Vascular endothelial growth factor (VEGF) signaling regulates hippocampal neurons by elevation of intracellular calcium and activation of calcium/calmodulin protein kinase II and mammalian target of rapamycin

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1. Introduction

Vascular endothelial growth factor (VEGF) was originally identified as a potent and selective endothelial cell mitogen implicated in vascularization and angiogenesis [1,2]. In rodents, four different forms, VEGF 188, 164, 120, and 115 are generated by alternate exon splicing of a single VEGF gene [3]. The predominant VEGF produced in most vertebrate tissues including the brain is VEGF164. VEGF and its tyrosine kinase...
receptors, VEGFR1 (also called Flt-1) and VEGFR2 (also called KDR or Flk-1), are also present in nonvascular cells including neurons, suggesting that VEGF has a wider physiological role [3]. In the nervous system, VEGF165, a human analog of murine VEGF164, is expressed in neurons in capillary-rich areas of the brain [4,5] and in astrocytes [6].

The following observations suggest that VEGF is a key component of nervous tissue [3]. First, it induces neurite outgrowth [7–9] and enhances nerve regeneration [10]. Second, it protects neurons from insults such as hypoxia [11] and glutamate toxicity [12], and defective VEGF signaling may contribute to motor neuron disease [13,14]. Third, it may have a role in hippocampal neuronal functions; it is induced by performing a learning task, as well as by environmental stimuli [15]. Finally, it has a potential role in neurogenesis within stem/progenitor cell populations: intraventricular VEGF application stimulates neurogenesis [16], and VEGF is necessary for the effects of learning on hippocampal neurogenesis [15,17].

Because environment and learning increase hippocampal VEGF levels and local changes in VEGF expression regulate neurogenesis, VEGF appears to be generated and act locally. In addition, it may act directly on mature neurons to produce neurotrophic effects. Because neuronal activity-dependent expression is required for a neurotrophic factor regulating neuronal function, characterization of the mechanisms by which neuronal activity regulates VEGF expression and secretion would be valuable. However, the manner in which learning triggers increased hippocampal VEGF expression is unclear, as is whether or how VEGF acts directly on neuronal cells.

Therefore, in the present study we examined the hypothesis that VEGF initiates successive events that lead to activation of signaling molecules required for increased neuronal functions including synaptic plasticity. Features of VEGF transcription, regulation of secretion upon neuronal stimulation, and the functional activation of signal molecules in response to neuronal activity were examined.

2. Materials and methods

2.1. Preparation of hippocampal neurons

Hippocampi from E16.5 Sprague–Dawley rat embryos were rapidly and aseptically dissected from each brain in ice-cold Ca2+/Mg2+-free Hank’s balanced salt solution (HBSS; Mediatech, Herndon, VA, USA), followed by removal of meninges and mincing into small pieces. The hippocampal tissue was then digested in 0.25% EDTA–trypsin ( Worthington Biochemical, Lakewood, NJ, USA), and dissolved in Ca2+/Mg2+-free HBSS for 10 min at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. The tissue was then digested in 0.25% EDTA (25 mg/ml in phosphate-buffered saline (PBS); Sigma-Aldrich, St Louis, MO, USA), and dissolved in Ca2+/Mg2+-free HBSS for 10 min at 37 °C in a 5% CO2/95% air humidified incubator. The neurons were used after 10 to 14 days. Animal care and experiments were conducted in accordance with the 2004 Guide for the Care and Use of Laboratory Animals (Korea National Institute of Health) and Hanyang University Veterinary committee.

2.2. Drugs

Drugs used included human recombinant VEGF165 from R&D systems (Minneapolis, MN, USA), (+)-MK801 maleate, LY-294002, PD98059, BAPTA-AM, nifedipine, α-conotoxin GVIA from Sigma (St. Louis, MO, USA), o-tocotrienol-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA), anisomycin, and rapamycin from Tocris (Ellisville, MO, USA) and KN62 from Alexis Biochemicals (San Diego, CA, USA). N-(−)-2-amino-5-phosphonopentanoic acid (α-AP5), thapsigargin, SKF-96365 and SU1498 were purchased from Calbiochem (Darmstadt, Germany). The drugs were prepared as stock solutions, stored at ~70 °C and diluted to their final concentrations in the perfusion solution immediately before use. Stock solutions of thapsigargin (2 mM), SU1498 (1 mM), KN-62 (1 mM), PD98059 (15 mM), anisomycin (20 mM) and rapamycin (1 mM) were prepared in dimethyl sulphoxide (DMSO). VEGF (10 μg/ml), MK801 (10 mM) and SKF-96365 (10 mM) were prepared in distilled water. The final concentration of DMSO was 0.02 or 0.1%. α-Conotoxin GVIA was dissolved in PBS and used at a final concentration of 1 μM. 6-N-(2-hydroxyethyl)-1,4-diaza-7-nitroso-2,3,4,5-tetrahydro-1H,4H-dione (CNOX) (Sigma) was dissolved in 0.1 M NaOH and used at 20 μM. BayK8644 and nifedipine were dissolved in DMSO and used at 3 μM and 10 μM, respectively with a final DMSO concentration of 0.02%.

2.3. Immunohistochemistry and Western blotting

For Western blot analysis, cells were prepared as previously described [18]. The primary antibodies used were: anti-phosphorylated mTOR (Ser2448) (1:1000), anti-mTOR (1:1000), anti-phosphorylated Akt (Ser473) (1:1000), anti-Akt (1:1000), anti-phosphorylated p70S6K (Thr389) (1:600), anti-p70S6K (1:1000), anti-phosphorylated CaMKII (Thr286) (1:1000), anti-CaMKII (1:1000), anti-phosphorylated p44/p42 MAPK (Thr202/Tyr204) (1:1000), anti-p44/p42 MAPK (1:1000), anti-phosphorylated CREB (Ser133) (1:1000), anti-phosphorylated 4E-BP1 (1:1000), anti-phosphorylated 4E-BP1/2/3 (Thr45) (1:200), anti-phosphorylated PLC-γ (Tyr773) (1:1000) and anti-β-actin (1:1000). All primary and secondary antibodies were from Cell Signaling Technology (Danvers, MA, USA) except for anti-phosphorylated 4E-BP1/2/3 (Thr45) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin (GencTex, San Antonio, TX, USA). After washing three times with TBST buffer, membranes were incubated with anti-rabbit or anti-mouse horseradish peroxidase-linked secondary antibody (1:1000) for 1 h at room temperature. They were washed twice with TBST and processed for chemiluminescence detection using a horseradish peroxidase substrate and enhanced chemiluminescence detection system (Amer sham Biosciences, HP790, NA, UK). Signals were captured on film.

