

Original Article

Silencing neuroglobin enhances neuronal vulnerability to oxidative injury by down-regulating 14-3-3 γ

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Aim: To explore the protective role and mechanism of endogenous neuroglobin (Ngb) in neuronal cells under oxidative stress.

Methods: A stable N2a neuroblastoma cell line expressing the Ngb-siRNA plasmid (N2a/Ngb-siRNA) was established by neomycin screening. Reverse transcription (RT)-PCR and Western blot analysis were used to detect Ngb gene and protein levels. Hydrogen peroxide was used to induce oxidative stress in N2a cells. Cytotoxicity and cell viability were measured by lactate dehydrogenase (LDH) and WST-8 assays. Apoptotic cells were detected by Hoechst staining.

Results: Cotransfection of Ngb-siRNA with Ngb-GFP plasmids suppressed the expression of Ngb-GFP in N2a cells. RT-PCR and Western blot analysis showed that the expression of endogenous Ngb was successfully knocked down to about 20% in N2a/Ngb-siRNA cells compared with control cells. A WST-8 assay demonstrated that viability was significantly decreased in N2a/Ngb-siRNA cells and N2a cells transiently transfected with Ngb-siRNA plasmids compared with controls following hydrogen peroxide treatment. An LDH assay demonstrated a time-dependent increase in the death of Ngb-siRNA-transfected N2a cells following hydrogen peroxide treatment. Hoechst staining demonstrated that the quantity of apoptotic cells among N2a/Ngb-siRNA cells following hydrogen peroxide treatment significantly increased compared with controls. In N2a/Ngb-siRNA cells, the expression level of activated caspase-3 significantly increased, whereas the expression of 14-3-3 γ decreased compared with that of N2a/vec cells. Transfection of 14-3-3 γ plasmids significantly enhanced the viability of N2a/Ngb-siRNA cells following hydrogen peroxide treatment compared with vector controls.

Conclusion: Ngb contributes to neuronal defensive machinery against oxidative injuries by regulating 14-3-3 γ expression.

Keywords: neuroglobin; siRNA; H₂O₂; 14-3-3 γ ; oxidative stress; N2a cells

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Introduction

Neuroglobin (Ngb), the first hexacoordinate hemoglobin (hxHb) identified in vertebrates, is a novel member of the globin superfamily that exists predominantly in the brain^[1] and retina^[2]. The binding affinity of Ngb to O₂ is similar to that of myoglobin (Mb) and thus is much higher than that of Hb^[1]. Therefore, an attractive hypothesis is that Ngb might store or transport O₂ in the brain as Mb does in the muscle^[3]. However, the concentration of Ngb in the brain is much lower than that of Mb in the muscle^[1]. Moreover, the hexacoordinated nature of the heme in Ngb makes the release of oxygen difficult^[4]. To date, whether Ngb plays such a role remains unclear^[5].

It has also been proposed that Ngb functions as a nitric oxide (NO) or reactive oxygen species scavenger, oxygen sensor and signal transduction regulator^[5, 6]. Neuroglobin binds and reacts to NO and H₂O₂ without cytotoxic ferryl protein generation^[7]. However, Ngb is devoid of O₂ and NO reductase activities^[8]. The expression of Ngb in most areas of the brain does not colocalize with neuronal nitric oxide synthase^[9]. In Ngb-transgenic mice, the expression of endothelial nitric oxide synthase increases^[10]. Therefore, determining whether NO-scavenging is an important physiological function of Ngb in the brain requires further study. The expression of Ngb should be sensitive to changes in oxygen concentration if it is an oxygen sensor. Indeed, the expression of Ngb is upregulated by severe hypoxia^[11, 12]. However, no significant changes in Ngb expression have been observed in the adult mouse brain in response to chronic hypoxia^[13, 14]. In goldfish, which can survive in aquatic environments with low oxygen partial pressures, the expression of Ngb is also not significantly

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altered by prolonged hypoxia exposure^[15]. This evidence argues against the role of Ngb as an important oxygen sensor *in vivo*. In addition to small molecules, Ngb binds to G-proteins and acts as a guanine nucleotide dissociation inhibitor, suggesting that Ngb may mediate intracellular signaling^[16, 17].

