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RESEARCH ARTICLE

Overexpression of Chemokine Receptors on Neural Stem Cells Pretreated with Valproic acid: Towards Improved Homing

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ABSTRACT

Neural stem cells (NSCs) have considerable capacity for self-renewing and also ability for generating neurons in the mammalian brain. However, one of the big challenges is the migration and targeted homing of transplanted NSCs into the injured site to treat neurodegenerative diseases including Alzheimer´s disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), brain ischemia (BI) and spinal cord injury (SCI). To improve homing capacity, pretreatment of NSCs with Valproic acid (VPA), which is supposed to cause diverse effects on migration ability of NSCs, is a strategy. More recently, hind brain and olfactory bulbs have been introduced as a good source of NSCs. So, NSCs were isolated from these two sources of postnatal day 1 (PND1) rats. These isolated cells were characterized by expressing neuronal markers such as Nestin and Sox2. The expression of four selected chemokine receptors (CXCR4, CXCR6, CCR1 and CCR7), which are important effectors in homing of stem cells, was investigated. It is concluded that VPA treatment enhances NSCs migration and homing showing its potential to be applied for cell-based therapies.

Keywords: Neurodegenerative diseases, Cell therapy, Regenerative medicine, Neural Stem Cell, Migration, Homing, Chemokine receptors.

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Introduction

Despite the various prevention programs, there are still many kinds of irremediable central nervous system (CNS) diseases, which have a lot of emotional and physical problems [1]. Thus, great researches have been made to eliminate these outcomes of degenerative diseases. At present, use of neural stem cells (NSCs) is proposed as a helpful way to treat CNS diseases [2,3]. Therefore, both endogenous and grafted NSCs (*in vitro* expanded) can be exploited to migrate into damaged sites after injury and differentiate into new neurons [4] or affect the lesion's environment by paracrine effects [5].

NSCs could mediate regeneration following nerodegeneration occurrence by inducing of neurogenesis and replacement of lost neurons. NSCs are located in and obtained from several niches of CNS such as hippocampus and subventricular zone (SVZ), white matter, olfactory bulb, cerebellum, spinal cord, retina, hypothalamus [6], and they are identified by several markers including brain lipid binding protein-positive (BLBP), Nestin, and glial fibrillary acidic protein (GFAP) [7], Musashi 1 and 2 and SOX2 [6] [8]. Ability of NSCs to migrate to a damage site, to survive, and potentially form new neurons, is important for their function but is often destroyed after stroke contributing to the insufficient capacity of the injury for intrinsic repairing and functional recovery [9]. Thus, supporting the proliferation, survival, and migration of endogenous NSCs seems to be a hopeful treatment in stroke. In CNS, usually NSCs are recruited to sites of damage by the cytokine called stromal cell-derived factor (SDF)-1α that is expressed in the damaged tissue, acting on the CXC chemokine receptor type 4 (CXCR4). SDF-1a/CXCR4 interaction mediates migration of stem cells, so overexpression of CXCR4 may amplified the migration potential [10]. CXCR4 is expressed on various types of stem cells, such as hematopoietic stem cells [11], mesenchymal stem cells [12], [13] and NSCs [14,15]. However, the amount of expression is low or impaired during *ex vivo* expansion [16]. Therefore, the strategies like pretreatment that could increase expression of surface receptors might be a good way to enhance homing of stem cells including NSCs [17].

For decades, valproic acid (VPA) has been usually used to treatment of bipolar disorder and epilepsy [18], additionally VPA have multiple pharmacological effects, such as enhancing γ-aminobutyric acid (GABA) neurotransmission, reducing glutamate and dopamine neurotransmission in the brain [19,20]. Recent experiments on animal model of stroke showed that VPA causes multiple beneficial effects including induction of neurogenesis, suppression of neuroinflammation and infarct volume reduction [10,21]. Additionally VPA acts as a histone deacetylas inhibitor that have effective role in transcriptional regulation and can increase the expression of CXCR4 in stem cells [22, 23].

In this study, VPA was used to treat NSCs in order to investigate its effects on expression of some important chemokine receptors in homing such as *CXCR4, CXCR6, CCR1* and *CCR7*. This would provide a clue as to what extent of gene manipulation is needed to improve the homing of NSCs in certain diseases such as neurodegenerative ones. We hypothesized that VPA promotes migration and homing of NSCs through overexpression of these receptors as a potential means of mobilizing and attracting NSCs for therapy of neurodegenerative diseases.

