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# Inhibition of class II histone deacetylase blocks proliferation and promotes neuronal differentiation of the embryonic rat neural progenitor cells

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Neural precursor cells (NPCs), which are capable of self-renewing, migrating to specific sites, and differentiating into the three main CNS lineages, neurons, astrocytes and oligodendrocytes, have been used experimentally to repair the damaged nervous system, either by grafting of cells grown *in vitro* or by activating endogenous NPCs. The grafting of NPCs, however, is limited by its lower viability and undesired glial differentiation. Understanding the mechanism underlying these events, therefore, is essential for the potential future use of NPCs. In the present study, we investigated the role of histone deacetylase (HDAC) inhibition on survival, proliferation, differentiation and migration of the rat NPCs. We observed that NPCs derived from the E14.5 rat brain constitutively expressed both class I and class II HDAC mRNA. Inhibition of HDAC by trichostatin A (TSA) blocked the proliferation, increased neuronal differentiation and decreased astrocyte differentiation of the NPCs. Meanwhile, TSA had no significant effects on survival and migration of the NPCs. Finally, we found that HDAC inhibition regulated proliferation and neuronal differentiation of the NPCs was associated with a reduction of class II and but not class I HDAC transcription. These findings collectively demonstrate that in the situation of not affecting survival and migration, HDAC inhibition may induce more neuronal differentiation.

Key words: histone deacetylase, trichostatin A, neural progenitor cells, survival, proliferation, differentiation, migration

## INTRODUCTION

Neural precursor cells (NPCs) are a heterogeneous population of mitotically active, self-renewing and multipotent cells of both the developing and the adult CNS, and show complex regulation patterns of gene expression that vary in space and time (Gage 2000, Temple 2001). NPC-based therapies for nervous system disorders – for example, stroke (Doeppner et al. 2010), Parkinson's disease (Cai et al. 2009), multiple sclerosis (Martino et al. 2010) and spinal cord injury (SCI) (Karimi-Abdolrezaee et al. 2006, Ziv et al. 2006, Xu et al. 2010) – have been successfully developed. Although most of these attempts have succeeded in experimental models, there are still important issues that need to be addressed before any potential applications of such promising therapies in humans can be foreseen (Martino and Pluchino 2006). One of the most important issues is to figure out how the cell lineage differentiation is regulated, because more neuronal differentiation to neurons in injured CNS is preferred for NPCs grafting. Previous studies, however, have indicated that most of the grafted NPCs differentiated into astrocytes but not neurons (Cao et al. 2001, Xu et al. 2010).

The reversible histone acetylation and deacetylation are epigenetic phenomena that play critical roles in the modulation of chromatin topology and the regulation of gene expression. The acetylation of the nucleosomal histone is controlled by a balance between histone acetyl transferase (HAT) and histone deacetylases (HDAC) enzymes. HAT cause chromatin relaxation

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and de-compaction, leading access to promoter DNA for transcriptional activators, while HDACs negatively control histone acetylation by removing covalent acetylation marks from histone tails, resulting in DNA compaction and repression of the genes (Peterson 2002, Saha and Pahan 2006). HDACs have been shown to regulate many important biological processes, including cell proliferation and differentiation (Humphrey et al. 2008, Montgomery et al. 2009) by forming complexes with various transcription factors and transcriptional co-regulators (Haberland et al. 2009). HDACs also act to regulate stem cell self-renewal and differentiation by controlling the activity of a network of downstream target genes (Hsieh et al. 2004). The ability to inhibit HDAC activity with small molecule HDAC inhibitors (HDACi) has attracted considerable therapeutic attention for nervous system diseases (Cheng et al. 2004). Recent work showed that valproic acid (VPA), an inhibitor of HDAC, increased neuronal differentiation of hippocampal neural progenitors (Hsieh et al. 2004, Yu et al. 2009). Other HDACi such as suberoylanilide hydroxamic acid (SAHA), MS-275 and M344 increased neuronal differentiation in adult forebrain precursor cells (Siebzehnrubl et al. 2007). Furthermore, the HDACi sodium butyrate (SB), promoted the differentiation of embryonic stem cells into neural cells in adherent monoculture (Martino et al. 2010). In addition, SAHA and SB suppressed the formation of neurospheres and blocked proliferation of adult mouse NPCs derived from subventricular zone (SVZ) *in vitro* by controlling cell cycle progression (Zhou et al. 2011). In addition to affecting cell proliferation and differentiation, SB and VPA enhanced retinal ganglion cells (RGC) survival at 0.1 mM. In contrast, Trichostatin A (TSA) at the dose of 0.5 to1.0 nM did not affect RGC survival but increased cell death at concentrations over 5 nM (Biermann et al. 2011). Previous study also demonstrated that TSA increased neuronal differentiation, with the decrease of astrocyte differentiation in embryonic mouse NSCs (Balasubramaniyan et al. 2006). However, the biological functions of TSA on NPCs derived from embryonic rat cortical cortex remain elusive. The purpose of this study is to determine the effects of TSA on survival, proliferation, differentiation, and migration of NPCs derived from embryonic rat neocortex, the possible mechanism underlying these TSA-mediated effects has also been clarified.

## METHODS

### Cell culture

NPCs were prepared from the cortex of embryonic E14.5 Sprague Dawley rats according to our previous method (Wang et al. 2007). Following removal of dura the cortexes were dissected out and dissociated to achieve single-cell suspensions. The suspension was filtered through a nylon mesh of 70 μm. After centrifugation at 1000×g for 8 min at 4°C, the cells were resuspended in serum-free Dulbecco's Modified Eagle's Medium/F12 medium (DMEM/F12, Gibco) supplemented with 0.06% glucose, 1.34 mM bicarbonate sodium, 0.5 mM HEPES, 2 μg/ml heparin (all from Sigma), 1×N2, 2 mM glutamine and 2% B27 (all from Gibco), which was described as a basal medium. Then, a total of 6 ml cells at a density of $0.1\times10^6$ cells/ml were plated in T25 uncoated culture flasks supplemented with 20 ng/ml of EGF (Sigma) and 20 ng/ml of bFGF (Gibco), which was described as a proliferation medium. Cells were incubated in a humidified atmosphere containing 5% $CO_2$ at 37°C. Culture medium was changed twice weekly by replacing half of the old medium by an equal volume of fresh medium containing the same concentrations of trophic factors. Spheres appeared within a few days and were grown for 3–5 days before collection for passage.

