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Valproic Acid and Other HDAC Inhibitors Induce Microglial Apoptosis and Attenuate Lipopolysaccharide- induced Dopaminergic Neurotoxicity

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Abstract

Valproic acid (VPA), a widely prescribed drug for seizures and bipolar disorder, has been shown to be an inhibitor of histone deacetylase (HDAC). Our previous study has demonstrated that VPA pretreatment reduces lipopolysaccharide (LPS)-induced dopaminergic (DA) neurotoxicity through the inhibition of microglia over-activation. The aim of this study was to determine the mechanism underlying VPA-induced attenuation of microglia over-activation. Other HDAC inhibitors (HDACIs) were compared with VPA for their effects on microglial activity. We found that VPA induced apoptosis of microglia cells in a time and concentration-dependent manner. VPA-treated microglial cells showed typical apoptotic hallmarks including phosphatidylserine externalization, chromatin condensation and DNA fragmentation. Further studies revealed that trichostatin A (TSA) and sodium butyrate (SB), two structurally dissimilar HDACIs, also induced microglial apoptosis. The apoptosis of microglia was accompanied by the disruption of mitochondrial membrane potential and the enhancement of acetylation levels of the histone H3 protein. Moreover, pretreatment with

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SB or TSA caused a robust decrease in LPS-induced pro-inflammatory responses and protected DA neurons from damage in mesencephalic neuron-glia cultures. Taken together, our results shed light on a novel mechanism whereby HDACIs induce neuroprotection and underscore the potential utility of HDACIs in preventing inflammation-related neurodegenerative disorders such as Parkinson's disease.

Keywords

HDAC inhibitors; Microglial Apoptosis; Neuroprotection

Introduction

Microglial cells play diverse roles and are highly dynamic surveillants in the brain (Nimmerjahn et al., 2005). They do not merely serve a passive role as scavengers. Instead, over-activated, uncontrollable microglial cells participate in the progressive neuronal degeneration in both animal studies and human diseases (Block et al., 2007). Hence, identification of compounds that modulate microglial reaction under pathological conditions is highly desirable for the development of therapeutic agents.

Valproic acid (VPA) is widely used for the treatment of seizures and bipolar disorder. Reports show that long-term administration of VPA results in neuroprotective effects that render neurons less susceptible to a variety of insults (Dou et al., 2003, Kanai et al., 2004, Ren et al., 2004, Peng et al., 2005, Chen et al., 2006, Leng and Chuang, 2006). These studies suggest that the neuroprotective mechanisms of VPA may involve, at least in part, in the inhibition of histone deacetylase (HDAC), a recently identified target of VPA (Phiel et al., 2001).

HDAC inhibition induces increased acetylation levels of histone proteins, causing chromatin to conform more openly such that transcriptional factors and RNA polymerase interact with DNA to modulate transcription (reviewed in (Camelo et al., 2005). HDAC inhibitors of diverse structures are capable of interrupting cell cycle progression, resulting in growth arrest, differentiation, and apoptotic cell death (Bolden et al., 2006). Previous studies have shown that HDACIs other than VPA also exert neuroprotective effects in various neurodegenerative models (Ferrante et al., 2003, Jeong et al., 2003, Gardian et al., 2004, Minamiyama et al., 2004, Ren et al., 2004, Gardian et al., 2005, Peng et al., 2005, Petri et al., 2006). However, most of these reports focused on the direct effect of HDACIs on neurons with little consideration for the role of microglia in neuroprotection.

Recent reports have demonstrated the immunomodulatory effects of HDACIs on microglia (Suuronen et al., 2003, Huuskonen et al., 2004, Kim et al., 2004b). Our previous study shows that VPA pretreatment robustly attenuates LPS-induced DA neuronal damage in mesencephalic neuron-glia cultures, and that this neuroprotection is in part associated with the suppression of microglia-mediated inflammation and a decrease in the microglial cell number (Peng et al., 2005). Dragunow et al. reported VPA induces apoptosis in microglia cell line, BV-2 (Dragunow et al., 2006). However, the mechanism underlying the decrease in number or apoptosis of microglia by VPA was not fully understood. This study was undertaken to address the following questions: (1) Do VPA and other HDACIs reduce the number of microglia by inducing apoptosis? (2) If so, is the induction of microglial apoptosis associated with HDAC inhibition? (3) What is the functional significance of microglial apoptosis in the neuroprotective effects of HDACIs?

