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Prenatal exposure to suberoylanilide hydroxamic acid perturbs corticogenesis

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ABSTRACT

Suberoylanilide hydroxamic acid (SAHA) is one of the epidrugs developed for cancer treatment that works epigenetically by inhibiting histone deacetylases (HDACs). SAHA has been reported to diffuse across the placenta and found in fetal plasma in preclinical study, implying that it can influence fetus if taken by pregnant cancer patients. However, report regarding this aspect and the study of *in utero* HDAC inhibition by SAHA especially on fate specification of neural stem/progenitor cells within the developing mammalian cortex, is yet unavailable. Here we show that transient exposure of SAHA to mouse embryos during prominent neurogenic period resulted in an enhancement of cortical neurogenesis, which is accompanied by an increased expression of proneuronal transcription factor *Neurog1*. Neurogenesis was enhanced due to the increase number of proliferating Tbr2+ intermediate progenitor cells following SAHA exposure. In this relation, we observed that SAHA perturbed neonatal cortical lamination because of the increased production of Cux1+ and Satb2+ upper-layer neurons, and decreased that of Ctip2+ deep-layer neurons. Furthermore, an upper-layer neuronal lineage determinant *Satb2* was also up-regulated, whereas those of deep-layer ones *Fezf2* and *Ctip2* were down-regulated by SAHA treatment. Taken together, our study suggests that proper regulation of HDACs is important for precise embryonic corticogenesis.

1. Introduction

The six-layered mammalian cerebral cortex comprises of two major neuronal subtypes with approximately 80% are pyramidal excitatory neurons and the remaining are inhibitory interneurons (Molyneaux et al., 2007). The pyramidal neurons are generated in a well-defined spatio-temporal manner during brain development and can be derived directly from neural stem/progenitor cells (NS/PCs) at ventricular zone (VZ), or indirectly through intermediate progenitor cells (IPCs) that reside in subventricular zone (SVZ) (Guillemot et al., 2006; Leone et al., 2008). In mouse, cortical neurogenesis (corticogenesis) starts around embryonic day (E) 10.5. The first wave of neurons occupy the preplate, which is subsequently split into two zones following the intercalation of later neurons. The upper zone of the splitted preplate (also known as marginal zone/MZ, which later become layer I) is populated by Cajal-Retzius neurons which are derived mostly from cortical hem (Zhao et al., 2006), while the lower zone becomes subplate (SP) which mainly functions to mediate axon targeting during development (Kanold and Shatz, 2006). A layer is created between MZ and SP, giving rises to the cortical plate (CP). Within CP, neurogenesis proceeds in an inside-out fashion establishing a six-layered laminar pattern, in which layer VI and V deep-layer (DL) neurons arise and migrate first, while layer IV-II neurons of the upper-layer (UL) are born and migrate later (Angevine and Sidman, 1961). Whereas the neurons in DL are generated mostly from mid-gestational NS/PCs (E11-E14), the neurons in the UL are suggested to derive from IPCs, which are derived from late gestational NS/PCs (E14-E17) (Tarabykin et al., 2001; Shen et al., 2006; Cubelos et al., 2008; Arnold et al., 2008).

Epigenetic mechanisms are among the critical intrinsic programs that dictate fate specification and differentiation of NS/PCs (Namihira et al., 2008; Juliandi et al., 2010a,b). We and others have identified one of such mechanisms, histone acetylation, that regulates fate specification of NS/PCs at different developmental stages (Hsieh et al., 2004; Balasubramaniyan et al., 2006; Asano et al., 2009; Yu et al., 2009; Abematsu et al., 2010; Juliandi et al., 2012). Increased global histone acetylation by histone deacetylase (HDAC) inhibitors drives NS/PCs toward neuronal lineage over

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glial ones (Hsieh et al., 2004; Abematsu et al., 2010). Moreover, taking advantage of the mouse embryonic stem cells (mESCs) culture system that mimics corticogenesis *in vitro* (Gaspard et al., 2008), we have previously shown that HDAC inhibition using valproic acid increases the production of cut-like homeobox 1 (Cux1)+ UL neurons and decreased that of B-cell leukemia/lymphoma 11B (Bcl11b; also called Ctip2)+ DL neurons, implying an important role of histone acetylation on the specification of UL neurons in late corticogenesis (Juliandi et al., 2012). However, the effect of increasing global histone acetylation by means of HDAC inhibition in NS/PCs during corticogenesis *in vivo* has hitherto remains elusive.

A new anti-cancer drug, suberoylanilide hydroxamic acid (SAHA), is one of the epidrugs that are capable of reversing improper histone acetylation by inhibiting HDACs in cancer cells (Marks et al., 2003; de Ruijter et al., 2003; Mei et al., 2004; Drummond et al., 2005; Kelly et al., 2005; Zhang et al., 2005; O'Connor et al., 2006; Marks, 2007; Duvic et al., 2007). However, in preclinical study, SAHA has been reported to cross placenta and can be found in fetal plasma, implying that this drug can influence cells in fetus during pregnancy if it is being taken by pregnant cancer patient or if the patient becomes pregnant while taking this drug. The administration of this drug also raises questions on its potential to affect epigenetic status of non-cancerous cells such as NS/PCs. Unfortunately, report regarding this aspect and its effects on NS/PC fate specification during embryonic corticogenesis is not yet available.

