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ORIGINAL ARTICLE

VGF, Which Is Induced Transcriptionally in Stroke Brain, Enhances Neurite Extension and Confers Protection Against Ischemia In Vitro

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Abstract Ischemic stroke is a devastating neural event as currently no therapies other than physical rehabilitation are available to enhance recovery after stroke. To identify endogenous mediators to repair stroke brain, we performed the expression profiling analysis of transcripts in the mouse photothrombotic stroke brain. Based on real-time PCR analysis, we found *VGF*, identified as a nerve growth factor (NGF)-regulated transcript, was induced transcriptionally in stroke brain at 1–7 days after insult. The immunoreactivites of VGF were observed in the neurons around the ischemic core of stroke brain. Experiments with various inhibitors and plasmid transfections indicated that cAMP response element binding protein-mediated complex signaling pathways are possibly implicated in the NGF-mediated VGF expressions in vitro. Furthermore, the over-expression of VGF promoted neurite extensions and conferred protections from ischemic stress in vitro. These findings raise the possibility the application of VGF could be one of the promising therapeutic strategies to enhance recovery after stroke.

Keywords Stroke · VGF · Photothrombosis · Ischemia

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Introduction

Stroke is one of leading causes of human death and serious long-term disability worldwide. Ischemic stroke resulting from focal brain infarction accounts for approximately 80 % of all human strokes and has a major impact on public health [1, 2].

Based on recent studies, adult brain maintains the capability for plasticity that promotes recovery after stroke damage, including cortical reorganization and neural regeneration. The developing brain is highly plastic, in which neural circuits are organized dependent on activity. The mechanisms of neural plasticity after stroke could be similar to those during development, including activity-dependent rewiring (dynamic axon and dendrite arbor growth) and synaptic plasticity (functional modulation of existing synapses as well as structural modulation) [3–8]. A variety of high-throughput expression analyses including microarray and real-time PCR using rodent, primate, and human stroke brain have shown that many of the genes and proteins critical for neural differentiation, apoptosis and survival, neurogenesis, synaptogenesis, and synaptic plasticity are induced after stroke as observed in the developing brain [9–13].

Various animal models have been established for the research of pathogenesis after ischemic stroke [14]. Rodent middle cerebral artery occlusion (MCAO) model has been the most widely used among them; however, it produces large ischemic lesions of varying size, which is a disadvantage for reproducibility and long period survival. Photothrombosis, based on the photochemically stimulated platelet aggregation by Rose bengal, can produce consistent cortex lesion size in specific regions of interest and enables animals to survive for longer period [15–17].

VGF (non-acronymic) was originally identified as a nerve growth factor (NGF)-regulated transcript in rat PC12

(pheochromocytoma cell), which encodes a secretory protein with multiple processing events resulting in a number of bioactive peptides such as NAPP129, TLQ-62, TLQP-21, and LQEQ-19 [18–22]. Various neurotrophins including NGF and brain-derived neurotrophic factor (BDNF) induce *VGF* gene expression in PC12 cells and cultured cortical neurons. *VGF* mRNA levels are regulated by neuronal activity, including long-term potentiation and seizure. *VGF* mRNA is synthesized widely by neurons in the brain, spinal cord, and peripheral nervous system with particularly abundant in developing neurons and in the hypothalamus. The analysis of *VGF* knockout mice has demonstrated that the secreted VGF polypeptide or processed peptides are critical in the control of energy homeostasis. Although extensively investigated, the receptor for this endogenous peptide has not been identified yet [21–24].

In the present study, we found that *VGF* transcript is induced in the brain of mouse photothrombotic stroke model and then examined the significance of this neurotrophin-induced secretory protein.

