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Histone deacetylase inhibitors up-regulate astrocyte GDNF and BDNF gene transcription and protect dopaminergic neurons

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Abstract

Parkinson's disease (PD) is characterized by the selective and progressive loss of dopaminergic (DA) neurons in the midbrain substantia nigra. Currently, available treatment is unable to alter PD progression. Previously, we demonstrated that valproic acid (VPA), a mood stabilizer, anticonvulsant and histone deacetylase (HDAC) inhibitor, increases the expression of glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) in astrocytes to protect DA neurons in midbrain neuron-glia cultures. The present study investigated whether these effects are due to HDAC inhibition and histone acetylation. Here, we show that two additional HDAC inhibitors, sodium butyrate (SB) and trichostatin A (TSA), mimic the survival-promoting and protective effects of VPA on DA neurons in neuron-glia cultures. Similar to VPA, both SB and TSA increased GDNF and BDNF transcripts in astrocytes in a time-dependent manner. Furthermore, marked increases in GDNF promoter activity and promoter-associated histone H3 acetylation were noted in astrocytes treated with all three compounds, where the time-course for acetylation was similar to that for gene transcription. Taken together, our results indicate that HDAC inhibitors up-regulate GDNF and BDNF expression in

astrocytes and protect DA neurons, at least in part, through HDAC inhibition. This study indicates that astrocytes may be a critical neuroprotective mechanism of HDAC inhibitors, revealing a novel target for the treatment of psychiatric and neurodegenerative diseases.

Keywords: Astrocytes, BDNF, dopaminergic neurons, GDNF, histone acetylation, neurotrophic

Introduction

Increasing evidence suggests that neurotrophic factors may be an ideal therapeutic target for the treatment of both neurodegenerative diseases and psychiatric diseases. Parkinson's disease (PD) is a neurodegenerative disease characterized by selective and progressive death of nigrostriatal dopaminergic (DA) neurons. Currently, only symptomatic treatment (e.g. L-dopa therapy) for PD patients is available. However, neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF), are a class of potentially neuroprotective compounds that have restorative effects on DA neurons (Grondin and Gash, 1998; Lapchak et al., 1997; Lin et al., 1993; Son et al., 1999). Consequently, identifying compounds that can induce endogenous secretion of neurotrophic factors would be of great significance in the treatment of PD.

In addition, neurotrophic factors have also been implicated for the treatment of mood disorders. For example, antidepressants have been associated with hippocampal neurogenesis in rodents (Santarelli et al., 2003), which was suggested to be facilitated by a number of trophic/growth factors, including brain-derived neurotrophic factor (BDNF) and GDNF (Chen et al., 2005; Duman, 2004; Scharfman et al., 2005). Chronic antidepressant treatment in animal models has been shown to induce both the expression of BDNF and activation of its receptor signalling pathways, which are critical for antidepressant efficacy (Duman, 2004). At the cellular level, studies using the rat astrocyte-derived cell line, C6 glioma, have shown that treatment with various antidepressants increases GDNF mRNA expression and protein release, where these effects are dependent upon activation of protein tyrosine kinases and extracellular signal-regulated kinases (ERK; Hisaoka et al., 2007). Notably, post-mortem and brain-imaging studies in patients with unipolar or bipolar disorder reveal a loss of brain grey-matter volume and decrease in the cellular density, particularly glia, in discrete brain regions (Manji et al., 2001). Thus, it has been proposed that a deficiency in trophic support to neurons and abnormal neuron—glia interactions may contribute to the pathophysiology of mood disorders.

