Effects of Retinoic Acid and Valproic Acid on differentiation of Embryonic Neural Stem Cells

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Neural stem cells (NSC) have a myriad of potential uses, such as the treatment for spinal cord injury. Both retinoic acid (RA) and valproic acid (VPA) have been proven to be involved in neurogenesis in mice model; VPA as an HDAC inhibitor to induce neuronal differentiation and RA which enhances histone H3 acetylation to induce astrocyte differentiation of NSC. The objective of this research is to find the most suitable substance and/or combination of substances to be used in neuronal differentiation of ESC-derived NSCs.

NS cells were derived first from mouse ES cells. DMEM Ham's F-12 along with Vitamin B27, PSF, EGF, and BFGF was used. For experiment cells were moved to a four-well dish with N2 medium and FGF; control, RA, RA+FBS, RA+VPA. Immunocytostaining was done with antibodies for Tuj1, GFAP, and nestin, and then observed under fluorescent microscope. Cell count was done to determine differentiation of cells in each dish.

In treatment of the VPA and RA, there is indication that neurogenesis enhanced compared to cells that was only treated with RA and the control dish. The enhancement is mostly shown in the Tuj1 immunofluorescence where it is more abundant. However the number of astrocyte differentiation was also increased in the combination of RA and VPA treatment.

This indicates that there is no specific fate preference from the treatment. It does however indicate that during the incubation period there was an increase in cell proliferation and differentiation of NSCs when treated with a combination of VPA and RA.

Keywords: *Neurogenesis, mESCs, HDAC inhibitor, Valproic acid, Histone acetylation, retinoic acid, astrocyte*

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ABSTRACT

Introduction and Objectives

Neural progenitor and stem cells was first described by Sally Temple in 1989 in the subventricular zone (SVZ) of the mouse brain[1]. They were then first isolated by Brent Reynolds and Samuel Weiss in 1992 from the SVZ. Weiss and Reynolds also discovered that neural stem cells could be maintained in culture via propagation of floating cell cultures termed "neurospheres"[2]. Nowadays, neural progenitor and stem cells have been isolated from various regions of the adult brain, including the hippocampus and also spinal cord. From these findings, it has since then hoped that it may be possible to stimulate endogenous Central Nervous System (CNS) repair mechanisms or to replace dead cells with cells generated in vitro[3,4]. The attempt to use in vitro cells leads to research in the most effective way to grow the expected cells of the CNS (neuron, astrocyte, or oligodendrocyte). The previous method of neurospheres proved to have certain limitations; the stem cells maintained within neurospheres are not directly identifiable and have an uncertain relationship to CNS precursor cells in vivo[5,6,7,8]. NSCs have a myriad of potential uses, such as the treatment for spinal cord injury. Even though neural stem cells have the ability to self-renew and generate various neural cell types, to use them as treatments the control of differentiation is vital[9]. Therefore, research on the mechanisms of NSC differentiation must be done. Both retinoic acid (RA) and valproic acid (VPA) have been proven to be involved in neurogenesis in mice model; VPA as an HDAC inhibitor to induce neuronal differentiation and RA which enhances histone H3 acetylation to induce astrocyte differentiation of NSC[10,11,12,13,14,15].The objective of this research is to find the most suitable substance and/or combination of substances to be used in neuronal differentiation of embryonic NSCs..

Material and Methods

NSCs were derived from embryonic day 14.5 mouse cortex. mESCs were grown at 37 °C in a 5% CO_2 incubator in ESC medium (ESM) containing 25ml Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM Ham's F-12), supplemented with 500µl Vitamin B27, 250µl antibiotic Penicillin Streptomycin Fungicide, 25µl growth factor Epidermal Growth Factor (EGF), and 25µl Basic Fibroblast Growth Factor (bFGF) on 9-cm pre-coated disc with Ornithine and Fibronectin (O/F disc). Medium was changed every day, and when the cells reached 60-70%

confluence they were passaged onto new dishes at a plating density of 1 x 10^6 cells per 9-cm dish.

mESCs were induced to differentiate to the neural lineage. One day later, the medium was replaced with DDM, which is composed of DMEM/F12 supplemented with freshly prepared modified N2-supplement, PSF, and FGF. The culture was maintained until differentiation day 4. At differentiation day 4, four experimental groups were used; control, Retinoic Acid, Retinoic Acid with Fetal Bovine Serum (FBS), Retionic Acid with Valproic Acid.

