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ORIGINAL PAPER

Synergistic Effects of Sodium Butyrate, a Histone Deacetylase Inhibitor, on Increase of Neurogenesis Induced by Pyridoxine and Increase of Neural Proliferation in the Mouse Dentate Gyrus

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Abstract We previously observed that pyridoxine (vitamin B_6) significantly increased cell proliferation and neuroblast differentiation without any neuronal damage in the hippocampus. In this study, we investigated the effects of sodium butyrate, a histone deacetylase (HDAC) inhibitor which serves as an epigenetic regulator of gene expression, on pyridoxine-induced neural proliferation and neurogenesis induced by the increase of neural proliferation in the mouse dentate gyrus. Sodium butyrate (300 mg/kg, subcutaneously), pyridoxine (350 mg/kg, intraperitoneally), or combination with sodium butyrate were administered to 8-week-old mice twice a day and once a day, respectively, for 14 days. The administration of sodium butyrate significantly increased acetyl-histone H3 levels in the dentate gyrus. Sodium butyrate alone did not show the significant increase of cell proliferation in the dentate gyrus. But, pyridoxine alone significantly increased cell proliferation. Sodium butyrate in combination with pyridoxine robustly enhanced cell proliferation and neurogenesis induced by the increase of neural proliferation in the dentate gyrus, showing that sodium butyrate treatment distinctively enhanced development of neuroblast dendrites. These results indicate that an inhibition of HDAC synergistically promotes neurogenesis induced by a pyridoxine and increase of neural proliferation.

Keywords Hippocampus · Neurogenesis · Vitamin B_6 · Histone deacetylase inhibitor · Ki67 · Doublecortin

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Introduction

The hippocampus is a complex and fascinating region of the brain and has enormous clinical significance regarding memory problems [1]. Because the hippocampus is thought to play a central role in memory, there has been considerable interest in the possibility that age-related declines could be caused by hippocampal deterioration [2, 3]. Neurogenesis in the brain is important to develop certain types of memory [4–6]. The hippocampal dentate gyrus is thought to be strongly related to cognitive decline in old age, although the exact contribution of neurogenesis is uncertain [7, 8]. Adult neurogenesis, which is an ongoing process throughout life, occurs in most mammalian species, including humans [9–12].

Factors that contribute to the birth, differentiation, maturation, migration, and survival of newly generated neurons have been poorly understood. The acetylation status of histones has been thought to act as a general regulator of chromatin structure and to enhance transcriptional activity [13]. Histone deacetylases (HDACs) modify the *N*-terminal tails of histones and alter their interaction

with DNA, thus serving as epigenetic regulators of gene expression [14, 15]. Some studies have shown that histone protein modifications such as acetylation and deacetylation play a key role in regulating gene expression during the processes of cell proliferation and differentiation [16]. In addition, changes in chromatin structure due to post-translational modifications of histones have been associated with a number of behavioral events, including memory formation [17–19].

There are many reports about the effects of HDAC inhibitors on cell proliferation and neuroblast differentiation in animal models [20–25]. However, a few studies have been conducted on these effects in combination with other factors. In a previous study, we observed that pyridoxine (Pyr, vitamin B_6) significantly increased cell proliferation and neuroblast differentiation in the mouse dentate gyrus without any neuronal damage [26]. In the present study, therefore, we observed effects of sodium butyrate (SB), a HDAC inhibitor, on neurogenesis induced by Pyr and increase of neural proliferation in the mouse dentate gyrus.