MATERIALS AND METHODS

Materials

LPS (strain O111:B4) was purchased from Calbiochem (San Diego, CA). Cell culture ingredients were obtained from Invitrogen (San Diego, CA). [^3H]-Dopamine (DA; 28 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). The polyclonal antibody against tyrosine hydroxylase (TH) was a kind gift from Dr. John Reinhard of Glaxo Wellcome (Research Triangle Park, NC) and the antibody diluent was a product of DAKO (Carpinteria, CA). Biotinylated horse anti-mouse and goat anti-rabbit secondary antibodies were purchased from Vector Laboratories (Burlingame, CA). Tumor necrosis factor-alpha (TNF- α) enzyme-linked immunosorbent assay (ELISA) kits were purchased from R & D Systems Inc. (Minneapolis, MN). All other reagents were obtained from Sigma (St. Louis, MO).

Animals

Timed-pregnant Fisher F344 rats were obtained from Charles River Laboratories (Raleigh, NC). Housing and breeding of the animals were performed in strict accordance with the guidelines of the National Institutes of Health.

Enriched Rat Microglial Culture

Primary microglial cultures were prepared from whole brains of 2-day-old Fisher F344 rat pups following a previously described protocol (Liu et al., 2000). For DNA fragmentation and flow cytometry studies, microglial cells were plated onto 12-well culture plates at a density of 5×10^5 cells/well. For TUNEL analysis, microglial cells were plated onto chamber slide. Cultures were washed 3 h later to remove unattached cells. The experiments were initiated 12 h after plating. The purity of microglia was greater than 98% at the time of this study.

Mesencephalic Neuron-glia Culture

The rat and mouse ventral mesencephalic neuron-glia cultures were prepared as described previously (Liu et al., 2000). Cultures were treated with various doses of HDACIs, either alone or in combination with LPS, 7 d after plating.

Cell Viability

The cell viability was measured with the blue formazan that was metabolized from colorless 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial dehydrogenases, which are active only in viable cells. The enriched microglial cells were incubated at 37°C for 2 h after the addition of 0.5 mg/ml MTT. The solubilizing reagent, dimethyl sulphoxide, was then added to extract the blue formazan product, followed by the measurement of the absorbance at 550 nm using SPECTRAmax PLUS384 (Molecular Devices, Sunnyvale, CA). The incubation time and the cell number used for the reaction were optimized for quantification.

Annexin V Staining

The changes in phosphatidylserine (PS) symmetry were determined using annexin V conjugated to phycoerythrin (PE) (BD, Pharmingen, San Diego, CA) and flow cytometry. Briefly, 5×10^5 control or treated cells, which were initially washed in 1x phosphate-buffered saline solution (PBS), were incubated with 5 μl of annexin V-PE and 7-amino-actinomycin D (7-AAD) for 15 min at room temperature, according to the manufacturer's instructions. Annexin V-PE/7-AAD-stained samples were diluted in 400 μl of 1x annexin V binding buffer and examined immediately using a BD LSRII flow cytometer with BD FACSDiVa software. Five thousand cells were examined by initially gating on a forward-scatter versus side-scatter

dot plot to exclude any debris. Cells were excited at 488 nm and examined at 575/26 and 695/40 nm for annexin V-PE and 7-AAD fluorescence, respectively.

DNA Fragmentation

Cells were detached from culture dishes, isolated by centrifugation, then followed by resuspension in 500 μ l of 10 mM Tris (pH 7.4), 10 mM EDTA, and 0.2% Triton X-100. The lysates were stored at -20°C overnight and then thawed and treated with 0.3 mg/ml proteinase K for 16 h at 55°C . The samples were extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1) followed by addition of NaCl to a final concentration of 0.1 M. The DNA was precipitated by addition of 2 volumes of ice-cold ethanol. The sample was kept overnight at -80°C . The DNA was pelleted, dried, and resuspended in 100 μ l of 10 mM Tris HCl, 1 mM EDTA buffer (pH 7.5) to which 0.1 mg/ml deoxyribonuclease-free ribonuclease (RNase) was added. The DNA samples were separated by electrophoresis on a 1.5% agarose gel and the DNA bands were identified using ethidiumbromide staining.

