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# Valproic acid, a mood stabilizer and anticonvulsant, protects rat cerebral cortical neurons from spontaneous cell death: a role of histone deacetylase inhibition

Mi Ra Jeong[a], Ryota Hashimoto[a], Vladimir V. Senatorov[a], Koichiro Fujimaki[a], Ming Ren[a], Min Soo Lee[b], De-Maw Chuang[a,*]

[a] *Molecular Neurobiology Section, National Institute of Mental Health, National Institutes of Health, Bldg. 10, Rm. 4C-206, 10 Center Dr MSC 1363, Bethesda, MD 20892-1363, USA*

[b] *Department of Psychiatry, College of Medicine, Korea University, Seoul 136-701, South Korea*

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**Abstract We studied the neuroprotective effects of valproic acid (VPA), a primary mood stabilizer and anticonvulsant, in cultured rat cerebral cortical neurons (CCNs). CCNs underwent spontaneous cell death when their age increased in culture. As shown by mitochondrial activity and calcein-AM assays, treatment of CCNs with VPA starting from day 9 in vitro markedly increased viability and prolonged the life span of the cultures. The neuroprotective action of VPA was time-dependent and occurred at therapeutic levels with a maximal effect at about 0.5 mM. LiCl (1 mM) also protected CCNs from aging-induced, spontaneous cell death but less effectively. VPA-induced neuroprotection in aging CCN cultures was associated with a robust increase in histone H3 acetylation levels and the protective effect was mimicked by treatment with a histone deacetylase inhibitor, trichostatin A, but not by VPA analogs which are inactive in blocking histone deacetylase. Our results suggest a role of histone deacetylase inhibition in mediating the neuroprotective action of VPA.**



*Key words:* Valproate; Neuroprotection; Cerebral cortical neuron; Histone deacetylase; Bipolar mood disorder; Lithium

## 1. Introduction

Valproic acid (VPA), a short-chained fatty acid, is widely used for the treatment of seizures. The mechanism(s) underlying its anticonvulsant activity is not well understood, but could be related to its ability to increase the level of γ-aminobutyric acid (GABA), an inhibitory neurotransmitter, and to enhance the sensitivity of GABA receptors in the brain (for review, [1]). Chronic treatment with VPA was also found to be beneficial for patients with bipolar mood disorder [1,2]. VPA is one of the primary drugs used in the treatment and prophylaxis for both manic and depressive phases of this mood disorder. Despite intensive research, the mechanisms underlying the therapeutic effects of VPA remain obscure. Since clinical efficacy for VPA requires chronic treatment, it has been postulated that drug-induced changes in gene expression are required for its therapeutic actions [3]. Recently, VPA was reported to directly inhibit histone deacetylase (HDAC) at therapeutic levels (with an $IC_{50} = 0.4$ mM), causing histone hyperacetylation [4,5]. HDAC has been strongly implicated in the modulation of gene expression as well as life span in a variety of organisms such as yeast, *Caenorhabditis elegans* and *Drosophila* [6]. In addition, VPA has been shown to activate the cell survival factor, Akt, presumably through inhibition of HDAC ([7], E. Chalecka-Franaszek and D.-M. Chuang, unpublished observations).

Recently, we found that lithium is neuroprotective against a variety of insults including excitotoxicity in cultured central nervous system neurons as well as animal models of diseases (for review, [8]). Our preliminary data also indicate that VPA protects mature cerebellar granule cells in cultures from glutamate-induced, *N*-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity, but is severely toxic to immature cerebellar granule cells (H. Kanai and D.-M. Chuang, unpublished observations). In this study, we used rat primary neuronal cultures prepared from embryonic cortex, a brain region where neuroanatomical abnormality has been implicated in the pathogenesis of bipolar mood disorder [9]. The goals of the present study were: (1) to investigate whether VPA has neuroprotective effects in cultured cortical neurons (CCNs) undergoing spontaneous cell death and (2) to characterize this VPA-induced neuroprotection and examine whether inhibition of HDAC is involved.