Experimental Procedures

Experimental Animals

Forty-eight male C57BL/6 mice were purchased from Japan SLC Inc. (Shizuoka, Japan). They were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12 h light/12 h dark cycle, and could freely access food and tap water. The handling and the care of the animals conformed to the guidelines established in order to comply with current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996), and were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University. All of the experiments were conducted with an effort to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Drug Treatment

The animals were divided into four groups ($n = 12$ in each group): (1) vehicle (physiological saline)-treated group, (2) 300 mg/kg SB (Sigma, St. Louis, MO)-treated group, (3) 350 mg/kg Pyr (Sigma, St. Louis, MO)-treated group and (4) Pyr in combination with SB-treated group (Pyr + SB-treated group). Vehicle or Pyr was intraperitoneally administered to mice (8 weeks of age) twice a day for 2 weeks and SB was subcutaneously treated to mice at the same age once a day for 2 weeks. The animals were sacrificed 2 h after the last Pyr and SB treatment. These schedules were adopted because doublecortin (DCX) is exclusively expressed in immature neurons from 1 to 28 days of cell age [27, 28].

Western Blot Analysis

To confirm the effects of Pyr and/or SB on acetylation levels of histone H3 and neurogenesis induced by increase of neural proliferation, five animals in each group were sacrificed and used for western blot analysis. After sacrificing them and removing the brain, the dentate gyrus was then dissected with a surgical blade. The tissues were homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol bis (2-aminoethyl Ether)-N,N,N′,N′ tetra-acetic acid (EGTA) (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylenediamine tetraacetic acid (EDTA) (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). After centrifugation, the protein level was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL). Aliquots containing 20 μg of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Corp., East Hills, NY). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with rabbit acetyl-histone H3 (1:1,000; Cell Signaling Technology, Danvers, MA) or goat anti-DCX (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), peroxidase-conjugated anti-rabbit IgG (Sigma) or anti-goat IgG (Sigma) and an enhanced luminol-based chemiluminescent (ECL) kit (Pierce Chemical). The blot was densitometrically scanned for the quantification of relative optical density of each band using Scion Image software (Scion Corp., Frederick, MD). These data were normalized against β-actin.

Immunohistochemistry

For histology, vehicle-, SB-, Pyr- and Pyr + SB-treated animals ($n = 7$ in each group) were anesthetized with 30 mg/kg Zoletil 50 (Virbac, Carros, France) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4), which was followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and postfixed in the same fixative

for 4 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. The 30-μm-thick brain sections were serially cut in the coronal plane using a cryostat (Leica, Wetzlar, Germany). The sections were collected in six-well plates containing PBS until further processing.

In order to obtain accurate data for immunohistochemistry, free-floating sections were carefully processed under the same conditions. The tissue sections were selected between −1.46 and −2.46 mm posterior to the bregma in reference to a mouse atlas [29] for each animal. Ten sections were in 90 μm apart from each other, and the sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS and 10% normal goat or rabbit serum in 0.05 M PBS. They were next incubated with diluted rabbit anti-Ki67 antibody (1:1,000; Abcam, Cambridge, UK) or goat anti-DCX antibody (1:50) overnight, and subsequently exposed to biotinylated rabbit anti-goat, or goat anti-rabbit IgG (diluted 1:200; Vector, Burlingame, CA) and streptavidin peroxidase complex (diluted 1:200, Vector). Then, the sections were visualized by reaction with 3,3′-diaminobenzidine tetrahydrochloride (Sigma).

The measurement of Ki67- and DCX-positive cells in all the groups was performed using an image analysis system equipped with a computer-based CCD camera (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ). In addition, images of all DCX-immunoreactive structures were taken from the dentate gyrus through a BX51 light microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP71, Olympus) connected to a computer monitor. The dendritic complexity of DCX-positive cells was traced using camera lucida at 100× magnification (Neurolucida; MicroBrightField, Williston, VT). DCX-positive cells were separated into two categories according to dendritic complexity. The first category contained cells that lacked dendrites or had immature dendrites with primary or secondary branches which did not extend into the outer molecular layer. The second category contained cells that had mature dendrites with tertiary branches which extended into the outer molecular layer. Then, the DCX-positive cells in each section of the dentate gyrus were counted using Optimas 6.5 software (CyberMetrics). The cell counts from all of the sections of all of the mice were averaged.

