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Neuroglobin Promotes Neurite Outgrowth via Differential Binding to PTEN and Akt

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Abstract Neuroglobin, the third mammalian globin with a hexa-coordinated heme, exists predominantly in neurons of the brain. Neuroglobin plays an important role in neuronal death upon ischemia and oxidative stress. The physiological function of neuroglobin remains unclear. Here, we report a novel function of neuroglobin in neurite development. Knocking-down neuroglobin exhibited a prominent neurite-deficient phenotype in mouse neuroblastoma N2a cells. Silencing neuroglobin prevented neurite outgrowth, while ectopic expression of neuroglobin but not homologous cytoglobin promoted neurite outgrowth of N2a cells upon serum withdrawal. In primary cultured rat cerebral cortical neurons, neuroglobin was upregulated and preferentially distributed in neurites during neuronal development. Overexpression of neuroglobin but not cytoglobin in cultured cortical neurons promoted axonal outgrowth, while knocking-down of neuroglobin retarded axonal outgrowth. Neuroglobin overexpression suppressed phosphatase and tensin homolog (PTEN) but increased Akt phosphorylation during neurite induction. Bimolecular fluorescence complementation and glutathione S-transferase pull-down assays revealed that neuroglobin and various mutants (E53Q, E118Q, K119N, H64A, H64L, and Y44D) bound with Akt and PTEN differentially. Neuroglobin E53Q showed a prominent reduced PTEN binding but increased Akt binding, resulting in decreased p-PTEN, increased p-Akt, and increased neurite length. Taken together, we demonstrate a critical role of neuroglobin in neuritogenesis or development via interacting with PTEN and Akt differentially to activate phosphatidylinositol 3-kinase/Akt pathway, providing potential therapeutic applications of neuroglobin for axonopathy in neurological diseases.

Keywords Ngb · Neuron · Axon · Regeneration · PI3K/Akt

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Introduction

Neuritogenesis or neurite outgrowth is essential for neuronal path findings and the establishment of synaptic connections during the development of the central nervous system (CNS) [1, 2]. Inducing neurite outgrowth is a crucial but

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tough step in treating diseases such as traumatic CNS injury, stroke, or neurodegenerative diseases [2–4]. Along with aging, regenerating neurite is of particular difficulty as neurons lose their ability to produce neurite after maturation [5]. Understanding clearly the molecular mechanisms regulating neurite outgrowth is required for searching effective drugs or improving treatments for various neurological diseases. Neurite outgrowth is an early neuronal differentiation process to establish neuronal connectivity during brain development. The mechanisms of neurite outgrowth in CNS are complicated and controlled by driving forces of neurite outgrowth in neurons and environmental neurite outgrowth-promoting or inhibiting forces. Key intracellular signaling molecules or pathways controlling neurite outgrowth include growth factors, Akt, phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinases, glycogen synthase kinase 3β (GSK-3β), protein kinase C (PKC), and Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) [6–10].

Neuroglobin (Ngb), a neuronal- or brain-specific respiratory protein, is the third heme-containing globin discovered in mammalian brains [11]. Until now, the physiological function of Ngb remains elusive. However, the differential expression of Ngb in developing and aging brains suggests an important role of Ngb in brain development and aging [12]. Previous studies of Ngb focus mainly on the role of Ngb in hypoxic of ischemic neurons, and most studies showed a neuroprotective effect of Ngb against hypoxic/ischemic injury [13, 14]. The increased expression of Ngb in the cortical peri-infarct region of stroke patients suggests a potential role of Ngb in neuronal function recovery after ischemia.

In addition to its native small ligands such as oxygen (O_2) and nitric oxide (NO), Ngb also interacts with proteins, suggesting its important role in coupling O_2/NO signal and intracellular signaling pathways. For example, the binding of Ngb with G-protein subunit, 14-3-3ζ, or cytochrome C contributes to Ngb's protection in neurons or astrocytes from hypoxia- or ischemia-caused cell death [15–17]. It is also reported that Ngb exerts a protective role upon H_2O_2 oxidative stress by increasing p-Akt [18]. In addition, Ngb attenuates Alzheimer-like tau hyperphosphorylation by activating Akt signaling [19]. These data suggest complicated biological functions and mechanisms of action of Ngb under physiological or pathological conditions as a representative of the hexa-coordinated globins.

In this study, we demonstrate that neuroglobin is required for neuronal development by promoting neurite outgrowth. To the best of our knowledge, we are the first to report such a physiological function of neuroglobin. Moreover, we find that Ngb promotes neurite development by interacting with Akt and its upstream regulator phosphatase and tensin homolog (PTEN).

Methods and Materials

The Ngb overexpression plasmid (p-Ngb-EGFP-N1) and its mutants (E53Q, E118Q, K119N, H64A, H64L, and Y44D) were constructed previously [20]. The Ngb short hairpin RNA (shRNA)-expressing plasmid (p-Ngb-shRNA-Genesil-1, sh-Ngb) for Ngb knock-down was constructed as in a previous study, and a nonspecific DNA fragment (scramble) was used as a negative control [20]. All other expression vectors were kindly provided by Prof. Haian Fu (Department of Pharmacology, School of Medicine, Emory University, Atlanta, GA, USA). All plasmids were confirmed by sequencing before use.

Cell Line Cultures and Transfection

N2a cells were cultured with 1:1 Dulbecco's modified Eagle's medium (DMEM) and OPTI-MEM supplemented with 5 % (*v*/*v*) fetal bovine serum (FBS) (GIBCO BRL, USA), at 37 °C with 95 % air/5 % CO_2 (*v*/*v*) and 95 % (*v*/*v*) humidity in an incubator (Heal Force Inc., Hong Kong, China). The human embryonic kidney 293T (HEK293T) cells were grown in DMEM containing 10 % FBS. N2a and HEK293T cells were seeded at a density of 5×10^5 cells per 35 mm dish for transfection with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Fresh OPTI-MEM media without serum or 20 μM retinoic acid (RA) (Sigma, USA) was used to induce N2a cell differentiation 24 h after transient transfection. Stably transfected cell lines were established by neomycin selection.

Primary Cultures and Nuclear Transfection

Primary cultures of rat cerebral cortical neurons were set as reported previously [21]. Briefly, cerebral cortices isolated from E18 rat embryos were kept in ice-cold Hanks' balanced salt buffer (Sigma, USA). The tissues were digested in 0.125 % (*w*/*v*) trypsin for 10 min at 37 °C and triturated with a flame-polished Pasteur pipette in plating medium (DMEM with 10 % (*v*/*v*) FBS) to disrupt cell–cell connections. Dispersed cells were filtered with a 200-mesh filer and then centrifuged at 800×*g* for 5 min. The cell pellets were resuspended in DMEM–10 % (*v*/*v*) FBS. Dissociated neurons were plated onto 35 mm dishes coated with poly-d-lysine (Sigma, USA) at a density of 0.5×10^5 cells/ml. After 2 h of cell attachment, media were changed to neurobasal medium supplemented with 2 % (*v*/*v*) B27 and 2 mM l-glutamine (Invitrogen, USA). Transfection of neurons was carried out immediately before cell seeding by using the Amaxa Nucleofector device (Lonza, Switzerland) according to the manufacturer's instructions. Transfected neurons were allowed to attach to the culture dishes for 3–4 h and then the culture media were replaced with neurobasal medium–2 % (*v*/*v*) B27–l-glutamine (2 mM). Primary

cultures of rat cerebral cortical astrocytes were set as reported previously [22].

Western Blotting Analysis

Total soluble proteins were extracted from cultured cells by using radioimmunoprecipitation assay lysis buffer (Applygen Technologies Incorporation, Beijing, China) containing phenylmethanesulfonyl fluoride (Sigma, USA), and equal amounts of total proteins were subjected to Western blotting analysis as previously described [23]. The membranes were blocked with 5 % (*w*/*v*) nonfat dried milk in Tris-buffered saline and then incubated with primary antibodies against Ngb, GFP, glutathione S-transferase (GST), His, β-actin (Santa Cruz Biotechnology, USA), p-PI3K p85 (Tyr458), PI3K p85, p-Akt Ser473, Akt, p-GSK-3β Ser9, GSK-3β, p-PTEN Ser380/Thr382/383, PTEN, p-Erk-1/2 (Thr202/Tyr204), and Erk-1/2 (Cell Signaling Technology, USA). After incubation with IRDye 800CW or IRDye 680CW conjugated goat anti-rabbit or anti-mouse IgG (LI-COR Biosciences, USA), the blots were visualized and quantified by using the Odyssey Infrared Imaging System (LI-COR Biosciences, USA).

Immunofluorescence Staining

Immunofluorescence staining was performed as described previously [23]. Briefly, cells in the cultures were fixed for 30 min in phosphate-buffed saline (PBS) containing 4 % (*w*/*v*) paraformaldehyde and permeabilized with 0.3 % (*v*/*v*) Triton X-100 for 15 min. The cells were then blocked with 3 % (*w*/*v*) bovine serum albumin (BSA) in PBS for 30 min at room temperature and incubated with primary antibody (rabbit anti-Ngb 1:100 (*v*/*v*), Santa Cruz; mouse anti-Ngb 1:100 (*v*/*v*), Abcam, USA; mouse anti-Tau-1 1:100 (*v*/*v*), Millipore, USA; rabbit anti-growth associated protein-43 (GAP-43) 1:100 (*v*/*v*), Abcam, USA) at 4 °C overnight. After washing three times with PBS, the cultures were incubated with secondary antibody conjugated with Dylight 488 or Dylight 594 (1:200 (*v*/*v*), Jackson, USA) for 1 h at room temperature. Hoechst 33342 (2 μg/ml, Sigma, USA) was used to stain nuclei. Micrographs were taken using a Zeiss 510 confocal microscope (Carl Zeiss, Germany). Fluorescence intensities of Ngb and GAP-43 of the same cell were measured by using Image-Pro Plus 6.0 software.

