Neurochem Res (2013) 38:1921–1934
DOI 10.1007/s11064-013-1098-9

ORIGINAL PAPER

Effect of HDAC Inhibitors on Neuroprotection and Neurite Outgrowth in Primary Rat Cortical Neurons Following Ischemic Insult

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Received: 26 February 2013 / Revised: 28 May 2013 / Accepted: 13 June 2013 / Published online: 22 June 2013
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Abstract Histone deacetylase inhibitors (HDACi)—valproic acid (VPA) and trichostatin A (TSA) promote neurogenesis, neurite outgrowth, synaptic plasticity and neuroprotection. In this study, we investigated whether VPA and TSA promote post-ischemic neuroprotection and neuronal restoration in rat primary cortical neurons. On 6 days in vitro (DIV), cortical neurons were exposed to oxygen-glucose deprivation for 90 min. Cells were returned to normoxic conditions and cultured for 1, 3, or 7 days with or without VPA and TSA. Control cells were cultured in normoxic conditions only. On 7, 9, and 13 DIV, cells were measured neurite outgrowth using the Axiovision program and stained with Tunel staining kit. Microtubule associated protein-2 immunostaining and tunel staining showed significant recovery of neurite outgrowth and post-ischemic neuronal death by VPA or TSA treatment. We also determined levels of acetylated histone H3, PSD95, GAP 43 and synaptophysin. Significant increases in all three synaptic markers and acetylated histone H3 were observed relative to non-treated cells. Post-ischemic HDACi treatment also significantly raised levels of brain derived neurotrophic factor (BDNF) expression and secreted BDNF. Enhanced BDNF expression by HDACi treatment might have been involved in the post-ischemic neuroprotection and neuronal restorative effects. Our findings suggest that both VPA and TSA treatment during reoxygenation after ischemia may help post-ischemic neuroprotection and neuronal regeneration via increased BDNF expression and activation.

Keywords HDAC inhibitors · Neurite outgrowth · Brain derived neurotrophic factor · Oxygen-glucose deprivation · Primary cortical neurons

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Introduction

Nucleosome, the fundamental unit of eukaryotic chromatin, is made up of approximately 146 bp of DNA wound around eight core histone proteins—a tetramer of H3/H4 and dimers of H2A and H2B [1]. N-terminal histone modifications can lead to an open DNA conformation (euchromatin), which promotes transcription, or a closed conformation (heterochromatin). Two groups of enzymes, histone deacetylases (HDACs) and histone acetyl transferases (HATs), determine the acetylation status of histones and thus regulate transcriptional activity. There are several families of HDACs, and HATs and HDACs and HATs target many non-histone proteins [2].

Histone deacetylase inhibitors (HDACi) are a heterogenous group of agents that inhibit HDACs and promote

acetylation by HATs, leading to an open DNA conformation and transcription. Structurally HDAC inhibitors can be grouped into several classes [1]. The inhibitory effects of HDACi are dependent on the type of HDACs. For example, MS-275 preferentially inhibits HDAC 1 and 3, while having little or no inhibitory effect on HDAC 6 and 8 [3].

Valproic acid (VPA), a short chain fatty acid having HDAC inhibitor properties, is an anticonvulsive and antimanic agent. Conversely, trichostatin A (TSA) is a member of the hydroximate class of HDAC inhibitors as well as an antifungal agent. VPA promotes neuronal differentiation in multi-potent adult neural progenitor cells [4] and neurite outgrowth in different cell types [5–9]. Cortical neuronal growth and neurogenesis promoted by VPA or TSA treatment involves activation of ERK pathway [7] as well as the JNK pathway [8] and regulation of GSK3 [6]. At the synaptic level, VPA regulates several types of receptors such as glutamate receptors, including both *N*-methyl-D-aspartate (NMDA) receptor and non-NMDA receptors, namely α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor, kainic acid receptor and quisqualate metabotropic glutamate receptor [10, 11], modifies spontaneous excitation and inhibition of cortical synapses [12], and promotes synaptic plasticity [13] via transcription factor, cAMP-response element binding protein [14]. VPA was recently shown to up-regulate promoter IV of BDNF [15] and to potentiate long-term memory in prefrontal rat cortex [16].

Trichostatin A does not alter gene expression globally, but rather increases the expression of specific genes during memory consolidation [14]. TSA also increases neuronal survival [17] and provides neuroprotection in rat stroke models [2]. Additionally, TSA produces anti-inflammatory effects in permanent ischemic rat models by inhibiting ischemia induced up-regulation of p53, inducible nitric oxide synthase and cyclooxygenase 2 [18].

Although there have been many in vivo and in vitro studies of HDACi related to neuroprotection [17–19], very little work has been done to examine the effect of HDAC on post-ischemic neuroprotection, in particular, and neuronal regeneration. On the other hand, neurite outgrowth related effects exerted by VPA and TSA have been observed in vitro without an ischemic insult [5–9].

In current study, we investigated whether HDACi promote neural restoration after ischemia, and what a novel molecular mechanism on post-ischemic neuroprotection and neural restoration by HDACi in rat primary cortical neurons.

We demonstrated that VPA and TSA treatment after oxygen and glucose deprivation (OGD) and reoxygenation in cortical neurons promotes neuroprotection and neuronal regeneration through up-regulation of BDNF expression.

Fig. 1 Post ischemic HDACi treatment promotes neurite outgrowth and neuroprotection. DIV 6 cortical neurons were treated with vehicle, VPA (0.5 mM) or TSA (30 nM) for 1, 3, or 7 days in normoxic or 90 min OGD plus reoxygenation condition. After treatment, cells were immunostained with MAP2 and stained with tunnel. **a** Representative images showing MAP2 immunocytochemistry of different groups. *Scale bar* = 50 µm. **b** Graph represents quantification of total neurite length, **c** graph represents quantification of mean neurite density, **d** representative images showing tunnel staining of different groups (tunel positive neurons: *red*; Topro3 nuclear maker: *blue*; overlay: *violet*). *Scale bar* = 20 µm. **e** Graph represents quantification of tunel positive neuron counts. Data are shown as the mean ± SEM of two independent experiments performed in triplicate. *Significantly different compared with control and #significantly different compared with OGD plus reoxygenation control (***$p < 0.001$, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$). Control: vehicle treated cells; OGD: vehicle treated OGD group. *Scale bar* = 50 µm