### Cytotoxicity assay

NPC Spheres were dissociated and single cells were transferred to 96-well microtiter plate ($1.0 \times 10^5$cells/100 μl/ well; Corning Costar Corp.) allowing them to grow under basal proliferation conditions for 24 h. Basal proliferation medium (100 μl) containing different concentrations of Trichostatin A (TSA, Sigma, St. Louis, MO, USA) was then added to the wells for another 24 h. Supernatants (50 μl) were transferred to an enzymatic assay plate and analyzed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Southampton, UK). This measures the release of lactate dehydrogenase (LDH) into the media by dead NPC cells. Results were expressed as the mean ± SD of the percentage of the absorbance of media from cells grown in different concentrations of TSA compared to that from cells grown in medium with no TSA (control) in eight independent experiments.

### Cell proliferation assay

The NPC proliferation assay was carried out by counting the viable cell number with Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). This assay is based on the conversion of water-soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disul fophenyl)-2H-tetrazlium, monosodium salt] to a water-soluble formazan dye upon reduction in the presence of an electron carrier by dehydrogenases. Briefly, NPC spheres were dissociated in DMEM/F12 and single cells were transferred to 96-well plates ($2.0 \times 10^4$ cells/100 µl/ well) allowing them to grow under basal proliferation conditions for 24 h. Basal proliferation media (100 µl) containing different concentrations of TSA was added to the wells for another 24, 48, and 72 h. The solution (10 µl) was added into each well, followed by incubation of the plates at 37°C for 5 h. The absorbance at 450 nm was determined by a multi-plate reader (Bio-Rad, Hercules, CA). Cell viability was expressed as a percentage of the absorbance values in different concentrations of TSA at three time-points compared to that in different concentrations of TSA immediately after TSA (control) in five independent experiments.

### Lineage fate assay

Dissociated NPCs in single-cell suspension were seeded onto poly-L-lysine (10 µg/ml; Sigma) pre-coated 12 mm coverslips in 35 mm dishes at a density of $1.5 \times 10^5$ cells/coverslip for 30 min. Then, a differentiation medium containing 1% fetal bovine serum (FBS, Gibco) and different concentrations of TSA, but no EGF and bFGF were added. The cells were allowed to differentiate for 7 days *in vitro* before fixed for immunofluorescence labeling. The percentage of cell specific marker positive cells over total number of all cells was measured. At least 10 random fields over 200 cells were counted for each condition. The results are presented as mean ± SD of three independent experiments.

### Immunofluorescence labeling

Floating spheres were harvested from the flasks and fixed using 4% paraformaldehyde (PFA) in 0.01M PBS (pH 7.4) at 4°C overnight (O/N) and were transferred to a solution containing 30% sucrose in 0.01M PBS for 2–3 days. NPC spheres were cut as tissue at 20 µm with a cryostat. Coverslips with differentiated cells were rinsed

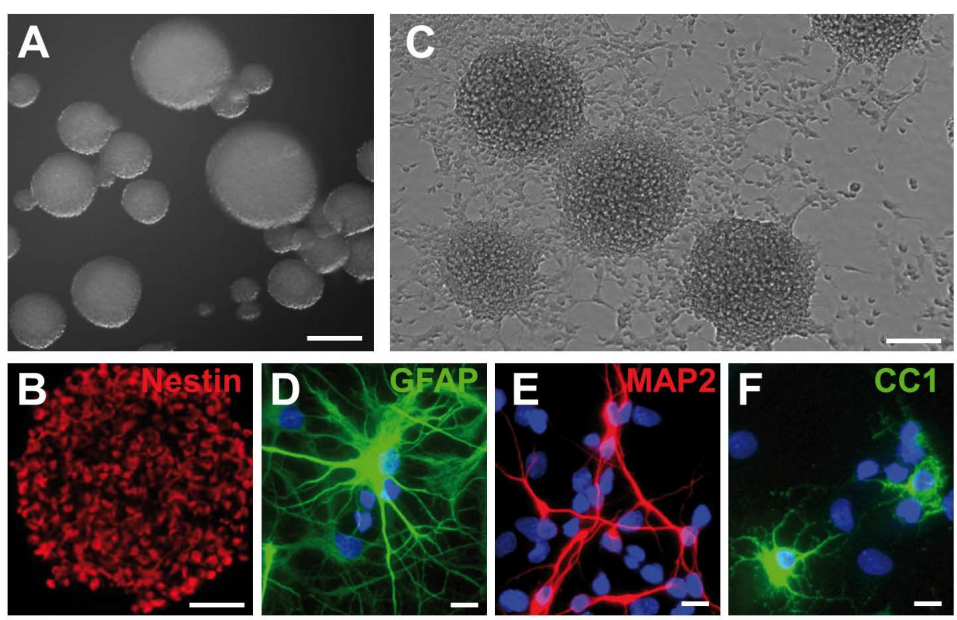


Fig. 1. Characterization of NPC spheres. (A) NPC spheres at passage 2. (B) Immunofluorescence staining of a typical NPC sphere showed that the cells were nestin$^+$. (C) After plating the NPC spheres on a coated coverslip, cells migrated out from the spheres. (D–F) These cells could differentiate readily into astrocytes (D, GFAP$^+$), neurons (E, MAP2$^+$) and oligodendrocytes (F, CC1$^+$). Nuclei counterstained with Hoechst. Scale bars are 200 µm (A), 100 µm (B), 50 µm (C), and 5 µm (D–F).