Immunohistochemistry

Rats were anesthetized with chloral hydrate (0.4 g/kg) and transcardiac perfusion was performed with 4 % (*w*/*v*) ice-cold paraformaldehyde solution. The brains were post-fixed in perfusate overnight and then cut into sections of 15 μm with a vibratome (Leica S100, Germany). The brain sections were permeabilized with 0.5 % (*v*/*v*) Triton X-100/0.3 % (*v*/*v*) H_2O_2 in PBS for 30 min. After blocking with 5 % (*v*/*v*) BSA for 1 h at room temperature, the sections were incubated with anti-Ngb antibody (1:100 (*v*/*v*), Santa Cruz, USA) overnight at 4 °C, followed by incubating with a biotin-labeled goat anti-rabbit IgG for 1 h at 37 °C. The immunoreaction was detected by incubating the sections with streptavidin–biotin–peroxidase complex for 1 h at 37 °C and visualized by using the DAB reagents. Micrographs were taken with a conventional microscope (Olympus BX60, Japan) [20].

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA from primary cortical neurons was isolated using TRIzol reagents according to the manufacturer's protocol (Invitrogen, USA). Total RNA (2 μg) was used to perform reverse transcription by using M-MLV transcriptase (Promega, USA), oligo (dT_{15}) primer (Promega, USA) in a total volume of 25 μl. Conventional PCR (25–30 cycles of 94 °C, 45 s; 55 °C, 30 s; 72 °C, 50 s plus final extension at 72 °C for 5 min) was performed in a volume of 20 μl containing 1× PCR buffer, 2 μl cDNA, 200 μM dNTPs, 400 nM primers, and 1 U Taq DNA polymerase, using β-actin as internal control. The primers used in PCR were as follows: Ngb—5′-CAT CGG GCA GTG GGA GTG AGG-3′ and 5′-TCC AGG CGG TCC TTG TAG CTG-3′; β-actin—5′-CAG CCT TCC TTC TTG GGT AT-3′ and 5′-GCT CAG TAA CAG TCC GCC TA-3′. Triplicate measurements were conducted for each sample and the Ngb mRNA level was normalized by β-actin [20].

Neurite Outgrowth Assay

Cultured cortical neurons or N2a cells were fixed with 4 % paraformaldehyde. For each culture, nine fields were micrographed randomly under a conventional microscope (Carl Zeiss, Germany). The longest neurite length in each neuron was measured by using the software Image-Pro Plus. The mean neurite length of 100 neurons from three independent experiments was used for statistical analysis. The fluorescence intensity in Ngb-GFP was plotted against the longest neurite length of the same cell and was subjected to Pearson's bivariate correlation analysis (SPSS statistical package).

Glutathione S-transferase Pull-Down Assay

After 48 h of transfection, HEK293T cells were washed once with cold PBS and lysed in 400 μl of binding buffer (20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 50 mM NaF, 0.5 mM EDTA, 0.5 % (*v*/*v*) Triton X-100, and protease inhibitors) per 35 mm dish. The cells were incubated in binding buffer for 15 min on ice, and supernatants were collected by centrifugation at 10,000×*g* for 10 min at 4 °C.

Glutathione sepharose beads (BioVision, USA) were prewashed with the binding buffer. Then, 200–400 μg of total soluble proteins was incubated with glutathione sepharose beads (20 μl per sample) for 4 h with gentle rotation at 4 °C. After three times of washing with ice-cold binding buffer, 20 μl of 2× SDS-PAGE gel loading buffer was used to dissociate proteins from precipitates by boiling for 5 min. The supernatants were collected and subjected to Western blotting analysis. Anti-GFP and anti-GST antibodies were used to probe corresponding fusion proteins.

Bimolecular Fluorescence Complementation Assay (BiFC)

BiFC assay is based on the reconstitution of a fluorescent protein molecule upon reassociation of its two splitted nonfluorescent fragments. When Venus yellow fluorescent protein (Venus, enhanced YFP) was cut into two fragments containing either the N-terminal or C-terminal, neither of the fragments displayed fluorescent property when expressed alone. Coexpression of the two fragments linked to interacting proteins allowed the partial reformation of Venus with the concomitant appearance of the fluorescent signal. N2a cells were transiently cotransfected with NV (Venus 1-157 N-terminal)- and CV (Venus 137-238 C-terminal)-tagged plasmids at a ratio of 1:1 for 24 h. Fluorescent micrographs were taken with a converted fluorescence microscope (Carl Zeiss, Germany) using the excitation (480 ± 30 nm) and emission (535 ± 25 nm) filter at the same conditions. The relative fluorescence intensity from nine fields per culture was measured by using the Image-Pro Plus 6.0 software, and the mean fluorescence intensity was used for statistical analysis. After photographing, cell lysates were extracted and the expression of each fusion protein was measured by Western blotting using antibodies against either GFP (for NV-tagged fusion proteins) or His (for CV-tagged fusion proteins, the vector contains a His tag) (Santa Cruz, USA).

Statistical Analyses

Experiments were repeated at least three times. Quantitative results were expressed as mean ± SEM. Statistical analyses were performed with ANOVA and Student's *t*-test; *p* values exceeding 0.05 were considered to be not significant.

Results

Endogenous Ngb Is Required for Neuritogenesis in N2a Cells

To explore the physiological function of Ngb in neuronal cells, we established a N2a cell line in which endogenous Ngb was stably knocked down by overexpressing short hairpin Ngb RNAi plasmids (p-shNgb-Gensil-1, sh-Ngb). The efficacy of Ngb knocking-down by various sh-Ngb constructs targeting to different positions in Ngb mRNA coding region was evaluated by cotransfecting sh-Ngb plasmids together with p-Ngb-EGFP-N1 plasmids (Ngb) in N2a cells (Fig. 1a). Since sh-Ngb 197 (targeting Ngb mRNA coding region position starting from 197) was most effective in reducing Ngb expression (Fig. 1b), we used this construct in the following studies. Using this sh-Ngb construct, we established corresponding stably transfected N2a cell line (N2a/sh-Ngb) as well as nonspecific control cell line (N2a/N-con, scramble control). Neurite outgrowth in N2a cells was induced by serum withdrawal, a common neurite inducing model. Serum deprivation induced a prominent neurite outgrowth in wild-type N2a cells (N2a/WT, without transfection) as well as N2a/N-con cells 3–12 h after media change; however, the neurite was not induced or significantly shorter in N2a/sh-Ngb cells after 3–12 h of induction (Fig. 1c). The neurite length (the longest one) of individual N2a cells in the cultures was measured (>100 cells/dish), and the percentages of N2a cells bearing different lengths of neurite were categorized into six groups (0–20, 20–40, 40–60, 60–100, 100–150, and>150 μm). The percentages of the six groups were plotted in Fig. 1d. The percentage distribution pattern of N2a/WT was similar to that of N2a/N-con cells (Fig. 1d). Obviously, N2a/sh-Ngb cells had much more cells in the group bearing the shortest neurite (0–20 μm) (>70 % in all time points) and much less cells in the longer neurite groups (especially, >60 μm) (Fig. 1d). The mean longest neurite length of N2a/sh-Ngb cells was significantly shorter than those of N2a/N-con and N2a/WT at various time points upon serum withdrawal (Fig. 1e). Therefore, endogenous Ngb was required for neuritogenesis or neuronal differentiation in N2a cells.

Ngb Overexpression Promotes Neurite Outgrowth in N2a Cells

To further establish the causative role of Ngb in neurite outgrowth in N2a cells, we examined the effect of Ngb overexpression on neurite growth. The transfection efficiency of N2a cells was around 77 % (Fig. 2a). Serum withdrawal induced neurite outgrowth in N2a/GFP cells (transiently transfected with p-EGFP-N1) as viewed by GFP fluorescence (Fig. 2b), verifying the neurite outgrowth inducing model in N2a cells. Overexpression of Ngb-GFP evidently increased neurite length in N2a cells at various time points (3, 6, 9, 16, and 24 h) upon serum withdrawal as compared to the corresponding GFP controls (Fig. 2b). Interestingly, overexpression of cytoglobin (Cygb), another hexa-coordinated globin closely related to Ngb and widely expressed in various tissues, had no effect on neurite outgrowth in N2a/Cygb cells (Fig. 2b). The morphometric analysis of mean neurite length was carried out by measuring the longest neurite of individual N2a cells (>100 cells/dish) in the culture.