2.4. Generation of adenovirus co-expressing VEGF and GFP

To generate an adenovirus that expresses VEGF and GFP in the E1 and E3 regions, respectively, the VEGF gene was first excised from the vector pcDNA3/VEGF with EcoRI and subcloned into the vector pCA14 (Microbes, Ontario, Canada) to generate pCA14-VEGF. Next, pCA14-VEGF was linearized with BspHI and co-transformed into E. coli B15183 with BstBI-digested pdl324; homologous recombination then yielded pdl-VEGF. To construct the E3 shuttle vector expressing GFP, the HindIII fragment cut from pcDNA-GFP was subcloned into pSP72-E3, generating pSP72-GFP. This was then linearized with XmnI and co-transformed into E. coli B15183 with SphI-digested pdl-GFP; homologous recombination then yielded the E3 shuttle vector expressing GFP. To verify the respective homologous recombinants, plasmid DNA purified from overnight E. coli cultures was digested with HindIII and analyzed. The confirmed homologous recombinant DNAs were digested with PaeI and antibiotic mixture. Half of the medium was replaced every 3 days and AraC (2 μM) was added on day 3. Cultures were maintained for 10–12 days at 37 °C in a 5% CO2/95% air humidified incubator. The neurons were used after 10 to 14 days. Animal care and experiments were conducted in accordance with the 2004 Guide for the Care and Use of Laboratory Animals (Korea National Institute of Health) and Hanyang University Veterinary committee.
transfected into 293 cells to generate AdVEGF-GFP, which expresses both VEGF and GFP under the control of CMV. Expansion, purification, titration and quality analysis of all adenoviruses were performed as previously described. Virus particle numbers were calculated from measurements of optical density at 260 nm (OD260), where 1 absorbency unit is equivalent to 10^{10} virus particles per milliliter, and infectious titers (PFU per milliliter) were determined by limited dilution on 293 cells.

2.5. Secretion of VEGF and AdVEGF from hippocampal neurons

For the analysis of VEGF and BDNF released from mature primary hippocampal neurons following treatment with various agents and their inhibitors, neurons (10–12 DIV) were treated with various stimuli with agonists for 30 min or 24 h under the given experimental conditions with or without 30 min pretreatment of inhibitors. Next, the culture media (300 μL) were collected and centrifuged at 1500 rpm to remove detached cells before ELISA. To obtain detectable levels of VEGF, VEGF was expressed by adenoviral vectors (AdVEGF), which produce approximately 4–10-fold higher levels than the endogenous levels (∼0.2 ng per 100,000 cells). For transfection of neurons with AdVEGF, primary neurons (4–5 DIV) were infected with concentrated AdVEGF (9.19×10^{10} pfu/ml) and empty adenovirus (3.61×10^{10} pfu/ml), respectively. Three days later, the neurons were treated with 20 mM KCl for 30 min or 24 h with or without 30 min pretreatment with specific inhibitors. VEGF and BDNF in the culture media were measured with Quantikine VEGF ELISA kits (R&D Systems, Minneapolis, MN) and Chemikine BDNF Sandwich ELISA kits (Chemicon, Temecula, CA, USA), respectively, using a standard curve of the absorbance of VEGF or BDNF vs concentration.

2.6. Luciferase reporter assay

Luciferase reporter assays of rat VEGF-A promoter activity were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The rat VEGF-A promoter (−921 to +11) was generated from rat genomic DNA by PCR and cloned into the luciferase reporter vector pG3L. Primary hippocampal neurons were plated at a density of 1–2×10^5 cells in 60-mm dishes, cultured for 3 days in NB media supplemented with B27, and transfected with the luciferase reporter plasmid containing the promoter sequence of rat VEGF-A in pG3L (firefly luciferase vector) and pRL-TK (Renilla luciferase vector, for normalization) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Cells were cultured for 48 h and then exposed to 24 h to 20 mM KCl with or without 30 min pretreatment with type-specific VACC inhibitors and harvested to measure luciferase activity with a luminometer (Berthold detection systems, Bleichehurst, Pforzheim, Germany) using a reagent kit (Promega). Transfection efficiency was normalized by the activity of Renilla luciferase.

2.7. Semi-quantitative RT-PCR and quantitative real time RT-PCR

Total RNA was prepared using Trizol reagent (Life Technologies Inc., Rockville, MD, USA). The following PCR primers were used: rat VEGF-A sense, 5′-GTACCTCCACCATGCCAAGT-3′, antisense 5′-CAAGGGTCACAGTGTATT TCTGG-3′; Hif-1α sense, 5′-GTCTCGAGATGCAGCCAGATCTCG-3′, antisense 5′-GTTGAGATGATCGAGTCCAAGG-3′; Flk-1 sense, 5′-AGAGATGCGG-GAAAACTACAGC-3′, antisense 5′-CTGTCCTCTGTTTGGATGG-3′; Flt-1 sense, 5′-TGGCCGATAAAAGCAGATGACG-3′, antisense 5′-GAGAGAGGCACGCA CACCAATTG-3′; rat BDNF sense, 5′-GTGACAGTATAGCCGATG-3′, antisense 5′-GGTATTGCTTGGCGATGC-3′; GAPDH sense 5′-CTGCTGTCATA TAGCCAAGATGCTGAAG-3′, antisense 5′-AGAGTCCACGACATCTAT-CAC-3′; β-actin sense, 5′-CAGAACCCTGCATGCG-3′, antisense 5′-ACCCAC- CACTTGGCCACTCA-3′. Transcript levels of each gene were normalized to the amount of the housekeeping genes GAPDH or β-actin in the individual samples, and average fold changes were compared using the Image J program (provided for free on the Wright Cell Imaging Facility’s website). For quantitative analysis, real time RT-PCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was performed with iCycler (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. A melting curve for each PCR product was obtained after each run to confirm that the signal corresponded to a unique amplicon of the predicted size. Statistical significance was tested using Student’s t-test.