Another proposed function of Ngb is the protection of neuronal cells from various pathological insults. Overexpression of Ngb prevents cell death from hypoxia, ischemia, NO, amyloid beta, and H₂O₂ insults^[11, 18–20]. However, the administration of penetrating Ngb directly to SH-SY5Y cells does not confer protection from oxygen and glucose deprivation^[21]. In Ngb-transgenic mice, although infarct volume is reduced, long-term improvement in limb deficits after stroke does not occur^[22]. We previously reported that Ngb gene expression is not altered in N2a cells following H₂O₂ treatment^[23]. These studies argue against the proposed protective role of Ngb. Here, we address the protective role and mechanisms of endogenous Ngb in response to H₂O₂ treatment by knocking down Ngb in N2a cells.

Materials and methods

Ngb-siRNA plasmid construction and identification

Ngb siRNA expression vectors (named Ngb-siRNA) targeted to different regions of mouse Ngb cDNA (gi:18999491) were constructed using a GFP-expressing siRNA vector (p-Genesil-1, Wuhan Genesil Biotechnology, China), as previously reported^[23]. The efficiency of Ngb-siRNA plasmids was confirmed by cotransfecting Ngb-siRNA and p-Ngb-EGFP plasmids^[18] at a ratio of 4:1 into N2a cells using LipofectAMINETM 2000 reagent (Invitrogen Life Technology, USA). All constructs were confirmed by DNA sequencing.

Stable N2a/Ngb-siRNA cell line

N2a cells were cultured with Dulbecco's modified Eagle's medium (DMEM)/Opti-MEM (*v/v*=1:1)/5% (*v/v*) fetal bovine serum (FBS) (GIBCO BRL, Life Technologies, USA) in an incubator (Precision Scientific, USA) at 37 °C with 95% air/5% CO₂ (*v/v*) and 95% humidity, as reported previously^[23]. N2a cells were transfected with Ngb-siRNA plasmids or control vectors, and stable cell lines were screened using a neomycin antibiotic (Sigma, USA) starting at a concentration of 800 µg/mL. The concentration of neomycin antibiotic was gradually reduced to a final concentration of 200 µg/mL within two weeks. Stable N2a cell lines expressing neomycin-resistant Ngb-siRNA (N2a/Ngb-siRNA) or control plasmids (N2a/vec) were obtained by subcloning. The cells were maintained in N2a medium plus neomycin antibiotic (200 µg/mL), and the medium was changed every 2 or 3 days. N2a cells within 50 passages were used in all experiments.

RT-PCR

The total RNA was isolated from N2a cells using TRIZOL[®] reagent according to the manufacturer's protocol (Invitrogen Life Technology, USA). The total RNA (2 µg) was then used to perform RT using M-MLV transcriptase (Promega, USA),

oligo (dT₁₅) primer (Promega, USA) and a total volume of 25 µL. Semi-quantitative PCR, 28 cycles of 94 °C, 45 s; 60 °C, 45 s; 72 °C, 45 s plus a final extension at 72°C for 10 min, was performed as previously reported, using β-actin as an internal control^[23, 24]. The primers used in PCR were Ngb: 5'-ctctggaa-catggcactgtc-3' and 5'-gcactggctgtcttact-3'; β-actin: 5'-cagc-cttctcttgggat-3' and 5'-gctcagtaacagctccgcta-3'; and 14-3-3γ: 5'-gttggctctggctcttcatcat-3' and 5'-aggtgcagagtagacttgggtg-3'. All PCR products were confirmed by DNA sequencing.

Western blot analysis

Cell extracts were prepared and Western blot analysis was performed, as previously described^[24, 25]. Briefly, 10–50 µg of total protein was resolved on 12%–15% reducing SDS-PAGE gels and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5% (*w/v*) nonfat dried milk in TBST buffer [0.1 mol/L Tris-HCl, pH 8.0, 0.9% (*w/v*) NaCl, and 0.1% (*v/v*) Tween-20] and probed with rabbit polyclonal antibodies to Ngb (Santa Cruz Biotechnology, USA) and 14-3-3γ (Immuno-Biological Laboratories, Japan) and mouse monoclonal antibody to β-actin (Santa Cruz Biotechnology, USA) in TBST overnight at 4 °C. After incubation with corresponding HRP-conjugate secondary antibodies (Chemicon International, USA), antigens were detected using a chemiluminescence kit (Amersham, France).