Statistical Analysis

The data presented represent the means of the experiments performed for each experimental investigation. The differences among the means were statistically analyzed by a two-way analysis of variance followed by a Bonferroni's pot-hoc method in order to compare two independent variables including sodium butyrate and pyridoxine.

Results

Deacetylation Activity in SB

In western blot analysis, acetyl-histone H3 levels were similar in the vehicle-, and Pyr-treated group (Fig. 1). However, in the SB- and Pyr + SB-treated group, acetyl-histone H3 levels were significantly increased by 389.1 and 415.2%, respectively, compared to that in the vehicle-treated group (Fig. 1).

Cell Proliferation

In the vehicle-treated group, Ki67-positive ($^+$) nuclei were observed in the dentate gyrus. These nuclei were clustered and mainly detected in the subgranular zone of the dentate gyrus (Fig. 2a). In this group, the mean number of Ki67$^+$ nuclei was 15.7 per section (Fig. 2e). In the SB-treated group, Ki67$^+$ nuclei were similar to that in the vehicle-treated group, and the mean number of Ki67$^+$ nuclei was 14.8 per section (Fig. 2b, e). In the Pyr-treated group, Ki67$^+$ nuclei were increased compared to those in the vehicle-treated group, and the mean number of Ki67$^+$ nuclei was 22.5 per section (143.3% of the vehicle-treated group) (Fig. 2c, e). In the Pyr + SB-treated group, Ki67$^+$ nuclei were much more increased: In this group, the number of Ki67$^+$ nuclei was 32.5 per section (207.0% of the vehicle-treated group) (Fig. 2d, e).

Neurogenesis Induced by Increase of Neural Proliferation

In all the groups, the somas of doublecortin (DCX)-immunoreactive ($^+$) neuroblasts were observed in the

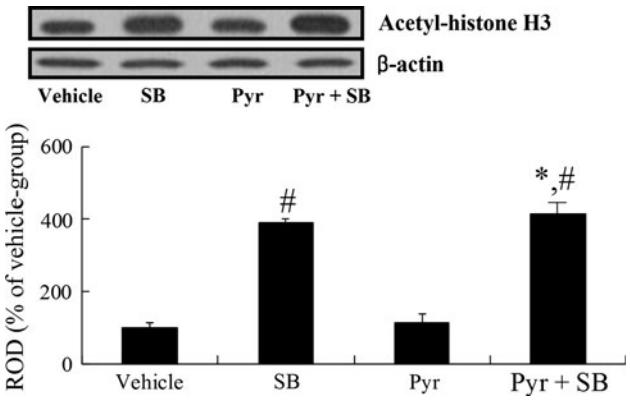

Fig. 1 Western blot analysis of acetyl-histone H3 levels in the dentate gyrus of the *vehicle*-, *SB*-, *Pyr*- and *Pyr + SB*-treated groups. The relative optical density (ROD) of the immunoblot bands are demonstrated as percent values ($n = 5$ per group; $^*P < 0.05$, indicating a significant difference compared to the vehicle-treated group; $^{\#}P < 0.05$, significantly different from the Pyr-treated group). *Bars* indicate standard errors (SE)