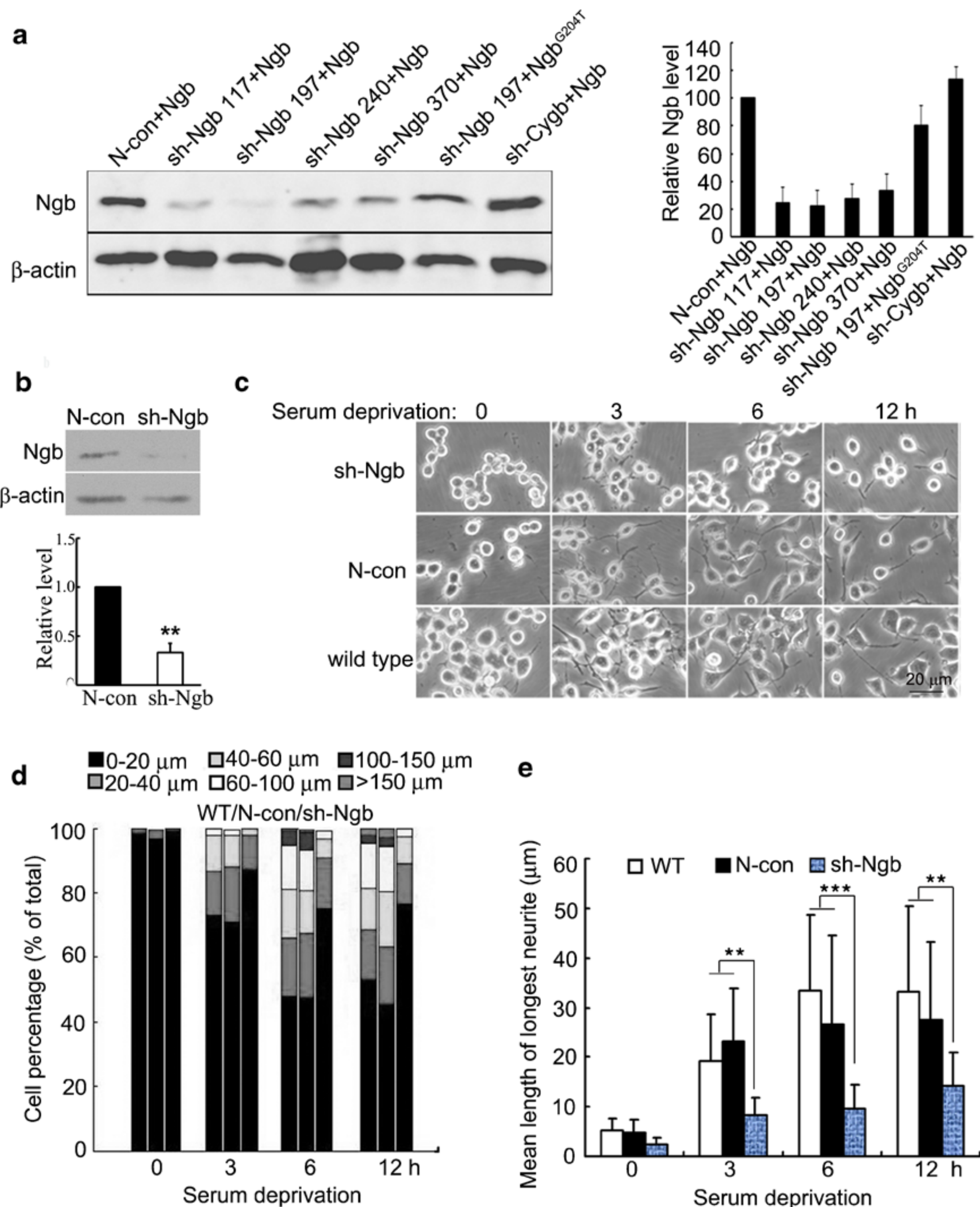

Fig. 1 Neurite-deficient phenotype in Ngb knocking-down N2a cells. **a** The effect of sh-Ngb plasmids on Ngb knock-down. Four sh-Ngb plasmids (targeting different Ngb mRNA coding regions) were co-transfected with p-Ngb-EGFP-N1 into N2a cells. The knocking-down efficiency of sh-Ngb was examined by measuring Ngb-GFP levels 2 days after cotransfection. Representative Western blotting results showed that Ngb-GFP levels were decreased in sh-Ngb-transfected cells as compared to scramble control (N-con). Ngb^{G204T} was a Ngb mutant resistant to sh-Ngb 197 (targeting Ngb coding sequence 197-217). sh-Cygb served as a nonspecific control of sh-Ngb and **β**-actin as a loading control. Relative Ngb level was expressed as Ngb/**β**-actin. Statistical analysis showed that sh-Ngb 197 was most effective in decreasing Ngb. **b** Effect of sh-Ngb on knocking-down endogenous Ngb in N2a cells. Representative Western blotting results and statistical analysis demonstrated that sh-Ngb overexpression efficiently decreased Ngb protein. $n=3$; $^{**}p<0.001$. **c** Phenotype of Ngb knock-down in N2a cells. N2a cells were stably transfected with sh-Ngb or N-con plasmids. Twenty-four hours after seeding, neurite outgrowth of N2a cells was induced by OPTI-MEM. Representative micrographs showed the morphological changes of N2a/sh-Ngb, N2a/N-con, and N2a/wild-type (WT) cells. **d** Cell percentage of N2a cells bearing different neurite lengths. N2a cells were categorized into six groups according to their longest neurite. Cell percentage of the six groups (100 % in total) was calculated from representative cultures. **e** Statistic analysis of the mean longest neurite length of N2a/sh-Ngb, N2a/N-con, and N2a/WT at indicated time points of serum withdrawal. Mean longest neurite length was determined by measuring the longest neurite of more than 100 cells per dish and three independent experiments were performed. $n=3$; $^{**}p<0.01$, $^{***}p<0.001$