Materials and Methods

Photothrombosis Stroke Model in Mice

Photothrombosis was performed in male 6 weeks old C57/BL6J mice (20–22 g) as described previously [16]. Briefly, mice were deeply anesthetized and 0.1 ml of 10 mg/ml Rose bengal (Wako, Japan) was injected intraperitoneally 5 min before illumination. Mice were placed in a stereotactic frame (NARISHIGE #SR-5M, Japan) and the skull was exposed by a median incision of the skin to identify bregma and lambda points. A fiber optic bundle of a cold light source (Zeiss Cold light source CL 1500) with ×20 objective lens (Olympus), centered using a manipulator at 2 mm posterior and 2 mm laterally from bregma (the sensorimotor cortex), illuminated the brain through the intact skull for 15 min initiating at 5 min after the injection of Rose bengal. The skin was then sutured. The body temperature was controlled during the operation by heating pad. Sham operations were performed in parallel without illumination by a cold light source. All efforts were made to minimize animal suffering and the number of animals used ($n=5$/group at each time point) in photothrombosis stroke model.

Cell Culture, Chemical Ischemia, and Viability Assay

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 5 % horse serum in 5 % CO_2 atmosphere at 37 °C. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Neural differentiation of PC12 was performed in the differentiation medium (DMEM with 2 % FBS and 100 ng/ml NGF). Reagents used in the present study were 10 μM H89 (Sigma-Aldrich, B1427), 10 μM U0126 (Focus Biomolecules), 50 μM LY294002 (Focus Biomolecules), 5 μM U73122 (Focus Biomolecules), and 100 ng/ml NGF (Sigma-Aldrich, N0513).

Chemical ischemia was performed in glucose-free DMEM with 5 mM sodium azide and 2 mM 2-deoxyglucose (TCI, #D0051). Viability were measured by a commercial kit (Cell Counting Kit-8[#CK04], Dojindo, Japan), a nonradioactive colorimetric assay based on water-soluble disulfonated tetrazolium salt.

Constructs

cDNA of human *VGF*, obtained from RIKEN cDNA bank (Tsukuba, Japan), was subcloned into FPC1-HA plasmid (HA-tagged protein expressing vector with the backbone of pEGFP-C1[Clontech]). pCF-cAMP response element binding protein (CREB), N-terminal FLAG-tagged CREB expression plasmid, was obtained from Addgene.org (Plasmid #22968).

Western Blot Analysis, Immunocytochemistry, and Immunohistochemistry

Western blot analysis, immunocytochemistry, and immunohistochemistry were performed as described previously [25, 26]. Briefly, brain lysates or cell extracts were prepared by lysis buffer [50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1 % NP-40, 100 mM NaCl, 10 mM β-glycerophosphate, Complete Protease Inhibitor Cocktail (Roche Diagnostics)]. The samples (30 μg/lane) were boiled in loading buffer [100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 4 % SDS, 0.2 % bromophenol blue, 0.2 % glycerol] for 5 min and subjected to electrophoresis on 10 % SDS-PAGE. After the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore Corp.), the membrane was incubated in blocking buffer [phosphate-buffered saline (PBS) containing 0.05 % Tween 20 (PBS-T) with 5 % nonfat dried milk] for 1 h at room temperature and then probed with a primary antibody in blocking buffer overnight at 4 °C. Detection of signal was performed with ECL chemiluminescence system (GE Healthcare Ltd). Five brains derived from each group (sham and photothrombosis) were used for Western blot analysis at each time point.

Mice were intracardially perfused with 4 % paraformaldehyde (PFA). Brains were harvested and post-fixed in 4 % PFA

overnight at 4 °C, followed by sucrose replacement. Sections were cut in 20-μm-thick sections with a cryostat. Cells were fixed with 4 % PFA for 15 min and then washed by PBS three times. Brain sections and cells were permeabilized in PBS containing 0.2 % Triton-X 100 with 5 % BSA and non-specific sites were blocked by incubating with blocking solution [PBS containing 0.1 % Triton X-100, 5 % bovine serum albumin (BSA) and 3 % goat serum]. The samples were incubated with primary antibodies diluted in a blocking solution overnight at 4 °C. Then, the samples were washed in PBS and incubated with Alexa488 or Alexa568 (Invitrogen) conjugated secondary antibodies for 1 h at room temperature and with DAPI (4′,6-diamino-2-phenylindole). Images were obtained using fluorescence microscopy (Nikon, E600) with digital camera DP72 (Olympus, Tokyo, Japan). The primary antibodies used were anti-VGF (D-20) (Santa Cruz Bio, #sc-10381), anti-NeuN (MILLIPORE, #MAB377), anti-FLAG (Sigma-Aldrich, M2), anti-HA (Sigma-Aldrich, HA-7), Neuronal Class III β-Tubulin (TUJ1) (Covance, #MMS-435P), and anti-Actin (I-19) (Santa Cruz Bio., sc-1616).

