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Research article

# Valproic acid and ASK1 deficiency ameliorate optic neuritis and neurodegeneration in an animal model of multiple sclerosis

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## HIGHLIGHTS

- We examine the effects of VPA on optic neuritis in EAE mice.
- VPA suppresses demyelination in the optic nerve and protects retinal neurons.
- VPA shows enhanced effects on retinal protection in ASK1-deficient EAE mice.
- VPA and ASK1 inhibition may be useful for treatment of optic neuritis and MS.

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## ABSTRACT

Optic neuritis, which is an acute inflammatory demyelinating syndrome of the central nervous system, is one of the major complications in multiple sclerosis (MS). Herein, we investigated the therapeutic potential of valproic acid (VPA) on optic neuritis in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. EAE was induced in C57BL/6 mice by immunization with $MOG_{35-55}$ and VPA (300 mg/kg) was administered *via* intraperitoneal injection once daily from day 3 postimmunization until the end of the experimental period (day 28). VPA treatment suppressed neuroinflammation and decreased the clinical score of EAE at an early phase (from day 12–14 after immunization). We also examined the effects of apoptosis signal-regulating kinase 1 (ASK1), an evolutionarily conserved signaling intermediate for innate immunity, in EAE mice. ASK1 deficiency strongly suppressed microglial activation and decreased the clinical score of EAE at a late phase (day 25, 27 and 28 after immunization). When VPA was administered to ASK1-deficient EAE mice, the clinical score was suppressed in both early and late phases (from day 12–28 after immunization) and showed synergistic effects on protection of retinal neurons. Our findings raise intriguing possibilities that the widely prescribed drug VPA and ASK1 inhibition may be useful for neuroinflammatory disorders including optic neuritis and MS.



## 1. Introduction

Optic neuritis is inflammation of the optic nerve and is the most common type of optic neuropathy. Patients usually present with an acute reduction of visual acuity, orbital pain exacerbated by eye movements, dyschromatopsia, and an afferent papillary defect, with or without swelling of the optic nerve head. Optic neuritis is the initial presentation in approximately 20% of multiple sclerosis (MS) cases and 30–70% of MS patients develop optic neuritis during the course of their disease [5,22]. Since optic neuritis can cause severe visual loss, especially in the optic-spinal form of MS or neuromyelitis optica [13,20], and this loss is irreversible currently, it draws much attention to finding a treatment that will restore the visual function.

Valproic acid (VPA) is a short-chain fatty acid and is used worldwide clinically for treatment of epilepsy, mood disorders, migraines and neuropathic pain [4,11,17,27]. The pharmacological action of VPA involves multiple mechanisms including those that affect intracellular signal transduction pathways. For example, VPA may modulate enzymatic activities such as extracellular-signal-regulated kinases (ERK), phosphatidylinositol 3-kinase/Akt-1, and glycogen synthase kinase 3β, as well as histone deacetylase (HDAC) [6,9,23,25]. Recently, the concept that VPA exerts neuro-

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protective effects has emerged [2,3,15,16,29] and in addition, VPA may ameliorate inflammation of the spinal cord in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, by suppressing the activation of T cells [18].

Another factor that is involved in the severity of EAE is apoptosis signal-regulating kinase 1 (ASK1), which is one of a growing number of mitogen-activated protein kinase (MAPK) kinase kinases identified in the upstream of the c-Jun N-terminal kinase and p38 MAPK pathways [12]. We previously reported that Toll-like receptor (TLR)-ASK1 signaling is required for chemokine productions in astrocytes and for recruitment of activated microglia into the lesion site during EAE [7]. In addition, the same signaling pathways in microglia seem to modulate progression of demyelination by altering the release of proinflammatory components. These results suggest that VPA and ASK1 may regulate the activity of different cell types that play important roles during EAE. To determine this possibility, in this study, we investigated the therapeutic potential of VPA against optic neuritis in EAE mice and examined whether ASK1 deficiency has synergistic effects with VPA.

## 2. Materials and methods

### 2.1. Animals

Female C57BL/6J and ASK1$^{-/-}$ (ASK1 KO) mice [10] were 6–8 weeks of age at the time of immunization. Animal treatments were performed in accordance with the Tokyo Metropolitan Institute of Medical Science Guidelines for the Care and Use of Animals.

### 2.2. EAE induction, VPA administration, and clinical scoring

EAE was induced with $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) as previously reported [7,21]. Briefly, mice were subcutaneously injected with 100 μg of $MOG_{35–55}$ mixed with 500 μg of heat-killed *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA) emulsified in complete Freund's adjuvant. Each mouse also received intraperitoneal injections of 500 ng pertussis toxin (Merck Millipore, Billerica, MA, USA) immediately and 48 h after the immunization. To evaluate the effect of VPA, mice were treated with either VPA (300 mg/kg) or vehicle (PBS) once daily by intraperitoneal administration from day 3 postimmunization until the end of the experimental period (day 28). Clinical signs were scored daily as follows: 0, no clinical signs; 1, loss of tail tonicity; 2, flaccid tail; 3, impairment of righting reflex; 4, partial hind limb paralysis; 5, complete hind limb paralysis; 6, partial body paralysis; 7, partial forelimb paralysis; 8, complete forelimb paralysis or moribund; 9, death.