The mood stabilizer and anti-epileptic drug, valproic acid (VPA), was recently shown to have activity as a histone deacetylase (HDAC) inhibitor (Gottlicher et al., 2001; Phiel et al., 2001). HDAC inhibitors are natural or synthesized compounds with diverse structures that can suppress HDAC activity (Miller et al., 2003). HDAC inhibition causes hyperacetylation in histone proteins that usually results in an 'open' chromatin structure and gene activation (Rodriquez et al., 2006). HDAC inhibitors can also change gene expression by increasing acetylation of non-histone proteins, such as transcription factors Sp1 and NFκB (Quivy and Van Lint, 2004; Ryu et al., 2003). Recent studies have shown that HDAC inhibitors could be neuroprotective agents, probably through the regulation of neuronal or glial gene expression (Langley et al., 2005). VPA promotes neuronal survival, neurite outgrowth and neurogenesis to protect neurons against various insults with multiple mechanisms proposed, including activation of ERK pathways and histone hyperacetylation (Hao et al., 2004; Jeong et al., 2003; Kanai et al., 2004; Laeng et al., 2004; Leng and Chuang, 2006; Ren et al., 2004; Yuan et al., 2001). Thus, while increasing evidence supports that HDAC inhibitors are neuroprotective, the reported mechanisms vary throughout the literature, perhaps depending upon the cell type in question and the disease model.

We have previously demonstrated that concentrations of VPA known to inhibit HDAC increases the expression of GDNF and BDNF in astrocytes, which leads to indirect neurotrophic and protective effects on DA neurons in midbrain neuron-glia cultures (Chen et al., 2006). Additionally, VPA has also been shown to increase GDNF and BDNF transcription in C6 glioma cells (Castro et al., 2005). However, the mechanisms underlying the effects of VPA remained unclear. In the present study, we explore whether HDAC inhibition and histone hyperacetylation mediate VPA-induced tropic factor transcription (BDNF and GDNF) in astrocytes and consequent DA neuroprotection.

Materials and methods

Chemicals and antibodies

All media and other reagents for cell cultures were purchased from Invitrogen (Carlsbad, CA, USA). The polyclonal antibody against tyrosine hydroxylase (TH) was a kind gift from Dr John Reinhard of Glaxo-SmithKline (Research Triangle Park, NC, USA). Rabbit anti-IBA-1 antibody was purchased from Wako Pure Chemical Industries (Osaka, Japan). Secondary antibodies and the Avidin—biotin Complex (ABC) kit were purchased from Vector Laboratories (Burlingame, CA, USA). VPA, sodium butyrate (SB) and trichostatin A (TSA) were purchased from Sigma-Aldrich (St Louis, MO, USA). [^{3}H]DA (28 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Boston, MA, USA).

Animals

Timed-pregnant Fisher-344 rats were obtained from Charles River Laboratories (Raleigh, NC, USA). Housing, breeding, and experimental use of the animals were performed in strict accordance with the National Institutes of Heath (NIH) guidelines and were approved by the NIEHS Animal Care and Use Committee.

Rat mesencephalic neuron-glia cultures

Neuron-glia cultures were prepared from the ventral mesencephalic tissues of embryonic day-14 Fisher-344 rats, as described previously (Gao et al., 2002). In brief, dissected mesencephalic tissues were dissociated into single cells by mechanical trituration and then seeded at a density of 5×10^5 cells/well in poly-D-lysine-coated 24-well plates. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, in Minimal Essential Medium, supplemented with 10% fetal bovine serum (FBS), 10% horse serum, 1 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 50 U/ml penicillin and 50 μg/ml streptomycin. Seven-day-old cultures were used for treatment, unless indicated otherwise. Immunocytochemical analysis indicated that the cultures contained approximately 11% micro-glia, 48% astrocytes, and 41% neurons, where approximately 1% of neurons were tyrosine hydroxylaseimmunoreactive (TH-IR).