TUNEL Assay

TUNEL assay was applied using the In Situ Cell Death Detection Kit, POD (Roche Diagnostics, Mannheim, Germany), as described previously (Wang et al., 2002). In brief, the cells were treated, washed with PBS, then fixed for 1 h with 4% paraformaldehyde and permeabilized for 2 min at 4°C with 0.1% Triton X-100 in 0.1% sodium citrate. After being washed twice with PBS, cells were labeled with fluorescence. The TUNEL reagent mixture was applied for 60 min at 37°C , according to the manufacturers' protocol. At the end, the color reaction with DAB lasted for about 5 min. Thereafter, the cells were counterstained with 4'-6-diamidino-2-phenylindole (DAPI). The labeled cells were visualized under the OLYMPUS 1X70 inverted microscope equipped with appropriate filters and a SONY DXC 5500 digital camera.

Mitochondrial transmembrane potential measurement

Cells were incubated with 5 μM rhodamine 123 (Sigma) for 30 min in a cultured medium. After being washed with PBS, cells were resuspended in cold PBS and immediately examined using a BD LSRII flow cytometer with BD FACSDiVa software. Cells were excited at 488 nm and examined at 530/30 nm for rhodamine 123 fluorescence. Five thousand cells were examined by initially gating on a forward-scatter versus side-scatter dot plot to exclude any debris. Changes in rhodamine fluorescence were determined by fluorescein isothiocyanate (FITC) fluorescence histograms.

Analysis of Histone Acetylation and Cell Cycle by Flow Cytometry

Cells were first fixed for 15 min with 1% formaldehyde in PBS on ice, then again with 70% ethanol. All washings were performed in a refrigerated centrifuge using cold PBS, and cells were finally fixed on ice. Cells, 5×10^5 per sample, were washed with PBS + 1% BSA and permeabilized with 200 μ l of 0.1% Triton X-100 in PBS for 10 min at room temperature. After being washed again with PBS+1% BSA, the samples were incubated with 500 μ l of 10% normal goat serum in PBS for 20 min. Histone acetylation levels were detected by incubation with the anti-tetra-acetylated histone H3 (1:250 dilution; Upstate, Chicago, IL). Detection was made by incubation with a FITC-conjugated, affinity purified F(ab')₂ fragment of goat anti-rabbit IgG (Jackson, West Grove, PA) for 1 h at room temperature in the dark. FITC-stained cells were pelleted and resuspended in 1 ml of propidium iodide (PI) solution containing 20 $\mu\text{g/ml}$ PI in PBS and 1 μ l of RNase (10 unit/ml in water stock) for 20 min at room temperature. Cells were immediately examined using a BD FACSort flow cytometer with CellQuest software. Cells were excited at 488 nm and examined at 530 and 585 nm for FITC and PI fluorescence,

respectively. Ten thousand cells were examined by initially gating on a PI area (DNA content) versus PI width dot plot for doublet discrimination. The samples were subsequently examined on a DNA content histogram and DNA content versus acetylated histone-FITC dot plot (Ronzoni et al., 2005).

TNF- α and Nitrite Assays

The release of TNF- α was measured with a rat TNF- α enzyme-linked immunosorbent assay kit (ELISA) from R&D System (Minneapolis, MN), and the production of nitric oxide (NO) was determined by measuring nitrite accumulations in the supernatant with Griess reagent, as described previously (Liu et al., 2000).

DA Uptake Assay

[^3H]-DA uptake assays were performed as described previously (Liu et al. 2000). In brief, the cells were incubated for 20 min at 37 °C with 1 μM [^3H]-DA in Krebs-Ringer buffer (16 mM NaH_2PO_4 , 16 mM Na_2HPO_4 , 119 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO_4 , 1.3 mM EDTA, pH 7.4). Nonspecific uptake was measured in the presence of 10 μM mazindol. After the cells were extensively washed with ice-cold Krebs-Ringer buffer and lysed with 1 N NaOH (0.5 ml/well), the lysate was mixed with 15 ml of scintillation fluid. The radioactivity was determined with a liquid scintillation counter. The specific [^3H]-DA was calculated by subtracting the amount of radioactivity obtained in the presence of mazindol from that obtained in its absence.