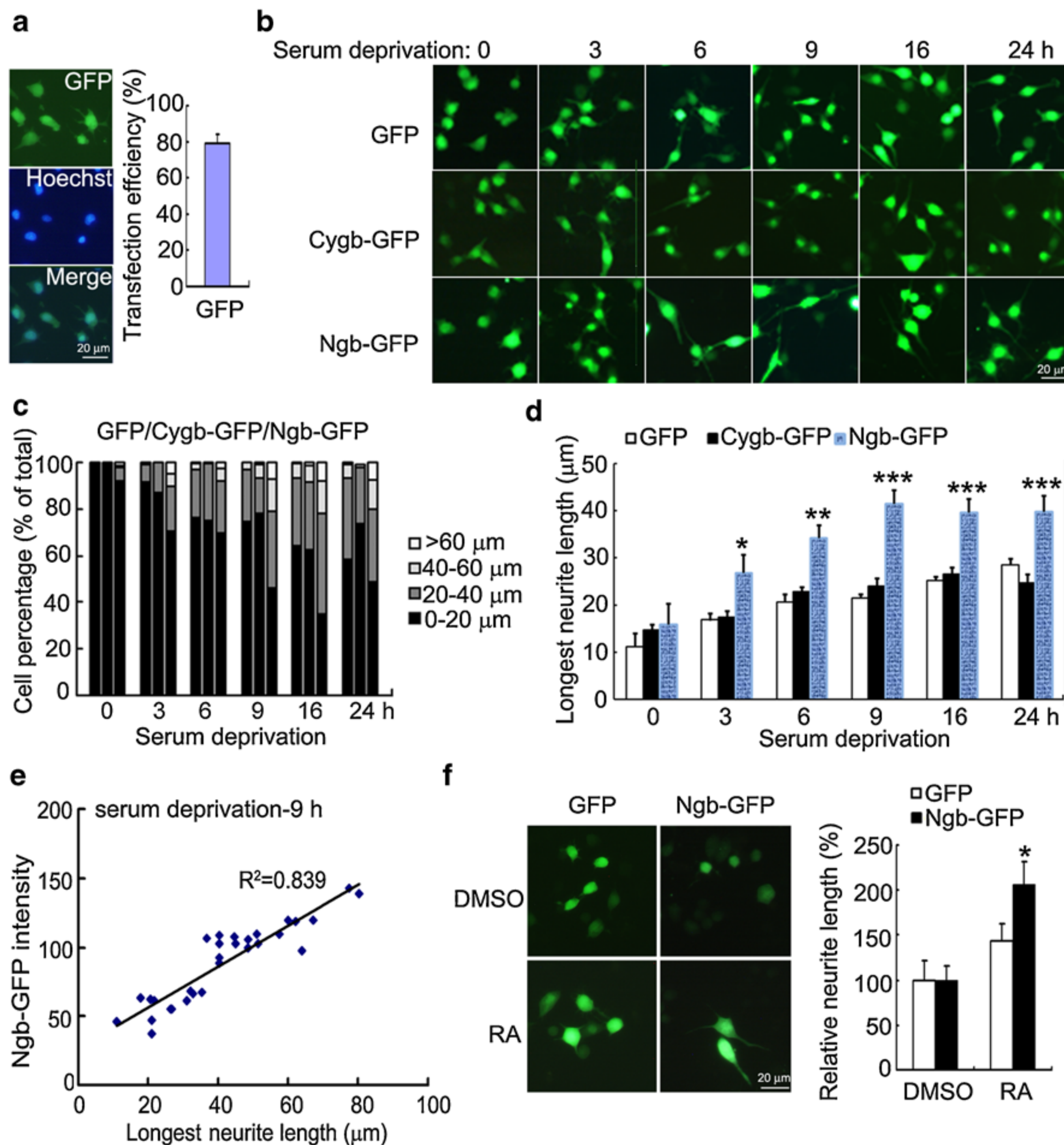

Fig. 2 Ectopic Ngb expression promotes neurite outgrowth in N2a cells. **a** Transfection efficiency of N2a cells. N2a cells were transfected with p-EGFP-N1 for 2 days and stained with Hoechst 33342. The percentage of GFP-positive cells represented the transfection efficiency. **b** Effect of Ngb overexpression on neurite outgrowth in N2a cells upon serum deprivation. N2a cells were transiently transfected with p-EGFP-N1, p-Cygb-EGFP-N1, or p-Ngb-EGFP-N1. Twenty-four hours after transfection, neurite outgrowth was induced by serum deprivation. Representative fluorescent micrographs showed the morphological change of N2a cells. **c** Percentage histogram of the six groups of N2a cells bearing different neurite lengths. **d** Statistical analysis of the mean neurite length of N2a/GFP, N2a/Cygb, and N2a/Ngb cells at indicated serum withdrawal time points. $n=3$; $*p<0.05$, $**p<0.01$, $***p<0.001$ vs corresponding GFP and Cygb controls. **e** Correlation of Ngb-GFP fluorescence intensity and neurite length in N2a cells upon 9 h of serum deprivation. **f** Effect of Ngb overexpression on RA-induced neurite outgrowth in N2a cells. N2a cells were transiently transfected with p-Ngb-EGFP-N1 or p-EGFP-N1 for 24 h and were then treated with 20 **μ**M of RA for 24 h. DMSO was used as vehicle control. Representative micrographs showed the morphological change of N2a cells (*right panels*). The *left panel* showed the results of the statistical analysis of relative longest neurite length. $n=3$; $*p<0.05$ vs corresponding GFP control

Cell percentage histogram clearly showed that there were much more cells bearing a longer neurite (>40 μm; 6, 9, 16, and 24 h) in Ngb-transfected N2a cells than GFP or Cygb controls (Fig. 2c). In the meantime, Ngb-transfected N2a cells had much less cells in the 0–20-μm group (Fig. 2c; 9 and 16 h). Statistical analysis demonstrated that the mean longest neurite length was significantly longer in the Ngb-transfected N2a cells as compared to that of GFP and Cygb controls at various time points (3, 6, 9, 16, and 24 h) upon serum withdrawal (Fig. 2d). The fluorescence intensity of Ngb-GFP in individual N2a cells was correlated positively to its longest neurite length ($R^2=0.839$, Fig. 2e). Further, the

effect of Ngb on neurite outgrowth was tested in retinoic acid-treated N2a cells. Overexpression of Ngb significantly increased the neurite length of N2a cells 24 h after RA treatment as compared to its corresponding GFP control (Fig. 2f). These results demonstrated that there is a neurite growth-promoting function of Ngb and that this function is specific to Ngb but not to other members of the globin family.

Ngb Is Upregulated and Preferentially Distributed in Developing Neurons In Vitro and In Vivo

The effects of Ngb silencing and overexpression on neurite outgrowth in N2a cells suggest a physiological function of Ngb in the brain. Therefore, we investigated the correlation of Ngb expression and neurite development in primary cultured cortical neurons. In cultured cortical and hippocampal neurons, neurite length was increased steadily along with the culture ages within 7 days in vitro (7 DIV) (Fig. 3a, b). Results of RT-PCR demonstrated that the expression levels of Ngb transcripts were significantly increased in cultured cortical neurons along with their culture ages in vitro (Fig. 3c, d). However, the expression levels of Ngb mRNA in cultured astrocytes did not differ at different culture ages (1, 2, 3, and 4 weeks; Fig. 3e, f). Consistently, the results of fluorescence cytoimmunostaining (Fig. 3g) showed that the expression levels of Ngb proteins were also increased in cultured cortical neurons along with their ages in vitro, correlating with the increasing axon length of cortical neurons during development. Statistical analysis demonstrated that the mean Ngb fluorescence intensity was significantly higher in neurons at 3, 5, and 7 DIV (Fig. 3h). Notably, Ngb was evenly distributed in neurons at 1 DIV but tropically distributed in neurons at 3 and 5 DIV with an apparent distribution in the neurites (Fig. 3g). The upregulation and distribution of Ngb in neurites were correlated well to the process of neurite outgrowth in cultured neurons.

We then analyzed the subcellular distribution of endogenous Ngb in the brain. Figure 4a showed that the Ngb antibody we used recognized Ngb protein specifically in Western blotting analysis, consistent with our previous studies [20]. Ngb existed not only in the cell body but also evident in the neurites of both cortical and hippocampal neurons (Fig. 4b, arrows indicated the neurites) in young adult (1 month) rat brains. In the developing cortical neurons (3 DIV), Ngb was largely distributed in the neurite, colocalizing well with Tau-1, a marker of axon (Fig. 4c). Notably, Ngb was accumulated in the proximal site of axons (indicated by arrows), the site of growth cone for axonal growth. During the earliest phase of neurite development, growth associated protein-43 (GAP-43) is expressed at the growth cone of the emerging neurite. Double fluorescence immunostaining showed that Ngb accumulation (indicated by arrows) was colocalized well to GAF-43 accumulation in cultured cortical neurons 6 h after initial seeding (Fig. 4d). The coefficient of determination (R^2) of the fluorescence intensities of Ngb and GAP-43 in individual neuron was 0.9068 (Fig. 4e). Above evidences together established a correlation between Ngb expression/distribution and neurite outgrowth during neuronal development.

Ngb Promotes Neurite Outgrowth in Primary Cultured Cortical Neurons

We then determined the functional role of Ngb in neurite outgrowth by overexpressing or knocking-down Ngb in primary cultured cerebral cortical neurons. The transfection efficiency of primary neurons was around 60 % by nuclear transfection (Fig. 5a). Cultured cortical neurons with Ngb overexpression possessed much longer neurites (the longest one, axon) as compared to GFP and Cygb controls 48 h after transfection (Fig. 5b). This evidence reassured the specific physiological function of Ngb in neurite outgrowth in neuronal cells. Statistical analysis demonstrated that the mean longest neurite length of Ngb-transfected neurons was significantly longer than that of GFP- and Cygb-transfected neurons (Fig. 5c). Complementary knocking-down experiments further demonstrated that silencing endogenous Ngb by overexpressing sh-Ngb significantly reduced the longest neurite length of cortical neurons as compared to its N-con 4 days after nuclear transfection (Fig. 5d). These data together demonstrated that Ngb was required for neurite outgrowth in primary cultured cortical neurons.

Ngb Promotes Neurite Outgrowth via Binding to PTEN and Akt

To understand the molecular mechanisms by which Ngb promotes neurite outgrowth, we investigated the effect of Ngb on several key proteins closely related to neurite outgrowth [24]. Western blotting analysis (Fig. 6a) and statistical analysis (Fig. 6b) showed that Ngb had prominent effects on PTEN/PI3K/Akt signaling cascades in N2a cells after serum withdrawal. Overexpression of Ngb evidently decreased p-PTEN Ser380/Thr382/Thr383 levels, increased p-Akt Ser473 levels in N2a cells upon serum deprivation but did not alter total PTEN and Akt levels as compared to their GFP controls. However, Ngb overexpression did not alter p-PI3K Thr458 and p-GSK3β Ser9 levels. Also, Ngb overexpression did not alter p-Erk-1/2 levels although Erk-1/2 activation was evident in N2a cells upon serum deprivation. The specificity of each phosphorylated antibody was verified by using phosphatase-treated samples (Fig. 6a). To further explore the mechanisms of Ngb action in the PTEN/PI3K/Akt pathway, the direct interaction of Ngb with PI3K or Akt was examined by the BiFC technique. Coexpression of NV-Ngb and CV-Akt reconstituted Venus signal in N2a