Primary cultures of rat cerebral cortical astrocytes

Primary rat cortical astrocytes were isolated as previously described (Cole and de Vellis, 2001) with a few modifications. Briefly, neonatal (1–3 d) Fisher rat pups were euthanized and whole brains isolated. The meninges were removed and the cerebral cortices were dissected and subjected to enzymatic digestion for 15 min in Dulbecco's Modified Eagle's Medium: Nutrient Mix F-12 (DMEM/F-12) without serum and containing 2.0 mg/ml porcine trypsin and 0.005% DNase I. The tissue was then mechanically disaggregated using a 60 μm cell dissociation kit (Sigma-Aldrich) to yield a mixed glial cell suspension. The cell suspension was centrifuged for 10 min at 300 *g* and resuspended in fresh complete culture medium (D-MEM/F-12 supplemented with 10% FBS, 100 μM non-essential amino acids, 100 μM sodium pyruvate, 200 μM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin). The cells were plated on 75 cm^2 polystyrene tissue culture flasks (BD Biosciences, Bedford, MA, USA) for 6 h, the medium was replaced and the cells were then incubated at 37 °C, 5% CO_2 and 95% air until confluency was attained (10–14 d). Fresh medium was replenished at 24 h and every 3–4 d thereafter. Following confluency, the cells were shaken at room temperature on an orbital shaker at 150 rpm for 6 h to remove contaminating cells (mostly microglia). The cells were harvested with 0.1% trypsin/EDTA in Hank's balanced salt solution and secondary cultures were plated in either 25 cm^2 flasks or 100 mM Petri dishes at a density of $0.35\text{-}1\times10^6$ cells. Medium was replaced every 2–3 d thereafter. Experimental studies were performed within 3–4 wk of initial seeding. Immunohistochemistry studies (see below) with ionized calcium-binding adaptor molecule 1 (microglial marker IBA-1) and glial fibrillary associated protein (astrocyte marker GFAP) confirmed that the purified secondary cultures contained <1% microgial contamination.

Rat C6 glioma cell culture

Rat glioma C6 cells were maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified incubator with 5% CO_2/95% air. The cells were grown in T75 flasks and used at passages 3–5 by seeding 12-well plates at a density of 1×10^5 cells/well. At ~80% confluency the cells were pre-conditioned in serum-free OptiMEM™ (Invitrogen) containing 0.5% BSA. Twenty-four hours later the medium was renewed and supplemented with the different HDAC inhibitors.

The C6 glioma rat astroglial cell line has been widely used for the study of pharmacological regulation of GDNF production by astrocytes (Armstrong and Niles, 2002; Caumont et al., 2006a; Hisaoka et al., 2001, 2004; Suter-Crazzolara and Unsicker, 1996) and the GDNF promoter assay has been well established in these same cells (Caumont et al., 2006a, b).

DA uptake assay

Cultures were washed twice with 37 °C Krebs-Ringer buffer (16 mM sodium phosphate, 119 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4) before incubation with 1 μM [^{3}H]DA (PerkinElmer Life Sciences) for 20 min in Krebs-Ringer buffer at 37 °C. After washing three times with ice-cold Krebs-Ringer buffer, cells were dissolved in 1 N NaOH. Radioactivity was determined by liquid scintillation counting with Packard TriCarb 2900TR scintillation counter. Non-specific DA uptake observed in the presence of 10 μM mazindol (a specific inhibitor of DA transport) was subtracted from total uptake to obtain the specific DA uptake component.

Immunocytochemistry

Cultures were fixed with 3.7% formaldehyde for 20 min at room temperature. After two washes with PBS, cultures were treated with 1% hydrogen peroxide (10 min) followed by sequential incubation with blocking solution [0.4% Triton X-100/PBS, 4% normal serum, 1% bovine serum albumin (BSA), for 30 min, at room temperature], primary antibody (overnight, 4 °C), biotinylated secondary antibody (2 h, room temperature) and finally ABC reagents (1 h, room temperature) according to the manufacturer's instructions (Vector Laboratories). Colour development was achieved by the addition of 3,3′-diaminobenzidine. For morphological analysis, the images were recorded with an inverted microscope (Nikon, Tokyo, Japan) connected to a charge-coupled device camera (Dage-MTI, Michigan City, IN, USA) operated with MetaMorph software (Universal Imaging Corporation, Downingtown, PA, USA). To study the effect of HDAC inhibitors on the cell number of DA neurons, all the TH-IR neurons in each well of the 24-well plate were counted under the microscope at 100 × magnification by two individuals in a blind experimental design. The average of these scores is reported.