## 2. Materials and methods

### 2.1. Animals

All procedures employing experimental rats were performed in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals.

### 2.2. Primary cultures of rat CCNs and drug treatments

CCNs were prepared from 17-day-old embryonic rats and cultured as described previously [10]. In brief, the cortical tissues were collected from embryonic rat brain and meninges were removed under sterile conditions. The cells were dissociated by trypsinization and trituration, followed by DNase treatment. The cells were resuspended in

*Correspondence author. Fax: (1)-301-480-9290.
*E-mail address:* chuang@helix.nih.gov (D.-M. Chuang).

*Abbreviations:* CCNs, cerebral cortical neurons; HDAC, histone deacetylase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 2M2P, 2-methyl-2-pentenoic acid; POI-1, propyl oligopeptidase inhibitor-1; TSA, trichostatin A; VPA, valproic acid

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neuron-defined, serum-free Neurobasal medium (Gibco BRL, Grand Island, NY, USA), supplemented with B-27, glutamine (500 μM) and glutamate (25 μM). The cells were plated at a density of $4.2\times10^5$ cells/cm$^2$ on polyethyleneimine-coated 96-well or 6-well plates. Cultures were then kept in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C. Over 95% of the cells present on day 5 in vitro were differentiated into neurons, as characterized by the appearance of long neurites expressing neurofilament protein. On day 9 in vitro, cultures were treated with 0.1–1 mM VPA and maintained until the measurements.

### 2.3. Measurement of cell viability

Cortical neuronal cultures were plated on 96-well plates and maintained for 23 days. Viability of cortical neurons was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which detects mitochondrial dehydrogenase activity [10]. Cell viability was expressed as a percentage of the value in untreated control culture. One-way ANOVA was used for statistical analysis, and significant differences in cell viability were determined by post hoc comparisons of means using the Bonferroni test. In some experiments, cell viability was also assessed in CCNs grown in 6-well plates using the membrane-permeable dye calcein-AM, which is hydrolyzed by an intracellular esterase in viable cells to yield a green fluorescent product. Cells were washed three times with phosphate-buffered saline (PBS) and then incubated with 2 μM calcein-AM for 10 min at 37°C before microscopic examination for green fluorescence.

### 2.4. Nuclear staining of DNA

For microscopic nuclear DNA analysis, cortical neuronal cultures were plated on 12-mm glass coverslips and maintained for 23 days. Cultures were washed with PBS and fixed with 4% formaldehyde in PBS for 10 min at 4°C. After fixation, cells were washed three times with PBS and then stained with 5 μg/ml Hoechst dye 33258 for 5 min at 4°C to detect chromatin condensation. Stained cells were washed again with PBS. Cells were visualized under UV illumination (400×) using a Zeiss Axiophot microscope.

### 2.5. Western blotting

All procedures of Western blotting analysis of protein have been described elsewhere [10]. Briefly, cells were lysed with sodium dodecyl sulfate (SDS) buffer (50 mM Tris–HCl, pH 7.5, 0.5% SDS, 1 mM dithiothreitol) and the lysates were sonicated and protein concentrations were determined by the BCA kit (Pierce, New York, NY, USA) using bovine serum albumin as the standard. Aliquots of 50 μg pro-