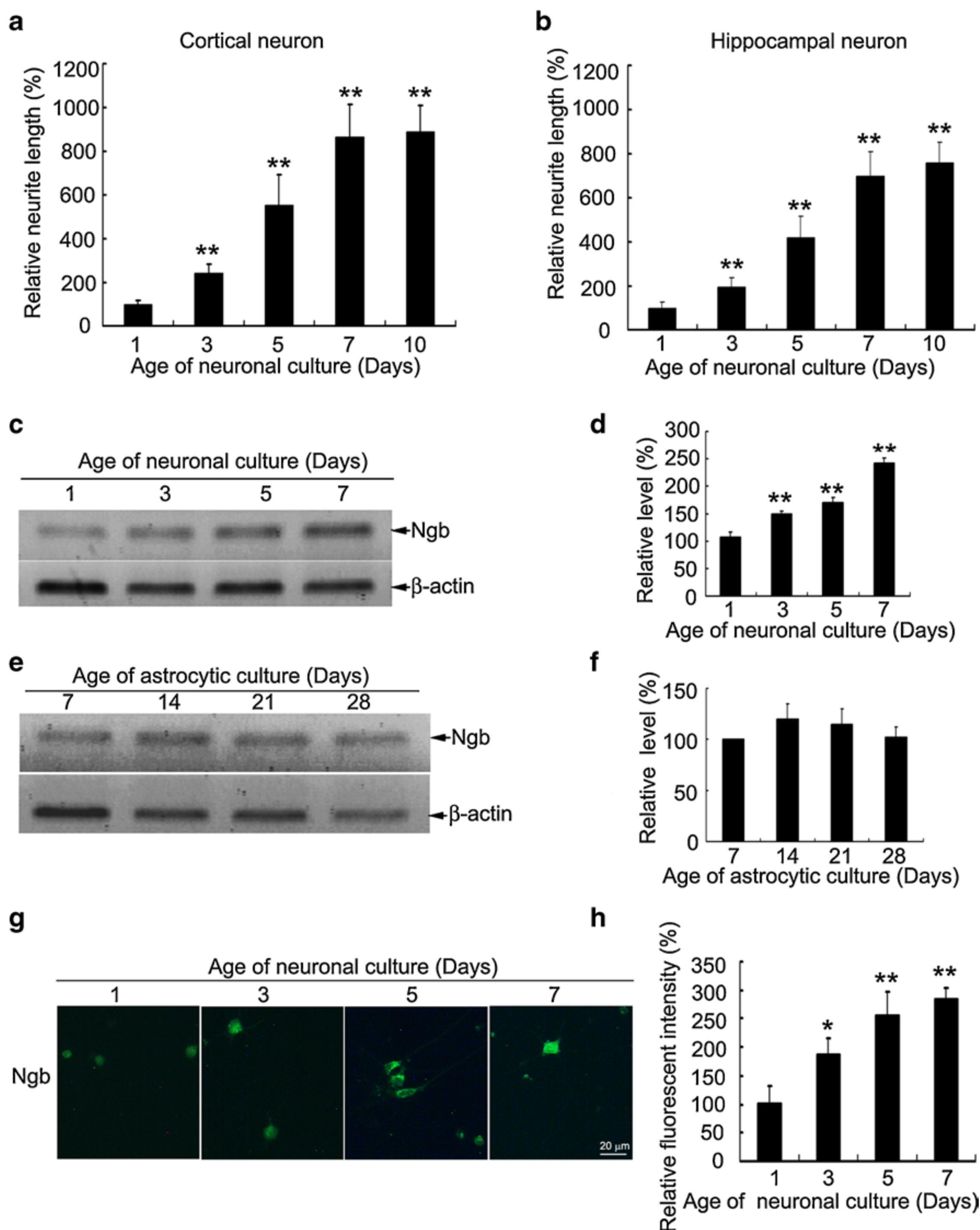

Fig. 3 Endogenous Ngb expression in developing cultured cortical neurons. **a**, **b** Statistical analysis of the mean longest neurite length of cultured cortical neurons (**a**) and hippocampal neurons (**b**) at the indicated culture ages (DIV). $n=3$; $^{**}p<0.01$ vs 1 DIV. **c** Representative RT-PCR results of Ngb mRNA in cultured neurons along with culture ages. **β**-Actin was amplified simultaneously as the internal control. **d** Statistical analysis of relative expression levels of Ngb mRNA after normalizing to **β**-actin. $^{**}p<0.01$ vs 1 DIV. **e** Representative RT-PCR results of Ngb mRNA in primary cultures of cerebral cortical astrocytes along with culture ages. **f** Statistical analysis of relative expression levels of Ngb. **g** Representative fluorescent micrographs showed the expression of endogenous Ngb in primary cultured cortical neurons. Experiments were repeated independently for three times. **h** Statistical analysis of the relative expression levels of Ngb in cultured neurons. The mean fluorescence intensity of Ngb was calculated by measuring more than 50 cells per dish. The experiments were repeated three times. $^{*}p<0.05$, $^{**}p<0.01$ vs 1 DIV

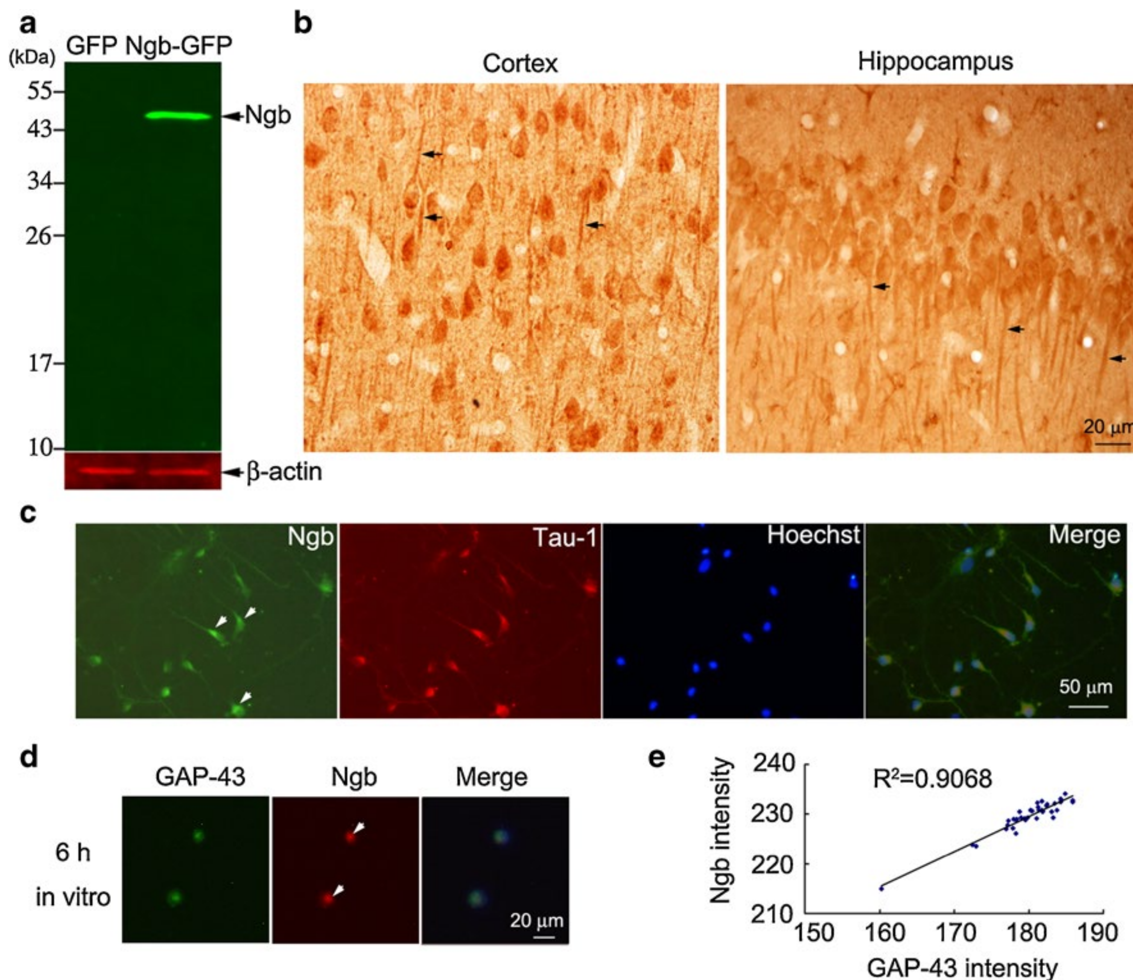

Fig. 4 Subcellular Ngb distribution in developing neurons in vitro and in vivo. **a** Identification of Ngb antibody specificity. p-Ngb-EGFP-N1 or p-EGFP-N1 was transiently transfected into N2a cells. Two days after transfection, cell lysates were subjected to Western blotting analysis with the Ngb antibody (1:500, Santa Cruz). **β**-Actin served as the loading control. **b** Ngb distribution in cerebral cortical and hippocampal neurons in rat brain. Sections from adult rat brains were subjected to immunohistochemical analysis with Ngb antibody. Representative micrographs showed the expression of Ngb in the cerebral cortex and hippocampus. *Arrows* indicated Ngb in neurites. The experiments were repeated three times. **c** Colocalization of Ngb and Tau-1 in developing cultured cortical neurons. Primary cultured rat cortical neurons at 3 DIV were double-stained with Ngb and Tau-1 antibodies. Representative fluorescence micrographs showed the subcellular distribution of endogenous Ngb (*green*) and Tau-1 (*red*) in cortical neurons. *Arrows* indicated Ngb aggregates. Hoechst 33342 stained the nuclei (*blue*). The experiments were repeated three times. **d** Colocalization of Ngb and GAP-43 in developing cultured cortical neurons. Primary cultured cortical neurons 6 h in vitro were double-stained with Ngb and GAP-43 antibodies. Representative micrographs showed that Ngb's aggregates (indicated by *arrows*, *red*) were colocalized to GAP-43's aggregates. The experiments were repeated three times. **e** Correlation of Ngb and GAP-43 intensities in cultured cortical neurons 6 h in vitro

cells, and its fluorescence was enhanced further upon 9 h of serum deprivation as compared to NV-Ngb/CV and NV-Ngb/CV-PI3K cotransfected cells (Fig. 6c). NV-MKK3/CV-p38 served as a positive control for BiFC assay. Moreover, fluorescent positive cells in NV-Ngb/CV-Akt cotransfected N2a cells were evidently increased upon 9 h of serum deprivation (Fig. 6c). Statistical analysis of the Venus fluorescence intensity demonstrated that Venus signal was significantly enhanced in NV-Ngb/CV-Akt cells upon 9 h of serum deprivation (Fig. 6d). Western blotting analysis showed similar expression level of NV- and CV-tagged proteins in NV-Ngb/CV-PI3K and NV-Ngb/CV-Akt cotransfected cells (Fig. 6e).

