A proapoptotic effect of valproic acid on progenitors of embryonic stem cell-derived glutamatergic neurons

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Valproic acid (VPA) is a branched-chain saturated fatty acid with a long history of clinical use as an antiepileptic drug (AED). VPA is also known to inhibit histone deacetylases (HDACs) and to cause diverse effects on neural progenitor cells (NPCs) and neurons. Although the neuroprotective or neurodestructive effects of VPA have been investigated in heterogeneous cell populations, in this study, we used homogeneous populations of NPCs and glutamatergic cortical pyramidal neurons, which were differentiated from embryonic stem (ES) cells. At therapeutic concentrations, VPA had a proapoptotic effect on ES cell-derived NPCs of glutamatergic neurons, but not on their progeny. This effect of VPA most likely occurred through the inhibition of HDACs, because similar phenotypes were observed following treatment with other HDAC inhibitors (HDACis) such as trichostatin A and sodium butyrate. The proapoptotic phenotype was not observed when cells were exposed to a structural analog of VPA, valproamide (VPM), which has the same antiepileptic effect as VPA, but does not inhibit HDACs. Western blotting confirmed that treatment with HDACis, but not VPM, significantly increased the levels of histone H3 acetylation in NPCs. HDACi treatments did not affect the survival of neurons, although the acetylation levels were increased to a limited extent. These results, which are based on a homogeneous culture system, suggest that VPA inhibits HDAC activity and induces the apoptosis of NPCs that are fated to differentiate into glutamatergic neurons. The dose-dependent effects of VPA both on apoptosis and hyperacetylation of histone H3 in NPCs supported this notion. These cell type- and differentiation stage-specific effects of VPA imply that dysfunction of HDACs during pregnancy significantly increase the risk of congenital malformations associated with VPA administration.

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Abbreviations: ES cell, embryonic stem cell; NPC, neural progenitor cell; VPA, valproic acid; TSA, trichostatin A; NaB, sodium butyrate; VPM, valpromide; DW, distilled water; DMSO, dimethyl sulfoxide; AED, antiepileptic drug; GABA, y-aminobutyric acid; HDAC, histone deacetylase; HDACi, HDAC inhibitor; ERK, extracellular regulated kinase; PKC, protein kinase C; GSK-3/y, glycogen synthase kinase-3/y (GSK-3/y)), peroxisome proliferator-activated receptor activation, proteasomal degradation of HDAC and DNA demethylation participate in its antitumor actions.8–14 Among these mechanisms, hyperacetylation of histones, as a result of HDAC inhibition, seems to be the most important one. VPA is also known as a human teratogen.15,16 Maternal ingestion of medication during pregnancy is associated with significantly increased risks for major congenital malformations, especially spina bifida.17 Several studies have proposed that HDAC inhibition by VPA is closely related to teratogenesis in vertebrate embryos.4,18,19 A growing body of evidence suggests that VPA has neuroprotective and neurotrophic effects on neural progenitor cells (NPCs) and neurons, apart from its function as an AED. It promotes neuroplasticity, neurogenesis and cell survival. However, the precise molecular mechanisms of these effects have not been fully understood. Recent studies suggest that HDAC inhibition, PKC inhibition, inhibition of GSK-3/y via Wnt-mediated signaling, ERK activation and phosphatidylinositol-3 kinase-protein

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kinase A activation by brain-derived neurotrophic factor, all
mediate the actions of VPA. Furthermore, many of these
pathways are interconnected.20–28 Despite numerous reports
citing evidence of the neuroprotective effects of VPA, there are
also some contradictory data, which show proapoptotic effects
of VPA and other HDAC inhibitors (HDACis) on NPCs and
neurons.29–32 Considering the antitumor activity and terato-
genic effects of VPA in embryos, one would predict, from the
standpoint of division potential, that VPA might possess
proapoptotic effects on NPCs.

