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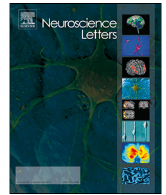

Research article

Enhancing the expression of neurotrophic factors in epidermal neural crest stem cells by valproic acid: A potential candidate for combinatorial treatment

Sareh Pandamooz[a,1], Mohammad Saied Salehi[a,b,1], Anahid Safari[c], Negar Azarpira[d], Mansooreh Heravi[a], Abolhassan Ahmadiani[a], Leila Dargahi[e,*]

[a] *Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[b] *Clinical Neurology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[c] *Stem Cells Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[d] *Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[e] *NeuroBiology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, 19615-1178, Velenjak, Iran*

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ABSTRACT

Effective delivery of trophic factors to cure neurological disorders and traumatic injuries is a major challenge. With promising therapeutic effects of epidermal neural crest stem cells (EPI-NCSCs) in preclinical spinal cord injury, there is an implication that these stem cells might provide supportive role through releasing various trophic agents. Hence, the present study was designed to assess the influence of valproic acid (VPA), a well-known histone deacetylases inhibitor, on mRNA expression of selected trophic factors. In this study, following stem cell migration from explanted hair bulges, immunostaining against Nestin, SOX-10, DCX, β-III tubulin and GFAP was carried out. Then, cells were treated with various clinically relevant concentrations of VPA and the survival rate was defined by MTT assay. Finally, stem cells were treated with 0.1 and 1 mM VPA and the drug impact on the transcription level of BDNF, GDNF, VEGF, NGF and NT3 at 6, 24, 72, 168 h was assessed by quantitative real-time PCR. The examined proteins expressions in the population of migrated cells confirmed the identity of stem cells as EPI-NCSCs. In addition, MTT assay showed that all three tested concentrations of VPA were suitable to treat these cells. Trophic factors assessment, following treatment revealed the mRNA expression level of BDNF, GDNF and VEGF could be significantly up- regulated at various time points, mainly by 1 mM VPA. However, NGF and NT3 transcripts were enhanced at few limited time points. Our findings showed that EPI-NCSCs due to secretion of various trophic factors are potential candidate to deliver the required trophic agents and their potential can be enhanced by 1 mM VPA, predominantly following 168 h treatment. Hence, these cells can be utilized to modulate destructive context of neurological disorders and injuries.

1. Introduction

In the past two decades we have witnessed a progress in the application of neurotrophic factors to treat neurological diseases and traumatic injuries [2,11,31,36]. Since neurotrophins have various functions in the central and peripheral nervous system, including neuronal differentiation and growth, synapse formation and plasticity, many researches have been dedicated to enhance the level of these neurotrophins in different neurological disorders and injuries [24]. If neurotrophins administered peripherally, most of them cannot pass the blood brain barrier (BBB) effectively, also their local injection or overexpression via gene therapy is challenging [15,32]; hence, the transplanted stem cells have been considered as potential delivery vehicles [17]. Amongst various types of stem cells that were investigated, epidermal neural crest stem cells (EPI-NCSC) exhibit several characteristics that make them suitable for nervous system cell-based therapies. Recently, EPI-NCSCs transplantation was proposed as a promising therapeutic approach that can replace the lost cells and/or release neurotrophic factors following spinal cord injury (SCI) [12]. These adult multipotent stem cells that persist in the bulge of skin hair follicle are readily accessible, and can be isolated via a minimal invasive procedure [28,29]. Regarding the ontological relationship of

* Corresponding author.
E-mail address: l.dargahi@sbmu.ac.ir (L. Dargahi).
[1] These authors contributed equally to this work.