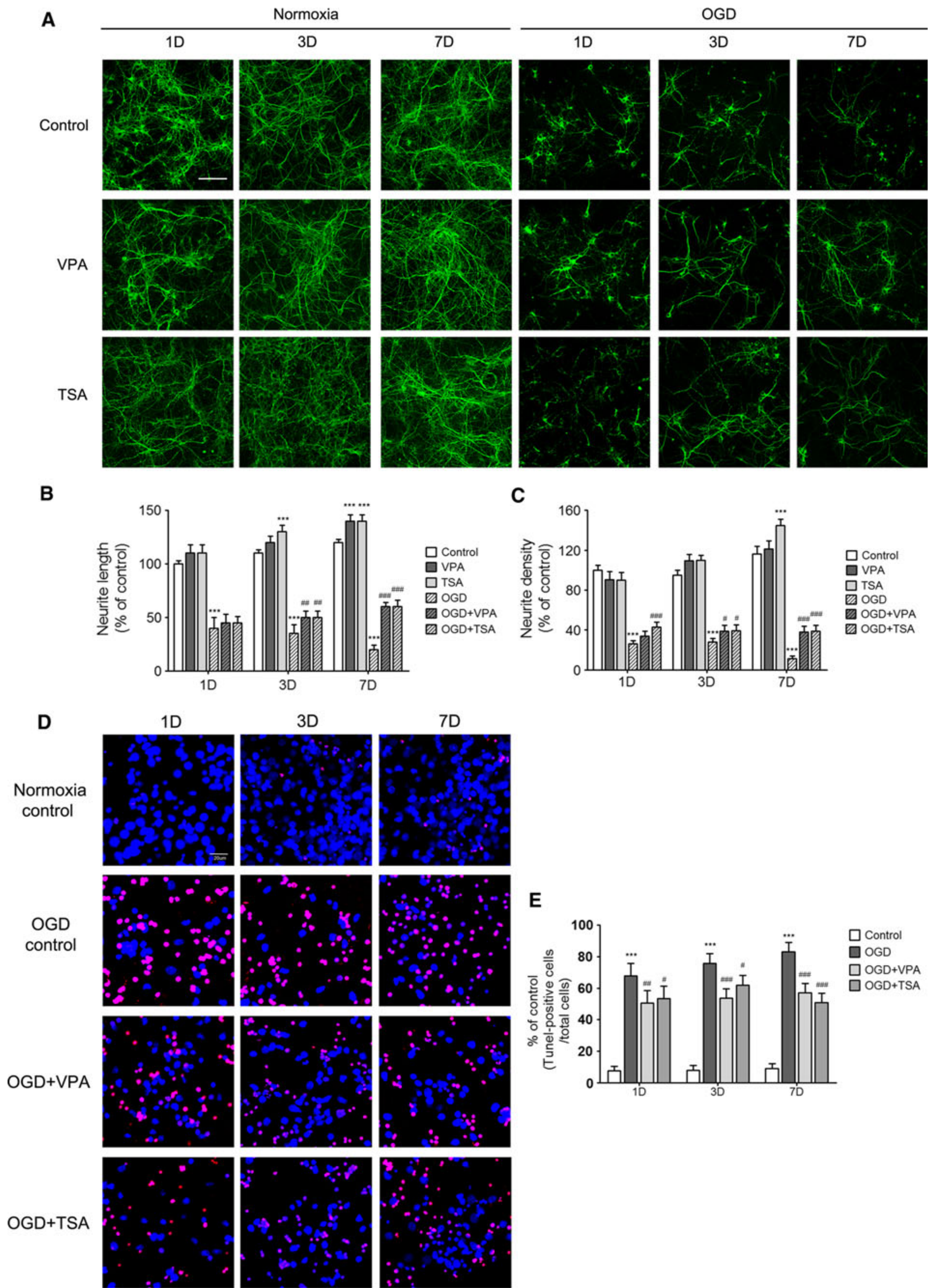
Materials and Methods

Materials

Pregnant Sprague–Dawley rats were obtained from Orient Bio Inc., Korea. Sodium valproate, trichostatin A, poly-D-lysine, L-Glutamine, protease inhibitor cocktail and phosphatase inhibitor cocktails were purchased from Sigma-Aldrich (St Louis, MO, USA). Neurobasal medium, B27 supplement, glucose and all other cell culture products were from invitrogen (Carlsbad, CA, USA), unless specified otherwise. Mouse monoclonal anti Synaptophysin antibody was obtained from BD Biosciences (Franklin Lakes, NJ, USA) and rabbit polyclonal anti PSD95, rabbit polyclonal anti GAP 43, rabbit polyclonal anti acetyl Histone H3, mouse monoclonal anti beta actin, mouse monoclonal anti MAP2 antibodies were purchased from Millipore (Temecula, CA, USA). Rabbit polyclonal anti Histone H3 and rabbit polyclonal anti ERK or anti pERK antibodies were obtained from cell signaling (Danvers, MA, USA) and normal rabbit immunoglobulin G (IgG) and rabbit polyclonal anti BDNF antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture

The cerebral cortices were collected from Sprague–Dawley rat embryos at embryonic day 18 and incubated with 0.01 % trypsin in Hank's balanced salt solution for 15 min at 37 °C. After trituration, primary cortical neurons were plated on each polystyrene cover slide that had been precoated with 100 µg/ml poly-L-lysine in a culture plate (density of 1×10^5 cells/cm^2). The cells were maintained at 37 °C in a humidified atmosphere with 5 % CO_2 in neurobasal medium supplemented with B-27, 2 mM glutamine, 100 IU/L penicillin, and 10 µg/mL streptomycin.


A
Normoxia
OGD
1D
3D
7D
1D
3D
7D
Control
VPA
TSA
B
Neurite length
(% of control)
150
100
50
0
1D
3D
7D
Control
VPA
TSA
OGD
OGD+VPA
OGD+TSA
C
Neurite density
(% of control)
160
120
80
40
0
1D
3D
7D
Control
VPA
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OGD+VPA
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Normoxia
control
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% of control
(Tunel-positive cells
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100
80
60
40
20
0
1D
3D
7D
Control
OGD
OGD+VPA
OGD+TSA

Highly pure cortical neurons (>99 and <1 % of astrocytes) were cultured for these experiments. On day 6 in vitro (DIV), the neurons were fed with fresh medium and treated. All experimental procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals and approved by the Medicine Animal Studies Committee of Konkuk University.

OGD Exposure

On DIV 6, the culture medium was replaced with a glucose-free, serum free MEM medium and cells were placed in an anaerobic incubator (Forma Anaerobic Systems, Thermo Electron, USA) ($pO_2 < 2$ mmHg) with an atmosphere of 95 % N_2, 5 % CO_2 and 98 % humidity at 37 °C for 90 min. OGD was terminated by removing the cultures from the anaerobic incubator, changing the medium and returning the cells to a normoxic condition. Control cultures were exposed to serum free MEM containing glucose for the same duration under the normoxic condition. The OGD exposure time used in this study resulted in a neuronal network of 40–50 % at 24 h after reoxygenation.

HDAC Inhibitors and Neutralizing Antibody Treatment

Except the control group, all cells were exposed to 90 min OGD treatment on DIV 6 followed by further subdivision into 7 groups (OGD—treated with vehicle 0.01 % ethanol, VPA—valproic acid 0.25, 0.5, or 1 mM and TSA—trichostatin A 10, 30, or 100 nM) according to the post OGD treatment for 1, 3, 7 days. 1,000× concentrated VPA and TSA was dissolved in distilled water (DW) and 100 % ethanol, respectively and then VPA or TSA was applied to the culture medium (0.1 % DW or 0.1 % ethanol final concentration). Appropriate concentrations of VPA and TSA determined form previous studies [7, 9] and our preliminary results. TSA is a specific HDACi in vitro and in vivo, working at nanomolar concentrations [20].

To neutralize BDNF expression, BDNF antibody (Santa Cruz Biotechnology, CA, USA) was treated (10 g/ml) for 1 week. IgG as a control against BDNF antibody was treated in the presence or absence of HDCAi in normoxic condition.

