Transcriptional regulation mechanisms of hypoxia-induced neuroglobin gene expression

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Ngb (neuroglobin) has been identified as a novel endogenous neuroprotectant. However, little is known about the regulatory mechanisms of Ngb expression, especially under conditions of hypoxia. In the present study, we located the core proximal promoter of the mouse Ngb gene to a 554 bp segment, which harbours putative conserved NF-κB (nuclear factor κB) and Egr1 (early-growth-response factor 1) -binding sites. Overexpression and knockdown of transcription factors p65, p50, Egr1 or Sp1 (specificity protein 1) increased and decreased Ngb expression respectively. Experimental assessments with transfections of mutational Ngb gene promoter constructs, as well as EMSA (electrophoretic mobility-shift assay) and ChIP (chromatin immunoprecipitation) assays, demonstrated that NF-κB family members (p65, p50 and cRel), Egr1 and Sp1 bound in vitro and in vivo to the proximal promoter region of the Ngb gene. Moreover, a κB3 site was found as a pivotal cis-element responsible for hypoxia-induced Ngb promoter activity. NF-κB (p65) and Sp1 were also responsible for hypoxia-induced up-regulation of Ngb expression. Although there are no conserved HREs (hypoxia-response elements) in the promoter of the mouse Ngb gene, the results of the present study suggest that HIF-1α (hypoxia-inducible factor-1α) is also involved in hypoxia-induced Ngb up-regulation. In conclusion, we have identified that NF-κB, Egr1 and Sp1 played important roles in the regulation of basal Ngb expression via specific interactions with the mouse Ngb promoter. NF-κB, Sp1 and HIF-1α contributed to the up-regulation of mouse Ngb gene expression under hypoxic conditions.

Key words: gene regulation, hypoxia, mouse, neuroglobin, promoter, transcription factor.

INTRODUCTION

Ngb (neuroglobin) is a novel tissue globin with a high affinity for oxygen which is predominantly expressed in the vertebrate brain [1,2]. The Ngb gene is evolutionarily extremely conserved, as human and mouse Ngb share 94% identity in amino acid sequence [1], implying that Ngb has a significant physiological role, but it remains poorly defined. Our laboratory and others have demonstrated that Ngb expression is increased in response to neuronal hypoxia, focal cerebral ischaemia and oxidative stress [3,4]. Ngb-overexpressing transgenic mice are resistant to focal cerebral and myocardial ischaemic injury, and protect against hypoxia and β-amyloid-mediated neurotoxicity [3–5]. These studies have demonstrated that Ngb is a novel endogenous neuroprotective molecule. The potential mechanisms of the neuroprotection by Ngb include maintaining mitochondria function, as well as scavenging ROS (reactive oxygen species) and RNS (reactive nitrogen species) [4,6,7]. Thus Ngb has been thought of as a therapeutic target for endogenous neuroprotection [8], and methods that can up-regulate endogenous Ngb gene expression may be novel approaches for intervention and treatment of neurodegenerative diseases and related disorders. Thus defining the transcriptional regulation mechanisms of Ngb gene expression would be fundamentally and translationally significant.

However, the transcriptional mechanisms of Ngb gene regulation are rarely investigated. Only one previous study, that from Zhang et al. [9], has described that transcription factors Sp1 (specificity protein 1) and Sp3 bind to the human Ngb promoter region and transactivate the Ngb promoter. However, detailed transcriptional regulation mechanisms of the Ngb gene under physiological and pathophysiological conditions remain unknown and need to be investigated further.

In the present study, in an attempt to further unravel the mechanisms involved in basal as well as hypoxia-induced Ngb gene regulation, we characterized the 5′-upstream region of the mouse Ngb gene promoter in order to identify regulatory elements and corresponding transcriptional factors that are involved in the basal expression of the mouse Ngb gene. Deletion analysis identified the 5′-upstream region in the Ngb promoter. −549 to +6 relative to the starting codon ATG, which showed relatively high promoter activity in N2a cells. Transcription-factor-binding site analysis of that region identified three conserved cis-elements, including two κB sites and one Egr1 (early-growth-response factor 1) site. Overexpression and knockdown approaches confirmed that NF-κB (nuclear factor κB), Egr1 and Sp1 were capable of controlling Ngb expression. Site-directed mutagenesis of κB2 and κB3 sites, as well as the Egr1 site, significantly reduced mouse Ngb promoter activity. EMSA (electrophoretic mobility-shift assay)
and ChIP (chromatin immunoprecipitation) analyses showed that NF-κB family members (p65, p50 and cRel), Egr1 and Sp1 bind directly to the proximal promoter region of the Ngb gene. Furthermore, the molecular mechanisms of hypoxia-induced Ngb up-regulation were further elucidated. Our results have shown that the κB3 site is required for hypoxia-induced Ngb up-regulation. Transcription factors NF-κB (p65) and Sp1, as well as HIF-1α (hypoxia-inducible factor-1α), are regulators responsible for the up-regulation of Ngb expression under hypoxic conditions. The results of the present study delineate a comprehensive regulation mechanism of Ngb gene expression under physiological resting and pathological conditions of hypoxia.