Table I

Primers used in RT-PCR and quantitative RT-PCR analysis

| Gene | Forward primers (5′-3′) | Backward primers (5′-3′) | Size (bp) |
|---|---|---|---|
| GAPDH | AAGTTCAACGGCACAGTCAAG | CCAGTAGACTCCACGACATACTCA | 137 |
| HDAC1 | GACATGCCAAGTGTGTGGAGT | ATATTGGAAGGGCTGATGTGA | 211 |
| HDAC2 | AAGGTCACGCTAAATGTGTAGAAG | AATCATTATATGGCAACTCATTGG | 162 |
| HDAC3 | GCTGCTTCAATCTCAGCATTC | TATAGGGAAGTTCCTCACTAATGG | 177 |
| HDAC4 | GCTGGATGAGCCCTACCTAGA | CCTCCATAGATGCCTGGTAGTT | 233 |
| HDAC5 | TGGCGAGAGTGGTCCTGAT | TGTGTGTTTCCGCACATACAC | 288 |
| HDAC6 | ATGCGGGATGCCGACTAC | GCCAAACCCATAAGGAAATG | 182 |
| HDAC7 | ACCGACCTTGCCTTCAAAGT | ATGGCGATGAAGGGATATGTA | 261 |
| HDAC8 | CAGCAAATCCTCAACTACATCAA | ACAAATCCCACAAACTGCTTG | 133 |
| HDAC9 | ATGTTCTCCACCAGTCTGTGAAT | GCTGTTCCACATCTACCGTCA | 192 |
| HDAC10 | TTCACAAGTCCCAGTTTCAGG | CTCTATGGGCTACACATCGCT | 168 |

with 0.01M PBS twice, fixed with 4% paraformaldehyde for 30 min, and rinsed with PBS for 10 min 3 times at room temperature (RT). The sections or coverslips were permeabilized and blocked with 0.3% Triton X-100/3% normal goat serum in 0.01M PBS for 30 min at 4°C. Primary antibodies were applied to the sections or coverslips at 4°C overnight. The cell specific primary antibodies included mouse anti-nestin monoclonal antibody (1:200, Chemicon International, Temecula, CA) to identify NPCs, mouse anti-$MAP_2$ antibody (1:400, Chemicon) to identify neurons, mouse anti-glial fibrillary acidic protein (GFAP; 1:400, Chemicon) to identify astrocytes, mouse anti-APC clone CCI antibody (1:20, Calbiochem) to identify oligodendrocytes. On the following day, the sections or coverslips were incubated with fluorescein-conjugated goat anti-mouse (FITC; 1:400, Chemicon) or Cy3-conjugated goat anti-mouse (1:50, Chemicon) antibodies for 2 hours at 37°C. To visualize nuclei, cells were counterstained with Hoechst 3342 (Sigma) (10 μg/ml, 10 min). Finally, sections or coverslips were mounted and examined under a Zeiss fluorescent microscope. Primary antiserum omission controls and normal mouse and goat serum controls were used to further confirm the specificity of the immunofluorescence labeling.

**Cell migration assay**

The scratch wound healing migration assay and transwell chamber transmigration assay were performed to analyze the effect of TSA on the migration of NPCs according to previous literature (Meintanis et al. 2001). Briefly, the dissociated NPCs were plated in 24-well plates coated with poly-D-lysine and fibronectin and maintained in serum-free NeuralBasal (NB) medium supplemented with 2% B27, 2 mM L-glutamine and 10 ng/ml bFGF (Ma et al. 1998, Lin et al. 2004) for 24 h. A scratch was made on the monolayer using a sterile 200 μl pipette tip. The cells were pretreated with DNA-polymerase inhibitor aphidicolin (12 mg/ml; Sigma) for 30 min and were then exposed to different concentrations of TSA for 48 h to allow cell migration into the scratched area. At the very beginning and 48 h following TSA treatment, cells were photographed using a low-magnification phase-contrast microscope. The migration distance was measured by subtracting the average gap width at the very beginning from that at 48 h following treatment using ImageJ software. The average speed of cells was also evaluated qualitatively by dividing gap width by time. To further confirm the effect of TSA on

NPCs migration, a transwell chamber analysis was performed according to our previous method (Wang et al. 2007). The transwell chambers with fibronectin-coated porous membrane in the bottom were immersed in wells half-filled with basal medium in the presence or absence of 10 ng/mL TSA. The NPC spheres were dissociated and plated into chambers for 48 h. The cells crossed the membrane were counted.

### RNA extraction, reverse transcription, RT-PCR and quantitative real-time PCR

Total RNA was isolated from the NPCs treated with TSA (10 ng/ml) or without TSA for 48 hours using TRIzol® Reagent (Invitrogen) according to manufacturer's instruction. Reverse transcription (RT) for complementary DNA (cDNA) synthesis was performed with 2 μg total RNA using RevertAidTM First Strand cDNA Synthesis Kits (Fermentas, Hanover, MD, USA) with random primers. RT-PCR was performed in total reaction volumes of 20 μl using DreamTaq Green PCR Master Mix (2X) (Fermentas, Hanover, MD, USA) in MyCycler™ Thermal Cycler 170-9703 instrument (Bio-Rad, Laboratories, Inc., USA) according to the manufacturer's protocol. Primer sets used for amplification of Class I and II HDAC genes, and sizes of PCR products are shown in Table I. The PCR conditions were as follows: 95°C for 2 min followed by 30 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and 72°C for 10 min. The specificity of the PCR product was verified by 1.5% agarose gel electrophoresis. Quantitative real-time PCR (qRT-PCR) was carried out in a 10 μL final volume and performed in triplicates using Power SYBR Green PCR Master Mix reagents (Fermentas, Hanover, MD, USA) in an ABI Prism 7500 sequence detection system (Applied Biosystems, Framingham, MA, USA) according to the manufacturer's protocol. Primer sets used for amplification of Class I and II HDAC genes, and sizes of PCR products are shown in Table I. The conditions for real-time PCR were as follows: 95°C for 10 min followed by 40 cycles at 94°C for 10 s, and 60°C for 1 min. Raw fold changes in target gene expression (ΔCt) were calculated by transforming the difference in Ct values of treated vs. untreated cells: $2^{-(\text{treated Ct} - \text{untreated Ct})}$. Fold changes in target gene expression were then normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) *via* the published comparative method using the formula: $2^{-\Delta\Delta Ct}$