Quantification for Western Blot Bands

Western blot images were analyzed by ImageJ software (National Institutes of Health), which evaluates the relative amount of protein staining with normalization to the corresponding controls.

Real-Time PCR Assay

Total RNA was extracted using Trizol (Invitrogen) according to the manufacturers' instructions. RNA (1 μg) was reverse transcribed to produce cDNA using ReverTra Ace® qPCR RT Kit (TOYOBO, Japan). Real-time PCR was performed using THUNDERBIRD® SYBR® qPCR Mix (TOYOBO, Japan). All real-time PCR assays were performed in triplicate using 7300 Real-Time PCR System (Applied Biosystems). Primers used in the study were listed on Supplementary Table 1. For standardization of relative mRNA expression, *GAPDH* primers were used. The results of cycle threshold values (*Ct* values) were calculated by the $\Delta\Delta Ct$ method to obtain the fold differences. Five brains derived from each group (sham and photothrombosis) were used for real-time PCR analysis at each time point.

Statistical Analysis

Quantitative data were analyzed by the Student's *t* test or ANOVA analysis with post-hoc multiple comparison tests, and *p* values less than 0.05 were considered as statistically significant. Data were expressed as means±S.D. or S.E. as indicated in the figure legends.

Results

VGF mRNA Is Induced at 1–7 Days After Injury in Post Stroke Brain

First, we prepared the brain and the sections of photothrombotic stroke model at 3 days (d) after insult and confirmed that brain infarction actually occurred in our stroke model as described in the previous report [16] (Fig. 1a). Then to identify novel therapeutic targets to promote recovery after stroke, we collected high-throughput experimental data on expression profiling of transcripts induced in stroke brain from published papers [9–13]. Based on these microarray data, we then picked up the representative transcripts of neural plasticity-related genes (*GAP43*, *Neuritin*, *VGF*, *Arc*, *PC3*, *hsp90*) induced after stroke mostly within 24 h after injury and performed the long-term (1–28 days) expression profiling analyses by real-time PCR assay using RNAs from the total cortices of photothrombotic or sham-operated brain on the ipsilateral side (Fig. 1b). Since a promising approach to increase recovery after stroke is the administration of pharmacological agents, neuronal growth factors or secreted peptides could be the ideal candidates. Then we determined to focus on *VGF*, which encodes a secretory protein [22, 24] and is induced transcriptionally at 1–7 days after stroke, a critical period for effectiveness of rehabilitative therapies [3].

To investigate whether VGF is induced at protein levels, we performed Western blot analysis using the lysate of total cortices of photothrombotic or sham-operated brain on ipsilateral side at the indicated time points (Fig. 1c). The expression levels of VGF protein were induced only at acute stage (1 day) after injury compared with sham (Fig. 1c). We then performed immunohistochemistry on the brain sections at 3 days after stroke, a critical period for effectiveness of rehabilitative therapies as described previously. The signals for VGF immunoreactivities were observed in cortex neurons on the ipsilateral side around ischemic core, co-stained with a neuronal marker NeuN (Fig. 1d).

VGF Is Induced by NGF Possibly Through CREB-Mediated Complex Signaling Pathways in PC12

VGF was originally identified as a NGF-regulated transcript in PC12 [18–20]. First to confirm that VGF is induced by NGF