EXPERIMENTAL

Materials

DMEM (Dulbecco’s modified Eagle’s medium), Lipofectamine™ 2000, pcDNA3.1-His/Myc-A vector, geneticin, penicillin and streptomycin were purchased from Invitrogen. FBS (fetal bovine serum), 0.05% trypsin/EDTA and L-glutamine were purchased from Gibco. The chicken anti-Ngb primary antibody was purchased from BioVendor, rabbit polyclonal anti-p65 and anti-p50/p105 antibodies were purchased from Abcam, and rabbit polyclonal anti-cRel, anti-Sp1 and anti-Egr1 antibodies, and all siRNAs (small interfering RNAs), were obtained from Santa Cruz Biotechnology. The pGL3-Basic vector, the pGEM®-T Easy Vector System II and the Dual-Luciferase® Reporter Assay System were purchased from Promega. LA Taq™ DNA polymerase with GC buffers was purchased from Takara. Expression vectors encoding the mouse gene including pcDNA3.1-FLAG-p65, pcDNA3.1-FLAG-p50, pcDNA3.1-FLAG-cRel and pcDNA3.1-FLAG-Egr1 have been described previously [10,11] and were purchased from Addgene. An expression plasmid encoding the mouse Sp1 gene, pCMV-HA-Sp1, was constructed as described previously [12].

Reporter plasmid construction

The proximal 5′ region of mouse Ngb which spanned from nucleotide +6 to nucleotide −2027 upstream of the starting codon ATG was amplified by PCR from genomic DNA isolated from the C57BL/6 mouse (the mouse Ngb sequence was obtained from GenBank® accession number NM022414), and then cloned into the promoter-less firefly luciferase reporter plasmid pGL3-Basic in the KpnI/HindIII site, denoted P-2033 (−2027/+6). Subsequently, a set of 5′-flanking fragments were amplified from P-2033 (−2027/+6) by PCR with different forward primers and subcloned into pGL3-Basic, designated P-1539 (−1533/+6), P-1004 (−998/+6), P-886 (−880/+6), P-554 (−549/+6), P-341 (−335/+6), P-247 (−241/+6), P-142 (−136/+6) and P-1692 (−2027/+335). Constructs with mutations to the NF-κB- and Egr1-binding sites were derived from the P-554 (−549/+6) construct. They were then generated by overlapping PCR and cloned into pGL3-Basic with a KpnI/HindIII site. The sequences of all constructs were confirmed by direct sequencing for deletions and mutations are listed in Table 1.

Cell cultures and transfections

The mouse Neuro2a (N2a) neuroblastoma cell line was cultured at 37°C in a humidified atmosphere of 5% CO2, 95% O2 in DMEM supplemented with 10% heat-inactivated FBS, 0.3 mM L-glutamine and 50 units/ml penicillin/streptomycin. The cells were seeded at a density of 1.5 × 105 cells/ml in 24-well plates or six-well plates. For transfection, N2a cells were grown on 24-well plates to approximately 70% confluence and then transiently transfected with DNA constructs or siRNAs using Lipofectamine™ 2000, following the manufacturer’s protocol.

Hypoxia

Hypoxia was induced as described previously [7,13]. Briefly, the cells were first incubated in a modular chamber (Billups–Rothenberg) perfused with 90% N2/5% CO2/5% H2 for 25 min at 37°C. The chamber was then sealed and placed at 37°C for the indicated periods of hypoxia. After hypoxia, cells were removed from the chamber and harvested for other studies. Control cultures were incubated under normoxic conditions for the corresponding lengths of time.

Luciferase assay

For the luciferase assay, in order to normalize the transfection efficiency, Renilla luciferase (PRL-TK) control plasmid was also co-transfected. After incubation for 24 h, cells were lysed in passive lysis buffer and harvested. Luciferase activities were measured using the Dual-Luciferase® Reporter Assay System. A 50 μl aliquot was used for luminescence measurements with 30 μl of substrate. All assays were carried out in a Veritas™ Microplate Luminometer (Turner BioSystems). Activity was normalized and defined as the firefly/Renilla luciferase ratio. Each experiment was performed at least three times.

Quantitative RT-PCR (real-time PCR)

Total RNA was isolated from N2a cell lines with the RNeasy Lipid Tissue Mini kit (Qiagen) according to the manufacturer’s protocol. mRNA was reverse-transcribed into cDNA using the SuperScript® system (Invitrogen). A RT-PCR primer for mouse Ngb was synthesized (Table 1). The mRNA level of Ngb was measured by quantitative RT-PCR using the SYBR Green kit according to the manufacturer’s protocol. Briefly, RT-PCR was performed on an ABI 7500 detection system (Applied Biosystems). Data were analysed according to the standard comparative threshold cycle method. Melting curves for each PCR were generated to ensure the purity of the amplification products.

Western blot analysis

N2a cells were rinsed with warm PBS and then lysed in Cell Lysis Buffer (Cell Signaling Technology) containing 100 × protease inhibitor (Sigma). Lysates were scraped and centrifuged at 16000 g at 4°C for 10 min. Then supernatants were carefully removed and the protein concentration was quantified using the Bradford method. In general, 30 μg of lysates were mixed with 4 × SDS loading buffer and 10 × reducing buffer, and then boiled for 10 min at 70°C. Electrophoresis was performed using SDS/PAGE (4–12%) and the samples were transferred on to nitrocellulose membranes. Membranes were blocked with 5% non-fat dried skimmed milk in 10 mM PBS buffer (PH 7.2) for 1 h at room temperature (20°C). Immunoblots were then performed overnight at 4°C by incubation with primary antibodies followed by incubation with hors eradish-peroxidase-conjugated goat anti-rabbit, anti-mouse or anti-chicken secondary antibodies for 1 h at 37°C. After washing with TTBS [Tris-buffered saline (250 mM Tris, 27 mM KCl and 1.37 M NaCl, pH 7.4) containing 0.1% Tween 20], immunolabelling was detected by ECL (enhanced chemiluminescence, GE Healthcare) according to
Table 1. Oligonucleotide probes used in the present study

<table>
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<th>Name</th>
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<th>Sequence (5′→3′)</th>
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<td>P-2033</td>
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<td>NF-κB consensus</td>
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<tr>
<td>Egr1 consensus</td>
<td>Sense</td>
<td>GGATCCACGGGGCGGAGCCG</td>
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</table>

the manufacturer’s protocol and then exposure to film (X-Omat; Eastman Kodak).