Cytotoxicity assays

N2a/Ngb-siRNA and N2a/vec cells were seeded in 96-well-plates at a density of 50 000 cells/100 µL per well. Twenty-four hours after incubation, cells were treated with hydrogen peroxide^[23]. An LDH assay was performed using a kit (Promega, USA). The WST-8 cell viability assay was performed using a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Japan)^[26]. The absorbance for the LDH and WST-8 assays was determined at 492 nm and 450 nm, respectively, using a microtiter plate reader (Synergy 2, BioTek, USA). Cell death and viability were calculated according to the formula provided by the manufacturers.

Identification of apoptosis by Hoechst staining

Cell cultures were prepared and Hoechst staining was performed as previously reported^[25]. Cultures of N2a/Ngb-siRNA and N2a/vec were fixed with 4% paraformaldehyde for 30 min. After being washed twice with PBS, Hoechst 33258 was used to stain the nuclei for 5 min at room temperature. After being washed five times with PBS, micrographs were taken with a conventional fluorescent microscope.

Statistical analysis

All data are presented as means±SEM of at least three individual experiments. RT-PCR and Western blot results were quantified by measuring the band intensity using a densitometer. The relative expression levels of Ngb, 14-3-3γ, and caspase-3 were normalized to those of β-actin and compared with controls. *P*<0.05 and *P*<0.01 were considered significant

compared with controls by Student's unpaired *t*-test.

Results

The expression of endogenous Ngb is efficiently knocked down in N2a/Ngb-siRNA neuroblastoma cells

To explore the biological function of neuroglobin, we used the siRNA technique to knock down the expression of endogenous Ngb in N2a cells. Three Ngb-siRNA expression vectors were constructed. The Ngb-siRNA plasmids were cotransfected with p-Ngb-EGFP-N1 plasmids^[18] into N2a cells to verify their inhibitory efficiency. The Ngb-siRNA plasmid targeting the gtgatcctagtgattgattca sequence within the Ngb cDNA inhibited the expression of the Ngb-GFP fusion protein (green under the fluorescent microscope) most effectively (Figure 1A). This effective Ngb-siRNA plasmid and control vector (p-Genesil-1) were then transfected into N2a cells to establish stable N2a/Ngb-siRNA and N2a/vec cell lines by neomycin screening. The RT-PCR results (Figure 1B) showed that the expression level of Ngb mRNA in N2a/Ngb-siRNA (lane 2) decreased (17.9%) compared with that of N2a/p-Genesil-1 (lane 3). A p-Ngb-EGFP-N1 plasmid was used as a positive control in the PCR. Furthermore, the results of the Western blot analysis show that the expression level of endogenous Ngb protein also decreased (21.5%) in N2a/Ngb-siRNA cells (Figure 1C). A statistical analysis demonstrates that the expression levels of Ngb mRNA and protein significantly decreased in N2a/Ngb-siRNA cells. These results together demonstrate that the expression of Ngb was efficiently suppressed in N2a/Ngb-siRNA cells.

Suppressing endogenous Ngb enhances neuronal vulnerability to oxidative stress-induced cell injury

N2a/Ngb-siRNA and N2a/p-Genesil-1 cells were exposed to H₂O₂. Cell viability was calculated by measuring the activity of dehydrogenase in living cells with a WST-8 assay^[26]. Both N2a/Ngb-siRNA and N2a/vec cells showed a concentration-dependent decrease in cell viability following H₂O₂ treatment (Figure 2A). Cell viability was significantly decreased in N2a/Ngb-siRNA cells compared with N2a/vec cells following 150 and 300 μmol/L treatments with H₂O₂ (Figure 2A). We then counted the apoptotic cells, which possessed shrunken and condensed nuclei visible with Hoechst staining, as previously reported^[18]. After treatment with 150 μmol/L of H₂O₂ for 12 h, apoptotic nuclei increased in N2a/Ngb-siRNA cells (50.8%) (indicated by arrow head, Figure 2B) compared with N2a/vec cells (20.2%).

