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Effect of anti-cancerous and histone deacetylase inhibitor suberoylanilide hydroxamic acid on brain development

(抗がん剤かつヒストン脱アセチル化酵素阻害剤スベロイルアニリドヒドロキサム酸の脳発達における影響)

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要旨

Stem cells drive the formation of tissues and organs through development and remain in adult niche settings for tissue repair and maintenance. In early development, embryonic stem cells (ESCs) present in the inner cell mass of the blastocyst sense gradients of differentiation factors which instruct the formation of mesoderm, endoderm and ectoderm. Specification of the central nervous system (CNS) begins with neural tube formation from ectoderm. ESCs develop into neural stem cells (NSCs), in addition to neurons, they can differentiate into glial cells (astrocytes, oligodendrocytes) in the CNS. Thus the study of NSCs has profound implications for brain form and function as well as the repair of damaged tissue, neurodegeneration, and brain tumors.

Histone deacetylases (HDACs) are the chromatin modifiers that can epigenetically regulate NSC fate choice. Our group and others have identified that the inhibition of HDACs by histone deacetylase inhibitors (HDACIs) *in vitro* increased global histone acetylation and drove NSCs toward neuronal lineage over glial ones. Moreover, we also found the role of histone acetylation on the specification of upper layer neurons in late corticogenesis through HDACI treatment of mouse ESCs culture system that mimics corticogenesis *in vitro*. However, the effect of increasing global histone acetylation by means of HDAC inhibition in NSC fate specification during corticogenesis *in vivo* is hitherto remains elusive.

Suberoylanilide hydroxamic acid (SAHA) is one of epi-drugs developed for cancer treatment that works epigenetically by inhibiting HDACs. SAHA has been reported to diffuse across placenta and found in fetal plasma in preclinical study, suggesting that it can influence fetuses if taken by pregnant cancer patients. However, reports regarding this aspect and study of *in utero* HDAC inhibition by SAHA especially on fate specification of NSCs within the developing mammalian cortex are as yet to be examined. Moreover, previous study reported that a series of HDACIs could interfere with proper embryonic development of numerous organisms suggesting the teratogenic potential of HDACIs. HDAICs also have exhibited substantial adverse side effects in patients such as troubled in

speaking, thinking, or walking, troubled in breathing with exertion, and unusual bleeding or bruising. Given that the intense clinical interest of SAHA as HDACI in many pathological condition, also the fact that HDACI raise profound adverse side effects in patients including fetus, it is therefore important to investigate the potential of SAHA in altering epigenetic status in particular histone acetylation status of non-cancerous cells during pregnancy especially in embryonic NSC fate examination.

In the present study, I demonstrate that transient exposure of mouse embryos to SAHA during prominent neurogenic period resulted in reduced NSC marker expression and an enhancement of excitatory cortical neurogenesis, which is accompanied by an increased expression of proneuronal transcription factor *Neurogenin1*. Neurogenesis was enhanced due to the increase of proliferating T-box brain protein 2 (Tbr2)-positive intermediate progenitor cells that committed to differentiation into neuron following SAHA exposure. An identical effect was observed *in vitro* upon SAHA application to differentiating embryonic NSCs derived from mouse cortices. In this relation, I observed that SAHA perturbed neonatal cortical lamination owing to the increased production of cut-like homeobox 1 (Cux1)-positive and Special AT-rich sequence-binding protein 2 (Satb2)-positive upper-layer neurons, and decreased that of Chicken ovalbumin upstream promoter transcription factor-interacting proteins 2 (Ctip2)-positive deep-layer neurons. Furthermore, a determinant of upper-layer neuronal lineage *Satb2* was also up-regulated, whereas those of deep-layer ones, *Fez family zinc finger protein 2* (*Fezf2*) and *Ctip2*, were down-regulated by SAHA treatment. Collectively, these results suggest that proper regulation of HDACs is critical for precise embryonic corticogenesis.

The defect affected by fetal exposure to SAHA apparently persisted in adult brain. The number of NSC was still lower in adult hippocampus of mice which exposed by SAHA during mid-gestation. Moreover, in mice with a history of prenatal SAHA treatment, the cortical astro-gliogenesis is suppressed likely owing to the decreased of the potency for astrocytic differentiation in mid-gestational NSC. Fetal SAHA exposure also increased cortical interneuron generation in adult brains presumably because SAHA increased number of proliferating NSCs cells committed to inhibitory interneurons. This may inconstant the normal ratio between excitatory and inhibitory neuron in the cortex. Taken together, these findings suggest that change in epigenetic status that is histone acetylation of non-cancerous cells by pharmacological inhibition of HDACs following anti-cancerous SAHA exposure during mid-pregnancy may alter fetal and adult brain structure. My findings might contribute to the pharmaceutical therapy guideline not only for cancer treatment but also for treatment of other diseases employing HDACIs. In the present study I provide information that SAHA has a potential hazard to the fetus and it should not be prescribed to pregnant patient or to patient who become pregnant while taking this drug.

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CNS	: central nervous system
ESC	: embryonic stem cell
NSC	: neural stem cell
NEC	: neuroepithelial cell
RGC	: radial glia cell
SNP	: short neural precursor cell
IPC	: intermediate progenitor cell
NRP	: neuron-restricted progenitor
GRP	: glial-restricted progenitor
CR	: Cajal-Retzius
VZ	: ventricular zone
SVZ	: subventricular zone
IZ	: intermediate zone
SP	: subplate
CP	: cortical plate
MZ	: marginal zone
TF	: transcription factor
PcG	: polycomb gene group
DNMT	: DNA methyltransferase
HAT	: histone acetyl transferase
HDAC	: histone deacetylase
MeCP2	: methyl-CpG binding protein 2
MBD	: methyl-CpG binding domain
GFAP	: glial fibrillary acidic protein
LIF	: leukaemia inhibitory factor
CNTF	: ciliary neurotrophic factor
IL-6	: interleukin-6
BMP	: bone morphogenetic protein
JAK	: Janus kinase
STAT	: signal transducer and activator of transcription
CBP	: CREB-binding protein
CREB	: cAMP response element-binding protein
SMAD	: Sma and Mad Related Family
gp130	: glycoprotein 130
PRC1/2	: polycomb repressive complexes 1/2
SUZ12	: suppressor of zeste 12
EZH1/2	: enhancer of zeste homolog 1/2
EED	: embryonic ectoderm development
S100β	: S100 calcium binding protein β
NICD	: notch intracellular domain
NFIA/B	: nuclear factor 1A/B
bHLH	: basic helix-loop-helix
Ngn1/2	: neurogenin1/2
TCF	: T-cell factor
SGZ	: sub granular zone
DG	: dentate gyrus
GCL	: granule cell layer
GABA	: gamma-aminobutyric acid

VPA	: valproic acid
SAHA	: suberoylanilide hydroxamic acid
TSA	: trichostatin A
VPM	: valpromide
HDACI	: HDAC inhibitor
AcH4/3	: acetylated histone H4/3
Olig2	: oligodendrocyte transcription factor 2
PADI4	: petidylarginine deiminase 4
JHDM	: JmjC-domain histone demethylases
LSD1	: lysine specific demethylase 1
KDM	: lysine-specific histone demethylase 1
KMT	: histone methyl transferase
FGF2	: fibroblast growth factor 2
EGF	: epidermal growth factor
DL	: deep-layer
UL	: upper-layer
Sox5	: SRY (sex determining region Y)-box 5
Fezf2	: FEZ family zinc finger 2
Satb2	: Special AT-rich sequence-binding protein 2
Tbr1/2	: T-box transcription factor 1/2
Ctip2	: chicken ovalbumin upstream promoter transcription factor-interacting protein 2
Brn1	: brain-specific homeobox/POU domain protein 1
NeuN	: neuronal nuclei
MTA2	: metastasis-associated gene family, member 2
Ski	: sarcoma viral oncogene homolog
Cux1	: cut-like homeobox 1
Pax6	: pair box domain 6
Dcx	: doublecortin
pH3	: phosphorylated histone H3
GAPDH	: glyceraldehyde 3-phosphate dehydrogenase
BrdU	: 5-bromo-2-deoxyuridine
SD	: standard deviation
ANOVA	: analysis of variance
βIII-tub	: βIII-tubulin
Div	: days in vitro
SCPN	: subcerebral projection neurons
CPN	: callosal projection neurons
SST	: somatostatin
PV	: parvalbumin
CR	: calretinin
LGE	: lateral ganglionic eminences
MGE	: medial ganglionic eminences
LMC	: lower active maternal care
ASD	: autism spectrum disorder
SZ	: schizophrenia

I. Introduction

I.1. Sequential development of neural stem cells

Stem cells drive the formation of tissues and organs through development and remain in adult niche settings for tissue repair and maintenance. Blood, skin and intestinal stem cells are prime examples of adult stem cells important throughout life. In early development, embryonic stem cells (ESCs) present in the inner cell mass of the blastocyst sense gradients of differentiation factors which instruct the formation of mesoderm, endoderm and ectoderm. Specification of the central nervous system (CNS) begins with neural tube formation from ectoderm. At embryonic day 5.5 (E5.5) ESCs develop into neural stem cells (NSCs), which eventually give rise to neurons and glial cells (astrocytes, oligodendrocytes) in CNS. Thus owing to the ability of NSC to self-renew and their potential to generate various neural cell types, NSCs has profound implications for brain form and function as well as the repair of damaged tissue (e.g. spinal cord injury), neurodegeneration (Parkinson disease), and brain tumors (Ramasamy et al., 2013).

Multipotent NSCs have been identified in both embryonic and adult CNS. They undergo fate restriction at various developmental stage which is attributable to their potency change. Throughout brain development, in the neurogenic phase, NSCs sequentially produce different types of neurons settled in the distinct cortical layer of neocortex. Termination of this phase marks the onset of gliogenic phase when NSCs transform into astrocyte and oligodendrocyte (Hirabayashi and Gotoh, 2005).

NSCs in the CNS begin as a single layer of columnal neuroepithelial cells (NECs) in the ventricular zone (VZ) of the neural tube. At an early stage of gestation, around E9.5 in the mouse, NECs continuously undergo symmetric division to produce two daughter cells resulted in the expansion of neural progenitor pool and lead to the increased surface area of

the VZ. Subsequently, at E11.5, NECs progressively switch into radial glia cells (RGCs) and short neural precursors (SNPs) (Fig. 1). These RGCs are proliferative and heterogenous population comprising neuronal and glial progenitors and other RGCs (Malatesta et al., 2000) thereby they are considered as the most predominant progenitor population. RGCs have ability to generate another RGCs as well as neurons *via* asymmetric division (Noctor et al., 2004), and later as gestation proceed to mid-stage (around E14.5), give rise to another RGC and one basal progenitor, thereby creating a new additional proliferative platform that lies above the VZ, the so-called subventricular zone (SVZ). Basal progenitor, or commonly known as intermediate progenitor cell (IPC), will subsequently divide symmetrically in one or two round amplifications to produce two neurons (Noctor et al., 2004).

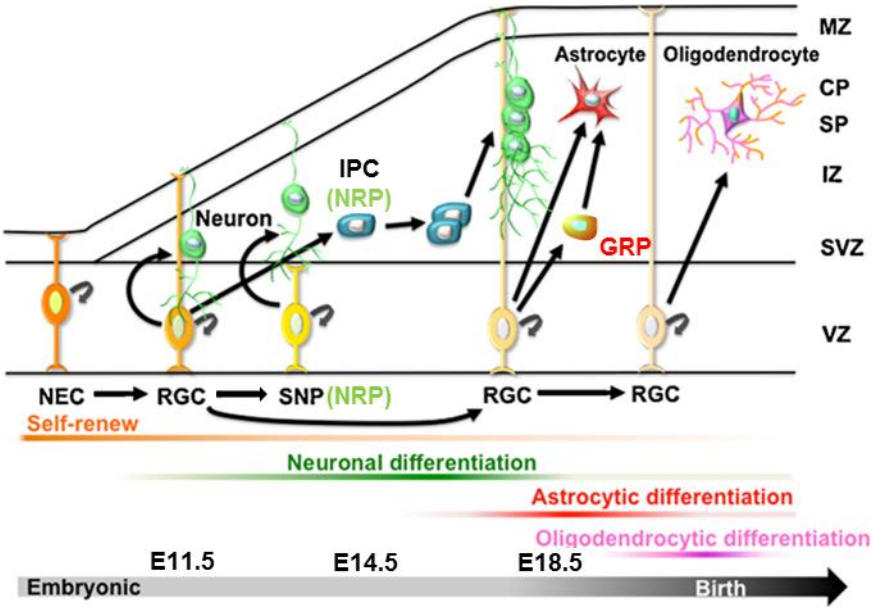


Fig. 1. Sequential developmental stages in mammalian cortex involved various types of progenitors that settled in respective proliferative niche and contributed to distinct cell types in mammalian CNS. At early embryonic stage prior to neurogenesis, the neocortex consists of a single layered neuroepithelium harboring NECs. NECs undergo self-renew and following the onset of neurogenesis, NECs transform into another types of progenitor namely RGCs and SNPs. RGCs subsequently generate to produce other progenitor cell type, IPCs. All RGC, SNP, and IPC are neurogenic progenitor that contributes to the neurogenesis phase during cortical development, but only SNP and IPC are NRP. At late embryonic stage, gliogenesis is initiated when progenitors (RGCs) progressively differentiate into GRP and glial cells, such as astrocytes and then oligodendrocytes (Muhchyi et al., 2013 with modification). NEC, neuroepithelial cell; RGC, radial glia cell; SNP, short neural precursor cell; IPC, intermediate progenitor cell; NRP, neuron-restricted progenitor; GRP, glial-restricted progenitor.

Thus, there are two types of neuron-restricted progenitors (NRP): SNP and IPC. It is thought that SNP is responsible for direct neurogenesis (Stancik et al., 2010) whereas IPCs involves in indirect neurogenesis (Noctor et al., 2008). Although IPCs have limited proliferative potential (Noctor et al., 2004), it is shown that IPCs are responsible for the production of majority of pyramidal-projection neurons for all layer within developing mammalian neocortex (MuhChyi et al., 2013). Towards the end of the neurogenic phase at late embryonic stage (E18.5) and perinatally, residual RGCs lose their neurogenic potential and acquire the multipotentiality to transform directly into astrocytes or give rise to glial-restricted progenitor (GRP) including astrocyte progenitors or generate progenitors for oligodendrocyte in addition to neurons (Qian et al., 2000; Fig. 1).

RGCs which are heterogenous population comprising neuronal and glial progenitors and other RGCs (Malatesta et al., 2000) recently identify as NSCs in many vertebrate species and in CNS regions (Malatesta and Gotz, 2013) because they possess key characteristic of NSCs, including the capacity for maintaining a copy of themselves (Shen et al., 2004) and multipotential, divide asymmetrically to give rise to at least four distinct cell type that include additional RGCs, IPCs, neurons, and astro-glial cells (Noctor et al., 2008).

I.2. Epigenetic regulation of sequential cell generation by neural stem cells

The best example of sequential cell generation by NSCs is demonstrated in corticogenesis, the developmental process of the cerebral cortex. The cerebral cortex is a six-layered structure derived from the anterior neuroectoderm. NSCs in the cerebral cortex first generate neurons, a process called neurogenesis, and then gliogenesis: produce astrocytes and oligodendrocytes. Within cortical neurogenesis, different neuronal subtypes are generated in a precise timing order, aligned into different layers in an “inside-out” manner: early born neurons form the deeper layers while later-born neurons form the upper layers (Hevner et al.,

2003). This timing program is preserved in cultured cortical-derived NSCs (Qian et al., 2000; Shen et al., 2006), and also in NSCs derived from mouse and human pluripotent ESCs (Gaspard et al., 2008). The molecular mechanisms driving the orderly generation of different types of neurons and glia from NSCs remain to be uncovered, however, it is believed that both extracellular factors, such as cytokine signaling, and intracellular processes including epigenetic modifications in concert with differential expression of certain transcription factors (TFs) contribute to the timing of NSC fate choice (Namihira et al., 2008; Okano and Temple, 2009; Juliandi et al., 2010a).

Epigenetic mechanisms signify heritable modifications in phenotype or gene expression, without alterations in DNA sequence (Yoder et al., 1997). During corticogenesis, key developmental genes are silenced or expressed to control neuronal-to-astroglial fate switch of NSCs. In the onset of neurogenic phase (around early to mid-gestation), proneuronal genes are activated by sequestered TFs whereas astrocytic genes in NSC are suppresed until appropriate cues trigger the execution of subsequent differentiation program that is astroglial differentiation. Later on, in astrocytic and oligodendrocytic phases (around late stage of gestation to postnatal), neuronal genes are repressed which is attributable to the loss of neurogenic capasity. Such repression or activation of developmental genes is epigenetic and is often achieved by the DNA methylation, modification of histones, polycomb gene group (PcG) and noncoding RNA expression (Hirabayashi and Gotoh, 2010).

I.2.a. DNA methylation suppresses astrocytic gene during the neurogenic phase

A family of DNA methyltransferases (Dnmts), including *de novo* DNA methyltransferases Dnmt3a and Dnmt3b and maintenance methyltransferase Dnmt1, mediates the methylation reaction, which adds a methyl group (-CH3) to the 5′ position of the pyrimidine ring of cytosine residues and primarily occurs at CpG dinucleotides. DNA

cytosine methylation is typically a repressive mark associated with transcriptional silencing. It can directly interfere with the binding of transcription factors to their target gene sequences or indirectly suppress gene expression through a family of methyl-CpG binding domain containing proteins (MBDs) such as MBD1-3 and methyl-CpG binding protein 2 (MeCP2), which further recruit repressor complexes containing histone deacetylases (HDAC) (Robertson et al., 2000).

The importance mechanism for timing the neuro-glial fate choice by DNA methylation has been discussed in excellent works and reviews by Nakashima's group (Kohyama et al., 2008; Juliandi et al., 2010a,b). A change in DNA methylation of the astrocyte-specific gene promoters (such as glial fibrillary acidic protein (*gfap*)) allows the NSCs to generate only neurons at midgestation, and then gradually switch to adopt glial fate during late stage of gestation in response to gliogenic signals such as leukaemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) (Kohyama et al., 2008; Namihira et al., 2008). Two well-studied pathways that act synergistically to promote astrocytic differentiation are those activated by interleukin-6 (IL-6) family of cytokines (such as LIF, CNTF, and cardiotrophin-1 (CT-1)) and bone morphogenetic protein (BMP) signaling (reviewed in Juliandi et al., 2010b; Fig. 2). LIF, CNTF and CT-1 can activate the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway upon binding to their cognate receptors, while BMPs activate the downstream TF SMAD through their serine/threonine kinase type cognate receptors. Synergistic activation of astrocytic genes is achieved by the formation of a complex involving activated STAT3 and SMADs bridged by the transcriptional coactivator p300/CBP (Nakashima et al., 1999; Fig. 2). It has been suggested that the irresponsiveness of early- and mid-gestational NSCs toward astrocyte-inducing stimulation such as LIF is reported to be attributeable to the hypermethylated DNA, a status which blocks binding of the STAT3-p300/CBP-SMADs complex to its target

sequence, in the promoter regions of astrocytic genes such as *gfap* (Takizawa et al., 2001; Fig. 3). In addition, there is a variation on methylation status of STAT3-binding element (TTC CGA GAA) within the *gfap* promoters in NSCs at different embryonic stages. The STAT3 binding site-containing *gfap* promoter in late-gestational (E14.5) NSC is barely methylated, while it is hypermethylated in mid-gestational (E11.5) one (Takizawa et al., 2001). Given that STAT3 cannot bind to sequences, thus it is conceivable that *gfap* promoter of NSC in mid gestation remains inert toward cytokine-inducible astrocyte differentiation.

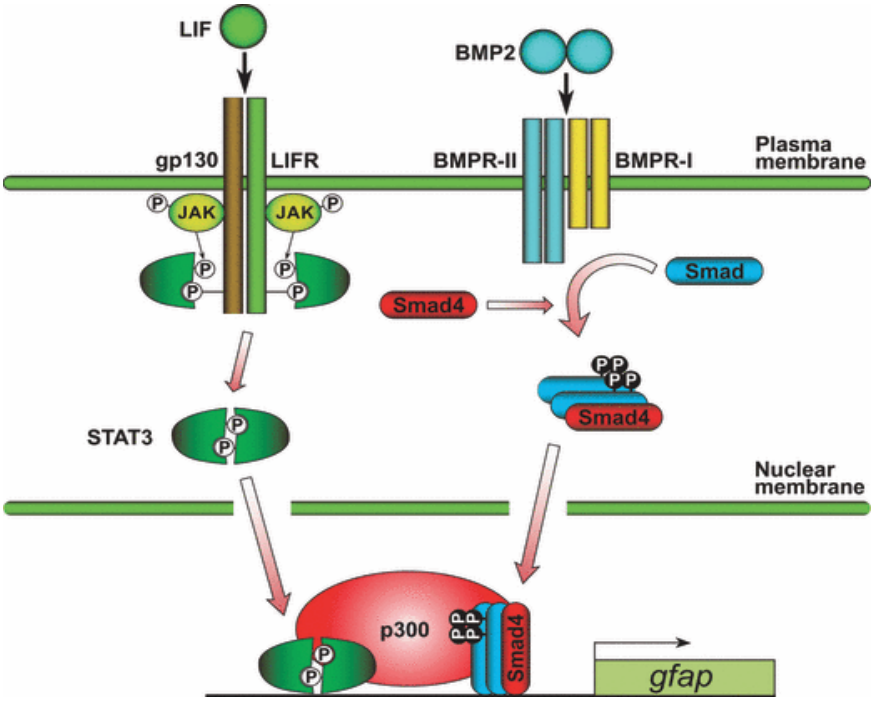


Fig. 2. Activation of astrocytic genes by crosstalk between two distinct cytokines. LIF, a member of the interleukin-6 (IL-6) cytokine family, binds to its specific receptor, LIFR, which dimerizes with a common signal transducer, gp130; this dimerization leads to activation of the JAK-STAT pathway. Activated STAT3 forms a homodimer and subsequently translocates into the nucleus. BMP2, a member of the transforming growth factor-β (TGF-β) superfamily, signals through a heterotetrameric serine/threonine kinase receptor complex composed of two type I (BMPR-I) and two type II (BMPR-II) receptor molecules. Activated BMPRs phosporylate the downstream transcription factors Smad1, -5, and -8, which then bind to the common mediator Smad4 before accumulating in the nucleus. There, STAT3 and Smads form a complex bridged by the transcriptional coactivator p300/CBP to activate the transcription of astrocytic markers such as *gfap* (Juliandi et al., 2010b).

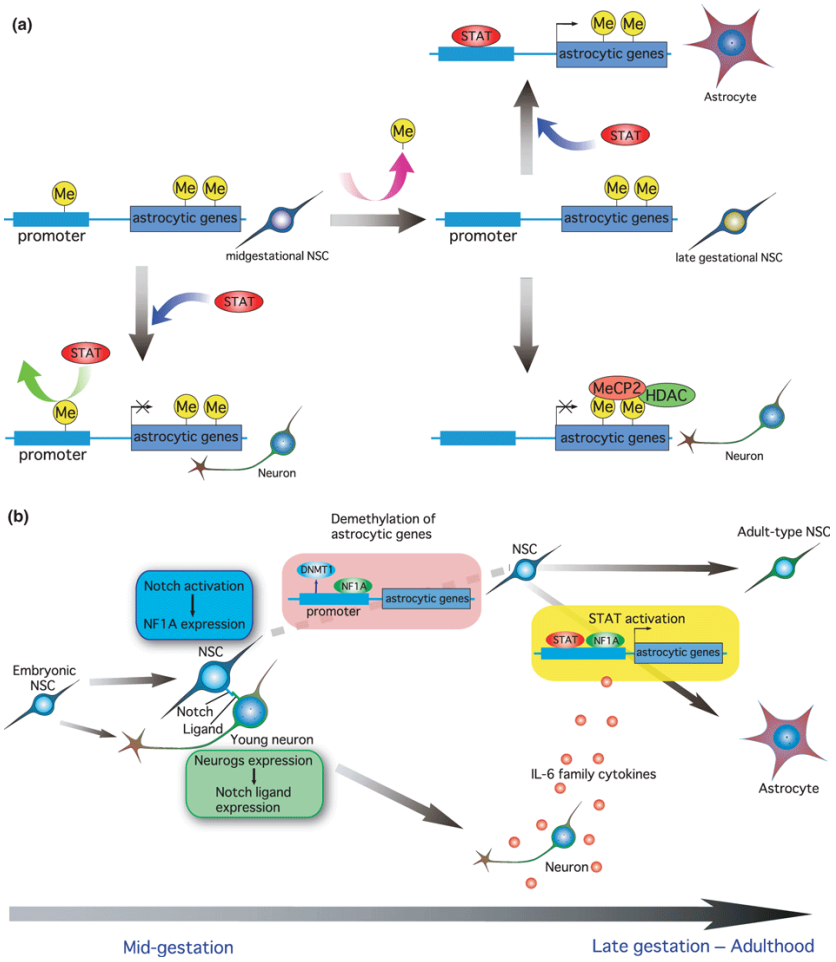


Fig. 3. (a) Astrocytic gene methylation status NSC development. Although STAT3 can be activated in midgestational NSCs, it cannot bind to astrocytic gene promoters such as *gfap* due to promoter hypermethylation, so these NSCs can only differentiate into neurons (left). As gestation proceeds, these promoters become demethylated, allowing STAT3 to bind and activate astrocytic genes, resulting in the differentiation of NSCs into astrocytes (upper right). MeCP2 blocks this activation in neurons (lower right). (b) Notch-induced demethylation of astrocytic genes. Activation of Notch signaling in residual NSCs by young neurons induces demethylation of astrocytic gene promoters by upregulation of NFIA and release of DNMT1 from astrocytic gene promoters. In turn, at late gestation, interleukin-6 (IL-6) family cytokines activate the STAT3 pathway and induce NSCs to differentiate into the astrocytic lineage (Juliandi et al., 2010b).

Conditional deletion of Dnmt1 in NSCs that led to accelerated demethylation in glial differentiation-related genes and precocious astroglial differentiation validated the function of DNA methylation (Fan et al., 2005). In contrast, ectopic expression of MeCP2, an MBD family gene mutated in Rett syndrome, in NSCs exposed to astrocyte-inducing factors prevents astroglia differentiation but promotes neuronal differentiation; however this is not

associated with methylation of the *gfap* promoter but the binding of MeCP2 to the hypermethylated exon region of the *gfap* gene (Setoguchi et al., 2006; Tsujimura et al., 2009; Fig. 3). Hence the status of DNA methylation in both promoter and exon regions of cell-specific genes is critical for cell-fate determining. Further, the precocious astrocytogenesis in the late stage NSC of Dnmt1 KO mouse was attributable not only of the STAT3-binding site demethylation in the *gfap* and *S100 calcium binding protein β* (*S100β*) promoter, but interestingly, also increased STAT-binding to the promoter of these astrocytic genes. Collectively, these data suggest that DNA methylation can regulate the timing and magnitude of astrocytic differentiation, through both direct inhibition of TFs binding to astrocytic genes promoter and exon regions and modulation of JAK-STAT activity (Fan et al., 2005). Nevertheless, many questions still remain such as: is demethylation a passive process that requires DNA replication or is it an active process? If it is an active process, does it function through either a DNA glycosylase or some other unknown enzyme that can remove methyl groups without base repair? How is the timing of this demethylation regulated? Is it the result of extracellular signals or through an intrinsic clock mechanism (Feng at al., 2007)?