EMSA

Nuclear extract was prepared from N2a cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). The oligonucleotides containing wild-type or mutated putative TFBSs (transcription-factor-binding sites) were synthesized (Table 1). Single-stranded oligonucleotides were 3′-end-labelled with biotin using a biotin 3′-end DNA-labelling kit according to the manufacturer’s protocol (Pierce), and were annealed to obtain double-stranded DNA probes. DNA–protein binding reactions were performed using the LightShift EMSA Optimization and Control kit (Pierce). Briefly, nuclear extract and binding buffer were incubated on ice for 10 min in a volume of 20 μl, and then labelled probes (20 fmol) were added to further incubate for an additional 20 min at room temperature. In competition experiments, nuclear extracts were pre-incubated with the indicated molar excess of unlabelled double-stranded oligonucleotides during the initial 10 min of incubation. In gel supershift analyses, nuclear extracts were pre-incubated with 2 μg of antibodies (Egr1, Sp1, p65, p50 or cRel-specific antibody) during the initial 10 min of incubation. The reaction mixtures were loaded on to a 6% non-denaturing polyacrylamide gel and electrophoresed in 0.5 × TBE buffer (1 × TBE = 45 mM Tris/borate and 1 mM EDTA) at 100 V at 4°C. Following electrophoresis, the DNA–protein complexes were then transferred on to a nylon membrane (Ambion) at a constant voltage of 30 V for 1 h using the XCell II™ Blot Module (Invitrogen). The membrane was cross-linked at 120 mJ/cm² for 60 s using a UV cross-linker (Stratagene). Biotin-labelled DNA was further detected using the Chemiluminescent Nucleic Acid Detection Module (Pierce) and exposure to film.

ChIP

The ChIP assay was performed using the EZChIP™ kit (Upstate Biotechnology) according to the manufacturer’s protocol.
RESULTS

Identification of the core regulatory region in the mouse Ngb promoter

We cloned the potential Ngb promoter, an approximate 2-kb genomic sequence upstream of the mouse Ngb ATG codon, into pGL3-Basic reporter vector, designated P-2033 (−2027/+6). Then a series of 5′-deletion fragments from P-2033 (−2027/+6) were generated by PCR. Each reporter construct was transiently transfected into N2a cells. Compared with empty vectors, transfection of vectors containing different promoter fragments increased the luciferase reporter expression. Only background levels of luciferase activity were detected with constructs P-142 (−136/+6) and P-1692 (−2027/−335). It is worth noting that P-554 (−549/+6) displayed a relatively high promoter activity, but it was gradually decreased following the progressive removal of 5′-regulatory flanking sequences, indicating the existence of several positive regulatory sequences within this 554 bp fragment. Moreover, the sequence between −241 and −136 contained cis-elements that are essential for the basal promoter activity of the mouse Ngb gene (Figure 1A). Therefore we went on to

Figure 1  Identification of the core regulatory region in the mouse Ngb promoter

(A) Deletion analysis of the mouse Ngb proximal promoter. Luciferase activity was normalized to Renilla activity and is shown as the fold induction of the promoter-less control vector pGL3-Basic. Data are expressed as relative luciferase values (means ± S.D.), n = 4, *P < 0.05 compared with P-247 (−241/+6). (B) Alignment and analysis of highly conserved TFBSs in the proximal promoter regions of mouse, rat and human Ngb using ClustalW (http://www.ebi.ac.uk/clustalw) and the JASPAR database (http://jaspar.genereg.net/). Sequences spanning from −549 to +6 relative to the translational start site are shown. One putative Egr1 site, four putative κB sites, one putative TATA box and the translation start codon (ATG) are shown as boxes.

Approximately 1 × 10⁶ N2a cells were cross-linked in 1% formaldehyde at room temperature for 10 min before terminating the cross-linking reaction with glycine. The cells were collected and resuspended in SDS lysis buffer for further sonication to shear the chromatin with an average size of approximately 300 bp. DNA–protein complexes were immunoprecipitated with anti-p65, anti-p50, anti-cRel, anti-Sp1 and anti-Egr1 antibodies, or a control rabbit polyclonal anti-IgG antibody. DNA–protein cross-linkers were reversed by heating at 65°C for 4 h. The DNA products were used for PCR with primers (Table 1) to amplify a 243 bp promoter region of mouse Ngb. Each of the experiments was repeated three times.

Statistical analysis

All values are expressed as means ± S.D. Student’s t test was performed for two-group comparisons. One-way ANOVA was performed for multiple group comparisons, followed by Tukey–Kramer tests for pairwise comparisons. P < 0.05 was considered statistically significant. At least three independent experiments were performed.
Figure 2  Effect of transcription factors NF-κB (p65, p50 and cRel), Egr1 and Sp1 on mouse Ngb promoter activity and mRNA and protein expression levels

(A) The luciferase activity (means ± S.D.) was determined 24 h after transfection; n = 4, *P < 0.05 compared with control cells transfected with pcDNA3.1-His/Myc. The expression of transcription factors was verified by Western blotting (right-hand panel). (B) The luciferase activity (means ± S.D.) was determined 24 h after transfection; n = 4, *P < 0.05, **P < 0.01 compared with cells transfected with control siRNA. (C) The relative (fold of control) Ngb mRNA level was examined by RT-PCR. Data are expressed as means ± S.D., n = 4, *P < 0.05 compared with control siRNA. (D) Representative Western blot showing Ngb protein expression. β-Actin served as the control for equal loading.

further identify the cis-acting regulatory elements within the P-554 (−549/+6) fragment.

