stress also triggers phosphorylation of mitogen-activated protein kinases (MAPKs), including extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (Ray et al., 2012). MAPK activation is involved in both cell growth and death, especially induction of apoptosis gene transcription (Choi et al., 2010). In addition, p38 and JNK signaling can be activated by neuronal injury, which results from various factors, e.g., inflammation or cytokines, and ERK signaling is responsive to apoptosis induced by oxidative stress (Tibbles and Woodgett, 1999; Chang and Karin, 2001; Nozaki et al., 2001).

Systemic inflammation, particularly due to proinflammatory cytokines, can cause learning and memory deficits and impair functioning of the dentate gyrus owing to synaptic plasticity (Kelly et al., 2003). During inflammation, the activation of $GABA_A$ receptors can increase from low levels under baseline conditions in the dentate gyrus (Glykys and Mody, 2007) and in the frontal cortex after a stroke due to ischaemic causes (Clarkson et al., 2010). In addition, a decrease in the overwhelming tonic inhibition induced by GABA after a stroke could promote functional recovery (Clarkson et al., 2010). In hippocampal neurons, the tonic inhibitory current caused by $GABA_A$ receptors was enhanced by the proinflammatory cytokine, interleukin-1β, through the p38 MAPK signaling pathway (Wang et al., 2012) and was decreased by inverse agonists for $GABA_A$ receptors (Wang et al., 2007; Wang et al., 2012). In the hippocampus, the inverse agonist of $GABA_A$ receptors is tolerated and can decrease memory loss due to the limited expressions of receptors (Pirker et al., 2000). In addition, GABA and its receptor agonist can improve cognitive decline in ageing monkeys (Leventhal et al., 2003). Administration of muscimol and baclofen, agonists of the $GABA_A$ receptor and $GABA_B$ receptor, respectively, in deprivation of oxygen and glucose either in global or focal cerebral ischaemia, exerted neuroprotection by attenuating the excitatory JNK3 apoptotic signaling pathway (Han et al., 2008). The use of inverse agonists for GABA receptors is therefore indicated as a promising treatment of choice.

Large amounts of GABA in polished rice or pregerminated brown rice can pass the blood-brain barrier (García et al., 1998) and enhanced memory and learning in mice during the Morris water maze test (Mamiya et al., 2004). Germinated brown rice (GBR), with its higher amount of GABA and total phenolic contents, has been previously reported as an antioxidant and anti-apoptotic substance (Park et al., 2010; Azmi et al., 2013) and exhibited neuroprotective effects regarding death of dopaminergic cells and deficits in motor functions in rats with Parkinson's-like disease induced by rotenone (Chompoopong et al., 2016) in mice with vascular cognitive impairment and in HT22 mouse hippocampal cells with toxicity induced by glutamate (Oo et al., 2020). Particularly in HT22 cells, the p38 and JNK pathways are MAPK signaling molecules involved in apoptotic death activated by glutamate (Fukui et al., 2009). Therefore, the protective effects of GBR via suppression of glutamate-induced oxidative stress in hippocampal neuronal HT22 cells may be a promising strategy for use as a nutraceutical for neurodegenerative diseases both therapeutically and prophylactically. In addition, it is important to elucidate whether the excitatory JNK3 apoptotic process is associated with the mediating effects of GBR via $GABA_A$ or $GABA_B$ receptors.

2. Materials and methods

2.1. Materials

The HT22 mouse hippocampal neuronal cell line (SCC129); glutamate (G5889), GABA (A5835); bicuculline ($GABA_A$ antagonist, B7686); phaclofen ($GABA_B$ antagonist, P118); and MTT (3-[4,5-dimethylthiazol-2-yl]−2,5-diphenyltetrazolium bromide) assays were purchased from Sigma—Aldrich (St. Louis, MO, USA). DMEM powder (1889145); foetal bovine serum (10270), Neurobasal™-A media (10888022); B27™ supplement (17504044); and L-glutamine (25030081) were purchased from Thermo Fisher Scientific (MA, USA). A Micro BCA™ Protein Assay Kit (23235) was purchased from Thermo Fisher Scientific (MA, USA). Anti-c-Jun mouse monoclonal antibodies (05–1076), anti-p-c-Jun rabbit polyclonal antibodies at serine 73 (06−659), anti-β-actin mouse monoclonal antibody clones AC-74 (A2228), affinity purified donkey anti-rabbit IgG, affinity purified peroxidase-conjugated secondary antibodies, and rabbit anti-mouse IgG antibodies with HRP conjugate were purchased from Millipore Sigma (Massachusetts, USA). Western ECL substrate (170–5060) was purchased from Bio-Rad (California, USA). Primary antibody-labelled polyclonal GABRG2 (PA5–23698) and secondary antibody-conjugated Alexa Fluor 488 (A11034) were purchased from Invitrogen (California, USA). An apoptosis detection kit (556547) was purchased from BD Pharmingen (San Diego, USA) and included propidium iodide (PI, 51–66211E) and fluorescein isothiocyanate (FITC)-labelled Annexin V (AV, 51–65874X) reagent.

2.2. Preparation of GBR and standard control of GABA

GBR was prepared from a paddy rice called Khao Dawk Mali 105 (KDML 105), provided by the Ban-Srangming community enterprise, Muang Sam Sip district, Ubon Ratchathani province, located in the northeastern part of Thailand. Briefly, according to a previous study by Oo et al. (2020); 500 g of paddy rice was prepared by germination processes, freeze-dried, and ground into fine powder. For this experiment, GBR powder was dissolved in sterile distilled water to obtain the GBR extract, warmed at 70 °C for 30 min, and centrifuged at 2990 g for 30 min at 4 °C. The supernatant was then harvested by passing the extract through a 0.45-μm filter and was stored at − 20 °C for the experiment. The protective effect of GBR at a concentration of 100 μg/ml on the viability of HT22 cells has been previously reported when compared to 0.125 μM pure GABA compound (Sigma-Aldrich, A5835, BioXtra, 99%, USA) (Oo et al., 2020). In addition, Wunjuntuk et al. (2016) has shown GABA composition at 11.9 mg/100 g of parboiled GBR in protection against oxidative stress in rats. In this study, the molarity of pure GABA was calculated from its molecular weight (103.12 g/mol) and used as a standard control at 0.115 μM with respect to the GABA content (11.9 ng/ml) in 100 μg/ml GBR.

2.3. HT22 cell culture and treatments

Immortalized hippocampal neurons derived from HT22 mice were grown to 80% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with a 1% antibiotic mixture (e.g., 100 U/ml penicillin and 100 μg/ml streptomycin), 10% foetal bovine serum (FBS) and L-glutamine in a humidified atmosphere at 37 °C supplemented with 5% CO_2. Cells were differentiated in Neurobasal™-A media with 2 mM L-glutamine, B27™ and 1% antibiotic mixture for 24–72 h before treatments (He et al., 2013). For each test sample treatment, differentiated HT22 cells were seeded in 6-well plates (5×10^5 cells/well). After 24 h of incubation to allow the cells to adhere, the cells were pretreated with GBR or GABA 24 h before exposure to 5 mM glutamate as a cotreatment for an additional 24 h. For blocking with GABA receptor antagonists, cells were blocked with bicuculline ($GABA_A$ antagonist, 12.5 nM) and phaclofen ($GABA_B$ antagonist, 1 mM) prior to pre- and cotreatment with GBR or GABA for 45 min (Fig. 1). After 24 h, the

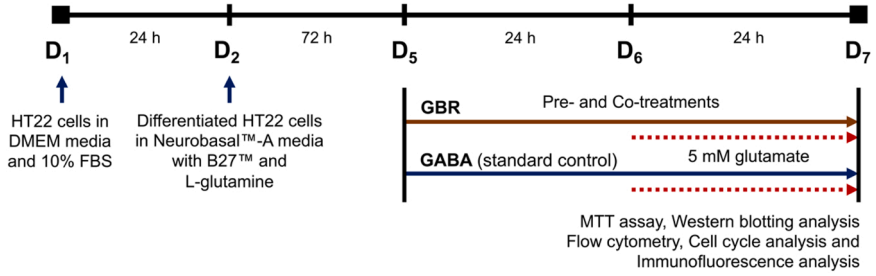

Fig. 1. Experimental design and timeline for 1 week (D1- D7).

cultures were assessed for neurotoxicity. The cell viabilities of neuronal cells were quantified by MTT, the proteins expressions in the JNK-mediated apoptotic pathway were obtained by Western blotting analysis, and apoptosis and cell cycles were analysed by using flow cytometry analysis.