Statistical Analysis

The data were presented as the mean $\pm$ SEM. ANOVA was used to make multiple comparisons of different pairs of groups. Statistically significant differences between groups was assessed by either paired or unpaired Student's t-test with Bonferroni's correction. A p value of less than 0.05 was considered statistically significant.

RESULTS

Treatment with VPA or Other HDACIs Trigger Microglial Apoptosis

To investigate whether VPA decreases the number of microglia by inducing apoptosis (Peng et al., 2005), we examined multiple parameters of apoptotic cell death in VPA-treated rat microglia-enriched cultures. The VPA-treated microglial cells showed a significant dose-dependent reduction in cell viability in the range of 0.3 mM to 1.2 mM, as determined by the MTT assay (Fig. 1A). Moreover, VPA treatment triggered the externalization of PS, an early hallmark of apoptosis, and increased the proportion of apoptotic cells by three-fold following treatment with 1.2 mM VPA for 24 h (Fig. 1B). In addition, internucleosomal DNA cleavage in a ladder-like pattern characteristic of apoptosis was detected by agarose gel electrophoresis in extracts of microglia treated with 0.6 mM or 1.2 mM VPA (Fig. 2A). The microglia which was treated with VPA for 24 h showed a progressive change in morphology characterized by a decrease in microglial cell size and distribution of small fragments around the VPA-treated microglial cell. (Fig. 2B). Furthermore, the TUNEL-positive apoptotic microglial cells were co-present with the living microglia in VPA-treated rat microglia-enriched cultures (Fig. 3). Together, these results demonstrated that increasing concentrations of VPA progressively increased the capacity to induce microglial apoptosis. The effective concentrations of VPA are at the high end of the therapeutic plasma levels for treating bipolar disorder (Allen et al., 2006), and within the dose range for inhibiting HDAC activity (Phiel et al., 2001).

To determine whether VPA-induced apoptosis of the microglia is related to its inhibitory effect on HDAC, a structurally dissimilar HDACI, TSA, and a similar HDACI, SB were tested. Treatment of the microglia with TSA (50 nM) or SB (1.2 mM) for 24 h also decreased the

microglial cell viability by 70% or 80%, respectively (Fig. 4A). This effect was associated with an approximately 7-fold increase in the number of apoptotic cells showing externalized PS (Fig. 4B). Thus, VPA-induced apoptotic death of the microglia was mimicked by other HDACIs with similar and distinct chemical structures.

VPA and Other HDACIs Induce a Loss of Mitochondrial Membrane Potential and Histone H3 Hyperacetylation in Microglia

Since it has been reported that mitochondria are involved in HDACI-induced apoptosis, we next examined the effects of VPA, TSA and SB on microglial MTP, determined by staining with the lipophilic cationic fluorochrome rhodamine 123. The results showed that treatment with VPA at 0.6 mM or 1.2 mM, but not 0.3 mM, caused a significant reduction in MTP (a decrease in rhodamine 123 fluorescence) following a 24-h treatment (Fig. 5). Moreover, TSA (50 nM) and SB (1.2 mM) induced an even greater (up to 60%) $\Delta\Psi_m$ reduction, reminiscent of their effects of increasing the population of apoptotic microglia.

To affirm that HDACIs did inhibit HDAC activity under our experimental conditions, levels of acetylated histone H3 protein in microglia were determined by flow cytometry analysis with rabbit antiacetyl histone H3 followed by FITC-conjugated anti-rabbit staining. The results showed that the acetylated histone H3 in the untreated microglia was detectable at the baseline level (Fig. 6). Treatment with VPA, TSA, or SB for 12 h increased the percentages of cells with acetylated histone H3 by more than four folds, suggesting the occurrence of HDAC inhibition under the aforementioned treatment conditions.