Alignment of the 554 bp sequence with corresponding sequences from mouse, rat and human by ClustalW revealed that this sequence was evolutionarily conserved. After analysis of this sequence with the bioinformatic TFBS-prediction tools MatInspector (http://www.genomatix.de) and Jaspar (http://jaspar.genereg.net/), we found several highly conserved regulatory elements including one potential Egr1-binding site and two potential NF-κB-binding sites, designated Egr1, κB2 and κB3 sites respectively. In addition, the region spanning −549 to + 6 also contained two unconserved NF-κB-binding sites, designated κB1 and κB4 respectively (Figure 1B). Interestingly, the Egr1-binding site of mouse Ngb overlapped directly with an Sp1-binding site within the human NGB promoter that was reported by Zhang et al. [9]. Taken together, the promoter region from −549 to + 6 may be the core proximal promoter region of the mouse Ngb gene. NF-κB, Egr1 and Sp1 are probably involved in mouse Ngb regulation by binding to their corresponding cis-elements.

Identification of NF-κB family members (p65, p50 and cRel), Egr1 and Sp1 in regulation of Ngb gene expression

To investigate the roles of NF-κB family members (p65, p50 and cRel), Egr1 and Sp1 in the regulation of mouse Ngb promoter activity, N2a cells were co-transfected with P-554 (−549/+6) and transcription factor expression vectors for p65, p50, cRel, Sp1 and Egr1. Corresponding empty vectors were used as controls. The results showed that overexpression of p65 or Egr1 significantly enhanced the luciferase activity, whereas other transcription factors, such as Sp1, p50 and cRel, only slightly increased the luciferase activity. Western blot analysis confirmed the overexpression of transcription factors in the transfected cells (right-hand panel of Figure 2A). To further examine the
roles of these transcription factors in regulation of Ngb gene expression, we used siRNAs to knock down these transcription factors, and then examined the Ngb promoter activity. The results showed that knockdown of p65, Egr1 or Sp1 significantly reduced Ngb promoter activity and knockdown of p50 resulted in a slight reduction, whereas knockdown of cRel did not alter Ngb promoter activity (Figure 2B). Western blot analysis confirmed clear suppression of the protein expression of these transcription factors by siRNAs (Figure 2D).

To further validate the involvement of NF-κB, Sp1 and Egr1 in Ngb promoter transactivation, endogenous Ngb mRNA and protein levels were examined by RT-PCR and Western blot analysis respectively. In accordance with the finding that knockdown of NF-κB, Sp1 and Egr1 led to a reduction in Ngb promoter activity, Ngb mRNA levels were decreased by 55\%, 60\% and 56\% in response to the decreased p65, Egr1 and Sp1 levels respectively (Figure 2C). Western blot analysis further confirmed that knockdown of p65, Egr1 and Sp1 significantly decreased Ngb protein levels (Figure 2D). Taken together, these findings suggested that NF-κB, Sp1 and Egr1 are involved in regulation of Ngb gene expression in N2a cells.

### Functional analysis of the putative NF-κB-, Egr1- and Sp1-binding sites within the mouse Ngb promoter

To further characterize the roles of NF-κB family members (p65, p50 and cRel), Egr1 and Sp1 in regulating Ngb gene expression, we located the cis-elements in the Ngb promoter region that are potential binding targets of these transcription factors by sequence mapping. Our results showed that overexpression of both p65 and Egr1 significantly increased the promoter activity of the Ngb promoter deletion construct P-554 (−549/+6). Progressive 5′-deletion from −549 to −242 gradually decreased Ngb promoter activity in response to p65 overexpression, and further deletion from −241 to −136 completely abolished the p65-increased promoter activity, indicating that this region is essential for both constitutive and p65-stimulated Ngb expression. We also found that progressive 5′-deletion from −359 to −335 did not alter Egr1-increased Ngb promoter activity, but further deletion from −334 to −242 dramatically reduced Egr1-increased Ngb promoter activity, suggesting that the region spanning −241 to −136 may contain p65-response elements, and that the region from −334 to −242 probably contains Egr1-response elements (Figure 3A).

We further performed site-directed mutagenesis of NF-κB- and Egr1-binding sites within the Ngb promoter. Our results showed that mutations of κB2-, κB3- and Egr1-binding sites dramatically silenced the Ngb promoter activity. Meanwhile, the promoter activity of the wild-type construct remained at approximately the same level as constructs with mutations of κB1- or κB4-binding sites (Figures 3B and 3C). To investigate whether NF-κB-, Egr1- and Sp1-induced Ngb promoter activity are dependent on their binding sites, we tested the effect of p65, cRel, Egr1 and Sp1 overexpression on their corresponding mutated Ngb promoter activity. Overexpression of p65 caused a strong stimulation of the Ngb promoter activity. The construct with mutation of the κB1 or κB4 site retained the ability to be stimulated by p65, whereas mutation of the κB3 site significantly reduced p65-stimulated Ngb promoter activity (Figure 3B). Moreover, co-transfection of P-554 (−549/+6) with the Egr1 or Sp1 expression vector increased the promoter activity by approximately 2- or 1.5-fold respectively, whereas the activity of Egr1M (an Egr1 site mutation) was not affected (Figure 3C). These results indicate that the κB3 site is required for p65-stimulated Ngb promoter activity, and the