1. Materials and methods

1.1. Isolation and culture of NSCs

NSCs were isolated from the hind brain and olfactory lobes of postnatal one-day old Sprague-Dawley rats. The animals were treated and maintained according to the standard guidelines of Animal Care and Use Committee of Mashhad University of Medical Science. Hypothermia is used to induce anesthesia in neonatal rats. To induce hypothermia, pups may be placed in a latex sleeve and immersed up to the neck in crushed ice and water (2° C-3° C) which requires a 5-8 minutes induction time (2-3 minutes to unconsciousness and 3-5 minutes to complete blockage of neural transmission). Under Laminar hood, rats were terminated by cervical dislocation. Brains were removed from the body and transferred to a petri dish. The olfactory bulb was cut off. Next behind brain was cut. These cells were digested by trypsin to produce single

cells. Then, they were seeded immediately into T25 cell culture flasks at a concentration of 5000 cells/cm^2 (~200,000 cells/ml), containing Dulbecco's Modified Eagle's Medium-High Glucose (DMEM-HG, Gibco) supplemented with 15% fetal bovine serum (FBS, Gibco) [24]. These flasks were incubated for 48 hours in a 5% CO2 incubator at 37° C. After this period, the cell adhered to a new flask and floating tissue and cells are removed. DMEM culture medium (3 ml) was then added to the adhered cells in the flasks. The medium was refreshed after 3 days and the cells were harvested at a sub confluency using 0.25% trypsin/EDTA (passage 0). Cell number and viability were determined by hemocytometer using trypan blue dye exclusion. After 3-5 passages NSCs were used for further analysis. In order to confirm the authenticity of NSCs, expression of *SOX2* and *Nestin* were analysed by PCR. The thermal profile for PCR was 94° C for 5 minutes followed by 30 cycles of (60 s at 94° C, 60 s at 60° C, and 60 s at 72° C), then followed by 1 minute extension at 72° C. PCR products were size-fractioned by electrophoresis agarose gels. The specific primers used have been shown in Table 1.

1.2. Pretreatment of NSCs with valproic acid (VPA)

NSCs in passage 4 with a confluency of about 80% were treated with 5 mmol/L VPA (VPA solvates in 10% ethanol) at 24 and 48 hours.

1.3. RNA extraction and reverse transcription-polymerase chain reaction

Cells in passages 4-6 were washed with PBS and used for reverse transcription-polymerase chain reaction (RT-PCR) assay. Total RNA was prepared using a modified guanidine isothiocyanate–phenol–chloroform method. Density and purity of extracted RNA was determined by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer. cDNA was synthesized by oligo (dT) primer and M-MuLV reverse transcriptase (Fermentas, Germany) according to the manufacturer's instructions. Sequences of the selective forward and reverse primers used for polymerase chain reaction (PCR) of GAPDH (internal control), *CXCR4*, *CXCR6*, *CCR1* and *CCR7* have been shown in Table 1.Quantitative real-time PCR was carried out using Biorad CFX96 thermal cycler. The reaction mixture contained 10 µl SYBR green Universal Master Mix (Pars Tous), each primer at 1 µl, and 2 µl of cDNA according to the manufacturer's instructions. The thermal profile for Real time PCR (94° C for 30 seconds, 60° C for 40 seconds and 72° C for 30 seconds) was done for 40 cycles after an initial single cycle of 94° C for 15 min (all experiments were performed from three donors and each test was at least duplicate). The sequences of primers have listed in Table 1. The melting curve for all amplification was considered to confirm that there were no nonspecific PCR products**.**

Table 1: Sequence of the primers used for RT-PCR and real time PCR analysis.

Target gene	Primer Sequence	Product size (bp)
Primers used for real time PCR		
CXCR4	**F: CGAGCATTGCCATGGAAA**	241
	R: AGGTGCAGCCGGTACTTG	
CXCR6	**F: GTAACAGCCAGGAACACAAAC**	242
	R: GCCAAAGACCCACTCATAGG	
CCR1	**F:ACTGGTGAGCACTGTGATGC**	158
	R: TCAAGGTTCAAGGTCCCAAC	
CCR7	**F: GCTGCGTCAACCCTTTCTTG**	142
	R: ACCGACGCGTTCCGTACAT	
Primers used for RT-PCR		
GAPDH	**F: AATGCATCCTGCACCACCAACTGC**	555
	R: GGAGGCCATGTAGGCCATGAGGTC	
SOX2	**F: ATGTATAACATGATGGAGACGGAGC**	960
	R: TCACATGTGCGACAGGGGCAGTTC	
Nestin	**F: GCTACATACAGGACTCTGCTG**	541
	R: AAACTCTAGACTCACTGGATTCT	

1.4. Data analysis

Statistical analysis was done with SPSS software version 22 and was set among the different groups to examine the expression of *CXCR4* and *CXCR6*, using real time PCR. The untreated and treated groups each were repeated three times and

the results were analysed using ANOVA and Tukey Post hoc test. $p < 0.05$ were considered statistically significant.