A developmental signal such as Notch that comes from the progeny of early- and mid-gestational NSCs (Fig. 3) has been reported to contribute to *gfap* promoter demethylation (Namihira et al., 2009). Notch signaling that present in most multicellular organisms is a highly conserved and fundamental signaling system used by neighboring cells to communicate with each other through direct cell-cell contact in which mediate critically important cellular function and governs cell fate determination in CNS in order to assume their proper developmental role. Upon Notch activation by its ligands, the Notch intracellular domain (NICD) is released from the plasma membrane and translocates into the nucleus, where it initiates a signaling cascade and converts a particular repressor complex into an activator complex (Wallberg et al., 2002; Nakayama et al., 2008). Namihira et al. (2009)

confirmed that Notch ligands are expressed in committed neuronal progenitor and their derived young neurons, and that these ligands activate Nocth signaling in the residual NSCs (Fig. 3). Notch activation in mid-gestational committed neuronal progenitor induced expression of nuclear factor IA (NFIA), which bind to astrocytic gene promoters and act as a crucial molecule downstream of the Notch signaling pathway to potentiate astrocytic differentiation of mid-gestational NSCs. Further, Notch activation impairs the association of Dnmt1 with the astrocytic gene promoter resulting in demethylation of astrocyte-specific genes, implying that its dissociation may be in part responsible for the Notch-induced demethylation astrocyte-specific gene promoters and thus allowed precocious astrocytic differentiation in response to LIF stimulation (Namihira et al., 2009).

Another molecular mechanism of astrocytic differentiation inhibition during neurogenic phase involving proneural a neurogenic basic helix-loop-helix (bHLH) TF Neurogenin1 (Ngn1), key determinant of neuronal fate in the neocortex (Ivaniutsin et al., 2009), to promote neuronal differentiation has been proposed. In the early to mid-stages of neocortical development, the Wnt pathway promotes the expression of Ngn1 in NSCs. NGN1 promotes neurogenesis by its transcriptional activity and represses astrogliogenesis by sequestering the transcription co-activator p300 from signal transducer and activator of transcription 3 (STAT3). In addition, DNA methylation at the astrocytic gene loci by DNMT1 suppresses astrogliogenesis (Hirabayashi and Gotoh, 2010; Fig. 4). This mechanism is further supported by the observation that a mutant of Ngn1 that cannot bind DNA is still capable of suppressing gliogenesis (Sun et al., 2001).

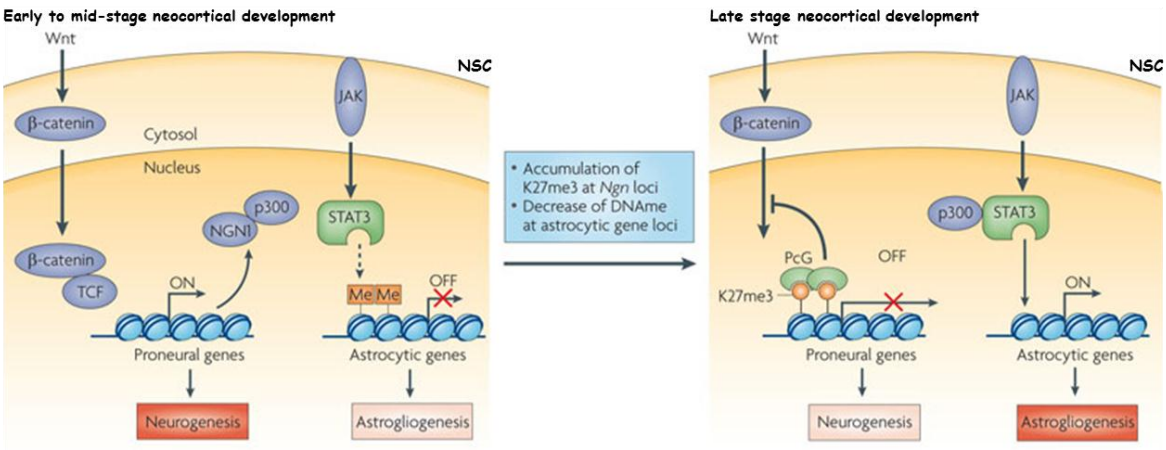


Fig. 4. Model for a switch in NSC differentiation from neurogenic to astrogenic. In the early to mid-stages of neocortical development, the Wnt pathway promotes the expression of neurogenin 1 (Ngn1) in NSCs. NGN1 promotes neurogenesis by its transcriptional activity and represses astrogliogenesis by sequestering the transcription co-activator p300 from signal transducer and activator of transcription 3 (STAT3). In addition, DNA methylation (DNAme) at the astrocytic gene loci by DNA methyltransferase 1 (DNMT1) suppresses astrogliogenesis. In the late stages of neocortical development, polycomb group (PcG) proteins repress the expression of Ngn1 and neurogenesis in NSCs. Moreover, the levels of DNAme at the astrocytic gene loci are reduced, which allows the binding of STAT3 to this locus and results in astrogliogenesis. JAK, Janus kinase; K27me3, lysine 27 trimethylation; TCF, T cell factor (Hirabayashi and Gotoh, 2010).

1.2.b. PcG and histone methylation silences neuronal genes during the astrocytic phase

Recent research has revealed that the polycomb-group (PcG) proteins play a major role in silencing important developmental genes. PcG acts as a transcriptional repressor by modulating chromatin structure (Schwartz and Pirrotta, 2008). PcG forms two major repressor complexes, polycomb repressive complexes, namely, PRC1 and -2. PRC2 contains Eed, Suz12, and Ezh1 or Ezh2 (which act as methyltransferase) trimethylate nucleosomes, which are positioned at genes encoding key developmental regulators, at trimethyl histone H3 lysine 27 (H3K27me3; Shen et al., 2008). This histone modification allows the recruitment of PRC1, consisting of ubiquitin ligase Ring1 that is able to effect the PcG-mediated repression (de Napoles et al., 2004).

Analogous to the suppression of astrogenic capacity during the early and mid-stage of neocortical development, the suppression of neuron production in the late stage of neocortical

development is attributable to the loss of neurogenic capacity (Hirabayashi and Gotoh, 2010). Whereas activation of the Wnt signalling pathway induces expression of Ngn1 and instructs neuronal differentiation in the early to mid-stage of neocortical development (Hirabayashi and Gotoh 2005; 2010; Ivaniutsin et al., 2009; Guillemot, 2007), it no longer does so in the late stage, indicating that NSCs in the late (astrocytic) phase have lost their capacity to respond to neurogenic signals (Hirabayshi and Gotoh, 2010; Fig. 4). As mentioned earlier, in addition to the promotion of neuronal differentiation, Ngn1 proteins suppress astrocytic differentiation by directly sequesters the p300/CBP-Smads complex from STAT3, leading to suppression of STAT3 target genes (Sun et al., 2001). Expression of NGN1 and NGN2 thus needs to be terminated at the onset of the astrocytic phase for both the termination of neurogenesis and the de-repression of astrogenesis. Ngn gene loci have recently been found to be suppressed by a PcG-mediated mechanism at the neuronal-to-astrocytic transition of NSC differentiation (Hirabayshi and Gotoh 2010; Fig. 4). The level of H3K27me3 correlated with transcriptional silencing at these gene loci gradually increase during neocortical development in parallel with downregulation of their expression suggesting interplay between different epigenetic mechanisms. The role of covalent histone modification including histone methylation and histone acetylation in the determination of NSC fate will be described in detailed below.

1.2.c. Role of covalent histone acetylation in NSC differentiation

1.2.c.i. Acetylation

Histone tails that normally have positive charges due to amine groups present on their lysine and arginine amino acids interact with and bind to the negative charged phosphate groups on the DNA backbone. There are two well-characterized mechanisms by which histone acetylation increases transcriptional activity (Kouzarides, 2007). Firstly, the addition

of acetyl moiety of acetyl coenzyme A by a member of the histone acetyl transferases (HATs) family results in the acetylation of lysine residue in histones (Hsieh and Gage, 2004). This neutralizes the positive charges on the histone by changing amines into amides and decreases the affinity of the histones to bind to DNA. This decreased binding allows chromatin expansion namely euchromatin configuration, permitting genetic transcription to take place (Kouzarides, 2007; Juliandi et al., 2010b; Fig. 5).

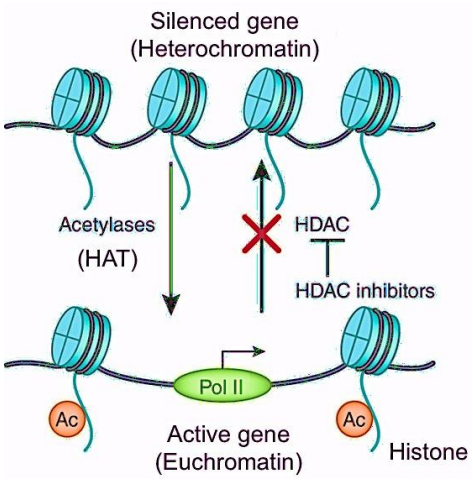


Fig. 5. The dynamic balance between histone acetylation and deacetylation influences whether a gene is active. When the histone proteins associated with the gene are unacetylated, chromatin is in a closed conformation that blocks access to key transcription factors (top). Acetylases (histone acetyl transferase (HAT)) attach acetyl groups (Ac) to histone tails, which contributes to a more open chromatin conformation that allows RNA polymerase II (Pol II) and specific regulators to access DNA (to bind to the promoter; bottom). HDAC removes acetyl groups and shifts this equilibrium toward silencing. HDAC inhibitors would block this reaction and thus promote the active state (Hyman et al., 2012, with modification).

Conversely, removal of those acetyl groups (deacetylation) from lysine residue in histone tails due to the activity of histone deacetylases (HDACs) increases the positive charge of histone tails and encourages high-affinity binding between the histones and DNA backbone. The increased DNA binding condenses chromatin structure (heterochromatin) thereby preventing transcription (Hsieh and Gage, 2005; Juliandi et al., 2010b; Fig. 5).

Secondly, histone acetylation mediates the recruitment of nonhistone proteins to the DNA. Modification of histone tails creates sites that are recognized by effector proteins,

which have bromodomains that specifically interact with the modified residues. Subsequently, the recruited effector proteins modulate DNA transcription (Izzo and Schneider, 2010). Interestingly, Wang et al. (2009) found that both HATs and HDACs are found at transcribed regions of active genes, and suggested that HDACs function to "reset" the conformational state of chromatin by removing acetylation at active genes (Wang et al., 2009).

Histone acetylation in neuronal and glial differentiation.

HDAC1 and HDAC2 act together in the CNS to maintain neuronal specification. Knockout of both genes result in a defective phenotype, with severe cortical disorganization, loss of hippocampal structure, and a lack of foliation in cerebellum, with lethality. This affected phenotype results from aberrant cell death in the neuronal progenitors and differentiation blockade restricted to neurons but not to astrocytes in vitro (Montgomery et al., 2009). However, a recent study on conditional deletion of HDAC2 in adult NSCs has shown that new neurons mature abnormally in the sub granular zone (SGZ) and the sub ventricular zone (SVZ), causing heightened apoptosis in both areas. A possible pathway for the regulation of adult neurogenesis by HDAC2 involves suppression of Sox2 after differentiation of NSCs (Jawerka et al., 2010). TLX (Tailless homolog) is an orphan nuclear receptor, which acts as a ligand-dependent transcription factor. TLX regulates NSC self-renewal by interacting with HDAC3, HDAC5, and HDAC7. TLX recruits HDACs to inhibit p21 and pten genes to regulate cell proliferation positively (Sun et al., 2007).

Pharmacological inhibition of HDACs with valproic acid (VPA) into adult hippocampal-derived NSCs in rat showed a decrease in the proliferation of adult NSC was coupled with an increase in neuronal differentiation and inhibited astrocyte and oligodendrocyte differentiation. Upon examination of gene expression levels, an increase in neuron-specific gene *NeuroD*, a neurogenic bHLH TF, occurs after VPA treatment. Correspondingly, over-expression of *NeuroD* in neural progenitors resulted in induction and

suppression of neuronal and glial differentiation, respectively. These results suggest that VPA promotes neuronal fate and inhibits glial fate simultaneously through the induction of neurogenic transcription factors including NeuroD (Hsieh et al., 2004). Interestingly, it is also found that HDAC inhibitor (HDACI) VPA promoted neuronal differentiation in adult hippocampal progenitor cells of mouse was accompanied by association of acetylated histone H4 (AcH4) with the proneuronal bHLH *Ngn1* promoter (Yu et al., 2009; Fig. 6A). As described earlier (Fig. 4), in cortical mid-gestational NSCs, TF NGN1 promotes neurogenesis by its transcriptional activity that may be mediated by histone acetylation regarding that acetylation of histone can loose the chromatin, permit specific regulators to access DNA and activate transcription. Since that the activation of *Ngn1/2* will set off a well-programmed cascade of NSC differentiation committed to all glutamatergic cortical neurons (Ngn1/2 → Tbr2 → NeuroD → Tbr1) in Notch-directed transcription during mid-corticogenesis (Kawaguchi et al., 2004; Englund et al., 2005; Sessa et al., 2008; Sansom et al., 2009; Fig. 6A), it is therefore tempting to address a question: does HDACI administration during mid pregnancy thereby caused acetylation of histone influence neocortical development (Fig. 6B)? VPA-mediated HDAC inhibition has also been shown to promote neurogenesis by activating the Ras-ERK pathway in the developing rat brain and in cultured E14 NSCs (Jung et al., 2008).

The presence of other HDACIs such as MS-275, M344, and suberoylanilide hydroxamic acid (SAHA) in adult forebrain precursor cell cultured not only increased the numbers of neurons, but also decreased the numbers of oligodendrocytes, accompanied by increasing the expression of proneural genes *NeuroD*, and reduced of oligodendrocyte lineage transcription factor 2 (*Olig2*) mRNA expression, respectively (Siebzehnrubl et al., 2007). Treatment of oligodendrocyte progenitors with trichostatin A (TSA), another HDACI, resulted in the blockage of differentiation in mature oligodendrocyte. Collectively, all these

findings revealed a necessity of HDAC activity for oligodendrocyte lineage progression (Marin-Husstege et al., 2002).

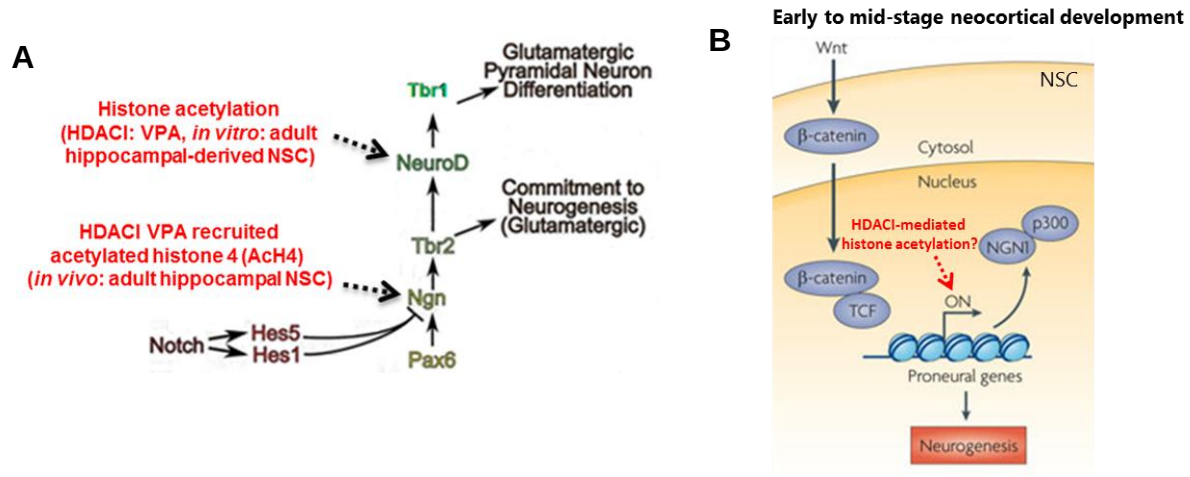


Fig. 6. *Histone acetylation in neuronal differentiation.* (A) *In vitro* study showed that the increased neuronal differentiation by HDACI VPA was coupled with increased histone H3 and H4 acetylation. Among the VPA-up-regulated, neuron-specific genes, a neurogenic bHLH TF, NeuroD, was identified. VPA promoted neuronal differentiation by recruiting AcH4 to the proneuronal bHLH *Ngn1* promoter, a core gene which initiate the cascade of NSC differentiation into pyramidal cortical neurons. (B) A proposed model depicting the roles of histone acetylation mediated by inhibition of HDAC during mid-corticogenesis (Hirabayashi and Gotoh, 2010 with modification).

Postnatal administration of VPA was also shown to delay the timing of NSC differentiation into myelin-forming oligodendrocytes in the developing forebrain (Shen et al., 2005); significant hypomyelination in the developing corpus callosum, together with sustained expression of progenitor markers and delayed expression of late differentiation markers, were observed in this study. However, HDAC inhibition by VPA after the onset of myelination resulted in comparable myelin gene expression to that seen in VPA-untreated rats, which was attributed to further transitions in nucleosomal histones from a state of reversible deacetylation to a more stably, repressed state by histone methylation. It has also recently been shown that HDAC1/2 contribute to the progression of murine oligodendrocyte differentiation by disrupting the β-catenin-TCF activator complex at *id2/4*, inhibitor of differentiation genes, thereby preventing the synthesis of Id2/4 proteins which can inhibit

myelin gene expression (Ye et al., 2009).

I.2.c.ii. Methylation

Unlike histone acetylation, which seems to be dynamic and reversible, the epigenetic modulation brought about by histone methylation was considered to be stable and could be reversed only by histone replacement. The discoveries of histone demethylases challenges this. PADI4, lysine-specific histone demethylase 1 (LSD1; KDM), and the JmjC-domain histone methylases (JHDM) can all remove methyl groups from histone residues (Whetstine et al., 2006) whereas histone methyl transferases (KMTs) catalyzed the methylated of histones. Inheritance of epigenetic modifications is believed to involve lysine methylation. Lysine methylation regulates transcriptional activity depending on the location and number of methyl-groups: histone H3 methylation at lysine 4 (K4), K36 and K79 leads to transcriptional activation (Zhao et al., 2005; Edmunds et al., 2008), whereas histone H3 methylation at K9 and K27 as well as histone H4 methylation at K59 leads to transcriptional silencing (Zhao et al., 2005).

Several extrinsic factors affect the histone methylation status of NSCs. For example, it has been suggested that fibroblast growth factor 2 (FGF2) signaling is directly responsible for the acquisition of glial competency during NSC culture by increasing H3K4 and reducing H3K9 methylation around the STAT3-binding sites of the *gfap* and *S100β* promoters so that, upon CNTF stimulation, NSCs can differentiate into astrocytes (Song and Ghosh, 2004). However, FGF2 is a common factor, when used either alone or together with epidermal growth factor (EGF), for maintaining NSCs in culture. Early gestational NSCs are initially responsive only to FGF2, and this signal then prime NSCs to become responsive to EGF later during development or culture *in vitro* (Tropepe et al., 1999; Lillien and Raphael, 2000; Ciccolini, 2001). How FGF2 and/or EGF signaling might influence KMTs and KDMs,

resulting in the aforementioned changes in histone methylation, therefore remains an open question.

I.3. Histone deacetylase (HDAC) and its inhibitors

There are currently 18 mammalian HDAC enzymes that have been identified. These enzymes are classified into four main classes, based on their homology to yeast (Gregoretti et al., 2004). The "classical," metal-dependent HDAC enzymes involve class I, II, and IV HDACs and the sirtuins; the nonmetal-dependent enzymes represent class III (Yang and Seto, 2008). The classical HDAC enzymes are metal-dependent as they contain zinc catalytic binding domains (Gregoretti et al., 2004). Class I enzymes contain HDAC1,2,3, and 8 and are expressed ubiquitously and share homology with the yeast transcriptional regulator RDP3 (Yang and Seto, 2008). These isotypes are usually expressed within the nucleus and act as transcriptional corepressors. The class II enzymes share homology with the yeast HDAC1 and are subdivided into class IIa, consisting of HDAC4,5,7, and 9, and class IIb, containing HDAC6 and 10 (Martin et al., 2007). These isotypes show tissue-specific distribution and are known to shuttle between the nucleus and cytoplasm, although histone proteins broadly represent their main target. The class IIb enzymes differ in that they primarily localize to the cytoplasm and differ structurally by containing two catalytic sites (Marks, 2010). HDAC11 shares homology with the class I isotypes, but shows more tissue-specific distribution with cytoplasmic localization. As it shares relationships with both class I and class II HDACs and structural homology to yeast, it has been designated a distinct class IV (Gregoretti et al., 2004).

Biological functions of individual HDACs have been difficult to determine due to the lack of specific pharmacological inhibitor compounds for particular HDACs (Haberland et al., 2009). HDACIs that target the classical HDAC enzymes are a diverse group of compounds,

which vary in structure, biological activity, and specificity. For example, the short-chain fatty acids VPA and butyrate inhibit class I and II HDACs (Hu et al., 2003; Go et al., 2011), while the hydroxamates include vorinostat (SAHA, brand name: Vorinostat®, Zolinza®), a prototypical HDACI TSA, primarily inhibits class I HDACs and limited class II HDAC enzymes (HDAC6) (Richon et al., 1998; Moradei et al., 2005; Witter et al., 2008; Guan et al., 2009; Rai et al., 2010). Other compounds can exert their properties specifically on class I HDACs, eg, the benzamide entinostat (MS-275) (Hu et al., 2003). Isotype-selective compounds are also increasingly becoming available, eg, tubacin, mocetinostat, and PC-34501 selectively inhibits HDAC6, −1, and −8, respectively (Tang et al., 2011).

HDACIs, which are mostly non-selective inhibitors, have demonstrated effectiveness in oncology and able to elicit a multitude of biological effects on cells, such as apoptosis, cell-cycle arrest, necrosis, autophagy, differentiation, and migration (Marks, 2010) lead to the multi targeted of HDACIs in treating some forms of cancer (Ververis et al., 2013). Unfortunately, they exhibit substantial adverse side effects in patients, presumably due to the broad dysregulation of gene expression in normal cells as well as in cancer cells. HDACIs have not only shown promising as anticancer agents, but also promising to treat in diverse disease state such as infectious and immunological diseases to traumatic shock, and from cardiac hypertrophy, to neurodegenerative disease, and airways disease implying that HDACIs target wide range of pathologies (Haberland et al., 2009). However, there has been much debate over whether isotype and class-specific HDACIs are preferred over broad-spectrum HDACIs to treat that diverse disease state (Morris and Monteggia, 2013).

Many evidences showed that HDAC isoform have specific targets *in vivo* (Morris and Monteggia, 2013) and governs different gene expression program in development and disease (Guan et al., 2009; Segré and Chiocca, 2011; Morris and Monteggia, 2013). All in all, given the intense clinical interest of HDACIs in many pathological conditions, also the fact that

HDACI profound adverse side effects in patients, and HDAC isoform have specific targets *in vivo* therefore it is important and plausible to discover isoform selective HDACIs that selectively block the pathological action of HDACs which should lead to improved drug safety or eliminate the side effects associated with non-selective HDAC inhibition.

I.4. Epigenetic modifications and teratogenic potential of HDAC inhibitor

Teratogen is defined as any substance, agent, or process that acts directly on the developing organism and interferes with normal prenatal development, causing the formation of developmental abnormalities in the fetus or indirectly, affecting such supplemental structures as the placenta or some maternal system. The type and extent of the defect are determined by the specific kind of teratogen, its mode of action, the embryonic process affected, genetic predisposition, and the stage of development at the time exposure occurred. The period of highest vulnerability in the developing embryo is from about early to middle stage of gestation, when differentiation of the major organs and systems occurs. Susceptibility to teratogenic influence decreases rapidly in the later periods of development, which are characterized by growth and elaboration.

Among the known teratogens are chemical agents, including such drugs as thalidomide, VPA, alkylating agents, and alcohol. A series of HDACI compounds such as VPA, butyrates, TSA, and SAHA had been shown also to interfere with proper embryonic development which lead to developmental anomalies of numerous organisms suggesting the teratogenic potential of HDACI (DiLiberti et al., 1984; Nau et al., 1991; Eikel et al., 2006; Phiel et al., 2001; Menegola et al., 2005; Wise et al., 2007; Ornoy, 2009). *In utero* exposure to VPA, widely prescribed broad spectrum antiepileptic agent, during early- and mid-pregnancy results in an increase in the expression of acetylated histones, which correlates with increased skeletal abnormalities and other congenital malformation observed at term

(Phiel et al., 2001; Menegola et al., 2005; Ornoy, 2009). Furthermore, the teratogenicity of VPA in vertebrate embryos is mimicked by the HDACI TSA (Phiel at al., 2001), whereas the structurally related VPA analogues lacking HDAC inhibitory activity, valpromide (VPM) and 2-methyl-2-propylpentenoic acid (2M2P), which function as anticonvulsants agent were shown to be less teratogenic (Nau et al., 1991; Menegola et al., 2005) indicating a HDAC induced teratogenesis. However, VPM is rapidly metabolized (80%) to VPA and hundred times more potent as an inhibitor of liver microsomal epoxide hydrolase. This makes it incompatible with other drugs and can affect the ability of the body to remove other toxins (Bialer, 1991), a fact that VPA is preferred than VPM in epileptic treatment. After *in utero* exposure to SAHA, the assessment of its developmental toxicity in fetal rats and rabbits also observed birth defects such as hematology and serum biochemistry change, skeletal defects, decreased fetal weight, and increases in the incidence of fetuses with incomplete ossification including skull bones (Wise et al., 2007).

HDACI has been known to be teratogenic for almost 30 years; however, the precise molecular mechanism by which HDACI exerts its teratogenicity is not yet fully elucidated. Because HDACs an important target of HDACI in the pathogenesis of birth defect (Phiel et al., 2001), and play important roles in embryonic development (Mannervik and Levine, 1999; Solari and Ahringer, 2000), it could be the target of HDAC induced teratogenesis. The first study done by Phiel et al. (2001) suggests that histone hyperacetylation induced by VPA may be a possible mechanism mediating teratogenesis. One evidences recently supported the possibility that epigenetic modifications caused by VPA during early mouse organogenesis results in congenital malformations (Tung and Winn, 2010). It has been reported that VPA, a direct inhibitor of HDAC, can induce histone hyperacetylation (Milutinovic et al., 2007) and other epigenetic changes such as histone (H3K4) methylation and DNA demethylation (Dong et al., 2007). In line with this study, maternal exposure to VPA in mouse has the ability to

cause these epigenetic alterations in the embryo such as increased acetylated histone H3 and -H4 (AcH3, AcH4), decrease in the expression of monomethyl histone H3 lysine 9 (H3K9Me) and increase in the expressions of di- and trimethyl histone H3 lysine 4 (H3K4Me2, H3K4Me3), and not changed in global or CpG island DNA methylation of embryo homogenates and thus these alterations contribute to mechanism for VPA-induced teratogenesis (Tung and Winn, 2010).