**Figure 3**  Functional analysis of conserved NF-κB- and Egr1-binding sites within the mouse Ngb promoter

(A) The luciferase activity (means±S.D.) was determined 24 h after transfection; n=4, *P<0.05 compared with control cells co-transfected with pCDNA3.1-His/Myc and P-554 (−549/+6). P-341 (−335/+5) or P-247 (−241/+6). (B) The luciferase activity (means±S.D.) was determined 24 h after transfection; n=4, *P<0.05 compared with control cells co-transfected with pCDNA3.1-His/Myc and P-554 (−549/+6). #P<0.05 compared with control cells co-transfected with pCDNA3.1-FLAG-p65 and P-554 (−549/+6). (C) The relative luciferase activities of P-554 (−549/+6) and P-554 (Egr1M) in N2a cells. *P<0.05 compared with N2a cells co-transfected with pCDNA3.1-His/Myc and P-554 (−549/+6).

Egr1 site is responsible for Egr1- and Sp1-induced Ngb promoter activity.

**Validation of NF-κB family members (p65, p50 and cRel), Egr1 and Sp1 binding to the mouse Ngb promoter in vitro by EMSA and gel supershift analyses**

Our previous site-directed mutagenesis suggested that mutation of κB2 and κB3 sites in the proximal promoter region of the mouse Ngb gene resulted in a remarkable decrease in mouse Ngb promoter activity. To determine whether NF-κB family members can physically bind to κB2 or κB3 sites, EMSAs were conducted using biotin-labelled probes corresponding to the potential κB2 or κB3 sites. Experimental data showed that two retarded complexes [SC-1 (specific complex 1) and SC-2 (specific complex 2)] were produced when κB2 probes were incubated with nuclear extract (Figure 4A, lane 2). Complex specificity was determined using a molar excess of WT (wild-type) or mutated unlabelled κB2 oligonucleotides as competitors. A dose-dependent increase in molar excess unlabelled WT κB2 oligonucleotides gradually abolished the observed SC-1 and SC-2, whereas a molar excess of unlabelled mutant κB2 oligonucleotides did not (Figure 4A, lanes 3–8). Furthermore, the presence of competitors that contain unlabelled NF-κB consensus oligonucleotides, but...
Figure 4 Interactions of NF-κB, Egr1 and Sp1 with the Ngb promoter in vitro

(A and B) EMSA analysis of p65 and cRel binding to the κB2 site. (A) Biotin-labelled κB2 probes were incubated with N2a nuclear extract with κB2 WT oligonucleotides (lanes 3–5), κB2 mutant oligonucleotides (lanes 6–8), NF-κB consensus oligonucleotides (lane 9) or Sp1 consensus oligonucleotides (lane 10) as indicated. (B) Biotin-labelled κB2 probes (lanes 1–5) or biotin-labelled NF-κB consensus probes (lanes 6–8) were incubated with nuclear extract from N2a cells with or without the addition of antibodies against p65, p50 and cRel. (C and D) EMSA analysis of p65 and p50 binding to the κB3 site. (C) Biotin-labelled κB3 probes were incubated with N2a nuclear extract and κB3 WT oligonucleotides (lanes 3–5), κB3 mutant oligonucleotides (lanes 6–8) or NF-κB consensus oligonucleotides (lane 9). (D) Biotin-labelled κB2 probes were incubated with N2a nuclear extract with or without addition of antibodies against p65, p50 and cRel. (E and F) EMSA analysis of Sp1 and Egr1 binding to the Egr1 site. (E) (Please note, lanes were rearranged from the same gel.) Biotin-labelled Egr1 probes were incubated with N2a nuclear extract with Egr1 WT oligonucleotides (lane 3), Egr1 mutant oligonucleotides (lane 4), Sp1 consensus oligonucleotides (lane 5), Egr1 consensus oligonucleotides (lane 6) or NF-κB consensus oligonucleotides (lane 7) as indicated. (F) Biotin-labelled Egr1 probes were incubated with N2a nuclear extract with or without the addition of antibodies against Sp1 or Egr1. Representative images of at least three independent experiments are shown; all had similar observations. IgG was added as a negative control.

not Sp1 consensus oligonucleotides, completely eliminated SC-2 (Figure 4A, lanes 9 and 10). These results indicated that both bands are specific, and that the κB2 site may be bound by NF-κB subunits. To identify the NF-κB subunits present in the specific DNA–protein complexes, a supershift assay was performed. Biotin-labelled κB2 probes were incubated with N2a cell nuclear extract in the presence or absence of antibodies that recognize p65, p50, cRel or IgG. As a positive control, biotin-labelled NF-κB probes (containing a consensus NF-κB-binding site) were also incubated with N2a cell nuclear extract and anti-p65 antibody, anti-p50 antibody or anti-IgG. Notable shifted bands were observed when antibodies against p65 were added (Figure 4B, lanes 3 and 8), whereas incubation with an anti-cRel antibody did not produce a supershift, but remarkably decreased both bands (SC-1 and SC-2) (Figure 4B, lane 5). Disappearance rather than supershifting of DNA–protein complexes can occur when specific antibodies interfere with the DNA–protein binding. These results therefore indicated that NF-κB family members p65 and cRel can bind to the κB2 site. Competition and supershift assays were also performed for the κB3 site. We found that three retarded complexes [SC-1, SC-2 and SC-3 (specific complex 3)] were observed when the biotin-labelled κB3 probes were incubated with N2a nuclear extract, whereas these complexes disappeared with incubation of a molar excess of unlabelled WT κB3 oligonucleotides, but the addition of a molar excess of mutant unlabelled κB3 oligonucleotides had no effect (Figure 4C, lanes 3–8). Moreover, the bottom complex (SC-3) was significantly diminished when a molar excess of NF-κB consensus oligonucleotides was added (Figure 4C, lane 9). These results indicated that these observed bands were specific,
and that the κB3 site might be bound by NF-κB subunits. A further supershift assay was performed with antibodies against p65, p50 and cRel. We observed shifted bands when anti-p65 and anti-p50 antibodies were added (Figure 4D, lanes 3 and 4). This result indicated that p65 and p50 can directly bind to the κB3 site.