The interaction between Ngb and Akt was further confirmed by GST pull-down assay (Fig. 7a). Interestingly, mutations of Ngb at various sites (E53Q, E118Q, K119N, H64A, H64L, and Y44D) evidently enhanced its binding to Akt (Fig. 7a). Since Ngb^{WT} bound little Akt and Ngb affecting both p-Akt and its canonical upstream negative regulator PTEN (Fig. 6a), we further examined the interaction of Ngb and PTEN. GST pull-down assay showed a clear Ngb–PTEN interaction. Notably, mutation of Ngb at various sites (E53Q, E118Q, K119N, H64A, H64L, and Y44D) notably reduced its binding to PTEN, contrary to the changes of Ngb–Akt interactions (Fig. 7b). Taken together, our data

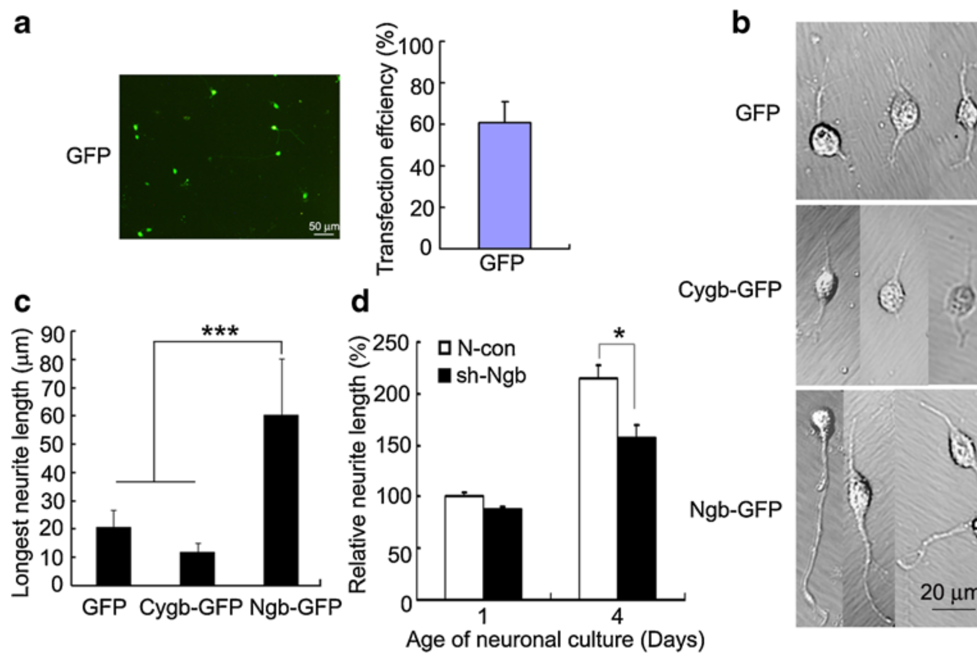

Fig. 5 Effects of Ngb overexpression and knock-down on axon outgrowth in primary cortical neurons. **a** Nuclear transfection efficiency in primary cultured cortical neurons. Cultured neurons were transiently transfected with p-EGFP-N1 plasmids by using the Nucleofector Kit and the Amaxa Nucleofector device. Representative micrograph (*left panel*) showed the expression of GFP in the culture 2 days after transfection. *Right panel* showed the transfection efficiency ($n=3$). **b** Effect of Ngb overexpression on axon outgrowth in primary cortical neurons. Cultured cortical neurons were transfected with either p-EGFP-N1, p-Cygb-EGFP-N1, or p-Ngb-EGFP-N1. Representative micrographs showed the morphological change of cultured cortical neurons at 3 DIV. The experiments were repeated independently at least three times. **c** Statistical analysis demonstrated that the mean longest neurite length of Ngb-transfected neurons was significantly longer than that of Cygb- or GFP-transfected neurons at 3 DIV. $n=3$; ***$p<0.001$. **d** Effect of Ngb knock-down on neurite outgrowth in cortical neurons. Statistical analysis demonstrated that the mean longest neurite length of sh-Ngb transfected neurons was significantly shorter than that of N-con at 4 DIV. $n=3$; *$p<0.05$

demonstrated that Ngb controls PTEN/PI3K/Akt signaling by interacting with PTEN and Akt selectively.

We then determined the effect of Ngb mutant (E53Q, decreasing Ngb–PTEN interaction most effectively) on PTEN/Akt signaling. Western blotting analysis (Fig. 7c) and statistical analysis demonstrated that Ngb^{E53Q} significantly decreased p-PTEN and increased p-Akt expression levels as compared to Ngb^{WT} controls in N2a cells upon 9 h of serum deprivation (Fig. 7c, d). Consistently, overexpression of Ngb^{E53Q} significantly increased the length of axons in primary cultured cortical neurons at 5 DIV as compared to Ngb^{WT} control (Fig. 7e).

Discussion

Neuroglobin, a highly conserved hemoprotein that evolved from a common ancestor to hemoglobin and myoglobin, is predominantly expressed in neurons of the brain and retina [25]. Current studies have revealed diverse potential functions of Ngb, which focus mainly on Ngb's neuroprotection upon ischemia or oxidative stress associating to its binding to small ligands such as O_2 and NO or macromolecules such as G protein and 14-3-3 [26]. Until now, the physiological function of Ngb is still not determined. The primary finding of the present study is that neuroglobin is essential for neuritogenesis or neurite (including axon) outgrowth during neuronal development or differentiation. The underlying mechanisms involve interactions among Ngb and PTEN or Akt.

Neurite length was gradually increased during neuronal development in primary cortical neurons in vitro. The expression levels of Ngb mRNA and protein were also increased, leading to the neurite growth process. During the earlier stage of axon growth, Ngb was even accumulated in the growth cones of axons (Fig. 4c). Ngb in the neurites of cortical and hippocampal neurons in young adult rats was also notable. These data clearly support a positive correlation of Ngb and neurite/axon development.

The differentiation of mammalian neurons during development is a highly complex process involving electrophysiological, morphological, and transcriptional changes [27]. This process is characterized by the initial formation of immature neurites, i.e., neurite outgrowth. By ectopic expression of Ngb, we demonstrated that elevated Ngb favored neurite outgrowth in both N2a cells and primary cortical neurons. Complementarily, reducing Ngb exhibited a prominent neurite deficiency or growth retardation pheno-

