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Valproic acid promotes the neuronal differentiation of spiral ganglion neural stem cells with robust axonal growth

Byoung-San Moon [a, b], Wange Lu [a, **, 1], Hong Ju Park [c, *, 1]

[a] Department of Stem Cell Biology and Regenerative Medicine, Broad Center for Regenerative Medicine and Stem Cell Research, Keck School of Medicine of the University of Southern California, Los Angeles, CA, USA
[b] Department of Neurosurgery, Keck School of Medicine of the University of Southern California, Los Angeles, CA, USA
[c] Department of Otorhinolaryngology-Head and Neck Surgery, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea

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ABSTRACT

Hearing loss occurs with the loss of hair cells of the cochlea and subsequent degeneration of spiral ganglion neurons (SGNs). Regeneration of SGNs is a potentially promising therapeutic approach to hearing loss in addition to the use of a cochlear implant (CI), because this device stimulates SGNs directly to restore hearing bypassing the missing hair cells. The presence of SGN-neural stem cells (NSCs) has been reported in adult human and mice. These cells have the potential to become SGNs and thus represent a cellular foundation for regeneration therapies for hearing loss. Valproic acid (VPA) has been shown to influence the neural differentiation of NSCs through multiple signaling pathways involving glycogen synthase kinase3β (GSK3β). Our present study therefore aimed to modulate the neural differentiation potential of SGN-NSCs by treatment with VPA. We here report that a clinically relevant concentration of 1 mM VPA induced the differentiation of basic fibroblast growth factor (bFGF)-treated P1- and P14-SGN-NSCs into neuronal and glial cells, confirmed by neuronal marker (Tuj1 and MAP2) and glial cell marker (GFAP and S100β) detection. VPA-treated cells also promoted much longer neurite outgrowth compared to differentiated cells cultured without bFGF. The effects of VPA on the regulation of differentiation may be related to the activation of the Wnt/β-catenin signaling pathway, but not the inhibition of histone deacetylases (HDACs). We propose that VPA has the potential to convert SGN-NSCs into SGNs and thereby restore hearing when combined with a CI.

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1. Introduction

The cochlea is a sensory organ that enables hearing. Cochlear hair cells transform the vibrating energy from sound into neural signals which are conveyed primarily by spiral ganglion neurons to the cochlear nucleus in the brainstem [1,2]. Sensorineural hearing loss is caused by the loss of the cochlear hair cells with the sequential degeneration of spiral ganglion neurons (SGNs), and this can be exacerbated by circumstances such as long-term or intensive noise exposure and aging [3]. Many individuals suffering from impaired hearing also experience a significant decrease in quality of life and are more likely to suffer from emotional, social, and communication dysfunction [4]. Moderate-to-moderately severe sensorineural hearing loss can usually be corrected with hearing aids; however, severe-to-profound hearing loss often requires a cochlear implant (CI) which stimulates SGNs directly bypassing the missing cochlear hair cells which cannot regenerate. Hearing loss is reported to affect 13% of people who are 12 years of age or older in the United States (~30 million people) [5], where roughly 58,000 CI devices have now been implanted in adults and 38,000 in children (https://www.nidcd.nih.gov/health/cochlear-implants). As of December 2012, approximately 324,200 registered CI devices had been implanted worldwide. Degeneration of the spiral ganglion neurons following hair cell loss, which leaves fewer neurons available for electrical stimulation, has critical implications for CI function because these devices stimulate the spiral ganglion cells

* Corresponding author. Department of Otorhinolaryngology-Head and Neck Surgery, Asan Medical Center, University of Ulsan College of Medicine, 86 Asanbyeongwon-gil, Songpa-gu, Seoul, 05505, South Korea.
** Corresponding author. Department of Stem Cell Biology and Regenerative Medicine, Broad Center for Regenerative Medicine and Stem Cell Research, Keck School of Medicine of the University of Southern California, Los Angeles, CA, 90033, USA.
E-mail addresses: wangelu@usc.edu (W. Lu), dzness@amc.seoul.kr (H.J. Park).
[1] W. Lu and H. J. Park contributed equally to this study and are the corresponding co-authors.

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directly. Hence, there is a pressing need to develop new strategies to regenerate SGNs.

Unlike cochlear hair cells, mouse and adult human SGN-neural stem cells (NSCs) have been reported [6–8]. These stem cells have a potential to become SGNs and thus represent a foundation for regeneration therapy against hearing loss. Valproic acid (VPA; 2-propyl-pentanoic acid) is a short-chain fatty acid that has been used for mood stabilization and the treatment of epilepsy for several decades [9]. VPA also exhibits potent *in vitro* and *in vivo* anti-tumor effects in leukemic cells, neuroblastomas, and gliomas [10–12]. VPA has been reported to regulate the differentiation and proliferation of various cell types, including mesenchymal and hematopoietic stem cells, neuroblastoma cells, primary neurons, and neural progenitor cells (NPCs) [13,14]. VPA can suppress the proliferation of neuroblastoma cells via the induction of the cell cycle regulator $p21^{Cip/WAF1}$ [15,16]. p21 is also known to be involved in the VPA-induced differentiation of hematopoietic cells [14]. VPA also acts as an epigenetic modifier by inhibiting histone deacetylases (HDACs), by regulating microRNA expression, and through its effects on various signaling systems, including the extracellular signal-regulated kinase (ERK), protein kinase C (PKC), PI3K/AKT/mTOR and the Wnt/β-catenin pathways [17–20]. VPA alters Wnt/β-catenin signaling by directly or indirectly inhibiting the activity of glycogen synthase kinase 3β (GSK3β) [21,22]. However, the role of VPA in regulating the differentiation and proliferation of SGN-NSCs is not well understood.