Since mutation of the Egr1 site reduced both Egr1- and Sp1- increased mouse Ngb promoter activity, EMSSAs were carried out with N2a nuclear extract using the biotin-labelled Egr1 probes, which cover the Egr1 site in the presence of different competitors, in order to discover whether Egr1 or Sp1 protein directly binds to Egr1 site. Four distinct complexes were observed when no competitors were added (Figure 4E, lane 2). Moreover, three complexes (SC-1, SC-2 and SC-3) were significantly diminished by a molar excess of unlabelled WT, but not mutant, oligonucleotides (Figure 4E, lanes 3 and 4), indicating that these complexes were specific. We also found that the top complex (SC-1) was eliminated when a molar excess of Sp1 consensus oligonucleotides were included in the binding reaction (Figure 4E, lane 5), and the bottom two complexes (SC-2 and SC-3) were significantly decreased when Egr1 consensus oligonucleotides were added (Figure 4E, lane 6), implying that both Sp1 and Egr1 may bind to the Egr1 site. This speculation was further confirmed by a supershift assay. Our results showed that both the top and the bottom complex (SC-1 and SC-3) were dramatically decreased when an anti-Sp1 antibody was added in the binding reaction, and the bottom complex (SC-3) was markedly decreased when an anti-Egr1 antibody was added. Serving as a negative control, IgG had no effect in the formation of the protein–DNA complexes (Figure 4F). These results indicated that both Sp1 and Egr1 were able to bind to the Egr1 site.

Validation of NF-κB family members (p65, p50 and cRel), Egr1 and Sp1 binding to the Ngb promoter in vivo using the ChIP assay

Using EMSSAs, we have shown above that p65, p50, cRel, Sp1 and Egr1 can bind to the promoter region of mouse Ngb in vitro, and we wondered whether these proteins can bind to the Ngb promoter in vivo. To address this question, we performed ChIP assays with chromatin isolated from N2a cells. The chromosomal DNA that was precipitated by different antibodies was subjected to PCR amplification for the mouse Ngb promoter. IgG served as a negative control. Precipitated DNA was determined by PCR which amplified a 243 bp product covering the region containing the κB2, κB3 and Egr1 sites. IP, immunoprecipitation.

The κB3 site is a pivotal cis-element involved in the up-regulated mouse Ngb promoter activity under hypoxic conditions

It has been reported that hypoxia can dramatically increase Ngb expression in neuronal cells [14,15]. To investigate the effect of hypoxia on Ngb expression in our system, N2a cells were subjected to hypoxia for a series of durations. Our results showed that 8 h or 16 h of hypoxia significantly increased Ngb protein levels, but prolonged 24 h hypoxia could no longer further increase the Ngb levels (Figure 6A). This finding was consistent with a previous observation [14], indicating that N2a cells can be used for investigating mechanisms of hypoxia-induced up-regulation of Ngb gene expression. Hypoxia for 16 h was the...
optimized condition for maximizing the increased Ngb expression in our model system.

To determine whether our cloned mouse Ngb promoter fragments exhibit hypoxia-induced luciferase activity, four deletion reporter constructs including P-554 (−549/+6), P-341 (−335/+6), P-247 (−241/+6) and P-142 (−136/+6) were transiently transfected into N2a cells for 24 h, and then subjected to hypoxia for 16 h. Our results showed that a clear stimulatory effect of hypoxia on luciferase activity was observed with all deletion reporter constructs, except for the shortest one, P-142 (−136/+6) (Figure 6B). These findings suggest that the κB3 site is probably involved in hypoxia-induced Ngb promoter activity.

To further determine contributions of NF-κB- and Egr1-binding sites in the Ngb promoter activity in response to hypoxia, their corresponding mutation reporter plasmids were transfected into N2a cells and then subjected to hypoxia for 16 h. Consistent with the results shown in Figure 6(B), hypoxic stimulation led to a robust increase in luciferase reporter activity in N2a cells transfected with WT P-554 (−549/+6) (Figure 6C). In contrast, mutation of κB2 sites only slightly decreased hypoxia-induced Ngb promoter activity, and mutation of the κB3 site significantly abrogated hypoxia stimulation of the Ngb promoter activity, supporting the important role for the κB3 site in the regulation of hypoxia-induced Ngb expression.

NF-κB (p65) and Sp1 are involved in hypoxia-induced up-regulation of mouse Ngb gene expression

The results described above show that NF-κB, Egr1 and Sp1 are transcriptional activators of Ngb under physiological resting conditions. To investigate their potential roles in hypoxia-induced activation of Ngb expression, we examined the effects of knockdown of NF-κB, Egr1 and Sp1 on Ngb promoter-driven luciferase expression upon hypoxic stimulation by co-transfection with P-554 (−549/+6), and NF-κB family members (p65, p50 and cRel), Egr1 or Sp1 siRNAs for 24 h followed by hypoxia for 16 h. Our results showed that hypoxia strongly induced Ngb promoter activity in control siRNA-transfected N2a cells; however, the hypoxia-induced increase in Ngb promoter activity was significantly attenuated by p65 and Sp1 siRNA (Figure 7). These results suggest that NF-κB (p65) and Sp1 are key transactivators of hypoxia-induced Ngb up-regulation.

