BRAIN RESEARCH 1596 (2015) 31–47

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Brain Research

Research Report

In vitro characteristics of Valproic acid and all-trans-retinoic acid and their combined use in promoting neuronal differentiation while suppressing astrocytic differentiation in neural stem cells

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ARTICLE INFO

Article history:
Accepted 13 November 2014
Available online 20 November 2014

Keywords:
Valproic acid
All-trans-retinoic acid
Neural stem cell
Cell fate determination
Time-dependent
Cross-talk

ABSTRACT

Multipotent neural stem cells (NSCs) are currently under investigation as a candidate treatment for central nervous system (CNS) injury because of their potential to compensate for neuronal damage and to reconstruct disrupted neuronal connections. To maximize the regenerative effect of the derived neurons and to minimize the side effects of the derived astrocytes, it is necessary to regulate the fate determination of NSCs to produce more neurons and fewer astrocytes. Both valproic acid (VPA) and all-trans-retinoic acid (ATRA), two clinically established drugs, induce neuronal differentiation and facilitate neurite outgrowth at the expense of astrocytic differentiation in NSCs. However, the time-dependent activities and the long-term treatment effects of these drugs have not been explored in NSCs. More importantly, the efficacies of VPA and ATRA in neuronal promotion and astrocytic suppression remain unclear. In this study, we compare the time-dependent characteristics of VPA and ATRA in NSC differentiation and neurite outgrowth *in vitro* and, for the first time, demonstrate the improved efficacy of their combined application in neuronal induction and astrocytic suppression. These significant effects are closely coupled to the altered expression of a neurogenic transcription factor, a Wnt signaling component, a cell cycle regulator and a neural growth factor, indicating an underlying cross-talk between the mechanisms of action of ATRA and VPA. These findings indicate that a novel strategy combining

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http://dx.doi.org/10.1016/j.brainres.2014.11.029

these two therapeutic drugs may improve the restorative effect of NSC transplantation by altering the expression of their interconnected targets for fate determination.

1. Introduction

Central nervous system (CNS) injury is a global burden that causes permanent neurological disabilities and, in some cases, death (Ackery et al., 2004; Corrigan et al., 2010; Mukherjee and Patil, 2011). Axonal regeneration rarely occurs after injury due to the limited ability of the adult CNS to replace the lost neurons and to reconstruct functional neural connections, resulting in incurable functional impairments (Sun and He, 2010; Yang and Yang, 2012). Because of their abilities to self-renew and differentiate into neurons and glial cells in the mammalian CNS, neural stem cells (NSCs) are considered to be a feasible candidate for repairing CNS injuries by integrating into the host neural tissue, rebuilding the disrupted neuronal circuitry, serving as a substrate for axonal regrowth, promoting remyelination, compensating for the lost cells and secreting trophic factors to support survival and regrowth (Abematsu et al., 2010a; Cummings et al., 2005; Lu et al., 2003; Pfeifer et al., 2004; Song et al., 2002; Yasuda et al., 2011). However, due to the hostile microenvironment at lesion sites, where high levels of inflammatory cytokines and other inhibitory growth components overwhelm the growth-stimulating factors, grafted NSCs exhibit a strong bias towards astrocytic differentiation at the expense of neuronal differentiation (Abematsu et al., 2006; Martino and Pluchino, 2006; Nakamura et al., 2003; Ogawa et al., 2002; Setoguchi et al., 2001, 2004; Vroemen et al., 2003). Outweighing the regenerative benefit of the occasionally generated neurons, these astrocytes substantially reduce and even impair functional restoration via aberrant host fiber sprouting and the resulting allodynia-like hypersensitivity (Hofstetter et al., 2005). Therefore, interventions are required to modulate this differentiation bias by improving neuronal differentiation and suppressing astrocytic differentiation, thereby reducing the side effects of NSC therapy (Abematsu et al., 2010a; Hofstetter et al., 2005).