Western Blot Analysis

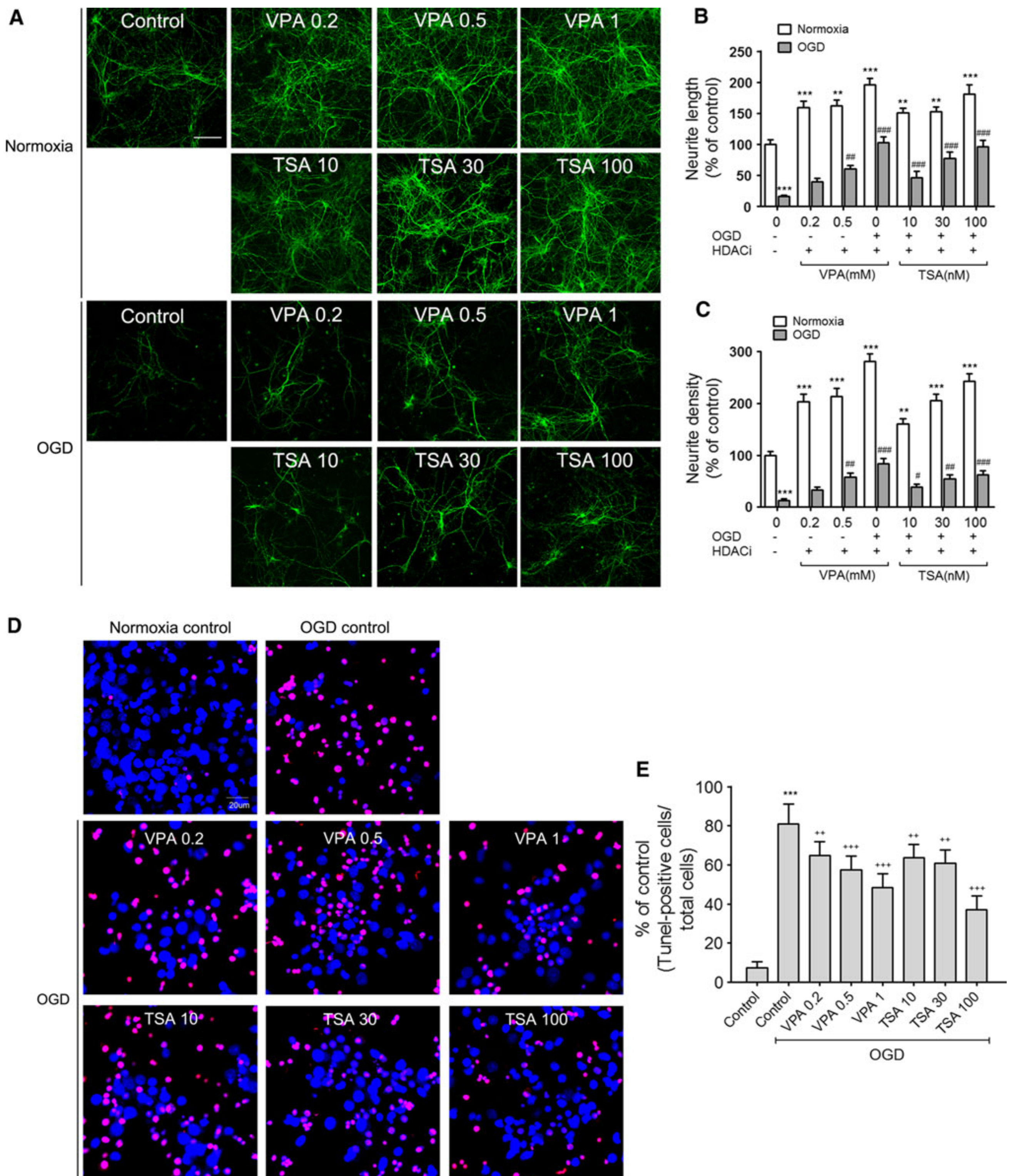
Western blotting was performed as described previously [7] to detect the expression of synaptic markers. The cells were washed with PBS and scraped off on ice. Cell lysates were collected with RIPA buffer containing protease and phosphatase inhibitor cocktails. 20 µg protein was subjected to SDS–polyacrylamide electrophoresis and transferred to a nitrocellulose membranes. The membranes were incubated overnight with the following primary antibodies: mouse monoclonal anti-synaptophysin (1:10,000), rabbit polyclonal anti-PSD95 (1:500), rabbit polyclonal anti-GAP 43 (1:5,000), rabbit polyclonal anti acetylated histone H3 (1:1,000), rabbit polyclonal anti histone H3 (1:1,000), rabbit polyclonal anti BDNF (1:200), mouse monoclonal anti beta actin (1:10,000), and rabbit polyclonal anti ERK or pERK (1:1,000). Reactive bands were visualized by detecting chemiluminescence.

Fig. 2 HDACi treatment in normorxic or post ischemic condition ▶ promotes neurite outgrowth and neuroprotection in dose dependent manner at 7 days. DIV 6 cortical neurons were treated with vehicle, VPA (0.2, 0.5, 1 mM) or TSA (10, 30, 100 nM) for 7 days in normoxic or 90 min OGD plus reoxygenation condition. After treatment, cells were immunostained with MAP2 and stained with tunnel. **a** Representative images showing MAP2 immunocytochemistry of different groups. *Scale bar* = 50 µm. **b** Graph represents quantification of total neurite length, **c** graph represents quantification of mean neurite density, **d** representative images showing tunnel staining of different groups (tunel positive neurons: *red* and Topro3 nuclear maker: *blue*). *Scale bar* = 50 µm. **e** Graph represents quantification of tunel positive neuron counts. Data are shown as the mean ± SEM of two independent experiments performed in triplicate. * Significantly different compared with control and # or + significantly different compared with OGD plus reoxygenation control ($^{***}p < 0.001$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$). Control: vehicle treated cells; OGD: vehicle treated OGD group

Immunocytochemistry

Cells grown and treated on a cover slide were fixed in cold 4 % paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4 for 30 min at room temperature. After washing twice in PBS, the cells were incubated for 1 h in blocking solution (5 % BSA and 0.3 % Triton X-100 in 0.1 M PBS). The cells were incubated overnight with primary antibody (mouse monoclonal anti-MAP2) diluted in incubation solution (2 % BSA and 0.2 % Triton X-100 in 0.1 M PBS) at 4 °C and washed twice in PBS. The samples were incubated at 24 °C for 1 h with biotinylated secondary antibody in the incubation solution, followed by avidin/biotin/peroxidase staining for 1 h in a humidified chamber. PBS (0.1 M, pH 7.4) containing 0.5 % BSA was used to wash cells on slides between all steps. The antigen–antibody complexes were visualized by incubation for 5 min in 0.05 % 3,3-diaminobenzidine and 0.003 % H_2O_2. Slide mounted sequentially in glass slides and images were captured by inverted light microscope (Carl Zeiss, USA). Data were expressed as the percentage of normoxia control representing neurite length and neurite density.

For fluorescent immunostaining, primary cortical neurons in the 4-well chamber slide were incubated for 1 h at 24 °C in PBS containing 5 % horse serum, and 0.03 % Triton-X100. The slides were incubated with primary mouse anti-MAP2



antibody (1:1,000) in PBS containing 2.5 % horse serum and 0.01 % Triton-X-100 overnight at 4 °C. Specific binding was detected by incubation for 60 min at room temperature with a 1:200 dilution of secondary antibodies conjugated to AlexaFluor 488 dyes. For determination of cell death, cells were stained with the TdT-mediated dUTP-X nick end labeling (TUNEL) reaction mixture (Roche Applied Science, USA) that contains TdT and tetramethylrhodamine red-labeled–dUTP for 60 min at 37 °C in a humidified atmosphere in the dark. Slides were washed with 0.1 M PBS