Acetylated histone tails allow TFs to access DNA by a conformational change to permissive or open chromatin. In addition, acetylated histones are recognized by other protein that allow for recruitment of ATP-dependent chromatin remodeling complex that consequently may lead to gene activation. VPA can induce a wide range of gene expression changes in the mouse embryo following maternal injection of the drug, including alterations in genes coding for structural and heat shock proteins, as well as transcription factors and regulators of translation (Kultima et al., 2004). Specifically in the head of mouse embryo, genes regulating cell cycle arrest and apoptosis were disrupted following VPA exposure suggesting that exencephaly could be caused by increased cell death (Okada et al., 2005). An increase in AcH3 and AcH4 staining were found in cranial mesenchyme and neuroepithelium in VPA-treated embryos (Tung and Winn, 2010). As HDAC activity is required during embryonic to maintain cell proliferation (Lagger et al., 2002), histone hyperacetylation in the cranial neural tube and surrounding tissues may result in in cell cycle arrest, cell death, and ultimately a neural tube defects (Tung and Winn, 2010). The increase AcH3 and AcH4 expressions were also found in the abnormal cardiac tissues of VPA-treated embryos suggesting the changes in histone acetylation are responsible for causing heart malformations (Tung and Winn, 2010).

I.5. HDAC expression and function during NSC differentiation

HDAC1 and HDAC2, belonging to the class I group, are clearly implicated in NSC differentiation. NSCs and their lineage-commited progenitor cells express high levels of HDAC1, while only some of them express low levels of HDAC2 (MacDonald and Roskams, 2008; Fig. 7). Interestingly, as NSCs commit to the neuronal lineage, expression of HDAC2 is upregulated but that of HDAC1 is down regulated to the extent that in most post-mitotic neurons it becomes undetectable, except only in some types of neurons (MacDonald and Roskams, 2008). On the other hand, HDAC1 expression is sustained in the majority of glial lineage cells (astrocytes and oligodendrocytes), in which HDAC2 is not detected (Shen et al., 2005; MacDonald and Roskams, 2008; Fig. 7). Moreover, HDAC2, but not HDAC1, was found to inhibit astrocytic differentiation (Humphrey et al., 2008). Intriguingly, despite the abundance of HDAC1 in NSCs, the level of histone acetylation in these cells is actually higher than that in their differentiated progeny (Hsieh et al., 2004). HDAC1 may begin to deacetylate histones, and thus to compact chromatin structure, at the onset of gliogenesis, as mature glial cells eventually display a very low level of global histone acetylation (Hsieh et al., 2004). The effect of HDAC1 on cell proliferation also probably differs among brain regions where NSCs reside, as HDAC inhibition using VPA has been reported both to inhibit (Hsieh et al., 2004; Jessberger et al., 2007) and to stimulate NSC proliferation (Higashi et al., 2008).

I.6. Cortical layer neuron generation in the cerebral cortex

Cerebral cortex is an essential part of neural tissue owing to its responsible for higher brain function, including sensory information, coordination of voluntary movement, and all cognitive functions (speech, math, learning, memory). It consists of up to six horizontal layers, each with a different composition in terms of neurons and connectivity. There are two

main populations of neurons in cerebral cortex: projection (or pyramidal) neurons which are glutamatergic and excitatory, and interneurons which are GABAergic and inhibitory (Molyneaux et al., 2007). The projection neurons originate from NSCs in the cortical VZ, and in contrast, almost all interneurons originate from NSCs located outside the cortex (Gorski et al., 2002).

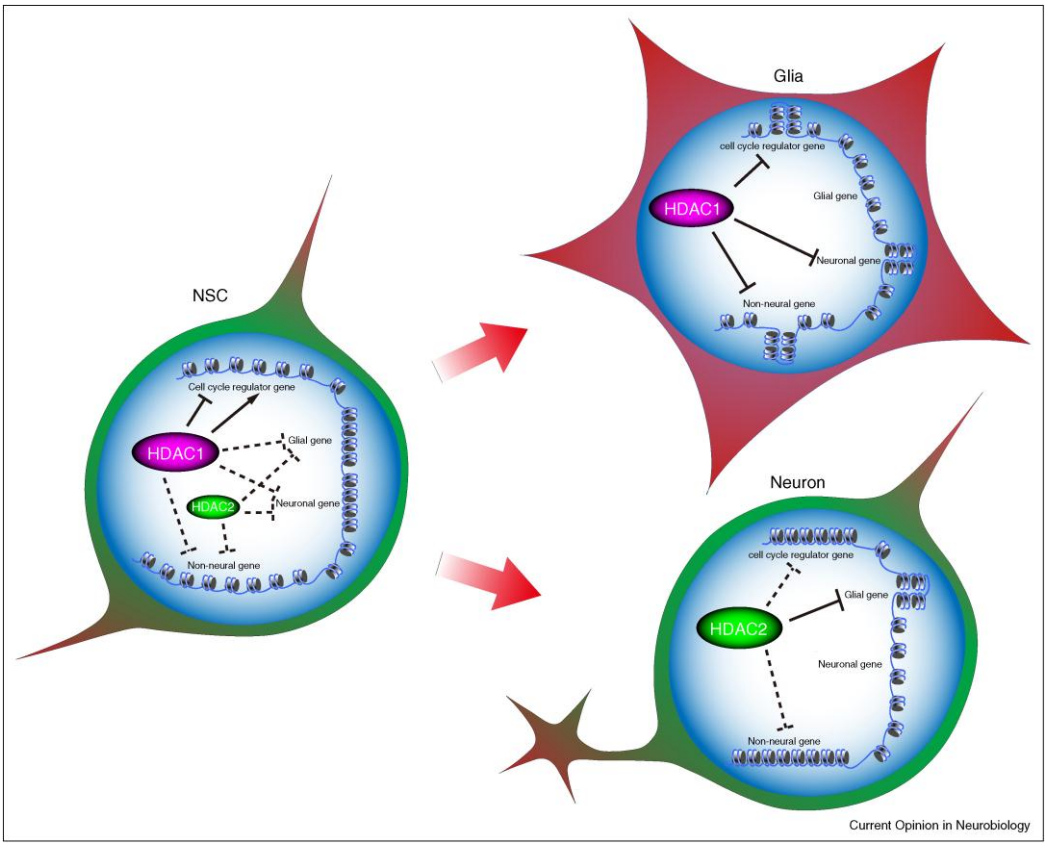


Fig. 7. A model of HDAC expression and function during NSC differentiation. In NSCs, HDAC1 (purple oval) is more abundant than HDAC2 (green oval) and probably functions mainly as a regulator of the cell cycle. During glial differentiation, global histone acetylation, and the amount of HDAC2 are reduced dramatically. In contrast, only a slight decrease of global histone acetylation occurs during neuronal differentiation with a persistent abundance of HDAC2. HDAC1 may contribute to global histone deacetylation in glial cells, while HDAC2 may have a specific function to repress glial genes in neurons. Solid and broken lines represent strong and moderate regulation of histone acetylation, respectively (Juliandi et al., 2010a).

Cortical projection neurons of different layers are generated in a well-defined spatio-temporal manner during brain development (Molyneaux et al., 2007) and can be derived directly from NSCs at VZ, or indirectly through IPCs that reside in SVZ (Guillemot et al.,

2006; Leone et al., 2008). In mouse, cortical neurogenesis (corticogenesis) starts around E10.5. The first wave neurons migrate out of the VZ and occupy the preplate, which is subsequently split into two zones by the intercalation of later neurons. The upper zone of the splitted preplate (also known as marginal zone/MZ, and later become layer 1/L1) is populated by Cajal-Retzius (CR) 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). Neurogenesis proceeds subsequently in an inside-out fashion to form a laminar pattern of six-layered CP, in which neurons of deep-layer (DL) cortex, corticothalamic neuron (L6) and subcerebral neuron (L5), arise and migrate first, while callosal projection neuron (L4-2) of the upper-layer (UL) cortex are born and migrate later (Angevine and Sidman, 1961; Molyneaux et al., 2007). Subcerebral projection neurons of L5 and corticothalamic neuron of L6 are generally classified as corticofugal neurons and project subcortically to different targets. On the other hand, callosal projection neuron falls into the class of commissural neurons and extends their axons only within the cortex (Molyneaux et al., 2007). Whereas the neurons in DL are generated mostly from mid-gestational NSCs (E11.5-E14.5), the neurons in the UL are suggested to be generated *via* IPCs, which are derived from late gestational NSCs (E14.5-E17.5) (Tarabykin et al., 2001; Zimmer et al., 2004; Shen et al., 2006; Guillemot et al., 2006; Cubelos et al., 2008; Arnold et al., 2008).The fate determination of DL and UL cortical neurons have been attributed to the key TFs Sox5, Fezf2, and Satb2 which have been implicated in neuronal migration and positioning (Kwan et al., 2008; Lai et al., 2008; Leone et al., 2008). The genetic variation of these TFs have been found within patients with developmental and language delays, intellectual disability, schizophrenia, and autism spectrum disorders (Cooper et al., 2011, Lamb et al., 2012, Potkin et al., 2009, Rosenfeld et al., 2010). It is likely that alterations in

these TFs not only change the neuronal identity of cortical neuron, but also contribute to a spectrum of cognitive and motor neurodevelopmental disorders.

The fate determination of multiple subtype projection neurons within DL developing cortex has been attributes to the direct transcriptional repression of each subtype cortical neuron fate: the zinc-transcription factor *Fezf2* (Leone et al., 2008), *Sox5*, and *Tbr1* (Fig. 8). Throughout embryonic development, SOX5 is absent from NSCs and its progenitors resided in VZ and SVZ, and newly born migrating neurons of the IZ. The expression of *Sox5* is enriched in SP neurons, L6 corticothalamic neurons and a subset of L5 subcerebral projection neurons (Fishell and Hanashima, 2008; Kwan et al., 2008; Lai et al., 2008; Muhchyi et al., 2013). *Fezf2* is expressed in NSCs and its progenitors starting on E8.5 and its expression is retained by DL neurons during corticogenesis (Chen et al., 2008). In order to maintain the co-existence of multiple subtypes of projection neurons in SP and L6 corticothalamic neurons, the earlier activation of *Fezf2* in NSCs and its progenitors and its continued expression in DL neurons is repressed by SOX5 and TBR1 (Shim et al., 2012), leading to a postnatal L5 with FEZF2-enriched pattern. Later on, L5 DL neurons acquire the expression of the second zinc-finger transcription factor *Ctip2* during postmitotic differentiation (Arlotta et al., 2005; Chen et al., 2008). Several studies have indicated that *Fezf2* expression in NSCs and its progenitors may promote the expression of *Ctip2* in young neurons, and together these genes confer a DL neuronal fate during differentiation (Leone et al., 2008).

In the case of UL neurons, its fate determination requires the chromatin remodeling protein Satb2 (Britanova et al., 2005; Leone et al., 2008). Fezf2 has been suggested to repress *Satb2* (directly or indirectly) thereby inhibiting generation of UL neurons (Chen et al., 2008) (Fig. 8). Satb2 has been also reported to inhibit *Ctip2* expression by direct binding to the matrix attachment regions in *Ctip2* locus (Alcamo et al., 2008; Britanova et al., 2008), *via* interacting with HDAC1 and MTA2, both are members of the nucleosome remodeling and

deacetylase (NuRD) complex (MuhChyi et al., 2013). In line with this finding, another transcription regulator SKI, appears to co-regulate the specification of UL neurons since the loss of SKI also leads to ectopic expression of *Ctip2* and thus suppression of UL neuron identity.

Accordingly, in UL but not DL neurons, SKI and SATB2 are largely co-expressed and constitute a repressor complex *in situ*. The formation of repressor complex consisting of SATB2, SKI, HDAC1 and MTA2, is essential for transcriptional repression of *Ctip2* in UL neurons (MuhChyi et al., 2013) (Fig. 8). While SATB2 binds to MAR-sequences in the *Ctip2* *cis*-regulatory region and recruits MTA2 to the same site, SKI is required for recruiting HDAC1, and subsequently allowing the formation of a functional NuRD complex (Baranek et al., 2011). Furthermore, consistent with the notion that SATB2 induces changes in chromatin state, Alcamo et al. (2008) revealed that in the absence of SATB2-containing NuRD complex binding to MAR regions, H3K4Me2 and histone H3 acetylation in the Ctip2 locus are markedly increased, both of which correlate with an 'open' chromatin status. Hence, it is conceivable that these transcriptionally active marks permit the ectopic activation of *Ctip2* in *Satb2*-deficient UL neurons (MuhChyi et al., 2013).

At later times during corticogenesis, when UL neurons are generated, Fezf2 expression is absent which relieves the repression of *Satb2* (Chen et al., 2008). This, in turn, enables Satb2 to actively repress *Ctip2* expression and promotes the adoption of UL neurons identity (Fig. 8).

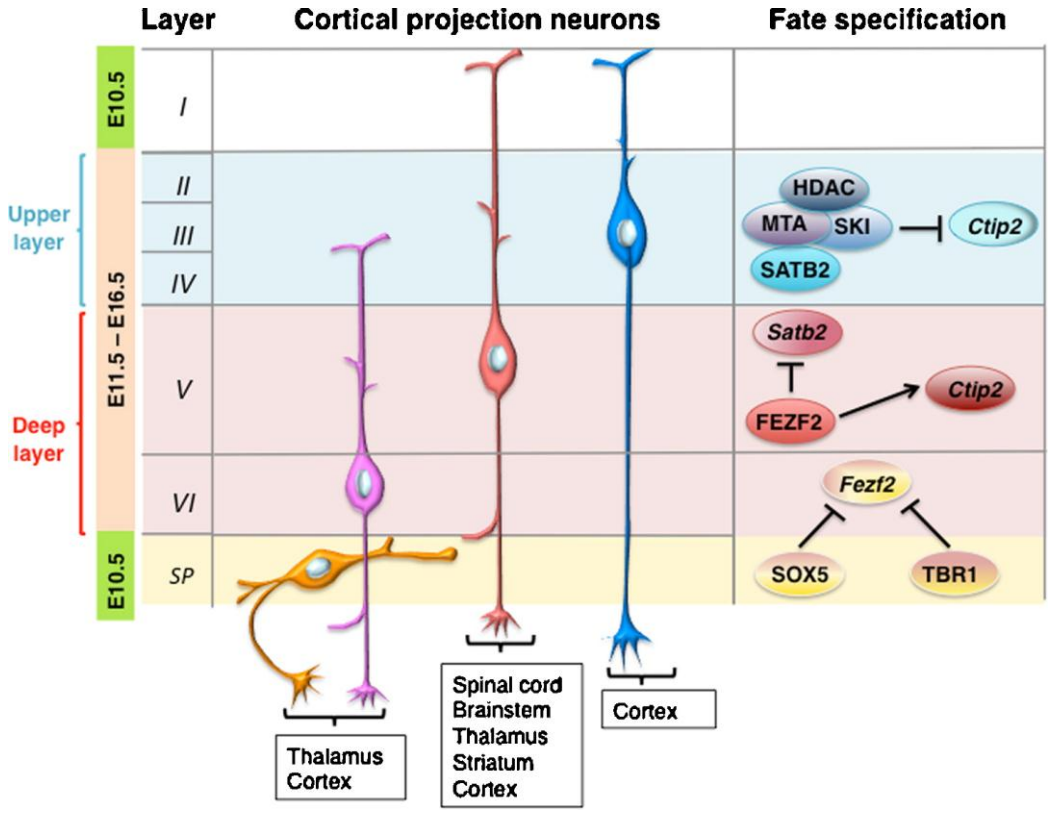


Fig. 8. Schematic representation of cortical neuronal subtype specification. After the acquisition of laminar- and neuronal-properties, postmitotic cortical neurons undergo subsequent specification programs that confer their projection subtype identity in a coordinated temporal order. Each neuronal subtype projects axon to different targets and their subtype identity is governed by distinct transcriptional factors together with epigenetic modifications that are responsible for progenitor expansion, neuronal fate specification, and neurogenic to gliogenic switch (Muhchyi et al., 2013).

I.7. Objectives of the researchs

Many cellular events and proper development of an organism required precise spatial and temporal controlled expression of all genes. The ability of epigenetic mechanism to confer active/repressive marks to DNA/chromatin and thus regulating the cellular outcomes has made such modifications one of the most effective intrinsic programs that govern developmental process including fate specification and differentiation of NSCs (Namihira et al., 2008; Juliandi et al., 2010a; 2010b). During brain development, the formation of CNS is initiated by self-renewal and differentiation of NSCs into three major cell types: neurons, astrocytes, and oligodendrocytes by which neurogenesis precedes astrogliogenesis occurs

only in mammalian neural development. Our group and other have identified that one of such mechanisms, histone acetylation, regulates fate specification of NSCs at different developmental stages (Hsieh et al., 2004; Laeng et al., 2004; Balasubramaniyan et al., 2006; Siebzehnrubl et al., 2007; Asano et al., 2009; Yu et al., 2009; Abematsu et al., 2010; Juliandi et al., 2012). Many evidences support that increasing global histone acetylation by histone deacetylase inhibitors (HDACI) treatment drives NSCs toward neuronal lineage over glial ones (Abematsu et al., 2010) in adult-derived NSC cultured (Hsieh et al., 2004; Siebzehnrubl et al., 2007; Yu et al., 2009). Moreover, taking advantage of mouse embryonic stem cells (mESCs) culture system that mimics corticogenesis *in vitro* (Gaspard et al., 2008), we have previously shown that HDAC inhibition using VPA increased the production of Cux1+ UL neurons and decreased that of 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 NSCs fate specification during corticogenesis *in vivo* is hitherto remains elusive.

In this study, I investigated the role of histone acetylation in the NSC differentiation by inhibiting HDAC pharmacologically. I used SAHA, which is one of epi-drugs developed for cancer treatment 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). Preclinical study, however, reported that SAHA could be found in fetal plasma due to its capacity to pass through the placenta, indicating that this drug can influence cells in fetuses during pregnancy if taken by pregnant cancer patient or if the patient becomes pregnant while taking this drug. The use of this drug raises questions on its potential to affect epigenetic status especially histone acetylation status of non-cancerous

cells. Unfortunately, report regarding this aspect and particularly its effects on brain development is not yet available.

In addition, previous studies have found that a series of HDACI such as VPA, butyrates, TSA, and SAHA can interfere with proper embryonic development which lead to developmental anomalies of numerous organisms suggesting the teratogenic potential of HDACI (DiLiberti et al., 1984; Nau et al., 1991; Eikel et al., 2006a; 2006b; Phiel et al., 2001; Menegola et al., 2005; Wise et al., 2007; Ornoy, 2009). *In utero* exposure to VPA during early- and mid-pregnancy results in an increase expression of acetylated histones in the embryo, which correlates with increased skeletal abnormalities, neural tube defect, and other congenital malformation (Phiel et al., 2001; Menegola et al., 2005; Ornoy, 2009) implying that histone hyperacetylation induced by VPA may be a possible mechanism mediating teratogenesis (Tung and Winn, 2010).

All in all, given the intense clinical interest of SAHA as HDACI in many pathological conditions, also the fact that HDACI profound adverse side effects in patients including negative effects on fetal growth, therefore it is important to investigate the potential of SAHA to effect on histone acetylation status of normal cells during pregnancy especially in embryonic NSC fate examination. Further, I also will elucidate its long-term consequence after postnatal considering that the effect of SAHA-mediated HDAC inhibition at embryos might also progressively affect brain structure, function, and related behavior through adulthood.

II. Materials and methods

II.1. Drug preparation

Suberoylanilide hydroxamic acid (SAHA; Cayman Chemical, Ann Arbor, MI) was dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) and then 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.

II.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 animals used and their suffering. Mice were individually housed on 12-h light/dark cycle at a constant temperature of 23-24°C, 50-70% humidity with free access to pellet diet 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.

Pregnant dams from Control and SAHA groups received single-pulse intraperitoneal (i.p.) 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 tracing the differentiation of NSCs at postnatal stage. These mice were sacrificed on postnatal day 7 (P7).

Another set of pregnant mice in Control and SAHA groups were allowed to give birth and their pups were weaned at 4 weeks of age. To analyze adult neurogenesis, 50 mg/kg BrdU were i.p. injected for 5 consecutive days starting on 12 weeks of age. Mice were sacrificed 1 day (proliferation groups) and 4 weeks (differentiation groups) after the last BrdU injection. For behaviour analysis, the same groups of mice 12 weeks of age were used.

II.3. Tissue preparation

Embryonic or postnatal brains were harvested at specified developmental days. Pregnant dams were sacrificed by cervical dislocation. Embryos were removed by cesarean section and immediately embryonic brains were collected in ice-cold phosphate buffered saline (PBS). Postnatal brains were collected after pups were deeply anesthetized by hypothermia before transcardially perfused with PBS and then 4% paraformaldehyde (PFA) in PBS. To isolate adult brains, mice were deeply anesthetized with i.p. injection of somnophentyl and perfused *via* the ascending aorta with PBS and PFA in PBS afterward. The dissected brains were fixed in 4% PFA in PBS, followed by incubation 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. Cryostat sections of embryonic brains (in coronal plane with 20 μm thickness) were serially cut on a Leica CM 1900 (Leica Microsystems, Wetzlar, German) and affixed to MAS-coated glass slides (Matsunami Glass), while postnatal and adult brains (40 μm) were sectioned and floated with PBS in 6-well or 12-well chamber slides (Nunc, Greiner).

II.4. 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 NSCs. To study the proliferation of NSCs, DMSO or 30 nM SAHA was added to individual culture 3 h after plating and the cells were expanded for 2 days (d). Later, 10 μg/ml BrdU (Sigma) was added 30 min before cell fixation. To induce neuronal differentiation, NSCs were cultured in the proliferating medium for 2 d and medium was changed into differentiation medium (N2-supplemented DMEM/F-12 without bFGF) and the

cells were cultured for 4 d. DMSO or 30 nM SAHA was added to individual cultures on the first day of differentiation period.

II.5. 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-β-tubulin isotype III (Tuj1; 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), rabbit anti-Sox2 (1:500; Millipore), rabbit anti-S100β (1:500; Swant), mouse anti-Reelin (1:500; Abcam), rabbit anti-cleaved Notch (1:100; Cell Signaling), 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), rabbit anti-cleaved caspase3 (1:100; Cell Signaling), 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 staining with anti-cleaved Notch, antigen retrieval was accomplished by autoclaving sections for 15 min at 105°C in Antigen Retrieval Solution and processing for immunostaining as described (Tokunaga et al., 2004). For BrdU immunohistochemistry, sections were pre-treated with 2N 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 anti-rat (all 1:500; Invitrogen). After 3 washes with PBS, nuclei were stained for 15 min at RT with Hoechst 33258 (Nacalai Tesque). Sections were washed with PBS, mounted on cover slips with Immu-Mount (Thermo Scientific, Pittsburgh, PA), and viewed and photographed using fluorescence microscope Axiovert 200M or Zeiss LSM 710 (Carl Zeiss, Gottingen, Germany) equipped with a camera and appropriate epifluorescence filters.

II.6. Quantitative real-time PCR (qRT-PCR)

Cells were prepared from E14.5 cortices 3 h after the last SAHA treatment. Total RNAs were isolated using Sepasol-RNA I Super G (Nacalai Tesque), and treated with DNase I (Promega, Madison, WI). cDNAs were synthesized from 1 μg total RNA with SuperScript VILO cDNA Synthesis Kit (Invitrogen) as recommended by the manufacturer. qRT-PCR was performed by MX3000p (Agilent Technologies, Santa Clara, CA) using KAPA SYBR FAST qPCR Master Mix Universal kit with ROX as a reference dye (KAPA Biosystems, Boston, MA). The expression of target genes was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The gene-specific primers were as follows (5′-3′): *Nestin*: Nestin-S, CCCTGAAGTCGAGGAGCTG; Nestin-AS, CTGCTGCACCTCTAAGCGA; *Pax6*: Pax6-S, CTGGAGAAAGAGTTTGAGAG; Pax6-AS, GGCTGTGGGATTGGCTGGTA; *βIII-tubulin*: βIII-tubulin-S, GGCCTCCTCTCACAAGTATG; βIII-tubulin-AS, TTGTTGCCAGCACCACTCTG; *Dcx*: Dcx-S, CATTGACGGATCCAGGAAG; Dcx-AS, GACATTCTTGGTGTACTCAACC; *Tbr2*: Tbr2-S, TTCGTGGAAGTGGTTCTG; Tbr2-AS, TTCAAGTCCTCCACACCGTC; *Fezf2*: Fezf2-S, GGCTACAAGCCCTTCGTCTG; Fezf2-AS, GTGCATTTGTACTGCTTCTC; *Ctip2*: Ctip2-S, CCCGACCCTGATCTACTCAC;

Ctip2-AS, TTCTCCTGCTTGGGACAGATG; *Satb2*: Satb2-S, GGAGAATCTCTGCACCATC; Satb2-AS, GAGCTCTCCTTAGTTGGCTG; *Hes5*: Hes5-S, AAGAGCCTGCACCAGGACTA; Hes5-AS, CGCTGGAAGTGGTAAAGCA; *NFIA*: NFIA-S, GGCATACTTTGTACATGCAGC; NFIA-AS, CCTGATGTGACAAAGCTGTCC; *NFIB*: NFIB-S, TTTTTGGCATACTACGTGCAGG; NFIB-AS, TCTGATACATTGAAGACTCCG; *Ngn1*: Ngn1-S, GGCTCTGCTGCACTCCCTG; Ngn1-AS, CCATAGGTGAAGTCTTCTGA AG; and *GAPDH*: GAPDH-S, ACCACAGTCCATGCC ATCAC; GAPDH-AS, TCCACCACCCTGTTGCTGTA.

II.7. Cell quantification

Coronal sections from at least 3 different levels of rostral-caudal axis were carefully matched between groups based on Hoechst staining. Cortical somatosensory area was selected for quantification of layer-specific marker expression in postnatal brain. The number of marker-positive cells was calculated in 8 different bins by which cortical L1 and UL (L2-4) were represented by bins number 1 and 2-4, respectively, and the remaining bins (5-8) were equivalent to DL (L5-6) of the neocortex. Counting was conducted manually by using a fluorescence microscope Axiovert 200M (Carl Zeiss) equipped with camera and appropriate epifluorescence filters within the indicated area. For BrdU counting in adult neurogenesis experiment, the total number of BrdU+ cells was calculated using every 6th section (240 μm apart). BrdU+ cells were counted throughout the rostrocaudal extent of the granule cell layer (GCL) using a 20x objective and a fluorescence microscope Axiovert 200M. Derived numbers were multiplied to 6 (slice series) to obtain total cell numbers per GCL. For BrdU double (or triple) immunostaining with other markers, 1-in-12 series of sections (480 μm apart) from the proliferation and the differentiation group were used. From each mouse, 50 BrdU+ cells were randomly picked throughout the GCL and analyzed for double (or triple) immunopositive.