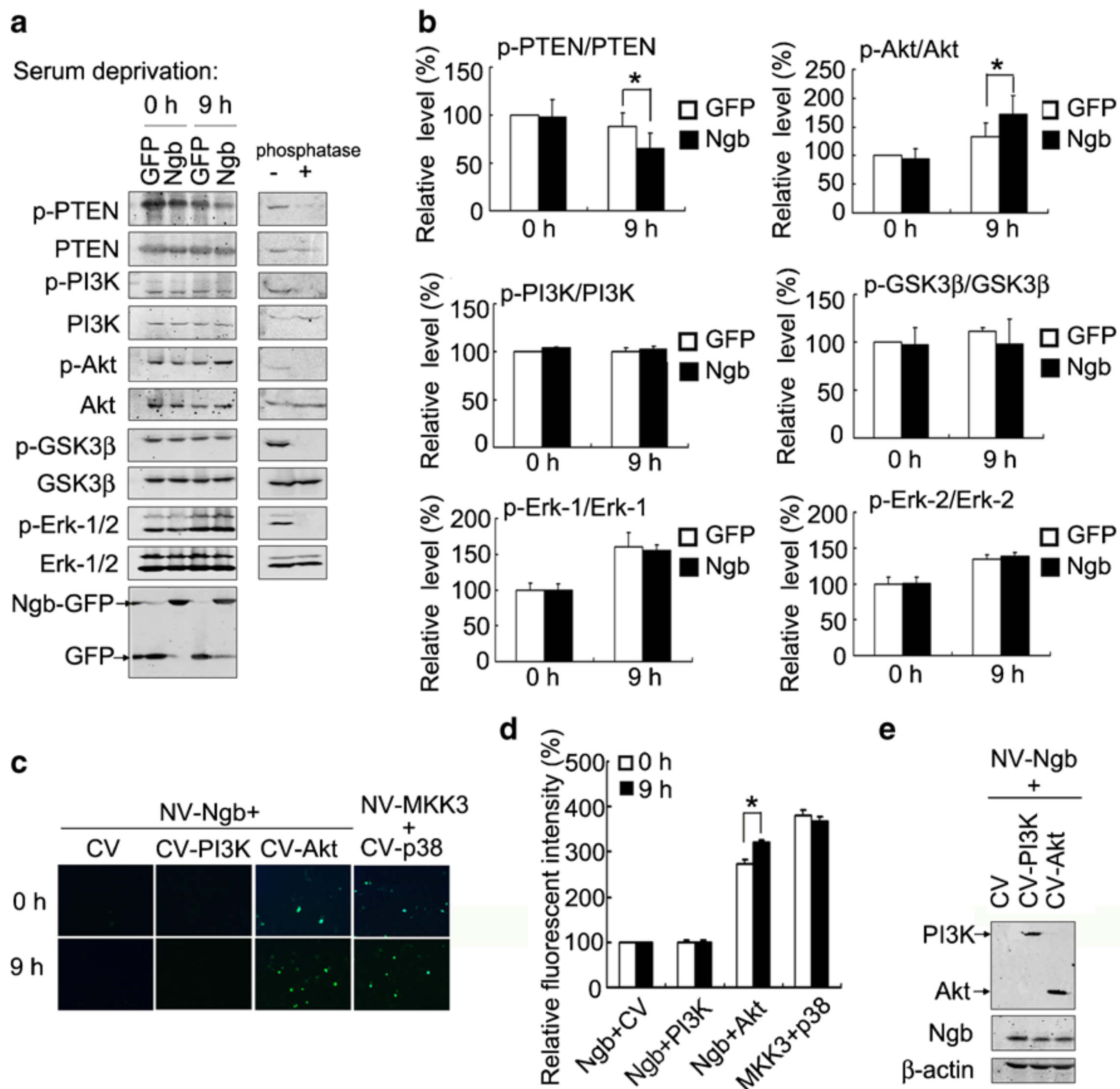

Fig. 6 Ngb regulates PTEN/PI3K/Akt signaling by direct binding to Akt in N2a cells. **a** Effects of Ngb overexpression on PTEN/PI3K/Akt activation. N2a cells were transiently transfected with either GFP or Ngb-GFP for 24 h and subjected to 9 h of serum deprivation. Representative Western blotting results showed the expression of p-Akt Ser473/Akt, p-PI3K Thr458/PI3K, p-PTEN Ser380/Thr382/383/PTEN, p-GSK3**β** Ser9/GSK3**β**, and p-Erk-1/2/Erk-1/2. The specificity of phosphorylated antibodies was verified by using **λ**-phosphatase-treated samples. The experiments were repeated three times independently. **b** Statistical analysis of relative p-Akt Ser473/Akt, p-PI3K Thr458/PI3K, p-PTEN Ser380/Thr382/383/PTEN, p-GSK3**β** Ser9/GSK3**β**, p-Erk-1/Erk-1, and p-Erk-2/Erk-2 levels from Western blotting results. $n=3$, $*p<0.05$ vs corresponding GFP control. **c** Representative results of BiFC showed that Ngb bound to Akt directly in living N2a cells. N2a cells were cotransfected with NV-Ngb (N-terminal Venus-fused Ngb) together with CV (C-terminal Venus), CV-PI3K, or CV-Akt plasmids at a ratio of 1:1. Twenty-four hours after transfection, neurite outgrowth was induced by 9 h of serum deprivation. Representative fluorescence micrographs showed the Venus signal in living cells. NV-Ngb+CV or NV-MKK3+CV-p38 served as negative or positive control, respectively. The experiments were repeated three times independently. **d** Statistical analysis of the relative Venus signals. Mean fluorescence intensity from nine fields of each culture was calculated and values higher than 1.5-fold of the background were considered positive. $n=3$; $*p<0.05$. **e** Expression of BiFC proteins in **c**. Representative Western blotting results showed the expression of NV-Ngb (anti-Ngb) and CV-fused proteins (anti-His, CV tag)

types in both N2a cells and primary cultured neurons. Therefore, we conclude that Ngb plays an important role in the regulatory machinery of neurite development.

To explore the molecular mechanisms underlying Ngb-induced morphological changes, we searched for Ngb's downstream players. Neuronal differentiation and neurite outgrowth are mediated by activation or inactivation of PI3K, Akt, GSK-3β, PKC, PKA, CaMKII, Erk, or p38 signaling pathways [28]. PI3K/Akt has been revealed as the key pathway regulating neurite development, including neurite genesis, elongation, branching, and caliber [29]. We demonstrated that Ngb positively regulated Akt phosphorylation, consistent with our previous and other results [18, 19]. PI3K is the most important upstream kinase activating

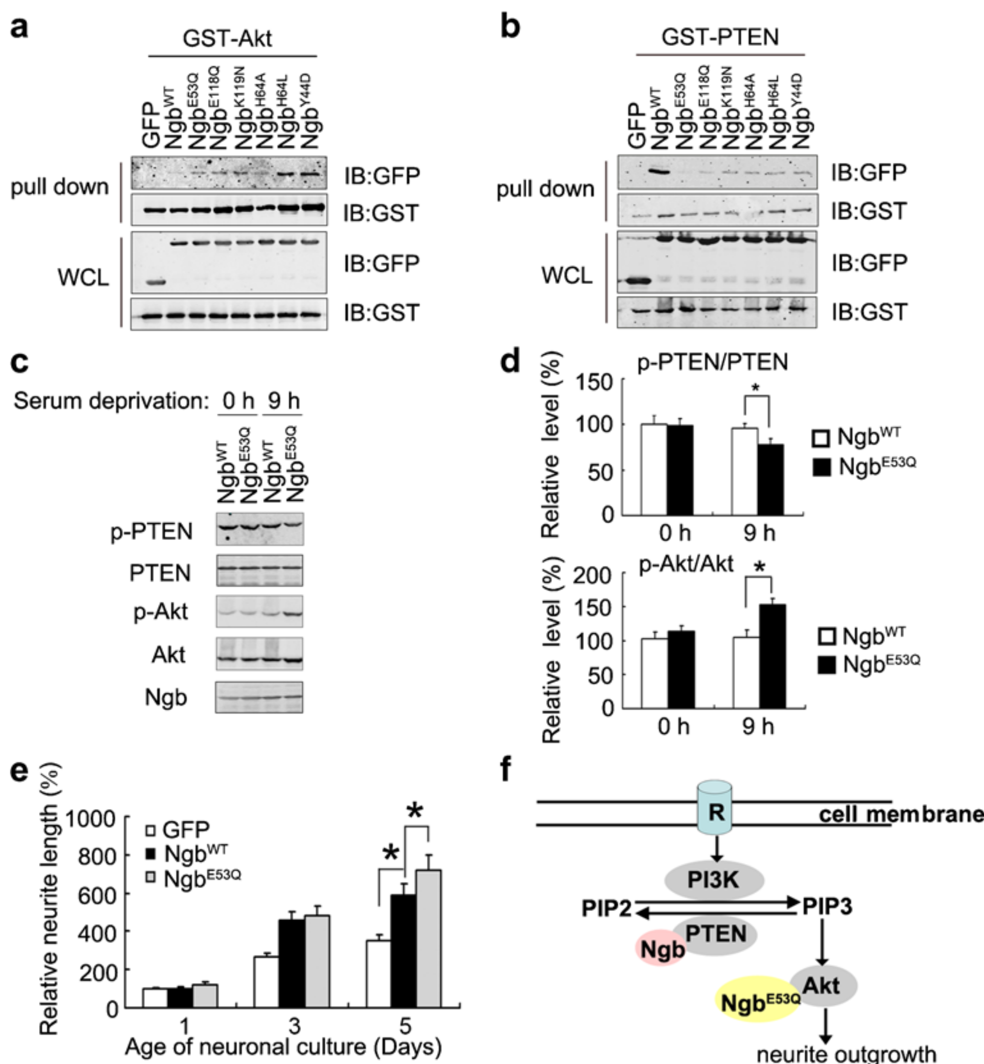

Fig. 7 Differential Ngb binding to Akt and PTEN regulates neurite outgrowth. **a**, **b** Ngb and its mutants interacted with Akt and PTEN. 293T cells were cotransfected with GST-Akt (**a**) or GST-PTEN (**b**) together with GFP-Ngb or GFP-Ngb^{mutant} at a ratio of 1:1 for 2 days. Equal amounts of total proteins were subjected for GST pull-down assay. Representative Western blotting results showed the binding of Ngb/Ngb^{mutant} to PTEN and Akt. *WCL* whole cell lysate, *IB* immunobloting. The experiments were repeated three times independently. **c** Representative Western blotting results showed the effects of Ngb^{E53Q} on PTEN and Akt phosphorylation. **d** Statistical analysis of **c**. $n=3$; $*p<0.05$. **e** Statistical analysis demonstrated that overexpression of Ngb^{E53Q} increased neurite length in cultured cortical neurons at 5 DIV. $n=3$; $*p<0.05$. **f** Proposed role of Ngb on PI3K/Akt pathway. Ngb promotes neurite outgrowth by preferential binding to PTEN under normal conditions and shifting a preferential Akt binding under pathological conditions as a result of conformational changes similar to Ngb^{mutant}

Akt by producing PIP3. However, Ngb overexpression did not alter p-PI3K Thr458 (the active form of PI3K). Ngb bound neither to PI3K. Therefore, Ngb modulates Akt activation via PI3K-independent mechanisms.

We then asked whether Ngb may interact directly to Akt or not. Indeed, Ngb directly bound to Akt in living N2a cells and their interactions were increased during neurite outgrowth. This finding suggests that direct binding of Ngb and Akt facilitates its phosphorylation, which is required for neurite outgrowth. However, we also noticed that Ngb–Akt interaction was weak as revealed by coimmunoprecipitation assays (Fig. 7a). This prompted us to search for other Ngb players in the PI3K/Akt pathway. In the canonical PI3K/Akt pathway, PTEN antagonizes PI3K function by dephosphorylating PIP3 and therefore negatively regulates Akt activity. Inhibition of PTEN activity facilitates regenerative outgrowth of adult peripheral axons [30]. We found that Ngb suppressed phosphorylation of PTEN during neurite outgrowth (Fig. 6a). Strikingly, Ngb–PTEN interaction was more prominent than Ngb–Akt interaction (Fig. 7b). Interestingly, point mutation of Ngb decreased its binding to PTEN but increased its binding to Akt. This shift binding property might be associated to the structural modulation of Ngb by cellular environment. Oxygen and NO are native

ligands of Ngb and its binding with these small ligands induces conformational changes [31]. It is documented that the interaction of Ngb with 14-3-3ζ and cytochrome C is affected by hypoxic incubation [32]. Conceivably, the interaction of Ngb with either PTEN or Akt is regulated dynamically by intracellular environment such as redox status, and this dynamic interaction might play an important role in balancing the positive or negative signals for neurite outgrowth.