2.4. Cell viability assay

The neuroprotective effect of GBR against glutamate-induced HT22 cell death was determined using the MTT assay. The differentiated HT22 cells were seeded onto 96-well plates (5×10^3 cells/well) and incubated for 24 h. After incubation, 5 mg/ml MTT solution in phosphate-buffered saline (PBS) was added to each well, and the cells were then incubated at 37 °C for 4 h. After removing the supernatant, 200 µl of DMSO was used to dissolve the MTT formazan crystals for 15 min at room temperature. The MTT formazan quantities were determined at 570 nm using a multidetection microplate reader (Biotek, USA, Synergy H1). All experiments were performed in triplicate. The cell viabilities were then compared with the control cells. Since GABA and total phenolic contents are known to improve the nutritional value of GBR, pure GABA was used as a control.

2.5. Western blotting analysis

After incubation as described above, a quantitative analysis of subsequent cell death after glutamate toxicity was performed to determine whether it was related to the JNK-mediated apoptotic pathway that could be protected by GBR. Cells were lysed in a standard cell lysis buffer, RIPA, with one tablet of cOmplete™ Mini Protease Inhibitor Cocktail on ice for 10 min, which was followed by scraping. The lysates were centrifuged at 17,200g for 10 min at 4 °C. The supernatant containing soluble proteins was collected. Total protein concentrations were measured by the Micro BCA™ Protein Assay Kit.

Protein samples (70 µg/lane) were analysed by SDS-PAGE (10% polyacrylamide gel) and blotted onto nitrocellulose membranes (100 volts, 1 h). The membranes were blocked with 5% BSA at room temperature for 1 h and then probed with primary antibodies (see Table 1) at 4 °C overnight. After washing, the membranes were incubated with secondary antibodies (Table 1) at room temperature for 60 min. Equal protein loading was achieved based on the amount of β-actin in each lane. Finally, the protein bands were visualized by developing with a Western ECL substrate detection system for 5 min in a dark room. The membranes were placed in a sheet protector and exposed to chemiluminescence by ImageQuant LAS 4000. The band intensities were determined using ImageJ software (National Institutes of Health, MD, USA).

2.6. Quantitative analysis of apoptotic cell death by flow cytometry

To study the protective effect of GBR, we examined whether it could decrease early and late apoptotic cell death. Differentiated HT22 cells were seeded in 24-well plates (3×10^4 cells/well) and incubated with the treatments mentioned above. Cells were collected using trypsin/EDTA, washed with PBS, and then centrifuged at 480 g for 5 min at 4 °C. Then, the cells were stained with FITC-labelled Annexin V and PI in Annexin V buffer for 15 min in the dark. The phosphatidylserine expressions on the cell membrane surfaces and permeabilities of DNA fluorescent dye in stained cells were measured within 1 h using a BD FACSCalibur flow cytometer with BD CellQuestTM Pro Software (BD Bioscience, CA, USA). At least ten thousand cells were collected for each data acquisition. Postacquisition analysis was performed using FlowJo™ software (Becton, Dickinson and Company, OR, USA).

2.7. Cell cycle analysis

PI staining was used to study the cell cycles to determine whether GBR could regulate and attenuate glutamate toxicity in differentiated HT22 cells. Cells were seeded in 24-well plates (5×10^4 cells/well) after incubation with the treatments mentioned above. Cells were harvested by trypsinization, fixed in cold absolute ethanol, vortexed, and stored at − 20 °C for 1 month. Then, the cells were washed with cold PBS and stained with 300 µl of PI-incubated reagent containing 200 µg/ml RNase-A and 50 µg/ml PI. After 30 min of incubation in the dark at 37 °C, the cells were centrifuged and resuspended in PBS. The PI-labelled DNA contents were measured by a BD FACSCalibur flow cytometer (Becton Dickinson, CA, USA) with BD CellQuest™ Pro Software (BD Bioscience, CA, USA), and the data were then analysed using FlowJo™ software (Becton, Dickinson and company, OR, USA).

Table 1
List of antibodies used in Western blotting (WB) and immunofluorescence (IF) analyses.

Antibodies	Applications	Hosts	MW (kDa)	Dilutions	Sources
Primary antibodies:					
Anti-c-Jun	WB	Mouse, monoclonal	~36–43 kDa	1:1000	MilliporeSigma
Anti-phospho-c-Jun (serine 73)	WB	Rabbit, polyclonal	~40 kDa	1:1000	MilliporeSigma
Anti-β-actin	WB	Mouse, monoclonal	~42 kDa	1:5000	MilliporeSigma
Anti-GABRG2	WB	Mouse, polyclonal	~55 kDa	1:1000	Invitrogen
Anti-GABRG2	IF	Mouse, polyclonal	–	1:100	Invitrogen
Secondary antibodies:					
Anti-rabbit, peroxidase conjugate	WB	Donkey	–	1:1000	MilliporeSigma
Anti-mouse, HRP conjugate	WB	Rabbit	–	1:1000	MilliporeSigma
Anti-mouse, Alexa-Fluor 488 conjugate	IF	Goat	–	1:200	Invitrogen

2.8. Immunofluorescence study

The $GABA_A$ receptor was used as a marker to demonstrate the reactive expressions in undifferentiated and differentiated HT22 cells and to support the interpretation of glutamate toxicity and GBR treatments. Cells were plated on 0.1 mg/ml poly-D-lysine-coated round coverslips (7×10^3 cells/coverslip), fixed with 4% paraformaldehyde at 4 °C for 15 min and then refixed with methanol at − 20 °C for 10 min, followed by permeabilizing incubation with 0.1% Triton X-100 in PBS at room temperature for 30 min. In antigen retrieval, citrate buffer at pH 6.0 and at a warm temperature was added to the cells for 15 min and then rewashed with 0.2% Tween 20 in PBS. Cells were then blocked with 5% normal goat serum for 2 h. Cells were probed with primary antibodies overnight at 4 °C. On the following day, autofluorescence quenching was blocked with 5% skim milk for 1 h at room temperature. After washing, the cells were incubated with secondary antibodies at room temperature in darkness for 2 h (Table 1) followed by DAPI (1:1000 dilution) for 5 min. Finally, the cells were observed using a fluorescence microscope (Nikon, Ti-S Intensilight).

2.9. Statistical analysis and graphs

All numerical experimental values are expressed as the mean ± standard error of the mean (SEM) calculated from the Statistical Package for Social Science (SPSS) program, version 18 (SPSS Inc., Chicago, USA). To compare two groups, one-way analysis of variance (ANOVA) was used, and Tukey's post-hoc multiple comparison test was then applied. Data are presented as the mean±SEM. Statistical significance was classified based on *P* values less than 0.001, 0.01, and 0.05. All results were displayed using SigmaPlot/SigmaStat 12.5 (Systat Software GmbH, Erkrath, Germany).