HIF-1α is involved in hypoxia-induced up-regulation of mouse Ngb gene expression

HIF-1α is known as an important regulator of gene expression in response to hypoxia [16–18]. In this experiment, we investigated the role of HIF-1α in hypoxia-induced up-regulation of mouse Ngb gene expression. We applied RNA interference to suppress endogenous HIF-1α protein expression. Our results showed that transfection of N2a cells with specific HIF-1α siRNA, but not control siRNA, led to a significant reduction of HIF-1α protein expression, as assessed by Western blot analysis (Figure 8A). Then we examined effects of HIF-1α on the Ngb promoter activity upon hypoxic stimulation by co-transfection with P-2033 (−2027/+6) and HIF-1α siRNA for 24 h followed by hypoxia for 16 h. HIF-1α siRNA, but not control siRNA, dramatically attenuated hypoxia-induced Ngb promoter activity (Figure 8B). We further tested whether endogenous HIF-1α is involved in hypoxia-induced up-regulation of mouse Ngb protein expression. Our results showed that the Ngb protein level was increased to approximately 1.5-fold upon hypoxic stimulation in control siRNA-transfected N2a cells. However, specific suppression of endogenous HIF-1α by transfection of HIF-1α siRNA significantly attenuated the hypoxia-induced increase in Ngb protein expression (Figure 8C). Taken together, these findings suggested that HIF-1α was also involved in hypoxia-induced up-regulation of mouse Ngb gene expression.

DISCUSSION

In the present study, for the first time, we characterized the mouse Ngb gene promoter and identified responsive transcription factors and their corresponding cis-elements. Furthermore, we also investigated regulatory mechanisms of hypoxia-induced mouse Ngb gene transcription, roles of putative binding sites and potential transcription factors. Important experimental findings from the present study are summarized below.

In the first set of experiments, we identified and characterized the core regulatory region of the mouse Ngb promoter. By luciferase reporter analysis for examining the relative promoter activities of a series of 5′-deletion fragments, we identified P-554 (−549/+6) as the core proximal promoter region (Figure 1A). Data obtained from a recent study analysed the human NGB promoter region in SHSY-5Y cells [9], and we found the results of the deletion analysis between the human and mouse Ngb promoter were largely similar. The human Ngb promoter construct P (−164/+306) (spanning from −164 to +306 relative to the transcription start site, −164 to +6 relative to the translational start site) exhibited the highest luciferase activity, whereas construct P (+102/+306) (−202 to +6 relative to the translational start site) showed the lowest activity. In the present study, the mouse Ngb promoter construct P (−549/+6) (relative to the translational start site) had a relatively high luciferase activity, but P-142 (−136/+6) (relative to the translational start site) had the lowest activity (Figure 1A). The great similarity is probably due to the high conservation between human and mouse proximal promoter region, and these results raise the possibility that similarity might exist in the regulation of the Ngb gene between mouse and human.

Cross-species conservation of cis-elements indicated the importance of their functionalities. On the basis of bioinformatics analysis of the Ngb promoter, we identified several conserved cis-elements, including two putative NF-κB-binding sites (κB2 and κB3) and one putative Egr1-binding site (Figure 1B).

We also found that κB2 and κB3 sites could be bound by two dimers, p65/cRel and p56/p50 respectively, suggesting that Ngb

Figure 7 Effect of NF-κB, Sp1 and Egr1 on regulating mouse Ngb expression under conditions of hypoxia

The relative luciferase activity (of control siRNA) of N2a cells was examined and compared. Values are means ± S.D., n = 3, * P < 0.05 compared with N2a cells transfected with the corresponding siRNA under normoxia.

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is a novel NF-κB-targeted gene. It is well known that NF-κB is constitutively expressed in the CNS (central nervous system), such as in hippocampal neurons and cerebral cortex neurons [19,20]. NF-κB subunits, including p65, p50 and cRel, are frequently studied. A number of studies have shown that knockout mice of p65, p50 or cRel genes result in a loss of neuroprotection, and defects in learning and memory [21–25], indicating that each NF-κB subunit is functionally important in the nervous system. However, for Ngb gene regulation, our data indicated that cRel was not necessary for maintaining basal Ngb expression levels, as knockdown of cRel had no significant effect on basal Ngb expression (Figures 2B–2D). One possible explanation is that cRel may contribute to Ngb expression only in response to certain stimulations. This is because previous studies documented that cRel-containing dimers cRel–p65 or cRel–p50 can be activated by IL (interleukin)-1β, mGlu5 (metabotropic glutamate 5) receptor agonists, S100B and leptin, and increase the resistance of neuronal cells to toxic insults [21,22,26,27]. However, whether the up-regulation of Ngb is mediated by activated cRel-containing dimers remains to be defined.