2.8. Preparation of hippocampal slices

For electrophysiological recordings, hippocampal slices were prepared from male Sprague–Dawley rats (4–5 week-old). Brains were removed and placed in cold (4 °C) oxygenated (95% O_2–5% CO_2) artificial cerebrospinal fluid (ACSF in mM; 124 NaCl, 2.5 KCl, 25 NaHCO_3, 1.25 NaH_2PO_4, 10 glucose, 1.3 MgSO_4, 2.0 CaCl_2). One hemisphere was immediately placed in 0.1 M PBS and processed for histological analysis. The hippocampus was then dissected from the second hemisphere, cut into 400 μm slices with a tissue chopper, and placed in an incubation chamber, which was continuously perfused at 28–29 °C with ACSF for at least 1.5 h. Field EPSPs were recorded from CA1 stratum radiatum in response to stimulation from Schaffer collaterals. All stimulations were at a stimulus intensity that evoked one-third of the maximal EPSP. For RNA extraction, slices were treated with KCl for the indicated time and then proceeded for RNA isolation.

2.9. Electrophysiological recordings

Field potential recordings were obtained in the medial molecular layer of the CA1–CA3 by stimulating the Schaffer collateral pathway. Baseline recordings were made by electrically stimulating the middle region of the molecular layer. Stimulation was performed with insulated bipolar tungsten electrodes of 40 μm tip diameter. Input–output curves were obtained after 20 min of stable recordings. The stimulation intensity that produced one-third of the maximum EPSP (EPSP; 200–400 μA, 10 μs) was chosen as the test pulse and high frequency stimulation (HFS). Only slices that produced field EPSPs 1 mV greater in amplitude were used. The baseline frequency for stimulation was 1 per 30 s. VEGF was infused over 10 min (10 ng/ml). KN62 (5 μM), a CaMKII inhibitor, or PD98059 (50 μM), an inhibitor of MEK, were present from 10 min before to 20 min after VEGF infusion. SU1498 (20 μM), an inhibitor of VEGFR2, was present from 15 min before to 5 min after HFS. Anisomycin was added to slices at least 20 min before VEGF and perfused for the whole recording period. Slices were continuously perfused with ACSF bubbled with 95% O_2 and 5% CO_2 at 3 ml/min. The temperature of the perfusion chamber was maintained at 28–29 °C.

2.10. Data processing and statistical analysis

Signals from hippocampus CA1–CA3 were amplified, filtered, digitized (for field potentials) and stored on a computer disk. Acquisition and analysis of field potentials were accomplished using National Instrument Labview software (National Instruments Co). The maximum slope of the EPSP was measured from its negative-going apex to the tangent line joining the first 2 positive peaks. Statistical analysis was performed using Statistica (Statsoft, 1996). Statistical analysis for LTP was carried out with pooled slices from individual rats. Data from all slices tested under the same conditions from the same rat were averaged. Statistical analysis of the electrophysiological data was based on comparison of the last 10 min of baseline recording with 10 min of recording during VEGF treatment. Group comparisons of immunohistochemical results were performed using the Student’s t-test (paired where indicated).

3. Results

3.1. Membrane depolarization activates VEGF transcription

The goal of the present study was to define the role of activity-dependent VEGF in hippocampal neurons. To see if membrane depolarization stimulates rat VEGF;_164 mRNA expression, we performed quantitative real time RT-PCR using cultured embryonic day 16.5 rat hippocampal neurons exposed to KCl (20 mM). Increased VEGF transcription was
detected within 1 h of membrane depolarization and it peaked within 3 h and remained moderately elevated for at least 6 h both by semi-quantitative RT-PCR (Fig. 1A) and quantitative real time RT-PCR (Fig. 1 a1). Membrane depolarization increases VEGF mRNA expression in hippocampal slices when slices were treated with KCl (50 mM) for 30 to 60 min (Fig. 1B and b1), indicating that expression of VEGF mRNA in response to membrane depolarization is coherent in hippocampus. Expression of VEGFR2 was also responsive to KCl but expression of VEGFR1 was not detected by semi-quantitative RT-PCR (Fig. 1A), probably because the level of VEGFR1 mRNA is very low. For comparison, we investigated brain-derived neurotrophic factor (BDNF) expression in cells exposed to similar KCl stimuli using primers detecting exon IX that is present in all BDNF isoforms. Membrane depolarization induced the BDNF mRNA expression with similar kinetics but greater fold increases (Fig. S1A and B). Depolarization by bath application of KCl induced an increase in [Ca^2+]i in neurons [19,20]. Therefore, the effects of KCl on the level of VEGF mRNA reflect VEGF transcription that might be, at least in part, Ca^{2+} influx-dependent.

To test if other stimuli that lead to Ca^{2+} influx also induce Ca^{2+}-dependent VEGF transcription, cells were exposed to NMDA and AMPA, which are glutamate receptor agonists, and BayK8644, a L-type voltage-activated Ca^{2+} channel (L-VACC) agonist. Expression of VEGF and VEGFR2 mRNA was increased by all three treatments when investigated by semi-quantitative RT-PCR (Fig. 1C) and quantitative real time RT-PCR (Fig. 1 c1). Expression of BDNF mRNA was also stimulated by all three treatments (Fig. S1C). Glutamatergic receptor activation-induced VEGF expression is restricted to neurons, because VEGF164 expression was not induced by glutamate in astrocytic cultures (Fig. 1D and d1) although the astrocytes contained VEGF transcripts (Fig. 1D) and protein (Fig. 1E). For VEGF to modulate neuronal function, it should be expressed in neurons, and, indeed, Northern analysis of several adult mouse hippocampal tissues has revealed VEGF expression in hippocampal neurons [21].

Fig. 1. Membrane depolarization-dependent expression of VEGF164 in hippocampal neurons. (A) Expression of VEGF164, VEGFR2 or VEGFR1 genes was investigated by semi-quantitative RT-PCR (A) and quantitative real time RT-PCR of VEGF164 (a1). Hippocampal neurons at 12 DIV were treated with KCl (20 mM) for the indicated times and mRNA was extracted. (B) Semi-quantitative and quantitative real time RT-PCR (b1) show that membrane depolarization by KCl (50 mM) induces VEGF164 mRNA in hippocampal slices. Hippocampal slices were prepared as described in Material and Methods, treated with KCl for the indicated time and then proceeded for RNA isolation. (C) Effects of BayK8644 (3 μM), an L-VACC agonist, and glutamate receptor subtype ligands, AMPA (10 μM) and NMDA (10 μM) treated for the indicated time on VEGF164 and VEGFR2 expression. Quantitative real time RT-PCR of VEGF164 and VEGFR2 following drug treatment shown in (c1) for 3 h. (D) VEGF164 mRNA expression is not induced by glutamate (10 mM) treated for the indicated time in astrocytes. Quantitative real time RT-PCR (d1). (E) Expression of VEGF in neurons and astrocytes. Cells expressing neuronal and astrocyte markers (Tuj1 and GFAP, respectively, arrows) express VEGF (green). The expression of each gene was normalized to the amount of GAPDH, and is shown relative to expression in non-stimulated cells. Values represent means± SE. Student’s t-test: *p<0.05, **p<0.01, ***p<0.001. Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3.2. VEGF expression is dependent on Ca\(^{2+}\) influx via either NMDA-R or L-VACC