To date, the effects of VPA on NPCs or neurons have been
analyzed in vivo or by using heterogeneous primary cell
cultures. Therefore, the different cell types (glutamatergic,
AChergic, GABAergic or DAergic) and/or differentiation
stages characteristic of the model systems used in earlier
studies might lead to a misinterpretation of the effects of VPA.
In this study, we have utilized a more homogeneous
population of cells by using a sophisticated in vitro differen-
tiation system in order to minimize problems associated with the
use of mixed-cell populations. Bibel et al.33,34 recently
established a retinoic acid-treated embryoid body-based
differentiation protocol that promotes the generation of highly
homogeneous glutamatergic cortical pyramidal neurons from
embryonic stem (ES) cells. The purity of this population is
known to reach 90–95%, which is the highest efficiency of
differentiation to glutamatergic neurons ever reported.35,36
Furthermore, our approach led to substantial gains in
neuronal survival.37 VPA did not have a proapoptotic effect on ES cell-derived glutamatergic neurons, but did demon-
strate proapoptotic effects on NPCs at therapeutic concentra-
tions (0.3–0.7 mM).4,11 The effect of VPA was most likely
through the inhibition of HDACs, because similar phenotypes
were observed following treatment with either of the two other
HDACis, trichostatin A (TSA) and sodium butyrate (NaB).
These phenotypes were not observed after treatment with
capomide (VPM), which is a structural analog of VPA having
the same antiepileptic effect as VPA but lacking the HDACi
activity. The levels of histone H3 acetylation was indeed
increased in NPCs by HDACis, but not with VPM. HDACi
treatments did not affect the survival of neurons, although the
acetylation levels were increased to a moderate degree.
Taken together with previous reports,25,27 these data suggest
that VPA, primarily by the inhibition of HDACs, suppresses
apoptosis and induces neuronal differentiation of heteroge-
neous NPCs, but contrarily induces apoptosis of homoge-
neous NPCs that are fated to differentiate into glutamatergic
neurons. We also observed that VPA had dose-dependent
effects both on apoptosis and hyperacetylation of histone H3
in NPCs, and this also strongly indicates a correlation
between hyperacetylation of histones and apoptosis.

Results

VPA, TSA and NaB, but not VPM, have proapoptotic
effects on NPCs of ES cell-derived glutamatergic neurons.
In our culture system, almost all cells are NPCs of ES
cell-derived glutamatergic neurons just after thawing and
plating (Figure 1a, upper scheme).33,34,37 The purity of this
culture is >97% when measured with immunocytochemistry
(ICC) using the glutamatergic neuronal marker vesicular
glutamate transporter 1 (VGLUT1) 7 days after plating
(Figure 1a, lower panels). To elucidate the effect of VPA on
NPCs of glutamatergic neurons at therapeutic levels (0.3–
0.7 mM),4,11 we treated our NPCs either with 0.5 mM VPA or
distilled water (DW; control) immediately after plating (day 0).
Remarkably, NPCs treated with VPA died within 24 h, so we
investigated caspase-3 activation using ICC to quantify
apoptotic neurons 15 h after VPA treatment (Figure 1b). The
percentage of cleaved caspase-3-positive neurons signifi-
cantly increased to 31% (cleaved capase-3+ cells per all
Tuj1+ neurons: 275.7 ± 48.3 per 862.4 ± 101.1 ) in VPA-
treated cultures, compared with 19% (cleaved capase-3+
cells per all Tuj1+ neurons: 231.9 ± 94.2 per 1223.1 ± 157.3 )
in the control (Figures 2a and b). These apoptotic cleaved
apoptosis, which are as extensive chromatin condensation and
nuclear fragmentation, as assessed by Hoechst staining (data
not shown). To determine the extent to which the proapoptotic
effects of VPA depended on HDAC inhibition,4,5 we also
treated NPCs at day 0 with either 100 nM TSA or 5 mM sodium
NaB (both HDACis), or 0.5 mM VPA, a structural analog of
VPA that lacks HDACi activity. The results were compared
with treatment with vehicle controls (dimethyl sulfoxide
(DMSO), DW and DMSO). At 15 h after the initiation of
treatment with these reagents, we assessed cultures for
apoptosis using the methods described above (Figure 1b).
Treatment with TSA or NaB dramatically increased the
percentage of cleaved caspase-3-positive neurons up to
45% (cleaved capase-3+ cells per all Tuj1+ neurons: 238.4 ± 7.4 per 562.0 ± 58.5 ) or 37% (cleaved capase-3+
cells per all Tuj1+ neurons: 256.7 ± 45.7 per 682.5 ± 103.7 ),
respectively, compared with controls (15% (cleaved capase-
3+ cells per all Tuj1+ neurons: 224.6 ± 38.1 per 1164.4 ± 94.7 ) and 20% (cleaved capase-3+ cells per all
Tuj1+ neurons: 237.8 ± 34.1 per 1180.7 ± 147.5 ), respectively; Figures 2a and b). In contrast, VPM did not have any effect on
apoptosis (Figures 2a and b). Thus, the proapoptotic effect
of VPA on NPCs of glutamatergic neurons was correlated with
the inhibition of HDACs. Moreover, to examine the dose-
response of VPA on apoptosis of NPCs, we also treated NPCs
at day 0 with 0.1–20 mM VPA for 9 h, and investigated
caspase-3 activation by ICC (Figure 1b). The proapoptotic
effect of VPA on NPCs was observed to be dose dependent
within the range of 0.1–20 mM VPA (Figures 2c and d). Proapopto-
tic effects of TSA, and NaB on NPCs were also dose
dependent within the range of 100–300 mM and 1–10 mM,
respectively (data not shown). Importantly, the effective dose
of VPA in this study (0.5 mM) lies within the therapeutic plasma
concentration levels (0.3–0.7 mM).4,11 and the doses of other
three reagents were also within the range of concentrations
common to those of many previous reports.23,25–27,29,30 Thus,
all of our remaining studies except for examinations for dose-
response of VPA were performed using the concentrations of
the four test reagents as mentioned above.