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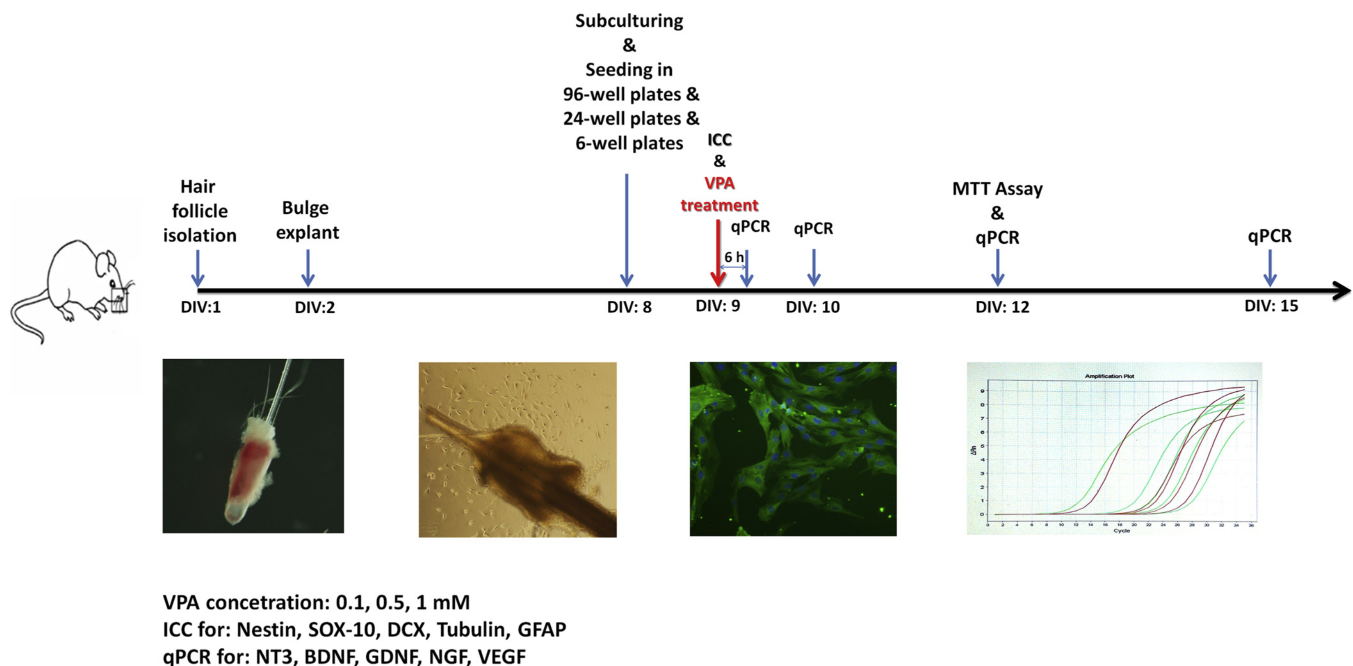

Fig. 1. The schematic protocol of study. In this investigation, EPI-NCSCs were isolated from the bulge area of hair follicle of adult rat's whiskers pad. Next, they were treated with various concentrations of VPA to find their impacts on transcription level of selected trophic factors at different time points. DIV: day *in vitro*.

Table 1
List of primers.

Gene	Forward primer (5´-3´)	Reverse primer (5´–3´)	Amplicon length
Bdnf	CGATTAGGTGGCTTCATAGGAGAC	CAGAACAGAACAGAACAGAACAGG	182
Gdnf	GCTGACCAGTGACTCCAATATGC	CCTCTGCGACCTTTCCCTCTG	192
Vegf	ACTTGAGTTGGGAGGAGGATGTC	GGATGGGTTTGTCGTGTTTCTGG	183
Ngf	CCCAATAAAGGCTTTGCCAAGGAC	GAACAACATGGACATTACGCTATGC	78
Nt3	GACACAGAACTACTACGGCAACAG	ACTCTCCTCGGTGACTCTTATGC	184
β-Actin	TCTATCCTGGCCTCACTGTC	AACGCAGCTCAGTAACAGTCC	122

these cells with spinal cord stem cells and their potential to express both neuronal and glial markers, their function was evaluated in various context of SCI [10,23]. Though much of the initial application of cell transplantation strategies was considered to be replacement of degenerating neurons, recent studies showed that the recovery following stem cell transplantation is mainly due to modulation of neurotrophins [18,33]. Therefore, the ongoing investigations have shifted toward the enhancement of neurotrophins in grafted stem cells to achieve an optimum recovery [8,31]. It has been established that EPI-NCSC can produce various neurotrophic factors that can prevent cell death and maintain cellular function [12]. According to several experiments, neurotrophins transcription can be regulated by drugs that can inhibit histone deacetylases (HDAC) [34]. Valproic acid (VPA) is a short-chain fatty acid used in treating epilepsy and bipolar disorder. Due to its neuroprotective and neurotrophic effects via inhibiting HDAC, it has attracted much attention and has been assessed in different neurological conditions [7]. In several investigations it was claimed that VPA functions through up-regulation of several trophic/growth factors in both *in vitro* and *in vivo* contexts [19,26].