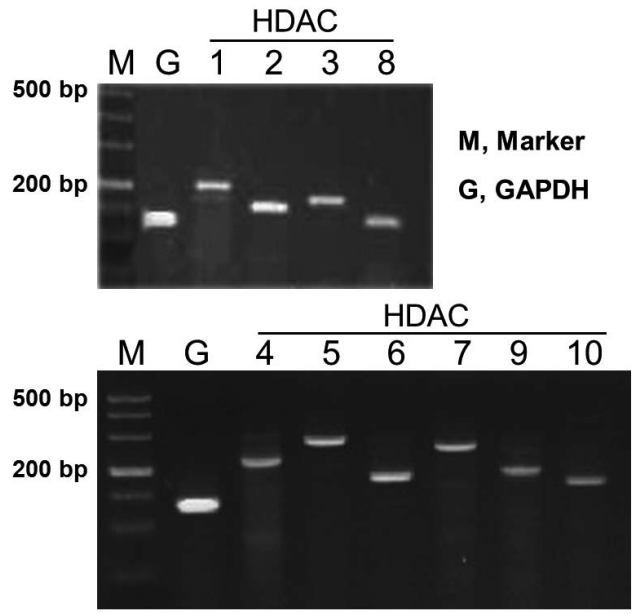


Fig. 2. RT-PCR shows gene expression of class I (1, 2, 3, and 8) and class II (4, 5, 6, 7, 9, and 10) HDAC in NPC spheres. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, served as an internal control. The resultant product sizes of each amplicon are: GAPDH, 137 bp; HDAC1-10, 211, 162, 177, 233, 288, 182, 261, 133, 192,168 bp, respectively. (M) DNA marker.

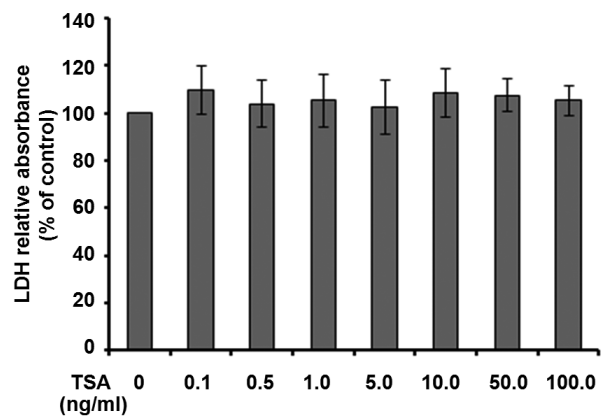


Fig. 3. Effect of TSA on the survival of NPCs. NPC spheres were dissociated and single cells were transferred to 96-well plates allowing them to grow under basal proliferation conditions for 24 h. Basal proliferation media (100 μl) containing 0.1–100 ng/ml of TSA was added to the wells for another 24 h. LDH concentrations in supernatants were detected to evaluate the survival of NPCs. There were no significant difference between TSA treatment groups and control group by one way ANOVA ($F$=0.34, $P$=0.93, $n$=8). The Dunnett's post hoc test revealed there were no significant differences between individuals groups ($P$=0.28–0.99, $n$=8). Data are presented as means ± SD.

### Statistical analysis

Statistical comparisons were made using Student's *t*-test, one-way or two-way ANOVA followed by Dunnett's *post hoc* test. Differences were assessed as significant when $P<0.05$. All data are presented as means ± SD.

## RESULTS

### Growth and fate of embryonic neural precursor cells expanded in spheres *in vitro*

Cerebral cortex from E14.5 embryonic rats were triturated, dissociated and plated in serum-free N2/B27 medium supplemented with EGF and bFGF. Under this condition, most cells died and a subpopulation of cells proliferated and formed clusters of small round cells that grew into floating spheres (Fig. 1A), these spheres were passaged by mechanical dissociation once every 4–5 days. Cell specific marker staining of sphere sections showed that they were nestin$^+$ cells (Fig. 1B). Potential of sphere cells to differentiate along the neural lineages was checked by their adherence to coated coverslips that promoted outward cell migration (Fig. 1C) and by their differentiation in 1% fetal bovine serum medium *in vitro*. Immunofluorescent staining performed after 7 days of differentiation showed that these spheres were multipotential and they generated cells of all three lineages, including glial fibrillary acidic protein positive astrocytes (GFAP$^+$, Fig. 1D), microtubule-associated protein 2 positive neurons (MAP2$^+$, Fig. 1E), and CC1 positive oligodendrocytes (CC1$^+$, Fig. 1F). Thus, we considered the spheres as neural precursor cell spheres (NPC spheres) or neural precursor cells (NPCs).