VPA and HDACIs Fail to Affect the Microglial Cell Cycle Progression

Because HDACIs can affect both cell proliferation and survival (Bolden et al., 2006), we assessed the effects of VPA, TSA, and SB on microglial cell cycle progression. The enriched rat microglial cells were treated with various HDACIs for 24 h and stained with PI, followed by flow cytometric analysis. The increase in the percentage of the sub-G1 population cells indicated an increase in apoptotic cells after treatment with HDACIs (Fig 7). However, the percentage of cells in other cell cycle stages was not significantly different between vehicle- and HDACI-treated microglia. These data suggest that HDACIs exert little or no effect on the proliferation of microglia.

Association between HDACI-induced Protection against LPS-induced DA Neurotoxicity and Microglial Apoptosis

Previous reports from our laboratory demonstrate a critical role for microglia in mediating inflammation-related neurodegeneration *in vivo* and *in vitro* (Block et al., 2007). We also showed that VPA protects DA neurons from LPS-induced neurotoxicity in neuron-glia cultures and that the phenomenon is related to the reduction in the number of activated microglia (Peng et al., 2005). To investigate whether the neuroprotective effects could be extended to TSA and SB, we treated rat mesencephalic neuron-glia cultures with TSA (50 nM) or SB (1.2 mM) for 24 h prior to LPS (10 ng/ml) exposure. Results here showed that, similar to VPA, TSA or SB pretreatment almost completely blocked the production of TNF- α and NO determined 3 h and 24 h after LPS stimulation, respectively (Fig. 8A and Fig. 8B). Seven days after LPS stimulation, the viability of DA neurons was assessed by the DA uptake assay. Pretreatment with TSA or SB almost completely prevented LPS-induced decrease in DA uptake (Fig. 8C). The morphological inspection also confirmed that the loss of neuronal processes of tyrosine hydroxylase-immunoreactive neurons in cultures was blocked by TSA or SB (Fig. 8D).

Discussion

In this study, we demonstrated that VPA induce the apoptosis of the cultured rat microglia. The microglial apoptosis is characterized by PS externalization, internucleosomal DNA fragmentation, and appearance of TUNEL-positive cells. The apoptosis of microglia is likely responsible for the reduction in the number of microglia in VPA-treated neuron-glia cultures (Peng et al., 2005). The finding that VPA exerted little effect on the proliferation of microglia further supports this notion. Evidence from this study also indicates that the apoptosis-related decrease in microglia number may be related to the reduced neuro-inflammatory responses in LPS-treated neuron-glia cultures, and may underlie, at least in part, the anti-inflammatory and neuroprotective effect of VPA.

Moreover, the above-mentioned effects of VPA were shared by TSA and SB, suggesting that this is a common action of HDACIs (Blanchard and Chipoy, 2005). Our results suggest that the induction of activated microglial apoptosis by HDAC inhibitors may terminate unwanted microglial neurotoxicity. The reduction of microglia by VPA was also observed in an animal study. Recently, using middle cerebral artery occlusion stroke model in rat, we showed that VPA pretreatment was effective in reducing the cerebral ischemia-induced neuronal damage, which was associated with a reduction in the microglia number in the damaged brain regions (Kim et al. in review)

Down-regulation of inflammation by the apoptosis of neuroimmune cells in the brain, such as microglia, has been proposed as a mechanism of regulating immune activity to protect brain cells from inflammation-mediated neurodegeneration (Elsisi et al., 2005, Pavese et al., 2006). In addition, microglia have demonstrated a high capacity to engulf apoptotic cells (Chan et al., 2003). As a result of the phagocytosis of apoptotic inflammatory cells, the secretion of TNF- α from microglia decreases significantly (Chan et al., 2001, Magnus et al., 2001). This process also occurs when peripheral macrophages recognize apoptotic cells through phosphatidylserine receptors (Reddy et al., 2002). Apoptosis is essential for clearance of potentially injurious inflammatory cells and subsequent efficient resolution of inflammation, thus, HDACIs might enhance the resolution of established inflammation by promoting apoptosis of microglia (Rossi et al., 2006).