To further confirm the protective role of endogenous Ngb from oxidative stress, we knocked down Ngb expression in N2a cells by transient transfection with Ngb-siRNA plasmids. Transfection of Ngb-siRNA plasmids into N2a cells significantly decreased cell viability following 150 and 250 μmol/L of H₂O₂ incubation (Figure 2C). We then determined the time window over which Ngb exerts its protection. A time-dependent increase in injury of both N2a/vec and N2a/Ngb-siRNA cells, measured by an LDH assay, was seen following 150 μmol/L of H₂O₂ incubation (Figure 2D). A statistical analysis

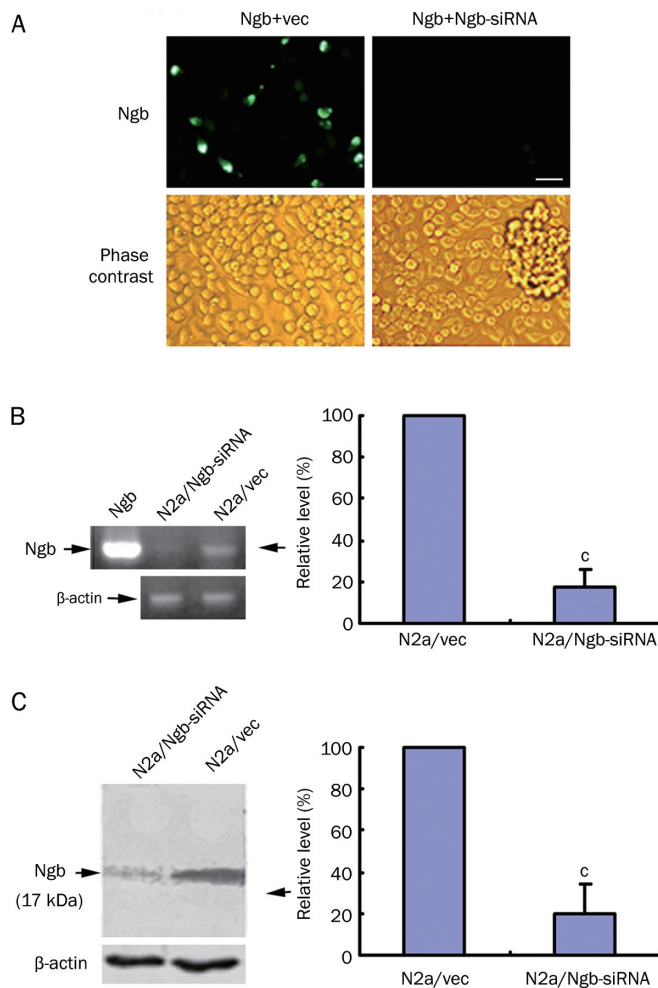


Figure 1. Knock-down of Ngb in N2a neuroblastoma cells. (A) Ngb-siRNA plasmids were co-transfected with p-Ngb-EGFP-N1 plasmids into N2a cells. The expression of Ngb-GFP fusion protein (green) was suppressed after Ngb-siRNA transfection (right panel). Bar, 20 μm. (B) Ngb-siRNA and control plasmids (vec) were transfected into N2a cells and stable cell lines were established by neomycin selection. A representative RT-PCR result shows that the expression level of Ngb mRNA decreased in Ngb-siRNA transfected (N2a/Ngb-siRNA) cells compared with vector transfected (N2a/vec) cells. β-actin served as an internal control and p-Ngb-EGFP-N1 plasmids served as positive controls. (C) A representative Western blot analysis shows decreased expression of Ngb protein in N2a/Ngb-siRNA cells. β-actin served as an internal control. All statistical results are expressed as means±SEM from three independent experiments. **P*<0.01 vs N2a/vec.

demonstrates that cell death was significantly increased in N2a/Ngb-siRNA cells compared with N2a/vec cells after 0.5, 1, 2, 3, 4, and 5 h of H₂O₂ treatment (Figure 2D). These data together demonstrate that endogenous Ngb can protect neuronal cell against oxidative insults.

Suppressing Ngb induces caspase-3 activation and down-regulates 14-3-3γ expression