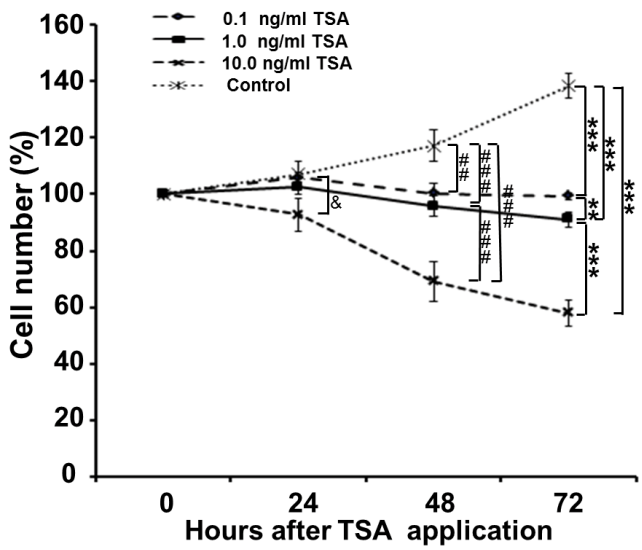


Fig. 4. Effect of TSA on the proliferation of NPCs. NPCs were treated with TSA (0.1, 1, 10 ng/ml) for 0, 24, 48, and 72 h and the total number of cells were counted at these time points. In normal culture medium, cell number significantly increased at 24, 48, and 72 h respectively compared to original plated cells. While high dose of TSA significantly inhibited the NPC proliferation ($P=0.04$, $n=5$), there were no significant differences between control, 0.1 ng/ml TSA, and 1.0 ng/ml TSA group at 24 h. TSA significantly inhibited the NPC proliferation in a dose dependent manner at 48 h ($F=19.99$, $P<0.001$, $n=5$) and 72 h ($F=129.05$, $P<0.001$, $n=5$). Data are presented as means ± SD. $^{\&}$ $P<0.05$, $^{\#\#}$ or $^{**}P<0.01$, $^{\#\#\#}$ or $^{***}$ $P<0.001$.

The expression of genes for class I HDAC (HDAC1, 2, 3, and 8) and class II HDAC (HDAC4, 5, 6, 7, 9, and 10) in NPC spheres was examined by reverse transcriptase polymerase chain reaction (RT-PCR). The analysis revealed the presence of mRNAs encoding all HDAC 1-10 genes in NPC spheres (Fig. 2), suggesting that HDAC may play a critical biological role in NPCs and conversely inhibiting the activity of HDACs in NPCs may potentially affect the normal biological functions of NPCs as examined in the experiments described below.

### Effect of TSA on NPC survival

To determine whether HDAC inhibition affects the survival of NPCs, NPC spheres were mechanically dissociated and 10 000 viable NPCs were plated into 96-well microplates for 24 h followed by the addition of different concentration of TSA (0.1, 0.5, 1.0, 5.0, 10.0, 50.0, 100.0 ng/ml) for another 24 h. Cell survival was evaluated by measuring LDH released into the medium from dead or dying cells. We found that there were no significant differences between TSA treatment groups and control group by one way ANOVA ($F=0.34$, $P=0.93$, $n=8$). The Tukey's *post hoc* test revealed there were no significant differences between individuals groups ($P=0.28$–$0.99$, $n=8$). (Fig. 3), indicating that inhibition of HDAC activity has no direct effect on the survival of NPCs.

### Effect of TSA on NPCs proliferation

To evaluate the effect of HDAC inhibition on NPCs proliferation, NPCs were treated with various con-

centrations of TSA (0.1, 1, 10 ng/ml) for 0, 24, 48 and 72 h. Cell proliferation was assessed by counting the total cell number using Cell Counting Kit-8. The proliferation curve can be clearly observed in normal culture medium with increased 107.2 ± 4.2%, 117.4 ± 5.6%, and 138.7 ± 4.4% cell at 24, 48, and 72 h respectively (Fig. 4). Although there were no significant differences between control, 0.1 ng/ml TSA, and 1.0 ng/ml TSA group at 24 h, 10.0 ng/ml TSA significantly inhibited the NPC proliferation (92.7 ± 13.2%, $t$=2.35, $P$=0.04, $n$=5) at 24 h. With increased interaction time, TSA significantly inhibited the NPC proliferation in a dose dependent manner (100.4 ± 7.3%, 95.8 ± 8.3%, and 69.2 ± 15.6% at 48 h, $F$=19.99, $P$<0.001, $n$=5; 99.4 ± 2.6%, 91.1 ± 5.9%, and 58.1 ± 10.4% at 72 h, $F$=129.05, $P$<0.001, $n$=5; respectively).

### Effect of TSA on NPCs differentiation

To examine the effect of HDAC inhibition on the lineage fate of NPCs, the NPC spheres were dissociated and adhered to PLL-coated coverslips, allowing them to differentiate in the absence of mitogens (EGF and bFGF) but in the presence of 1% FCS with or without the addition of TSA (0.1, 1.0, 10.0 ng/ml) for 7 days. Immunofluorescent staining showed that, in the control cultures (no TSA), there were 14.3 ± 0.7% $MAP2^+$ cells, 47.0 ± 5.0% $GFAP^+$ cells, 3.3 ± 0.5% $CC1^+$ cells (Fig. 5, $n$=3). Inhibition of HDAC with low dose of 0.1 ng/ml TSA significantly increased the neuronal differentiation (22.9 ± 0.9%, $P$<0.01, $n$=3) of NPCs, moderate (1.0 ng/ml) and high (10 ng/ml) dose of TSA further increased the percentage of $MAP2^+$ cells in a dose-dependent manner (25.9 ± 1.4% and 30.0 ± 1.2%, respectively). However, with the inhibition of HDAC in NPCs the differentiation of astrocytes decreased in moderate (28.5 ± 2.0%, $P$<0.05, $n$=3) and higher concentration of TSA (33.0 ± 4.1%, $P$<0.05, $n$=3) compared to control group, although we did not observe significant differences of the $GFAP^+$ cells between 0.1 ng/ml TSA group and control group ($P$=0.28). Finally, we compared the percentage of $CC1^+$ cells following induction of graded HDAC inhibition with TSA and found that there was no significant difference ($F$=1.59, $P$=0.26).