VPA, TSA, NaB and VPM have no proapoptotic effects
on ES cell-derived glutamatergic neurons. Almost all of
our NPCs are designed to differentiate into glutamatergic
neurons within 48 h (Figure 1a, upper scheme)33,34,37

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Therefore, to elucidate the effects of VPA on glutamatergic neurons, we treated the neurons with VPA 3 days after plating (day 3). There were no apparent morphological changes in neurons treated with VPA compared with controls, as assessed by visual inspection. We examined the hallmarks of apoptosis by using the methods described above 24 h after VPA treatment (Figure 1c). ICC analyses revealed that VPA had no proapoptotic effects on these neurons (Figures 3a and b). We also treated the neurons with TSA, NaB or VPM at day 3 and analyzed 24 h later. Compared with the controls, none of the treatment groups showed morphological changes or elicited proapoptotic effects (Figures 3a and b). Furthermore, neurons treated with either of the four reagents remained viable for at least 1 week (data not shown). These data demonstrated that all four reagents had no proapoptotic effects on glutamatergic neurons. To assess the dose-response of VPA on apoptosis of glutamatergic neurons, we also treated neurons at day 3 with 0.1–20 mM VPA for 9 h, and investigated caspase-3 activation by ICC (Figure 1c). VPA had no proapoptotic effect on glutamatergic neurons up to 20 mM (Figures 3c and d).

VPA, TSA and NaB, but not VPM, rapidly and dramatically enhance the levels of histone H3 acetylation in NPCs. To investigate the HDACi activity of VPA, TSA and NaB in NPCs of glutamatergic neurons under the experimental conditions described above, we quantified the levels of histone acetylation in NPCs. We treated our NPCs with VPA, TSA, NaB or their vehicle controls at day 0, and harvested cell lysates from each culture 3, 6, 9 and 12 h after the initiation of treatment (Figure 1b). We analyzed cell lysates by western blotting (WB) using antibodies specific for total histone H3 (C-terminus, pan) and acetylated histone H3. The levels of histone H3 acetylation immediately and substantially increased following treatment with VPA, TSA or NaB, and remained elevated for up to 12 h (Figures 4a–c). In contrast, VPM did not affect the levels of histone H3 acetylation (Figure 4d). These data support the notion that the proapoptotic effect of VPA on NPCs of glutamatergic neurons is most likely due to the inhibition of HDACs. To examine the dose-response of VPA on the level of histone acetylation in NPCs, we also treated NPCs at day 0 with 0.1–20 mM VPA for 9 h, and investigated the level of histone acetylation.
H3 acetylation by WB (Figure 1b). VPA enhanced the level of histone H3 acetylation in NPCs of ES cell-derived glutamatergic neurons in a dose-dependent manner within the range of 0.1–20 mM (Figure 5). Taken together with the similar dose-dependent tendency in the proapoptotic effect of VPA on NPCs (Figures 2c and d), these results strongly suggest a correlation between hyperacetylation of histones and apoptosis. Similar increases in histone H4 acetylation were also observed (data not shown), although its quantitative analyses has been hindered by the low sensitivity and specificity of the anti-acetyl histone H4 antibody.