3. Results

3.1. Neuroprotective effect of GBR on differentiated HT22 cell toxicity caused by glutamate

Neuroprotection of GBR on differentiated HT22 cell toxicity caused by glutamate was determined by a cell viability assay (MTT). In this study, pure GABA at 0.115 μM was employed as a standard control with respect to the GABA content in 100 μg/ml GBR. All tested compounds demonstrated no cytotoxicity on HT22 cells (data not shown).

The addition of 5 mM glutamate to induce toxicity for 24 h resulted in a reduction in HT22 cell viability to 49.80 ± 3.95% ($p < 0.001$) in greater amounts than the untreated control (Fig. 2). In the pre- and cotreatment of HT22 cells, GBR cells were protected from glutamate-induced toxicity and preserved cellular viability ($p < 0.001$) significantly (90.52 ± 7.04%) when compared to the group without GBR treatment at a concentration of 100 μg/ml.

To investigate whether GABA receptor inhibition would perturb the preserved cellular viability, antagonistic bicuculline and phaclofen prior to pre- and cotreatment with GBR and GABA were used. Bicuculline, a $GABA_A$ antagonist, significantly reversed the decrease in cell viability of HT22 cells induced by glutamate toxicity in pre- and cotreated GBR cells to 63.15 ± 4.23% ($p < 0.001$). However, phaclofen, a $GABA_B$ antagonist, did not modify the preserved cellular viability in pre- and cotreated GBR cells. Similarly, in cells pre- and cotreated with GABA, the standard control indicated that the GABA contents in GBR also reversed their decrease in cell viability of HT22 by bicuculline but was not affected by phaclofen, as shown in Fig. 2.

3.2. Effects of GBR on JNK-mediated apoptosis caused by glutamate in differentiated HT22 cells

Quantitative analysis of subsequent cell death after glutamate toxicity to determine whether GBR regulated activation of the JNK-mediated apoptotic pathway in response to glutamate was performed by Western blotting analysis. The transcription factor, AP-1, contains c-Jun, which is a major component. Meanwhile, p-c-Jun at serine 73 is its active form can activate the expressions of various pro-apoptotic proteins and trigger cell death; hence, c-Jun and active p-c-Jun are involved in apoptotic cell death.

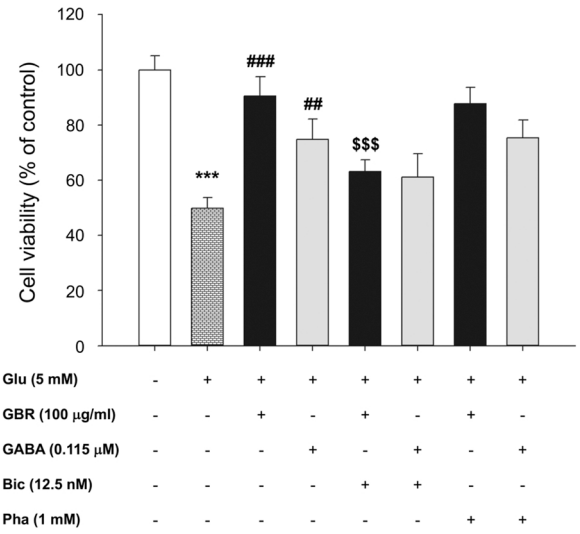

Fig. 2. MTT viability assay demonstrated the protective effects of GBR against glutamate toxicity on differentiated HT22 cells with/without the GABA antagonists, bicuculline (Bic) and phaclofen (Pha). $^{***}p < 0.001$ compared with the control; $^{###}p < 0.001$ and $^{##}p < 0.01$ compared with the cells treated with glutamate (Glu) only; $^{\$\$\$}p < 0.001$ compared with the cells treated with GBR.

After being treated with GBR for 24 h, HT22 cells were treated with both 100 μg/ml GBR and glutamate insults (5 mM). Twenty-four hours after glutamate toxicity, the c-Jun and p-c-Jun protein expression levels were analysed. Fig. 3 shows remarkable increases in c-Jun and p-c-Jun expressions of 0.90 ± 0.03 and 0.87 ± 0.04, respectively, at 24 h after glutamate treatment when compared to the control treatment ($p < 0.001$), whereas the pre- and cotreatments of GBR reversed this increase significantly to 0.48 ± 0.00 for c-Jun and 0.60 ± 0.01 for p-c-Jun when compared to cells treated with glutamate only ($p < 0.001$).

In addition, to evaluate whether the neuroprotection of GBR by regulating JNK-mediated apoptosis activation responds to glutamate mediated through activation of the GABA receptor, cells were blocked with bicuculline and phaclofen prior to pre- and cotreatment with GBR for 45 min. After 24 h of glutamate toxicity, the effects of GBR on decreasing the remarkable expressions of c-Jun and p-c-Jun were eliminated significantly by bicuculine ($p < 0.001$) but this effect was not shown when blocking with phaclofen. Similarly, cells pre- and cotreated with GABA, the standard control indicating GABA contents in GBR, their significant increases in c-Jun and p-c-Jun expressions were also reversed, and these effects were also blocked significantly by bicuculline but were not affected by phaclofen, as shown in Fig. 3.

3.3. Effect of GBR on glutamate-induced apoptosis in differentiated HT22 cells

The protective effect of GBR was examined to determine whether it could attenuate glutamate-induced apoptotic cell death or growth inhibition. Differentiated HT22 cells pre- and cotreated with GBR (100 μg/ml) were subjected to neurotoxicity induced by 5 mM glutamate with or without GABA receptor antagonists. After 24 h, photomicrographs were obtained (Fig. 4D). GBR-treated cells were not susceptible to cell death induced by glutamate toxicity, as observed in cells with higher expressions of triangular-like shapes and prolonged neurites, similar to healthy cells in the untreated control group, than in glutamate-only treated cells