RNA extraction and quantitative real-time PCR

Total cellular RNA from purified primary astrocyte cultures was extracted using the Qiagen RNeasy kit (Qiagen Inc., Valencia, CA, USA). cDNA was synthesized using the High Capacity cDNA Archive kit with random primers according to the manufacturer's protocol. Quantitative real-time PCR was performed on an Applied Biosystems 7900HT Sequence Detection System (Foster City, CA, USA). GDNF and BDNF gene expression was examined by Assays-on-Demand™-Gene Expression products containing TaqMan® probes, forward primers and reverse primers specific to GDNF, BDNF or the housekeeping gene β-actin cDNA respectively. TaqMan® Universal PCR Master Mix, No AmpErase® UNG was used in the PCR reaction. The total reaction volume was 20 μl and contained 2 μl cDNA. The reaction cycle consisted of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. All reactions were run at least in duplicate. A standard curve using pooled cDNA from each cDNA sample was included for each gene to calculate relative amounts of the genes of interest. β-actin was used as a control for normalization. Effects of HDAC inhibitors on the transcripts of the neurotrophic factors were expressed as percentage of the non-treated control groups. All reagents and kits for RT—PCR were obtained from Applied Biosystems, unless stated otherwise.

GDNF promoter activity assay

A DNA fragment of 1436 kb (-1412/+24) upstream of the putative transcription initiation site +1 (Caumont et al., 2006a) of the rat GDNF gene (GenBank accession no. AJ011432) was generated by PCR of newborn rat genomic DNA using PfuTurbo DNA Polymerase (Strategene Corporation, La Jolla, CA, USA). The forward and reverse primers used are listed in Table 1 (GDNF $P^{1412/+24}$). The fragment was amplified a second time to generate *Mlu*I and *Xho*I restriction sites on the 5′ and 3′ ends, respectively, with primer GDNF $P^{1412/+24}$-RS. The amplification product was cleaved at the *Mlu*I and *Xho*I restriction sites and inserted into the multi-cloning site of vector pGL3-Basic (Promega, Madison, WI, USA) harbouring the firefly luciferase reporter gene to construct a pGL3-GDNF$^{-1412/+24}$ plasmid. C6 cells were transfected with pGL3-Basic or pGL3-GDNF plasmids along with the control phRL vector (harbouring the *Renilla luciferase* gene; Promega) to correct for variations in transfection effciency. Transfection was performed with 80% confluent cells using 0.2 μg GDNF pGL3-GDNF or pGL3-Basic plus 20 ng phRL plasmid and 3 μl TransIT reagent (Mirus, Madison, WI, USA) agent in 100 μl OptiMEM™. At 24 h after transfection, cells were treated with the indicated HDAC inhibitor for an additional 24 h. This was followed by luciferase assay, using the Dual-Luciferase Reporter Assay System (Promega) in conjunction with a Luminometer (Dynex Technologies, Chantilly, VA, USA), allowing independent measure of the activity of both the firefly and Renilla luciferases in the same samples. Relative activity of luciferase is shown. The C6 astroglioma cell line was used to measure GDNF promoter activity as previously reported (Caumont et al., 2006a, b).

Table 1

Sequences of primers used to amplify GDNF promoters in PCR

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GDNF, Glial cell line-derived neurotrophic factor.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out with the Acetyl-H3 ChIP assay kit (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's instructions with slight modifications. In brief, astrocytes in 10 cm culture dishes with differing treatments were cross-linked with 1% (v/v) formaldehyde in culture medium at 37 °C for 10 min. Cells were then washed, lysed, collected, and sonicated (eight times, 10 s each) to shear chromatin into 200–1000 bp fragments. After a 6-fold dilution, 0.5 ml of the sheared chromatin was incubated at 4 °C overnight with 1 μg of either acetylated histone H3 antibody or normal rabbit IgG, the latter being used as a negative control to exclude non-specific binding. The protein—DNA complex was collected with protein A agarose beads, eluted, and reverse cross-linked. DNA was then purified at a final volume of 50 μl using the QIAquick PCR Purification kit (Qiagen). The immunoprecipitated DNA was used as the ChIP DNA. Total DNA from 100 μl of nonimmunoprecipitated chromatin was also purified as input DNA for normalization. ChIP and input DNA were analysed using PCR or quantitative real-time PCR. For quantitative real-time PCR reactions, 5 μl ChIP DNA or 10-fold diluted input DNA was mixed with SYBR Green PCR Master Mix (Applied Biosystems) to a final volume of 25 μl and PCR was run under the same conditions as those used for real-time PCR of cDNA. For regular PCR, PfuTurbo DNA Polymerase (Stratagene Corporation, La Jolla, CA, USA) was used to amplify the ChIP or input DNA under the following conditions: 30 cycles of 94 °C, 15 s; 59 °C, 30 s; 72 °C, 30 s followed by 72 °C for 5 min. The PCR products were run on a 2% agarose gel and stained with ethidium bromide for visualization.