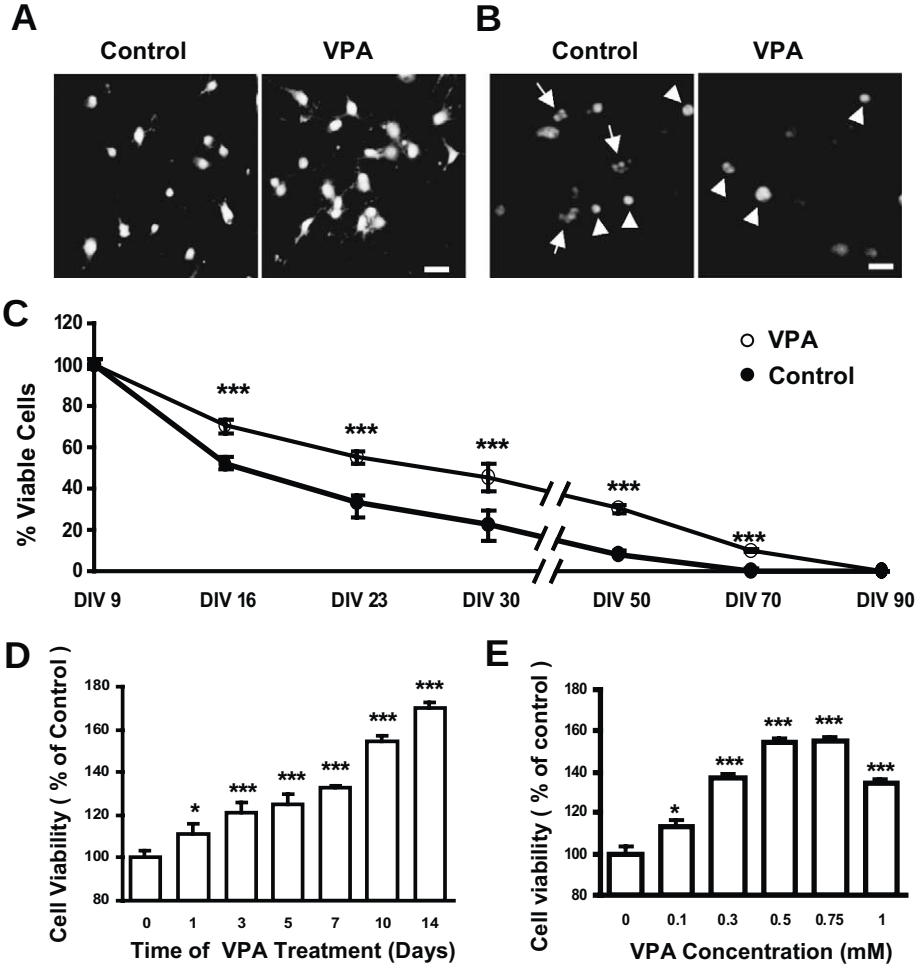


Fig. 1. VPA promotes cell survival in aging cortical neuronal cultures. A: VPA-induced neuroprotection revealed by fluorescent microscopy. CCN cultures were treated with 0.5 mM VPA or its vehicle for 14 days starting after day 9 in vitro. Viable cells were stained with calcein-AM yielding green fluorescence. Bar: 30 μm. B: VPA inhibits aging-induced chromatin condensations. CCN cultures were treated with 0.5 mM VPA from day 9 in vitro for 14 days then treated with Hoechst 33258 for nuclear DNA staining. Photographs of cell nuclei are from a typical experiment of three independent repeats with similar results. Arrows show nuclear fragmentation and arrowheads show nuclear chromatin condensation. Bar: 10 μm. C: Life span extension by VPA. Cortical neurons were treated with 0.5 mM VPA from day 9 in vitro and viable cells stained with calcein-AM were counted at various times thereafter. Data are the means ± S.E.M. of the percent of viable cell number on day 9 in vitro in the control culture and are derived from three independent experiments. ***$P<0.001$ compared with respective control. D,E: Time- and dose-dependent neuroprotection by VPA. CCN cultures were exposed to 0.5 mM VPA for various times (0–14 days) (D) or to indicated concentrations of VPA for 14 days (E). All neuronal viabilities were determined on day 23 in vitro using the MTT assay. Data are the means ± S.E.M. of the percent of vehicle control from five independent experiments. *$P<0.05$, ***$P<0.001$ compared with control.

tein were subjected to SDS–polyacrylamide gel electrophoresis. Separated proteins were transferred onto a polyvinylidene difluoride membrane, which was incubated with a primary polyclonal antibody against acetyl histone H3 (Upstate, Lake Placid, NY, USA). Membranes were washed and then exposed to the secondary antibody. Immunoreactive protein bands were visualized by using horseradish peroxidase-conjugated anti-rabbit IgG and ECL detection reagent (Amersham Biosciences, Piscataway, NJ, USA). Protein intensities were quantified using a CCD camera (Sierra Scientific, Sunnyvale, CA, USA) and Macintosh NIH Image 1.62 software. Data were evaluated for significance by one-way ANOVA and Student's *t*-test.