To investigate whether endogenous Ngb is involved in apop-

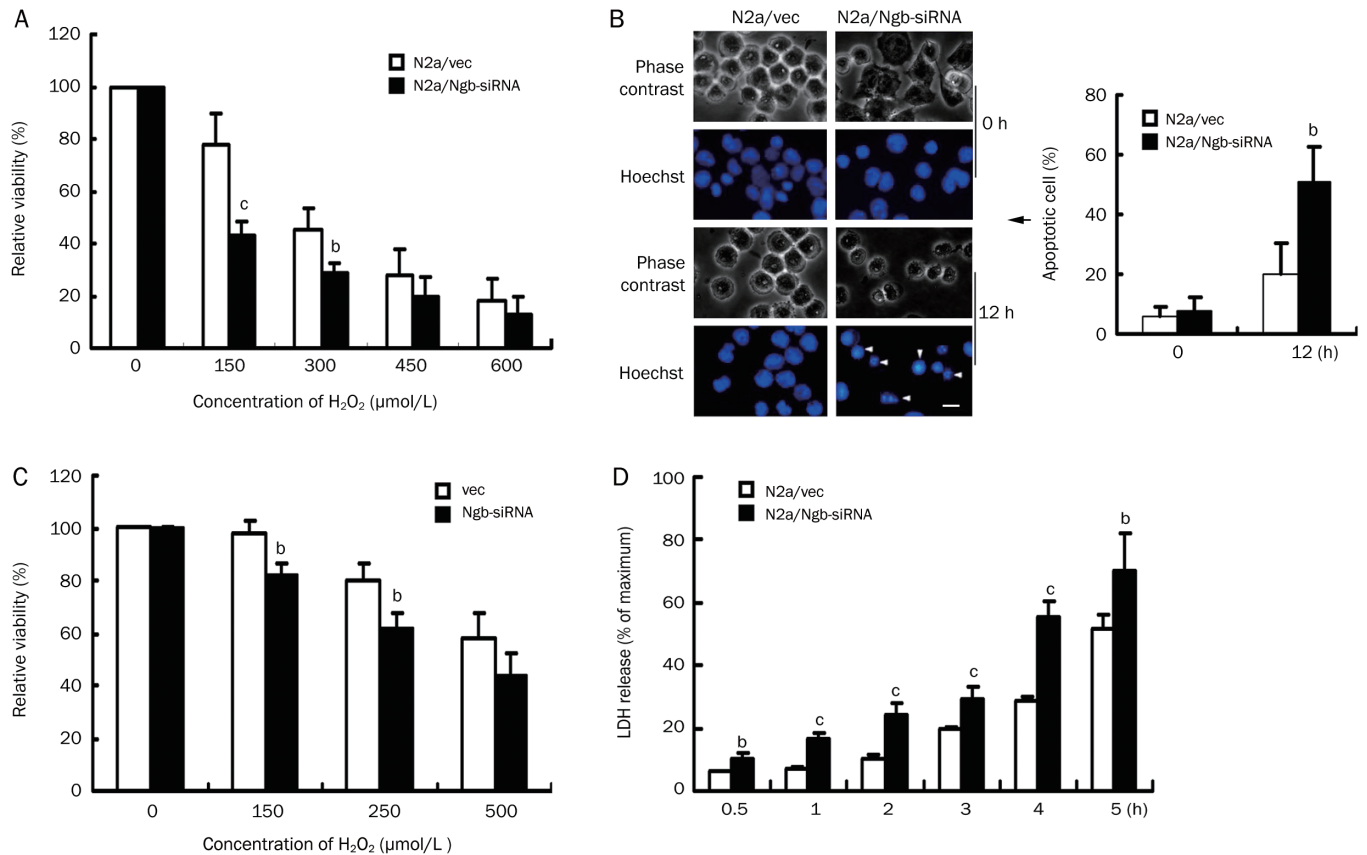


Figure 2. Silencing of Ngb enhances cell death following H_2O_2 treatment. (A) N2a/Ngb-siRNA and N2a/vec cells were exposed to various concentration of H_2O_2 for 12 h. A WST-8 assay demonstrates that the viability of N2a/Ngb-siRNA cells significantly decreased compared with N2a/vec cells following treatment with 150 and 300 $\mu\text{mol/L}$ of H_2O_2 . (B) N2a/Ngb-siRNA and N2a/vec cells were exposed to 150 $\mu\text{mol/L}$ of H_2O_2 for 12h and the nuclei were stained with Hoechst 33258. Apoptotic nuclei (condensed heavy staining nuclei indicated by arrow heads) increased in N2a/Ngb-siRNA cells after 12 h of H_2O_2 incubation. Bar, 10 μm . (C) Wild-type N2a cells were transiently transfected with Ngb-siRNA plasmids or control vectors and exposed to various concentrations of H_2O_2 for 12 h. A WST-8 assay demonstrates that the viability of N2a cells after Ngb-siRNA transfection significantly decreased following treatments with 150 and 250 $\mu\text{mol/L}$ of H_2O_2 compared with N2a cells transfected with vectors. (D) N2a/Ngb-siRNA and N2a/vec cells were exposed to 150 $\mu\text{mol/L}$ of H_2O_2 for 0.5, 1, 2, 3, 4, and 5 h. An LDH assay demonstrates that cell death significantly increased in N2a/Ngb-siRNA cells. All statistical results are expressed as means \pm SEM from at least three independent experiments. ^b $P < 0.05$, ^c $P < 0.01$ vs corresponding control cells.