II.8. Behavioral test

Experimental apparatuses and image analyzing software were obtained from O'Hara and Co., Ltd., Japan. Image analyzing software (Image OF4, Image LD2, Image EP2 and Image FZ2) was developed from the public domain ImageJ software. All experiments were done with 12 mice per group and were conducted between 13:30 and 16:30. The level of background noise during behavioral testing was about 50 dB. After each trial, the apparatuses were wiped and cleaned.

II.8.a. Open field test

The locomotor activity was measured for 10 min using an open field apparatus made of white plastic (50 x 50 x 40 (H) cm). An LED light system was positioned 50 cm above the centre of the field (50 lux at the centre of the field). Total distance travelled (cm), time spent in the central area (30% of the field)(sec), and the frequencies of movement were measured (Tanemura et al., 2002).

II.8.b. Light/dark transition test

The apparatus used for the light/dark transition test consisted of a cage (21 x 42 x 25 (H) cm) divided into two chambers by a partition with an opening. One chamber was brightly illuminated (250 lux), whereas the other chamber was dark (2 lux). A mouse was placed into the dark area and allowed to move freely between the two chambers through the opening for 5 min. The latency for the first move to the light area, the total number of transitions and the time spent on each side were measured.

II.8.c. Elevated plus maze test

The plus-shaped apparatus consisted of four arms (25 x 5 cm) connected to a central square area (5 x 5 cm). Opposite two arms were enclosed with 20 cm-high transparent walls and other two were left open. The floor of the maze was made of white plastic plate and was elevated 60 cm above the room floor (200 lux at the centre of the apparatus). A mouse was

placed to the central square area of the maze, facing one arms, and the behavior was recorded for 10 min: total distance traveled (cm), total time on open arms and central square area (sec) and the total number of entry to any of the arms (Tanemura et al., 2002).

II.8.d. Contextual/cued fear conditioning test

The apparatus consists of a conditioning chamber (or a test chamber) (17 x 10 x 10 (H) cm) made of clear plastic with ceiling and placed in a sound proof box. The chamber floor has stainless steel rods (2-mm diameter) spaced 5 mm apart for electric foot shock (0.1 mA, 3 sec duration) to the mouse. The sound proof box consists of white-coloured wood, and was equipped with an audio speaker and light source (35 lux at the centre of the floor). A CCD camera was positioned 20 cm above the ceiling of the chamber. During the conditioning trial (Day 1), mice were placed individually into the conditioning chamber in the sound proof box and, after 90 sec, they were given three tone-shock pairings (30 sec of tone, 75 dB, 10 kHz followed by 3 sec of electric shock at the end of the tone, 0.1 mA) separated by 90 sec. Then they were returned to their home cage. Next day (Day 2), as a "contextual fear test", they were returned to the conditioning chamber without tone and shock for a 6-min. On the third day (Day 3), they were brought to a novel chamber of different make without stainless steel rods place in the sound proof box and, after a period of 3 min, only the conditioning tone was presented for 3 min (no shock was presented, 35 lux at the centre of the floor). The freezing response of mice was defined as a consecutive 2 sec period of immobility. Freezing rate (%) was calculated as [time freezing/session time] x 100 (Tatebayashi et al., 2002).

II.8.e. Prepulse inhibition test

A startle reflex measurement system was used. A test session was started by place a mouse in a Plexiglas cylinder, where it was left undisturbed for 10 min. The duration of white noise that was used as the startle stimulus was 40 msec for all trial types. The startle response was recorded by accelerometer for 140 msec (measuring the response every 1 msec) starting

with the onset of the prepulse stimulus. The background noise level in each chamber was 70 dB. The peak startle amplitude recorded during the 140 msec sampling window was used as the dependent variable. A test session consisted of 6 trial types (i.e. two types for startle stimulus-only trials, and four types for prepulse inhibition trials). The intensity of the startle stimulus was 120 dB. The prepulse sound was presented 100 msec before the startle stimulus, and its intensity was 90 and 95 dB. Two combinations of prepulse and startle stimuli were employed (90-120 and 95-120). Six blocks of the 6 trial types were presented in pseudorandom order such that each trial type was presented once within a block. The average inter-trial interval was 15 sec (range: 10-20 sec).

II.8.f. Tail suspension test

Mice were suspended above the floor (60 cm) for 3 min by holding their tail. The immobility time during this period was scored. Immobility was defined as the absence of all movement except for those required for respiration.

II.8.g. Y-maze alternation test

The Y-maze is made of clear plastic. The maze has 3 identical arms (40 x 9 x 16 cm) placed at 120° from each other. The centre platform is a triangle with 9 cm side-length. Each mouse is placed at the end of one arm; the head directed to the walls and allowed to explore freely the apparatus for 5 min. Sequential alternations in entering the arms were recorded.

II.9. Western blot analysis

Western blot analysis was performed as described previously (Kohyama et al., 2010). In brief, the E15.5 and P7 cortices were isolated, lysed, and the protein concentration in each sample was determined by the Bradford method harnessing Coomasie Protein Assay Reagent (Thermo Scientific). Twenty μg protein samples of each total cell extract were separated by 5–20% gradient polyacrylamide gels (e-PAGEL; ATTO Corp., Tokyo, Japan), transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, Pittsburgh, PA), and probed with

anti-AcH3 (rabbit IgG, Millipore), anti-histone H3 (rabbit IgG, Abcam), anti-gfap (mouse IgG, Millipore), anti-Brn1 (rabbit IgG, Santa Cruz), anti-Satb2 (mouse IgG, Abcam), anti-Tbr1 (mouse IgG, Abcam) or anti-GAPDH (mouse IgG, Millipore) antibodies. Signals were detected with anti-rabbit and anti-mouse IgG of horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Lab.) using an ECL kit (GE Healthcare, Buckinghamshire, UK). Expression of AcH3 (~17 kD) and histone H3 (~15 kD) were detected at E15.5, and gfap (~50 kD), Brn1 (~50 kD), Satb2 (~81 kD), Tbr1 (~74 kD) at P7. The amounts of proteins loaded in each slot were normalized to those of GAPDH (~38 kD). The relative density of the protein expression was analyzed by ImageJ software (NIH, Bethesda, MD).

II.10. Statistical analysis

Analysis was performed from at least 3 embryos or pups of each group. Statistical comparisons were made by Student's *t*-test (for 2-groups comparison) and ANOVA (for multiple groups' comparison) with Tukey post hoc tests. Data represent mean + SD.

III. Results

III.1. SAHA enhances embryonic cortical neurogenesis

To examine the role of histone acetylation in the developing brain particularly during mid-gestation, the period of prominent neurogenesis (Qian et al., 2000), I orally administered SAHA to pregnant mice from E12.5 to E14.5 and performed histological analysis on E15.5 (Fig. 9A). I found that SAHA treatment led to a global increase of acetylation of both histones H3 and H4 in the cortex (Fig. 9B). To confirm the changes of histone acetylation, protein extracts were also harvested from E15.5 cortex and analyzed by Western blot analysis with antibody specific for acetylated histone H3. The level of acetylated histone H3 was still relatively high in SAHA-treated extracts as compared with Control extracts (Fig. 9C).

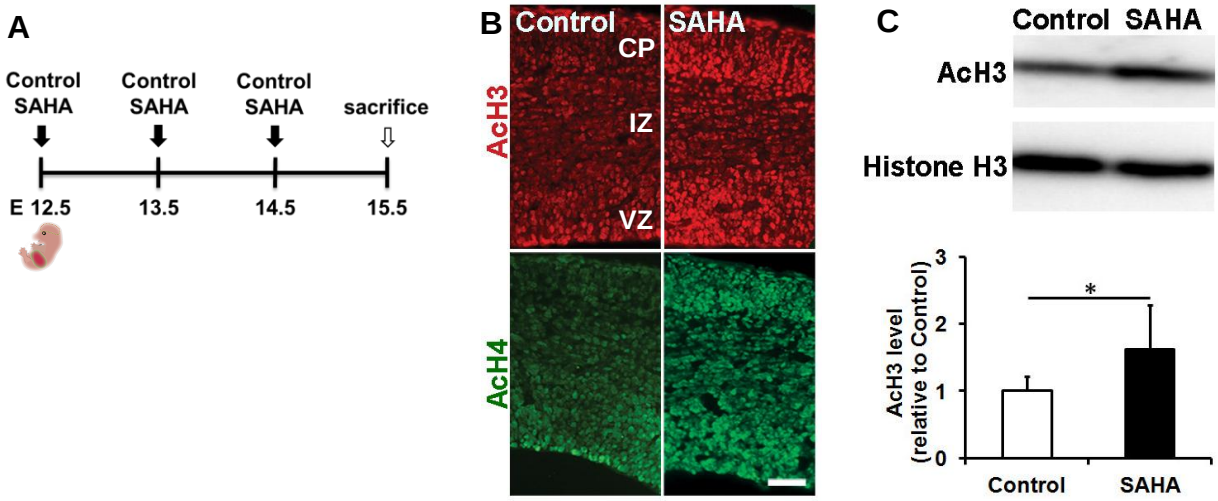


Fig. 9. SAHA increases histone acetylation of embryonic cortex. (A) SAHA or methyl cellulose (Control) was orally administered to pregnant mice from embryonic day (E) 12.5 until E14.5 and sacrificed at E15.5. (B) Acetylation of histone H3 and H4 were higher in SAHA-treated cortex compared to that of control, as shown by immunostaining of acetylated-histone H3 (AcH3; red) and acetylated-histone H4 (AcH4; green), and (C) Western blot and relative density expression of AcH3 and total histone H3 protein. Data are represent as means + SD. *$p < 0.05$, Student's *t*-test; n = 3. Scale bars: 50 μm. CP: Cortical Plate, IZ: Intermediate Zone, VZ: Ventricular Zone.

To exclude the possibility that the change in histone acetylation observed during neuronal lineage progression was due to changes in overall histone level, I also performed Western

blot analysis with antibody againts total histone H3. As shown in Fig. 9C, the steady-state level of histone H3 did not change by SAHA administration.

I next asked whether this global increase of histone acetylation accompanied by increased neuronal differentiation as our group and others already observed *in vitro* (Hsieh et al., 2004; Yu et al., 2009; Abematsu et al., 2010). Indeed, I observed that cortical area (cortical plate and intermediate zone) positive for immature neuron markers, βIII-tubulin and Dcx, was thicker and denser in SAHA-treated embryos than in control (Fig. 10A, B). Furthermore, I also verified the enhancement of neuronal differentiation of embryonic NSCs exposed to SAHA by BrdU single-pulse injection to pregnant dams on E14.5 and fate analysis on P7 when six-layered cortical lamination had been largely completed (Fig. 10C). Immunohistological examination showed that embryonic SAHA administration induced higher production of mature neurons (BrdU+/NeuN+) that were born at E14.5 (Fig. 10D, E), thus supporting my earlier findings on E15.5.

Because newborn neurons were derived from NSCs in VZ which then migrate into CP during midgestation and the enhancement of neurogenesis, or differentiation in general, usually coupled with the reduction of NSCs number because these cells have to exit cell cycle before they differentiate, I next examined the expression of NSC markers such as Nestin and Pax6 (Sansom et al., 2009). I found that Nestin expression in VZ was lower (Fig. 10B), and the proportion of Pax6+ among Hoechst+ cells was reduced in VZ of SAHA-treated cortex (Fig. 11A, B). In order to check whether transcription level of these neuronal and NSCs markers also influenced by SAHA administration, I conducted qRT-PCR against E14.5 SAHA-treated cortices, 3 h after the last SAHA treatment. I found that mRNA levels of *βIII-tubulin* and *Dcx* were up-regulated, whereas those of *Nestin* and *Pax6* were down-regulated (Fig. 11C, D). Taken together, these results indicate that HDAC inhibition by SAHA treatment promotes neuronal differentiation of NSCs in the developing cortex.

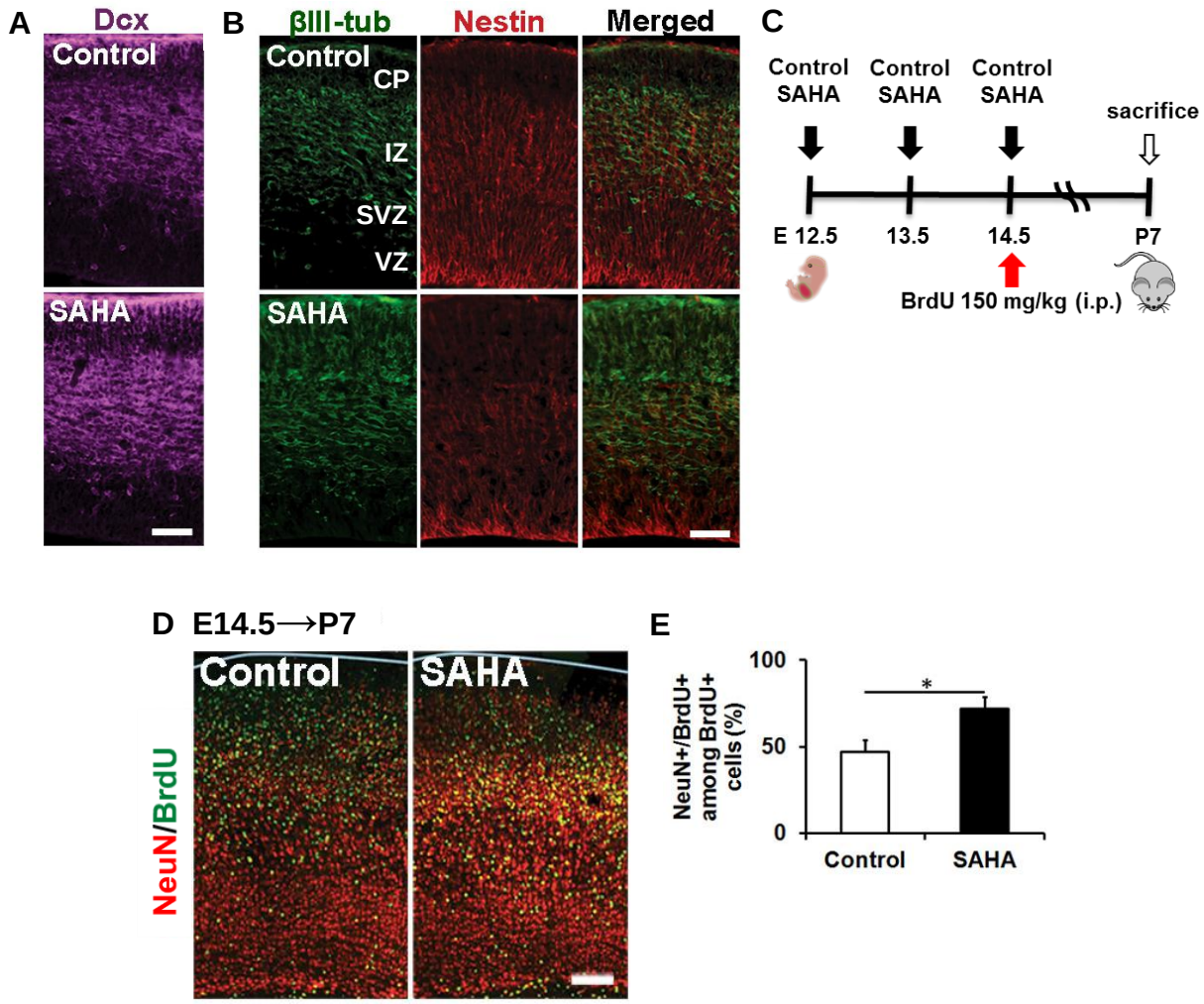


Fig. 10. SAHA promotes embryonic neurogenesis. (A, B) Immunohistochemistry showed a thicker area and denser distribution of immature neuronal markers Dcx (magenta; A) and βIII-tubulin (green; B), while a thinner area and more sparse distribution of NSCs marker Nestin (red; B) E15.5 cortex of SAHA-treated mice. (C) To track the specification of embryonic NSC, BrdU was injected into pregnant dams on E14 and analyzed at P7. (D, E) An increased proportion of E14.5-born mature neurons (BrdU+, green; NeuN+, red) on postnatal day 7, emphasized the enhancement of embryonic neurogenesis at E15.5 after SAHA treatment. Data are represent as means + SD. *$p < 0.05$, Student's *t*-test; n = 3. Scale bars: 50 μm (embryonic brains) and 100 μm (postnatal brains). SVZ: Subventricular Zone.

III.2. SAHA promotes indirect neurogenesis in the embryonic forebrain

Embryonic NSCs differentiate into neurons by two main pathways: directly by forming neurons and indirectly by forming IPCs, a neurogenic basal progenitor (Noctor et al., 2008). IPCs are highly proliferative and express Tbr2 (Englund et al., 2005). Given that SAHA enhanced embryonic neurogenesis, I investigated which pathway was used

dominantly by NSCs to generate neurons after SAHA treatment. SAHA-treated mice showed increased number of T-box brain 2 (Tbr2)+ cells and higher proportion of Tbr2+/BrdU+ among BrdU+ cells in the cortical VZ and SVZ, after 30 min of single-pulse of BrdU injection (Fig. 12A-D). Moreover, I detected a two-fold increase of *Tbr2* mRNA level when I performed qRT-PCR analysis for E14.5 SAHA-treated cortices, 3 h after the last SAHA administration (Fig. 12E).

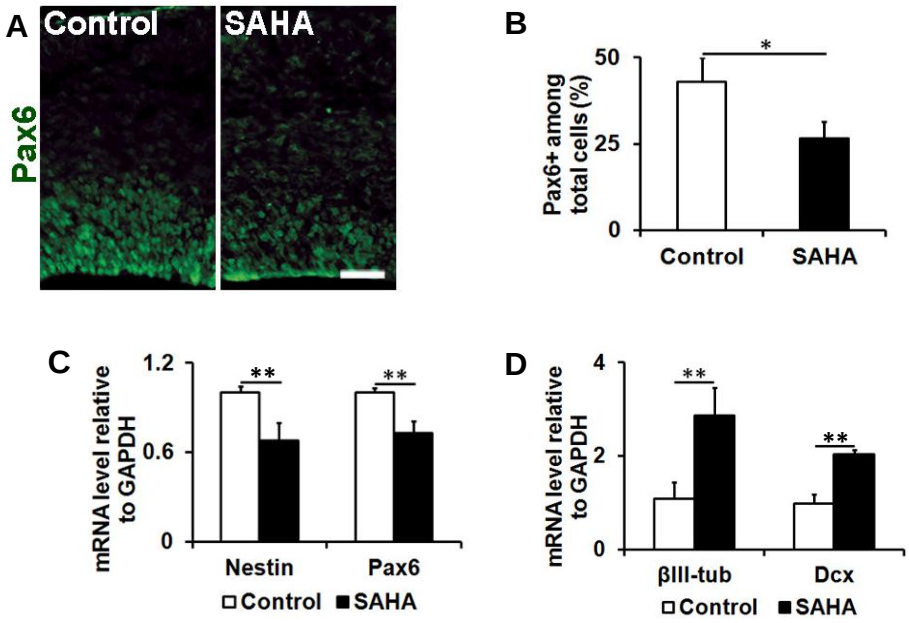


Fig. 11. SAHA treatment led to the decreased of NSC and increased of young neuron markers, respectively. (A, B) The number of NSCs marker Pax6+ cells (green) was reduced in SAHA-treated cortex. (C, D) Quantitative RT-PCR data of neuronal genes *βIII-tubulin* and *Dcx*, and NSCs genes *Nestin* and *Pax6* showed an increased expression level for neuronal genes but decreased expression level for the NSCs ones. Expression level was normalized to GAPDH expression. Data are represent as means + SD. *$p < 0.05$; ANOVA; n = 3; **$p < 0.01$; Student's *t*-test; n = 3. Scale bars: 50 μm.

As described previously in Introduction section (Fig. 4), during early to mid-stage of neocortical development, the Wnt pathway promotes the expression of Ngn1, key determinant of neuronal fate in the neocortex (Guillemot, 2006), in cortical mid-gestational NSCs. TF NGN1 promotes neurogenesis by its transcriptional activity that may be mediated by histone acetylation regarding that acetylation of histone can loose the chromatin, permit

specific regulators to access DNA and activate transcription. Interetingly, here I also found that expression of a proneural gene *Ngn1* was increased (Fig. 12E) following HDAC inhibition by SAHA thereby increasing histone acetylation. In addition, *Neurogenin* gene family is an epistatic regulator of *Tbr2* and has been shown implicated in the specification of neuronal phenotype at the expenses of glial cell fates (Bertrand et al., 2002; Ochiai et al., 2009). Taken together, these results suggest that SAHA enhances neurogenesis by promoting indirect pathway that produce proliferating IPCs.

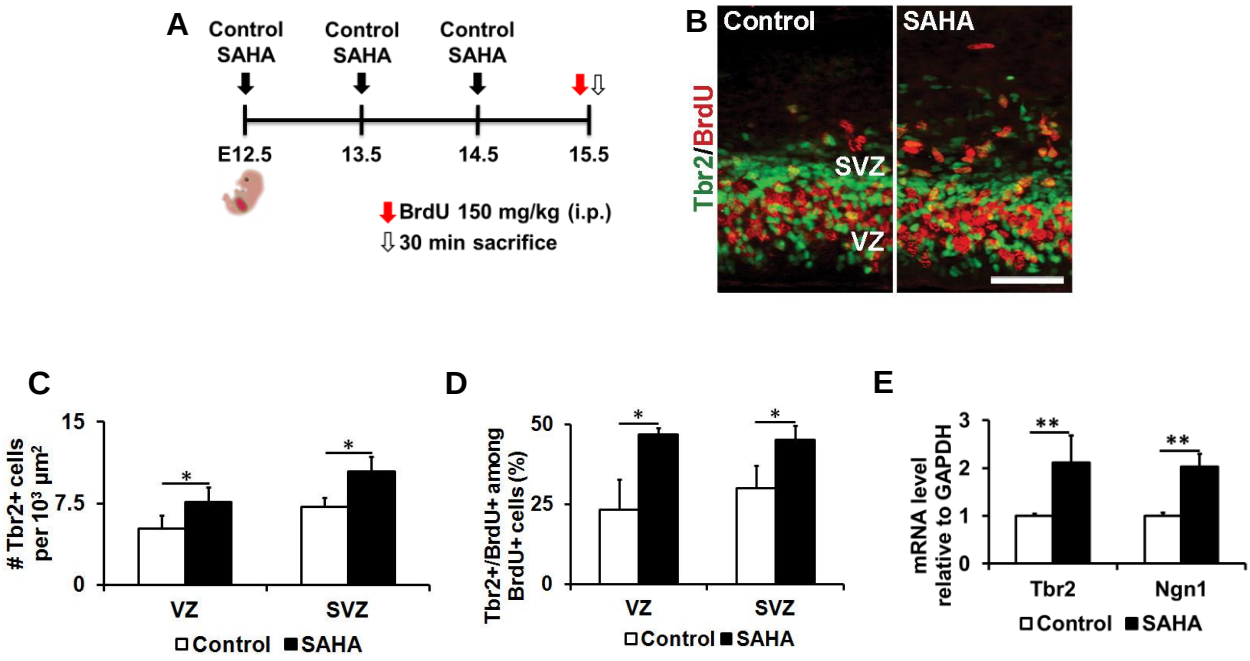


Fig. 12. SAHA enhanced neurogenesis *via* indirect pathway by increasing proliferative Tbr2+ IPCs. Immunostaining of E15.5 cortex for Tbr2 (green) and BrdU (red; B) and its quantification (C, D), following 30 min single-pulse of BrdU injection (A) showed increasing number of Tbr2+ cells (C) and higher proportion of Tbr2+BrdU+ cells among BrdU+ cells (D) in SAHA-treated mice. (E) SAHA up-regulated *Tbr2* and *Ngn1* expression level in the cortex. Data are represent as means + SD. *$p < 0.05$; ANOVA; n = 3; **$p < 0.01$; Student's *t*-test; n = 3. Scale bars: 50 µm. VZ: ventricular zone, SVZ: subventricular zone.

In order to further assess the effect of SAHA on the proliferation of NSCs, I performed phosphorylated histone H3 (pH3) immunostaining that can label mitotic cells. I found that the number of pH3+ cells in VZ and SVZ was decreased and increased,

respectively in SAHA-treated mice (Fig. 13A, B). I also observed that higher number of mitotic Tbr2+ cells (Tbr2+/pH3+) was existed in SVZ of SAHA-treated mice (Fig. 13A). To examine whether the increased number of mitotic IPCs in SVZ was due to the shortened cell cycle or increased production of IPCs, I performed BrdU and Ki67 immunostaining 30 min after single-pulse of BrdU injection (Chenn and Walsh, 2002). Consistent with an increased number of Tbr2+ and pH3+ cells in SVZ, I found a higher number of Ki67+ cells in SVZ of SAHA-treated mice (Fig. 13C, D). However, there was no significant difference in the ratio of BrdU+/Ki67+ double-labeled cells over total Ki67+ cells, indicating that NSCs cycling at a similar rate regardless of SAHA treatment (Fig. 13C, E). Therefore, I concluded that the increased number of proliferating cells in SVZ of SAHA-treated mice was mostly due to the increased production of proliferating Tbr2+ IPCs.

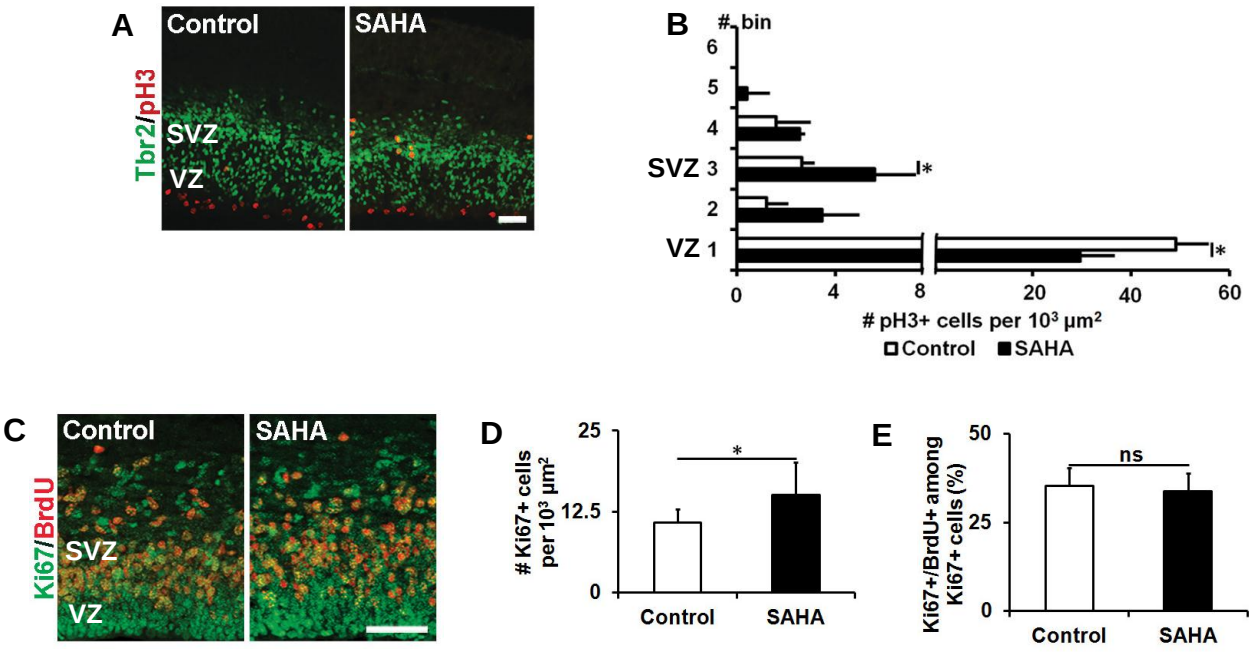


Fig. 13. Effect of SAHA on the proliferation of NSCs. (A, B) The number of mitotic cells (pH3+, red) was decreased and increased respectively in VZ and SVZ of SAHA-treated mice. Note also a higher proportion of Tbr2+ cells (green) that mitotically active (pH3+, red) in SVZ of SAHA-treated mice. (C-E) Ki67 (green) and BrdU (red) immunostaining showed an increased numbers of Ki67+ cells (D) and similar cell cycle length of NSCs (E). Data are represent as means + SD. *p < 0.05; Student's *t*-test; n = 3. Scale bars: 50 µm. VZ: ventricular zone, SVZ: subventricular zone.