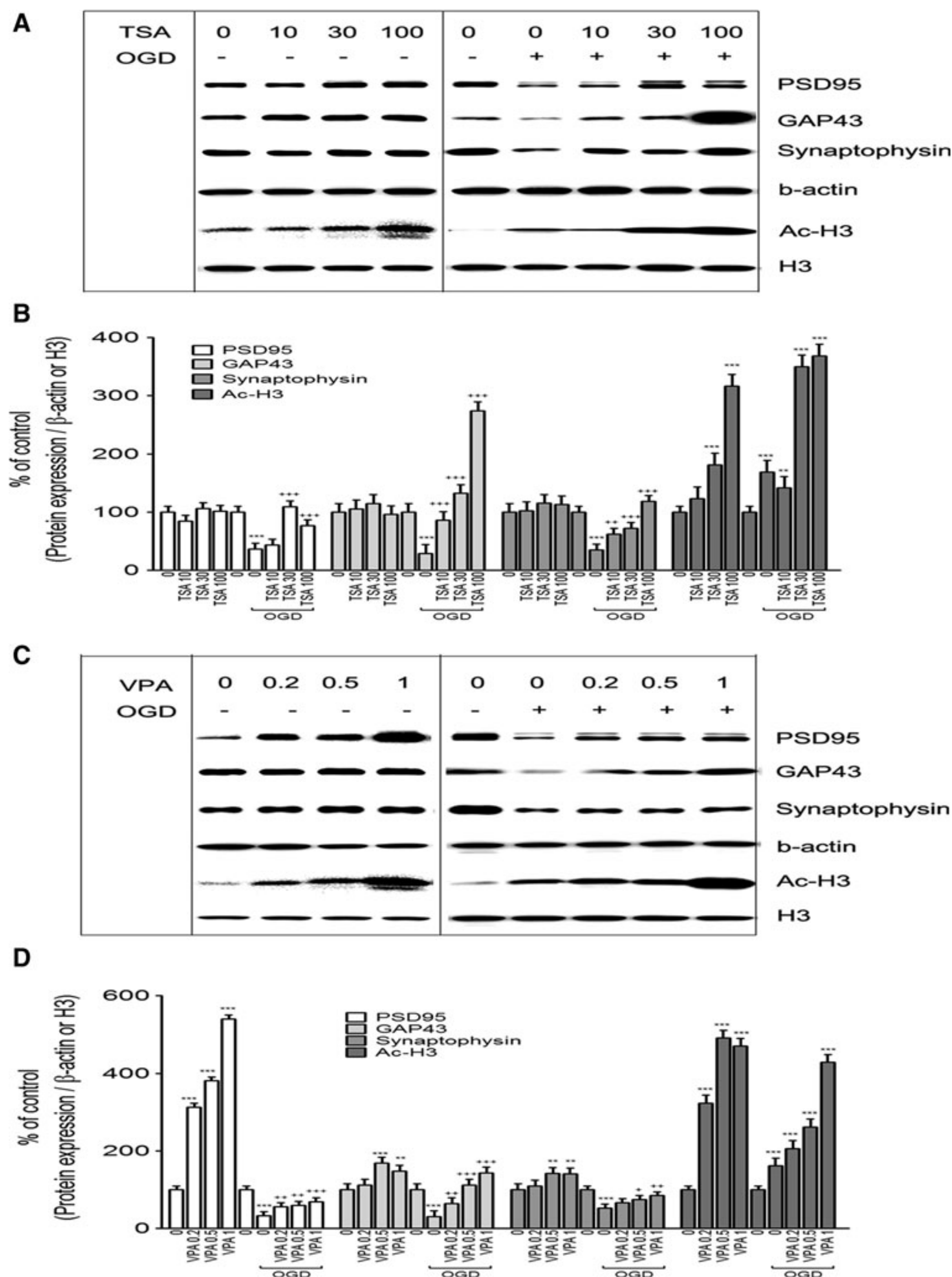


Fig. 3 HDAC inhibitors promote expression of synaptic markers after OGD and acetylation of Histone H3. DIV 6 cortical neurons were treated with vehicle, VPA (0.2, 0.5, 1 mM) or TSA (10, 30, 100 nM) for 7 days in normoxic or 90 min OGD plus reoxygenation condition. After treatment, acetylation of histone H3 and synaptic marker protein expressions were measured by Western blot as described. **a, c** Representative Western blots against PSD95, GAP 43, synaptophysin, and acetylated hisone H3 (Ac-H3). **b, d** Graphs represent quantification of PSD95, GAP 43, synaptophysin, and Ac-H3 band intensity normalized against β-actin or Histone H3(H3). Data are shown as the mean ± SEM of four independent experiments performed in triplicate. * Significantly different compared with control and $^{+}$ significantly different compared with OGD plus reoxygenation control (** $p < 0.01$, *** $p < 0.001$, $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$). Control: vehicle treated cells; OGD: vehicle treated OGD group

and then stained with ToPro3 (Invitrogen, USA) and mounted sequentially in glass slides using Vectashield (Vector Labs, USA). Mounted slices were evaluated for fluorescence under settings for 546 and 647 nm fluorescence emissions on a confocal microscope (Carl Zeiss, USA). 5 or more randomly captured images were analyzed for each group. Cells were counted in $255 \times 345\ \mu m^2$ area—the dimension of the captured images at 40× magnification. Data were expressed as the percentage of cell counts of TUNEL-positive cells/ToPro3-positive total cells representing cell death.

Capturing Images and Analysis

Images were captured by inverted light microscope or confocal microscopy and analyzed with Axio Vision software using a CCD camera attached to an inverted light microscope and a 40× objective (Carl Zeiss, USA). Mean density of the neurites and total fiber length were quantified by an observer blind to the grouping using an automated program wizard from 'measurement' plug-in of Axio Vision software. Objects of interest (neurites) were selected by performing the segmentation command. Artifacts