## 3. Results

### 3.1. Protective effects of VPA on aging-induced spontaneous neuronal cell death

CCNs underwent spontaneous cell death as their age increased in culture. Treatment of CCNs with 0.5 mM VPA starting from day 9 in vitro markedly suppressed spontaneous cell death assessed by viable cell staining with calcein-AM within a time frame of 23 days after plating (Fig. 1A). The aging cultures showed extensive chromatin condensation and nuclear fragmentation, a hallmark of apoptosis (Fig. 1B). The occurrence of these apoptotic markers was also inhibited by VPA treatment. The life span of CCNs was approximately 90 days in VPA-treated cultures, compare with about 70 days in saline-treated controls (Fig. 1C). The number of viable neurons was increased to 140%, 166% and 210% of the respective control values after VPA treatment for 1, 2, and 3 weeks, respectively. The neuroprotective effect of VPA was time-dependent with a progressive increase in the extent of neuroprotection between 1 day and 14 days of treatment (Fig. 1D). The VPA neuroprotection was evident in the concentration range of 0.1–1.0 mM following treatment for 14 days with maximal protection at 0.5–0.75 mM (Fig. 1E).

### 3.2. Drug selectivity for neuroprotection in cortical neuronal cultures

Other drugs were examined for their neuroprotective effects in the CCN cultures. The mood stabilizer lithium protected CCNs from spontaneous cell death but less robustly (Fig. 2A). Another mood stabilizer, carbamazepine (10–100 μM), neuroleptics such as haloperidol (1–100 nM) and risperidone (10–300 nM) as well as antidepressants such as desipramine (0.1–3 μM) and fluoxetine (0.1–3 μM) did not have significant effects (Fig. 2B,C).

### 3.3. Role of HDAC inhibition in VPA-induced neuroprotection

The neuroprotective effect of VPA in the aging CCN cultures was associated with a marked hyperacetylation of histone H3. Following treatment for 14 days with VPA (0.5 mM) or trichostatin A (TSA) (30 nM), a HDAC inhibitor, the levels of acetylated histone H3 were increased by more than two-fold (Fig. 3A), suggesting the occurrence of HDAC inhibition. The VPA-induced elevation of histone H3 acetylation was time-dependent with a significant increase after treatment for 1–7 days, and an even more robust elevation after 2 weeks of treatment (Fig. 3B). Exposure of CCN cultures to up to 30 nM TSA for 14 days also induced a concentration-dependent neuroprotection; further increase of concentration resulted in reversal of the protective effect (Fig. 4A). In contrast, treatment with valpromide and 2-methyl-2-pentenoic acid (2M2P), two structural analogs of VPA devoid of HDAC inhibitory activity [4], were without effect on neuronal viability in the examined concentration range of 0.1–1 mM (Fig. 4B). The spontaneous cell death in CCN cultures was also unaffected by the presence (from 9 to 23 days in vitro) of inositol (1–10 mM), glucose (1–10 mM), an inhibitor of caspase-3 (DEVD-fmk; 10–100 μM), antagonists of NMDA re-