To determine histone acetylation at the GDNF promoter, three pairs of primers, GDNF Pa (-1357/-1158), GDNF Pb (-625/-426) and GDNF Pc (-248/-9) (Table 1), were designed to amplify the 1.4 kb fragment described in the GDNF promoter activity assay. The three different sites were chosen to investigate whether histone acetylation regulation occurs on the whole 1.4 kb promoter after HDAC inhibitor treatment.

Statistical analysis

The data are expressed as mean±S.E.M. and statistical significance was assessed by one-way ANOVA followed by Bonferroni's *t* test using the StatView program (Abacus Concepts Inc., Berkeley, CA, USA). A value of $p<0.05$ was considered statistically significant.

Results

SB and TSA exert neurotrophic and protective effects on DA neurons in neuron-glia cultures

To investigate the role of HDAC inhibition in DA neuroprotection, SB and TSA were tested in neuron-glia cultures in the presence and absence of the DA neurotoxin 1-methyl-4-phenylpyridinium (MPP^+). MPP^+ is the active metabolite of the selective DA neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPP^+ alone caused a 50–60% decrease in DA uptake ability (Figure 1a, b), consistent with earlier studies (Chen et al., 2006). SB alone augmented DA uptake in a dose-dependent manner in the neuron-glia cultures compared to vehicle controls, with 50% and 200% increases over basal uptake in the presence of 0.6 mM and 1.2 mM SB, respectively (Figure 1a, white columns). Similarly, a 49% and 119% increase in DA uptake were noted in the cultures treated with 50 nM and 100 nM TSA, respectively (Figure 1b, white columns). The decrease in DA uptake observed in the presence of MPP^+ was attenuated significantly by pretreatment for 30 min with either SB or TSA, with complete protection observed at the two highest concentrations of SB or TSA tested (Figure 1a, b, black columns). The effects of SB and TSA on DA uptake closely resembled those of VPA previously reported (Chen et al., 2006). Furthermore, using TH staining, cultures treated with VPA, SB or TSA at the highest concentrations tested showed comparable effects on absolute cell numbers of DA (TH-IR) neurons when compared to that observed with the DA uptake assay. Specifically, cell loss caused by MPP^+ was also blocked by each of the HDAC inhibitors (Figure 2a). Higher densities of TH-IR neurons with more complex neurite branching were noted in the presence of VPA, SB or TSA, compared to vehicle controls. Additionally, DA neurites severely damaged by MPP^+ were ameliorated by the presence of the HDAC inhibitors (Figure 2b), further confirming the ability of the HDAC inhibitors to provide a neuroprotective effect against a known DA neurotoxin. However, it remains to be studied whether other possible effects, e.g. promotion of cellular differentiation and neurogenesis, might also play a role here.

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Figure 1

Treatment with histone deacetylase (HDAC) inhibitors preserves dopaminergic (DA) neuronal function in 1-methyl-4-phenylpyridinium (MPP^+)-treated neuron-glia cultures. Midbrain neuron-glia cultures were treated with vehicle, sodium butyrate (SB) (a) or trichostatin A (TSA) (b) at the indicated concentrations in the presence (■) or absence (□) of 0.5 μM MPP^+. MPP^+ was added 30 min after SB and TSA pretreatment. DA neuronal function was evaluated by the [^{3}H]dopamine uptake assay 7 d after the varying treatments. Results are expressed as percent of control and represent means ±S.E. of three separate experiments. * $p<0.05$, ** $p<0.01$, compared to untreated control groups; $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$, compared to MPP^+ alone group.

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Figure 2

Histone deacetylase (HDAC) inhibitors prevent cell loss of dopaminergic (DA) neurons in 1-methyl-4-phenylpyridinium (MPP^+)-treated neuron-glia cultures. Midbrain neuron-glia cultures were treated with vehicle, 1.2 mM valproic acid (VPA), 1.2 mM sodium butyrate (SB), or 100 nM trichostatin A (TSA) in the presence (■) or absence (□) of 0.5 μM MPP^+.MPP^+ was added 30 min after SB and TSA pretreatment. DA neuronal cell loss was assessed by tyrosine hydroxylase (TH) immunostaining 7 d after treatment (a). Results are expressed as percent of control and are the means±S.E. of three experiments. * $p<0.05$, ** $p<0.01$, compared to untreated control groups; # $p<0.05$, compared to MPP^+ alone group. (b) Representative micrographs of morphological changes observed following treatment, as indicated in panel (a).