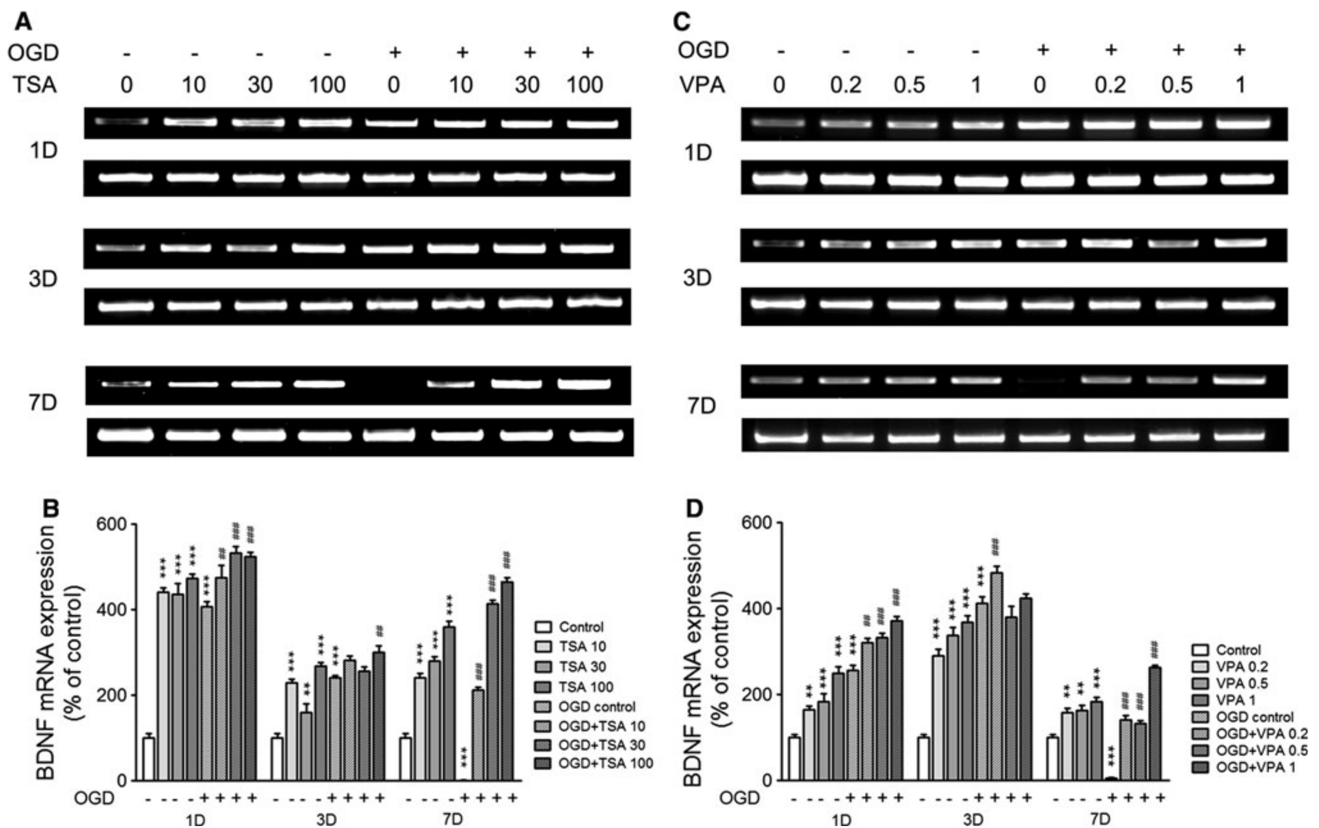


Fig. 4 HDAC inhibitors promote expression of BDNF mRNA in cortical neurons DIV 6 cortical neurons were treated with vehicle, VPA (0.2, 0.5, 1 mM) or TSA (10, 30, 100 nM) for 7 days in normoxic or 90 min OGD plus reoxygenation condition. **a, c** After treatment, cellular BDNF mRNA level was measured by RT-PCR procedures and GAPDH was used as a loading control. **b, d** Graph represent quantification of quantified analysis normalized against GAPDH. Data are shown as the mean ± SEM of two independent experiments performed in duplicate. * Significantly different as compared with control and # significantly different as compared with OGD plus reoxygenation control ($^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$)

and objects of non-interest (cell bodies) were manually deleted from the selected group of objects. The overall neurite density (μm^2) and total fiber length (μm) were measured from the total area of a captured image using the 'density' and 'fiber length' tools. 5 or more randomly captured images were analyzed for each group.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNAs were prepared from 1 μg total RNA with Revert Aid First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instructions. Oligonucleotide primers were designed based on Genebank entries for rat BDNF (accession number EF125679.1, sense, 5′-ATAGGAGACCCTCCGCAACT-3′; antisense, 5′-CTGCCATGCATGAAACACTT-3′) and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH, accession number M17701sense, 5′-ATCACCATCTTCCAGGAGCG-3′; antisense, 5′-GATGGCATGGACTGTGGTCA-3′).

PCR mixes contained 10 μl of 2 × PCR buffer, 1.25 mM of each dNTP, 100 pmol of each forward and reverse primer, and 2.5 U of Taq polymerase in a final volume of 20 μl. Amplification was performed in 35 cycles at 60 °C, 30 s; 72 °C, 1 min; 94 °C, 30 s. After the last cycle, all samples were incubated for an additional 10 min at 72 °C for the final extension step. PCR fragments were analyzed on a 1.2 % agarose gel in 0.5× TBE containing ethidium bromide and their amounts were normalized against GAPDH amplified, in parallel. The primer set specifically recognized only the gene of interest as indicated by amplification of a single band of the expected size.

ELISA Assay

The amount of released BDNF was quantified from medium of treated neuron by using the Emax ImmunoAssay system (Promega, Madison, WI, USA). BDNF enzyme-

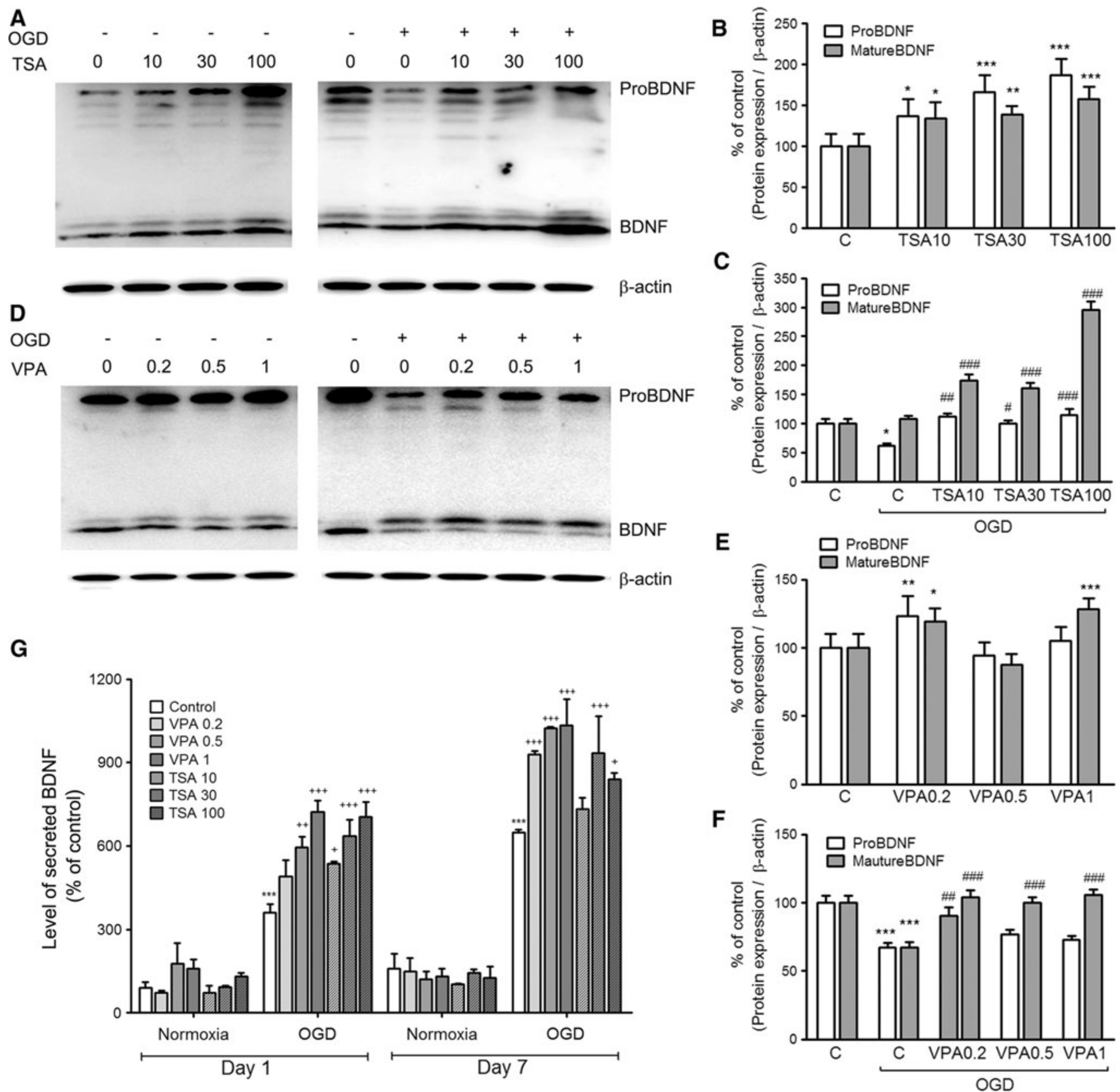


Fig. 5 HDAC inhibitors promote BDNF protein expression and the levels of released BDNF in cortical neurons. DIV 6 cortical neurons were treated with vehicle, VPA (0.2, 0.5, 1 mM) or TSA (10, 30, 100 nM) for 1 or 7 days in normoxic or 90 min OGD plus reoxygenation condition. **a, d** After treatment, we measured BDNF protein expression by Western blot as described. **b, c, e, f** Each graph represents quantification of cellular preform of BDNF (proBDNF) and mature BDNF (BDNF) band intensity normalized against β-actin. **g** The level of released BDNF was measured from cell supernatant using ELISA assay kit as described in "Materials and Methods" Data are shown as the mean ± SEM of three independent experiments performed in triplicate. * Significantly different compared with control and # or + significantly different compared with OGD plus reoxygenation control. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$)