We previously reported that the combined therapy of an *ex vivo* model of SCI with VPA and EPI-NCSC ameliorated the injury [23]; however, the direct impact of this drug on these stem cells is still unclear. Therefore, this study was designed to examine the effects of VPA on various trophic transcription factors that are already expressed in EPI-NCSC, in order to enhance their therapeutic effects.

2. Materials and methods

In this study, adult male Wistar albino rats were used. All animal experiments were performed in accordance with the Ethical Committee for the use and care of laboratory animals of Neuroscience Research Center, Shahid Beheshti University of Medical Sciences (IR.SBMU.PHNS.REC.1397.027) compliance with the standards of the European Communities Council directive (86/609/EEC).

2.1. Cell culture

The EPI-NCSCs were obtained from the hair bulge of adult rat whiskers pad as described earlier [22,29]. Here, the isolated hair bulges were explanted on the collagen-coated 4-well cell culture plate and fed with alpha-modified minimum essential medium (α-MEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Gibco), 5% day-11 chick embryo extract, and 1% penicillin/streptomycin (P/S) and cultured at 37 °C with 5% CO_2. Following EPI-NCSCs migration and reaching the confluent density, cells were detached using 0.25% Trypsin/EDTA (Bio-idea, Iran) and further evaluations were performed as illustrated in Fig. 1.

2.2. Immunofluorescent staining

The cultured EPI-NCSCs were fixed with 4% paraformaldehyde for 12 min at room temperature. After three washes with TPBS (0.05% Tween-20 in PBS), cells were treated with 0.2% Triton X-100 for 10 min followed by sequential incubation with blocking solution (1% BSA in