### Effect of TSA on NPCs migration

To further address the effect of HDAC inhibition on NPCs migration, a scratch wound-healing assay was performed to examine the migration distance and speed as previously described (Meintanis et al. 2001). We found that adherent culture NPCs displayed weak migration capability, in control group, only few cells migrated into the scratch area after 48 hour of culture (Fig. 6A, second row), treatment with TSA did not result in more cells migrating into the scratched area, or increase their migration distance. Quantification of the average migration distance (Fig. 6B) and corresponding speed rate (Fig. 6C) indicated that there were no sig-

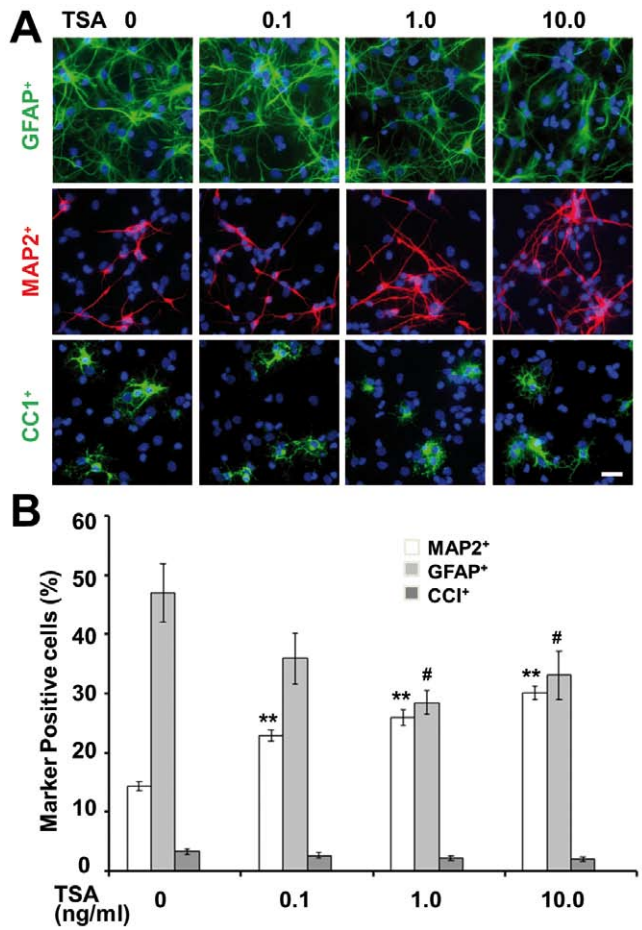


Fig. 5. Effect of TSA on the differentiation of NPCs. NPC spheres were dissociated and adhered to poly-lysine-coated coverslips allowing them to differentiate in the presence of 1% FCS with or without the addition of TSA (0.1, 1.0, 10.0 ng/ml) for 7 days. Inhibition of HDAC with TSA significantly increased the $MAP_2^+$ neuronal differentiation ($F$=38.71, $P$<0.001) and decreased the $GFAP^+$ astrocyte differentiation ($F$=3.92, $P$=0.04) of NPCs in a dose dependent manner. ** $P$<0.01 vs. control; $^{\#}P$<0.05 vs. control. However, no significant difference were observed between the percentage of $CC1^+$ oligodendrocytes following induction by graded TSA and control group ($F$=1.59, $P$=0.26). Data are presented as means ± SD.

nificant differences between TSA treatment groups and control group by one way ANOVA ($F$=0.86, $P$=0.48, $n$=8). The Tukey's *post-hoc* test revealed there were no significant differences between individuals groups ($P$=0.17–0.79, $n$=8, Fig. 6B,C), indicating that inhibition of HDAC activity has no direct effect on the migration of NPCs. To further confirm the effect of TSA on NPCs migration, a transwell chamber analysis was performed according to our previous method (Wang et al. 2007). There were no significant changes in transmigration in response to TSA, compared with control, evaluated by the number of cells that crossed the membrane (Fig. 6A, third row) which was consistent with the results in scratch wound-healing assay, indicating that inhibition of HDAC did not affect the migration of NPCs.

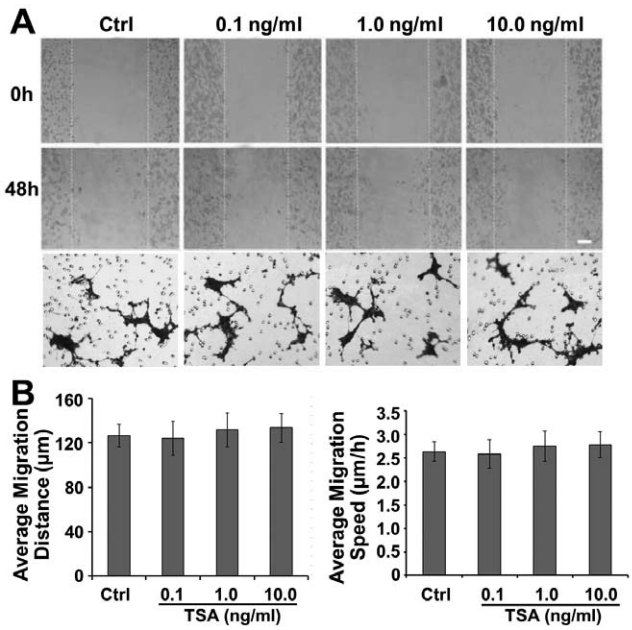


Fig. 6. Effect of TSA on the migration of NPCs. A scratch wound-healing assay and a transwell chamber analysis were performed to examine the migration of the cells into the scratched area and the speed of migration. (A) Immediately after scratch (first row) or plating on chamber, NPCs were treated with different concentration of TSA for 48 hours, TSA did not change the number or the migrated distance of cells (second row) or the number of cells that crossed the membrane (third row). Quantification of the average migration distance (B) and corresponding speed rate **(**C**)** indicated that there were no significant differences between TSA treatment groups and control group by one way ANOVA ($F$=0.86, $P$=0.48, $n$=8). The Dunnett's *post-hoc* test indicated there were no significant differences between individuals groups ($P$=0.17–0.79, $n$=8). Data are presented as means ± SD. Scale bar is 100 μm.