Because our results showed that membrane depolarization triggers VEGF expression at the transcriptional level, we investigated the regulation of VEGF promoter activity in neurons by Ca\(^{2+}\) influx. The promoter region of rat VEGF cDNA, containing the transcriptional activation domain was cloned, and a reporter plasmid containing the promoter region of VEGF cDNA was fused to the firefly luciferase reporter gene in pGL3. The reporter plasmid was transiently transfected into hippocampal neurons together with a plasmid containing the Renilla luciferase reporter gene driven by the Herpes simplex virus (HSV) TK promoter. After 48 h, transfected cultures were treated with NMDA (10 μM), BayK8644 (3 μM), or KCl (20 mM). Luciferase activity was induced 8- to 10-fold following NMDA, BayK8644 and KCl treatment. Since Ca\(^{2+}\) influx is commonly increased as NMDA receptor (NMDA-R), L-VACC and VACC are activated by NMDA, Bayk8644 and KCl, respectively, our results suggest that Ca\(^{2+}\) influx might be involved in the transcriptional activation of VEGF (Fig. 2A). Ca\(^{2+}\) influx through L-VACC has previously been shown to mediate membrane depolarization-induced BDNF expression [22,23]. To determine if the induction of the VEGF\(_{164}\) promoter-driven luciferase gene following membrane depolarization requires Ca\(^{2+}\) influx through L-VACC, the promoter’s response to KCl in the presence of nifedipine was tested. The addition of nifedipine, a specific L-VACC antagonist, completely blocked KCl-driven VEGF reporter gene induction (Fig. 2A), indicating that KCl induction of the VEGF\(_{164}\) promoter-driven luciferase gene is dependent upon Ca\(^{2+}\) influx through L-VACCs. MK801 (20 μM), an NMDA receptor antagonist, blocked KCl-driven VEGF reporter gene induction (Fig. 2A), indicating that KCl induction of the VEGF\(_{164}\) promoter-driven luciferase gene is dependent upon NMDA receptor activation.

Consistent with the promoter assay, KCl induction of VEGF mRNA expression was also completely inhibited by nifedipine (10 μM) by RT-PCR (Fig. 2B and b1); in addition it was inhibited by MK801 (20 μM) or D-AP5 (200 μM), an NMDA antagonist (Fig. 2B and C, c1), indicating that Ca\(^{2+}\) influx through NMDA-R contributes to VEGF expression in response to chronic membrane depolarization with 20 mM KCl. KCl-induced VEGF transcription was completely repressed by coapplication of nifedipine and MK801 (Fig. 2B and b1). However, ω-conotoxin, an N-type VACC blocker, had no effect on VEGF transcription (Fig. 2B and b1). KCl induction of BDNF mRNA expression was inhibited by MK801, but not either by nifedipine or ω-conotoxin (Fig. S2A). Consistent with the involvement of NMDA-R in the VEGF mRNA expression, treatment of neurons with NMDA increased VEGF\(_{164}\) mRNA (Fig. 2Ca and c1) or BDNF (Fig. S2B) expression 2 to 3-fold, and these effects were completely blocked by MK801,
3.3. Activity-dependent release of VEGF

Previous studies of activity-dependent neurotransmitter release demonstrated that chronic depolarization is effective at releasing both classical transmitters and peptide co-transmitters such as BDNF [24,25]. To see whether VEGF release is similarly regulated, we tested whether 30 min of continuous membrane depolarization by 20 mM KCl caused the release of native VEGF from hippocampal neurons. This treatment proved to be ineffective (See Fig. 3B). However, we detected increases of VEGF (Fig. 3A: 684.1 ± 36.6 in control vs 775 ± 30.3 pg/ml in KCl; \( p < 0.05 \)) or BDNF (Fig. S3: 5.3 ± 0.7 in control vs 12.8 ± 3.4 pg/ml in KCl; \( p < 0.05 \)) concentrations following chronic depolarization for 24 h. Chelation of extracellular Ca\(^{2+}\) with EGTA (5 mM) completely abolished KCl-induced VEGF (Fig. 3A) and BDNF release (Fig. S3), as did loading of the neurons with BAPTA-AM, indicating both extracellular and intracellular Ca\(^{2+}\) is necessary for the activation step in secretion (Fig. 3A and B). The increases in BDNF and VEGF levels were not attributable to increased neuronal survival (1234 ± 71 neurons per well in control cultures vs 1430 ± 91 neurons per well in KCl-treated cultures; \( p > 0.05 \)).

To further characterize cellular mechanisms underlying activity-dependent release of native VEGF, we examined the role of particular voltage-activated Ca\(^{2+}\) channels and NMDA receptor activation. VEGF release increased from 684 pg/ml in the control to 773 and 756 pg/ml in NMDA (10 \( \mu \)M) and BayK8644 (3 \( \mu \)M)-treated cultures, respectively (Fig. 3A). BDNF release increased from 5.3 pg/ml in the control to 7.9 pg/ml upon 24 h stimulation with NMDA (Fig. S3). However, BayK8644 did not induce a significant increase in BDNF release (Fig. S3). The release of VEGF induced by NMDA and BayK8644 was abolished by pretreatment of the cultures with EGTA or BAPTA-AM, demonstrating that VEGF is released in response to elevation of [Ca\(^{2+}\)].

To see if activation of voltage-dependent Ca\(^{2+}\) channels is required for VEGF release, we examined release in the presence of nifedipine and \( \omega \)-conotoxin GVIA. Because endogenous VEGF was too low to detect secretion after short periods of stimulation, we used hippocampal neurons transduced with an adenoviral vector carrying VEGF (AdVEGF). VEGF secretion from control hippocampal neurons was not affected by 30 min stimulation with KCl, NMDA, or BayK8644, as previously mentioned (Fig. 3B). However, VEGF release from cultures transduced by AdVEGF was increased by 24 h stimulation with these agents (Fig. 3B). The KCl-induced VEGF release was blocked by nifedipine, but not by \( \omega \)-conotoxin GVIA, indicating that it requires Ca\(^{2+}\) entry specifically through L-VACCs. In addition, the fact that VEGF release was not affected by \( \omega \)-conotoxin GVIA demonstrates that it is induced by a different range of stimuli from BDNF release, which was previously shown to be N-VACC dependent [26]. After stimulation for 24 h there was no significant difference in the number of cells in the control and the stimulated cultures (per well: control, 15,750 ± 60.35; KCl-stimulated, 14,800 ± 57.15; \( p > 0.05 \); data not shown).