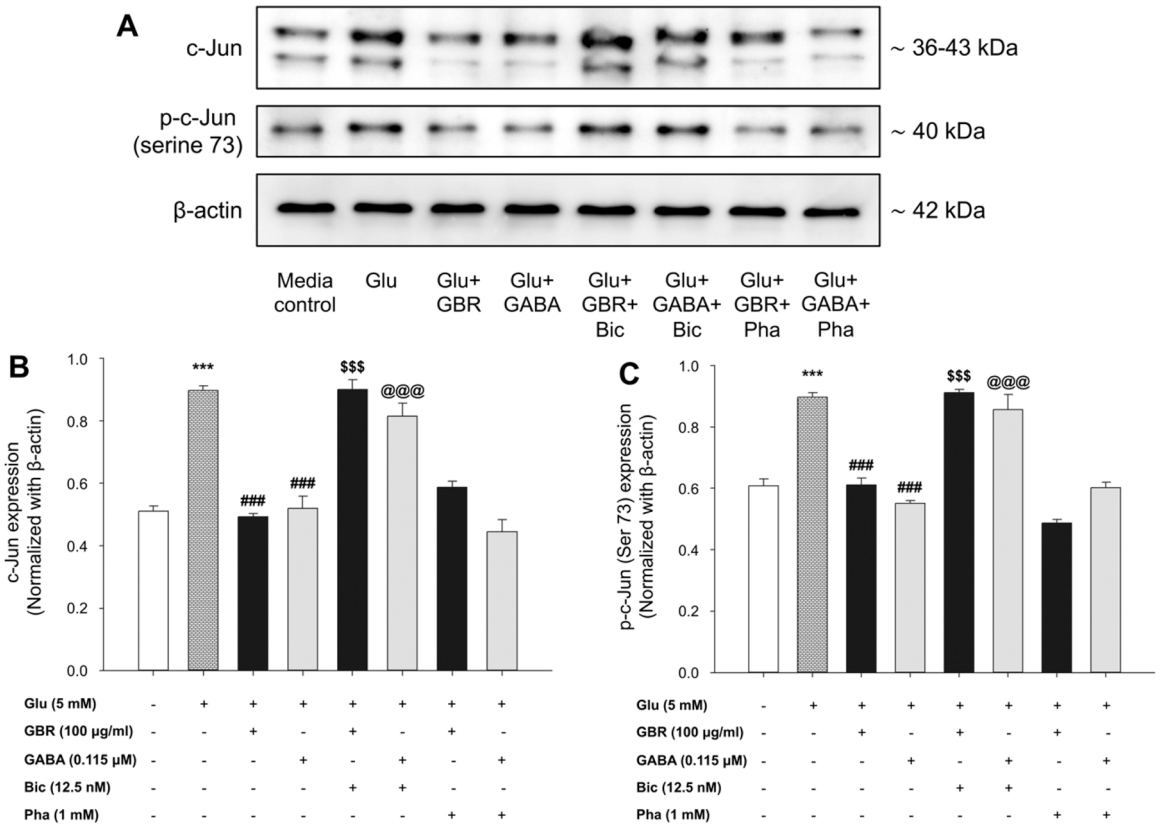

Fig. 3. The effects of GBR on JNK-mediated apoptosis activation in response to glutamate induction in HT22 hippocampal cells. After being treated with GBR or GABA for 24 h, HT22 cells were exposed to 5 mM glutamate as a cotreatment for another 24 h. The c-Jun or phosphorylated c-Jun contents in total cell lysate protein (70 μg/lane) were determined by Western blotting assays using JNK-specific antibodies. The results are presented as the mean±SEM. $^{***}p < 0.001$ compared with untreated cells, $^{\#\#\#}p < 0.001$ compared to glutamate-treated cells, $^{\$\$\$}p < 0.001$ compared to pre- and cotreated GBR cells, $^{@@@}p < 0.001$ compared with the cells treated with GABA; Bic, bicuculline; Pha, phaclofen.

with round shapes and short processes due to cell damage.

To determine ongoing cell apoptosis, cells were stained with Annexin V-FITC and PI, which was followed by FACS analysis. During glutamate-mediated apoptosis and necrosis, typical apoptotic cell death shows phosphatidylserine translocation to the outer membrane for active Annexin V binding. As shown in Fig. 4 A, the four quadrants show populations of viable cells (lower left), necrotic cells (upper left), early apoptotic cells (lower right) and late apoptotic cells (upper right). In Fig. 4B, the number of apoptotic cells significantly increased with glutamate toxicity ($p < 0.01$), while the number of apoptotic cells significantly decreased in GBR-treated cells ($p < 0.001$). These data demonstrated the ability of GBR to attenuate cell injury induced by glutamate toxicity, as evidenced by the percentage of dead cells shown in Fig. 4 C. Interestingly, GBR might act through the $GABA_A$ receptor due to significant blocking by bicuculline ($p < 0.05$) to attenuate the increase in cell apoptosis. Similarly, cells pre- and cotreated with GABA, the standard control indicating GABA contents in GBR, the significant increases in cell apoptosis were reversed, and these reversed effects were also blocked significantly by bicuculline but were not affected by phaclofen, as shown in Fig. 4B and Fig. 4 C.

3.4. GBR arrested differentiated HT22 cells at the G1 phase of the cell cycle

Recent evidence shows the relationship between apoptosis and the cell cycle; thus, cell cycle manipulation may either suppress or activate an apoptotic response depending in the cellular context. The proteins functioning in proliferation may promote cell apoptosis (Evan et al., 1995; Pucci et al., 2000). To investigate whether DNA fragmentation can be induced by glutamate, G1- and S-phase cell populations were quantitated by measuring their PI-labelled DNA contents via flow cytometry. The cell populations were gated to exclude debris cells and were normalized to untreated control cells, as shown (red dot) in Fig. 5 A. In this study, differentiated HT22 cells were evaluated, while some studies used undifferentiated HT22 cells instead. Moreover, a previous report by Liu et al. (Liu et al., 2009) suggested that proliferation of differentiated HT22 cells was suppressed and that they showed obvious outgrowth of neurites, which were morphologically similar to postmitotic neurons. Notably, after treatment with 5 mM glutamate for 24 h, the G1 cell population significantly decreased from 64.9% to 41.5% compared with the control ($p < 0.001$) (Fig. 5B). In addition, the cell cycle might be driven by glutamate from the G1 to S phase, so the number of S cells increased significantly from 17.4% to 39.2% when compared to untreated cells ($p < 0.05$). The effects of GBR on glutamate toxicity in differentiated HT22 cells showed a significant increase in the G1 cell population to 57.8% ($p < 0.01$) but a significant decrease in the S cell population to 21.3% ($p < 0.05$). These results indicated that the G1 phase of the cell cycle in differentiated HT22 cells might be arrested by GBR, leading to a decrease in S-phase entry of the cell cycle. GBR might act through the $GABA_A$ receptor due to significant blocking by bicuculline of 46.3% of G1 cells, $p < 0.05$% and 36.5% of S cells.

Similarly, in cells pre- and cotreated with GABA, the standard control indicated that the GABA contents in GBR were also reversed due to their significant decrease in G1 cells and increase in S cells, and these reversed effects were also blocked significantly by bicuculline but were not affected by phaclofen, as shown in Fig. 5 A and Fig. 5B.