otic machinery, we analyzed the level of activated caspase-3, which is the molecular marker for activation of the apoptotic signaling pathway. Western blot analysis demonstrated that the level of cleaved caspase-3 in N2a/Ngb-siRNA cells was 1.7 fold and significantly higher than the level in N2a/vec cells (Figure 3A, $P < 0.01$). The activation of caspase-3 after Ngb silencing indicates that the overall effect of endogenous Ngb in neuronal cells is anti-apoptotic.

We then investigated the mechanism by which Ngb inhibited caspase-3 activation. 14-3-3 proteins exist predominantly in the brain and mediate essential apoptotic signaling pathways^[28, 29]. We have previously reported that 14-3-3 γ is up-regulated and protects astrocytes from ischemia-induced apoptosis^[24, 25]. We compared the expression of 14-3-3 γ transcripts (Figure 3B) and proteins (Figure 3C) in N2a/Ngb-siRNA and N2a/vec cells. The results of RT-PCR show that the expression level of 14-3-3 γ transcripts decreased (25.4%) in N2a/Ngb-siRNA cells compared with N2a/vec cells (Fig-

ure 3B). A statistical analysis demonstrates that the level of 14-3-3 γ transcripts was significantly lower than that of controls (Figure 3B). A Western blot analysis showed that the expression level of 14-3-3 γ protein decreased (28.8%) in N2a/Ngb-siRNA cells compared with N2a/vec cells (Figure 3C). We further confirmed the protective role of 14-3-3 γ from oxidative stress by transfecting 14-3-3 γ plasmids^[24] into N2a/Ngb-siRNA cells. A WST-8 assay demonstrated that over-expression of 14-3-3 γ in N2a/Ngb-siRNA cells significantly enhanced cell viability following treatment with 150 $\mu\text{mol/L}$ of H_2O_2 compared with vector controls (Figure 3D).

Discussion

Neuroglobin may serve as an oxygen store/transporter, gas sensor, reactive oxygen species scavenger, and signal transduction modulator based on its ability to bind to O_2 , NO, H_2O_2 , and G-protein^[3, 6]. However, there has been no clear physiological function that can be assigned to Ngb until now. Here,