3.4. Intracellular Ca\(^{2+}\) is increased by VEGF

For the released VEGF to act as a neurotrophic factor, VEGF could possibly modulate [Ca\(^{2+}\)], elevation either by direct Ca\(^{2+}\) influx or through intracellular Ca\(^{2+}\) release. To test whether VEGF increases [Ca\(^{2+}\)], experiments were designed to characterize VEGF-induced Ca\(^{2+}\) signaling in cultured neurons. Bath application of 50 and 100 ng/ml VEGF induced an elevation
of [Ca\textsuperscript{2+}]\textsubscript{i}, which gradually increased with time during application in whole cell parts including cell bodies and dendrites of hippocampal neurons (Fig. 4A). BDNF (100 ng/ml) led to a marked elevation of [Ca\textsuperscript{2+}]\textsubscript{i} in cultured hippocampal neurons (Fig. S4). To examine whether these signals are associated with an influx of extracellular Ca\textsuperscript{2+} ions, cells where VEGF was added to a Ca\textsuperscript{2+}-free buffer containing EGTA were tested. Perfusing the cells with the Ca\textsuperscript{2+}-free buffer completely blocked the VEGF-induced Ca\textsuperscript{2+} response, indicating VEGF-mediated Ca\textsuperscript{2+} response largely depends on extracellular Ca\textsuperscript{2+} influx (Fig. 4B). Removal of intracellular Ca\textsuperscript{2+} with BAPTA-AM blocked VEGF-induced Ca\textsuperscript{2+} response (Fig. 4C), indicating that VEGF-induced Ca\textsuperscript{2+} response is controlled by intracellular Ca\textsuperscript{2+}. The slow kinetics of the intracellular Ca\textsuperscript{2+} increase in response to VEGF shown in Fig. 4A may be initially due to Ca\textsuperscript{2+} release from intracellular stores. Thapsigargin, a blocker of calcium reuptake into endoplasmic reticulum (ER), depletes internal stores \cite{27}. Treatment with thapsigargin (2 μM) completely inhibited the calcium responses to VEGF (Fig. 4D), indicating that intracellular calcium release from ER is involved in VEGF-mediated Ca\textsuperscript{2+} response. There might be the possibility that other members of Ca\textsuperscript{2+} channels in addition to VACCs mediate the Ca\textsuperscript{2+} influx involved in the VEGF-mediated Ca\textsuperscript{2+} increases. Therefore, the involvement of voltage-independent transient receptor potential canonical (TRPC) channels, which mediate BDNF-induced slow nonselective cationic currents \cite{28}, in the VEGF-mediated Ca\textsuperscript{2+} responses was checked. SKF96365 (30 μM), an inhibitor known to block TRPC channels, reduced VEGF-induced Ca\textsuperscript{2+} responses (Fig. 4E) at significantly higher than those measured under control conditions, suggesting that TRPC channels play a partial role in VEGF-mediated responses. To determine whether the VEGF-induced calcium influx is dependent on the specific receptor, the VEGFR2-specific receptor kinase inhibitor SU1498 was applied to neurons before stimulation with VEGF. The VEGF-induced calcium influx was eliminated by SU1498 (Fig. 4F). Thus, VEGFR2 activation is a key factor in the initiation of VEGF-induced changes in cytosolic calcium in hippocampal neurons. Together, these observations demonstrate that VEGFR2, full intracellular Ca\textsuperscript{2+} stores, and Ca\textsuperscript{2+} influx through TRPC channels in part are required for VEGF-induced Ca\textsuperscript{2+} responses.

Fig. 4. VEGF elevates [Ca\textsuperscript{2+}]\textsubscript{i} in hippocampal neurons. (A) Continuous fluorometric [Ca\textsuperscript{2+}]\textsubscript{i} recording in hippocampal neurons loaded with 5 μM fura-2/AM. Neurons were exposed to VEGF at the indicated time for 10 min. The effects of bath application of 50 and 100 ng/ml VEGF on [Ca\textsuperscript{2+}]\textsubscript{i} were measured in the whole cell by the ratio of fluorescence at 340 and 380 nm excitation wavelength (F/F\textsubscript{0}). The mean values of all the loaded cells are shown from 3 discrete cultures. Bath application of 50 and 100 ng/ml VEGF for 10 min caused a time-dependent and sustained elevation in [Ca\textsuperscript{2+}]\textsubscript{i} when Ca\textsuperscript{2+}-containing buffer was used. Application of 50 ng/ml VEGF still caused a [Ca\textsuperscript{2+}]\textsubscript{i} elevation, although it was reduced, indicating that VEGF-induced Ca\textsuperscript{2+} influx is concentration-dependent (50 ng/ml VEGF, 0.56±0.03 vs control, 0.42±0.01 in F/F\textsubscript{0}, p<0.001, n=73 cells; 100 ng/ml VEGF, 0.71±0.02 vs control in F/F\textsubscript{0}, p<0.001, n=74 cells from 3 discrete cultures). The images are of a field of neurons at the time points corresponding to 1 and 2. (B) No change in fluorescence ratio was observed in the absence of extracellular Ca\textsuperscript{2+} (0 calcium, 10 μM EGTA; 0.42±0.00 vs control; p=0.05, n=18). BAPTA-AM (10 μM) (C) and thapsigargin (2 μM) (D) prior to VEGF blocked VEGF-induced Ca\textsuperscript{2+} responses (BAPTA-AM: 0.44±0.01 vs control; p=0.05, n=10; thapsigargin: 0.48±0.01 vs control; p=0.05, n=19 from 3 discrete cultures). BAPTA-AM and thapsigargin were present from 30 min before to 10 min after VEGF. (E) SKF96365 (30 μM) inhibited VEGF-induced Ca\textsuperscript{2+} responses (0.54±0.01 vs 0.71±0.02 in 100 ng/ml VEGF; p<0.01, n=11). (F) Effect of inhibiting VEGFR2 with SU1498 (50 μM) on VEGF-induced calcium influx (0.41±0.01 vs control, p>0.05, n=10).
3.5. Signaling molecules activated by VEGF