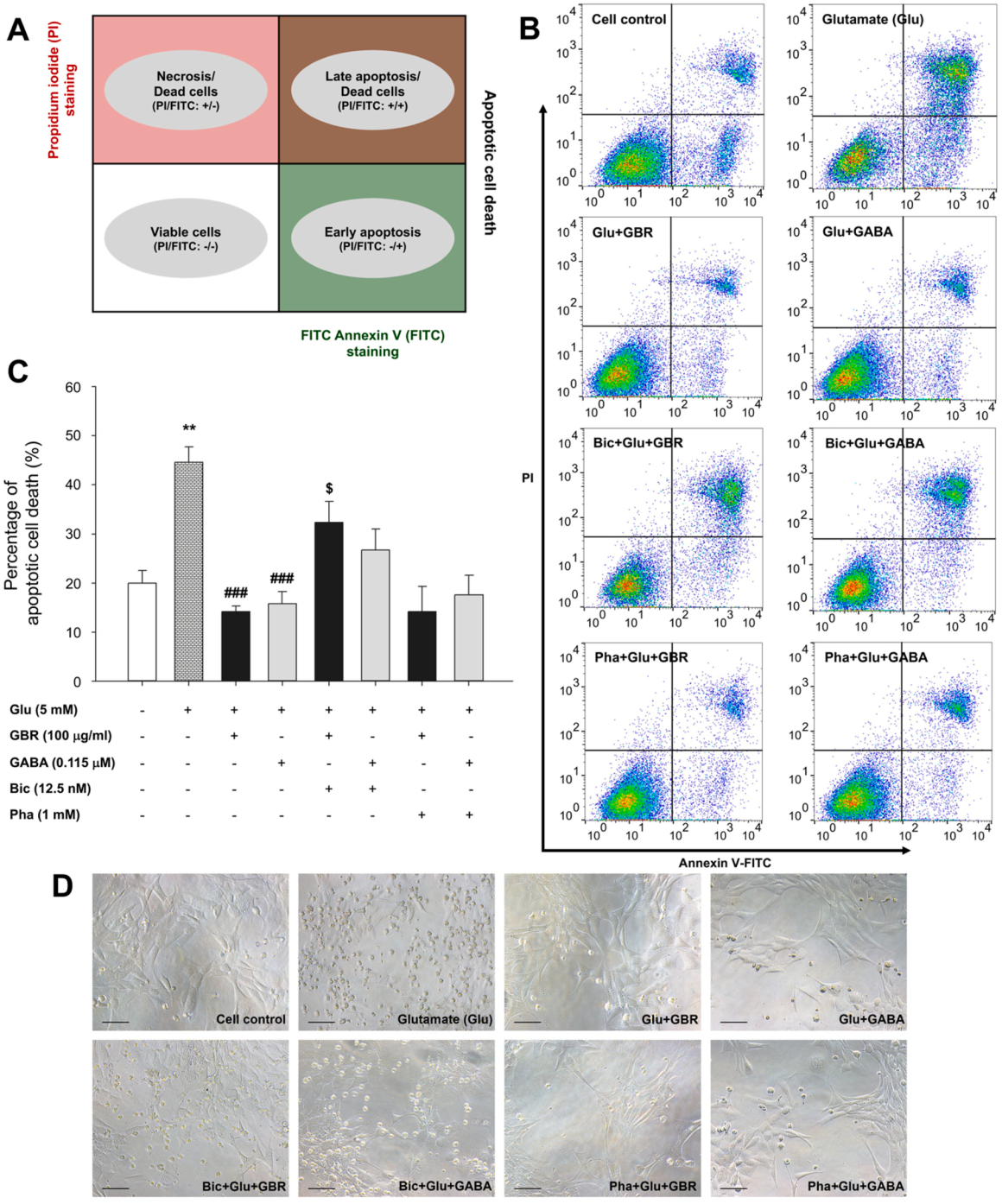

Fig. 4. GBR protected differentiated HT22 cells from apoptosis induced by glutamate. (A) Flow cytometric analyses of apoptotic and necrotic cells by Annexin V and PI staining is shown as four quadrants. The x-axis represents the loss of plasma membrane asymmetry by Annexin V binding, and the y-axis represents the loss of selective membrane permeability by PI. (B) Representative flow cytometric analyses of apoptosis assays of control cells, Glu-treated cells with GBR or GABA in the presence of Bic or Pha. (C) The percentages of apoptosis of the control and treated cells. (D) Inverted microscopic results showed the morphological changes of differentiated HT22 cells, i.e., rounder shapes with short processes in damaged cells with glutamate toxicity but fewer damaged cells under Glu+GBR treatment with/without GABA receptor antagonists. The bars indicate 100 μm. Each value is presented as the mean±SEM. $^{**}p < 0.01$ compared with the untreated one; $^{###}p < 0.001$ compared with glutamate toxicity (Glu); $^{\$}p < 0.05$ compared with *p*re- and cotreated GBR cells; Bic, bicuculline; Pha, phaclofen.

3.5. Differentiated HT22 cells showed high expression of $GABA_A$ receptors

Previous reports suggested that expressions of the strong γ2 subunit of the $GABA_A$ receptor could be observed in pyramidal cells, granule cells, neuropil in the DG and in all subregions of the hippocampus in both rats (Wisden et al., 1992; Fritschy and Mohler, 1995) and mice (Heldt and Ressler, 2007). Moreover, the protective effect of GBR on differentiated HT22 cells against glutamate toxicity, as mentioned above, was mediated through the $GABA_A$ receptor since all results exhibited blocking by bicuculine but not by phaclofen. Therefore, we evaluated whether the $GABA_A$ receptor was expressed in differentiated HT22 cells. In this study, the γ2 subunit of the $GABA_A$ receptor (GABRG2) total protein was evaluated using Western blotting analysis

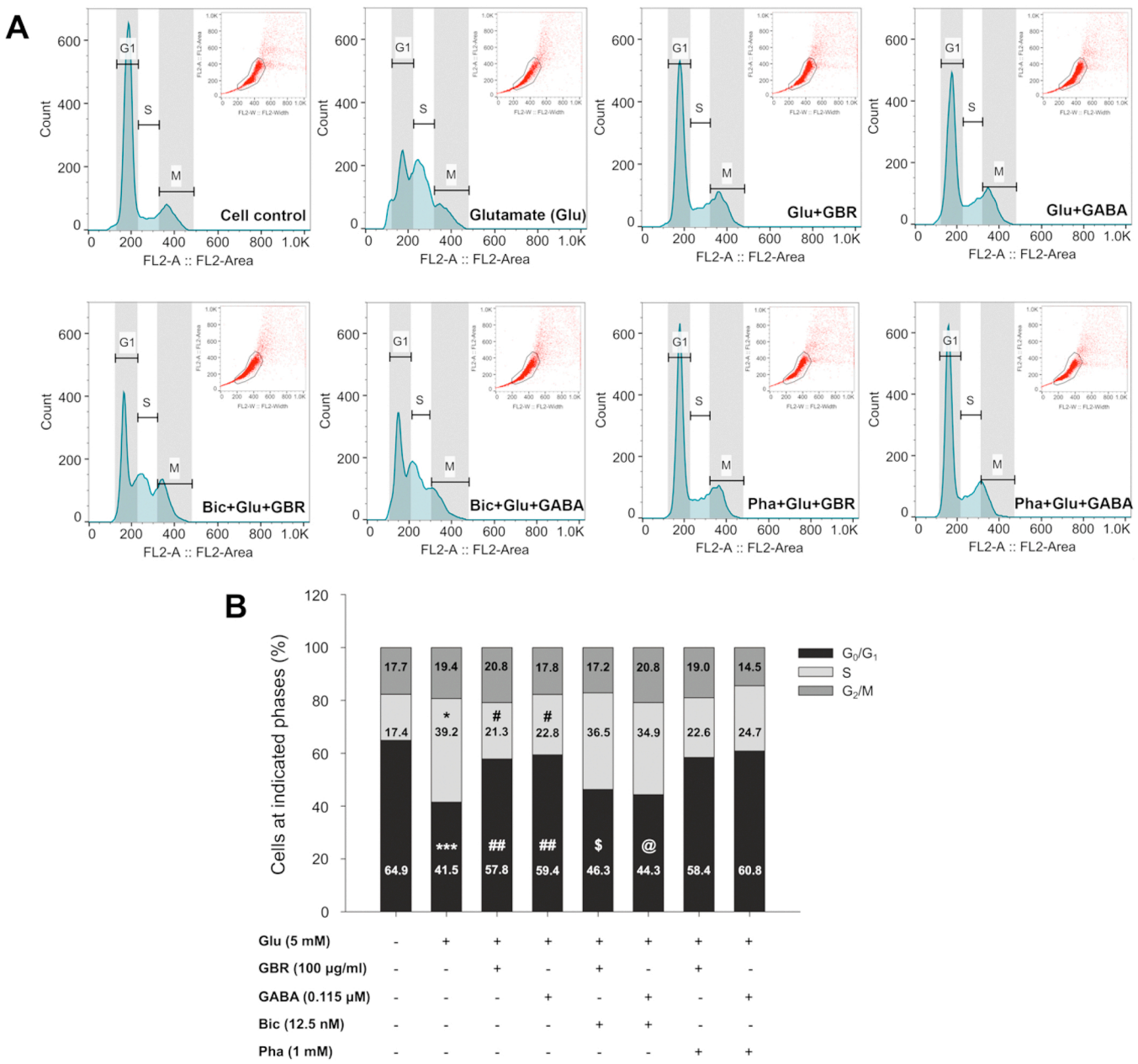

Fig. 5. The effect of GBR on differentiated HT22 cell damage by glutamate toxicity contributed to the arrest of the cell cycle in the G1 phase, as shown by flow cytometry. (A) Representative flow cytometric analysis of the cell cycle assay of control cells. Glu treated cells with GBR or GABA in the presence of Bic or Pha. The DNA content histograms represent different phases of the cell cycle: G1 population, living cells; S population, cells undergoing DNA synthesis; and M population, dividing cells. (B) Cell percentages in different phases of the control and treated cells. ***p < 0.001 and *$p < 0.05$ compared with untreated cells; $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ compared with glutamate toxicity (Glu); $^{\$}p < 0.05$ compared with pre- and cotreated GBR cells; $^{@}p < 0.05$ compared with pre- and cotreated GABA cells; Bic' bicuculline; Pha, phaclofen.