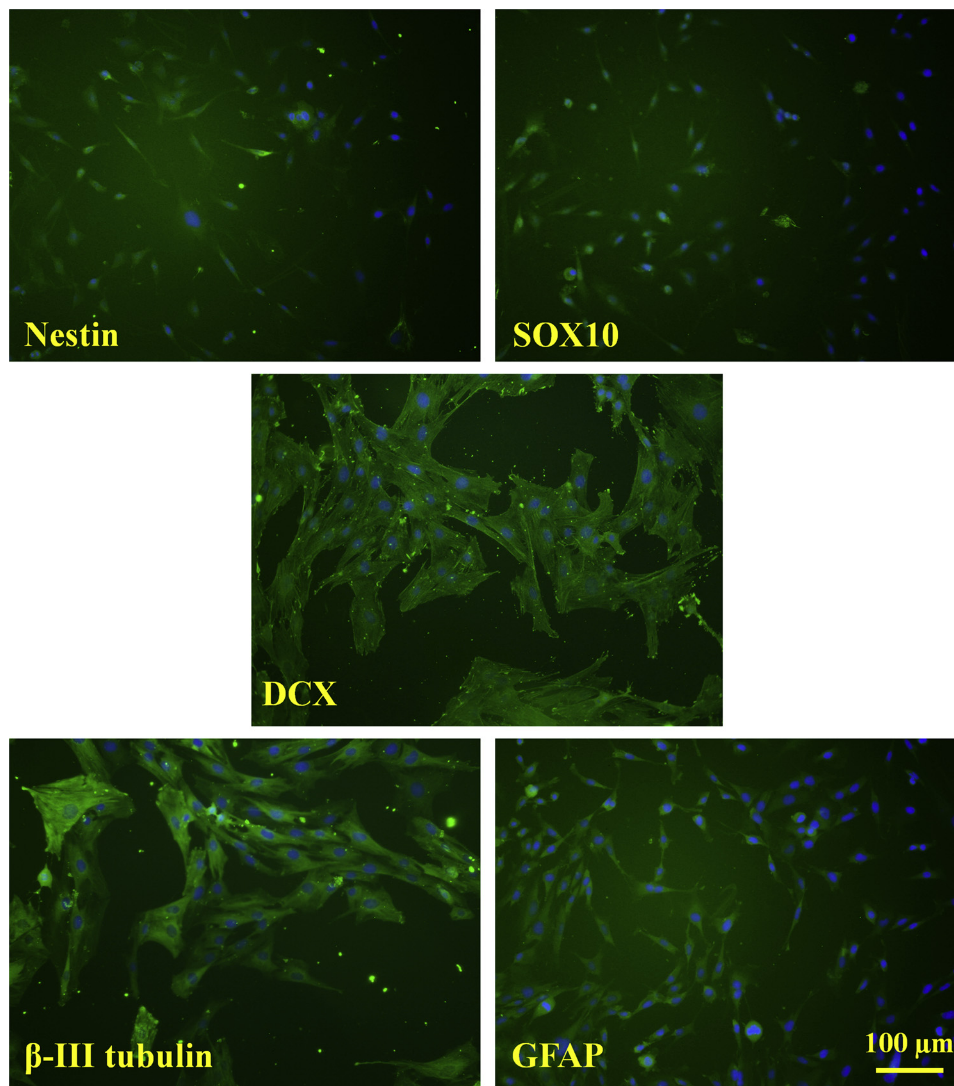

Fig. 2. The characterization of migrated stem cells in primary culture. Indirect immunofluorescent staining of cells upon their migration from the bulge area of hair follicle revealed the expression of Nestin, SOX-10, DCX, β-III tubulin and GFAP which confirmed the characteristic of these cells as EPI-NCSCs. Cell nuclei were counterstained with DAPI. Images are examples of three performed assessments for each immunostaining (n = 3).

0.2% Triton X-100, 30 min) and primary antibody (at 4 °C, overnight). Primary antibodies were used as follow: rabbit anti-Nestin (Abcam, Cat No: ab93157, 1:200), SOX-10 (Abcam, Cat No: ab155279, 1:250), rabbit anti-Doublecortin (DCX) (Abcam, Cat No: ab77450, 1:200), rabbit anti- β-III tubulin (Abcam, Cat No: ab18207, 1:200), rabbit anti- and rabbit anti-GFAP (Abcam, Cat No: ab7260, 1:1000). After three washes, cells were incubated with FITC conjugated secondary antibody (Sigma-Aldrich, Cat No: F1262, 1:100) and the cells' nuclei were counterstained with DAPI (Sigma-Aldrich, Cat No: D9564). Images were captured with the Olympus invert florescence microscope.

2.3. Cell viability assay

To define the suitable concentration of VPA to treat EPI-NCSC, the MTT assay was carried out based on the previously introduced doses of VPA, which were employed to treat other types of stem cells [1,3,6,34]. Here, 24 h ahead of drug treatment, stem cells were seeded in 96-well plate at density of $5{\times}10^3$ per each well. Next day, plates medium was replaced with fresh culture medium containing 0.1, 0.5 and 1 mM VPA (prepared in complete medium), purchased from Darou Pakhsh Pharma. Chem. Co., Tehran, Iran. Cells were incubated for 72 h at 37 °C in the Co_2 incubator. On the third day, the medium was removed and 0.5 mg/ml MTT (Sigma- Aldrich, Cat No: M5655) prepared in α-MEM was added to each well. Following 4 h of incubation, the MTT solution was discarded and acidic isopropanol (0.01 N HCl in absolute isopropanol, 100 μl/well) added to dissolve the blue formazan crystals. Finally, the developed color was measured at 570 nm using microplate reader (BioTek, USA).

2.4. Stem cell treatment with proper dose of VPA

To assess the effect of VPA on mRNA expression of the selected trophic factors in EPI-NCSCs, these cells were treated with 0.1 or 1.0 mM VPA. The day before treatment, cells were seeded at density of 6.6×10^5 and 3.3×10^5 cells/cm^2 in two 6-well plates for 6 and 24 h, and 5.3×10^4/cm^2 and 1.3×10^4/cm^2 cells in two different 6-well plates for 72 and 168 h, respectively. Also each time point has its own corresponding control group, which were incubated with α-MEM containing 10% FBS plus 1% P/S in the same condition.