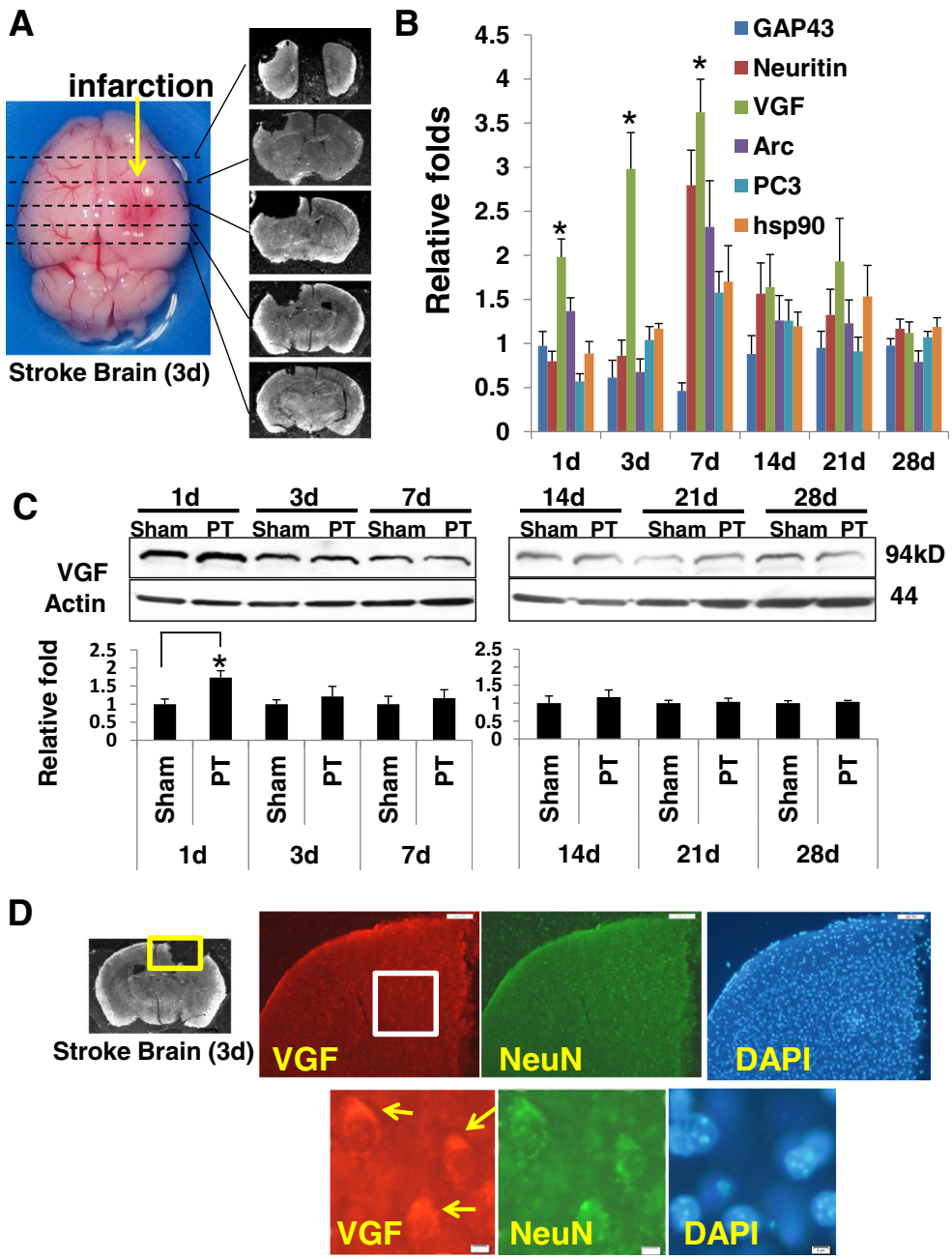

Fig. 1 *VGF* transcript is induced at 1–7 days after injury in photothrombotic stroke brain. **a** Pictures showing the whole mouse brain (*left*) and the sections (*right*) at 3 days (d) after photothrombosis. Right panels are 30 μm thick brain coronal cryostat sections around the ischemic region at 3 days after photothrombosis, scanned with a digital microscope camera (Olympus DP72). **b** Expression profiles of transcripts induced in the brain lysates of photothrombotic stroke model by real-time PCR analysis using RNAs from total cortices on the ipsilateral side of sham or photothrombosis samples (n=5). Values were shown as mean± S.E. (n=5) of relative fold changes compared with sham samples at each time point. $^{*}p<0.05$, sham vs photothrombosis at the same time point. **c** Brain lysates from total cortices on the ipsilateral side of sham (*Sham*) or photothrombosis (*PT*) brains were processed for Western blot analysis with the indicated antibodies at the indicated time points (days after injury). Bar graph shows the relative fold changes (mean±S.E., n=5) at the indicated time points (days after injury) compared with sham. $^{*}p<0.05$, sham vs photothrombosis at 1 day after stroke. **d** *Left panel* is 30 μm thick brain coronal cryostat section around the ischemic region at 3 days after photothrombosis, scanned with a digital microscope camera (Olympus DP72). *Right panels* show brain sections of photothrombotic stroke brain at 3 days after injury, which were co-stained with anti-VGF and -NeuN antibody and then incubated with diamidino-2-phenylindole (DAPI). *Arrows* indicate the signals for the co-staining of VGF and NeuN. *Bars*, 200 μm (*upper panels*) and 5 μm (*lower panels*)