HDAC inhibitors up-regulate GDNF and BDNF gene expression in primary cortical astrocyte cultures

Analysis by quantitative real-time PCR showed significantly increased levels of GDNF and BDNF mRNA in astrocytes treated with these three HDAC inhibitors, except for a surprising decrease in GDNF mRNA at 3 h induced by SB or TSA, compared to vehicle-treated control cultures (Table 2). GDNF transcript in astrocytes treated with 1.2 mM VPA increased to 260% of the control at 6 h, and remained at a high level at later time-points, with a slight decrease noted at 48 h, i.e. 223% of the control (Table 2). The time-course of GDNF mRNA induced by SB was similar to VPA. However, the magnitude of the GDNF mRNA expression induced by SB was less than that induced by VPA (Table 2). TSA induced a more transient increase in GDNF transcript that peaked at 12 h (233%) and returned to control levels by 48 h (Table 2). In the presence of the three inhibitors, a similar bell-shaped pattern of BDNF transcript induction was noted (Table 3). That is, BDNF mRNA had already begun to increase at 3 h, peaked at 12 h and then continued to decrease towards baseline levels by 24–48 h (Table 3). At the 12 h time-point, VPA, SB and TSA increased the expression of BDNF transcript by 457%, 187% and 450%, respectively, compared to the vehicle control.

Table 2

GDNF mRNA levels in astrocytes treated with HDAC inhibitors

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GDNF, Glial cell line-derived neurotrophic factor; HDAC, Histone deacetylase; VPA, valproic acid; SB, sodium butyrate; TSA, trichostatin A.

Results are expressed as percent of untreated control and are the means±S.E. of at least three separate experiments.

* $p<0.05$
** $p<0.01$, compared to untreated controls.

Table 3

BDNF mRNA levels in astrocytes treated with HDAC inhibitors

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BDNF, Brain-derived neurotrophic factor; HDAC, Histone deacetylase; VPA, valproic acid; SB, sodium butyrate; TSA, trichostatin A.

Results are expressed as percent of untreated control and are the means±S.E. of at least three separate experiments.

*$p<0.05$
**$p<0.01$, compared to untreated controls.

HDAC inhibitors increase promoter activity of GDNF and induce hyperacetylation of GDNF promoter-associated histone H3

The C6 cells were used to confirm that HDAC inhibitors triggered an increase in GDNF promoter activity in astrocytes. Significant increases in the GDNF promoter activity in C6 cells were observed in the presence of all three HDAC inhibitors, compared to that in control cells (Figure 3).

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Figure 3

Histone deacetylase (HDAC) inhibitors enhance glial cell line-derived neurotrophic factor (GDNF) promoter activity. C6 glioma cells were transfected with a pGL3-GDNF$^{-1412/+24}$ reporter construct and treated with 1.2 mM valproic acid (VPA), 1.2 mM sodium butyrate (SB) or 100 nM trichostatin A (TSA) for 24 h before the GDNF promoter activity was assayed in the luciferase reporter system. Results are the means±S.E. of three independent experiments and expressed as relative luciferase activity (firefly luciferase relative to Renilla luciferase activity) compared to untreated cells. * $p<0.05$, compared to control groups.