Another important finding is that NF-κB may also function as a dominant transactivator of the Ngb gene under hypoxic stress, as knockdown of NF-κB (p65) significantly attenuated the hypoxia-induced increase in the Ngb promoter activity (Figure 7). Furthermore, we found that the κB3 site is a pivotal cis-element responsible for hypoxia-up-regulated mouse Ngb promoter activity (Figure 6C). It has been shown that NF-κB can be activated by hypoxia [28–30] and is involved in a series of hypoxia-induced gene regulations [31,32]. Previous studies have suggested that early NF-κB activation contributes to hypoxia/ischaemia-induced brain damage, and late NF-κB activation provides endogenous neuroprotection by up-regulating anti-apoptotic molecules [33], and neuronal cell fate in response to pro-apoptotic or neuroprotective agents may rely on the recruitment of different NF-κB dimers [21,22,34]. The results of the present study show that hypoxia gradually increased mouse Ngb expression; however, prolonged hypoxia resulted in reduction of Ngb expression (Figure 6A), possibly caused by hypoxia-induced neurotoxicity. A previous study from Qiu et al. [35] reported that hypoxia produced different binding patterns of NF-κB dimers on the κB site within the Bcl2 gene promoter at different time points of hypoxia [35]. Thus it will be important to further clarify whether different NF-κB dimers modulate Ngb expression during the hypoxic process.

One more interesting finding is that both Egr1 and Sp1 also play important roles in maintaining Ngb gene expression under physiological resting conditions (Figures 2B and 2C). We found that the Egr1 site could be bound by Egr1 and Sp1 proteins (Figure 4F), implying that the Egr1–binding site probably recruit both Egr1 and Sp1 for synergistically activating the Ngb promoter. This speculation has been supported by our data, which show that mutation of the Egr1 site partially abrogated both Egr1- and Sp1-induced Ngb promoter activity (Figure 3C). Previous studies from others have indicated that Egr1 often binds to the GC-box which is preferentially bound by Sp1 [36,37], and Egr1 regulates expression of some genes by displacing Sp1 from its binding site in response to external stimuli [38,39]. Thus it is possible that Egr1 is involved in Ngb expression under certain pathological conditions. Interestingly, the results of the present study suggest that Sp1, but not Egr1, is responsible for hypoxia-induced Ngb up-regulation, as knockdown of Sp1, but not Egr1, clearly decreased hypoxia-induced Ngb promoter activity (Figure 7). It has been reported that Sp1 can stimulate a variety of genes’ expression under hypoxic conditions [40,41]. However, the mechanism of how Sp1 mediates hypoxia-induced Ngb up-regulation is not clear, and the results of the present study did not show that Sp1 is involved in hypoxia-induced Ngb up-regulation by the Egr1 site (Figure 6C). It is possible that, under hypoxic conditions, Sp1 might bind to other undetected cis-elements within the Ngb promoter. Another possibility is that Sp1 is indirectly involved in hypoxia-induced Ngb expression. For example, it has been reported that Sp1 could contribute to the activation of NF-κB [42], implying that Sp1 may indirectly increase Ngb promoter activity by promoting the binding of NF-κB to the corresponding site. However, these issues need to be defined in future studies.

In addition to the investigations of potential roles of transcriptional factors in the Ngb promoter region in the regulation of Ngb, HIF-1α is known to be a sensitive and important regulator
in hypoxia-triggered gene regulation [16–18]. Previous studies by others have indicated that Ngb is up-regulated by CoCl2 and defereroxamine, which could stabilize HIF-1α under normoxic conditions [14], suggesting the possible involvement of HIF-1α in up-regulation of Ngb. In the present study, we have demonstrated that HIF-1α was also a key regulator for hypoxia-induced Ngb up-regulation. Although the results of the present study show that the promoter activity of mouse Ngb could be inhibited by specific HIF-1α siRNA, the exact mechanism of how HIF-1α mediates hypoxia-induced Ngb expression remains unknown. It has been suggested that regulation of the target genes by HIF-1α depends on the interaction of the transcription factor and its consensus sequence 5′-RCGTG-3′ which is located in the proximal promoter region of the target gene [43–45]. However, there are no highly conserved HREs (hypoxia-response elements), analysed by bioinformatics, within the Ngb proximal promoter region. In the absence of the conserved HRE, it is still possible that HIF-1α might regulate Ngb by binding to the non-conserved HIF-1α elements. More supporting evidence is that a previous study has reported that Sp1 could serve as a molecular switch by recruiting HIF-1α to the gene promoter upon hypoxic stress stimulation [46], suggesting that HIF-1α probably cross-talks with other transcription factors to co-operatively transactivate the Ngb gene. However, further studies are necessary to elucidate the exact mechanisms of HIF-1α in participating in Ngb regulation under hypoxic and other pathological conditions.

In summary, the present study has identified and characterized the promoter of the mouse Ngb gene, and defined the role of NF-κB (p65, p50 and cRel), Egr1 and Sp1 and their corresponding binding sites in the regulation of Ngb expression under physiological resting conditions. NF-κB (p65) and Sp1, as well as HIF-1α, were involved in hypoxia-induced Ngb expression. For the first time, the present study has provided a molecular basis in understanding the transcriptional regulation mechanism of mouse Ngb gene expression. We confidently believe that characterization of the mouse Ngb promoter and the fundamental insights into mouse Ngb transcriptional regulation will not only aid future elucidation of Ngb expression regulation and biological functions, but also help in the development of Ngb-targeting therapeutics for intervention of hypoxic/ischaemic CNS disorders, including stroke and neurodegenerative diseases.

AUTHOR CONTRIBUTION
Ning Liu designed and performed the experiments, analysed the data and prepared the paper. ZhanYang Yu performed some of the experiments, analysed the data and prepared the paper. Shuqian Liang designed the experiments and analysed the data. Song Zhao performed some of the experiments and analysed the data. Anna Tjärnlund-Wolf designed the experiments. Changhong Xing performed some of the experiments. Jian Zhang and Xiaoying Wang designed the experiments and prepared the paper.

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