(Fig. 6 A) and visualized in HT22 cells by immunofluorescence staining and fluorescence microscopy (Fig. 6B). GABRG2-conjugated Alexa 488 (green) was expressed in the membranous soma and neurites and was more highly expressed in differentiated HT22 cells, especially at 72 h, than in undifferentiated cells.

4. Discussion

The present study aimed to investigate the neuroprotective effect of GABA receptor stimulation by using the high GABA contents in GBR and its underlying mechanisms The effects of GBR and a pure commercial GABA compound against apoptotic cell death induced by 5 mM Glu in differentiated HT22 cells were studied. GBR (100 µg/ml) suppressed Glu-mediated neurotoxicity and preserved cellular viability. GBR reduced c-Jun expression and p-c-Jun (its active form) expression, while c-Jun is the downstream product of the JNK-mediated apoptotic pathway and subsequent cell death. The percentage of cells with apoptosis decreased remarkably in the group treated with Glu+GBR and Glu+GABA ($p < 0.001$). Moreover, understanding the molecular signals that regulate cell cycle changes is valuable, and glutamate toxicity induced a decrease in the G1 phase and increase in cell cycle re-entry in differentiated HT22 cells. However, the G1 phase of the cell cycle might be arrested by GBR with a decrease in S-phase entry of the cell cycle. This regulation of cell cycle changes in differentiated HT22 cells might mitigate glutamate toxicity in the adult brain, and how the decision to exit or re-enter the cell cycle provides valuable insight into the

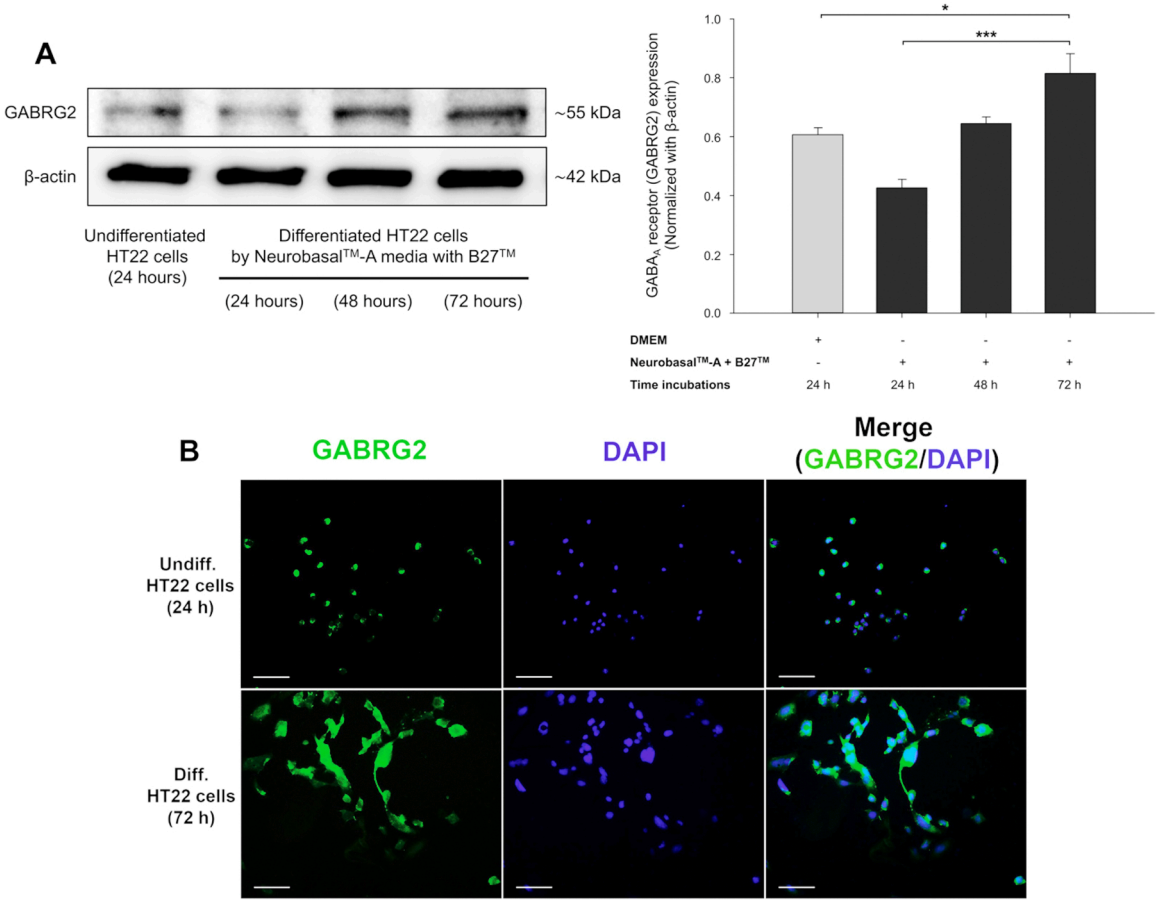

Fig. 6. Expressions of $GABA_A$ receptor (GABRG2) protein in differentiated HT22 cells. (A) Western blotting analyses and histograms show significant increases in $GABA_A$ receptor expression in a time-dependent manner at 24, 48 and 72 h) when compared to undifferentiated cells at 24 h. (B) Immunofluorescence staining. Data are presented as the mean±SEM, ***p < 0.001, differentiated cells compared between 24 h and 72 h; *$p < 0.05$ compared between undifferentiated cells at 24 h and differentiated cells at 72 h. GABRG2 protein was visualized by Alexa Fluor 488 (green) in the membrane, cytoplasm and DAPI (blue)-stained nuclei. The bars indicate 100 μm.

mechanisms underlying injury-induced neurogenesis. In addition, bicuculline, a $GABA_A$ antagonist, could eliminate all effects, but phaclofen could not. During differentiation, significantly increased expression of the $GABA_A$ receptor (GABRG2) protein in a time-dependent manner has been observed in HT22 cells. Therefore, the high GABA contents in GBR may stimulate the $GABA_A$ receptor in differentiated HT22 cells and provide neuroprotection against glutamate toxicity through the JNK-mediated apoptotic pathway.

GBR was produced from the germination process of Thai rice variety, KDML105, and was obtained from the same source as in previous studies by Oo et al. (Oo et al., 2020). In this study, 100 μg/ml GBR with high GABA content (11.5%) after germination was used according to Wunjuntuk et al. (Wunjuntuk et al., 2016), Butsat and Siriamornpun (Butsat and Siriamornpun, 2010). With respect to the GABA content in 100 μg/ml GBR, pure commercial GABA was also used as a standard control at 0.115 μM. Moreover, other bioactive constituents with antioxidant activities, including total phenolic compounds (e.g., γ-oryzanol, tocopherols, and phenolic acids) and proanthocyanidins, have been previously shown to quench unpaired oxygen radicals, resulting in a decrease in free radicals (Krinsky, 1992). Therefore, the neuroprotective effects of GBR may include GABA receptor and antioxidant activities.