2.5. Quantitative RT-PCR

The total RNA was extracted from EPI-NCSC culture with YTzol, as described by supplier (Yekta Tajhiz Azma, Iran). After DNase I

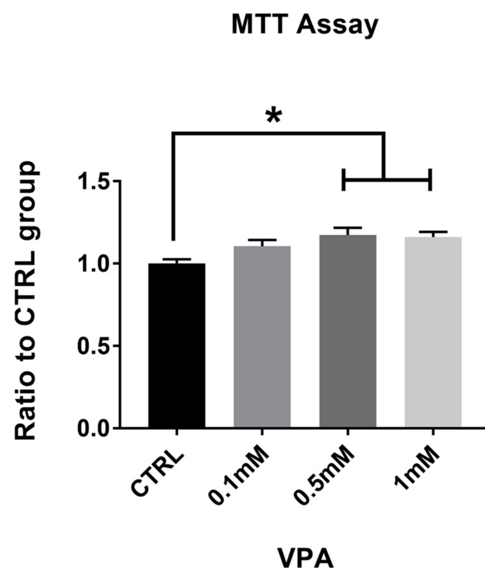

Fig. 3. The effect of VPA on the survival and proliferation of EPI-NCSC. Performing MTT assay 72 h following the EPI-NCSCs treatment with various clinically relevant concentrations of VPA indicated that 0.5 and 1 mM concentrations of VPA significantly increased the proliferation of these stem cells compared to non-treated control group while 0.1 mM did not increase significantly. The data are expressed as mean ± SEM and their analysis was performed using one-way ANOVA (n = 3).

treatment (Thermo Fisher Scientific), the optical density of samples was defined using spectrophotometer (Thermo Fisher Scientific) and cDNA was synthesized from 1 μg total RNA using reverse transcriptase kit (Yekta Tajhiz Azma, Iran) and oligo (dT) primers. For real-time PCR, analysis was performed in triplicate, using ABI StepOne Real-Time PCR system (Applied Biosystems, USA) with RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) and primers listed in Table 1. The reaction cycle consisted of 95 °C for 15 min, followed by 30 cycles of 95 °C for 15 s, and 60 °C for 1 min. Gene expression analysis was performed using relative cycle threshold. In this experiment the β-actin was used as the control for normalization. In addition, the impacts of VPA on the neurotrophic factors transcripts are shown as the percentage of the non-treated control groups.

2.6. Statistical analysis

Data on the relative expression of target genes were subjected to the test of normality. The statistical analysis was performed on GraphPad Prism (Version 7.03, GraphPad Software, Inc., San Diego, CA) using two-way ANOVA and Tukey post hoc test. $P < 0.05$ was considered to be statistically significant. The data are presented as means ± S.E.M.

3. Results

3.1. The characteristic of migrated stem cells was confirmed as an EPI-NCSCs

In this experiment, 2–3 days after explantation, the migrated stem cells were detected around the bulges. Here, immunostaining of *in vitro* expanded stem cells against specific markers of EPI-NCSC, showed the expression of nestin (a marker of neural crest stem cell), SOX-10 (a neural crest stem cell marker), doublecortin (DCX, a neuronal precursor marker), β-III tubulin (immature neurons marker) and GFAP (traditional astrocyte marker) in their population. This finding verified the identity of migrated stem cells as an EPI-NCSC (Fig. 2).

3.2. VPA can enhance survival rate and EPI-NCSC proliferation

Although previous studies had assessed the impact of VPA on different cell types, in the current investigation, the effect of three concentrations of VPA were evaluated on EPI-NCSCs via MTT assay to confirm the safety of VPA and its suitable treatment dose. The results showed that 0.5 and 1 mM doses of VPA can significantly increase the survival rate of EPI-NCSC in comparison with the non-treated group (CTRL group) while 0.1 mM VPA did not show significant effects. Accordingly, both low and high doses of VPA were selected for further evaluation of their impacts on the mRNA expression of various trophic factors (Fig. 3).

3.3. VPA up-regulates the mRNA expression of BDNF, GDNF and VEGF in EPI-NCSC

The quantitative real-time PCR analysis showed that treating EPI-NCSC with 1 mM VPA could significantly increase the mRNA expression level of brain-derived neurotrophic factor (BDNF) in the all examined time points and highest level of expression was detected at 72 h post VPA treatment. This increase was almost 2 times higher than non-treated control group, whereas 0.1 mM VPA did not change the BDNF mRNA expression significantly, except for the time point of 24 h treatment (Fig. 4A). In addition, data showed significantly enhanced level of glia cell line-derived neurotrophic factor (GDNF) mRNA following 72 and 168 h treatment with 1 mM VPA, and also significant overexpression of this transcript was observed at 24 and 72 h after utilizing 0.1 mM VPA (Fig. 4B). The vascular endothelial growth factor (VEGF) mRNA was the other target that its expression was significantly induced at 24 and 168 h time points by 1 mM VPA treatment. However, none of the 0.1 mM time points were able to significantly change the level of VEGF transcript (Fig. 4C). It is worth mentioning that following VPA treatment with both concentrations, a slight decrease in VEGF expression was detected at 72 h, which significantly peaked at 168 h in 1 mM VPA treated group.