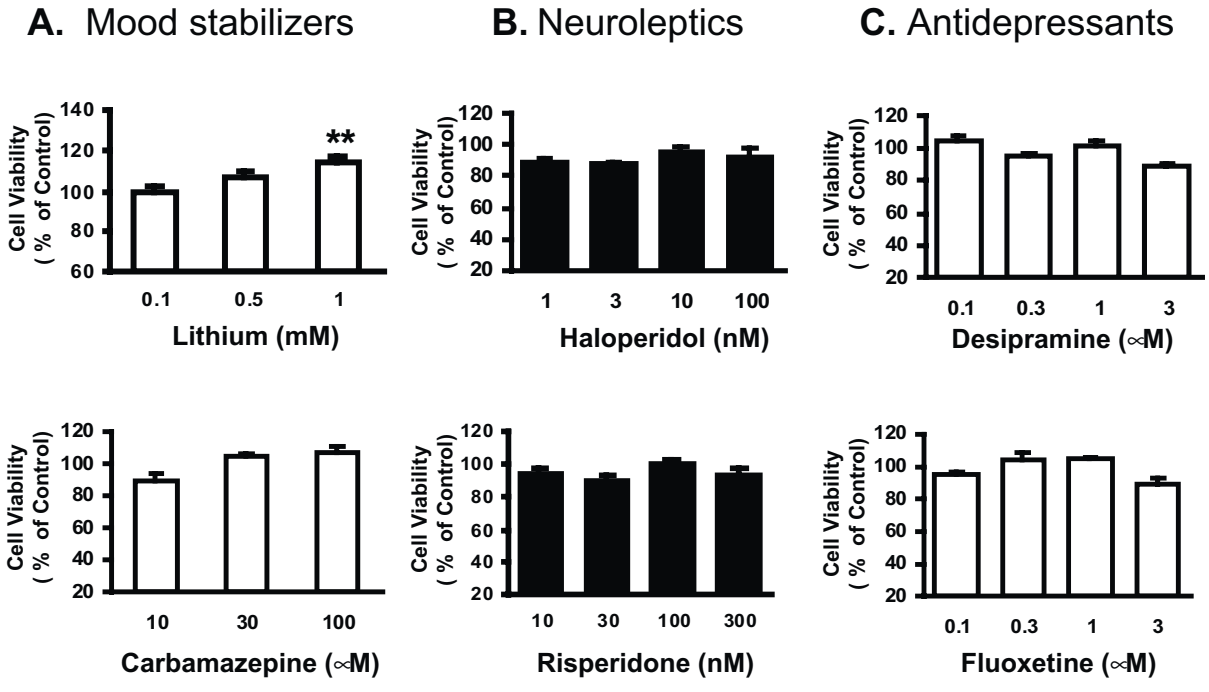


Fig. 2. Selectivity of drug-induced neuroprotection in cortical neuronal cultures. CCN cultures on day 9 in vitro were treated with indicated concentrations of mood stabilizers (A), neuroleptics (B), or antidepressants (C). Cell viability was determined on day 23 in vitro using the MTT assay. Data are the means ± S.E.M. of the percent of vehicle control from three independent experiments. $^{**}P < 0.01$ compared with control.