III.3. SAHA induces upper-cortical layer neurons

Given that SAHA enhanced embryonic neurogenesis indirectly *via* increased production of Tbr2+ IPCs that have been suggested to predominantly generate neurons destined to comprise upper cortical layers (Arnold et al., 2008), I looked at P7 cortices to examine the types of cortical neurons produced after embryonic SAHA treatment. I found that postmitotic neurons expressing Cux1 and Satb2 in UL (L2-4) of SAHA-treated cortex were more abundant than control (Fig. 14A, B and 14E, F). Furthermore, higher number of Satb2+ neurons was also observed in DL of SAHA-treated cortex (Fig. 14E, F). These neurons were considered to be generated primarily after SAHA treatment because I found that cells labeled with BrdU at E14.5 were mostly differentiated into Cux1+ and Satb2+ cortical neurons (Fig. 14C, D and 14G, H).

Regarding DLs of the cortex, I found that the number of T-box brain 1 (Tbr1)+ subcortical projection neurons was increased in L5 and 6 of SAHA-treated cortex compared to that of control (Fig. 15A, B). Moreover, I found that protein levels of POU domain class 3 transcription factor 3 (Pou3f3; also called Brn1), Satb2, and Tbr1 were also increased in postnatal cortices after SAHA-treatment (Fig. 15G). Meanwhile, the number of Ctip2+ L5 neurons was decreased with SAHA treatment (Fig. 15C, D), and similar reduction in the fraction of E14.5-born neurons double-labeled by BrdU and Ctip2 was also observed (Fig. 15E, F). Analysis of immunohistology in E15.5 cortices displayed identical effect that SAHA increased and decreased the number of Tbr1+ and Ctip2+ neurons, respectively (Fig. 15H, I).

SAHA can induce apoptosis in cancer cells and tumor (Vrana et al., 1999), thus I addressed the possibility that neurons were being produced upon SAHA treatment but then dying and lead to the depletion of DL neurons. Here, I ruled out the possibility of apoptosis as the cause of the reduction of Ctip2+ DL neurons by SAHA given that no difference was

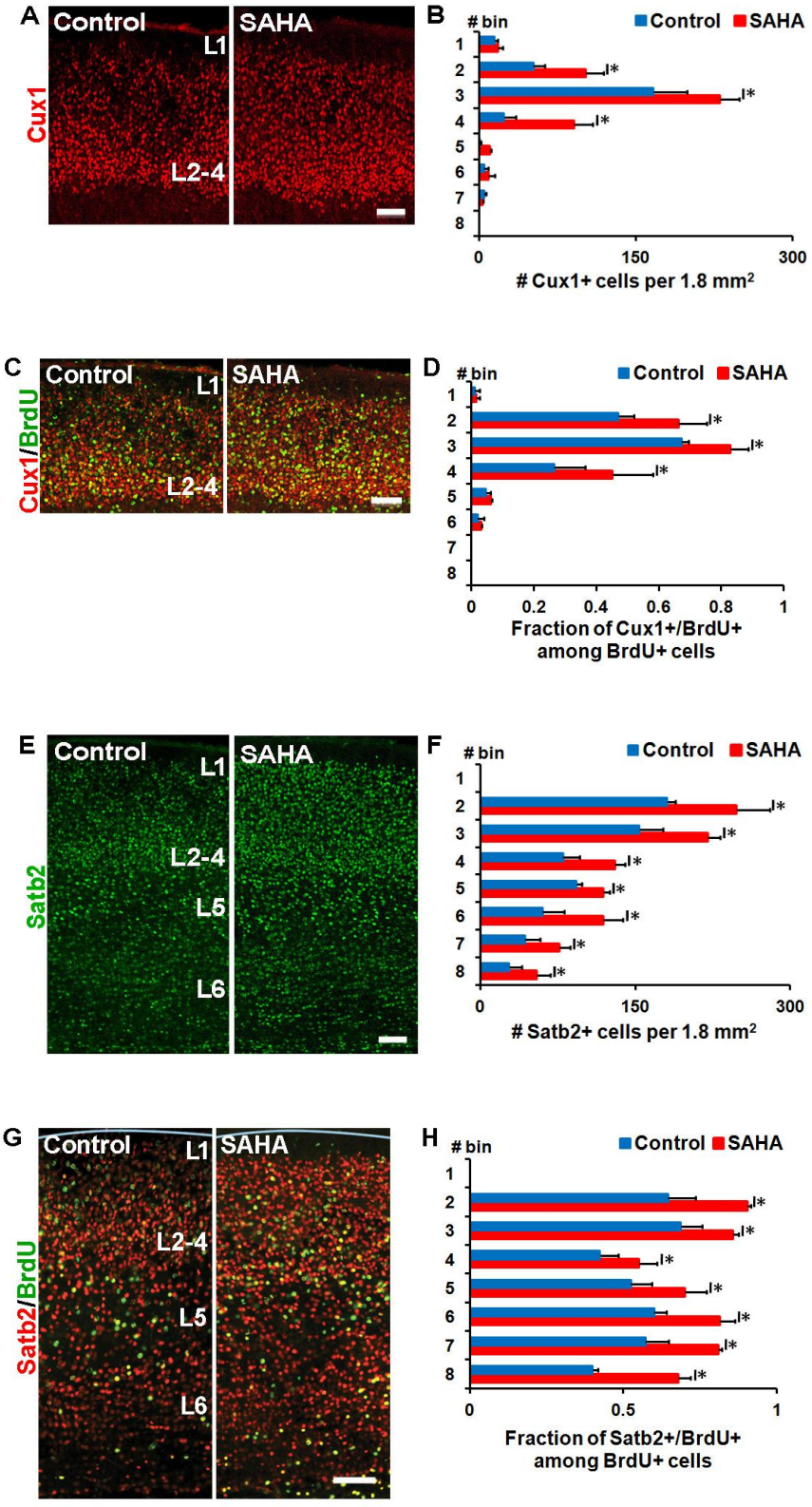


Fig. 14. SAHA treatment increased the number of UL neurons on postnatal cortex. Coronal section of neonatal cerebral cortex (P7) containing somatosensory area was immunostained for cortical layer markers as indicated (A, C, E, G). The number of postmitotic neurons expressing Cux1 (red) (A, B) and Satb2 (green) (E, F) in UL of SAHA-treated cortices were higher compared to control. Data are represent as means + SD. *$p < 0.05$; ANOVA; n = 3. Scale bars: 100 μm.

observed in the number of cells harboring cleaved caspase-3 apoptotic protein between control and SAHA-treated cortices at E15.5 and P7 (Fig. 16A, B).

To further support this phenotype, I carried out quantitative analyses of mRNA expression levels for several layer-specific markers for E14.5 cortices, 3 h after the last SAHA treatment (Fig. 17A). Three hours was chosen because histone acetylation and expression of *Ngn1* mRNA were increased at this time (Fig. 9B, 12E). SAHA administration up-regulated the expression of genes specific for UL neuronal lineage (*Satb2*), while it down-regulated those of DL neuronal lineage (*Fezf2*, *Ctip2*) (Fig. 17B-C). Collectively, these results indicate that prenatal SAHA treatment results in the perturbation of postnatal cortical lamination due to the increased and decreased production of UL and DL neurons, respectively.

I next examine whether SAHA oral administration during mid-pregnancy causes the disturbance of SP and newborn L6 corticothalamic neurons. It is described above that Sox5 is TF required for the neuronal identity (Fig. 8), migration and axonal projection of SP and newborn L6 corticothalamic neurons (Fishell and Hanashima, 2008; Kwan et al., 2008; Lai et al., 2008; Muhchyi et al., 2013). Here I observed that the expression of *Sox5* was not changed in SAHA-treated cortices (Fig. 18A) suggesting proper differentiation, normal migration and axonal projection of SP and newborn L6 neurons. This is may be due to Sox5 is absent from VZ and SVZ progenitors and have been expressed in post-mitotic SP, L6 neurons and in a subset of L5 projection neurons during mid-gestational SAHA administration (at E12.5) by which the expression of Sox5 is started from E14.5 to the first postnatal week (Kwan et al., 2008; 2012). In addition, I found normal Reelin (marking the early born neurons in SP neurons and in the MZ (L1)) staining in SAHA-treated cortices (Fig. 18B).

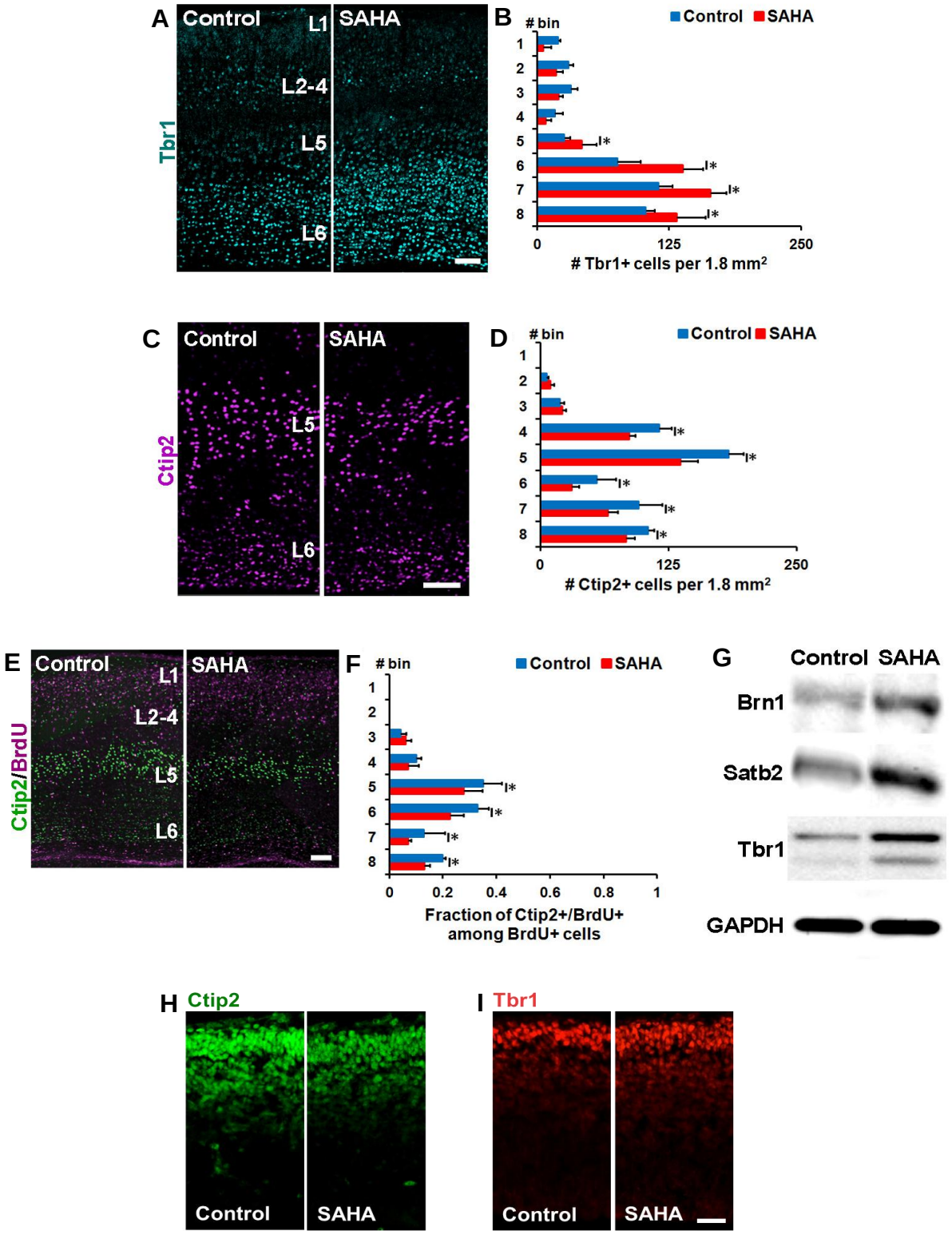


Fig. 15. (A, B) The number of Tbr1+ neurons (blue) in deep layer (DL) of SAHA cortex was increased, however, Ctip2+ neurons (magenta) was decreased (C, D). SAHA treatment led to the decreased proportion of BrdU+Ctip2+ cells (E, F) in P7 cortices after single-pulse of BrdU injection on E14.5 (Fig. 10C). (G) Western blot of several cortical layer neuronal markers. (H, I) Immunostaining of E15.5 brains verified the decreased of Ctip2+ neurons (green) (H) and increased Tbr1+ neurons (red) (I) in SAHA-treated cortices. Data are represent as means + SD. *$p < 0.05$; ANOVA; n = 3. Scale bars: 100 and 50 μm (neonatal and embryonic brains).

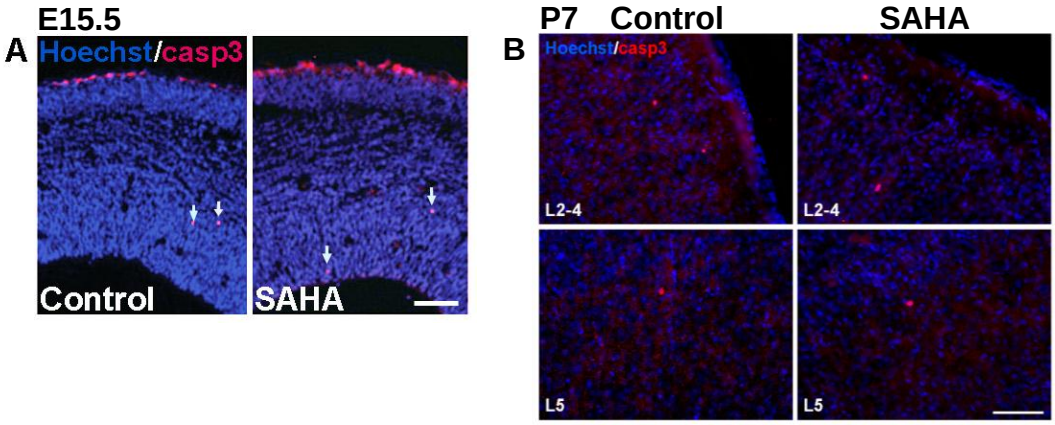


Fig. 16. Activated caspase3 staining of the E15.5 (A) and UL, DL P7 (B) cortices demonstrated apoptosis was unaffected by SAHA treatment. The death cells were pointed out with white arrows. Scale bars: 50 μm (embryonic brains) and 100 μm (postnatal brains).

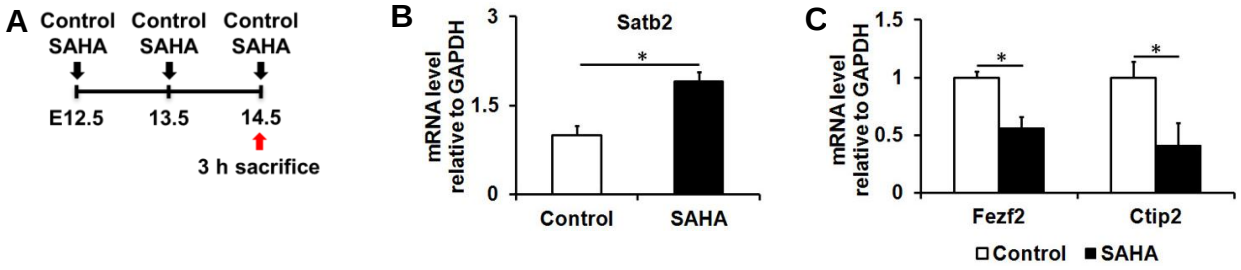


Fig. 17. Gene expression analyses of layer-specific transcription factors in response to SAHA. E14.5 cortices were isolated 3 h after SAHA or Control treatment to pregnant mice (A). (B, C) Using Satb2, Fezf2, and Ctip2 primers, the quantification of RNA were performed. SAHA up regulated expression of gene specific for UL neuronal lineage (*Satb2*) (B), while it down regulated genes for DL neuronal lineage (*Fezf2*, *Ctip2*) (C). GAPDH was used to normalize each value. Values are averages ± SD (*$p < 0.05$; Student's t-test; n = 3).

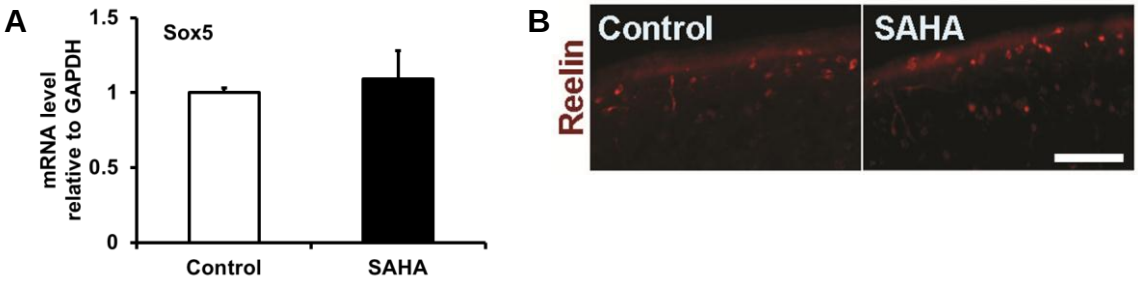


Fig. 18. Oral administration of SAHA during mid-pregnancy did not change the SP and newborn L6 corticothalamic neuron identity. The expression of *Sox5* (A; n = 3), key determinant of SP and L6 neuron specification in DL cortex, and reelin (B; red), marker for these neurons, were similar between SAHA and Control-treated embryonic cortices.

III.4. SAHA promotes indirect neurogenesis *in vitro*

In order to elucidate the molecular biological and biochemical analysis of SAHA-induced change in differentiation of NSCs during corticogenesis, an *in vitro*-based approach was at first selected. To generate a uniform population of NSCs directly from embryonic brain, I turned to establish an adherent culture as a model of cortical layer neurogenesis. I performed *in vitro* differentiation upon NSC derived from embryonic cortex. Monolayer cultures were prepared from the E11.5 forebrain. Owing to the differentiation of NSCs start around E9.0 with neurogenesis followed by gliogenesis (Ramasamy et al., 2013), the E11.5 cortex harbors heterogenous cells. However, Qian et al. (2000) described that at E11.5, cortical cells are mostly consist of NSCs which are Nestin+ (96%), and only 14% are βIII-tubulin+ neuron indicating the high purity of NSCs at E11.5 mouse cortical cells. Later on, peak of neurogenesis occur from E12.5 to E14.5, while the first glial cells were seen at E17 accompanied with the decline of NSC numbers after E14.5 (Qian et al., 2000) and very few remaining of NSC around birth (8%) (Qian et al., 2000; Ramasamy et al., 2013).

The E11.5 NSCs used here are likely to fulfill the definition of multipotent NSCs: they self-renew and can differentiate into neuron, astrocytes, and oligodendrocytes (Qian et al., 2000). To maintain NSCs in culture, FGF is a commonly used factor either alone or together with EGF. Early gestational (E8.0-13.0) NSCs are initially responsive only to FGF, and this signal then prime NSCs to become responsive to EGF later during development (E13.0-18.0) or culture *in vitro* (Tropepe et al., 1999; Lillien and Raphael, 2000; Ciccolini, 2001; Ramasamy et al., 2013). In the absence of FGF, the cells divided very little and differentiated into neurons arose after 3 days in vitro (div) cultures (Qian et al., 2000).

In my study, cells were plated directly in differentiated medium (in the omission of FGF) after dissociation from E11.5 cortex and cultured for 4 d (Fig. 19A). SAHA or control (DMSO) was treated to the cells 3 h after plating to let the cells attached on dish. This 3 h

incubation is needed because cell growth in culture generally undergoes a decline after plating. The decrease in viable cells after plating is due to "anchorage-dependence"-cells must attach to the bottom of the culture dish (Mather and Roberts, 1998). Here I found that in the presence of SAHA, both RNA level of *βIII-tubulin* and *Dcx* were upregulated, but *Tbr2* expression level was tend to downregulate (Fig. 19B). In addition, immunostaining of SAHA-treated cells showed higher proportion of βIII-tubulin+ neurons compared to DMSO-treated one (Fig. 19C, D). To examine the neuron subtypes generated from these E11.5 cortical stem cells, cells were stained for Tbr1 and Ctip, early pyramidal neuron markers that preferentially labels projection neurons (Hevner et al., 2001). Among these neurons are Tbr1+ and Ctip2+ cells and SAHA treatment increased the number of these cells (Fig. 19E, F). Taken together, these results suggest that the E11.5 NSC cultured can generate early projection neurons that resided in DL cortex and SAHA increased this DL neurogenesis.

This embryonic cortical-derived NSCs model came in line with Shen et al (2004) work. They also can generate Tbr1+ projection neurons from E10-11 cortical stem cells expanded in endothelial coculture secreting soluble factors that stimulate the self-renewal of NSCs, inhibit their differentiation, and enhance their neuron production. Interestingly, upon endothelial cell removal, they found few Tbr1+ neurons produced from E15.5 cortical stem cells and none from adult SVZ cells, indicating that the neurogenic potential of the cortical stem cells declines so that it generates fewer neurons with age (Qian et al., 2000; Shen et al., 2004) and endothelial cells as a critical component of the NSC niche (Shen et al., 2004).

Because *Tbr2* expression was tend to downregulate in SAHA-treated culture after 4 div probably due to most progenitors have differentiated into neurons, I then perform 2 div culture to model SAHA-promoted indirect neurogenesis that involve production of proliferating Tbr2+IPCs. To study the proliferation of NSCs, BrdU was added 30 min before cell fixation (Fig. 20A).

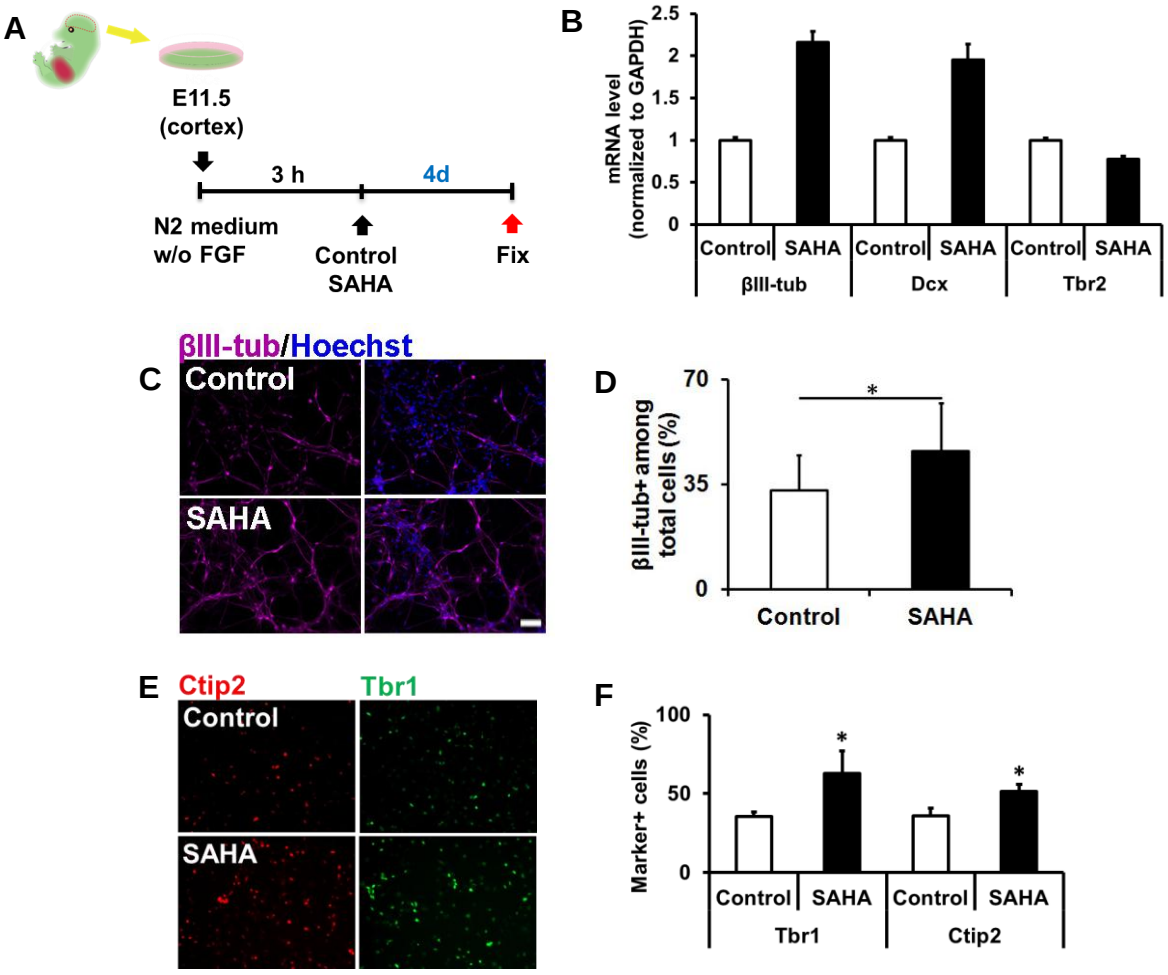


Fig 19. SAHA enhanced neurogenesis in primary culture of NSCs from E11.5 embryonic forebrain (A) as shown by an increase proportion of cells expressing immature neuronal marker βIII-tubulin (B-D). (E) The E11.5 NSC cultured can generate early projection neurons that resided in DL cortex and SAHA increased this DL neurogenesis. Immunostaining of E11.5 mouse embryonic cortex NSCs cultures for Ctip2 (red) and Tbr1 (green; E) and its quantification (F) displayed the increased production Ctip2 and Tbr1+ cells following SAHA application *in vitro*. Data are represent as means + SD. *p < 0.05; Student's *t*-test; n = 3. Scale bars: 50 μm.