3.4. VPA increases the NGF and NT3 transcripts with limited action in EPI-NCSC

To provide accurate information about the impact of VPA on two other members of neurotrophins family, nerve growth factor (NGF) and neurotrophin-3 (NT3) transcripts were evaluated following VPA treatment. The quantification of the obtained data revealed that NGF mRNA level significantly increased in the stem cells exposed to 1 mM VPA after 24 and 168 h. However, 0.1 mM concentration of VPA was only able to enhance mRNA expression following 24 h incubation (Fig. 5A). Moreover, NT3 transcript in EPI-NCSC treated with 1 mM VPA significantly increased only after 6 h and all time points of 0.1 mM VPA treatment surprisingly decreased the expression of NT3 mRNA (Fig. 5B). It should be noted that the rate of both NGF and NT3 mRNA expression induced by both concentrations of VPA, was less than other transcripts.

4. Discussion

The effective delivery of trophic factors to treat neurological disorders and traumatic injuries is a challenging issue. The present study was carried out to define the efficacy of combined EPI-NCSC and VPA therapy for future treatment of various neurological conditions such as SCI. According to the findings, the mRNA expression level of BDNF, GDNF and VEGF can be significantly up-regulated in EPI-NCSC following treatment with VPA at various time points, predominantly by 1 mM concentration for 168 h. However, NGF and NT3 transcripts in these stem cells were increased by VPA at limited time points.

Currently, there is an increasing body of evidence suggesting the replenishment of endogenous neurotrophic factors by supplementing various neurotrophins has therapeutic effects in different neurological