Medium was removed and cells were washed with phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde in PBS for 15 minutes. After 3 washes with PBS, the cells were incubated for 30 minutes at room temperature (RT) in blocking solution (PBS containing 3% FBS and 0.1% Triton X-100). They were then incubated overnight at 4 °C with the appropriate primary antibodies. The following primary antibodies were used: rabbit anti-β-tubulin (Tuj1; 1:1000), chick anti-nestin (1:500), and mouse anti-GFAP (1:500). After 3 washes with PBS, the cells were incubated for 30 minutes at RT with the appropriate secondary antibodies. The following secondary antibodies were used: Hoechst, FITC-conjugated donkey anti-rabbit, Cy5-conjugated donkey anti-chick and Cy3-conjugated donkey anti-mouse (all 1:500). After 3 washes with PBS, cells are mounted on cover slips with Immu-Mount and examined and photographed using a fluorescence microscope equipped with a camera and appropriate epifluorescence filters. Cell counting was done manually by using a fluorescence microscope Axiovert 200M equipped with a camera and appropriate epifluorescence filters, within the indicated areas.

Results and Discussion

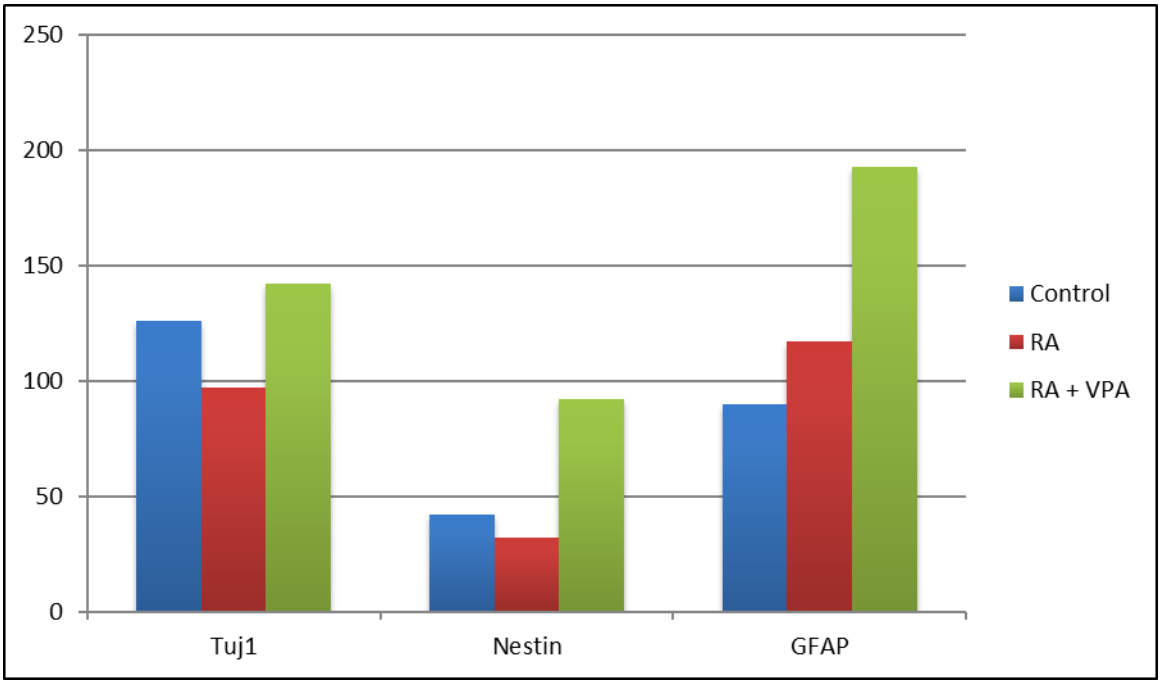
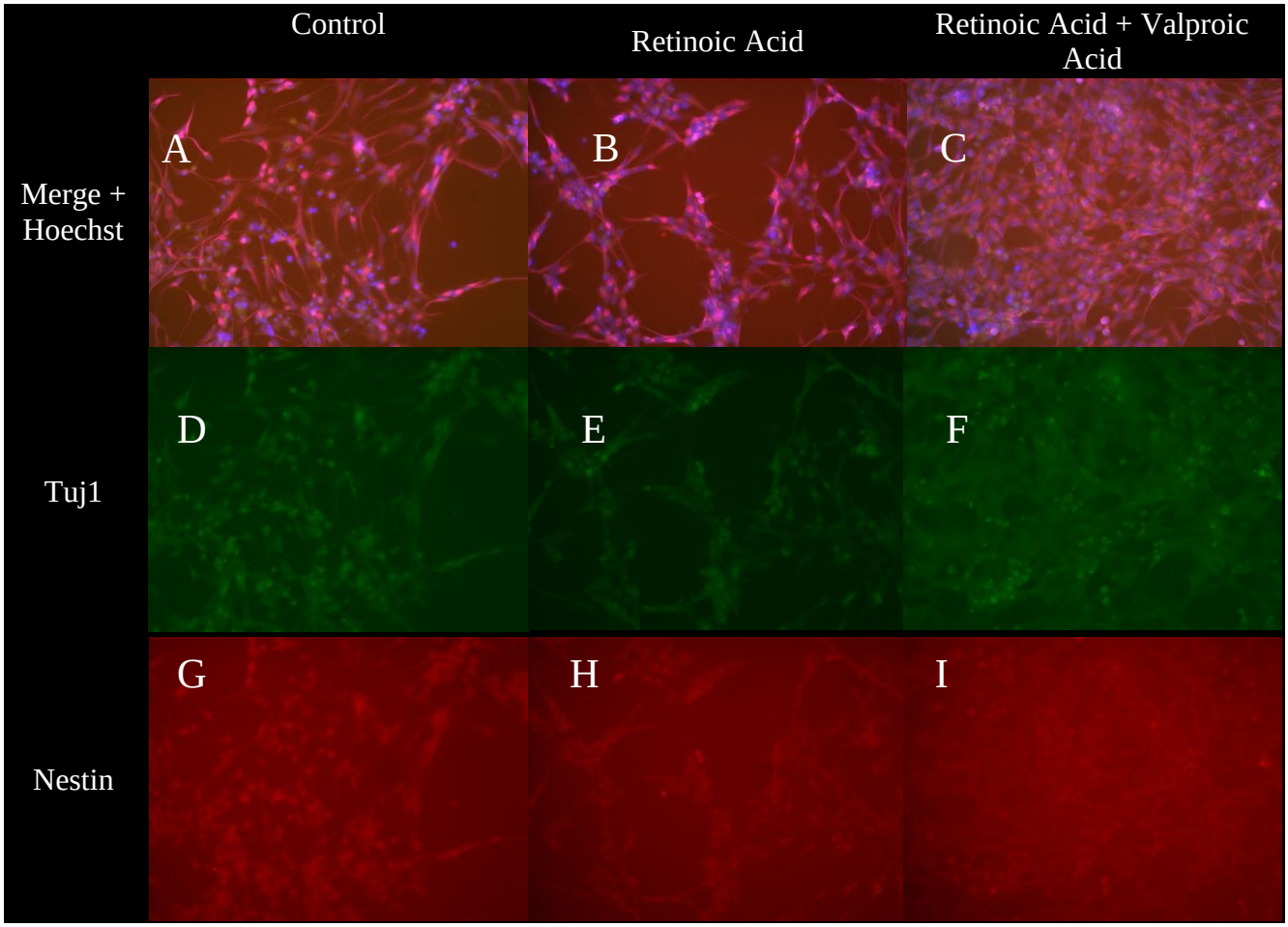