in our culture system, we treated PC12 cells with 100 ng/ml NGF and performed immunocytochemisty and Western blot analysis. The expression of approximately 90 kD VGF protein, which corresponds to non-processed full-length form, was detected at 24 h after NGF treatment (Fig. 2a, upper panels). Neurite extensions, stained with anti-Neuronal Class

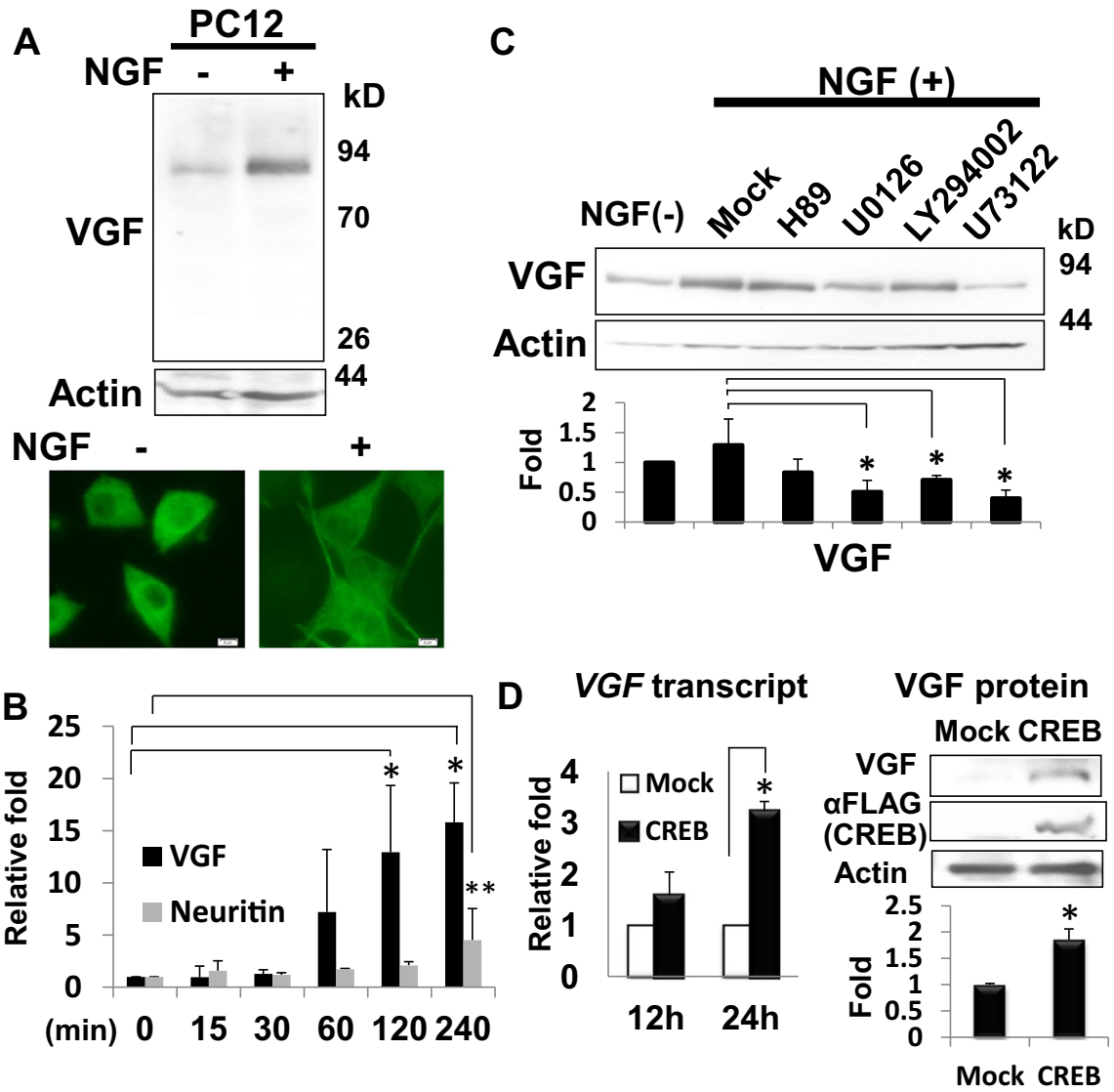

Fig. 2 VGF is induced in PC12 by NGF possibly through CREB-mediated complex signaling pathways. **a** PC12 cells, treated with 100 ng/ml NGF (NGF +) or mock (NGF −) for 24 h in the differentiation medium, were lysed for Western blot analysis with anti-VGF and anti-actin antibody (*upper panels*). PC12 cells were treated with NGF (NGF +) or Mock (NGF −) for 48 h in the differentiation medium, fixed and then stained with anti-Tuj1 antibody (*lower panels*). *Bars*, 5 μm. **b** PC12 cells, treated with NGF for 240 min in the differentiation medium, were processed for real-time PCR at the indicated time points. Experiments were performed in triplicate and data were shown as relative fold changes (mean±S.D., $n=3$) compared with value at 0 min. *Asterisks* show significant differences (*$p<0.05$ vs 0 min sample of *VGF*; **$p<0.05$ vs 0 min sample of *Neuritin*). **c** PC12 cells, pretreated with various inhibitors (Mock, 10 μm H89, 10 μm U0126, 50 μm LY294002, 5 μm U73122) for 30 min and then applied with NGF for 24 h in the differentiation medium, were lysed for Western blot analysis with anti-VGF and anti-actin antibody. Bar graph shows the relative fold changes (mean±S.E., $n=3$) of VGF expression compared with sample of NGF(−). *Asterisks* show significant differences ($p<0.05$ vs Mock). **d** PC12 cells were transfected with Mock (empty vector) or FLAG-tagged CREB plasmid. At 12 h after transfection, medium was