To further investigate whether histone hyperacetylation contributed to the induction of GDNF transcripts in astrocytes by HDAC inhibitors, we performed a ChIP assay to examine histone H3 acetylation at the GDNF promoter. Levels of histone H3 acetylation were examined at three sites within a 1.4 kb GDNF promoter fragment using acetylated H3 antibody and normal rabbit IgG. The relative positions of these sequences (GDNF Pa, Pb and Pc) are shown in Figure 4a. ChIP and input DNA first analysed by PCR (Figure 4b), demonstrated that VPA, SB and TSA treatment for 24 h did increase histone H3 acetylation at the astrocyte GDNF promoter, with most prominent changes in the region closest to the transcriptional initiation site (GDNF Pc) (Figure 4b). PCR of normal IgG control DNA indicated that the acetylated H3 antibody was specific. To better quantify the changes in acetylation, quantitative real-time PCR was then performed with the GDNF Pc primer set to examine H3 acetylation at different time- points (Figure 4c). VPA, SB and TSA all caused an increase in H3 acetylation levels at 5 h, 24 h and 48 h with a maximal elevation of more than 4-fold (Figure 4c). Notably, a robust increase in GDNF Pc-associated histone H3 acetylation was already observed 5 h after initial VPA, SB or TSA treatment, suggesting that histone hyperacetylation occurs before GDNF transcriptional activation. These results suggest that promoter-associated histone hyperacetylation contributes to the induction of GDNF by HDAC inhibitors.

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Figure 4

Glial cell line-derived neurotrophic factor (GDNF) promoter-associated histone H3 is hyperacetylated in astrocytes treated with histone deacetylase (HDAC) inhibitors. (a) Schematic representation of the rat GDNF promoter. The locations of GDNF promoter primers are indicated with arrowheads. (b) Enriched cortical astrocyte cultures were treated with 1.2 mM valproic acid (VPA), 1.2 mM sodium butyrate (SB), or 100 nM trichostatin A (TSA) for 5, 24 or 48 h. Chromatin immunoprecipitation (ChIP) assay was performed using an anti-acetyl-histone H3 antibody. The amount of immunoprecipitated (ChIP DNA) and non-immunoprecipitated genome DNA (input DNA) following 24 h treatment was measured by PCR with GDNF Pa, GDNF Pb and GDNF Pc primer sets. PCR products were run on a 2% agarose gel and stained with ethidium bromide. Representative results from gels for each primer set are shown. (c) Real-time PCR was performed with the GDNF Pc primer set to quantify the ChIP DNA and input DNA after 5 h (□), 24 h (■) or 48 h (■) treatment using a relative standard curve method. The values of the ChIP DNA were normalized to the input DNA. Data are expressed as fold-change over the control and are the means±S.E. of three independent experiments. * $p<0.05$, ** $p<0.01$, compared to control groups.

Discussion

HDAC inhibitors exert complex effects on multiple cell types through diverse cellular mechanisms. While our earlier report on the HDAC inhibitor VPA (Chen et al., 2006) suggested that HDAC inhibitors regulate astrocyte function to confer neuroprotection, the mechanisms through which this occurred were largely unknown. Here, we addressed two aspects of the cellular and molecular mechanisms underlying VPA regulation of astrocyte function: (1) the ability of structurally diverse HDAC inhibitors to regulate the production of astrocyte growth factors and confer neuroprotection ; (2) the role of histone acetylation in the regulation of astrocyte growth factor expression. We now show that HDAC inhibition by VPA, SB, and TSA is associated with DA neuroprotection and increased BDNF and GDNF transcription in astrocytes, where histone hyperacetylation in the astrocyte GDNF promoter may contribute to both the enhanced GDNF promoter activity and gene transcription.

HDAC inhibitors and neuroprotection

Histone acetylation is an important aspect of gene expression, where HDAC and histone acetyltransferase (HAT) are the two enzymes that reciprocally regulate the acetylation of core histones and some non-histone proteins (Kuo and Allis, 1998). In general, HDAC inhibition causes hyperacetylation of core histones and subsequent chromatin remodelling, which leads to gene activation or suppression (Rodriquez et al., 2006). However, because HDAC inhibitors can also mediate changes in gene expression by regulating acetylation of non-histone proteins, the potential mechanisms through which these compounds exert their effects are often unclear.

In the present study, we demonstrated that SB (HDAC inhibitor structurally similar to VPA) and TSA (HDAC inhibitor structurally unrelated to VPA) promote DA neuronal survival and protect DA neurons from MPP^+ in neuron-glia cultures (Figures 1, 2), which paralleled the effects of VPA reported in our previous study (Chen et al., 2006). In addition, optimal concentrations of VPA, SB and TSA for inducing the neurotrophic and protective effects on DA neurons were shown to induce significant increases in GDNF and BDNF mRNA in enriched astrocytes (Tables 2, 3). Taken together, this study indicates that HDAC inhibition, and not just VPA, regulates the expression of growth factors from and the neuroprotective characteristics of astrocytes.