Having obtained evidence that VEGF increases [Ca^{2+}]_i, we questioned whether VEGF regulates neuronal functions. Therefore, the intracellular signaling molecules potentially activated by VEGF were then investigated with an attempt to characterize the possible contribution of VEGF to hippocampal synaptic plasticity. A link between VEGF and the phosphoinositide 3-kinase (PI3K)/Akt pathway has been demonstrated by numerous groups [2,29], and therefore the potential link between VEGF and Akt downstream pathway using a variety of antibodies was investigated initially. Treatment of hippocampal neurons with human recombinant VEGF_{165} (50 ng/ml) showed an increase in the expression of p-Akt with a peak expression between a 30 min and 1 h application. Since the mammalian target of rapamycin (mTOR) is a downstream component of the VEGF-PI3K-PKB-Akt pathway [29], we examined levels of p-Ser^{2448}mTOR, which reflect activation of mTOR. Activation of mTOR is increased upon VEGF stimulation with similar kinetics to p-Akt (Fig. 5A and a1). p-Thr^{389}p70S6K is a major determinant of p70S6K activation and

![Fig. 5. VEGF-dependent activation of signaling molecules by Western blotting analysis.](image-url)

**A** VEGF_{165} (50 ng/ml) activates PI3K, mTOR, p70S6K, ERK and CaMKII in a time-dependent manner, with peaks at approximately 0.5 to 1 h after treatment (p-Akt: 30 min, 188.3±8.1% vs 100% in control, \(p<0.01\); 1 h, 176.8±2.2% vs control, \(p<0.05\), \(n=6\); p-mTOR: 30 min, 148.2±21.7% vs control, \(p<0.05\); 1 h, 153.1±9.4% vs control, \(p<0.01\), \(n=7\); p-p70S6K: 30 min, 143.3±10.1% vs control, \(p<0.05\); 1 h, 171.8±16.2% vs control, \(p<0.05\), \(n=5\); p-CaMKII: 30 min, 690.8±335.1% vs control, \(p<0.05\); 1 h, 683.8±336.2% vs control, \(p<0.05\), \(n=3\); p-ERK: 30 min, 189.8±29.2% vs control, \(p<0.05\); 1 h, 168.8±9.92% vs control, \(p<0.01\), \(n=5\)). (B) A rapid increase in the phosphorylation of PLCγ (Tyr783), CREB(Ser133) and CaMKII(Thr286) following VEGF_{165} stimulation (PLCγ: 10 min, 148.0±15.6% vs control, \(p<0.05\), \(n=4\)). (C) Application of VEGF in the presence of SU1498 (50 μM) inhibits the phosphorylation of Akt(Ser473), mTOR(Ser2448), p70S6K(Thr389), ERK1/2(Thr202/Tyr204) and CaMKII. SU1498 was added 30 min prior to VEGF_{165} treatment for the indicated time (\(n=3\)). (D) Representative blots show the effects of various inhibitors on the VEGF-induced activation of signaling molecules. (D1) Quantitative analyses of signaling molecules shown in D. LY294002 (10 μM), rapamycin (25 μM), KN-62 (5 μM), PD98059 (50 μM), anisomycin (20 μM) were added 30 min prior to VEGF_{165} in the continuous presence of VEGF_{165} for 1 h (VEGF+LY294002: 12.7±5.0% in p-Akt, \(p<0.01\) vs VEGF, \(n=4\); VEGF+rapamycin, 19.1±4.8% vs VEGF, \(p<0.001\); p4E-BP1: VEGF+rapamycin, 12.0±5.1% vs VEGF, \(p<0.01\), \(n=4\)). Optical density (OD) of each molecule was normalized to the OD of β-actin to calculate the relative amount of the molecule (\(n=3\) to 7 per group from 7 discrete cultures for A and B; \(n=4\) per group from 5 discrete cultures). Expression levels at each time point (or treatment) are depicted relative to the level of the non-treated control for comparison. Cells preincubated with vehicle and not exposed to VEGF_{165} were defined as control cells. Student’s t-test: *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\).
is regulated in a complex manner by mTOR and PI3K [30]. The level of p-Thr389-p70S6K also increased in a similar manner to p-Akt and p-Ser2448mTOR (Fig. 5A and a1).

The phosphorylation of 4E-BPs by mTOR results in their dissociation from eIF-4E and initiates translation [30,32]. Western blot analysis using antisera detected all three forms of phosphorylated 4E-BP (4E-BP1, 2, and 3), and demonstrated that the 4E-BPs were phosphorylated following VEGF stimulation (data not shown). Hyperphosphorylation of 4E-BP1 decreases its binding to eIF4E and increases translation rates [31], and PI3K and its downstream effector, Akt, have been shown to lead to 4E-BP1 phosphorylation [33]. We observed a slight, but not significantly high, increase in phosphorylation of 4E-BP1 following VEGF (Fig. 5A). These results together indicate the Akt/mTOR/p70S6K pathway is activated following VEGF stimulation, consistent with previous reports in other type of cells [33].

Calcium/calmodulin protein kinase II (CaMKII) and extracellular signal-regulated protein kinase (ERK) are activated in response to neuronal activity and are required for synaptic plasticity [34]. Since VEGF induces Ca2+ influx, we tested whether CaMKII and ERK were responsive to VEGF. In fact VEGF had a greater effect on the phosphorylation of CaMKII and ERK than of mTOR and p70S6K (Fig. 5A and a1), implying that activation of the CaMKII or ERK signaling pathways may play a significant role in the VEGF-mediated responses. In human umbilical vein endothelial cells (HUVEC), VEGF activates phospholipase C-γ (PLC-γ) and PI3K independently of each other [35]. In hippocampal neurons, VEGF appears to activate PLC-γ within a rapid (less than 10 min) time period (Fig. 5B and b1). Phosphorylation of CaMKII and cAMP responsive element binding protein (CREB) appears to be greatly increased within a similar rapid time period (Fig. 5B). To determine whether the VEGF-induced calcium influx is dependent on VEGFR2, we added SU1498 at the time of stimulation with VEGF. The activation of mTOR, p70S6K, ERK, CaMKII and PLC-γ was completely eliminated by SU1498 and the inhibition effect was highly reproducible in successive analyses (n=3), demonstrating VEGFR2 activation is required for the activation of these molecules (Fig. 5C).