linked immunosorbent assay (ELISA) was performed according to the manufacturer's manual. 96 well plates were pre-coated with anti-BDNF monoclonal antibody diluted with carbonate coating buffer (pH 9.7) at 4 °C for 24 h. The plates were blocked by block and sample 1× buffer at RT. After 1 h blockade, the plates were incubated with BDNF standard and culture spent medium sample for 2 h at RT followed by another 2 h incubation with anti-human BDNF polyclonal antibody. Anti-IgY HRP conjugate was incubated for 1 h. The reaction was developed with tetramethylbenzidine developer (TMB One Solution) solution and the absorbance was read at 450 nm

with a spectrophotometric microplate reader (Spectra Max 340 pc; Molecular Devices, Menlo Park, CA) after stopping the reaction with 1 N hydrochloric acid (HCl). All BDNF values are subtracted by the mean value of culture media (neurobasal medium supplemented with B-27, 2 mM glutamine, 100 IU/L penicillin, and 10 μg/mL streptomycin), before result interpretation. Culture media has contained BDNF of 165.00 ± 0.32 pg/ml. Data were expressed as the percentage of control representing secreted BDNF activity.

Lactate Dehydrogenase Assay

Degrees of cell death were assessed by activity of LDH released into the culture medium using the cytotoxic assay kit (Promega Bioscience, San Luis Obispo, CA, USA). Cells were treated with vehicle, VPA (1 mM) or TSA (100 nM) with BDNF neutralizing antibody (10 μg/ml) or IgG control (10 μg/ml) for 7 days in normoxic or 90 min OGD plus reoxygenation condition. Briefly, aliquots (50 μl) of cell culture medium were reacted with an equal volume of LDH substrate solution (Promega, Madison, WI, USA) for 30 min. The reaction was stopped by the addition of 1 M of acetic acid (1/2 volume), and the absorbance at 492 nM was recorded (Spectra Max 340 pc; Molecular Devices, Menlo Park, CA). IgG control-treated cells in normoxia condition used as the control for LDH activity assay. Data were expressed as the percentage of control representing LDH activity.

Statistical Analysis

Statistical analyses were performed by one way ANOVA with Newman–Keuls multiple comparison post hoc test using GraphPad Prism 5 software. Null hypotheses of no difference were rejected if p values were less than .05. Data were expressed as mean ± SEM.

Results

Post Ischemic HDACi Treatment Promotes Neurite Outgrowth and Neuroprotection

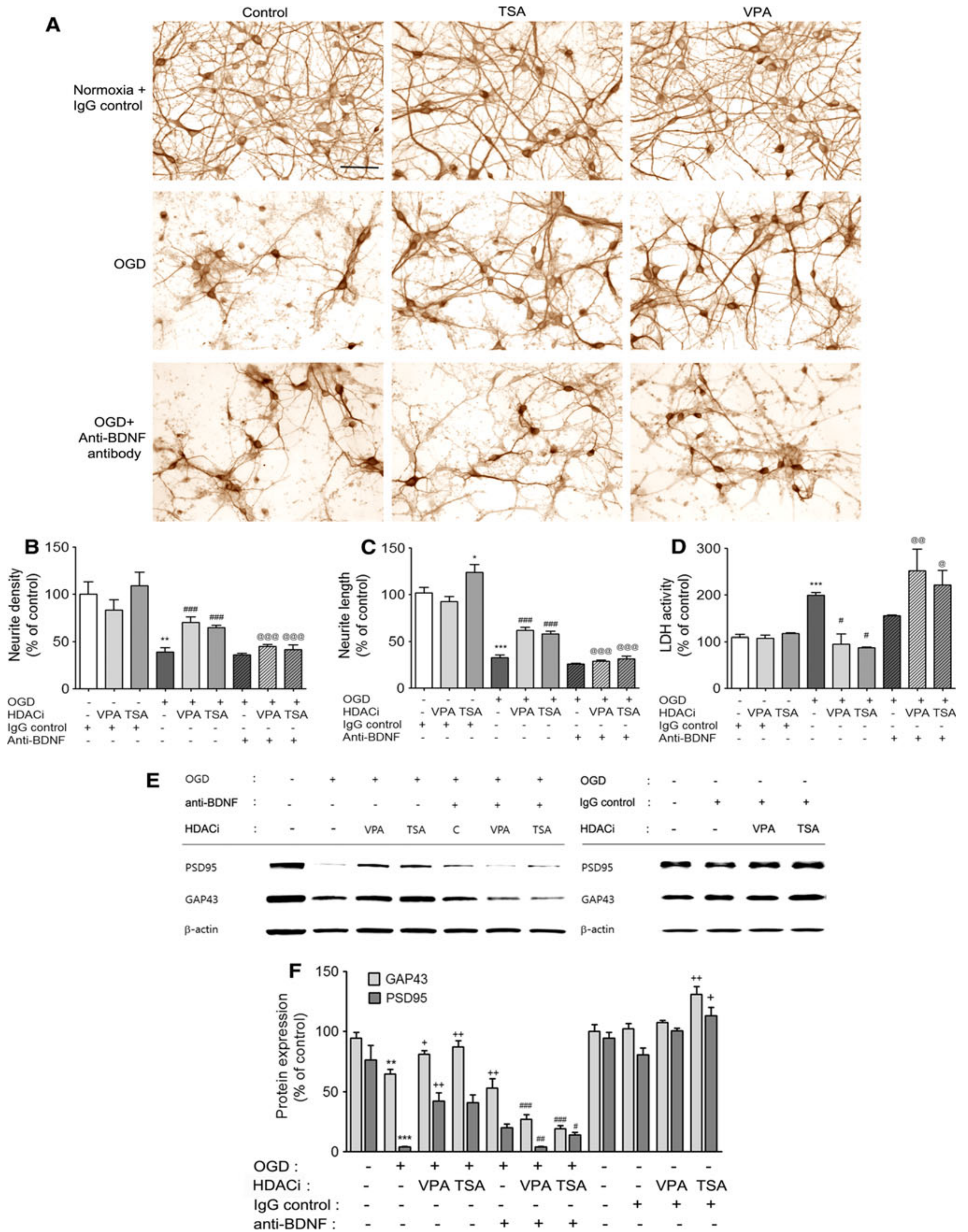
We tested the effect of neurite outgrowth and neuroprotection of VPA and TSA in vitro model of ischemic cell death. OGD followed by reoxygenation caused cell death of primary rat cortical neurons. Cells were exposed to OGD for 90 min on 6 DIV and then neurons were treated with VPA (0.5 mM) and TSA (30 nM) for various durations (1, 3, 7 days) in normal oxygen conditions. As shown in Fig. 1, neurons exposed to OGD showed a significantly decrease in neurite outgrowth (neurite length and neurite density), confirmed by images captured following MAP2 immunocytochemistry, at 1, 3, 7 days after OGD plus reoxygenation. In the presence of VPA or TSA for 3 or 7 days after OGD plus reoxygenation, on the other hand, the decrease in neurite outgrowth significantly recovered (Fig. 1a–c). Neurite outgrowth of neurons in normoxic condition was also enhanced in the presence of VPA and TSA for 7 days. We found that HDACi treatment provided post-ischemic neuroprotection (Fig. 2). Tunel stained cell number in OGD plus reoxygenation condition was increased at post-OGD 1, 3, or 7 days ($p < 0.001$). In the presence of VPA (0.5 mM) or TSA (30 nM), conversely, the increase in apoptotic cell death was significantly inhibited (Fig. 1d, e). Next, neurons were treated with different concentrations of VPA (0.2, 0.5, or 1 mM) and TSA (10, 30, or 100 nM) for 7 days in normoxic and OGD puls reoxygenation condition.