VPA, TSA and NaB, but not VPM, rapidly increase the levels of histone H3 acetylation in glutamatergic neurons, but less efficiently than in NPCs. To examine whether VPA, TSA, NaB and VPM altered the levels of histone H3 acetylation in glutamatergic neurons, we treated our neurons with each of the four reagents at day 3, and...
harvested cell lysates from each culture 3, 6, 9 and 12 h after the treatments (Figure 1c). WB analyses revealed that the levels of histone H3 acetylation was also enhanced rapidly, but less efficiently than in NPCs, after treatment with VPA, TSA or NaB for up to 12 h (Figures 6a–c). In contrast, VPM did not affect acetylation levels (Figure 6d). These data suggested that VPA also inhibited HDACs in glutamatergic neurons, albeit weakly compared with its effect on NPCs, yet it did not induce apoptosis. To examine the dose-response of VPA on the level of histone acetylation in glutamatergic neurons, we also treated neurons at day 3 with 0.1–20 mM VPA for 9 h, and investigated the level of histone H3 acetylation by WB (Figure 1c). VPA subtly increased the level of histone H3 acetylation also in glutamatergic neurons in a dose-dependent manner within the range of 0.1–20 mM, but the effect was much smaller than in NPCs (Figure 7). Approximately ~2-fold increase was observed at 20 and 0.1 mM in glutamatergic neurons and in NPCs, respectively (Figures 5 and 7), and apoptosis was not induced under these conditions (Figures 2c,d and 3c,d), whereas histone acetylation was increased up to ~10-fold in NPCs concomitantly with the increase in apoptotic cells (Figures 2c,d and 5). Taken together, glutamatergic neurons appear to be more resistant to induction of apoptosis because they are less vulnerable to inhibition of HDACs than their NPCs.

Discussion

Many previous studies have suggested that VPA has neuroprotective and neurotrophic effects both on NPCs and
neurons apart from its function as an AED. However, contradictory data indicate that VPA and other HDACis may have proapoptotic effects on NPCs and neurons. This study demonstrated that VPA has no proapoptotic effects on ES cell-derived glutamatergic neurons, but does have proapoptotic effects on their NPCs at therapeutic concentrations (0.3–0.7 mM). Similar phenotypes were induced by treating cells with either of two other HDACis, TSA and NaB, but not with VPM, a structural analog of VPA, which has the same antiepileptic effect as VPA, but does not inhibit HDAC activity. These results suggest that the proapoptotic effect of VPA on NPCs of glutamatergic neurons might result from the inhibition of HDACs. Consistent with this view, we found a robust increase in the levels of histone H3 acetylation in NPCs treated with VPA, TSA or NaB, but not with VPM. The results of dose-dependent effects of VPA both on apoptosis and hyperacetylation of histone H3 in NPCs of ES cell-derived glutamatergic neurons. Values represent the mean ± S.E.M. of 10–13 separate experiments. TSA and its control (n = 10); NaB and its control (n = 10–12); VPA, VPM and their own controls (n = 13). *P < 0.05; **P < 0.01, by one-way ANOVA followed by Tukey-Kramer’s post hoc test. NS, not significant.

**Figure 4** VPA rapidly and dramatically enhances the level of histone H3 acetylation in NPCs. NPCs treated with VPA, TSA, NaB, VPM or their vehicle control at day 0 were subjected to WB analysis to assess histone H3 acetylation 3, 6, 9 and 12 h after treatments. Each panel consists of a typical immunoblot image detected by antibodies against acetylated (upper) and total histone H3 (middle). Quantification of the relative levels of histone H3 acetylation was done using double normalizations (lower): first, by measuring the ratio of acetyl-H3 to total H3 and second by normalizing against the controls 3 h after the treatment. (a) Change in the levels of histone H3 acetylation after TSA exposure. (b) Change in the levels of histone H3 acetylation after NaB exposure. (c) Change in the levels of histone H3 acetylation after VPA exposure. (d) Change in the levels of histone H3 acetylation after VPM exposure. Note that VPA, TSA and NaB, but not VPM, rapidly and dramatically increased the levels of histone H3 acetylation in NPCs of ES cell-derived glutamatergic neurons. AED, antiepileptic drug; HDAC, histone deacetylase; ES, embryonic stem; NPCs, neural progenitor cells; WB, Western blot; ANOVA, analysis of variance; N.S., not significant.
important factor for such drug treatments. In contrast to NPCs that rapidly underwent apoptosis, neurons were resistant to VPA treatment for 24 h. Although it was possible that the duration of exposure was too short for neurons, we have also confirmed that neurons treated with either of the four reagents remained viable for at least 1 week.