changed to the differentiation medium. Then cells were processed for real-time PCR assay at 12 and 24 h (left graph) and for Western blot analysis with the indicated antibodies at 24 h (*right panels and graph*) after medium change. Experiments were performed in triplicate and data were shown as relative fold changes (mean±S.D.) compared with Mock. *Asterisks* show significant differences ($p<0.05$, vs Mock)

III β-Tubulin (Tuj1) antibody, were promoted in PC12 treated with NGF at 48 h after treatment (Fig. 2a, lower panels). We then examined the time course of NGF-induced expression of *VGF* transcript by real-time PCR assay using *neuritin*, a NGF-inducible transcript, as a positive control [27]. *VGF* transcript was increased rapidly at 30–60 min and raised up to 15 folds of baseline at 4–6 h after NGF treatment (Fig. 2b). The expression pattern of *VGF* transcript was similar to that of *neuritin*.

The promoter region of *VGF* gene contains cyclic AMP response element in rodent and human [28, 29]. CREB (ser133) is phosphorylated by multiple signaling pathways,

including cAMP-dependent protein kinases (PKA), phosphoinositide 3-kinases (PI3Ks), Ras-Mitogen-activated Protein Kinase (MAPK), and phospholipase C (PLC) downstream of NGF-TrkA receptor [21, 30]. To examine which signaling pathways are critical on VGF expression, we performed Western blot analysis using PC12 pretreated with various inhibitors including H89 (PKA inhibitor), U0126 (MEK1/2 inhibitor), LY294002 (PI3Ks inhibitor), and U73122 (PLC inhibitor) before NGF application. Among them, U0126, LY294002, and U73122 significantly reduced the NGF-induced VGF expression compared with mock in our system (Fig. 2c), suggesting the complex signaling pathways including MAP kinases, PI3Ks, and PLC are implicated in NGF-mediated VGF expression.