A potential interaction between the PI3K/Akt/mTOR and CaMKII/ERK pathways was investigated using a variety of

Fig. 6. Effects of VEGF on basal neurotransmission and synaptic plasticity in hippocampal CA1. (A) VEGF165 (10 ng/ml) induced a rapid and persistent enhancement of synaptic strength (n=8). (B) KN62 (5 μM), or PD98059 (50 μM) was perfused from 10 min before and until 20 min after VEGF (10 ng/ml) infusion for the entire recording period. KN-62 blocked the VEGF-induced synaptic potentiation (107.1±3.6% vs 277.0±15.1% in VEGF; p<0.05, n=4). PD98059 also blocked VEGF-induced synaptic potentiation (118.6±7.7% vs 277.0±15.1% VEGF; p<0.05, n=5). Test pulses were applied at a rate of 1 every 30 s. The inset shows traces of averaged field responses (5 sweeps) recorded at the times indicated. (i–ii) Activation of CaMKII and ERK by VEGF165. Immunoreactivities for p-CaMKII and p-ERK were examined after hippocampal slices incubated in ACSF for 2 h at 30 °C and stimulated with VEGF165 (10 ng/ml) for 10 min. KN62 (5 μM) or PD98059 (50 μM) was perfused for 30 min before and during 10 min of VEGF treatment. Scale bar: 50 μm. (C) HFS (2 trains of 100 Hz at an interval of 10 s) induced LTP. HFS in the presence of VEGF induced an enhancement of LTP. HFS was applied 5 min after VEGF perfusion with VEGF perfusion lasting 10 min. Treatment of SU1498 (20 μM) reduced the VEGF-induced synaptic enhancement to the level of LTP obtained in the absence of VEGF SU1498 (131.8±12.4% vs 151.1±5.2% of LTP in the presence of VEGF, p<0.05, n=6). Representative traces show the fEPSP recorded before and after LTP at the indicated times. (D) Effects of anisomycin on the VEGF-induced synaptic transmission. Pretreatment of slices with anisomycin (20 μM) markedly attenuated the late phase of synaptic potentiation (106.6±1.8% vs 277.0±15.1% in VEGF, p<0.05, n=8). The average fEPSP amplitude at each time point was normalized to the averaged baseline for 10 min before HFS, which was represented as 100%. Test pulses were applied at a rate of 1 every 30 s. The inset shows traces of averaged field responses (5 sweeps) recorded at the time points indicated. Calibration: 0.5 mV, 30 ms.
inhibitors. Pretreatment of cells with the PI3K inhibitor LY294002 entirely abolished activation of Akt, mTOR and p70S6K by VEGF (50 ng/ml) (Fig. 5D). Rapamycin inhibited mTOR activation as shown by reduced phosphorylation of p70S6K and 4E-BP1 (Fig. 5D and d1). Interestingly, PD98059 and KN62 also inhibited the phosphorylation of 4E-BP1 to a comparable extent to rapamycin (Fig. 5D), suggesting a dual requirement for ERK and mTOR signaling for VEGF-dependent translation. The effects of VEGF on CaMKII and ERK phosphorylation were decreased to control level by either LY294002 or rapamycin (Fig. 5D and d1), implying a potential crosslink between the mTOR and CaMKII/ERK pathways.

3.6. VEGF induces protein synthesis-dependent long-lasting synaptic enhancement in the hippocampal CA1 region

Since membrane depolarization-induced VEGF expression is observed in hippocampal slices, endogeneous VEGF might have influence on the hippocampal neuronal activity such as synaptic plasticity. BDNF has been shown to cause synaptic potentiation in hippocampal neurons [31]. We performed electrophysiological experiments to see whether VEGF had the same effect, by continuous recording from hippocampal slices perfused with VEGF (10 ng/ml). A 10 min application of VEGF led to an increase in the slope of the fEPSP that began 3–5 min after perfusion and reached a plateau after 90–120 min (Fig. 6A: 277.0±15.1% vs 99.1±6.3% in control; p<0.05). Since our pharmacological experiments showed that VEGF had a greater effect on CaMKII and ERK than on mTOR (Fig. 5A), we investigated the involvement of CaMKII and ERK in VEGF-induced synaptic enhancement. Preincubation with KN-62 (5 μM) blocked the VEGF-induced synaptic enhancement (Fig. 6B) as did PD98059 (Fig. 6B), suggesting that activation of either CaMKII or ERK is required for the VEGF-induced synaptic potentiation. We observed a marked increase in the levels of p-CaMKII and p-ERK in the cell bodies and dendrites of VEGF-treated slices (Fig. 6B).

To investigate if VEGF has effects on LTP, VEGF (10 ng/ml) was perfused for 10 min prior to and during high frequency stimulation (HFS). The slices perfused with VEGF exhibited a persistent and significant enhancement of LTP (Fig. 6C: CTL, 151.1±5.2% vs VEGF, 232.0±43.1%, p<0.05, n=6). To determine whether the VEGF-induced synaptic enhancement is dependent on the VEGFR2, SU1498 was applied to slice before stimulation with VEGF. Although SU1498 (20 μM) alone did not change basal synaptic transmission (data not shown), pretreatment of SU1498 reduced the VEGF-induced synaptic enhancement to the level of LTP obtained in the absence of...
VEGF (Fig. 6C), indicating that VEGFR2 activation is responsible for the VEGF-induced synaptic enhancement in hippocampal neurons. Pretreatment of slices with anisomycin (20 μM), a protein synthesis inhibitor, did not affect the early phase of synaptic enhancement (Fig. 6D) but it markedly attenuated the late phase (Fig. 6D), indicating VEGF-induced synaptic enhancement is likely to involve de novo protein synthesis.

Overall, these results demonstrate that VEGF, when expressed and released from hippocampal neurons in an activity-dependent manner, plays a role in the Ca\(^{2+}\) influx and the activation of signaling pathways, possibly CaMKII and ERK pathways. These pathways in turn, may influence the generation of long-term changes in synaptic efficacy of hippocampal neurons.

4. Discussion

The present study provides three novel insights into the functions of VEGF in hippocampal neurons. First, endogeneous, native VEGF is expressed and released from hippocampal neurons in response to neuronal activity. Second, a brief pulse of exogenously applied VEGF is shown to induce a sustained elevation of \([\text{Ca}^{2+}]_i\), caused by both mobilization from intracellular Ca\(^{2+}\) stores and Ca\(^{2+}\) influx, and a substantial and sustained increase in basal neurotransmission in hippocampal CA1 neurons. Third, the effects of VEGF on hippocampal neurons might result from activation of the CaMKII/ERK pathway as well as PI3K/mTOR processes. The steps in the VEGF-initiated signaling cascades leading to the activation of neurons are summarized in the model shown in Fig. 7.