Our previous findings demonstrated the significant neuroprotective effects of GBR when applied both in vivo and in vitro (Oo et al., 2020). Several studies showed that GABA receptor activation exerted a protective action against the neuronal injury induced by ischaemia or exogenously added excitatory amino acids (Jackson-Friedman et al., 1997; Lyden and Lonzo, 1994; Muir et al., 1996; Ohkuma et al.,1994). Conversely, other studies failed to show such neuroprotection (Erdö and Michler, 1990). Similar to previous reports, Han et al. (Han et al., 2008) suggested that inhibitory GABA receptor stimulation by the muscimol and baclofen agonists of $GABA_A$ and $GABA_B$ receptors, respectively, exerted neuroprotection by attenuating the excitatory JNK3 apoptotic signaling pathway in global cerebral ischaemia (4-vessel occlusion, 4-VO) and focal cerebral ischaemia (middle cerebral artery occlusion, MCAO), particularly in the hippocampal CA1 region.

Alzheimer's disease and other neurodegenerative diseases can be caused by neuronal cell death induced by glutamate at high concentrations. Therefore, a substance that can suppress glutamate-induced neuronal cell death is a good candidate for preventing such pathologies. Several studies have focused on HT22 cells to study the oxidative glutamate toxicity model because HT22 cells derived from an immortalized murine hippocampal neuronal cell line can mimic potentiation both short- and long-term and do not have ionic glutamate receptors for transducing apoptotic signals induced by glutamate. HT22 cells also do not have NMDA receptors, and excitotoxity therefore may not occur in this cell line (Zhang and Bhavnani, 2006). A previous report by Zhao et al. (Zhao et al., 2012) suggested the important differentiation process of HT22 cells due to their features similar to primary hippocampal neurons, whereas undifferentiated HT22 cells were used as neuronal precursor cells in the proliferation stage. With the changes in neuronal morphology and neurochemical features when becoming cholinergic cells (Liu et al., 2009), differentiation also induces expression of NMDA receptors in these cells. Therefore, differentiated HT22 cells were used in this study to evaluate the toxicity of glutamate, which induces altered

pro-/anti-apoptotic regulator expressions.

Tan et al. suggested that the cytotoxicity induced by glutamate in HT22 cells was considered "oxytosis" and was distinguished from classical apoptosis (Tan et al., 2001). A previous report by Fukui et al. demonstrated that glutamate could induce both apoptosis and necrosis, and the distinctiveness of either type of cell death occurs exclusively at dissimilar intervals after exposure to glutamate and is partially influenced by glutamate concentrations (Fukui et al., 2009). At a relatively early period (8–12 h), necrosis mostly occurred, whereas at a late period (16–24 h), apoptosis mainly occurred (Fukui et al., 2009). In this study, administration of 5 mM glutamate to differentiated HT22 cells for 24 h (Sato et al., 2016) was therefore designed to induce apoptosis.

Since it has been reported previously that HT22 cells also do not express caspase-3, apoptosis induced by glutamate in this cell line was described to be mediated via the caspase-independent pathway (Tan et al., 2001; Zhang and Bhavnani, 2006). Moreover, the kinetics of the apoptotic pathway indicate that calpain plays a crucial role in apoptosis induced by glutamate. Fukui et al. also reported the neurotoxicity mechanism induced by glutamate in HT22 cells. Mitochondrial dysfunction and oxidative stress induced by glutamate are important early events necessary for apoptotic initiation via mitochondrial AIF to accelerate ATP-independent DNA fragmentation (Fukui et al., 2009). Their report suggested that the absence of caspase activation would result from ATP shortages in cells due to mitochondrial dysfunction following oxidative stress (Fukui et al., 2009). Moreover, it was also reported that JNK and p38 activation also contributed to glutamate-induced apoptosis in HT22 cells.

Oxidative stress may activate JNK, an MAPK. JNK phosphorylation occurs in response to stress-activated cytotoxicity and cytokines as potent regulators of apoptotic cells (Takeda and Ichijo, 2002). After mitochondrial dysfunction, AIF is translocated to the nucleus, and apoptosis is induced. Fukui et al. also reported JNK activation by phosphorylation after glutamate treatment for at least 12 h in HT22 cells. According to this activation period, JNK likely did not play a role at earlier time points of glutamate treatment for necrosis induction (Fukui et al., 2009). Therefore, we investigated in this study whether JNKs were activated after treatment with glutamate for 24 h in differentiated HT22 cells and whether GBR with or without GABA receptor antagonists can prevent glutamate toxicity in differentiated HT22 cells through the JNK-mediated apoptotic pathway.

In the present study, 5 mM glutamate for 24 h induced toxicity, resulting in an approximately 50% decrease in the viability of differentiated HT22 cells. GBR (100 μg/ml) suppressed glutamate-mediated neurotoxicity to preserve cellular viability. The neuroprotective effects of GBR on cell death and the cell cycle were characterized by flow cytometry. In agreement with Fukui et al. (Fukui et al., 2009), who used Annexin V/propidium iodide staining, glutamate toxicity (5 mM, 24 h) in this study resulted in approximately 50% apoptotic cells, with no related necrotic cells observed, while GBR attenuated apoptotic cell death and increased cell viability up to approximately 80%.

Neuronal apoptosis is one of the crucial steps in both the normal development of the nervous system (Oppenheim, 1991) and in neuronal disease or injury (Cheng and Mattson, 1991). A previous report by Park et al. suggested that proteins that physiologically function to control the cell cycle may also function in the death of neurons (Park et al., 1997). The likelihood of neuronal death owing to deprivation of trophic factors and neuronal apoptosis caused by DNA-damaging agents, such as camptothecin, may be due to uncontrolled cell cycle signals (Park et al., 1997). Moreover, it was found that the signaling pathways of the cell cycle have noncanonical roles in apoptosis control in nondividing neurons following either DNA damage or deprivation of trophic factors (Park et al., 1997).

In this study, in cell cycle analysis, 5 mM glutamate decreased the population of G1 cells but increased the population of S cells. Similar to a previous study by Park et al. (Park et al., 1997), they reported that neuronal PC12 apoptotic cell death caused by camptothecin triggered cell death involving cell cycle signals that promote entry into the S phase and underlie the checkpoint to significantly control apoptosis. Therefore, in this study, the high population in the S phase of the cell cycle is likely because with forced entry into S phase, cells begin replicating DNA.

As mentioned above, differentiated HT22 cells exited their proliferative stage and expressed evident neurite outgrowth, which was similar to postmitotic neuronal morphology (Liu et al., 2009). In addition, the differentiated HT22 cells showed the improvement of synaptic plasticity (Cullen et al.,2010). Previous study by Oo et al. (Oo et al., 2020) also pre-treated with GBR that exhibited the extended neurites in HT22 cell morphology as the mature cells, it was hypothesized that GBR might provide the neurotrophic factors for differentiation into mature neurons. Therefore, the fully differentiated condition has been used in this study prior to the GBR treatment.

The differentiated CNS neurons were also categorized as postmitotic cells and were thought to lose their proliferative properties (Liu et al., 2009). In neurodegenerative diseases, a relationship between apoptosis and re-entry into the cell cycle has been shown, especially cell cycle reactivation, and could be observed both in Alzheimer's disease mouse models and brains with Alzheimer's disease (Becker and Bonni, 2004). Therefore, it is necessary to elucidate the cell cycle signaling pathways that stimulate re-entry into the cell cycle because this may be beneficial for developing potential treatments.