Valproic acid (2-propylpentanoic acid; VPA), a drug demonstrated to be effective for the treatment of bipolar disorder and epilepsy (Blaheta and Cinatl, 2002; Chiu et al., 2013; McElroy et al., 1989), was recently reported to promote cell differentiation, improve neuroprotection, and facilitate neurite initiation and elongation in NSCs (Abematsu et al., 2010a; Go et al., 2011; Hsieh et al., 2004; Jung et al., 2008; Lv et al., 2012). Importantly, VPA induces the neuronal differentiation of NSCs at the expense of astrocytic and oligodendrocytic differentiation both *in vitro* and *in vivo* (Abematsu et al., 2010a; Hsieh et al., 2004; Jung et al., 2008). The mechanism for this differentiation bias is closely associated with the VPA-mediated inhibition of histone deacetylase (HDAC) and alterations in gene transcription that are relevant to the fate determination of NSCs, including the up-regulation of the neurogenic basic helix–loop–helix (bHLH) transcription factor NeuroD, the pivotal Wnt signaling component β-catenin, and the cell cycle regulator $p21^{Cip/WAF1}$ (Chateauvieux et al., 2010; Hsieh et al., 2004; Jung et al., 2008; Rocchi et al., 2005; Yu et al., 2009). Moreover, the expression levels of neural growth factors, especially brain-derived neurotrophic factor (BDNF), are also up-regulated by VPA, resulting in improved neuronal survival and maturation and neurite outgrowth (Chen et al., 2006; Lv et al., 2012; Yasuda et al., 2009). All-trans-retinoic acid (ATRA) also induces neuronal differentiation while blocking astrocyte production in NSCs (Christie et al., 2010; Henion and Weston, 1994; Takahashi et al., 1999; Wohl and Weiss, 1998), and ATRA facilitates neuronal survival and neurite outgrowth in cultured neurons (Chen et al., 2008; Erceg et al., 2008; Quinn and De Boni, 1991; Wuarin and Sidell, 1991). The underlying mechanism involves the stimulation of β-catenin expression (Israsena et al., 2004), the transcriptional activation of NeuroD (Kuwabara et al., 2009) and the subsequent up-regulation of $p21^{Cip/WAF1}$ (Takahashi et al., 1999; Zhang et al., 2009). Moreover, ATRA augments the beneficial effects of BDNF on neuronal maturation and neuroprotection via the up-regulation of TrkB in NSCs (Takahashi et al., 1999).

Although much progress has been made in modulating NSC differentiation by treating these cells with either VPA or ATRA (Abematsu et al., 2010a; Christie et al., 2010; Hadinger et al., 2009; Hsieh et al., 2004; Jung et al., 2008; Laeng et al., 2004; Takahashi et al., 1999; Wohl and Weiss, 1998; Yu et al., 2009), the differentiation efficiency remains unsatisfactory; the neuronal differentiation ratios of VPA and ATRA range from 20% to 50% or less (Hadinger et al., 2009; Hsieh et al., 2004; Takahashi et al., 1999; Wohl and Weiss, 1998; Yu et al., 2009), and their astrocytic differentiation ratios range from 20% to 30% (Hadinger et al., 2009; Hsieh et al., 2004; Takahashi et al., 1999; Wohl and Weiss, 1998). However, the shared benefits and interconnected mechanisms of ATRA and VPA strongly suggest the potential superiority of their combined use in inducing neuronal differentiation and promoting cell survival and neurite outgrowth while simultaneously suppressing astrocytic differentiation. Impressively, both of these clinically established drugs display optimal pharmacokinetics and penetrate the blood–spinal cord barrier (Chateauvieux et al., 2010; Chiu et al., 2013; Kern et al., 2007; Mey et al., 2005), which guarantee the future clinical application of their combined use for treatment of CNS injuries. However, no study has focused on the potential combined effects of VPA and ATRA on NSC differentiation. Moreover, the reported differentiation effects of ATRA and VPA have primarily been based on a 4-day drug treatment without investigating the time-course of the changes in NSC differentiation and neurite outgrowth or the long-term treatment effects, which are valuable pieces of evidence for the characterization and treatment timing of these two pharmacological agents.

Therefore, NSCs were isolated from the forebrains of embryonic rats for *in vitro* differentiation induced by ATRA and VPA, either alone or in combination. During a 14-day period of differentiation, the cells were characterized via immunocytochemistry (ICC), western blot and quantitative real-time PCR (qRT-PCR) at various time points. The aim of this study was to characterize the effects of ATRA and VPA on NSC fate determination and neurite outgrowth over an extended time-course, to evaluate the efficacy of their