Increased acetylation of GDNF promoter

One of the major functions of astrocytes is the production of a host of neurotrophic factors, including GDNF and BDNF, which support neuronal development, plasticity and survival (Koyama, 2002; Seifert et al., 2006). In fact, the presence of astrocytes and astrocyte-conditioned medium have potent and selective survival-promoting effects on cultured DA neurons (O'Malley et al., 1992; Takeshima et al., 1994). Exogenous GDNF and BDNF prominently protect DA neurons from both natural and induced cell death (Beck et al., 1995; Burke et al., 1998; Canudas et al., 2005; Lin et al., 1993; Tomac et al., 1995), where protein levels of both factors are decreased in PD brains (Chauhan et al., 2001). Moreover, decreased production of neurotrophic factors including BDNF have been implicated in the pathogenesis of other neurological disorders, including Alzheimer's disease (AD) (Connor et al., 1997; Ferrer et al., 1999; Phillips et al., 1991), schizophrenia (Durany et al., 2001) and mood disorders (Duman, 2004; Hashimoto et al., 2004). It is also noteworthy that chronic-defeat stress induces down-regulation of BDNF mRNA and this deficiency is normalized by the antidepressant imipramine, an effect that appears to involve down-regulation of HDAC 5 isoform (Tsankova et al., 2006). Recently, it was also reported that SB induces antidepressant-like effects in mice following chronic administration (Schroeder et al., 2007). On the other hand, status epilepticus is also reported to increase histone acetylation at specific BDNF promoters and up-regulate BDNF expression in the brain (Huang et al., 2002; Tsankova et al., 2004). In the present study, we show for the first time that HDAC inhibitors induce BDNF production in astrocyte cultures, suggesting that histone acetylation also regulates BDNF expression in astrocytes. Until the present study, the mechanisms through which HDAC regulated astrocyte growth factors such as GDNF and BDNF, were poorly understood.

As mentioned earlier, HDAC inhibitors are known to affect gene expression through increased acetylation of either histones and/or non-histone proteins, such as transcription factors, at selective gene promoters (de Ruijter et al., 2003). To investigate whether the increases in GDNF gene expression reported previously (Chen et al., 2006) are related to HDAC blockade by VPA, we examined GDNF promoter activity in an astroglial cell line and promoter-associated histone acetylation in primary astrocyte cultures treated with VPA, SB and TSA. We found that all three HDAC inhibitors induced a marked increase in GDNF promoter activity and promoter-associated histone H3 acetylation, and that the time-course of the change in acetylation correlated with that of the increased GDNF mRNA levels. Thus, histone hyperacetylation at the GDNF promoter mediates, at least in part, the activation of GDNF transcription by HDAC inhibitors in astrocytes. Together, these results suggest that HDAC inhibition is involved in VPA-elicited DA neuroprotection, and more importantly, that up-regulation of GDNF and BDNF in astrocytes through histone hyperacetylation is probably a mechanism by which HDAC inhibitors exert their neuroprotective effects.

Collectively, the present research suggests that HDAC inhibitors up-regulate GDNF and BDNF expression in astrocytes through histone hyperacetylation. Our results also suggest that the neuroprotective/neurotrophic effects of HDAC inhibitors on DA neurons are mediated, at least in part, through the induction of these growth factors. Further studies are needed to determine whether the effect of HDAC inhibitors on cultured astrocytes can be replicated using an animal model of PD. However, our findings are strongly supported by a recent study showing that the HDAC inhibitor phenylbutyrate significantly attenuated MPTP-induced depletion of striatal dopamine and loss of DA neurons in mouse substantia nigra (Gardian et al., 2004). Moreover, the present study also suggests the prevention of brain volume reduction by VPA in patients with bipolar disorder may be mediated through increased neurotrophic support from astrocytes. As such, astroglial GDNF and BDNF may represent ideal therapeutic targets for the development of novel neuroprotective drugs in PD and other neuropsychiatric/neurodegenerative diseases.

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