Many studies have suggested that VPA suppresses apoptosis and induces neuronal differentiation of NPCs through the inhibition of HDACs. Among them, Abermatsu et al. specifically examined neurons differentiated from the mouse embryonic forebrain NPCs, which were transplanted into spinal cord injury model mice followed by VPA treatment, and found that 17% were glutamatergic and 70% were GABAergic. Laeng et al. also demonstrated that VPA stimulates GABA neurogenesis from rat forebrain NPCs, but this was not apparently through the inhibition of HDACs. Unlike heterogeneous NPCs isolated from animal brains for primary culture, NPCs used in this study are strongly committed to differentiate into homogeneous glutamatergic neurons, and are most likely induced to undergo apoptosis with VPA through the inhibition of HDACs. These differences can be explained by the action of VPA, which acts primarily to inhibit HDACs, suppress apoptosis, and induce neuronal differentiation of heterogeneous NPCs (especially into GABAergic neurons), but contrarily induces the apoptosis of homogeneous NPCs that are fated to differentiate into glutamatergic neurons.

In this study, VPA, TSA and NaB also increased histone H3 acetylation levels of neurons, albeit weakly compared with their effects on NPCs, but did not significantly affect neuronal survival. Thus, glutamatergic neurons are more resistant to induction of apoptosis because they are less vulnerable to inhibition of HDACs than their NPCs. HDACis are reported to cause acetylated histones to accumulate in tumors (including neuroblastoma cells) as well as in normal tissues (including post-mitotic neurons), but often act selectively to inhibit cell growth of tumors at levels that have little to no toxicity for normal cells. Laeng et al. also demonstrated that the induction of GABA neurogenesis by VPA treatment was effective only in the undifferentiated population of NPCs. Kataoka et al. showed that proapoptotic and antiproliferative effects of VPA on NPCs in the embryonic neocortex, which might be due to the inhibition of HDACi, was exerted transiently only during early embryonic brain development, especially around E12.5. Thus, the proliferation potentials of cells during HDACi exposure appear to be important for the cellular response. Our observation of the differentiation stage-specific proapoptotic effect of VPA can be also explained by the division potentials of NPCs and neurons. For further confirmation, we examined the proliferation potentials of our NPCs and neurons by using ICC for the proliferation marker Ki-67, 2 h and 3 days after plating. Expectedly, among the living cells, 80% of the cells after 2 h of plating, which are thought to be mostly still NPCs, were Ki-67-positive, and the percentage decreased to 7% after 3 days, at which point most cells are thought to be neurons (data not shown).

Several studies have proposed that the HDACi action of VPA is closely related to teratogenesis in vertebrate embryos. However, there are contradictory reports regarding the effects of VPA on apoptotic death of NPCs at developmentally critical periods. Studies have shown that the reduced (Go et al.) or increased (Kataoka et al.) apoptotic deaths of NPCs by VPA treatment underlie neurodevelopmental defects. The findings of our study are consistent with those of Kataoka et al.

Although further studies are required to clarify the detailed mechanism of how HDAC inhibition causes apoptosis, this is the first report demonstrating the cell type- and differentiation stage-specific proapoptotic effects of VPA on homogeneous NPCs of glutamatergic neurons, which is most likely due to the inhibition of HDACs. Our study implies that the dysfunction of HDACs during pregnancy might be responsible for a high risk of congenital malformation.