### TSA regulate proliferation and neuronal differentiation of the NPCs by reducing the transcription of class II and but not class I HDAC

To further clarify the type of HDACs related to the TSA application and observed changes of proliferation and neuronal differentiation following TSA, we performed real-time quantitative RT-PCR analyses. Total RNAs were isolated from NPCs cultured in the absence or presence of 10 ng/ml of TSA for 48 hours. Our result showed that HDAC1-10 are constitutively expressed in NPCs (Fig. 2). Although TSA treatment reduced the mRNA expression of Class I HDAC (1, 2, and 3) and increased the mRNA expression of HDAC 8 at 48 h, these differences were not significant compared to control group (Fig. 7A) (HDAC1, $t$=0.42, $P$=0.69; HDAC2, $t$=2.00 $P$=0.12; HDAC3, $t$=3.87, $P$=0.07; HDAC8, $t$=1.62, $P$=0.18) ($n$=3). We then tested the expression of class II HDAC and found that TSA significantly inhibited the expression of HDAC 4-7, 9 and 10 following 48 h TSA treatment (Fig. 7B) (HDAC4, $t$=19.96, $P$<0.001; HDAC5, $t$=9.53 $P$=0.001; HDAC6, $t$=15.11, $P$<0.001; HDAC7, $t$=15.54, $P$<0.001; HDAC9, $t$=8.61, $P$=0.001; HDAC10, $t$=14.87, $P$<0.001) ($n$=3).

## DISCUSSION

In the present study, we extensively investigated the role of HDAC inhibition on survival, proliferation, differentiation and migration of NPCs derived from embryonic rat cortical cortex. We observed that NPCs expressed both Class I and Class II HDAC, inhibition of HDAC by TSA blocked the proliferation and increased neuronal differentiation of the NPCs. However, TSA had no significant effects on survival and migration of the NPCs. Finally, we found that TSA regulated proliferation and neuronal differentiation of the NPCs mainly by reducing the transcription of class II and but not class I HDAC. These findings collectively demonstrated that in the condition of no direct effects on the survival and migration, HDAC inhibition may induce more neuronal differentiation.

HDAC inhibition can induce apoptosis by linking death receptor and mitochondrial pathway of apoptosis. For example, HDAC inhibition may significantly affect the survival of a type-specific cell by regulating the activity of other cells *in vivo* or co-culture system *in vitro*. For instance, valproic acid (VPA), one of the HDAC inhibitors and mood stabi-

lizers, prolongs the survival of midbrain DA neurons in lipopolysaccharide (LPS) or 1-methyl-4-phenylpyridinium ($MPP^+$)-treated neuron-glia cultures through the inhibition of the release of pro-inflammatory factors from microglia or by regulating the expression of neurotrophic factors, including glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) from astrocytes (Chen et al. 2006). In this study, by evaluating the LDH release, we first demonstrated that inhibition of HDAC with TSA had no direct effects on the survival of the rat NPCs. Similar results have been observed in embryonic mouse NSC by analyzing the apoptosis following TSA treatment (Balasubramaniyan et al. 2006). The survival response to TSA implies that the effects of HDACi might be multiple and complicated in the nervous system and may depend on epigenetic status, cell type, tissue specificity, and microenvironment.

HDACi have emerged as the accessory therapeutic agents for multiple human cancers, since they can induce specific changes in gene expression and influence a variety of other processes, including growth arrest, differentiation, cytotoxicity and induction of apoptosis (Humphrey et al. 2008). However, due to the different inhibition mechanism and complicated regulation network, the effect of HDAC inhibition on the cell proliferation may be inhibitor-, cell type-, and environment- specific. In the CNS, for example, it has been reported that VPA, and sodium butyrate (NaB) block the proliferation of adult (Hsieh et al. 2004) and embryonic (Yu et al. 2009) hippocampal NPCs. VPA, however, stimulates the proliferation of NPCs derived from cortex (Laeng et al. 2004). In this study, we demonstrated that TSA significantly blocks the proliferation of NPCs derived from embryonic cortex, an observation that is comparable with the report that TSA reduces the proliferation of adult hippocampal NPCs (Hsieh et al. 2004). Our finding that TSA significantly reduced the proliferation of NPCs from embryonic rat cortical brain suggests that HDAC may play an important regulatory role in NPCs proliferation.

Neurogenesis during development depends on the coordinated regulation of self-renewal and differentiation of neural precursor cells (NPCs). HDACs play redundant and essential roles in the progression of neuronal precursors to mature neurons *in vivo* (Montgomery et al. 2009). It has been reported that VPA increased neuronal differentiation, inhibited astrocyte and oligodendrocyte differentiation in NPCs derived from adult (Hsieh et al. 2004) and embryonic (Yu et al. 2009) hippocampus. Second generation HDACi such as MS-275, M344 and SAHA, mainly increased β-III tubulin positive neurons and decreased oligodendrocytes while astrocyte quantity remained unaffected after HDACi treatment in NPCs from postnatal Wistar SVZ (Siebzehnrubl et al. 2007). Zhou and coauthors (2011) reported that SAHA significantly reduced glial and oligodendrocyte differentiated cell fate, whereas it did not lead to a compensatory increase in neuronal cell fate in culture of adult mouse NSCs harvested from SVZ. By constructing "dominant-negative" stem cell lines, Humphrey and others (2008) proved that HDAC activity inhibits differentiation to oligodendrocytes, and that HDAC2 activity specifically inhibits differentiation to astrocytes, while HDAC1 activity is required for differentiation to neurons. We found in this study that TSA promoted