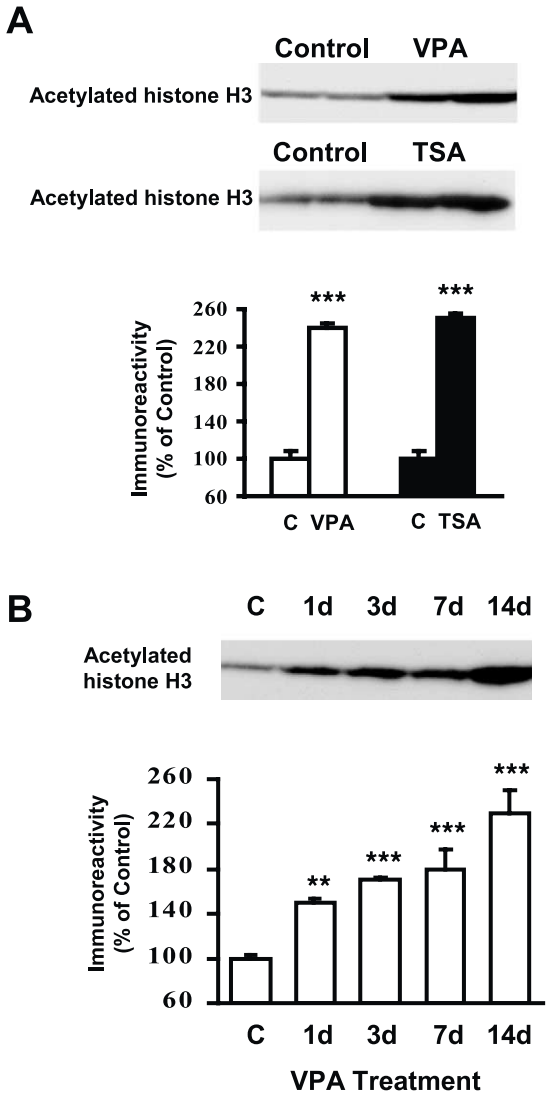


Fig. 3. Effects of VPA and TSA on levels of histone H3 acetylation in cortical neuronal cultures. A: VPA and TSA increase levels of acetylation of histone H3. CCN cultures after 9 days in vitro were treated with 0.5 mM VPA or 30 nM TSA for 14 days and then harvested for Western blotting for levels of acetylated histone H3. Upper panels: acetylated histone H3 immunoblots in duplicate. Lower panel: quantified relative immunoreactivity of acetylated histone H3 protein bands expressed as means ± S.E.M. of the percent of vehicle control averaged from three independent experiments. B: VPA-induced increase of acetylated histone H3 levels is time-dependent. Cortical cultures were treated with 0.5 mM VPA or its vehicle for the indicated times. All cultures were harvested on day 23 in vitro for Western blotting. Upper panel: acetylated histone H3 immunoblot. Lower panel: quantified relative immunoreactivity of acetylated histone H3 protein band expressed as means ± S.E.M. of the percent of untreated control averaged from three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control.

ceptors (MK-801; 10–50 μM) and non-NMDA receptors (CNQX and DNQX; 10–50 μM) as well as propyl oligopeptidase inhibitor-1 (POI-1; 1–100 μM) (data not shown).

## 4. Discussion

The present study shows that VPA markedly prolonged the life span of CCNs when added to maturing cultures. The neuroprotective effect was concentration-dependent with a maximal effect occurring at 0.5–1.0 mM which is within the therapeutic dose range of this drug used for the treatment of manic-depressive illness and seizure. The selectivity of the protection was examined using other mood stabilizers. Lithium was also neuroprotective against spontaneous cell death but less robustly, while carbamazepine was ineffective. Moreover, the neuroleptic haloperidol and clinical antidepressants were unable to protect these neurons from cell death, thus indicating the selectivity of the neuroprotection. In the case of cerebellar granule cells in cultures, the spontaneous, aging-induced apoptotic death is robustly suppressed by NMDA receptor antagonist [11]. However, the death of CCNs was unaffected by antagonists of NMDA and non-NMDA receptors, suggesting that it is not due to excitotoxicity induced by glutamate excessively released into the medium. Although the aging culture of CCN is characterized by the appearance of the apoptotic marker chromatin condensation, the culture was not protected by a caspase-3 inhibitor, implying that other form(s) of caspases are involved. It has been recently reported that VPA and lithium inhibit the collapse of growth cones and increase the growth cone area in sensory neurons [12]. Moreover, these VPA and lithium effects are blocked by supple-