**Materials and Methods**

**Antibodies and reagents.** The nerve-cell culture medium (SBM, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) was used for culture of glutamatergic neurons. All other cell culture reagents have been described previously. The following reagents were used for experiments investigating the effects of VPA on NPCs and neurons: VPA (Sigma-Aldrich, St. Louis, MO, USA), TSA (Millipore Co., Billerica, MA, USA), NaB (Sigma-Aldrich), VPM (Sigma-Aldrich) and DMSO (Sigma-Aldrich). For ICC, the following antibodies were used: mouse monoclonal antibodies to neuronal class III β-tubulin (TuJ1, 1:1000; Covance Laboratories, Inc., Berkeley, CA, USA), and Ki-67 (1:500; BD Pharmingen, San Diego, CA, USA); rabbit polyclonal antibodies to cleaved caspase-3 (1:200, Cell Signaling Technology, Danvers, MA, USA); and Alexa Fluor 488- or 568-conjugated secondary antibodies: Alexa Fluor 488- and 568-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (1:400; Invitrogen, Carlsbad, CA, USA), rabbit polyclonal antibodies to TuJ1 (1:1000; Covance Laboratories, Inc.), VGLUT1 (1:1000; Synaptic System, Co., Billerica, MA, USA), and cleaved caspase-3 (1:200, Cell Signaling Technology, Danvers, MA, USA). Fluorescent mounting medium was purchased from DakoCytomation Inc., Fort Collins, CO, USA. We used the following fluorescence-conjugated secondary antibodies: Alexa Fluor 488- or 568-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (1:400; Invitrogen, Carlsbad, CA, USA), rabbit polyclonal antibodies to histone H3 C-terminus, pan (1:50,000; Cat. # 07-690, Millipore Co.), and acetyl-histone H3 (1:10,000; Cat. # 06-599, Millipore Co.); and horseradish peroxidase (HRP)-conjugated anti rabbit IgG (1:5000; Cell Signaling Technology).
**Figure 6** VPA rapidly increases the level of histone H3 acetylation in glutamatergic neurons, but less efficiently than in NPCs. Glutamatergic neurons treated either with VPA, TSA, NaB, VPM or their vehicle controls at day 3 were subjected to WB analysis to determine levels of histone H3 acetylation 3, 6, 9 and 12 h after treatments. Each panel portrays a typical immunoblot image detected by antibodies against acetylated (upper) and total histone H3 (middle). Quantification of the relative levels of histone H3 acetylation was done using double normalizations (lower); first, by measuring the ratio of acetyl-H3 to total H3, and second by normalizing against the controls 3 h after the treatments. (a) Change in the levels of histone H3 acetylation after TSA exposure. (b) Change in the levels of histone H3 acetylation after NaB exposure. (c) Change in the levels of histone H3 acetylation after VPA exposure. (d) Change in the levels of histone H3 acetylation after VPM exposure. Note that VPA, TSA and NaB, but not VPM, rapidly enhanced the levels of histone H3 acetylation also in ES cell-derived glutamatergic neurons. Values represent the mean ± S.E.M. of 9–11 separate experiments. TSA and its control (n = 10); NaB and its control (n = 9–10). VPA, VPM and their own controls (n = 11). *P < 0.05; **P < 0.01, by one-way ANOVA followed by Tukey–Kramer’s post hoc test. NS, not significant.

**Cell culture.** The ES cell line we selected was E14TG2a (CRL-1821; American Type Culture Collection, Manassas, VA, USA). ES cell-derived glutamatergic neurons were differentiated essentially as we have previously described with a minor modification (see below).33,34,37 We adjusted the CO₂ content in the incubators to maintain the pH of the cell culture medium to around 7.4 as originally described.34 By changing the medium for glutamatergic neurons from the original medium (complete medium, glutamatergic neuronal marker VGLUT1 confirmed that this modification did not decrease the purity (> 98%).37 Purity in our study was even higher than the purity of cultures grown in original CM (90–95%).33,34 Subsequent to this report, we also tried changing to SBM instead of N₂ medium (N₂M) 24 h earlier. After confirming that this second modification improved neuronal health and survival without decreasing their high purity (> 97%; Figure 1a, lower panels), we decided to use SBM from 24 h after plating for all of the following experiments. In all of the experiments investigating the effects of VPA on NPCs and glutamatergic neurons except for those for dose-response of VPA, cells were treated either with 0.5 mM VPA, 100 nM TSA, 5 mM NaB, 0.5 mM VPM, or their vehicle controls either immediately after plating (day 0) or 3 days after plating (day 3). In the experiments for dose-response of VPA, cells were treated with VPA (0.1, 0.5, 3, 20 mM) or its vehicle control.