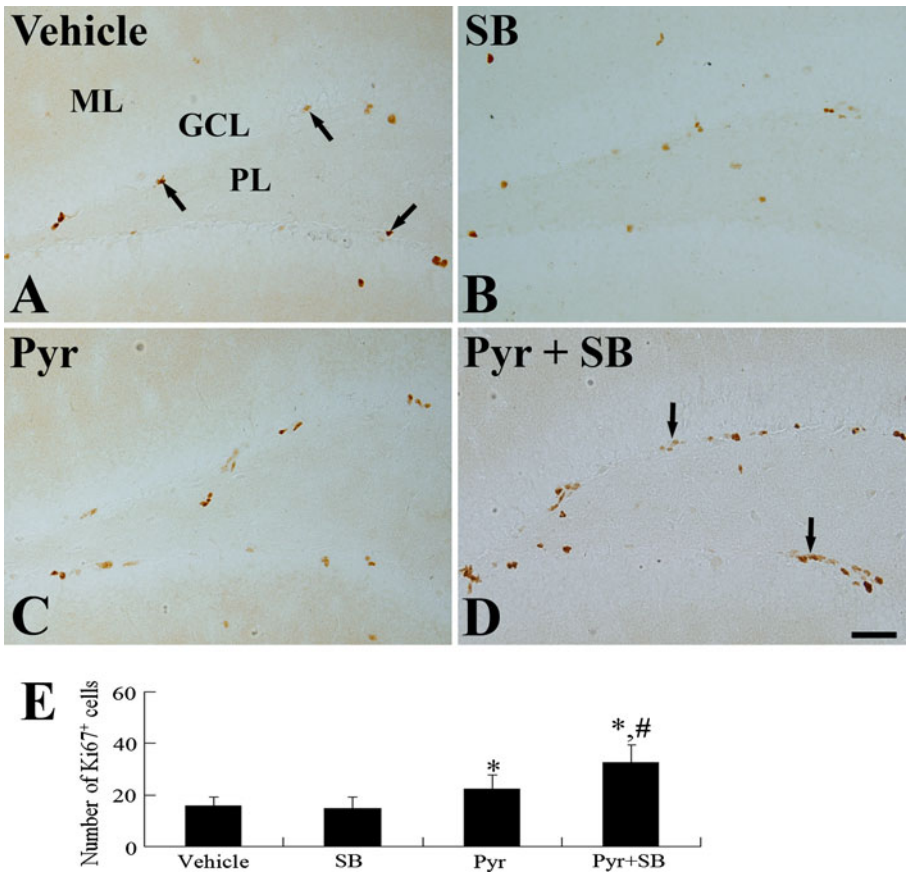

Fig. 2 Immunohistochemistry for Ki67 in the dentate gyrus of the *vehicle* (**a**)-, *SB* (**b**)-, *Pyr* (**c**)-, and *Pyr* + *SB* (**d**)-treated groups. Ki67$^+$ cells (*arrows*) are observed in the dentate gyrus. Ki67$^+$-nuclei are slightly increased in the Pyr-treated group, and they are most abundant in the Pyr + SB-treated group. *GCL* granule cell layer, *ML* molecular layer, *PL* polymorphic layer. *Scale bar* = 50 μm. **e** The number of Ki67$^+$ per section in all the groups ($n = 7$ per group; $^*P < 0.05$, significantly different from the vehicle-treated group, $^{\#}P < 0.05$, significantly different from the Pyr-treated group). The bars indicate the standard errors of the mean (SEM)

subgranular zone of the dentate gyrus, and their dendrites extended into two-thirds of the molecular layer of the dentate gyrus (Fig. 3a–h). However, there were significant differences in the morphology and in the number of DCX$^+$ neuroblasts among the groups. In the vehicle-treated group, DCX$^+$ neuroblasts were mainly distributed in the subgranular zone of the dentate gyrus, and the number of DCX$^+$ neuroblasts with and without tertiary dendrites was 72.4 and 125.4 per section, respectively, in the dentate gyrus (Figs. 3a, b, 4a). In the SB-treated group, the number of DCX$^+$ neuroblasts with tertiary dendrites was slightly increased compared to the vehicle-treated group, while the number of DCX$^+$ neuroblasts without tertiary dendrites was similar to the vehicle-treated group (Figs. 3c, d, 4a). In the Pyr-treated group, dendrites of DCX$^+$ neuroblasts were more complex compared to those in the vehicle-treated group (Fig. 3e, f): In this group, the number of DCX$^+$ neuroblasts with and without tertiary dendrites was 198.2 and 71.3%, respectively, of the vehicle-treated group (Fig. 4a). In addition, some DCX$^+$ neuroblasts were also observed in the granule cell layer of the dentate gyrus (Fig. 3e, f): This finding was not shown in the vehicle-treated group. In the Pyr + SB-treated group, DCX$^+$ neuroblasts were more abundant than in the Pyr-treated group, and they were observed in the granule cell layer as well as in the subgranular zone of the dentate gyrus (Fig. 3g, h). In this group, the number of DCX$^+$ neuroblasts with and without tertiary dendrites was 278.2 and 125.1%, respectively, of the vehicle-treated group (Fig. 4a).