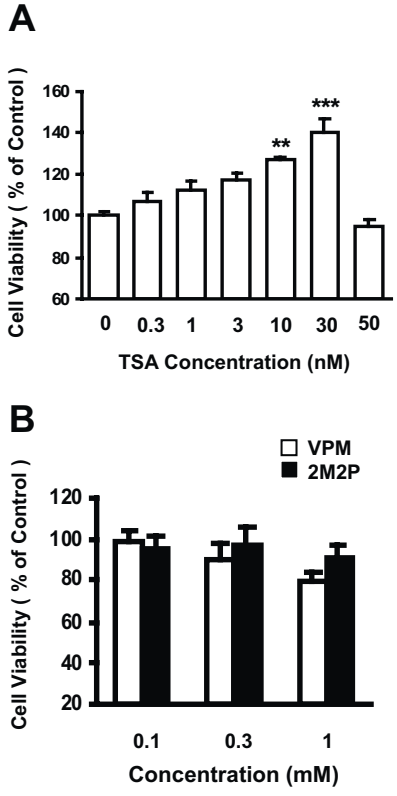


Fig. 4. TSA, but not valpromide or 2M2P, increases viability of cortical neurons in aging cultures. A: TSA-induced neuroprotection. CCN cultures were treated with indicated concentrations of TSA for 14 days. Cell viability was determined on day 23 in vitro using MTT assay. Data are the means ± S.E.M. of the percent of vehicle control from three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control. B: Lack of effect of valpromide and 2M2P on cell viability. CCN cultures were treated with indicated concentrations of valpromide or 2M2P for 14 days. Cell viability was determined on day 23 in vitro using the MTT assay. Data are the means ± S.E.M. of the percent of vehicle control from three independent experiments.

mentation with inositol or addition of propyl oligopeptidase inhibitors. However, in the present study, we found that inositol and POI-1 affected neither the spontaneous cell death nor the neuroprotective action of VPA. Thus, inositol depletion and propyl oligopeptidase activity do not seem to participate in neuroprotection by VPA. VPA appears to have subtle effects of increasing the length and number of neurites of CCNs after 2 weeks of treatment (Fig. 1A). These effects could be related to the observations by Hall et al. [13] that VPA induces axonal remodeling and synapsin clustering in developing neurons. It has been suggested that these effects are due to indirect inhibition by VPA of the activity of glycogen synthase kinase-3 (GSK-3) [13]. The role of GSK-3 in mediating the neuroprotective effects of VPA in CCNs requires further studies.

VPA-induced neuroprotection was concentration- and time-dependently associated with an increased level of histone H3 acetylation, thus consistent with the ability of this drug to inhibit HDAC [4,5] and the role of this action in prolonging the life span of our neuronal cultures. This notion is further supported by our observation that TSA, a prominent HDAC inhibitor, mimics the neuroprotective effect of VPA on cortical neurons. Additionally, valpromide and 2M2P, which are structurally related to VPA but unable to inhibit HDAC [4], were ineffective in protecting CCNs. It should be noted that both valpromide and 2M2P are anticonvulsants with no teratogenic effects, dissimilar to VPA [14]. Based on these observations, it has been suggested that HDAC inhibition is related to the teratogenic effect of VPA [4,5]. Our results provide the novel information that HDAC inhibition can contribute to the neuroprotective effect of VPA.

HDACs such as RPD3 and SIR2 are known to be involved in the regulation of life span of yeast and the nematode *C. elegans* [15,16]. A HDAC inhibitor, phenylbutyrate, also significantly increases the life span of *Drosophila* [6]. It has been suggested that induction and/or repression of genes involved in longevity through HDAC inhibition is a determining factor for increasing the life span in those organisms [6]. Some of the prominent genes markedly induced by phenylbutyrate in *Drosophila* are superoxide dismutase which has a central role in antioxidation and detoxification, and the cytoprotective heat shock response proteins [6]. Our preliminary results showed that the expression of superoxide dismutase was unaffected by VPA exposure for 2 weeks in cortical neurons, but heat shock protein 70 appeared to be significantly up-regulated. Interestingly, HDAC inhibition results in down-regulation of glyceraldehyde-3-phosphate dehydrogenase [6]. We have reported that this so-called housekeeping gene is in fact up-regulated and plays a prominent role in aging-induced apoptosis of cultured cerebellar granule cells [17]. Future studies will be necessary to identify precise VPA-targeted gene(s) involved in the neuroprotection of CCN cultures. In this context, VPA has been reported to induce anti-apoptotic proteins such as Bcl-2 and GRP-78 (78-kDa glucose-regulated protein) [18,19]. It remains to be investigated whether these genes are under the regulation of histone deacetylation and if they have a major role in VPA-elicited neuroprotection in cortical neuronal cultures.

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