### 2.3. Histopathology and immunohistochemistry

At the end of the experimental period, mice were perfused with Zamboni's Fixative (2% paraformaldehyde and 15% picric acid in 0.1 M phosphate buffer). Eyes were enucleated and post-fixed in 3% glutaraldehyde solution (3% glutaraldehyde, 9% formaldehyde, 37.5% ethanol and 12.5% acetic acid in distilled water) for 2 h. Paraffin embedded retinal sections of 7 μm thickness were cut through the optic nerve and stained with hematoxylin and eosin (HE). The extent of retinal degeneration was quantified by counting the number of neurons in the ganglion cell layer (GCL) from one ora serrata through the optic nerve to the other ora serrata [10]. Optic nerves and spinal cords were post-fixed in Zamboni's Fixative for 2 h and cut into 10 μm thick sections. Immunohistochemistry was performed using the following primary antibodies: rabbit anti-GFAP (1:2; Abcam, Cambridge, MA, USA), goat anti-Iba1 (1:400; Abcam), and mouse anti-CD3 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). To quantify the stained region, the spinal cord sections were divided into four quadrants and the immunointensity in the area of one quadrant of the dorsal lateral region was calculated using ImageJ 1.50c4 (NIH, Bethesda, MD, USA). To analyze the optic nerve, the area 2 mm from the optic nerve head (0.04 mm$^2$), was evaluated. GFAP and Iba1 immunointensity are expressed as fold changes relative to the WT non-EAE mice. The number of T cells is expressed as percentage of the WT EAE mice. To evaluate the extent of demyelination, optic nerve sections were stained with Luxol fast blue (LFB) followed by HE. Demyelination is expressed as percentage decrease in myelinated (stained) area relative to WT non-EAE mice.

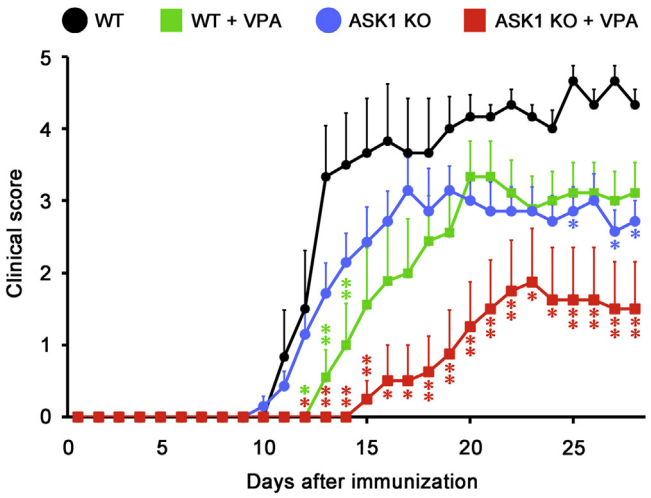


**Fig. 1.** Effects of VPA on clinical evaluation of EAE in WT and ASK1 KO mice during a period of 28 days after MOG immunization.
Mice were treated with either VPA (300 mg/kg) or vehicle (PBS) for 25 days (once daily, *i.p.*) from 3 days after MOG immunization. Data are presented as mean ± S.E.M. WT; $n = 6$, WT + VPA; $n = 9$, ASK1 KO; $n = 7$, ASK1 KO + VPA; $n = 8$. $^{**}p < 0.01$; $^{*}p < 0.05$.

### 2.4. Statistics

Data are presented as means ± S.E.M. When statistical analyses were performed, the one-way ANOVA with Tukey-Kramer post hoc test was used to estimate the significance of the results. $P < 0.05$ was regarded as statistically significant.

## 3. Results

### 3.1. Effects of VPA and ASK1 deficiency on disease severity during EAE

Mice were observed for a period of 28 days after MOG immunization. Wild-type (WT) EAE mice started to show disease signs about 10 days after disease induction (Fig. 1), and reached an incidence of 100%. Daily treatment of VPA significantly suppressed the severity of clinical symptoms in WT EAE mice from day 12–14. As we previously reported [7], the clinical score in ASK1 KO EAE mice was decreased at day 25, 27 and 28 compared with WT EAE mice. When ASK1 KO mice were treated with VPA, the average clinical score was decreased from day 12 through to the end of the experimental period compared with WT EAE mice.

We then examined the spinal cords histopathologically at day 28 (Fig. 2A). The immunointensity of glial fibrillary acidic protein (GFAP)-positive astrocytes (Fig. 2B) and Iba1-positive microglia (Fig. 2C) was increased in WT EAE mice, but this increase was milder in VPA-treated WT EAE mice. The immunointensity of Iba1-positive microglia in ASK1 KO EAE mice was significantly lower than those in VPA-treated WT EAE mice (Fig. 2C). Since T cells play critical roles in EAE, we also examined the infiltration of CD3-