I observed that *Tbr2* is increased by SAHA treatment in 2 div culture (Fig. 20B). This E11.5 cortical NSCs cultures produced higher proportion of Tbr2+ among Hoechst+ cells and Tbr2+BrdU+ among BrdU+ cells in response to SAHA treatment (Fig. 20C-E), consistent with *in vivo* result that SAHA enhances neurogenesis by promoting indirect pathway that produce proliferating IPCs. The NSC expression markers such as *Nestin* and *Pax6* were reduced and unchanged, respectively following SAHA application (Fig. 20F).

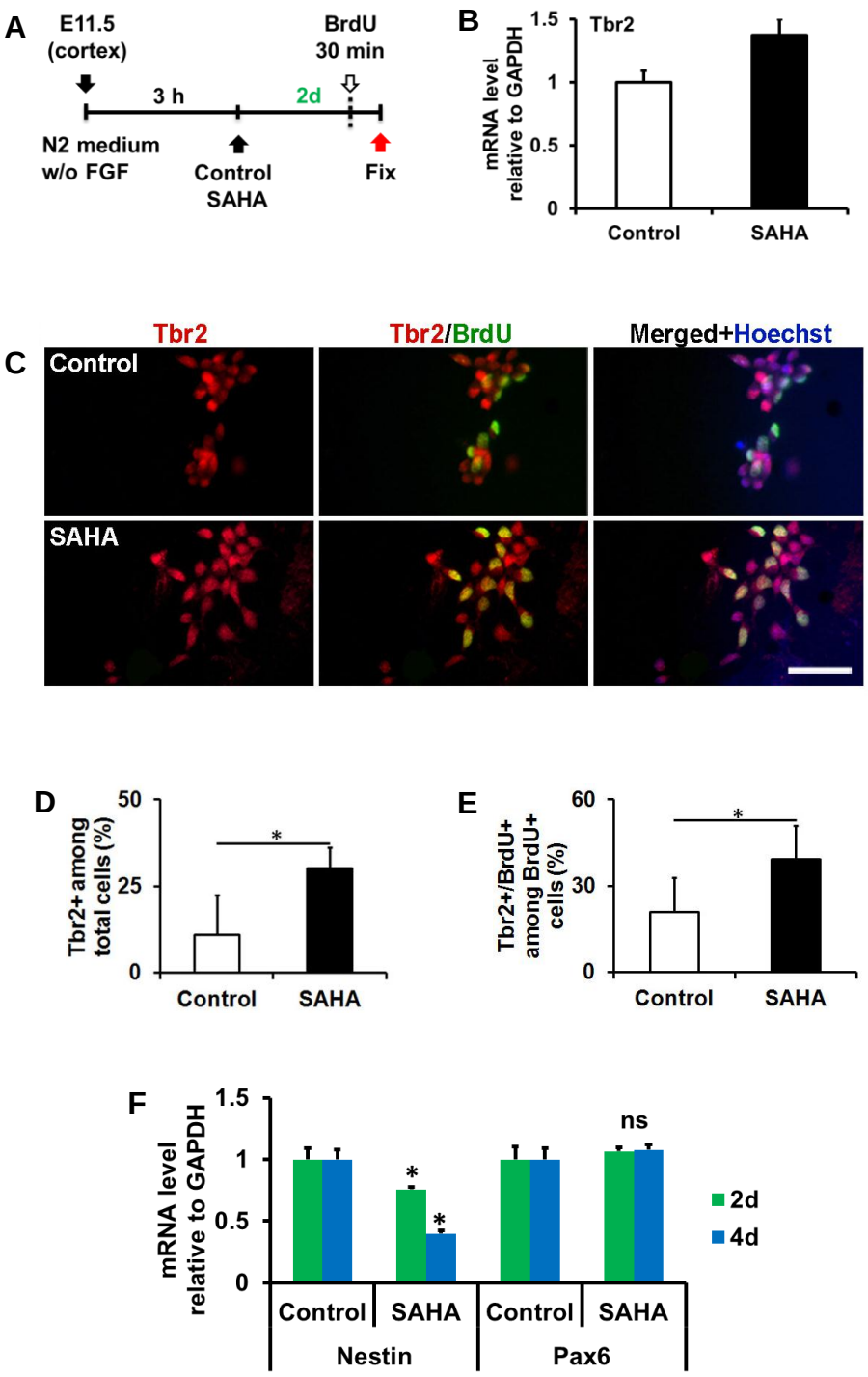


Fig 20. SAHA enhanced neurogenesis by promoting indirect pathway that produce proliferating Tbr2+ IPCs in primary culture of NSCs from E11.5 embryonic forebrain (A) as shown by upregulation of *Tbr2* expression (B; n = 3) and increased production (D) and proliferation (E) of Tbr2+ IPC by SAHA treatment *in vitro*. Immunostaining of E11.5 mouse embryonic cortex NSCs cultures for Tbr2 (red) and BrdU (green; C). In the presence of SAHA, NSC expression markers such as *Nestin* and *Pax6* were reduced and unchanged, respectively (F; n = 2). Data are represent as means + SD. *p < 0.05; Student's *t*-test; n = 3. Scale bars: 50 μm.

During the first 2 days of differentiation *Nestin* expression was decreased in SAHA-treated cells (Fig. 20F). This expression was reduced over the 4 div cultures in the SAHA cells reflecting the production of differentiated neurons.

Analysis of gene expression cortical layer TFs to the culture in response to HDAC inhibition by SAHA demonstrated that genes for DL neuronal lineage (*Fezf2*, *Ctip2*) were upregulated (Fig. 21). Meanwhile, protein markers for UL neurons did not express both in the control and SAHA cultured. These results implied that SAHA increased DL neurogenesis, but the progenitors in this culture system did not have potency to generate UL neurons, only produce DL neurons. Qian et al. (2000) suggested that E10 cortical NSCs when cultured in medium for 14 days of culture with 10 ng/ml FGF as mitogen have the capasity to reconstitute the normal timing program of neural cell production, neurons before glia. At the day 3 time point (equivalent to E13), 85% βIII-tubulin+ neurons were detected. Even by 6-7 div (equivalent to E16-17), the majority of cells in the culture contained βIII-tubulin+ neurons, only few gfap+ astrocyte (4.7%), but no O4+ oligodendrocyte. By 10 days in culture (equivalent to P2), the cells still contained neurons, predominantly gfap+ astrocyte (90%) and O4+ oligodendrocytes were begun to emerge. I then hypothesized that by culturing the E11.5 cortical NSCs first in proliferating medium (with FGF) before performing neuronal differentiation (by mitogen withdrawal) will change the potency of these cells and result in normal temporal order of cortical layer neuron production, DL first followed by UL afterwards. I designed other condition for cultures to induce UL neuron differentiation. Because the neurons in the UL are derived from late gestational NSCs (E14-E17) (Tarabykin et al., 2001; Shen et al., 2006) during corticogenesis, E11.5 NSCs were cultured in the proliferating medium for 2 d (equivalent to E13.5, starting point of UL neuron generation) and medium was changed into differentiation medium (without FGF) and the cells were

cultured for 4 d. DMSO or 30 nM SAHA was added to individual cultures on the first day of differentiation period (Fig. 22A).

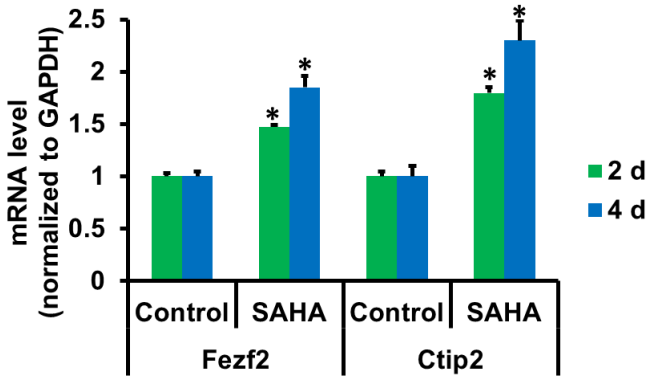


Fig. 21. Gene expression analyses of DL neuronal lineage in response to SAHA from E11.5 cortical NSC primary culture. *Fezf2* and *Ctip2* were upregulated in SAHA-treated cells (n = 3).

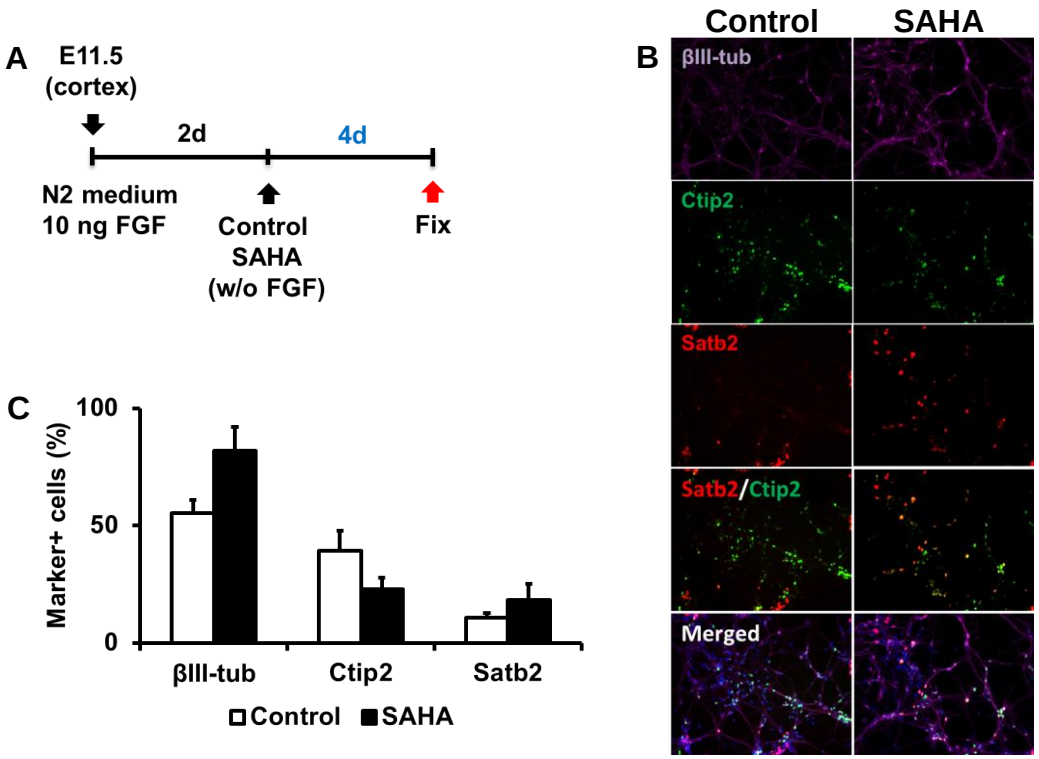


Fig. 22. Both DL and UL neuron marker expression can be detected after 6 div of E11.5 NSC primary culture (A). SAHA increased βIII-tubulin+ neurons (magenta) (B, C). The number of Ctip2+ (green) deep- and Satb2+ (red) upper-layer neurons were decreased and increased, respectively by SAHA application (B, C; n = 2).

In this culture model, both DL and UL neuron marker expression can be detected (Fig. 22B). I observed again the increased of neuronal protein βIII-tubulin expression in SAHA-

treated culture (Fig. 22B, C) and among these neurons, the number of Ctip2+ deep- and Satb2+ upper-layer neurons were decreased and increased, respectively (Fig. 22B, C). However, to establish this method, I further need to confirm the result with other cortical layer specific markers such as Reelin (L1), Cux1 (L2-4), and Tbr1 (Subplate) and to evaluate the genes expression of those markers.

III.5. Effect of SAHA on developmental Notch signaling

At the start of neurogenesis, Notch signaling controls NSC and its progenitor self-renewal *via* induction of *Hes* genes (Kageyama and Ohtsuka, 1999). *Hes1*/*5* negatively regulates transcription of neurogenesis-promoting genes *Ngns1* and *2*, thereby affecting maintenance of NSC and its progenitor and inhibiting neurogenesis (Ochiai et al. 2009; Fig. 6A). It is reported that Notch ligands are expressed in NSC and its committed neuronal progenitor and their derived young neurons, and these ligands activate Nocth signaling in the residual NSCs (Namihira et al., 2009; Juliandi et al., 2010b). Regarding that SAHA increased number of neurogenic basal progenitor (IPCs) and young neuron, I hypothesis that SAHA might effect on the Notch signaling. First, I examine the expression of Notch ligand (NICD) employing cleaved-Notch antibody in E15.5 cortices. Antigenically recognizable NICD were increased by SAHA treatment (Fig. 23A) indicating the increased of Notch ligand expression. This probably because the increased of IPC as the neurogenic progenitor of NSC since it has been reported that NSC and its committed neuronal progenitor expressed Notch ligand (Campos et al., 2001; Kawaguchi et al., 2008; Yoon et al., 2008; Namihira et al., 2009). This idea, however, needs investigated further by double immunohistochemistry of cleaved-Notch and Tbr2. Notch ligands expressed in NSC and its neurogenic progenitors subsequently may activate Notch signaling in the residual NSC. Thus the increased of IPC should be accompanied with increased of Notch signaling activation in midgestational NSCs of SAHA-exposed cortices. Nonetheless, here I found that the expression of Notch signaling pathway

downstream molecules such as *Hes5*, *NFIA*, and *NFIB* were downregulated after SAHA administration (Fig. 23B). The downregulation of *Hes5* expression is likely due to the increased of Brn1 in SAHA-treated postnatal cortices (Fig. 15G). Brn1 is one of Pou3f factors those are proneural genes capable of biasing progenitor cells toward a neural fate at the expense of progenitor maintenance. It has been reported that Pou3f factors such as Brn1/2 facilitated neuronal differentiation by diminishing Notch-directed transcription of Hes5 (Dominguez et al., 2012). Brn1/2 expressed in NSC and its progenitors starting at E14.5 and necessary for migration of L5 cells, and for the production of L2-4 cells (Sugitani et al. 2002); however, the mechanisms carrying out the latter process have remained elusive. In SAHA-exposed embryonic brains, the increased of Brn1 expression might repress Hes5 and drive the differentiation of NSC toward neuronal lineage. Therefore, to reinforce this issue it would be interesting to examine the expression of Brn1 in SAHA-exposed embryonic cortices.

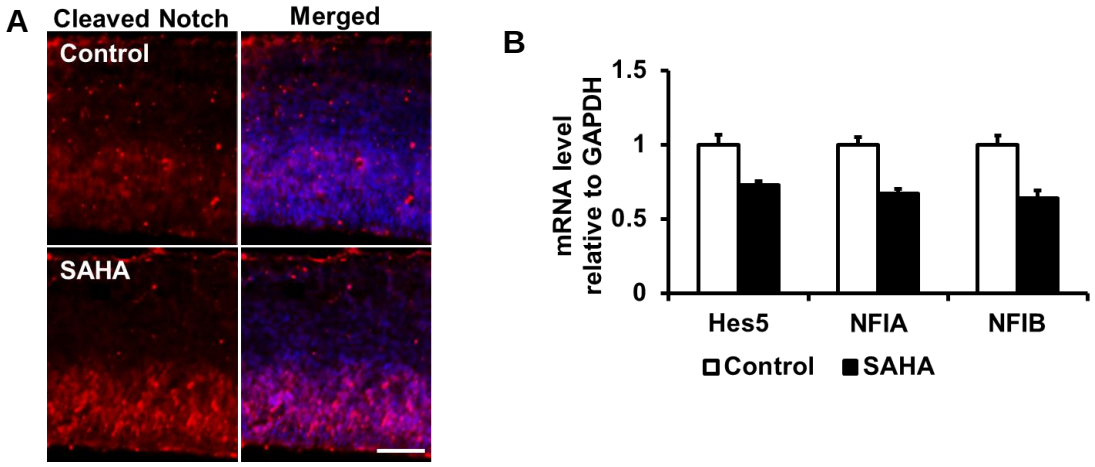


Fig. 23. Antigenically recognizable Notch ligand NICD was increased by SAHA treatment (red) (A) probably owing to the increased of IPC number in embryonic cortex. The expression of Notch signaling pathway downstream molecules including *Hes5*, *NFIA*, and *NFIB* were reduced by SAHA administration (B; n = 3).

In term of reduction of *NFIA* and *NFIB* expression, it seems that the potency to astrocytic differentiation in mid-gestational NSCs following SAHA exposure is reduced regarding that NFIA and NFIB are downstream of the Notch signaling pathway to potentiate astrocytic differentiation of mid-gestational NSCs by accelerating demethylation of astrocytic

gene promoters. NFIA and NFIB prevent DNMT1 from binding to astrocytic gene promoters (Fig. 3b) and thus allowed precocious astrocytic differentiation (Namihira et al., 2009; Juliandi et al., 2010b).

III.6. SAHA reduces cortical astrocytogenesis

It is suggested that the activation of Notch in mid-gestational committed neuronal progenitor induced expression of NFIA, which bind to astrocytic gene promoters and act as downstream of the Notch signaling pathway to potentiate astrocytic differentiation of mid-gestational NSCs (Namihira et al., 2009; Juliandi et al., 2010b). Thus, the effect of SAHA on Notch signaling during mid-gestation (Fig. 23B), perhaps it also effect on the potency of NSC to differentiate into astrocyte. Additionally, considering the fact that NSCs lining the embryonic neural tube give rise to the entire repertoire of neurons, astrocytes and oligodendrocytes of the adult CNS, the decreased of NSCs in embryonic SAHA-treated brains (Fig. 11A-C) would influence the subsequent generation of glial cells, astrocytes and oligodendrocytes that generally follows neurogenesis in the developing mammalian brain. To determine the effect of *in utero* HDAC inhibition by SAHA on gliogenesis particularly astrocytogenesis, I quantify astrocyte markers, gfap and S100β, expression of postnatal SAHA cortices using western blot and RT-PCR, respectively. Immuno blotting analysis and relative band density of gfap protein in SAHA-treated neocortex is reduced until 0.4 and 0.3 compared to the Control one (Fig. 24A, B). mRNA level of *S100β* astrocytic gene confirmed this phenotype (Fig. 24C). Collectively, this result indicates that SAHA repress the production of cortical astrocyte. I assume that this phenotype is the consequence of the reduction of NSC number (Fig. 11A-C) thus lead to the reduced number of astrocyte progenitor in SAHA-treated cortices. Other possibility related to Notch signaling is the reduction of *NFIA* expression by SAHA administration (Fig. 23B) may cause decreased the potency of astrocytic differentiation of mid-gestational NSC as explained earlier.

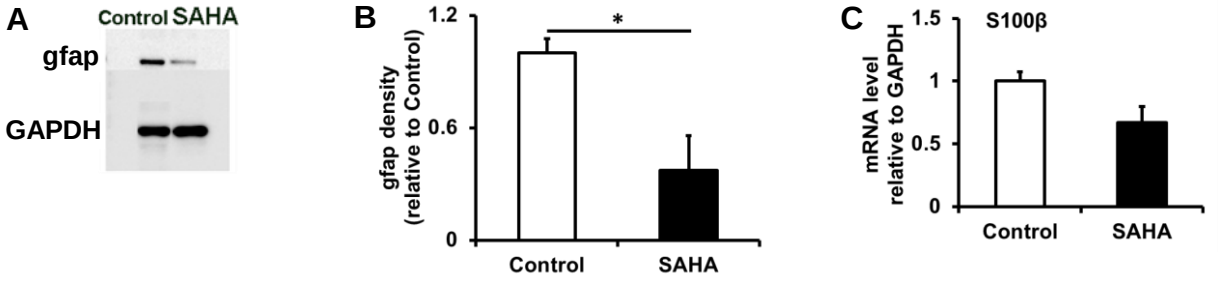


Fig. 24. Mid gestational HDAC inhibition by SAHA led to the suppression of astrocyte differentiation in P7 cortices. Western blot (A) and its quantification (B) showed lower expression of gfap protein in SAHA-treated neonatal cortices (n = 3). Data represent as means + SD. *p < 0.05; Student's *t*-test; n = 3. RT-PCR of astrocytic gene *S100β* supported this result (C) (n = 2).

III.7. Effect of prenatal SAHA administration on adult hippocampal neurogenesis and related behavior

Epidemiological and experimental studies have shown that alterations in the intrauterine occurring during critical periods of development may have adverse consequences in later life (Fowden and Forhead, 2004). In rodent, during the perinatal of life, take place during the gestational period up into the first two postnatal weeks until weaning, is a period of unique sensitivity during which experience can confer enduring effects on brain structure and function, thus it represents a critical period for an individual. In this period, the rodent brain is plastic and sensitive enough to environmental changes to be subject to permanent alteration (Korosi et al., 2012). Prenatal stress due to drug treatment, alcohol exposure or prenatal repeated restraint lead to lasting reductions of adult neurogenesis in hippocampus, certain regions of the adult brain retain neurogenic potential, consistently associated with cognitive and emotional deficits, indicating that perinatal life is a critical period during which the environment and experience program brain and behavior (Mandyam et al., 2008; Bosch et al., 2006; Lemaire et al., 2000).

The hippocampus, part of the limbic system, has a major role in cognition and mood regulation (Kessels et al., 2001). Multipotent NSC have been identified in both embryonic

and adult CNS. The mammalian hippocampus, in particular the dentate gyrus (DG), is one of the few sites in the brain where NSC generate new neurons continues up to adulthood and this process is termed adult hippocampal neurogenesis. Adult neurogenesis is a multi-step process starting with proliferation of NSCs, followed by the selection of a subset of these cells through apoptotic cell death, migration and subsequent differentiation into fully functional neurons, which incorporate in the pre-exisiting hippocampal network (Kempermann et al., 2004). Adult neurogenesis is a form of brain plasticity that has an important role in circuit development, cognition and emotional regulation. Its deregulation has been suggested to be involved in cognitive impairments, mood disorders and addiction. However, none of molecules served as the mediators of neurogenesis as well as epigenetic mechanism considered so far can by themselves fully account for the long lasting reduction of adult neurogenesis observed in rodents that experienced adverse early-life experiences (Korosi et al., 2012).

Recent *in vitro* studies by Bose and colleagues (2010) exhibited that rat embryonic cortical NSC exposed to corticosteroid drug such as dexamethasone (Dex) at critical periods of development results in decrease NSC number without altered in cell differentiation, and deficit in mitochondrial functions accompanied with increased occurrence of apoptotic cell death. Dex reduced the proliferation of NSC by up-regulated cell-cycle regulating genes *p16* and *p21*, which represent potent inhibitors of cyclin-dependent kinases and D-type cyclins in various cell types, thereby leading to cell cycle arrest. All these features are retained in daughter NSCs (never directly exposed to Dex) and are associated with a higher susceptibility to oxidative stress support the idea that fetal exposure to drug is likely to result in long-term consequences that may predispose to neurodevelopmental disorders (Bose et al., 2010).

Having found that *in utero* SAHA administration reduced the number of embryonic cortical NSC (Fig. 11 A,B,D) and considering that fetal exposure to drug is likely to result in long-term consequences including lasting reductions of adult neurogenesis in hippocampus that may predispose to disorder of brain function that affects emotion, learning ability and memory and that unfolds as the individual grows, therefore, it would be quite interesting to know whether this reduction and the change of epigenetic that is histone acetylation state mediated by prenatal SAHA treatment could cause long-term consequences after the mice reach adult, in particular on proliferation and differentiation of NSC in hippocampus. I will also investigate the functional deficit of it related to cognition by conducted several hippocampal related behavior including learning and memory test.

To examine the effect of prenatal SAHA treatment on proliferation and differentiation of adult hippocampal DG NSCs, SAHA or methylcellulose as Control was administered orally to pregnant mice on E12.5-14.5. After the embryos become adult (3 months old), BrdU was injected for 5d to label proliferating NSCs in DG of SAHA-treated and Control mice. Mice were then sacrificed 1 d and 4 week after the last BrdU injection to analyze the proliferation and differentiation of NSC, respectively (Fig. 26A, B). Here, I found that the number of residual NSCs was still lower in adult DG of SAHA-treated mice as showed by immunostaining and quantification of Sox2+ cells as NSC marker (Fig. 25A, B).

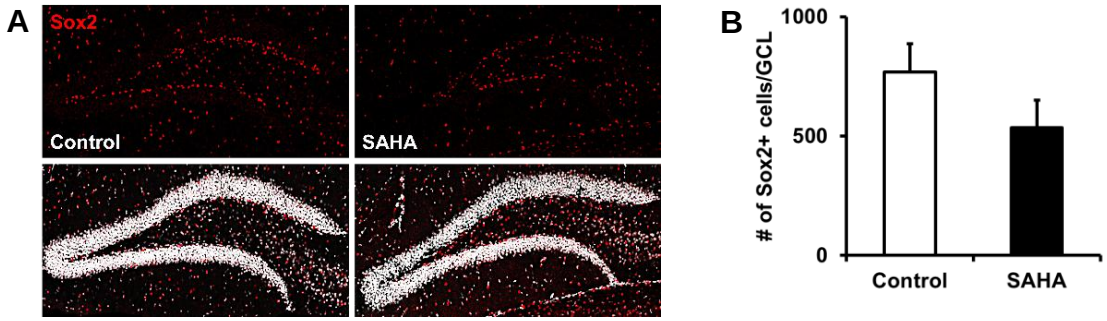
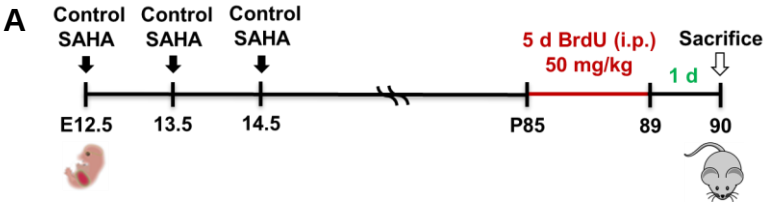


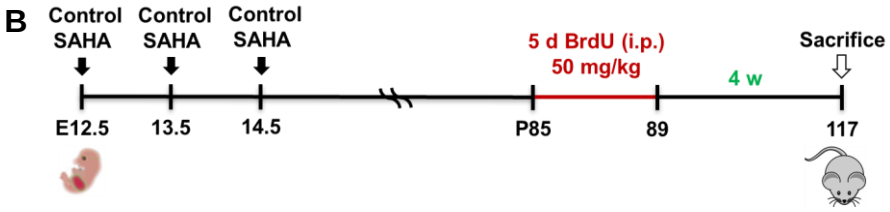
Fig. 25. The decreased number of residual NSCs persisted in the adult DG of SAHA-treated mice following mid-gestational SAHA treatment as displayed by immunohistochemical image (A) and quantification of Sox2+ NSC (B; n = 2).