Among the VACCs, only L-VACCs were shown to contribute to the increase in KCl-induced VEGF mRNA expression since nifedipine, but not α-conotoxin GVIA, inhibited the KCl-induced VEGF mRNA expression (Fig. 2A and B). The KCl-induced VEGF mRNA expression was partially repressed by MK801, indicating that Ca\(^{2+}\) influx through NMDA-R in part contributes to VEGF expression in chronic membrane depolarization with 20 mM KCl. Our observation that Ca\(^{2+}\) signals evoked via L-VACC are more effective in inducing VEGF\(_{165}\) expression than those evoked via NMDA-R could be due to a difference in the localization of these channels in neurons or to different intracellular signaling mechanisms associated with these channels [36].

The similarity in expression between BDNF and VEGF mRNAs with regard to time course following membrane depolarization indicates that neuronal activity may also control VEGF expression. It is possible that the BDNF and VEGF promoters are coordinately activated by Ca\(^{2+}\) signals, but real time RT-PCR demonstrated that the extent of activity-dependent VEGF induction was lower than that of BDNF (Fig. 1B and Fig. S1). In addition, like BDNF secretion, VEGF secretion is regulated by both neuronal activity including glutamate receptor activation and membrane depolarization. However, Ca\(^{2+}\) influx through L-VACC was markedly more effective at evoking activity-dependent release of AdVEGF than influx through N-VACCs, whereas adenovirally overexpressed BDNF (AdBDNF) from hippocampal neurons is regulated mainly by Ca\(^{2+}\) influx through N-VACCs [37].

VEGF\(_{165}\) induced a sustained \([\text{Ca}^{2+}]_i\) rise, which appears to be mediated by an influx of extracellular Ca\(^{2+}\) (Fig. 4). Consistent with this, VEGF alone without HFS was found to progressively increase synaptic transmission, and brief application of VEGF prior to HFS increased the magnitude of LTP. The mechanism by which VEGF increases Ca\(^{2+}\) influx in neuronal cells is unclear. There is evidence that it regulates channel activation in HUVECs; Application of VEGF\(_{165}\) to HUVECs elicits a rapid rise in cytosolic Ca\(^{2+}\) and sustained membrane depolarization [27]. However, in hippocampal neurons we did not observe a rapid rise in cytosolic Ca\(^{2+}\) following VEGF stimulation. Instead, we detected slowly rising Ca\(^{2+}\) signals (Fig. 4A). The slow kinetics of the intracellular Ca\(^{2+}\) increase may be initially due to Ca\(^{2+}\) release from intracellular stores. This possibility is supported by our observation that VEGF activates PLC\(\gamma\), which in turn can induce inositol 1, 4, 5-triphosphate (IP3) production with consequent release of Ca\(^{2+}\) from the ER via the IP3 receptor. The importance of store-emptying in response to VEGF is supported by the effect of thapsigargin shown in Fig. 4D. The initial increase in \([\text{Ca}^{2+}]_i\) may induce membrane depolarization and activate VACCs such as L-VACC, leading to Ca\(^{2+}\) influx (Fig. 7). Our results also indicate that VEGF might induce Ca\(^{2+}\) influx, at least in part, through TRPC channels. Given that VEGF stimulates Ca\(^{2+}\) influx through L-VACC, NMDA channels and TRPC channels and activates the PLC\(\gamma\)-IP3 pathway, in hippocampal neurons, our results are in accord with the hypothesis that VEGF plays a role in regulating membrane potential and neuronal excitability.

Our finding that the inhibitory effects of PD98059 or KN62 were comparable to those of rapamycin, suggest a dual requirement for CaMKII/ERK and mTOR signaling in VEGF-dependent translation. In support of this concept, field-recording studies in hippocampal slices revealed a general dependence of VEGF-induced synaptic potentiation on ERK and CaMKII (Fig. 6). CaMKII is likely to act upstream of ERK since KN62 inhibits VEGF-induced phosphorylation of ERK, but not vice versa, as shown in Fig. 5. ERK activation is required for inducible phosphorylation of multiple factors involved in translation initiation [37]. This, together with the results of the present study, suggests that VEGF regulates translational efficiency affecting synaptic plasticity and memory through the functional interplay of ERK- and mTOR-dependent signaling mechanisms.

If VEGF serves as a signaling modulator in neurons, it could act via Ca\(^{2+}\) influx and/or activation of intracellular tyrosine kinase domains. VEGF-induced increase in \([\text{Ca}^{2+}]_i\), and membrane depolarization in HUVECs is inhibited by blockers of nonspecific Ca\(^{2+}\) channels, VEGFR2, src kinase, IP3 receptor, or PI3K [27]. In the present study, VEGF2 was expressed much more strongly than VEGFR1 (Fig. 1) and SU1498 blocked VEGF-induced increase in \([\text{Ca}^{2+}]_i\), and synaptic potentiation following HFS, supporting the hypothesis that VEGF plays the dominant role in signaling in neurons, as previously reported [3]. Noticeably, SU1498 reduced the LTP in the presence of VEGF to a control LTP level that is obtained in the absence of VEGF, suggesting that VEGF2 is involved in an increase in VEGF-induced synaptic potentiation on top of control LTP.
The source of VEGF in the mammalian brain could be local, peripheral, or both [3,15,17]. Consistent with the proposed function of VEGF in hippocampal neurons, hippocampal expression of VEGF was shown to be locally increased by both an enriched environment and the performance of a spatial maze [15]. In addition, VEGF, as one of the circulating factors that originate in the periphery, may be involved in running-induced hippocampal neurogenesis [17]. These results suggest a model in which the VEGF originates from both the periphery and from neurons and in which the peripheral VEGF affects neurogenesis and the neuronal VEGF affects both neuronal signaling (see Fig. 7) and neurogenesis. Since hippocampal neurogenesis is regulated by glutamate acting through NMDA receptors [38], our results indicate that VEGF modulates hippocampal synaptic plasticity in addition to its role in neurogenesis in response to NMDA receptor activation.

In summary, our data suggest that VEGF is expressed in, and released from, neuronal cells in an activity-dependent manner and plays a role in the increase of Ca²⁺ influx and synaptic transmission that influence long-term changes of synaptic efficacy. Our experiments highlight the importance of VEGF in signal processing in hippocampal neurons and confirm that neuronal excitability is controlled by locally produced VEGF.

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Appendix A. Supplementary data


References