2. Results

2.1. Characterization of NSCs by *Nestin* and *Sox2* expression

NSCs from hindbrain and olfactory bulbs of postnatal day 1 (PND1) rats were isolated successfully and formed a monolayer about 5 days after initial plating. These cells have irregular and polygonal shapes. After one week NSCs covered the surface of flask T25 and thus were passaged and maintained their shapes during culture (Figure 1).

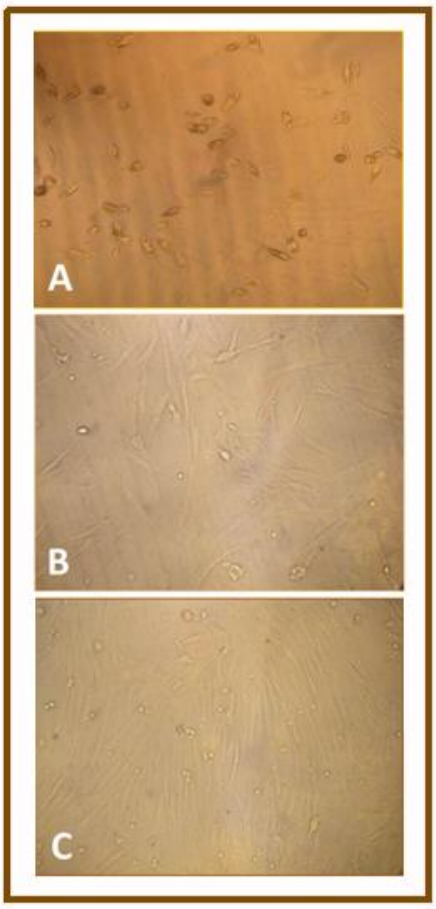


Figure 1: **Microscopic images of neural stem cells (NSCs) derived from the brain of rat.** Images showing the cell at passage 1 (A), NSCs with irregular shape at end of passage 1 (B), and at passage 3 (C).

Primary fetal rat NSCs were grown in monolayer cultures and characteristically expressed *SOX2* as a marker for undifferentiated cells (Figure 2) and also these cells expressed *Nestin*.

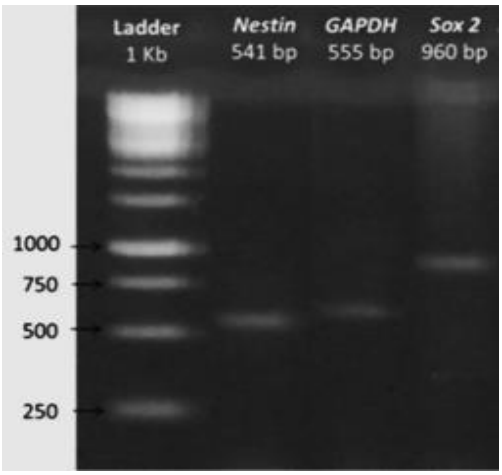


Figure 2: **Characterization of neural stem cells (NSCs) derived from the brain of rat by RT-PCR**. Expression of *Nestin* and *Sox2* genes confirm the identity of NSCs. *GAPDH* was used as an internal control in all cases.

2.2. Chemokine receptor's expression

Data in real time showed that *CXCR4* had a high expression in NSCs treated for 24 hours with VPA. There was no significant increase in 48 h treated sample. Additionally, *CXCR6* had a high expression in samples which that NSCs were treated for 24 h and 48 h. However, *CCR1* and *CCR7* had no expression at all (Figure 3). *GAPDH* was used as an internal control (Figure 4A and B).