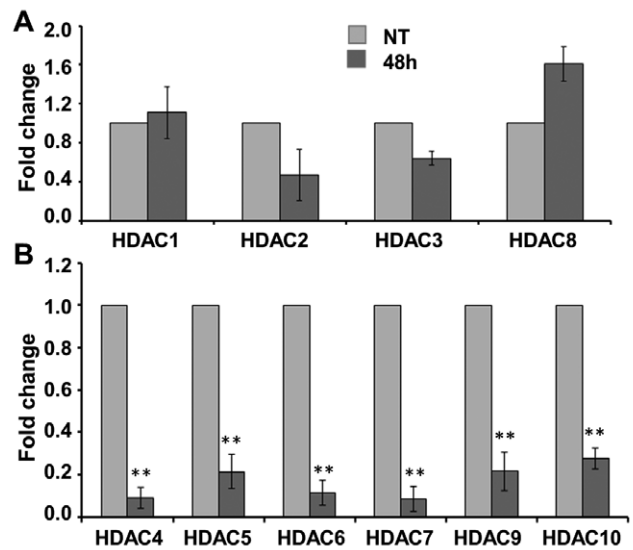


Fig. 7. TSA treatment is associated with a reduction of class II and but not class I HDAC transcription. Total RNAs were isolated from NPCs cultured in the absence (NT) or presence of 10 ng/ml of TSA for 48 hours. (A) TSA treatment did not affect the mRNA expression of Class I HDAC significantly ($t$=0.42, $P$=0.69; $t$=2.00 $P$=0.12; $t$=3.87, $P$=0.07; $t$=1.62, $P$=0.18, for HDAC1,2,3 and 8) ($n$=3). (B) By contrast, TSA significantly inhibited the expression of class II HDAC following 48 h of treatment ($t$=19.96, $P$<0.001; $t$=9.53 $P$=0.001; $t$=15.11, $P$<0.001; $t$=15.54, $P$<0.001; $t$=8.61, $P$=0.001; $t$=14.87, $P$<0.001, for HDAC4-7, 9 and 10) ($n$=3). Data are presented as means ± SD.

neuronal differentiation, reduced astrocyte differentiation and had no direct effect on oligodendrocytes fate of NPCs derived from embryonic rat cortex. These results are comparable to the effect of TSA on differentiation of NPCs derived from embryonic mouse (Balasubramaniyan et al. 2006). Collectively the effect of HDAC inhibition on the differentiation of NPCs is dependent on not only the HDACi (TSA,VPA, NaB, SAHA), the sites NPCs are isolated from (brain, hippocampal or SVZ), but also developmental stages NPCs derived from (embryonic or adult) and the media [10% FBS (Siebzehnrubl et al. 2007), neurobasal medium (Yu et al. 2009), and proliferation culture medium (Zhou et al. 2011)]. The mechanism of HDACi induced neuronal differentiation is usually related to the upregulation of several neuron-specific genes. For example, VPA treatment during the progenitor stages resulted in strong induction of neuronal differentiation accompanied by increases in the expression of Ngn1, Math1 and p15, pointing to a shift towards neuronal fate (Yu et al. 2009). MS-275, M344 and SAHA increased the β-III tubulin positive neurons by a reduction of Olig2 mRNA expression and an increased expression of NeuroD, Cyclin D2 and B-lymphocyte translocation gene 3 (Btg3) (Siebzehnrubl et al. 2007).

It is apparent that migration of NPCs is an essential process for the development of the CNS as well as the ongoing neurogenesis that occurs in the mature CNS (Hatten 1999, Gage 2000). For appropriate migration to the correct site, cell adhesion/traction, competency to migrate, and ability to respond to directional signals is required. For NPCs, molecules such as integrins (Murase and Horwitz 2002) and cell adhesion molecules, such as the NPC marker, the polysialated neural cell adhesion molecule (PSA-NCAM) (Hu et al. 1996, Lois et al. 1996), are involved in providing the framework for migration, whereas a number of growth factors, such as epidermal growth factor (EGF), confer the capacity for migration (Aguirre et al. 2005). These factors make the cells competent to migrate and may be permissive factors allowing NPCs to respond to directional cues. Currently ample evidence suggest that HDAC regulate migration of the cancer cells (Cho et al. 2010) and other cells such as pericyte cell (Karen et al. 2011). Here, using a scratch wound-healing assay and a transwell chamber analysis, we demonstrate for the first time that HDAC inhibition with TSA have no significant effect on the migration of NPCs *in vitro*, indicating HDAC may not be the key factor of NPC migration.

Chromatin regulation is a critical step in self-renewal activity and fate decision of NPCs. However, the underlying molecular mechanism is not fully understood. In this study, we observed that all types of HDAC (1–10) were expressed in embryonic rat NPCs. Inhibition of HDAC with TSA predominantly blocked the mRNA expression of class II HDACs and had no significant effect on the expression of class I HDACs.

## CONCLUSION

In conclusion, chromatin structure modulation plays a pivotal role in the regulation of the biologic properties of embryonic NPCs. Blocking of HDAC inhibits proliferation, increases neuronal differentiation and decreases astrocyte differentiation of the NPCs. TSA have no significant effects on survival and migration of the NPCs. TSA regulate proliferation and neuronal differentiation of the NPCs by reducing the transcription of class II and but not class I HDAC. These findings collectively demonstrate that although not directly affecting the survival and migration, a combined treatment with HDAC inhibition may induce more neuronal differentiation following NPCs grafting.

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