Here we report that HDAC inhibition using SAHA promotes embryonic neurogenesis, hence later perturbs cortical lamination of mouse neocortex. SAHA increased the production of Cux1+ and special AT-rich sequence-binding protein (Satb2)+ UL and decreased that of Ctip2+ DL neurons. SAHA treatment up- and down-regulated the expression of genes specific for UL (*Satb2*) and DL (*Fez family zinc finger 2; Fezf2*, and *Ctip2*) lineage, respectively. Our results suggest that precise control of global histone acetylation by HDACs is vital for the regulation of corticogenesis during brain development.

2. Materials and methods

2.1. Drug preparation

Suberoylanilide hydroxamic acid (SAHA; Cayman Chemical, Ann Arbor, MI) was dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) and dose formulations were prepared as suspensions in 0.5% (w/v) methyl cellulose (Wako, Osaka, Japan). The formulations were freshly prepared and briefly mixed before use.

2.2. Animals treatment

All mice used in this study were handled according to the animal experimentation guidelines of Nara Institute of Science and Technology that comply with National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of mice used and their suffering. Mice were housed on 12-h light/dark cycle with free access to food and water. Time-pregnant C57BL/6J mice were orally administered with SAHA (50 mg/kg) or equal volume of 10% (v/v) DMSO in methyl cellulose as control, once a day for 3 consecutive days starting from embryonic day (E) 12.5 until E14.5, a period of prominent cortical neurogenesis.

2.3. 5-Bromo-2′-deoxyuridine (BrdU) labeling

Pregnant dams received single-pulse intraperitoneal injection of 150 mg/kg BrdU (Sigma, St. Louis, MO) in saline (0.9% NaCl), either 30 min before sacrifice on E15.5 for cell cycle analysis, or on E14.5 for the analysis of NS/PCs differentiation at postnatal stage.

2.4. Tissue preparation

Embryonic or postnatal brains were harvested at specified developmental days. Pregnant dams were sacrificed by cervical dislocation and embryonic brains were collected in ice-cold phosphate buffered saline (PBS). Postnatal brains were collected after pups were deeply anesthetized by hypothermia and further perfused with PBS followed by 4% paraformaldehyde (PFA) in PBS. Brains were fixed in 4% PFA in PBS and incubated in a series of sucrose gradient in PBS before being embedded in Optimal Cutting Temperature compound (Sakura Finetek, Torrence, CA) and stored at −80 °C until use. Frozen samples were serially sectioned with Leica CM 1900 (Leica Microsystems, Wetzlar, German) in coronal plane of 20 μm (embryonic brains) or 40 μm (postnatal brains) thickness.

2.5. Cell culture

E11.5 mouse forebrains were dissected and triturated in calcium- and magnesium-free Hanks' Balanced Salts solution (Sigma) and plated on poly-ornithine/fibronectin-coated chamber slides at a density of 2.5×10^4 cells/well in proliferating medium (N2-supplemented DMEM/F-12; Invitrogen, Carlsbad, CA), containing 10 ng/ml bFGF (PeproTech, Rocky Hill, NJ) to expand the NS/PCs. To study the proliferation of NS/PCs, DMSO or 30 nM SAHA was added to individual culture 3 h after plating and the cells were expanded for 3 days (d). Later, 10 μg/ml BrdU (Sigma) was added 30 min before cell fixation. To induce neuronal differentiation, NS/PCs were first cultured in the proliferating medium for 2 d and then later in differentiation medium (N2-supplemented DMEM/F-12 without bFGF) for another 4 d. DMSO or 30 nM SAHA was added to individual cultures during the first day of differentiation.

2.6. Immunohistochemistry

Brain sections were washed 3 times with PBS and then incubated in blocking solution (PBS containing 3% FBS and 0.1% Triton X-100) for 3 h at room temperature (RT). Next, sections were incubated overnight at 4 °C with appropriate primary antibodies. The primary antibodies used were as follows: chick anti-Nestin (1:500; Aves Labs, Tigard, OR), rabbit anti-βIII-tubulin (1:1000; Covance, Berkeley, CA), rabbit anti-Pax6 (1:250; Covance), rabbit anti-AcH3 (1:1000; Millipore, Temecula, CA), rabbit anti-AcH4 (1:1000; Millipore), goat anti-Dcx (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-CDP/Cux1 (1:250; Santa Cruz), mouse anti-pH3 (1:1000; Cell Signaling, Danvers, MA), rat anti-BrdU (1:1000; AbD Serotec, Oxford, UK), mouse anti-Ki67 (1:500, BD Pharmingen, Franklin Lakes, NJ), rabbit anti-Tbr2 (1:500; Millipore), mouse anti-NeuN (1:250; Millipore), rat anti-Ctip2 (1:500; Abcam, Cambridge, MA), rabbit anti-Tbr1 (1:500; Abcam), and mouse anti-Satb2 (1:500; Abcam). Antigen retrieval was conducted by Antigen Retrieval solution (Dako, Glostrup, Denmark) for 15 min at 90 °C in water bath for detection of some antigens. For BrdU immunohistochemistry, sections were pre-treated with 2 N HCl for 30 min at 37 °C. After 3 washes with PBS, the sections were incubated for 1–2 h at RT with the appropriate secondary antibodies. The following secondary antibodies were used: FITC-conjugated donkey anti-mouse, FITC-conjugated donkey anti-rat, Cy3-conjugated donkey anti-mouse, Cy3-conjugated donkey anti-chick, Cy3-conjugated donkey anti-rabbit, Cy5-conjugated donkey anti-rat, Cy5-conjugated donkey anti-goat (all 1:500; Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa Fluor 488-conjugated donkey anti-mouse, Alexa Fluor 488-conjugated donkey anti-rabbit, and Alexa Fluor 488-conjugated donkey

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