We here investigated the role of VPA in the neural differentiation of SGN-NSCs in relation to the Wnt/β-catenin pathways which play roles in the proliferation and differentiation of various cell types [7,23,24]. We have found that a 1 mM VPA exposure induces the differentiation and inhibits the proliferation of SGN-NSCs by overcoming the effects of basic fibroblast growth factor (bFGF), a factor which inhibits the differentiation of NSCs [18,25–27]. We further found that the Wnt/β-catenin signaling pathway is involved in generating the mutually exclusive differentiation and proliferation phenotypes of SGN-NSCs.

2. Materials and methods

2.1. Experimental animals and ethics statement

C57BL/6 mice were used in the experiments. All of the animals were housed in filter-topped shoebox cages equipped with a computerized environmental control system. All of the animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Southern California (Protocol Number, 11489) and the National Institutes of Health.

2.2. Isolation, culture, and propagation of sphere-forming stem cells from the postnatal spiral ganglion

The otic capsule was dissected from decapitated mice after removal of the brain and immersed in ice-cold phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA). The bony otic capsule was then opened and removed to visualize the membranous labyrinth of the cochlea. The cochlear duct was microdissected from the modiolus where the spiral ganglion resides. The isolated spiral ganglion cells were cultured as described previously [25,27,28]. To maintain stem cell characteristics, SGN-NSCs were cultured in N2 medium containing basic FGF (bFGF: 20 ng/ml; purchased from PeproTech, Rocky Hill, NJ) for 4 days and then evaluated for nestin and Sox2 expression. To induce differentiation, the cells were seeded and further cultured without bFGF.

2.3. Antibodies and reagents

The antibodies used in this study were anti-Ki67 (rabbit polyclonal 1:500; Abcam Ltd, Cambridge, MA), anti-Nestin (mouse monoclonal 1:350; BD Biosciences, San Jose, CA), anti-Sox2 (rabbit polyclonal 1:500; Abcam Ltd), anti-Tuj1 (mouse polyclonal 1:200; Covance, Princeton, NJ), anti-MAP2 (rabbit polyclonal 1:200, Chemicon, Temecula, CA), and anti-GFAP (rabbit 1:500; Cell Signaling Technology Inc., Danvers, MA). The secondary antibodies were anti-rabbit Alexa Fluor 488-conjugated, anti-mouse Alexa Fluor 488-conjugated, anti-rabbit Alexa Fluor 555-conjugated, and anti-mouse Alexa Fluor 555-conjugated IgG (1:200 dilution; Molecular Probes, Eugene, OR). The protease inhibitor cocktail was obtained from Roche Applied Science (Indianapolis, IN). The HDAC inhibitors valproic acid (VPA), trichostatin A (TSA) and 4-(dimethylamino)-N-[6-(hydroxyamino)-6-oxohexyl]-benzamide (DHOB) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.4. Immunohistochemistry and immunocytochemistry

For the immunocytochemistry analysis, cells were cultured on coverslips, fixed with 4% PFA/PBS for 30 min and immunostained after permeabilization with 0.2% Triton X-100. This was followed by incubation with secondary antibodies at room temperature for 1 h and counterstaining in 4′-6-diamidino-2-phenylindole (DAPI) (Boehringer Mannheim, Mannheim, Germany). The images then were visualized using confocal microscopy (LSM 700; Zeiss, Jena, Germany).

2.5. Quantitative PCR

Cells were harvested and total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). A SuperScript III qRT-PCR kit (Invitrogen) was used to synthesize cDNA from the total RNA. Quantitative PCR (qPCR) was performed using a ViiA7 system (Applied Biosystems) with an iTaq Universal SYBR Green Master Mix (BioRad) at 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s and 60 °C for 3 s. The samples were run in triplicate and *nestin*, *Dlx1*, *Dlx2*, β-tubulin III (*Tuj1*), glial fibrillary acidic protein (*Gfap*), S100β, myelin basic protein (*Mbp*), myelin oligodendrocyte glioprotein (*Mog*), and myelin associated glioprotein (*Mag*) transcripts were quantified by comparing their cycle threshold (Ct) values for each reaction with a *Gapdh* reference. The primer sets used in these qPCR analyses are listed in Table S1.

2.6. Transient transfections and reporter assays

SGN-NSCs were transfected using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA) in accordance with the manufacturer's protocol. Cells were transfected with either Topflash or Fopflash luciferase reporter plasmids and co-transfected with a β-galactosidase reporter plasmid to measure transfection efficiency. At 24 h post-transfection, fresh N2 medium was used containing basic fibroblast growth factor (bFGF: 20 ng/ml; purchased from PeproTech, Rocky Hill, NJ). The cells were harvested 24 h post-treatment using Promega reporter lysis buffer. Lysates were assayed for luciferase and β-galactosidase activity using appropriate kits from Promega. Luciferase values were corrected for transfection efficiency using the β-gal reference values and representative values were calculated as pTopflash/pFopflash.

2.7. Statistical analysis

Statistical differences among the groups were analyzed using the Student *t*-test or analysis of variance (ANOVA: Tukey's multiple

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