Moreover, Ye and Blain also reported that in postmitotic neurons in the developing brain, DNA synthesis may act as a death signal because cell cycle protein activation and DNA synthesis were not observed during apoptosis (Ye and Blain, 2010). They demonstrated that the in vitro homocysteine-treated neurons from the cortex that were differentiated but quiescent were reactivated to express the proteins in the G1 phase of the cell cycle, leading to pursuit of the S phase, by using staining with BrdU, and synthesizing DNA during the S phase while undergoing apoptosis (Ye and Blain, 2010). Similarly, Martin et al. previously reported that the death of nerve growth factor (NGF)-deprived (for 30–48 h) neurons was entirely prevented by inhibiting protein or RNA synthesis (Martin et al., 1988). Therefore, apoptosis resulting from glutamate toxicity might be involved in a similar mechanism that leads to S-phase re-entry, which showed increased cell populations in this study. The GBR property for relieving the toxicity of glutamate in differentiated HT22 cells was shown to increase the G1 cell population but decrease the S cell population that experienced a decrease in synthesizing DNA during the S phase and could attenuate apoptosis.

Recent evidence by Miller et al. revealed that p53 tumor suppressor protein played a crucial role in apoptotic control in injured mature nervous systems or even in developing ones. The increase in p53-dependent neuronal apoptosis involves JNK pathway activation and dysregulation of the cell cycle via the CDK4/6-pRb-E2F-p53 pathway, which involves regulation of the G1-S transition (Miller et al., 2000). Consequently, our study showed that glutamate-initiated apoptosis occurred via the c-Jun and p-c-Jun signaling pathways in differentiated HT22 cells, and GBR decreased JNK activation after glutamate toxicity for 24 h. In agreement with Azmi et al., it was shown that treatment with GBR extract prior to H_2O_2 induction of oxidative stress in differentiated SH-SY5Y cells also downregulated apoptotic signaling pathways, such as JNKs, p38 and p53, leading to prosurvival and antiapoptotic effects on neuronal cells (Azmi et al., 2013).

Under differentiated conditions, HT22 cells not only presented morphologies similar to those of postmitotic hippocampal neurons but also increased NR1 mRNA and protein expressions of the NMDA receptor subunit 1, indicating that differentiation acts through the HT22 cell sensitivity to glutamate excitotoxicity via the expressed NMDA receptor (Zhao et al., 2012; He et al., 2013), which causes activation of oxidative stress and transcription factors provoking apoptotic cell death (Maher and Schubert, 2000; Nicholls, 2004). In addition, not only NMDA receptors (Zhao et al., 2012) but also GABA receptors were

reported in differentiated HT22 cells in the present study. Western blotting analysis showed a significant increase in expression of the $GABA_A$ receptor (GABRG2) protein in differentiated HT22 cells in a time-dependent fashion starting from 24 h. Similar to a previous study by Behuet et al. (Behuet et al., 2019), during neuronal development, a redefining process of neuronal networks occurred due to synapse elimination in postnatal brains from P30 to P90, which showed a decreased density of NMDA receptors, especially in the hippocampus and striatum. However, there was an increase in the density of the $GABA_A$ receptor from P10 to P20, which coincides with the shift in the developmental phase of GABA transmission from excitatory to inhibitory types in the postsynaptic area. Previous findings by Schweizer et al. (Schweizer et al., 2003) reported the importance of the clustering gamma 2 subunit (γ2) for both maintenance of $GABA_A$ receptors at mature synapses and being specifically required for chloride channel function, which contributed to postsynaptic localization of GABRG2 in mature neurons. Similar to a previous report by Cullen et al. (Cullen et al., 2010), neuronal differentiation in mature neuronal cultures enhanced synaptic neurons; therefore, in the present study, the increased expression of the $GABA_A$ receptor (GABRG2) protein in differentiated HT22 cells after 24 h is interesting and may be involved in an inhibitory mechanism that is counteracted by overactivation of glutamate-mediated toxicity as an excitation in differentiated HT22 cells.

According to recent studies, there is supporting evidence that still conflicts with the notion that GABA receptor activation is directly neuroprotective. Various neurodegenerative conditions are mostly associated with oxidative damage via apoptotic neurons, especially imbalances in glutamate concentrations. In our study, the GBR molecular mechanisms underlying pathological conditions were investigated in glutamate-induced differentiated HT22 cells and demonstrated that GBR showed neuroprotection mediated through the $GABA_A$ receptor. In all determinations after using GBR with a $GABA_A$ receptor antagonist (bicuculline), the decreased levels of pro-apoptotic proteins, c-Jun and p-c-Jun, at serine 73, the arrested G1 phase of the cell cycle that upregulated living cells, the increased cell viability and decreased apoptotic index were eliminated in the $GABA_A$ antagonistic group, but phaclofen showed no significant effects. GBR exhibited a better protective effect than the pure GABA compound in standard control cells. Therefore, the present study supported the evidence that activating GABA receptors, especially the $GABA_A$ receptor, by using high GABA contents, such as GBR, might be neuroprotective.

There was a limitation of this study, the increased expression of the $GABA_A$ receptor (GABRG2) protein in differentiated HT22 cells was shown after 24 h as a time dependent manner, the changes in the $GABA_A$ receptor expression when treated with GABA or GBR has not been studied. According to previous study by Behuet et al. (behuet et al., 2019), they mentioned that the early peak of metabotropic receptors, $GABA_B$ densities was shown between P0 and P10, in contrast to the later peak of $GABA_A$ receptors in the adult stage. Metabotropic receptors tend to be expressed early in brain development and play a more modulatory role in adult brains. However, in HT22 cell line remains less identified. In this study, phaclofen blocking, $GABA_B$ receptor antagonist, showed non-significant outcome after GBR treatment, it was hypothesized that the $GABA_B$ receptor might be activated slower than $GABA_A$ receptor because of the modulatory role of metabotropic receptors. However, $GABA_B$ receptor expression in this study should be determined and shown in the differentiated HT22 cells. In general, the caspase cascade pathway is important in the apoptotic pathway. This begins with the release of cytochrome c from the mitochondria, which is regulated by the Bcl-2 family. In the glutamate-induced neuronal cells, the amount of these proteins; Bcl-2, Bax, p53, caspase-3, caspase-9, and PARP or mitochondrial membrane potential should be studied to confirm previous report concerning the caspase-independent pathway. In addition, no data supported that differentiated HT22 cells do not express caspase-3, apoptosis induced by 5 mM glutamate for 24 h for supporting the caspase-independent pathway which was described by Tan et al. (Tan et al., 2001), Zhang and Bhavnani (Zhang and Bhavnani, 2006).

5. Conclusion

The findings of the current study suggest that GBR possesses neuroprotective and anti-apoptotic properties against glutamate toxicity induced in differentiated HT22 cells through the $GABA_A$ receptor. The molecular mechanism mediated by the JNK pathway, c-Jun, p-c-Jun, and the G1/S transition in cell cycle analysis may provide novel therapeutic targets for treating neuronal apoptosis in various pathological brain functions. GBR could be further designed as a valuable added nutritional supplement to prevent neurodegenerative diseases caused by apoptosis.

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CRediT authorship contribution statement

Sukrit Promtang: Writing – original draft, review & editing, Investigation, Data curation, Formal analysis. **Chairat Turbpaiboon:** Writing – original draft, Writing – review & editing, Visualization, Methodology, Formal analysis. **Eve Mon Oo:** Investigation, Visualization, Formal analysis. **Ladawan Khowawisetsut:** Methodology, Writing – review & editing, Formal analysis. **Panapat Uawithya:** Methodology, Visualization, Formal analysis. **Supin Chompoopong:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Validation, Funding acquisition, Project administration.

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Declaration of Competing Interests

The authors declare no conflict of interests.

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