DCX Protein Levels

In western blot study, we found that DCX levels in the dentate gyrus of the SB-treated groups were similar to the vehicle-treated group. In the Pyr- and Pyr + SB-treated group, DCX protein levels were increased to about 145.3 and 211.9% of the vehicle-treated group, respectively (Fig. 4b).

Discussion

Pyr, which is a water-soluble vitamin, is rapidly taken up by circulating erythrocytes and converted into its active forms, pyridoxamine and pyridoxal phosphate. These substances, which act as coenzymes for transaminase [30] and in the biosynthesis of the neurotransmitters GABA, dopamine, and serotonin, are important for the development and function of the central nervous system [31].

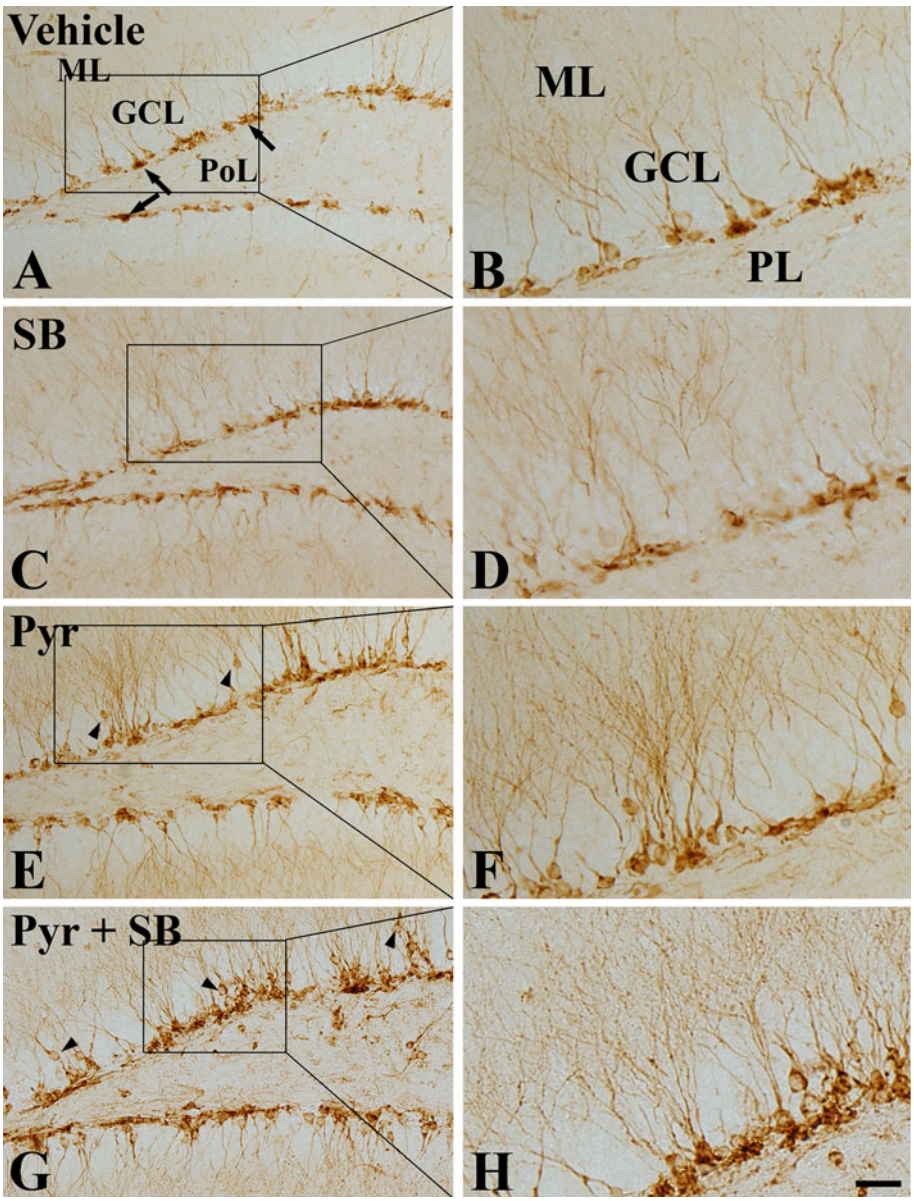

Fig. 3 Immunohistochemistry for DCX in the dentate gyrus of the *vehicle*- (**a**, **b**)-, *SB* (**c**, **d**), *Pyr* (**e**, **f**)-, and *Pyr* + *SB* (**g**, **h**)-treated groups. DCX^+ neuroblasts (*arrows*) are observed in the vehicle-treated group, and they are not changed in the SB-treated group. Some DCX^+ neuroblasts (*arrowheads*) are additionally observed in the granule cell layer (*GCL*) in the Pyr- and Pyr + SB-treated groups, showing that DCX^+ cells are most abundant in the Pyr + SB-treated group. *ML* molecular layer, *PL* polymorphic layer. *Scale bar* = 50 μm (**a**, **c**, **e**, **g**) or 25 μm (**b**, **d**, **f**, **h**)