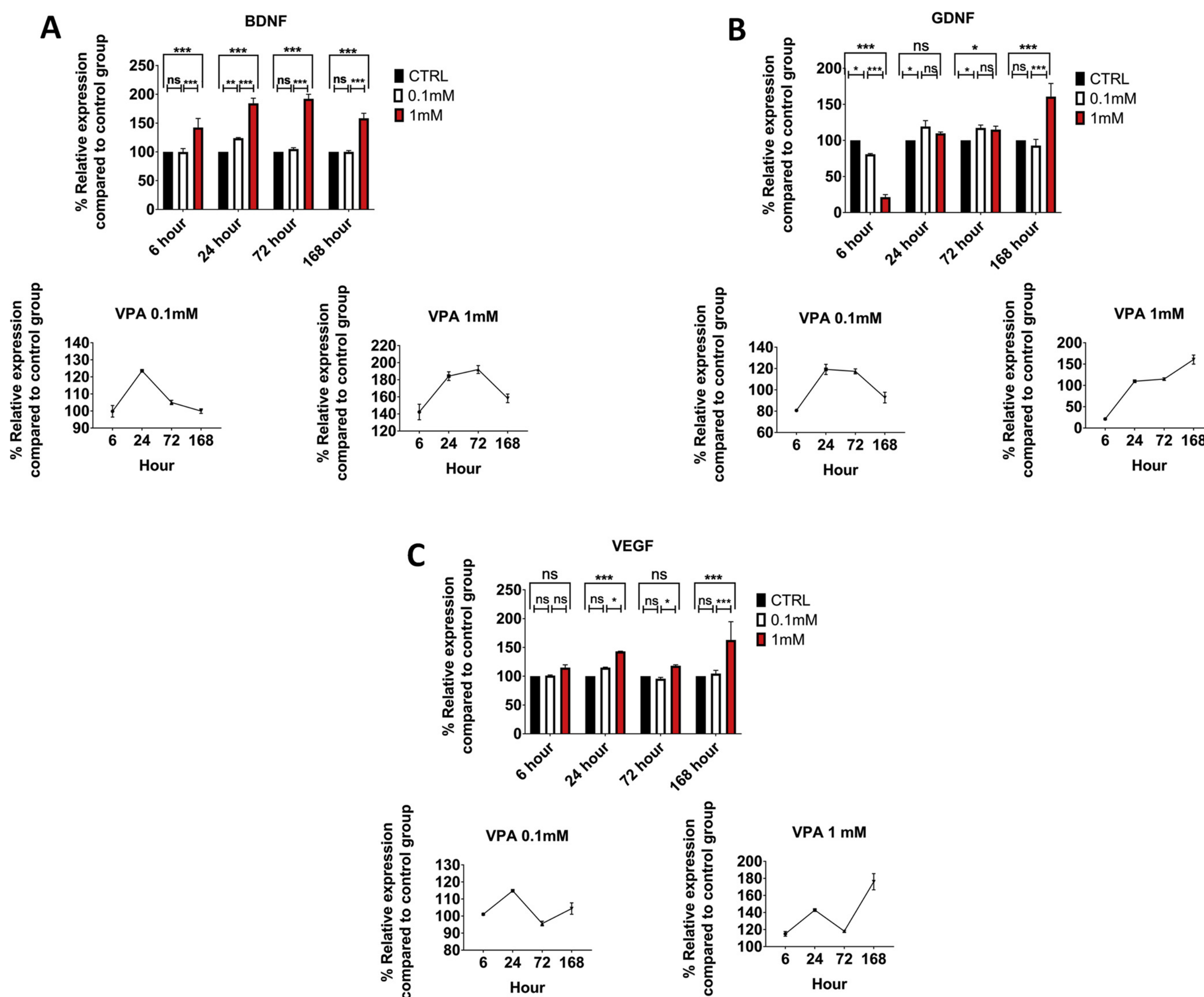

Fig. 4. The impact of VPA treatment on the BDNF, GDNF and VEGF mRNA levels in EPI-NCSCs. The assessment of BDNF (A) GDNF (B) and VEGF (C) expression level following treatment with 0.1 and 1 mM VPA was carried out by quantitative RT-PCR at 6, 24,72 and 168 h. The expression of target genes was normalized against the housekeeping gene β-actin. The data are expressed as mean ± SEM and their analysis was performed using Two-way ANOVA and Tukey *post hoc* test (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

diseases such as Alzheimer's disease, Amyotrophic lateral sclerosis [20], Huntington disease [9], Parkinson's disease [14] and SCI [5,30]. It is established that neurotrophic factors can regulate synaptic activity within seconds following secretion, modify synaptic structure within minutes, and in the following hours and days can alter gene expression and protein synthesis in the nervous system [24]. Owing to relatively large molecular size of trophic factors and their polarity, they cannot readily cross the BBB; hence, have to be administered directly. To circumvent this issue, various strategies were employed such as infusion of recombinant trophic factors, which yielded disappointing results [4]. Also, transplantation of genetically modified cells to express neurotrophins [5,27] and delivering engineered viral vectors encoding the neurotrophic factors are two other ongoing strategies [13,16]. Despite, the delivery of trophic factors through the above mentioned strategies, recent application of genetically modified stem cells that overexpress different neurotrophins is growing. In this field, many researches were dedicated to evaluate the efficacy of genetically manipulated bone marrow-derived mesenchymal stem cells in various neurological conditions [17,25]. Based on the findings in the present investigation, EPI-NCSCs with the aid of VPA can be implicated as a potential source of trophic factor release that can migrate to the damaged areas and independently impose several therapeutic impacts.

While viral vectors are widely used to induce overexpression of trophic factors in the target stem cells; here, we used VPA to enhance neurotrophins expression in the EPI-NCSCs. It has been well established that HDAC inhibitors such as VPA can significantly increase the activity of BDNF and GDNF promoter through alterations in histone acetylation as was described thoroughly in the research of Almutawaa et al. They revealed the induction of neurotrophic factors expression in neural stem cells by VPA [3]. Also the neuroprotective function of VPA in the *in vitro* context was reported earlier as it can up-regulate the BDNF and GDNF gene expression and their release from astrocytes in the *in vitro* context [6,34]. Regarding the facts that glial subtype predominantly produces key growth factors in the nervous system and EPI-NCSCs population is a combination of neuronal and glial cells, it can be assumed that the glial cells mainly respond to the VPA stimulation.