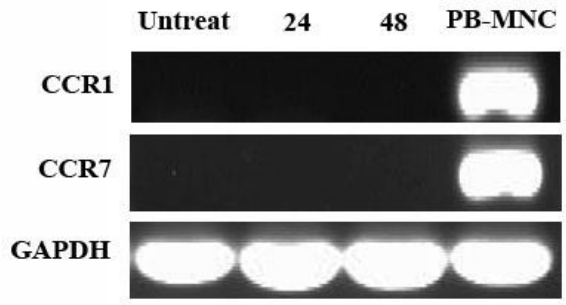
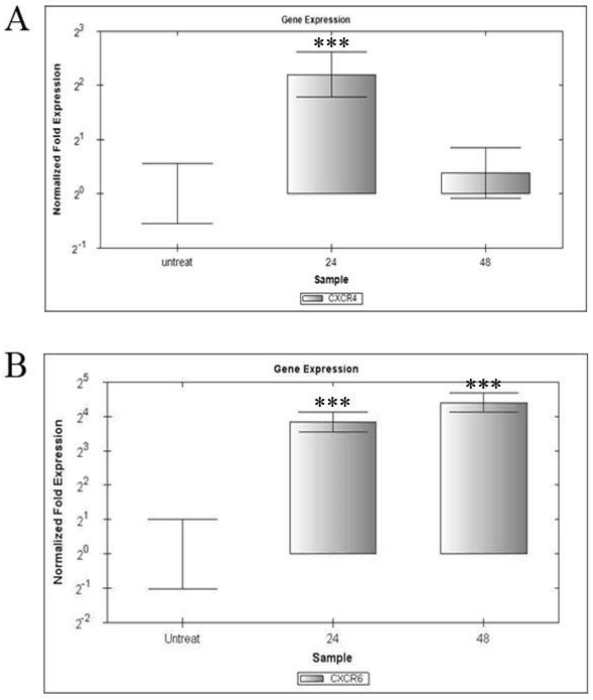


Figure 3. Chemokine receptor's expression *by RT-PCR.* *CCR1* and *CCR7* had no expression at all.



Figurer 4: **Expression of chemokine receptors in neural stem cells (NSCs) treated with valproic acid.** NSCs treated with VPA at 24 h have the highest expression of *CXCR4*. Results showed that there is significant difference between untreated cell groups and 24 h treated cell groups. However there is no significant difference in expression of *CXCR4* between untreated group and 48 h treated group (A). Regarding *CXCR4* (B), the results showed that there is a significant difference in expression between untreated cell groups and both of 24 h and 48 h treated cell groups, but there is no significant difference between 24 h and 48 h treated groups (B). (Stars show significant difference at $p \leq 0.05$)

3. Discussion

One of the strategies to improve homing capacity is pretreatment of NSCs with chemical agent in such a way that they could home around the damaged tissues and modify them to differentiate into various cell types through employment of growth factors or signalling molecules [7]. Efficient homing is closely related to the expression of chemokine receptors [25]. Therefore, deciphering the level of expression of various chemokine receptors on stem cells' membrane would be helpful for scientists; providing better vision and improved cell source concerning NSCs for therapeutic use. For example, by modulating of the expression of such receptors it could affect their homing efficiency to the damaged sites [22, 26].

The finding of several studies revealed that, neural stem cell migration and homing into damaged sites are restricted [20-22]. Therefore, researchers around the world are looking for strategies which can improve migration and homing of NSCs into damaged region. In this study, NSCs were isolated from hind brain and olfactory bulbs of postnatal one-day old rat and treated with 5mM concentration of VPA at passage 4 for 24 and 48 hours to see effect of VPA on expression of chemokine receptors which are involve in migration and homing of NSCs. Our result showed that expression of *CXCR4* was increased at 24h after treatment and expression of *CXCR6* was high at 24h and 48h after treatment compare with untreated group. We know that elimination half-life of VPA is about 4-16 h, [27] and studies showed that the best effect of VPA on *CXCR4* expression on hematopoietic stem cells has been after 24 h treatment [22].

Although VPA has a transient effect on *CXCR4* expression, it seems to be enough for increase homing of hematopoietic stem cells that occurs at 24 h after treatment. Additionally, it was demonstrated that CXCR4 is a major molecule facilitating MSC homing [26] and migration in cerebral ischemia [29, 30] also CXCR6, CCR1 and CCR7 may play a main role in migration and homing of MSCs [31]. Consistently, our results showed that overexpression of these chemokine receptors may enhance migration and homing potentials of NSCs [32]. It has been shown that pretreatment with VPA enhance stem cells migration through the activation of the histone deacetylase (HDAC)-CXCR4 signaling pathway [33, 34].

4. Conclusion

Our preliminary results demonstrated that VPA could considerably improve the migratory potential of NSCs through overexpression of *CXCR4* and *CXCR6*. It is concluded that VPA

treatment might be used as a strategy to enhance migration and homing of NSCs; a potential improved way for cell-based therapy of neurodegenerative diseases.

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Conflict of interest

The authors have no conflict of interest to declare.

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