PERSPECTIVE

Valproic acid as a microRNA modulator to promote neurite outgrowth

Valproic acid (VPA) has been a first-choice drug for clinical treatment of epilepsy and manic disorder. For decades, its pharmacological action was believed to act on inhibition of gamma-aminobutyric acid (GABA) transaminase, in turn, increasing GABA in inhibitory synapses. However, in recent years, VPA has been investigated on other therapeutic actions. Those investigations demonstrate that VPA shows neuroprotective effects by promoting neurogenesis, neuronal differentiation, and neuroregeneration (Foti et al., 2013). Those VPA efficacies had been reported to induce epigenetic changes such as inhibiting histone deacetylases (Tremolizzo et al., 2005; Jessberger et al., 2007) that modulate gene expression changes transcriptionally. To unravel other epigenetic modulators, our research group, together with others, have recently reported that VPA treatment induces microRNAs (miRNAs) expression in the brain (Goh et al., 2011; Hunsberger et al., 2012; Oikawa et al., 2015).

miRNAs are functional nucleic acid molecule about 22 nucleotides in length and that is encoded in the genome. These small chain lengths RNAs are classified into non-cording RNA and silence RNA and subsequent post-transcriptional regulation of gene expression, without changes in DNA sequence. The humane genome has encoded over 1,000 miRNAs, and many miRNAs are expressed in the central nervous system (CNS). miRNAs in the brain have been reported to regulate neuronal development, differentiation, synaptogenesis, and plasticity (Fiore et al., 2011). miRNA-132 has been reported to regulate the cAMP response element binding (CREB) protein pathway that repressed translation of the Rho family GTPase-activating protein, p250GAP and neuronal morphogenesis in developing neurons (Wayman et al., 2008). miRNA-124 contributes to the control of neurite outgrowth during neuronal differentiation through regulation of cytoskeleton to also partially effect *via* Rho GTPase family pathway (Yu et al., 2008). In fact, VPA up-regulated a network of inter-related miRNAs that are intimately linked with neural network development. This fact has more strongly supported the potential of critical period reactivation. Our previous study also shows that miRNAs interact with components of the protein-protein interaction networks that affect dendritic growth and synaptic plasticity in VPA treated mouse brain (Goh et al., 2011).

In our recent study, we propose to add a new function of VPA to modulate miRNAs to affect neurite outgrowth (Oikawa et al., 2015). We demonstrated VPA induced miRNA-124 that down-regulated a novel protein target, guanine nucleotide binding protein (Gi protein), alpha inhibiting 1, or sometimes termed as Gia1 or GNAI1. GNAI1 is primarily expressed in the brain and functions to inhibit adenylate cyclase activity and cAMP dependent pathways. By using a global miRNA microarray analysis, we observed 188 miRNAs differentially changed after 200 mg/kg VPA intraperitoneal infusion over a period of two days. A previous report also supported our findings that VPA induced miRNAs (Hunsberger et al., 2012). Most of the miRNAs were up-regulated (136 out of 188 miRNAs) while 52 miRNAs were down-regulated. We first validated our microarray data with RT-qPCR method in both mouse cortical tissues and mouse primary neuronal culture. We then narrowed it down on four up-regulated miRNAs, miRNA-22, 33a, 124 and 132 and by far, these four produced the most robust results.

We next detected differential protein changes by using iTRAQ method. Approximately more than half of the proteins were up-regulated (83 out of 147 proteins) while 64 proteins were down-regulated. Based on our hypothesis that miRNA functions in RNA silencing and post-transcriptional regulation of gene expression, we focused on down-regulated proteins by VPA. A combination of three different *in silico* algorithm analyses: Target Scan, PicTar, and DIANA, predicted the likely candidate miRNAs that target to down-regulate proteins from our iTRAQ result and miRNA-124 was found to have the highest probability to target at *GNAI1* protein. miRNA-124 is one of the richest miRNA in the brain and it is known to cause neuronal differentiation, maturation, and neurogenesis in normal brain growth.

Next, we investigated if GNAI1 changes were indeed caused by VPA treatment. By treating mouse cortical tissue and primary neuronal culture with VPA treatment, GNAI1 protein was significantly decreased. However, no change in Gnai1 mRNA was observed. VPA down-regulated the expression level of miRNA-124 target protein GNAI1 without Gnai1 mRNA reduction and this suggests that GNAI1 protein was silenced post-transcriptionally by VPA induced miRNA-124. To strengthen our *in silico* prediction, we then investigated GNAI1 protein's regulation by using selective miRNA-124 inhibitor. We performed both experiments in primary neuronal culture and found that miRNA-124 inhibitor markedly increased the GNAI1 protein expression whereas the miRNA-124 mimic significantly decreased the GNAI1 protein expression.