Although our findings in the case of BDNF and GDNF was in line with other investigations, interestingly the increase of VEGF mRNA following VPA treatment was strikingly in contrast with the prevailing reports, indicating that VPA has the ability to reduce the VEGF level in various malignant cells [21,35]. Furthermore, the evaluation of NGF and NT3 mRNA levels revealed that in spite of their relatively abundant

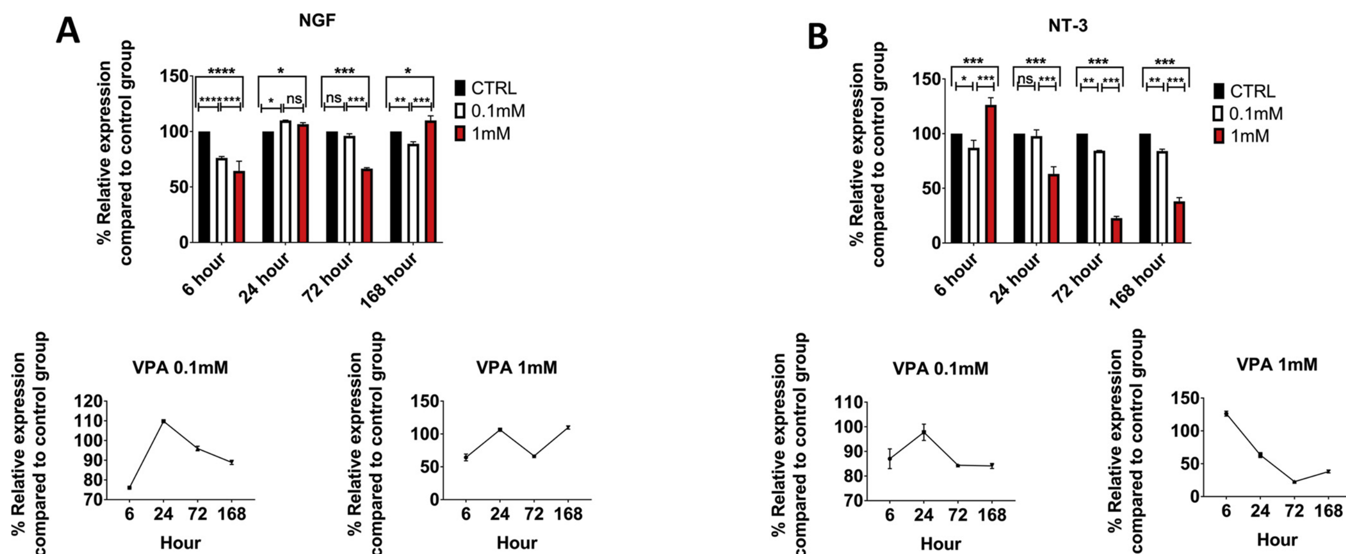

Fig. 5. The effect of VPA on the transcripts of NGF and NT3 in EPI-NCSCs. Following treatment of EPI-NCSCs with 0.1 and 1 mM VPA for various times (6, 24,72 and 168 h), the transcripts of NGF (A) and NT3 (B) were measured by quantitative RT-PCR and β-actin was used as a control for normalization. The data are expressed as mean ± SEM and statistically analyzed using Two-way ANOVA and Tukey post hoc test (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

expression in the EPI-NCSCs, the VPA treatment generally did not improve their transcription.

Since repair capability of adult nervous system following disease and injury is limited, providing trophic factor might increase the survival rate of uninjured axons and/or rescue severed neurons from degenerative atrophy and apoptosis. Hence, grafted stem cells due to secretion of various factors can play a vital role to modulate devastating condition of neurological disorders. Here, based on acquired data, it can be suggested that EPI-NCSCs are suitable candidate for delivering required trophic agents and their potential can be enhanced by VPA. Since the expression of most important trophic factors increased following one week (168 h) treatment with 1 mM VPA, this treatment can be employed as a suitable strategy ahead of stem cells transplantation. Consequently, further investigations are warranted to evaluate the efficacy of this combinatorial therapy in the *in vivo* context and also to compare their potential with other stem cells that are widely used in a range of neurological diseases.

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Conflict of interest

The authors declare that they have no competing interests.

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