We found that OGD exposed cells treated with HDACi significantly promoted neurite outgrowth and arborization when compared to non-treated groups, confirmed by images captured following MAP2 immunocytochemistry (Fig. 2). Significant differences in the mean neurite density (μm^2) and total neurite length (μm) were observed for the HDACi treated groups. On DIV 6, the neuronal network was destroyed after 90 min of OGD treatment. Our findings show that on DIV 13, the neuronal network was destroyed by up to 35.42 ± 7.36 % (μm^2) relative to the control after OGD plus reoxygenation without any treatment (Fig. 2a, b, c). VPA and TSA treatment significantly increased the total fiber length (Fig. 2a, b) and mean neurite density (Fig. 2a, c) in a dose-dependent manner. Neurite outgrowth of neurons in normoxic condition was also improved in the presence of various doses VPA and TSA for 7 days.

Increased tunel stained cell numbers of total cells were observed in OGD plus reoxygenation without HDACi treatment when compared with the same aged control cells (DIV 13). In the presence of VPA or TSA, on the other hand, the increase in apoptotic cell death was significantly inhibited in a dose dependent manner (Fig. 2d, e).

VPA and TSA Promotes Acetylation of Histone H3

Treatment with HDACi for 1 week after 90 min OGD treatment significantly increased acetylation of Histone H3 by VPA and TSA (Fig. 3). We found that VPA (0.2, 0.5, and 1 mM) and TSA (10, 30 and 100 nM) significantly increased the acetylated status of Histone H3 proteins when compared to control cells or those exposed to OGD without HDACi treatment (Fig. 3).



A
Control
TSA
VPA
Normoxia + IgG control
OGD
OGD+ Anti-BDNF antibody
B
Neurite density (% of control)
C
Neurite length (% of control)
D
LDH activity (% of control)
OGD
HDACi
IgG control
Anti-BDNF
VPA TSA
E
OGD
anti-BDNF
HDACi
IgG control
PSD95
GAP43
β-actin
F
GAP43
PSD95
Protein expression (% of control)
OGD :
HDACi :
IgG control :
anti-BDNF :

◀**Fig. 6** Reductions of neurite outgrowth and synaptic marker expressions induced by HDACi after treatment of BDNF neutralizing antibody in ischemic condition DIV 6 cortical neurons were treated with vehicle, VPA (1 mM) or TSA (100 nM) with BDNF neutralizing antibody (10 μg/ml) or IgG control (10 μg/ml) for 7 days in normoxic or 90 min OGD plus reoxygenation condition. **a** After treatment, cells were immunostained with MAP2 antibody. Images showing MAP2 immunocytochemistry of different groups (n = 5), *Scale bar* = 50 μm. **b** Graph represents quantification of mean neurite density, **c** graph represents quantification of total neurite length, **d** cytotoxicity was measured by LDH assay as indicated in ''Materials and Methods''. Data are shown as the mean ± SEM of two independent experiments performed in triplicate. *Significantly different compared with control, # or + significantly different compared with OGD plus reoxygenation control, and @ significantly different compared with VPA or TSA in OGD plus reoxygenation condition (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.05$, ### $p < 0.001$, @ $p < 0.05$, @@ $p < 0.01$, @@@ $p < 0.001$). **e** Synaptic marker protein expressions were measured by Western blot as described. Representative Western blots against PSD95 and GAP 43. **f** Graph represents quantification of PSD95 and GAP 43 band intensity normalized against β-actin. Data are shown as the mean ± SEM of two independent experiments performed in triplicate. * Significantly different compared with control, + significantly different compared with OGD plus reoxygenation control, and # significantly different compared with VPA or TSA with BDNF neutralizing antibody in OGD plus reoxygenation condition (** $p < 0.01$, *** $p < 0.001$, + $p < 0.05$, ++ $p < 0.001$, ## $p < 0.01$, ### $p < 0.001$). Control: vehicle treated cells; OGD: vehicle treated OGD group

HDAC Inhibitors Promote Expression of Synaptic Markers After OGD Plus Reoxygenation

To determine a change in the synaptic marker expression by HDACi-induced histone H3 acetylation, cortical neurons were treated with or without VPA (0.2, 0.5, and 1 mM) and TSA (10, 30 and 100 nM) for 1 week after 90 min of OGD pretreatment. Figure 3 shows that three synaptic markers (PSD95, GAP 43, and synaptophysin) were markedly reduced after OGD plus reoxygenation without any treatment, while expression of these markers was remarkably recovered by VPA and TSA treatment in a dose dependent manner. Figure 3 also showed a dose dependent increase in PSD95, GAP 43, and synaptophysin expression by VPA treatment relative to non-treated cells ($p < 0.001$) in normoxic condition.

HDAC Inhibitors Promote Expression of BDNF After OGD Plus Reoxygenation

We found that post-OGD HDACi treatment increased in the expression of BDNF (Brain derived neurotrophic factor). RT-PCR was used to determine the BDNF mRNA level. In this analysis, HDACi treatment for 1, 3, or 7 days increased expression of BDNF mRNA in normoxic condition. Fig. 4 shows that ischemic insult increases expression of BDNF mRNA at 1 and 3 days. Post-OGD HDACi treatment significantly ($p < 0.01$) enhanced BDNF mRNA expression. OGD plus reoxygenation for 7 days causes decreased expression of BDNF mRNA, but treatment with TSA and VPA significantly ($p < 0.001$) up-regulated BDNF mRNA expression (Fig. 4). Western blot analysis was used to determine if the BDNF protein level was similar to the mRNA expression level. We confirm that OGD plus reoxygenation for 7 days causes decreased expression of preform of BDNF (proBDNF) and mature BDNF (BDNF), but treatment with TSA and VPA significantly ($p < 0.01$) up-regulated ProBDNF and mature BDNF expressions (Fig. 5a–e). TSA and VPA treatment also increased expression of proBDNF and mature BDNF in normoxic condition (Fig. 5a, b, d, e).