As the function of GNAI1 is to inhibit adenylate cyclase activity, we tested our hypothesis by checking the amount of cAMP concentration in primary neuron culture with the following parameters: with or without VPA, with or without miRNA-124 inhibitor, and with or without *Gnai1* siRNA. Our results showed that cAMP levels were markedly increased by VPA stimulation, markedly decreased by using selective miRNA-124 inhibitor, and significantly increased in *Gnai1* siRNA. We also looked at other downstream effectors and investigated brain-derived neurotrophic factor (BDNF) expression level as our proteomics data suggested VPA aids in neutrophic growth. *Bdnf* mRNA was concomitantly expressed with cAMP activity in each treatment but the up-regulation of GNAI1 and Bdnf were abolished with the application of selective miRNA-124 inhibitor and even with addition of VPA. Conversely, under the application of selective miRNA-124 mimic, GNAI1 was significantly decreased and BDNF was significantly increased by VPA treatment for primary neuron culture. These results suggest that VPA treatment enhanced miRNA-124-GNAI1-BDNF pathway.

We postulated that the cAMP level increased by the inhibition of GNAI1 at downstream cascade. Indeed, VPA treatment also increased PKA expression level and CREB phosphorylation in primary neuronal culture. Similarly, *Gnai1* siRNA treatment increased PKA expression level and CREB phosphorylation. However, with the selective miRNA-124 inhibitor, PKA and CREB phosphorylation expression levels were not affected. These results suggest that the inhibition of GNAI1 at the downstream of miRNA-124-GNAI1 pathway enhanced PKA-pCREB-BDNF cascade. In summary, we have identified a

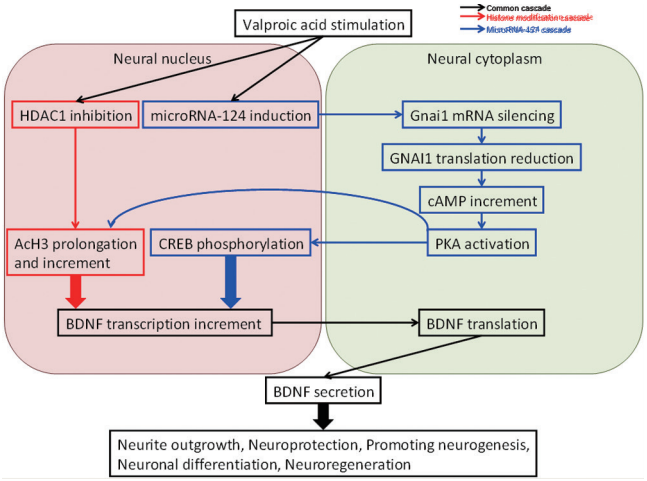

Figure 1 Cross-talk of epigenetic modulation by valproic acid in neurons.
We propose that new function of valproic acid (VPA) as a microRNA modulator and epigenetic modulation of VPA in brain. From this miRNA-124 cascade, brain-derived neurotrophic factor (BDNF) transcription is doubly supported by acetylated histone H3 (AcH3) increment of protein kinase A (PKA) and cAMP response element binding (CREB) phosphorylation. By combining with histone deacetylase 1 (HDAC1) inhibition cascade, secretion of BDNF by VPA is supported. Consequently, it is promoted that neurite outgrowth, neuroprotection, neurogenesis, neuronal differentiation, and neuroregeneration.

molecular mechanism through VPA that induces BDNF under miRNA-124 control and its target protein, GNAI1.

As a follow-up to our study, we used VPA in adult mice and looked at the dendritic arborization in primary visual cortex after VPA treatment. Surprisingly, VPA induced dendritic branching and cortical rewiring in a circuitry, theoretically, should not have such dynamic rewiring at adulthood. We also show that it has potential in reactivating critical period plasticity (Lim et al., in preparation). One of the epigenetic mechanistic actions of VPA is also acting as a HDAC inhibitor. Neurodevelopment, synaptic plasticity, and memory formation are dynamically regulated by histone acetylation and histone deacetylation (Ballas et al, 2005; Jessberger et al, 2007; Lubin and Sweatt, 2007). Additionally, it has been shown that PKA-induced histone H3 acetylation could play a role in synaptic plasticity (Chwang et al., 2007). To modulate histone acetylation within mammalian brain, we and other groups proposed treating the adult brain with VPA, though its exact mechanism is still relatively unknown. It could be transcription of the BDNF protein sustained by the persistence of acetylation of histone H3 or by the inhibition of histone deacetylases. There could be crosstalk between miRNA and HDACs to consequently control transcription of the BDNF protein *via* the pCREB and PKA acting through histone H3 acetylation (**Figure 1**). BDNF is well established to direct growth and differentiation in the developing nervous system, and promote neurogenesis, dendritic spine reorganization, and activity-dependent plasticity in adult brain (Nishino et al., 2012). It is speculated that the transcribed BDNF enhances the neurite extension and neurite outgrowth as a functional neurotrophic factor (**Figure 1**). How they specifically target at each other or crosstalk is of interest in the neurochemistry field. We would like to propose the role and the function of VPA as a miRNA inducer, in addition to being an HDAC inhibitor, and that it may become the prospective innovative therapeutic drug of various refractory CNS diseases.

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