In the present study, we first investigated whether SB properly inhibits HDAC by western blot analysis. The results showed that SB significantly increased acetyl-histone H3 levels in the mouse dentate gyrus. Epigenetic regulation by histone modifications such as acetylation, methylation, and phosphorylation is dynamically and coordinately regulated in many physiological processes including development [32]. HDAC inhibitors, such as valproic acid, trichostatin-A, and SB, thereby promote histone acetylation, relax DNA, and promote transcription of genes involved in important cellular processes [22, 33, 34].

Next, we investigated whether Pyr in combination with HDAC inhibitor promotes neurogenesis induced by Pyr and increase of neural proliferation in the dentate gyrus. SB, one of HDAC inhibitors, did not show any significant increases in cell proliferation. However, SB slightly increased DCX^+ neuroblasts with tertiary dendrites in the dentate gyrus. However, there are some contradictory results about effects of HDAC inhibitors on cell proliferation in the dentate gyrus: Most of the studies have demonstrated that HDAC inhibitors promote neuroblast differentiation in the hippocampus [20, 23]. In embryonic stem (ES) cells, HDAC inhibitors, in combination with basic fibroblast growth factor, can promote ES-to-neural conversion without the formation of embryonic bodies or the co-culture with other types of cells [24]. Valproic acid decreases proliferation of progenitor cells [22] and SB significantly decreased the number of 5-bromodeoxyuridine $(BrdU)^+$ cells in embryonic hippocampal neural