**ICC and nuclear staining.** Cells cultured on glass coverslips in 24-well plates were washed with phosphate-buffered saline (PBS). After transferring coverslips to a fresh 24-well plates, we fixed cells with 4% paraformaldehyde in...
We quantified the percentage of cleaved caspase-3-positive neurons (cleaved caspase-3, red) and nuclei were counterstained with Hoechst 33342 or DAPI (blue). We investigated caspase-3 activation by using ICC and WB analysis to determine levels of histone H3 acetylation after a 9-h exposure. Typical immunoblot images with antibodies against acetylated (upper) and total histone H3 (middle) are shown with quantitative data (lower). The relative levels of histone H3 acetylation were quantified using double normalizations: first, by measuring the ratio of acetyl-H3 to total H3, and second by normalizing against the control. Note that VPA enhanced the level of histone H3 acetylation also in ES cell-derived glutamatergic neurons in a dose-dependent manner within the range of 0.1–20 mM, albeit the effect was much weaker than in NPCs. Values represent the mean ± S.E.M. of four separate experiments.

**Detection of apoptosis.** Hallmarks of apoptotic cell death include activation (cleavage) of caspases, condensation, and fragmentation of nuclei and formation of apoptotic bodies. We investigated caspase-3 activation by using ICC and examined extensive chromatin condensation and nuclear fragmentation by using Hoechst staining. Neurons were analyzed by immunofluorescence labeling with the neuronal marker TuJ1 (green) and the apoptotic marker cleaved caspase-3 (red), and nuclei were counterstained with Hoechst 33342 or DAPI (blue). We randomly obtained four representative images per well under the microscope with a 10× objective, and counted all of the cells in those images for one experiment.

**Analysis of histone acetylation levels by WB.** NPCs or glutamatergic neurons were treated with VPA, TSA, NaB, VPM or their vehicle controls, and harvested 3, 6, 9 and 12 h after the treatment. Whole-cell lysates were prepared by boiling samples in SDS-PAGE sample buffer for 5 min, separated by SDS-PAGE, and transferred electrophoretically onto polyvinylidene difluoride membranes (Millipore Co.). The blotted membranes were blocked for 1 h with 5% non-fat dry milk in PBS containing 0.05% Tween-20 (PBS-T) and incubated for 2 h at room temperature or overnight at 4°C, with the primary antibody against anti-acetyl-histone H3 diluted in PBS-T containing 1% non-fat dry milk. After washing in PBS-T, the membranes were incubated for 1 h at room temperature or overnight at 4°C, with an HRP-conjugated anti-rabbit IgG antibody (1:5000). The immune complexes were visualized using the ECL chemiluminescence system (GE Healthcare, Buckinghamshire, UK) with the LAS-3000 image analyzer (Fuji Film, Tokyo, Japan). Then, the membranes were stripped for 30 min at 37°C in Restore Plus Western blot stripping buffer (Thermo Scientific, Rockford, IL, USA) and reprobed with anti-histone H3 and HRP-conjugated anti-rabbit IgG antibodies as above. Note that no signal was detected when the second primary antibody (anti-histone H3) was added in the second blots, indicating that both the first primary and secondary antibodies were efficiently stripped off from the membrane. We measured the intensity of acetyl-histone H3 and total signals using Multi Gauge (Fuji Film), and the acetylation level in each time point was expressed as the relative value to each vehicle control at 3 h after the treatment by double normalization: first by measuring the ratio of acetyl-H3 to total H3 and secondly by normalizing against the control. We performed 9–13 independent experiments for quantification. For Figures 5 and 7, the acetylation level in each VPA concentration was expressed as the relative value to vehicle control by double normalization; first by measuring the ratio of acetyl-H3 to total H3 and secondly by normalizing against the control. Although we also tried to perform similar analyses for histone H4 acetylation, we experienced difficulty with quantitative analyses because of the poor sensitivity and specificity of the anti-acetyl histone H4 antibody.

**Statistical analysis.** The quantitative data are expressed as mean ± S.E.M. of at least three (indicated in the figure legends, when the number was > 3) independent experiments. All statistical analyses of these values were performed using one-way ANOVA followed by Tukey–Kramer’s post hoc test. Values of P < 0.05 were considered statistically significant.

**Conflict of Interest**

The authors declare no conflict of interest.
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