Then to confirm the direct effect of CREB on VGF expression, we transiently transfected PC12 cells with plasmid encoding FLAG-tagged CREB or mock (empty vector) and performed real-time PCR assay and Western blot analysis. CREB over-expression induced the expression of *VGF* transcript (Fig. 2d, left graph) and VGF protein compared with mock (Fig. 2d, right panel).

VGF Over-expression Promoted Neurite Extensions and Viability Under Ischemia in PC12

To gain insights into the significance of VGF in neurons, we investigated the effects of VGF on neurite extension and viability in ischemic stress. We transiently transfected PC12 cells with plasmid encoding HA-tagged VGF or mock and performed Western blot analysis and neurite extension assay. PC12 cells, transfected with *VGF* plasmid, expressed HA-tagged VGF (Fig. 3a, upper panels) and increased the ratio of neurite-harboring cells compared with mock (Fig. 3a, lower panels). Next to examine whether VGF could protect neurons from ischemic stress, we assessed viability under chemical ischemia. The viabilities of PC12 cells were declined in a time-dependent manner (2 h, 36.7±3.3 %; 4 h, 21.2±3.7 %; 6 h, 11.8±3.4 %) in ischemic stress (Fig. 3b, upper graph).

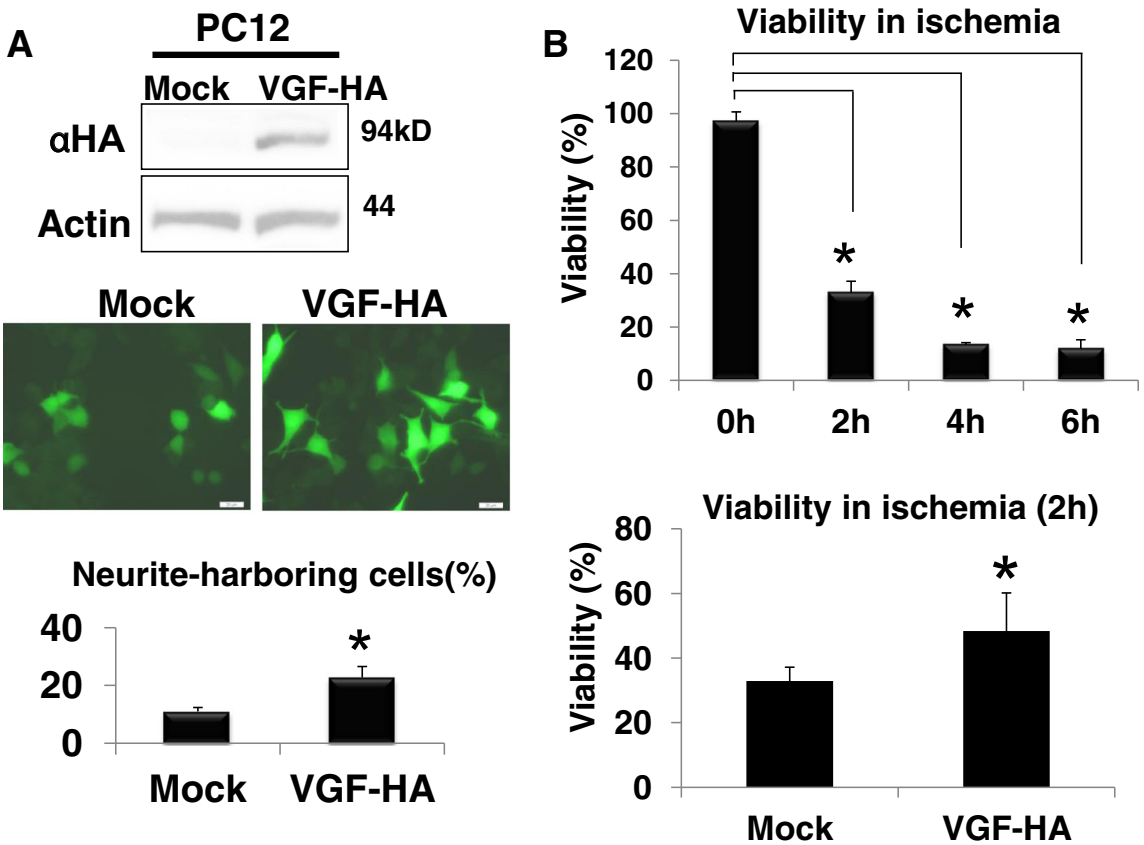

Fig. 3 VGF over-expression promoted neurite extensions and viability under ischemia in PC12. **a** PC12 cells were co-transfected with plasmids encoding HA-tagged VGF and GFP at the ratio of 5:1. At 12 h after transfection, medium was changed to in the differentiation medium. Then cells were fixed at 48 h after medium change. Cells with GFP signals harboring neurites longer than cell body were counted as neurite-harboring cells. Bar graph indicates the ratio (%) of neurite-harboring cells (mean±S.D). The experiments were performed in triplicate at least 40 cells counted per experiment. $^{*}p<0.05$ vs Mock. **b** PC12 cells in 96 well plates were exposed to chemical ischemia. In the upper graph, viabilities were assessed at the indicated time point (0, 2, 4, 6 h) after ischemia. *Asterisks* show significant differences ($p<0.05$ vs 0 h). In the lower graph, PC12 cells were transfected with plasmid encoding HA-tagged VGF or mock, and then exposed to chemical ischemia at 24 h after transfection. Viability assay was performed at 2 h after ischemia. Bar graph indicates the viability (%) (mean±S.D, $n=10$). $^{*}p<0.05$ vs Mock

Then we transfected PC12 cells with mock or *VGF* plasmid and performed viability assay after exposing them to chemical ischemia for 2 h. The viability of PC12 cells over-expressing VGF was higher (48.3±11.7 %) than that of mock (32.9± 4.2 %) (Fig. 3b, bottom graph).

Discussion