HDACIs have been reported to induce apoptosis in immune cells (Wen and Wu, 2001, Moreira et al., 2003, Kim et al., 2005). However, the effects of HDACIs on microglia remain inconclusive (Suuronen et al., 2003, Huuskonen et al., 2004, Kim et al., 2004a, Park et al., 2005). Kim et al. has used BV2 murine microglial cells to demonstrate that SB suppresses interferon-gamma but not LPS-mediated induction of nitric oxide or TNF- α Suuronen et al. first reported that simultaneous treatment 15nM TSA with LPS may enhance the LPS-induced inflammatory response in both murine N9 and rat primary microglial cells (Suuronen et al., 2003). They subsequently reported that SB may be anti-inflammatory in primary microglial cells but proinflammatory in N9 microglial cells (Huuskonen et al., 2004). Moreover, they reported that different treatment schedules may cause different results using primary microglia. Using rat primary microglia, we observed that SB or TSA treatment by itself did not induce an inflammatory response. In addition, we noted that pretreatment with SB or TSA before LPS stimulation blocked the stimulus-induced TNF- α and nitrite production. We also observed that SB or TSA could strongly enhance the LPS-induced microglial inflammatory response, if microglia were treated with VPA and LPS simultaneously (data not shown). Although the underlying mechanisms for these conflicting results remain unclear, we speculate that the effects of HDACIs may be cell type-selective and that pretreatment with HDACIs may induce cellular feedback HDAC responses that induce adaptive changes in the microglial activation system.

Results of the current study revealed that the increase in levels of acetylated histone H3 precedes the induction of microglial apoptosis in HDACI-treated microglia. However, our results also suggest that VPA, TSA and SB share the capacity to induce microglial apoptosis via a mitochondria-related pathway. The effects of VPA on the mitochondrion may be related to its ability to interfere with several mitochondrial metabolic pathways, including fatty acid oxidation (Neuman et al., 2001). Interestingly, SB and TSA displayed more robust effects on inducing microglial apoptosis and reducing mitochondrial membrane potential than VPA, while the extents of histone H3 hyperacetylation induced by these three HDACIs were similar. These observations suggest that substrates other than histone H3 may be more critically involved in HDAC-mediated disruption of mitochondrial membrane potential and the apoptosis of microglia (Martirosyan et al., 2006).

In conclusion, the present study provides strong evidence that VPA and other HDACIs markedly induce the apoptosis of the microglia. This apoptosis-inducing effect is associated with a loss of microglial mitochondrial transmembrane potential and an increase in histone hyperacetylation. The HDACI-induced microglial apoptosis likely contributes to their neuroprotective effects in response to pro-inflammatory stimuli. Since microglia could potentially act as both protector and attacker, future *in vivo* studies using VPA and other HDACIs to test the functional consequences of this microglial apoptosis-inducing effect seem warranted.

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Abbreviations

7-AAD	7-amino-actinomycin D
DA	dopaminergic
DAPI	4'-6-diamidino-2-phenylindole
FBS	fetal bovine serum
FITC	Fluorescein isothiocyanate
HBSS	Hanks' balanced salt solution
HDAC	histone deacetylase
HDACIs	HDAC inhibitors
LPS	lipopolysaccharide

MTT	3- (4 , 5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NO	nitric oxide
PI	propidium iodide
PS	phosphatidylserine
RNase	ribonuclease
SB	sodium butyrate
TNF-α	tumor necrosis factor-alpha
TSA	trichostatin A
VPA	valproic acid

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Fig. 1.

VPA treatment triggers apoptosis in primary rat microglial cultures. Enriched rat microglial cells were treated with various concentrations of VPA for 24 h. **A:** Cell viability was determined by the MTT analysis. The percentages of viable cells from triplicate determinations are shown as mean $\pm$ SEM compared with vehicle control, **B:** Externalization of PS in microglia exposed to various concentrations of VPA for 12 h or 24 h, as determined using PE-conjugated annexin V followed by flow cytometry analysis. The percentages of apoptotic cells from five independent experiments are shown as mean $\pm$ SEM. # $p < 0.05$, * $p < 0.001$ compared with vehicle control.