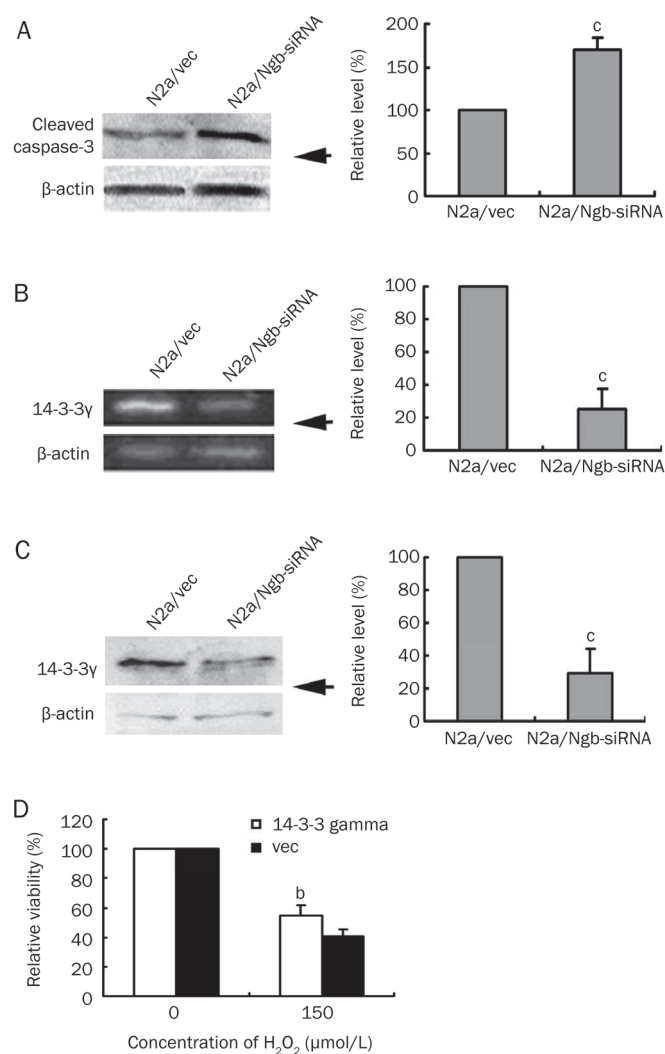


Figure 3. Silencing of Ngb induces caspase-3 activation and inhibits 14-3-3 γ expression. (A) A Western blot analysis demonstrates that cleaved caspase-3 increased significantly in N2a/Ngb-siRNA cells. (B) RT-PCR demonstrates that the expression level of 14-3-3 γ mRNA decreased significantly in N2a/Ngb-siRNA cells. (C) Western blot analysis demonstrates that the expression of 14-3-3 γ protein decreased significantly in N2a/Ngb-siRNA cells. (D) A WST-8 assay demonstrates that overexpression of 14-3-3 γ significantly enhanced the viability of N2a/Ngb-siRNA cells following treatment with 150 μ mol/L of H₂O₂ for 12 h compared with vector controls (p-EGFP-N1). All statistical results are expressed as means \pm SEM from three independent experiments. ^b P <0.05, ^c P <0.01 vs corresponding control cells.

we successfully knocked down the Ngb expression level using an siRNA technique and demonstrated that endogenous Ngb plays an important protective role in oxidative injuries by upregulating the expression of 14-3-3 γ , a protein that mediates essential apoptotic signaling^[27, 28].

Our results demonstrate that the death of N2a/Ngb-siRNA cells increases following H₂O₂ treatment. An interesting finding is that the silencing of Ngb increases cell death with lower concentrations (150 and 300 μ mol/L) but not higher concentrations (450 and 600 μ mol/L) of H₂O₂. As estimated

by microdialysis in the ischemic striatum, the concentration of H₂O₂ can rise as high as 100 μ mol/L during the reperfusion phase^[29]. Our data supports a physiological or pathological role of endogenous Ngb in protecting cells from oxidative stress in the central nervous system. Moreover, we found that increased injury of N2a/Ngb-siRNA cells occurred within 30 min following H₂O₂-administration, indicating that Ngb has a direct protective effect against H₂O₂-induced cell damage.

Although the silencing of Ngb did not cause obvious cell death, it did induce caspase-3 activation, indicating that endogenous Ngb suppresses apoptotic signal transduction pathways. This is consistent with the hypothesis proposed by Fago *et al*^[30] that Ngb increases the apoptosis-triggering threshold by maintaining cytochrome C in the nonapoptotic ferrous oxidative state. Furthermore, we have identified an important apoptotic signaling protein^[27] through which Ngb may exert its anti-apoptotic function. 14-3-3 γ protein mediates essential apoptotic signaling pathways by binding to proapoptotic proteins such as Bad, Bax, ASK-1, and p53^[28]. We previously demonstrated that 14-3-3 γ is up-regulated in and protects astrocytes from ischemic injury^[24, 25]. In ischemic neurons, Ngb is up-regulated and exerts a protective role^[11]. We propose that Ngb might exert its protection by regulating 14-3-3 γ expression in ischemic neurons. Considering that Ngb has a regulatory role for the G-protein^[16, 17], it is likely that Ngb may control 14-3-3 γ expression through G-protein-mediated signaling. In addition to controlling 14-3-3 γ levels, Ngb may exert its protective role via other mechanisms such as lipid raft-mediated signaling^[31].

In conclusion, we are the first to demonstrate that the suppression of Ngb enhances neuronal vulnerability to oxidative injuries and that the underlying mechanism involved is 14-3-3 γ down-regulation.

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Author contribution

Xiao-qian CHEN designed research; Xin-yu ZHOU, Shi-qiao YE, Xiao-jing LAI, and Li ZHENG performed research; Xiao-qian CHEN and Shi-qiao YE wrote the paper.

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