In summary, we demonstrate for the first time that Ngb controls neurite genesis and extension. Furthermore, we propose that Ngb promotes neurite outgrowth by binding to PTEN under physiological conditions and retains this function under pathological conditions (e.g., hypoxia) by increasing binding to Akt (Fig. 7f). Our findings provide insights for understanding the biological functions of Ngb and for improving therapeutic strategy for promoting neurite regeneration in various neurological diseases such as stroke, trauma, and neurodegenerative diseases.

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References

1. da Silva JS, Dotti CG (2002) Breaking the neuronal sphere: regulation of the actin cytoskeleton in neuritogenesis. Nat Rev Neurosci 3(9):694–704. doi:10.1038/nrn918
2. Lin Y, Jones BW, Liu A, Tucker JF, Rapp K, Luo L, Baehr W, Bernstein PS, Watt CB, Yang JH, Shaw MV, Marc RE (2012) Retinoid receptors trigger neuritogenesis in retinal degenerations. FASEB J 26(1):81–92. doi:10.1096/fj.11-192914
3. Hill JJ, Jin K, Mao XO, Xie L, Greenberg DA (2012) Intracerebral chondroitinase ABC and heparan sulfate proteoglycan glypican improve outcome from chronic stroke in rats. Proc Natl Acad Sci U S A 109(23):9155–9160. doi:10.1073/pnas.1205697109
4. Dasari VR, Spomar DG, Gondi CS, Sloffer CA, Saving KL, Gujrati M, Rao JS, Dinh DH (2007) Axonal remyelination by cord blood stem cells after spinal cord injury. J Neurotrauma 24(2):391–410. doi:10.1089/neu.2006.0142
5. Gonzenbach RR, Schwab ME (2008) Disinhibition of neurite growth to repair the injured adult CNS: focusing on Nogo. Cell Mol Life Sci 65(1):161–176. doi:10.1007/s00018-007-7170-3
6. Zhou FQ, Snider WD (2006) Intracellular control of developmental and regenerative axon growth. Philos Trans R Soc Lond B Biol Sci 361(1473):1575–1592. doi:10.1098/rstb.2006.1882
7. Hafner A, Obermajer N, Kos J (2012) gamma-Enolase C-terminal peptide promotes cell survival and neurite outgrowth by activation of the PI3K/Akt and MAPK/ERK signalling pathways. Biochem J 443(2):439–450. doi:10.1042/BJ20111351
8. Namekata K, Harada C, Guo X, Kimura A, Kittaka D, Watanabe H, Harada T (2012) Dock3 stimulates axonal outgrowth via GSK-3beta-mediated microtubule assembly. J Neurosci 32(1):264–274. doi:10.1523/JNEUROSCI.4884-11.2012
9. Conrad S, Genth H, Hofmann F, Just I, Skutella T (2007) Neogenin-RGMa signaling at the growth cone is bone morphogenetic protein-independent and involves RhoA, ROCK, and PKC. J Biol Chem 282(22):16423–16433. doi:10.1074/jbc.M610901200
10. Bodrikov V, Sytnyk V, Leshchyns'ka I, den Hertog J, Schachner M (2008) NCAM induces CaMKIIalpha-mediated RPTPalpha phosphorylation to enhance its catalytic activity and neurite outgrowth. J Cell Biol 182(6):1185–1200. doi:10.1083/jcb.200803045
11. Burmester T, Weich B, Reinhardt S, Hankeln T (2000) A vertebrate globin expressed in the brain. Nature 407(6803):520–523. doi:10.1038/35035093
12. Sun Y, Jin K, Mao XO, Xie L, Peel A, Childs JT, Logvinova A, Wang X, Greenberg DA (2005) Effect of aging on neuroglobin expression in rodent brain. Neurobiol Aging 26(2):275–278. doi:10.1016/j.neurobiolaging.2004.03.006
13. Li SQ, Li WB, Zhang M, Wu YZ, Hu YY (2013) The role of neuroglobin in the neuroprotection of limb ischemic preconditioning in rats. Mol Neurobiol 47(1):197–208. doi:10.1007/s12035-012-8373-7
14. Jin K, Mao Y, Mao X, Xie L, Greenberg DA (2010) Neuroglobin expression in ischemic stroke. Stroke 41(3):557–559. doi:10.1161/STROKEAHA.109.567149
15. Wakasugi K, Nakano T, Morishima I (2003) Oxidized human neuroglobin acts as a heterotrimeric Galpha protein guanine nucleotide dissociation inhibitor. J Biol Chem 278(38):36505–36512. doi:10.1074/jbc.M305519200
16. Jayaraman T, Tejero J, Chen BB, Blood AB, Frizzell S, Shapiro C, Tiso M, Hood BL, Wang X, Zhao X, Conrads TP, Mallampalli RK, Gladwin MT (2011) 14-3-3 binding and phosphorylation of neuroglobin during hypoxia modulate six-to-five heme pocket coordination and rate of nitrite reduction to nitric oxide. J Biol Chem 286(49):42679–42689. doi:10.1074/jbc.M111.271973
17. Raychaudhuri S, Skommer J, Henty K, Birch N, Brittain T (2010) Neuroglobin protects nerve cells from apoptosis by inhibiting the intrinsic pathway of cell death. Apoptosis 15(4):401–411. doi:10.1007/s10495-009-0436-5
18. Antao ST, Duong TT, Aran R, Witting PK (2010) Neuroglobin overexpression in cultured human neuronal cells protects against hydrogen peroxide insult via activating phosphoinositide-3 kinase and opening the mitochondrial K(ATP) channel. Antioxid Redox Signal 13(6):769–781. doi:10.1089/ars.2009.2977
19. Chen LM, Xiong YS, Kong FL, Qu M, Wang Q, Chen XQ, Wang JZ, Zhu LQ (2012) Neuroglobin attenuates Alzheimer-like tau hyperphosphorylation by activating Akt signaling. J Neurochem 120(1):157–164. doi:10.1111/j.1471-4159.2011.07275.x
20. Zhang J, Lan SJ, Liu QR, Liu JM, Chen XQ (2013) Neuroglobin, a novel intracellular hexa-coordinated globin, functions as a tumor suppressor in hepatocellular carcinoma via Raf/MAPK/Erk. Mol Pharmacol 83(5):1109–1119. doi:10.1124/mol.112.083634
21. Chen XQ, Liu S, Qin LY, Wang CR, Fung YW, Yu AC (2005) Selective regulation of 14-3-3η in primary culture of cerebral cortical neurons and astrocytes during development. J Neurosci Res 79(1–2):114–118. doi:10.1002/jnr.20323
22. Chen XQ, Qin LY, Zhang CG, Yang LT, Gao Z, Liu S, Lau LT, Fung YW, Greenberg DA, Yu AC (2005) Presence of neuroglobin in cultured astrocytes. Glia 50(2):182–186. doi:10.1002/glia.20147
23. Chen XQ, Chen JG, Zhang Y, Hsiao WW, Yu AC (2003) 14-3-3gamma is upregulated by in vitro ischemia and binds to protein kinase Raf in primary cultures of astrocytes. Glia 42(4):315–324. doi:10.1002/glia.10185
24. Raivich G, Makwana M (2007) The making of successful axonal regeneration: genes, molecules and signal transduction pathways. Brain Res Rev 53(2):287–311. doi:10.1016/j.brainresrev.2006.09.005
25. Trent JT 3rd, Watts RA, Hargrove MS (2001) Human neuroglobin, a hexacoordinate hemoglobin that reversibly binds oxygen. J Biol Chem 276(32):30106–30110. doi:10.1074/jbc.C100300200
26. Wakasugi K, Morishima I (2005) Identification of residues in human neuroglobin crucial for guanine nucleotide dissociation inhibitor activity. Biochemistry 44(8):2943–2948. doi:10.1021/bi0477539

27. Zorina Y, Iyengar R, Bromberg KD (2010) Cannabinoid 1 receptor and interleukin-6 receptor together induce integration of protein kinase and transcription factor signaling to trigger neurite outgrowth. J Biol Chem 285(2):1358–1370. doi:10.1074/jbc.M109.049841
28. Vaudry D, Stork PJ, Lazarovici P, Eiden LE (2002) Signaling pathways for PC12 cell differentiation: making the right connections. Science 296(5573):1648–1649. doi:10.1126/science.1071552
29. Read DE, Gorman AM (2009) Involvement of Akt in neurite outgrowth. Cell Mol Life Sci 66(18):2975–2984. doi:10.1007/s00018-009-0057-8
30. Christie KJ, Webber CA, Martinez JA, Singh B, Zochodne DW (2010) PTEN inhibition to facilitate intrinsic regenerative outgrowth of adult peripheral axons. J Neurosci 30(27):9306–9315. doi:10.1523/JNEUROSCI.6271-09.2010
31. Giuffre A, Moschetti T, Vallone B, Brunori M (2008) Neuroglobin: enzymatic reduction and oxygen affinity. Biochem Biophys Res Commun 367(4):893–898. doi:10.1016/j.bbrc.2008.01.021
32. Dong Y, Zhao R, Chen XQ, Yu AC (2010) 14-3-3gamma and neuroglobin are new intrinsic protective factors for cerebral ischemia. Mol Neurobiol 41(2–3):218–231. doi:10.1007/s12035-010-8142-4