HDAC Inhibitors Promote Neurite Outgrowth and Neuroprotection Through Secreted BDNF

In addition, post-OGD HDACi treatment increased the level of secreted BDNF in culture supernatant fraction, which was determined by BDNF ELISA (Fig. 5g). To examine the importance of HDACi-induced BDNF expression in neurite outgrowth, we blocked the function of BDNF using neutralizing antibody. IgG as a control against BDNF antibody was treated in the presence or absence of HDCAi in normoxic condition. Interestingly, the effects of VPA (1 mM) and TSA (100 nM) on neurite outgrowth in rat primary cortical neurons were abolished when we treated the cultured rat primary cortical neuron with BDNF neutralizing antibody (Fig. 6a–c). BDNF neutralization diminished the neurite density as well as the neurite length suggesting that the BDNF expression plays a critical role as a mediator in neurite outgrowth induced by HDAC inhibitor. BDNF neutralization also significantly reversed cell death reduction effect of HDACi treatment after OGD (Fig. 6d). In addition, BDNF neutralization considerably reduced the level of PSD95 and GAP 43 expression in HDAC inhibitor-treated cells after OGD (Fig. 6e, f). These results indicated that secreted BDNF by HDACi is related to the expressions of PSD95 and GAP 43 and neuroprotection after OGD plus reoxygenation.

HDACi Treatment Does not Alter Phosphorylation of ERK 1/2 on OGD Plus Reoxygenation Condition

To investigate the role of HDACi on ERK signaling pathways, we examined the phosphorylation status of ERK1/2 after treatment with HDACi. VPA increased the phosphorylation of ERK1/2 in rat primary cortical neuron of normoxic condition at 7 days after treatment (Fig. 7a, $p < 0.001$). On the other hand, TSA treated cells the same as control. While OGD plus reoxygenation for 7 days significantly increased the phosphorylation of ERK1/2

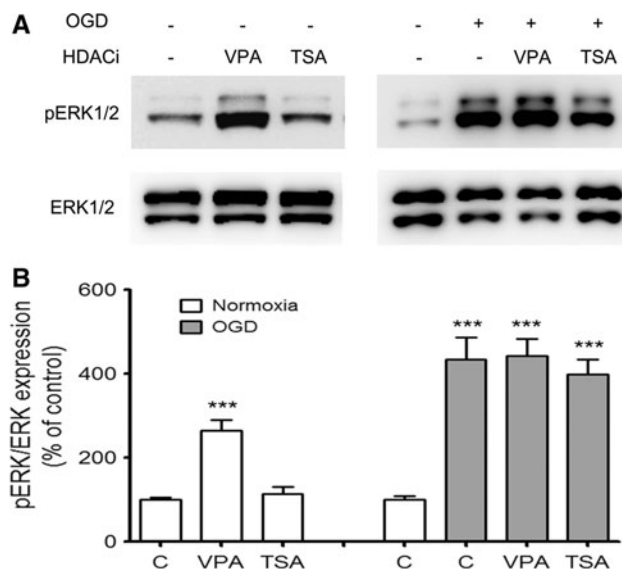


Fig. 7 HDACi treatment does not alter phosphorylation of ERK 1/2 on OGD plus reoxygenation condition DIV 6 cortical neurons were treated with vehicle, VPA (1 mM) or TSA (100 nM) for 7 days in normoxic or 90 min OGD plus reoxygenation condition. **a** After treatment, cells were analyzed by Western blot against phospho and total ERK1/2. **b** Graph represents quantification ratio of phospho and total ERK1/2 band density. Data are shown as the mean ± SEM of three independent experiments performed in triplicate. * Significantly different compared with control (***$p < 0.001$). C: vehicle treated control; OGD: vehicle treated OGD

($p < 0.001$), phosphorylation status of ERK 1/2 did not altered by HDACi treatment in OGD plus reoxygenation condition (Fig. 7).

Discussion

In this study, we examined the effect of HDACi treatment on the acetylation status of histone H3 after prolonged OGD exposure. We found that both VPA and TSA treatment significantly increased the level of acetylated Histone H3, and HDACi treatment significantly restored neurite outgrowth through upregulation of BDNF expression.

Valproic acid is currently in clinical trials as a monotherapy or in combination with other anticancer compounds for the treatment of solid and haematopoietic malignancies [21–23]. Furthermore, HDACi are emerging as a novel class of potentially therapeutic agents for treating acute injuries of the central nervous system [24]. A growing body of evidence indicates beneficial effects of these agents in both stroke and trauma [24–26].

Ischemia causes significant changes in the expression patterns of several proteins and ischemia itself can stimulate dendritic sprouting and axonal arborization [27]. Within minutes of the ischemic insult, dendritic spines at excitatory synapses are lost. Ischemic cortical injury promotes the expression of growth factors in the peri-infarct zones and behavioral recovery occurs with increased dendritic branching and synaptogenesis peaking 2–4 weeks after a stroke [28]. In addition, axonal sprouting significantly increases several months after focal ischemia [29].

However, the effect of HDACi on the outgrowth of neurites after severe disruption by prolonged OGD was not previously examined. In this study, we examined neurite outgrowth 1 week after prolonged hypoxic exposure. We found that HDACi treatment significantly helped restore neurite arborization when compared with the non-treated group. This demonstrated that HDACi treatment far exceeded the hypoxic stimulation effect. These findings are supported by the increased expression of GAP 43, a protein expressed by growing axons, in the HDACi treated groups.

VPA and TSA display neuroprotective effects [7, 18, 19]. However, the long term neuroprotective effects of post-ischemic HDACi treatment have not yet been systematically examined. Our study shows that HDACi was efficient to save dying cells during reoxygenation even after 1 week of prolonged hypoxia. Thus, post ischemic HDACi treatment may provide neuroprotection as well as neuronal regeneration.

In this study, PSD95, a postsynaptic marker, synaptophysin, a presynaptic marker and GAP 43, another presynaptic marker and important marker of neurons with growing axons, were used. Our study shows that following prolonged hypoxia, 1 week treatment with HDACi significantly restored all three of these synaptogenetic markers.

Although HDACi such as VPA, sodium butyrate, or TSA exhibit anti-inflammatory and neurogenesis effects via multiple mechanisms of action in a rat permanent ischemic model of stroke [30], it has not been demonstrated effect of HDACi on BDNF expression after stroke. BDNF regulates synaptogenesis and neurite outgrowth. We examined the expression of BDNF at both the protein level and mRNA level. Our western blot results show that ischemia caused an early increase in the expression of BDNF. We also found that treatment with 1 mM VPA and 100 nM TSA further upregulated BDNF expression when compared with vehicle treatment after OGD. This is a novel finding of this study.

Some studies have reported that VPA was shown to increase glial cell derived neurotrophic factor and BDNF in rat C6 glioma cells [31] as well as in astrocytes and thereby protects dopaminergic neurons in the midbrain [32]. Chronic administration of VPA increases BDNF levels in the rat frontal cortex [33].

Recent reports have demonstrated that post-insult treatment with HDACi robustly reduced infarct volume, cell death, neuroinflammation, and improved neurological performance in rats subjected to middle cerebral artery

occlusion (MCAO) [30, 34] In addition to, decreased levels of both high and low molecular weight forms of BDNF by permanent MCAO in the ischemic hemisphere were blocked by HDAC inhibitor treatment [30].

Based on the combined findings of this study, it may be postulated that post ischemic HDACi treatment increases promoting post-ischemic neuroprotection, synaptogenesis and neurite outgrowth via mechanism of ERK independent up-regulation of BDNF expression in ischemia exposed cortical neurons.

There are limitations of the present study. In vitro models were used to study HDACi effects after ischemia in this study. We are still not aware of translational studies assessing HDACi effects on BDNF expression after stroke. Thus, the in vivo significance of BDNF upregulation as well as unraveling particular mechanisms contributing to functional benefit by HDACi should be further investigated.

Acknowledgments This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010–0025382 grant to DHC and 2012R1A1A4A010137 grant to JL).

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