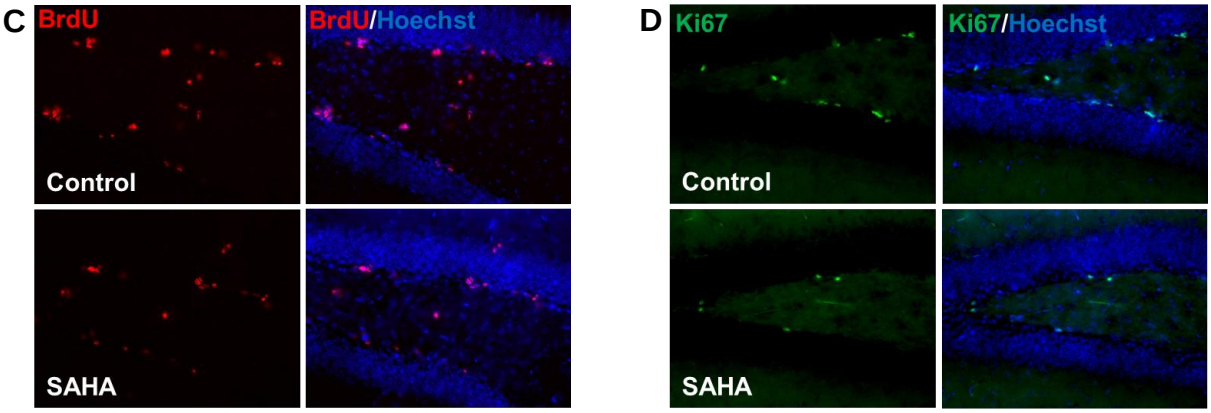
The decreased number of NSC could be caused by decreased proliferation rate or increased in the number of differentiated cells. In order to clarify this, I quantify the number of proliferating cells that are BrdU+ in 1 d and 4 w after the last BrdU injection cortices. The number of BrdU+ cells in SAHA-treated mice on both time points was slightly lower than Control (Fig. 26C, E, F) indicating lower number of NSCs in SAHA-exposed mice relative to Control ones. To confirm these results, I also checked the number of proliferating cells by using immunostaining of Ki67. As observed in experiment 1d after the last BrdU injection, the number of proliferating Ki67+ cells was also lower in SAHA-treated mice (Fig. 26D, E). Next, I investigated the fate of the labeled BrdU+ cells 4 weeks after the last BrdU injection (Fig. 26B). The percentage of BrdU+ cells that differentiated into NeuN+ neurons and S100β+ astrocytes are similar between Control- and SAHA-treated mice (Fig. 26G, H). These results suggest that SAHA decreased NSC proliferation thereby reduced NSC number. The absence of a reduction in adult neurogenesis in animals with a history of prenatal SAHA administration could indicate that SAHA did not have lasting consequences on adult hippocampal neurogenesis in mice and perhaps there is an adaptive mechanism preventing this structural plasticity to be further diminished.

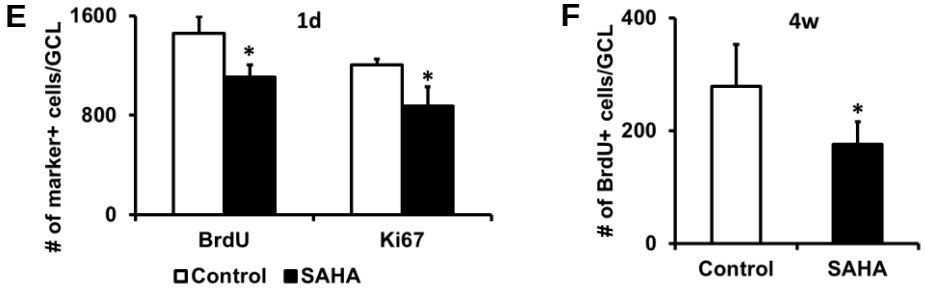
In order to investigate whether the changes in adult hippocampus (Fig. 25, 26C-F) caused by embryonic treatment of SAHA could cause alteration in hippocampal function related to cognitive function such as learning ability and memory, in collaborations with National Institutes of Health Sciences (Tokyo) we conducted several behavioral tests on 12 weeks old male mice (Fig. 27). We focused on hippocampal related behavior that is learning and memory test because it has been reported that adult NSCs in the DG of hippocampus, which probably come from embryonic NSCs, have a functional role in learning and memory processes by undergo neurogenesis to generate adult born neural cells which later integrate into the existing neural circuit (Zhao et al., 2008) indicating the responsibility of

hippocampus for learning and memory test.









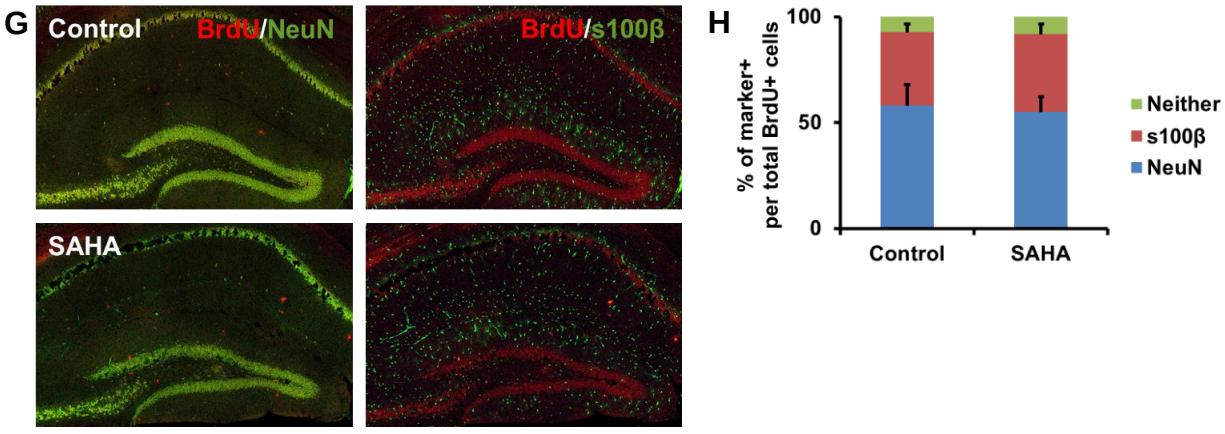


Fig. 26. SAHA-treated mice have fewer proliferating cells in adult DG. (A, B) SAHA or Control was orally administered to pregnant mice from E12.5 until E14.5. BrdU was injected once a day for 5 days starting at P85. Mice were sacrificed one day (1d, A) and 4 weeks (4w, B) after the last BrdU injection. (C-D) Immunostaining of 1d and quantification of BrdU+ and Ki67+ cells (E) at adult DG show that SAHA-treated mice have fewer proliferating cells. (F) SAHA-tretaed mice showed similar reduction of BrdU+ cell number after 4w last BrdU injection. Immunohistochemical image (G) and quantification of newly generated NeuN+ neurons and S100β+ astrocytes (H) display no difference differentiation of NSCs at DG of SAHA-treated mice, 4w after the last 5d BrdU injection. *$P < 0.05$, Student t-test. Data represent as means + SD. *p < 0.05; Student's *t*-test; n = 3.

Here we found that there is no significant difference between Control and SAHA-treated mice in behavior test related to hippocampal neurogenesis listed in Table 1 including the tests that assessed learning and memory behavior such as contextual/cued fear conditioning, Y-maze alternation tests, and etc.

Table 1. List of tests used in this study to assess behavioral alterations on 12 weeks of age, after embryonic administration of SAHA on E12.5-14.5

Test name	Behavior assessed	Results
Open field	general activity level, gross locomotor activity, exploration habits	total distance, center time, move episode, distance per movement
Light/dark transition	anxiety-like	dark distance, dark time, transition, light distance, light time, latency to enter light
Elevated pluz maze	anxiety-like	total distance, open time, close time, open entry, close entry, total entry
Per-pulse inhibition	sensorimotor gating	pre 90db/120db, pre 95db/120db
Tail suspension	depression	immobility time
Contextual/cued fear conditioning	learning and memory	conditioning, contextual, cued
Y-maze alternation	learning and memory	correct alternation

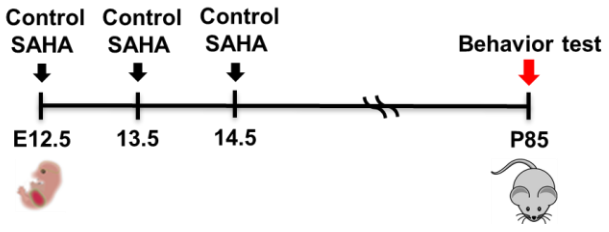


Fig. 27. Schematic animal treatment to conduct behavior test. SAHA or Control was orally administered to pregnant mice from E12.5 until E14.5. Behavior study was performed after the mice were on 12 weeks of age.

III.8. SAHA increased cortical interneuron generation

High-level cortical function including cognition, sensory perception, and motor function relies on the coordinated assembly of local microcircuitry between pyramidal projection neurons which are glutamatergic and excitatory, and interneurons which are GABAergic and inhibitory (Molyneaux et al., 2007). Across many cortical regions and species, approximately one in five neurons in the adult neocortex is inhibitory and uses the neurotransmitter GABA to hyperpolarize pyramidal projection neurons, their target neurons (Hendry et al., 1989), thus modulate locally the firing activity of these projection neuron types. To balance the local circuitry thereby regulates neuronal activities and gain normal cerebral cortical function, the interconnection of excitatory and inhibitory neurons is needed and the constant fraction of cortical excitatory/inhibitory (E/I) neurons circuit balance in 5/1 from near the start of cortical neurogenesis to adulthood (Sahara et al., 2012) is necessary as well as their correct reciprocal positioning. Recent work demonstrated that projection neuron subtypes were required for the laminar distribution of their selected interneuron partners, a process that is critical for the development of the local inhibitory microcircuitry (Lodato et al., 2011).

Fezf2 is a critical transcription factor for forebrain development in several species (Molyneaux et al., 2007; Shimizu et al., 2010). In mice, loss of *Fezf2* results in the exclusive absence from the DL cortex of subcerebral projection neurons (SCPN), which are replaced by another population of excitatory neurons: Satb2+ callosal projection neurons (CPN) (Chen et al., 2005; 2008; Molyneaux et al., 2005). Lodato and colleagues (2011) found that within the neocortex of *Fezf2* KO mice, this absence of L5 SCPN and their replacement by CPN cause distinctly abnormal location of interneurons and altered GABAergic inhibition. Loss of *Fezf2* does not affect the birth or ventral migration of interneurons indicate that projection neurons are cell-extrinsically required for the normal lamination of cortical interneurons. In *Fezf2*$^{-/-}$

L5, reduced percentages of interneurons were observed accompanied by increased interneuron percentages in the *Fezf2*$^{-/-}$ UL (L2-4) in somatosensory area. This defect is projection neuron type-specific, since the generation of CPN in place of the missing SCPN (L5) cannot compensate for the interneuron abnormalities. In this mutant, somatostatin (SST) and parvalbumin (PV)+ interneuron populations are sensitive to the absence of SCPN, but calretinin (CR)+ interneurons are unaffected suggesting that the identity of the projection neurons generated, rather than strictly their birthdate, determines the specific types of interneurons recruited (Lodato et al., 2011).

Having found that the phenotype of prenatal exposure to SAHA is similar to *Fezf2* muntant (Shimizu et al., 2010) that is perturbed of postnatal cortical lamination due to the diminished Ctip2+ SCPN in DL neocortex (Fig. 15C-F) and also downregulation of *Fezf2* expression (Fig. 17C), I next address a hypothesis that the disturbance of these cortical SCPN laminar distribution would effect interneuron (especially PV and SST) expression following midgestational SAHA treatment.

During development, pyramidal projection neurons and interneurons are born in separate locations. The pyramidal neurons are generated from NSC in the VZ and SVZ of embryonic cortex and typically migrate radially in an ‘inside-out’ sequence to form layers in the CP. Most, if not all, interneurons, however, are born from NSC located outside the cortex, in the lateral- and medial-ganglionic eminences (LGE and MGE) (Fig. 28A) in ventral telencephalon, and migrate first tangentially from their birthplace to the proper cortical region, and then radially through the CP to reach their final laminar location. It seems that the LGE and MGE of the subcortical telencephalon also contribute cells to the neocortex and they are the primary sources of cortical interneurons in rodents (Anderson et al., 2002) including PV- and SST-expressing interneurons (Xu et al., 2004). MGE NSC give rise to early born and PV and SST-expressing interneurons, which distribute at higher densities in deep cortical layers

(Butt et al., 2005; Fogarty et al., 2007). In contrast, vasoactive intestinal peptide and calretinin interneurons originate in the caudal ganglion eminence and preferentially populate the UL (L2-3) (Miyoshi et al., 2010; Xu et al., 2004).

Because the generation of PV and SST interneurons from NSC start emerged around E12.5-13.5 in the MGE (Butt et al., 2005) and this is the time window when SAHA exposed to the pregnant dams, I first examined the cells in the MGE and LGE. Here I demonstrated that SAHA-treated mice have greater proliferating cells in embryonic LGE and MGE than Control ones (Fig. 28B, C). PV and SST interneurons can later be found throughout the brain, including hippocampus, thalamus, and cortex (Butt et al., 2005). Thus next, I investigated the PV and SST expression within the adult cortex. Here I found that SAHA treatment lead to the increased percentage of cortical PV+ cells (Fig. 28E, F). Immunoblotting supported the increased expression of PV and SST-expressing interneurons in SAHA-treated adult cortices (Fig 28D). Because the interneuron cortical lamination defect in *Fezf2* KO mice appeared without accompanied by a change in the total cell number of interneurons between wild-type and mutant cortices (Lodato et al., 2011), these features are presumably caused by increased number of proliferating cells in E15.5 LGE and MGE that might be NSC committed to PV and SST interneurons. This notion, however, need to be support by observation of (1) double label marker for proliferative cells such as pH3 or BrdU and Nkx2.1 as a PV and SST progenitor marker (Xu et al., 2004) in LGE and MGE of E15.5 brains, (2) double label of pH3 or BrdU and PV and SST in adult cortices and quantification of PV and SST-expressing cells in every layer of adult cortices following prenatal SAHA administration.

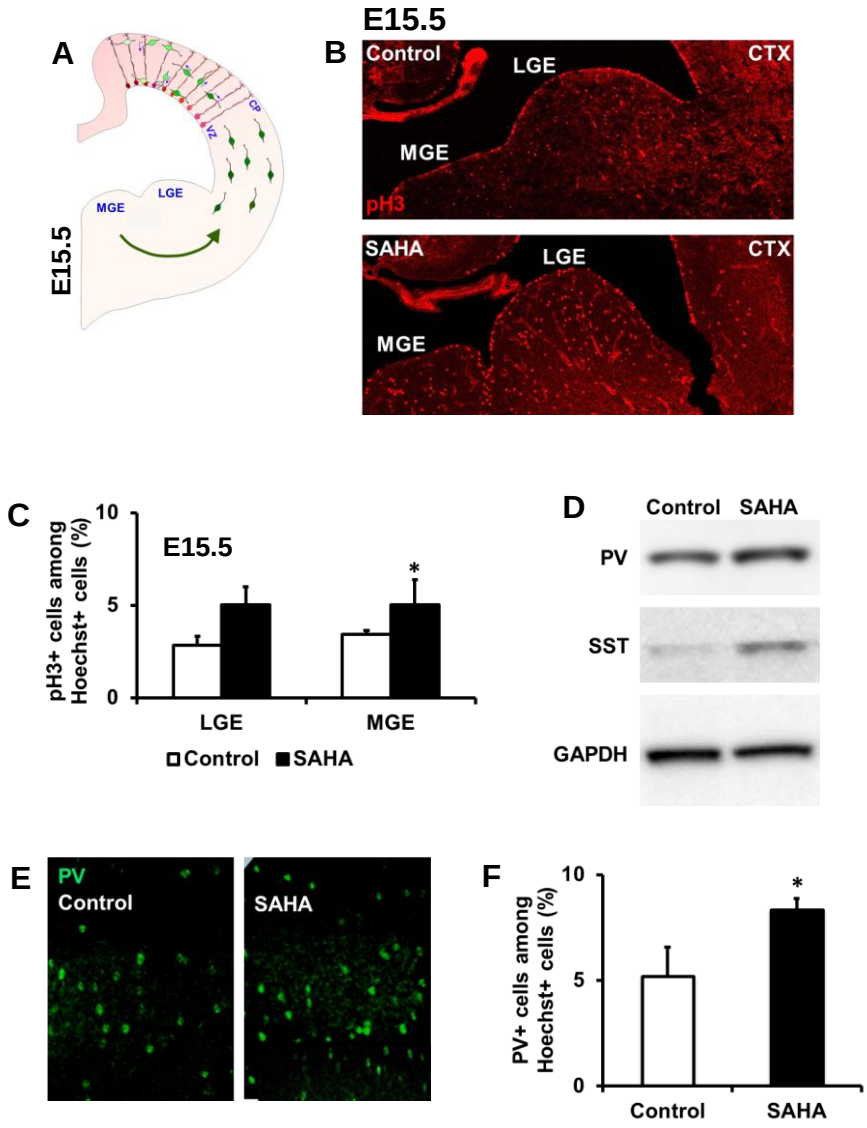


Fig. 28. SAHA increased cortical interneuron in adult brains perhaps due to increased number of proliferating cells within the embryonic LGE and MGE. (A) Interneurons (green) originate from NSC located outside the embryonic cortex, in the LGE and MGE of ventral telencephalon. Interneurons migrating into the cerebral wall from the MGE and LGE interact with NSC (red) and can exhibit changes in direction of migration after contacting NSCs. Interneurons can use glia fiber of NSCs as a scaffold upon which to migrate as they ascend to the cortical plate (CP) or descend in the direction of the ventricular zone (VZ). Once the interneurons invade the cortex from the MGE and LGE, differential interactions between interneurons and NSC scaffold and localized multidirectional migration of interneurons influenced by local guidance cues may facilitate interneuronal positioning within distinct domains of the developing cerebral cortex. Blue arrows indicate direction of migration. (B-C) Immunostaining (B) and number of cells (C) that are proliferative (pH3+) during embryonic development in the LGE and MGE increased following SAHA application. (D-F) SAHA-treated adult cortices showed increased PV- and SST-expressing interneurons relative to Control ones. Data represent as means + SD. *$p < 0.05$; Student's *t*-test; n = 3. Ctx: Cortex.

IV. Discussion

IV.1. Regulation of cortical NSCs development by HDAC inhibition

It is well-known that the dynamic level of histone acetylation is regulated by two opposing enzyme groups called histone acetyl transferases (HAT) and HDACs (Hsieh and Gage, 2004). In the present study, I have demonstrated that proper embryonic cortical neurogenesis during development is controlled by precise regulation of HDACs activity. I found that treatment with SAHA, an anti-cancer drug and HDAC inhibitor (HDACI), during prominent embryonic neurogenesis period induced higher histone H3 and H4 acetylation in both NSCs and neurons (Fig. 9B, C). This result was consistent with previous report that acetylated histones H3 and H4 in NSC and neuronal cultures were more abundant after HDACI treatment (Hsieh et al., 2004). Furthermore, the level of acetylated histones in the presence of HDACI was exceptionally higher in neuronal culture than in astrocytes and oligodendocytes cultures, underscoring that histone acetylation are particularly important for neuronal lineage progression of NSCs (Hsieh et al., 2004).

The increased histone H3 and H4 acetylation, together with enhanced neuronal differentiation following SAHA treatment (Fig. 10, 11D), strongly support my and other's findings that increasing global histone acetylation by HDACI promotes neuronal differentiation both *in vivo* and *in vitro* (Fig. 10, 11D, 19, 21, 22; Hsieh et al., 2004; Balasubramaniyan et al., 2006; Yu et al., 2009; Abematsu et al., 2010; Aizawa and Yamamuro, 2010; Juliandi et al., 2012). Given that embryonic NSCs are multipotent, I proposed that histone acetylation plays an important role in the fate decision of NSCs. The maintenance and/or upregulation of the acetylated histone state, revealed by HDAC inhibition, may have global and dominant effects on neuronal lineage progression.

The mechanisms of neurogenesis-promoting effect of SAHA could be *via* direct or indirect activation of neurogenic transcription factors. My present study shows that SAHA

treatment up-regulated *Ngn1* and its downstream *Tbr2* (Fig. 12E, 29), genes which play crucial role in neurogenesis, suggesting that SAHA could, in fact, induce neuronal differentiation through the transcriptional activation of neurogenic transcription factors (Fig. 29A). The activation of *Ngn1* subsequently will set off a well-programmed cascade of NSC differentiation committed to all glutamatergic cortical neurons (Ngn1/2 → Tbr2 → NeuroD → Tbr1) in Notch-directed transcription during mid-corticogenesis (Kawaguchi et al., 2004; Englund et al., 2005; Sessa et al., 2008; Sansom et al., 2009; Fig. 29B). In line with this, previous study showed that histone acetylation is pivotal in the activation of *Ngn1* in retinoic acid-induced neuronal differentiation (Wu et al., 2009). Other examination on neuronal-specific genes such as *NeuroD*, showed that treatment with HDACI, such as trichostatin A and valproic acid, could activated and increased the expression of these genes (Lunyak et al., 2002; Hsieh et al., 2004; Fig. 6A). Vertebrate neurogenesis is driven by proneural bHLH transcription factors including Ngn1, which promote neuronal differentiation by inducing the expression of *NeuroD* (Bertrand et al., 2002), and these genes were up regulated in the presence of HDACI (Fig. 6A). Therefore, it seems that there is a causal relationship between neuronal differentiation mediated by histone acetylation, revealed by HDAC inhibition, and neurogenic bHLH transcription factor expression (Fig. 29B). Does acetylation of histone selectively activate specific master control genes, such as *Ngn1*, that trigger downstream genes important for neuronal differentiation, or does *Ngn1* itself possess the ability to regulate the maintenance of histone acetylation? Future studies are necessary to clarify the relationship between histone acetylation and neuronal differentiation of NSCs mediated by proneural bHLH transcription factors.

Other mechanism of neurogenesis-promoting effect of SAHA could be related to the Notch signaling-directed transcription of neurogenesis-promoting genes *Ngns* (*1* and *2*). At the start of neurogenesis, Notch signaling control NSC self-renewal *via* induction of *Hes* gene

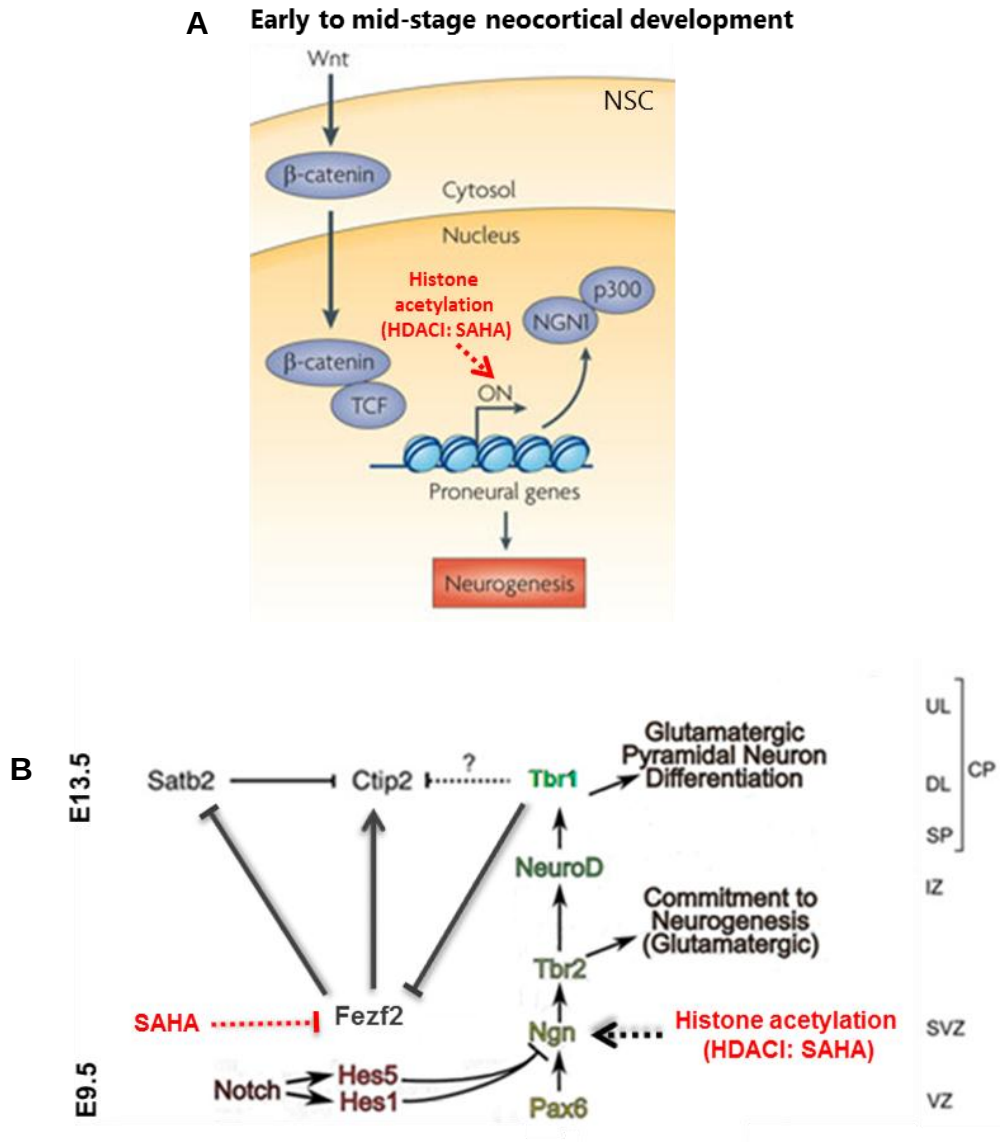


Fig. 29. (A) Mid-gestational HDAC inhibition by SAHA thereby induced histone acetylation up-regulated *Ngn1*, genes which play crucial role in neurogenesis, suggesting that SAHA could, in fact, induce neuronal differentiation through the transcriptional activation of neurogenic TFs (Hirabayashi and Gotoh, 2010; with modification). (B) A model depicting the mid-corticogenesis roles of HDACI SAHA in Notch-directed transcription and cascade of NSC differentiation. At start of neurogenesis (E9.5), Notch signaling controls self-renewal of NSC via induction of Hes genes by repressing proneural gene Ngn1/2. In NSC and its progenitor, if Notch signaling is low, Ngn1/2 becomes highly active, setting of a well-programmed cascade of differentiation. Hence, the initial step of differentiation in cortical pyramidal neurons is the expression of Ngn in lockstep with the appearance of Tbr2 that committed to pyramidal projection neurogenesis followed by direct transcriptional of NeuroD and Tbr1. This model is confined since SAHA treatment increased *Ngn1*, *Tbr2*, and Tbr1 expression (indicated by black dashed line). Tbr1 directly repress Fezf2. The reduction of Fezf2 expression presumably by SAHA itself (represented by red dashed line) or owing to higher expression of Tbr1 may relieve the repression of UL neuron identity regulated by Satb2 and enable Satb2 to repress Ctip2 expression. This will promote the UL neuron identity that project callosally (Satb2+). Arrows indicate activation, whereas 'T'-shaped lines represent inhibition.