Table 1 Cell Counting of Marker; Tuj1, Nestin, GFAP in three different conditions; Control, Retinoic Acid (RA), and Retinoic Acid with Valproic Acid (RA+VPA)

By manual cell counting with the help of Adobe Photoshop CS6 for Mac, it was found that there are 126 Tuj1 positive cells, 42 Nestin positive cells, and 90 GFAP positive cells in the control group. In the Retinoic Acid (RA) only group there were 97 Tuj1 positive cells, 32 Nestin positive cells, and 117 GFAP positive cells. In the Retinoic Acid with Valproic Acid group (RA+VPA) there were 142 Tuj1 positive cells, 92 Nestin positive cells, and 193 GFAP positive cells. Each group's result as well as the merged photo can be seen in figure 1.



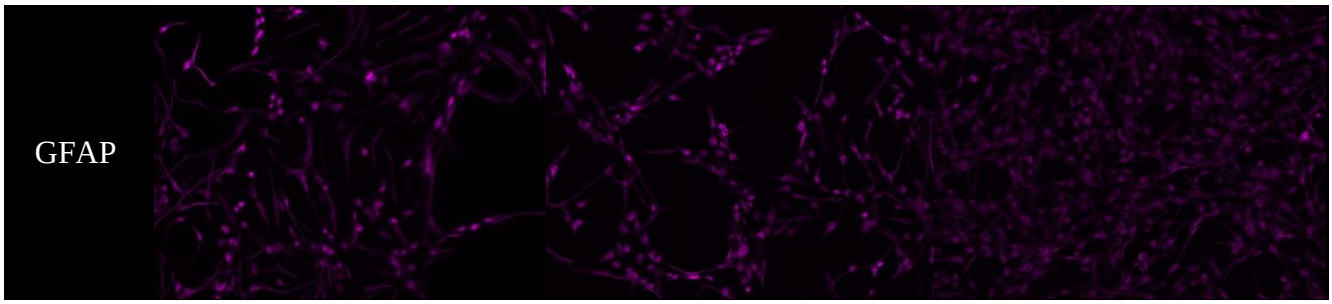


Figure 1 A-C: Fluorescent Imaging of NSCs with antibody markers Hoechst, Tuj1, Nestin, and GFAP in Control medium, Retinoic Acid medium, and Retinoic Acid and Valproic Acid; D-F antibody marker Tuj1; G-I antibody marker Nestin; J-L antibody marker GFAP

Inhibition of HDAC activity by valproic acid (VPA), a widely used anticonvulsant and mood-stabilizing drug, has been shown to drive mESCs to differentiate into the ectodermal lineage at the expense of mesodermal and endodermal lineages[10]. This ectodermal lineage differentiation is further biased in favor of neuronal rather than glial fates by the VPA treatment. Prior to this finding in mESCs, Hsieh et al. (2004) and Balasubramaniyan et al. (2006) had also found a similar tendency for neuronal over glial fate preference when they cultured neural progenitor cells (NPCs) in the presence of HDAC inhibitors such as VPA and trichostatin A. The types of neurons produced in these studies were not examined in detail, however, and the effects of HDAC inhibition on the differentiation of mESC-derived NPCs generated from culture condition for cortical projection neurons production have not yet been studied[16,17,18].

In treatment of the VPA and RA, there is indication that the neurogenesis is enhanced compared to the NSCs that was only treated RA and control, which is shown on all three markers. The enhancement was also the same as in previous findings that retinoic acid is involved in astrocyte differentiation but not in neuronal differentiation, which is shown in the data comparison between control group and retinoic group for Tuj1 and Nestin marker. The increase of GFAP marker count in the presence of retinoic acid and valproic acid is more than difference between control group and retinoic acid group. Overall cell proliferation was also increased in the retinoic acid and valproic acid group compared to the control group.

RA facilitates astrocyte differentiation of Neural Progenitor Cells[19,20]. RA activates the promoter of GFAP, which encodes the astrocytic marker glial fibrillary acidic protein (GFAP), and is activated by a putative RA response element in the promoter. The mechanism behind this is that histone H3 acetylation around the STAT-binding site in the GFAP promoter was increased in NPCs treated with RA, allowing STAT3 to gain access to the promoter more efficiently. The result from this experiment correlates with this by showing that GFAP markers in both retinoic acid and retinoic acid with valproic acid conditions increased compared to the control group.

The interaction between VPA and RA has been explained in a previous research. The research done by Chuang et al found that VPA treatment on chicken embryos showed downregulations of ovotransferrins, carbonic anhydrase-2, retinol binding protein-4 (RBP4), NADH cytochrome b5 reductase 2 (CYB5R2), apolipoprotein A1, and protein SET, with the most significant being RBP4, which is assumed to be involved in the generation of RA. In the same research, downregulation of RBP4 tends to induce hypervitaminosis A, which will induce a high concentration of RA by favoring retinol flux into RA generating tissue[20]. This explains not only that the separate effects of VPA and RA are involved in neuronal differentiation, but also VPA

induces an increase of RA; therefore enhancing the effect when used together as one medium. However the increase of neuron in VPA and RA condition that is higher than RA alone still cannot be explained just from this experiment.

Conclusion

Although the treatment of VPA and RA in combination seems to be enhancing both the differentiation and proliferation of the NSCs, from the result of only immunocytochemistry and its poor image quality, its effect can not be accurately determined, due to limited time for the research. The combined treatment does not seem to cause a negative effect on the NSCs. However they do need to be studied more with large number of samples for their actual effect on proliferation and neuronal differentiation of NSCs. The result for the sample with RA and FBS was excluded because the data gained was invalid.

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