In the present study, we found that the expression levels of *VGF* transcript were induced in the photothrombotic stroke brain especially at 1–7 days after injury, a critical period for effectiveness of rehabilitative therapies [3]. The experiments using inhibitors and plasmid transfections indicated VGF is induced possibly in part through CREB-related complex signaling pathways downstream of NGF in PC12 cells. Since CREB-mediated pathways were activated downstream of various growth factors and neurotrophins, including NGF, NT-3, BDNF, and IGF-1 [21, 24, 30], VGF could be one of the targets of these growth factors and neurotrophins induced in stroke brain. In addition, VGF over-expression increased the ratio of neurite harboring cells and conferred protections from chemical ischemia in PC12. Taken together, these results suggest that various growth factors and neurotrophins in stroke brain could induce the expression levels of *VGF* transcript to enhance recovery after stroke. Despite the sustained up-regulation of *VGF* transcript, the expression levels of VGF protein were increased only at acute stage (1 day) after injury in this stroke model. Since one of the first cellular functions to be affected by cerebral ischemia is protein synthesis in rodent stroke model [31], one of reasons for this discrepancy is the repressed protein synthesis of VGF in the damaged brain despite the up-regulation of the mRNA. Another reason for that is we using the lysates of total cortices of brain on the ipsilateral side for Western blot analysis.

VGF undergoes multiple processing events, resulting in a number of bioactive peptides such as NAPP129, TLQ-62, TLQP-21, and LQEQ-19 [22, 24]. In the present study, we used the anti-N terminal VGF antibody, which detects approximately 90 kD VGF protein corresponding to the full-length VGF protein. Therefore, one of future problems for the translational stroke research is to identify which bioactive peptides processed from VGF are critical for neuroprotective or neurite-extending effects. Another future problem for the translational stroke research is to identify the receptor and the downstream signaling pathways of VGF-derived bioactive peptides for the functions of neuroprotection and neural plasticity. Recently, possible candidates for receptors of VGF-derived bioactive peptides were reported [32, 33]. Taken together, we here propose the application of VGF could be one of the promising therapeutic targets for stroke therapy to promote recovery when those problems are solved in the future.

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Conflict of Interest Muneki Sakamoto, Yuta Miyazaki, Keiko Kitajo, and Atsushi Yamaguchi declare that they have no conflict of interest.

Compliance with Ethics Requirements All institutional and national guidelines for the care and use of laboratory animals were followed. Specifically, procedures followed the guidelines approved by the Chiba University Animal Care and Use Committee.

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