(Kageyama and Ohtsuka, 1999). *Hes1/5* negatively regulates transcription of neurogenesis-promoting genes *Ngns1* and *2*, thereby effecting progenitor maintenance and inhibiting neurogenesis (Ochiai et al. 2009). The low expression of Notch signaling pathway downstream molecules such as *Hes5* by SAHA treatment (Fig. 23B) could allow relieve of *Ngns* suppression. This, in turn, enables activation of *Ngns* and promotes neurogenesis (Fig. 29B). However, I found that SAHA reduced the number of NSCs (Fig. 11A-C) which seemingly it caused the decline expression of *Hes5*. Additionally, it has been reported that Brn1 facilitated neuronal differentiation by diminishing Notch-directed transcription of Hes5 in NSC and its progenitors during midgestation (Dominguez et al., 2012). Thus the increased Brn1 expression in SAHA-exposed brains (Fig. 15G) could in turn suppressed *Hes5*. Taken together, these results excluded the possibility of *Hes5* gene as SAHA target.

The decreased of NSCs in embryonic SAHA-treated brains (Fig. 11A-C) also influenced the subsequent generation of glial cells, astrocytes and oligodendrocytes that generally follows neurogenesis in the developing mammalian brain. The production of cortical astrocytes was diminished following prenatal SAHA exposure. This is likely the consequence of the reduction of NSC number (Fig. 11A-C) thus lead to the reduced number of astrocyte progenitor in SAHA-treated cortices. Other possibility related to Notch signaling is the reduction of *NFIA* expression by SAHA administration (Fig. 23B) may cause decreased astrocytic differentiation potency of mid-gestational NSC since it has been suggested that NFIA, which bind to astrocytic gene promoters and act as downstream of the Notch signaling pathway to potentiate astrocytic differentiation of mid-gestational NSCs, accelerated demethylation of astrocytic gene promoters by preventing DNMT1 from binding to them (Fig. 3b) and thus allowed precocious astrocytic differentiation (Namihira et al., 2009; Juliandi et al., 2010b).

SAHA enhanced neurogenesis indirectly by increasing the number of proliferating

IPCs (Fig. 12A-D, 20A-E) which suggested by several reports to mainly differentiate into cortical UL neurons (Tarabykin et al., 2001; Cubelos et al., 2008). Indeed, I observed an increase number of UL neurons (Cux1+ and Satb2+) in SAHA-treated mice (Fig. 14A-B, E-F). It is conceivable that these SAHA-induced UL neurons production due to the increase in the number of proliferating IPCs. It has been reported that in mice, Satb2 confers UL identity and is initially present in the neurons that project callosally (Alcamo et al., 2008; Britanova et al., 2008; Chen et al., 2008). SAHA-treated mice also showed higher number of Satb2+ neurons in DL than in control (Fig. 14E-F), suggesting that SAHA expanded callosal projection neurons in all layers of cortex. In DL, I also observed a decrease number of Ctip2+ neurons with SAHA treatment (Fig. 15C-D). A similar phenotype was observed in the *Fezf* knock out mice (Shimizu et al., 2010).

Recent studies unraveled that *Fezf2*, which acts upstream of *Ctip2*, are a master regulator in postmitotic specification of subcortical DL neurons in the developing cerebral cortex (Chen et al., 2008; Leone et al., 2008). *Fezf2* is expressed in NSCs and its progenitors starting on E8.5 and its expression is retained by DL neurons during corticogenesis (Chen et al., 2005; 2008). Later on, DL neurons acquire the expression of *Ctip2* during postmitotic differentiation (Arlotta et al., 2005; Chen et al., 2008). Several studies have indicated that *Fezf2* expression in NSCs and its progenitors may promotes the expression of *Ctip2* in young neurons, and together these genes confer a DL neuronal fate during differentiation (Leone et al., 2008). Indeed, treatment with SAHA during mid-gestation resulted in the repression of genes specific for DL neuronal lineage (*Fezf2* and *Ctip2*) and the induction of genes for UL ones, such as *Satb2* (Fig. 17). Although it is currently unknown whether SAHA suppress *Fezf2* expression directly or indirectly, the suppression could in turn reduce the generation of DL neurons and derepress UL neuron production regulated by *Satb2*, and enables Satb2 to inhibit *Ctip2* expression. Satb2 is highly enriched in post-mitotic L2-5 projection neurons,

starting at E13.5 in newly born neurons as they migrate out of the germinal zones and into the IZ (Alcamo et al., 2008; Britanova et al., 2008). It has been previously shown that Satb2, functions as a repressor of *Ctip2* by direct binding to the regulatory region of *Ctip2* (Alcamo et al., 2008), supporting this senario (Fig. 29B). In this regard, I found that SAHA treatment increased *Satb2* while decreased *Fezf2* and *Ctip2* mRNA levels in the E14.5 cortex (Fig. 17). I also found an increase number of Tbr1+ neurons at postnatal cortices (Fig. 15A-B, G). Tbr1 is selectively expressed in projection neurons positioned in L6 and in the SP, and in Cajal-Retzius neurons of the MZ, starting from E12.5 (Hevner et al., 2001). It has been reported that Tbr1 directly binds to a region in the 3' of *Fezf2*, and this binding leads to suppression of the gene to regulate the development of corticothalamic-corticospinal neurons in DL (Han et al., 2011; McKenna et al., 2011). The increased number of Tbr1+ and Satb2+ in DL cortex (Fig. 14E-F and 15A-B) apparently in line with the recent notion that IPCs are responsible for the production of majority (>80%) of pyramidal projection neurons for all layer (MuhChyi et al., 2013) which against the earlier hypothesis that IPCs generated only UL neurons (Tarabykin et al., 2001; Zimmer et al., 2004).

My data provide critical insight into the significant role of histone acetylation as a key regulator of neuronal differentiation. Nevertheless, elucidation of the HDAC-mediated mechanisms that trigger NSCs to produce more UL than DL neurons after prenatal exposure of SAHA is an important challenge for future study. Recently, it has been proposed that DL and UL neurons are produced by distinct population of NSCs that co-existed since early gestation, rather than from a sequential developmental progression of NSCs (Franco et al., 2012). Therefore, SAHA might induce the untimely differentiation of UL-producing NSCs and/or accelerates the temporal transition of DL-producing NSCs into UL-producing ones within specific time-window when SAHA was being administered to the mice (Fig. 30). My findings might also contribute to the pharmaceutical therapy guideline for cancer treatment.

In the present study, I provide information that SAHA has a potential hazard to the fetus and should not be prescribed to pregnant cancer patient or to patient who planned to become pregnant while taking this drug.

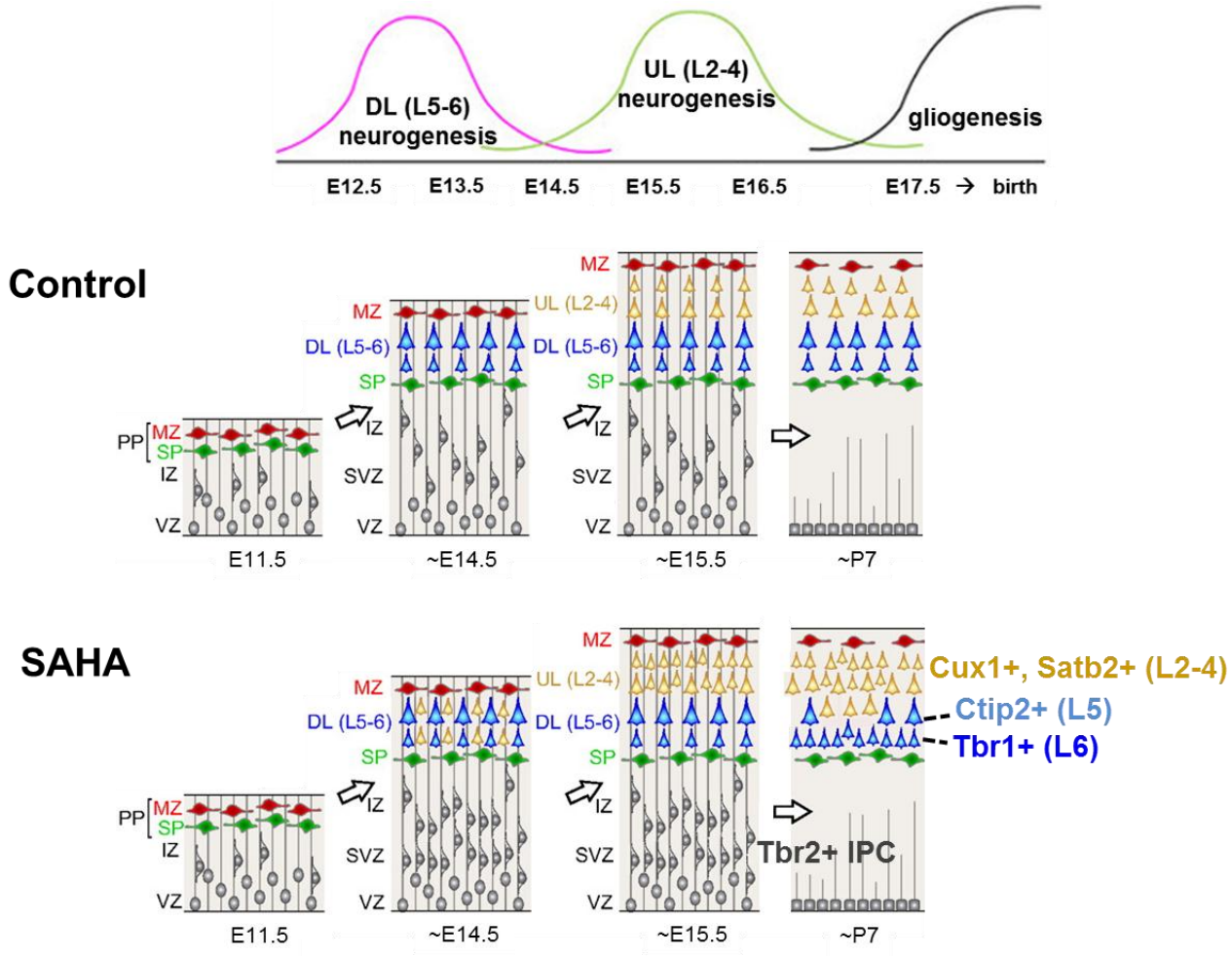


Fig. 30. SAHA might induce the untimely differentiation of UL-producing NSCs and/or accelerates the temporal transition of DL-producing NSCs into UL-producing ones within specific time-window when SAHA was being administered to the mice.

IV.2. Adult neurogenesis and related behavior after prenatal HDAC SAHA exposure

Perinatal events in rodents taking place during the gestational period up into the first few postnatal weeks until weaning appear to be crucial for the lasting programming of brain structure and function because in this period, the rodent brain is plastic and sensitive enough to environmental changes to be subject to permanent alteration (Korosi et al., 2012).

Neurogenesis, a unique form of structural plasticity reflecting the generation of new neurons from NSC emerged since the embryonic stage and continued up to adulthood and their functional integration into existing circuitries in an adult brain. Increasing evidence points to an important role for neurogenesis in critical functions related to cognition and emotional regulation such as spatial and contextual learning and memory, pattern separation and anxiety regulation. The perinatal period including prenatal hence represents a time window during which lasting, and often non-reversible changes occur in brain structural development, programming and shaping the adult phenotype as well as behavior. Perturbations during this critical time window may change developmental programming events such that they can contribute to disease susceptibility. For instance, prenatal stress due to drug treatment, alcohol exposure or prenatal repeated restraint lead to lasting reductions of adult neurogenesis in hippocampus, certain regions of the adult brain retain neurogenic potential, (Mandyam et al., 2008; Bosch et al., 2006; Lemaire et al., 2000) consistently associated with cognitive and emotional deficits appeared in numerous neuropsychiatric disorders, including depression, anxiety, schizophrenia and autism (Gillot and Standen, 2007; Koenig et al., 2002; Nestler et al., 2002).

Recently, an *in vitro* and *in vivo* study particularly in the proliferation and differentiation of NSCs supported the idea that fetal exposure to drug is likely to result in long-term consequences that may predispose to neurodevelopmental disorders (Bose et al., 2010). Rat embryonic cortical NSC exposed to corticosteroid drug such as dexamethasone (Dex) at critical periods of development results in (1) decrease NSC number without altered in cell differentiation, and (2) deficit in mitochondrial functions accompanied with increased occurrence of apoptotic cell death. All these effects are retained in daughter NSCs (never directly exposed to Dex) and are associated with a higher susceptibility to oxidative stress. Mice treated with VPA, an anti-epileptic drug and also have HDACI property, during mid-

gestation also showed an impaired adult neurogenesis in the DG of hippocampus accompanied with more less proliferative of adult NSCs and slowly differentiated into neural lineages (more quiescent) resulting lower absolute number of proliferating and differentiating NSCs in DG of VPA-treated mice, which then caused the deficiencies in learning and memory (data from Dr. Berry Juliandi). However, in the present study I found that prenatal HDAC inhibition by SAHA thereby increased acetylation of histone in the embryonic forebrain did not show the significant difference in behaviour test related to hippocampus-dependent memory such as light/dark transition test, tail suspension, and Contextual/Cued Fear Conditioning to assess anxiety, depression, learning and memory (Table 1). SAHA did not effect on the differentiation (Fig. 26G, H) and decreased on the proliferation (Fig. 26C-F) of adult NSC in the DG of hippocampus.

One model for the anti-cancer action of vorinostat is that vorinostat inhibition of HDAC activity, and subsequent accumulation of acetylated histones, leads to the activation of genes whose expression causes induction of differentiation or apoptosis, thus inhibiting cancer growth. This model is based on the finding that the expression of genes is regulated following exposure of cancer cells to SAHA. One of the most commonly induced genes is the cell cycle gene inhibitor $p21^{WAF1}$ (reviewed in Richon et al., 2009). In agreement with this result, in the present study I found that SAHA reduced NSC that is presumably because SAHA decreased proliferation of NSC by inducing cell cycle inhibitor gene p21, or because the NSC is used to differentiate in this respect neurogenesis back to control levels in SAHA-treated mice. This result is likely explain why there is no significant difference between Control and SAHA-treated mice in behaviour test related to hippocampus-dependent memory (Table 1) since it has been reported that adult NSCs proliferation and differentiation in DG are important for learning and memory (Zhao et al., 2008). The changes in adult hippocampus (Fig. 25, 26C-F) caused by embryonic treatment of SAHA may not be

sufficient to induce behavior changed after the mice reach adult. The absence of a reduction in adult neurogenesis, or differentiation in general, in animals with a history of prenatal SAHA administration could indicate that SAHA did not have lasting consequences on adult hippocampal neurogenesis in mice and perhaps there is an adaptive mechanism preventing this structural plasticity to be further diminished. This adaptive mechanism in SAHA-treated mice might bring the neurogenesis back to normal level. This could in turn to make the mice were less anxious, less sensitive to stress in adult life. It could be induced by the active maternal care during perinatal life regarding the fact that offspring raised by a mother that exhibit lower active maternal care (LMC) impaired hippocampal learning and memory in adulthood (Liu et al., 2000). Additionally, stressful conditions in adult rats raised by LMC (induced by daily repeated maternal separation from the pups during the first three week of life of the pups) results in animals with behavioral and neuroendocrine signs of elevated stress activity, associated with significant cognitive deficits in adulthood (Aisa et al., 2007).

In contrast, prenatal VPA treatment, widely uses as anti epileptic drug and also have HDACI property, showed reduced proliferation and differentiation of NSCs thus led to impaired adult neurogenesis in the DG of hippocampus accompanied with more less proliferative of adult NSCs and slowly differentiated into neural lineages (more quiescent) resulting lower absolute number of proliferating and differentiating NSCs in DG of VPA-treated mice, which then caused the deficiencies in learning and memory (data from Dr. Berry Juliandi). Prenatal SAHA administration may not induce the adult NSCs become more quiescent thereby resulting in the normal differentiation of NSC into neural lineages (Fig. 26G, H) and no significant difference on the behaviour test. Since that recent study displayed the role of imprinted gene p57 to control adult NSC quiescence (Furutachi et al., 2013) and this gene is novel HDAC1 target (Zupkovitz et al., 2006), it is tempting to further examine

and compare the difference expression of p57 and HDAC1 after prenatal VPA and SAHA application.

The impairment of adult neurogenesis which then lead to the deficiencies in learning and memory in mice with a history of mid-gestational VPA administration (data from Dr. Berry Juliandi) which did not observe in SAHA ones might associated with 'fetal valproate syndrome' since that this syndrome were found during clinical studies on VPA to treat epilepsy, bipolar disorders, and migraine suggested that VPA administration in pregnant mother (Williams et al., 2001).

Mid gestational SAHA administration disturbed cortical layer formation by altering *Fezf2* and *Satb2* expression, TFs that determine DL and UL cortical neuron identity and also have been found within patients with developmental and language delays, intellectual disability, schizophrenia and autism spectrum disorder (ASD) (Cooper et al., 2011; Potkin et al., 2009; Rosenfeld et al., 2010). Clinical studies on one of HDACI VPA, widely used to treat epilepsy, bipolar disorders, and migraine, suggested that VPA administration during pregnancy may result in a 'fetal valproate syndrome', which has feature similar to ASD (Williams et al., 2001). For instance, autistic children exhibit impaired language, abnormal social interactions and repetitive behaviors (Canitano, 2007). Along this line, previous studies in mice showed that prenatal exposure to VPA displayed ASD-like behavioral abnormalities including social interaction and communication deficits, resistance to change in routine, altered ultrasonic vocalization, and restricted interests and activities (Losh et al., 2008; McFarlane et al., 2008; Kataoka et al., 2013), and these behavioral abnormalities were accompanied with transient histone hyperacetylation and lower number of DL cells in the somatosensory cortex (Kataoka et al., 2013; Hara et al., 2012). It is therefore important to further investigate the behavior test related to corticogenesis disruption on the future offspring of SAHA-treated mice.

IV.3. SAHA increased cortical interneuron generation

A prominent trait of the mammalian cerebral cortex is its complex cellular architecture, which relies on the development of a diversity of neurons extensively interconnected into functional networks. Cortical excitation and inhibition are executed by highly heterogeneous populations of glutamatergic excitatory pyramidal projection neurons and GABAergic inhibitory interneurons, respectively. The establishment of correct reciprocal positioning and interactions between these two broad neuronal classes is very important for balanced electrical activity and normal cortical function. Thus, the constant fraction of cortical excitatory/inhibitory (E/I) neurons circuit balance in 5/1 from near the start of cortical neurogenesis to adulthood (Sahara et al., 2012) is necessary. The disruption of E/I circuit balance due to reduced expression of cortical GABAergic neurons such as *GAD67*, *reelin*, *SST*, *PV* in the hippocampus, basal ganglia, or upper to L5 cortical layers of psychotic patients including schizophrenia (SZ), ASD, and bipolar disorder patients markedly reduces the effectiveness of the GABAergic inhibitory transmission that impinges on the dendrite and on the initial axon segments of pyramidal neurons (Grayson et al., 2011; Fig. 31). This deficit of inhibitory neurotransmitter disrupts the intermittent synchronization of pyramidal neuron firing that is critical for normal neuronal function (Gonzales-Burgos and Lewis, 2008).

My finding about the perturbation of postnatal cortical laminantion due to the increased and decreased production of excitatory pyramidal projection neuron in UL and DL neocortex, respectively, and also the increased number of cortical inhibitory interneurons following midgestational SAHA exposure might cause the inconstant of E/I fraction thus lead to the alteration of pyramidal neuron firing and induce the abnormality of neuronal function in the cortex. This defect reinforce the necessity of performing behavior test related to corticogenesis disruption on the future offspring of SAHA-treated mice. The increased number of interneurons in the embryos exposed to SAHA in my study is in agreement with

Grayson et al. (2011) by which treatment of neuronal progenitor cultured cells with various HDACIs (MS-275, VPA, TSA) led to a robust induction of interneuron mRNAs such as *reelin* and *GAD67* as well as DNMT inhibitors.

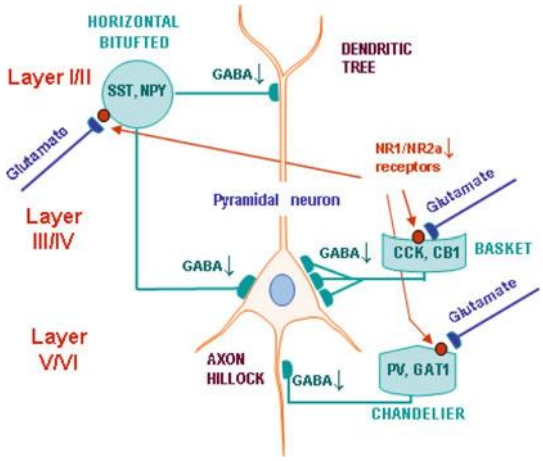


Fig. 31. Cortical circuitry links multiple neuronal subtypes. Phenotypically distinct neurons reside in different cortical layers. GABAergic interneurons (aqua) function to modulate the output of pyramidal (tan) and other neurons. We propose that the reduced expression of GAD1 (also known as GAD67) and other GABAergic markers (SST, NPY, CCK, CB1, PV, and GAT1), along with reduced levels of GRIN1- and GRIN2A-containing glutamate receptors, contributes to reduced GABA release. This GABA hypofunction causes decreased pyramidal neuron synchronization (Grayson et al., 2011).

DNMT and HDAC inhibitors target DNMT1 and HDAC1 and facilitate the dissociation of DNMT-containing repressor complexes from interneuron (as proposed for the *reelin* and *GAD67* genes) promoters led to DNA demethylation, histone acetylation, and a relaxation of chromatin allows the recruitment of specific TFs, and the general transcriptional machinery to the promoters. Collectively, this results in the drug-induced epigenetic changes leading to promoter activation providing a new approach for the treatment of SZ employing epigenetic drugs (Grayson et al., 2011; Fig. 32) since that similar regulatory mechanisms are suggested to be operative in adult GABAergic neurons (Costa et al., 2006; Szyf et al., 2008). Thus the present invention in my study that SAHA increased PV- and SST-expressing interneurons in adult cortices (Fig. 28 D) may provide a preliminary result to enrich pharmacological approach employing HDACIs for correcting reelin and GAD67 mRNA

levels, and the GABAergic deficits associated with SZ (Guidotti et al. 2005; Levenson et al., 2008).

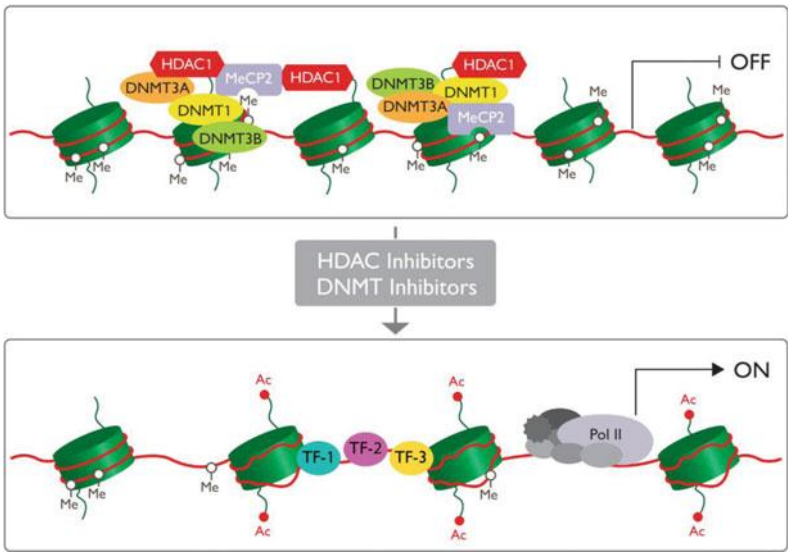


Fig. 32. Model for promoter activation of interneuron genes by HDAC and DNMT inhibitors. Promoters are shown as either silent (repressed–OFF) or fully active (ON). The transcriptionally inactive chromatin structure surrounding the indicated promoter region (upper panel) is the consequence of cytosine methylation and subsequent recruitment of repressor proteins, including DNMT1, DNMT3A, DNMT3B, MeCP2, and HDAC1 (most likely others, also). The downregulation of DNMT protein (by DNMT and HDAC inhibitors), together with the inhibition of HDAC enzymatic activity and the decrease in MeCP2 expression (in the case of the HDAC inhibitors), result in dissociation of these repressor complexes. This leads to DNA demethylation, histone acetylation, and a relaxation of the chromatin surrounding the respective regulatory regions (lower panel). The more open chromatin configuration allows the recruitment of specific TFs, such as Sp1 and the general transcriptional machinery (gray shapes) to the promoters. Collectively, these results in the drug-induced epigenetic changes lead to promoter activation (as proposed for the *reelin* and *GAD67* genes) (Grayson et al., 2011).

IV.4. Concluding remarks

The ability of SAHA, a promising anti-cancer drug, to inhibit HDAC thereby increase histone acetylation and promote neurogenesis provides critical insight into the significant role of histone acetylation as a key regulator of NSC differentiation. The mechanism of neurogenesis-promoting effect of SAHA is through the transcriptional activation of proneural bHLH transcription factors such as *Ngn1* and its downstream *Tbr2*. It is becoming clear that there is a causal relationship between histone acetylation and neuronal differentiation of NSCs mediated by proneural bHLH transcription factors. However, it is unclear why a global increase of histone acetylation upregulates only specific genes such as bHLH neuronal genes in NSCs. Does acetylation of histone selectively activate specific master control genes, such as *Ngn1*, that trigger downstream genes important for neuronal differentiation, or does *Ngn1* itself possess the ability to regulate the maintenance of histone acetylation? Could this be due to a unique subunit composition of HDAC-containing chromatin remodeling complexes at the promoter of this neuronal gene? If so, what are these subunits and what are their specific features?

The defect affected by fetal exposure to SAHA apparently persisted. In adult mice which exposed by SAHA during mid-gestation, the number of NSC was still lower in hippocampus; certain regions of the adult brain retain neurogenic potential. Moreover, in mice with a history of prenatal SAHA treatment increased embryonic neurogenesis and further perturbed postnatal cortical lamination due to increased and decreased production of UL and DL excitatory neurons, respectively and suppresed cortical astro-gliogenesis. SAHA might induce the untimely differentiation of UL-producing NSCs and/or accelerates the temporal transition of DL-producing NSCs into UL-producing ones within specific time-window when SAHA was being administered to the mice. Nevertheless, elucidation of the

HDAC-mediated mechanisms that trigger cortical NSCs to produce more UL than DL neurons after prenatal exposure of SAHA is an important challenge for future study. Furthermore, midgestational SAHA administration also increased cortical inhibitory interneuron generation in adult brains. This may deregulate the normal ratio between excitatory and inhibitory neuron in the cortex. But, are all these features responsible for the lasting corticogenesis disturbance? Does this defect can be depicted with the abnormalities of behavior employing mice model? Given the intense clinical interest of SAHA and the use of HDACI for treating CNS disorders and diseases, answers to these and other outstanding questions are eagerly awaited.

All in all, these findings might contribute to the pharmaceutical therapy guideline not only for cancer treatment but also for treatment of other diseases employing HDACIs. Regarding that SAHA has a potential hazard to the fetus, it should not be first choice medicine for pregnant patient or for patient who become pregnant while taking this drug.

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