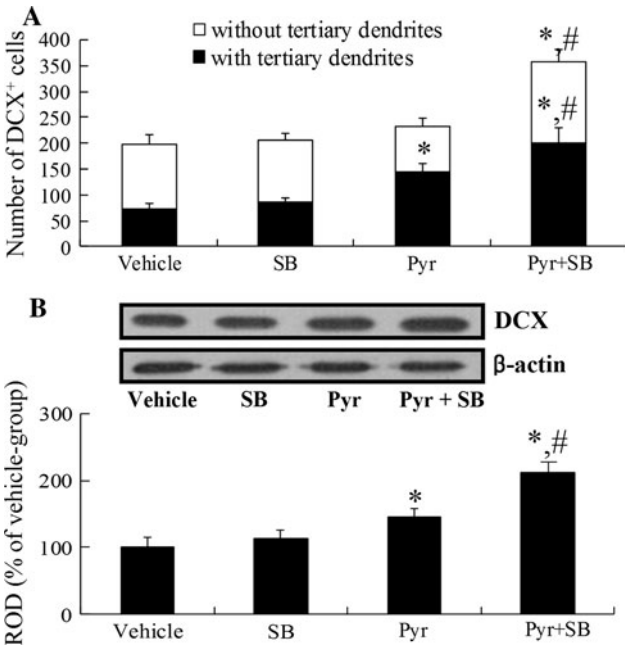

Fig. 4 **a** The number of DCX^+ neuroblasts with and without tertiary dendrites per section in all the groups ($n = 7$ per group; $^*P < 0.05$, significantly different from the vehicle-treated group, $^{\#}P < 0.05$, significantly different from the Pyr-treated group). **b** Western blot analysis of DCX in the dentate gyrus from all the groups. The relative optical density (ROD) of immunoblot band is represented as % values ($n = 7$ per group; $^*P < 0.05$, significantly different from the vehicle-treated group; $^{\#}P < 0.05$, significantly different from the Pyr-treated group). The *bars* indicate the SEM

progenitor cells [25]. In addition, valproic acid treatment in mice for 2 weeks significantly decreases the number of BrdU-labeled cells in the hippocampal dentate gyrus [25]. However, SB and trichostatin-A further enhances ischemia-induced increase of BrdU-labeled cells in the dentate gyrus [35]. In addition, chronic treatment with valproic acid in adult rats for 6 weeks stimulates BrdU-positive cells [21]. These discrepancies may be associated with duration of treatment and animal models used.

In this study, the administration of Pyr significantly enhanced neural proliferation in the dentate gyrus. In addition, Pyr administered with SB robustly increased the number of $Ki67^+$ nuclei (cell proliferation) and DCX^+ neuroblasts in the dentate gyrus. In particular, Pyr, in combination with SB, significantly increased DCX^+ neuroblasts in the granule cell layer of the dentate gyrus.

This result is coincided with our previous study that Pry significantly increased cell proliferation and neuroblast differentiation probably due to the upregulation of GAD67 expression and increase of $GAD67^+$ cells in the dentate gyrus [26]. In addition, a previous study showed that deficits of vitamin B_6 in utero significantly reduced the number of total neurons and normal neurons in the neocortex, with an increase in the number of shrunken neurons (700–1,500% of controls) [36]. In our previous study, we administered Pyr and observed the GAD67 immunoreactivity in the dentate gyrus [26]. This result is supported by previous studies using electrophysiological analysis that functional GABA receptors is expressed in neural progenitor cells and their neuronal progeny in both juvenile and adult animals [37–41]. In addition, GABA initially depolarizes these immature cells in the adult brain during the first 2–3 weeks of their neuronal development [37, 38, 42–44].

In this study, we did not examine behavioral changes after treatment with Pyr with SB. However, some studies have demonstrated that HDAC inhibitors such as trichostatin-A and SB increase contextual fear conditioning and extinction [18, 19]. In addition, SB promotes an increase in histone acetylation caused by exposure to cocaine [45], as well as decreases anxiety-like symptoms associated with alcohol withdrawal [46]. However, in mouse models of chronic antidepressant responses, SB fails to cause any changes despite causing changes in the level of histone acetylation in the hippocampus [47]. In the present study, we did not elucidate the mechanisms of synergistic effects of Pyr and SB. It has been reported that HDAC inhibition activates BDNF promoter IV and increases BDNF mRNA levels in dissociated rat cortical neurons [48] and activates BDNF promoter 1 in the rat hippocampal neurons [49]. In contrast, the administration of Pyr significantly increases serotonin levels in the hippocampus of diabetic animals induced by streptozotocin [50].

In conclusion, our findings indicate that SB robustly enhanced neurogenesis induced by Pyr and increase of neural proliferation in the mouse dentate gyrus, and these drugs may be helpful to patients with neurodegenerative diseases.

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