Fig. 2. VPA induces DNA fragmentation and morphological changes in microglia-enriched culture. **A:** DNA fragmentation in cells treated with 0.6 or 1.2 mM of VPA for 24 h was analyzed by agarose gel electrophoresis. The left lane (M) represents the molecular weight marker (100 bp). **B:** Enriched rat microglial cells treated with vehicle or 0.6 mM VPA for 24 h. The cell morphology was observed using light microscope.

Fig. 3. VPA induces DNA damage in microglia-enriched culture: A TUNEL study. The VPA-treated microglia were fixed with 4% paraformaldehyde followed by TUNEL assays (red) and DAPI staining (blue). Merge: merged images of TUNEL assay and DAPI staining. The photographic figures are from a representative experiment. Arrow: TUNEL-positive cells.

Fig. 4.

Treatment with HDACIs induces microglial apoptosis. The enriched microglial cells were treated with 50 nM of TSA or 1.2 mM of SB for 24 h. **A:** The cell viability was determined by the MTT analysis and the percentages of viable cells from triplicate determinations are shown as mean $\pm$ SEM. **B:** The apoptotic cells were identified using PE-conjugated annexin V followed by flow cytometry analysis at 12 h and 24 h after TSA or SB treatment. The dot plots are from a representative experiment at 24 h (*upper panel*), and the percentages of apoptotic cells are shown as mean $\pm$ SEM from three independent experiments (*lower panel*). # $p < 0.05$, * $p < 0.001$ compared with vehicle control.

Fig. 5.

Treatment with HDACIs causes a decrease in MTP in the microglial cells. The microglial cell cultures were treated with the indicated concentrations of VPA, TSA, or SB for 24 h. The loss of MTP ($\Delta\Psi_m$) was determined by rhodamine 123 staining. The histograms are from a representative experiment (*upper panel*). A decrease in rhodamine 123 fluorescence (shift to the left) indicates a decrease in MTP. The percentages of cells with $\Delta\Psi_m$ (cells to the left of the white line) are shown as means $\pm$ SEM from three independent experiments (*lower panel*). # $p < 0.05$, * $p < 0.001$ compared with vehicle control.

Fig. 6.

HDACIs increase histone H3 acetylation in the microglial cells. The levels of histone H3 acetylation in the microglial cells treated with 1.2 mM VPA, 50 nM TSA, or 1.2 mM SB for 12 h were analyzed by flow cytometry using specific acetyl-histone H3 antibody conjugated with FITC and PI staining for DNA content as described in the Method. The dot plot (*upper panel*) of DNA content versus FITC fluorescence for each sample was used to calculate the percentage of histone H3-acetylated cells (green cells). The percentages of histone H3-acetylated cells (*lower panel*) are shown as mean $\pm$ SEM from four independent experiments done in triplicate. * $p < 0.001$, compared with vehicle control.

Fig. 7.

HDACIs failed to change the cell cycle in the microglial cells. The microglial cells were treated with 1.2 mM VPA, 50 nM TSA, or 1.2 mM SB for 24 h and stained with PI. DNA content was analyzed by flow cytometry. The percentages of cells in the sub-G1, G1, S, and G2-M phases of the cell cycle from experiments done in duplicate are shown as mean $\pm$ SEM. # $p < 0.05$, compared with vehicle control.

Fig. 8.

HDACIs attenuate LPS-induced production of microglial pro-inflammatory factors and reduce microglial dopaminergic neurotoxicity. Mesencephalic neuron-glia cultures were pretreated with 1.2 mM SB or 50 nM TSA for 24 h prior to stimulation with 10 ng/ml LPS. **A:** The release of TNF- α into the supernatant was determined 3 h post-LPS treatment. **B:** The level of nitrite in the supernatant, an indicator of NO production, was determined 24 h after LPS treatment. Results of four independent experiments done in triplicate are shown as means $\pm$ SEM. * $p < 0.001$ compared with cultures treated with LPS alone. **C:** Seven days after LPS stimulation, the viability of DA neurons was assessed by DA uptake assays. The ratios of DA uptake capacity from cultures treated with LPS to those without LPS in the same treatment groups. The results of four independent experiments in triplicate are shown as means $\pm$ SEM. * $p < 0.001$ compared with LPS alone. **D:** The morphology of tyrosine hydroxylase-immunoreactive neurons. The figures shown are from a representative experiment.