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RESEARCH ARTICLE

Multifaceted Regulation of Neural Stem Cell Fate in the Developing Brain

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ABSTRACT

Stem cells are the source of diverse cell types, and therefore their fate decisions are tightly regulated by multiple layers of controls in each tissue. Without a doubt, the brain is one of the most complex and highly functional tissues, as we now know more than 70 million neurons and even more non-neuronal cells are distributed across dozens of cortical areas in the mouse cerebral cortex, and a single region of cortex contains more than 40 cell types. These diverse neuronal cells emerge from initially homogeneous neural stem cells during embryonic development. In the course of differentiation, neural stem cells undergo cellular division to produce daughter cells with new cellular identities, during which epigenetic and transcriptional regulations determine their fate. Recent advances in the field of neural stem cell biology have revealed that not only epigenetic regulators and transcription factors but also specialized intracellular organelles regulate many aspects of stem cell functions and fate choices, and therefore it is timely to review the mechanisms of sophisticated changes of the properties of neural stem cells during development and how they impact the function of the daughter cells.

Keywords: Neural stem cells, fate specification, epigenetics, transcription factors, organelles

1.Introduction

In the development of the central nervous system (CNS), multipotent neural stem cells (NSCs) of ectodermal origin are responsible for the production of all types of neurons and macroglial cells, i.e., astrocytes and oligodendrocytes.[1] In vertebrates, NSCs arise from the neuroepithelium lining the nascent neural tube. As typical epithelial cells, NSCs exhibit a distinct apical-basal polarity, with their apical side facing the lumen of the neural tube and the basal membrane touching the pial surface. In the early stages before neurogenesis, NSCs self-renew in a symmetric division manner, expanding their pool, and then they start producing neurons that migrate to the basal direction and gradually construct the six-layered cortex.[1] With the onset of neurogenesis, specialized intermediate progenitor cells are generated at the apical/ventricular surface from asymmetrically dividing NSCs, which gradually elongate radial fibers and are termed apical radial glial cells (aRGCs). aRGCs produce transient amplifying progenitors, which delaminate from the ventricular surface while retaining the basal radial fiber and migrate to the germinal zone consisting of cells named basal RGCs (bRGCs).[1] bRGCs are enriched in gyrencephalic species such as humans, contributing to the production of more neurons to form a brain with complex and higher-order functions.[2] Finally, after the formation of the six-layered cortex, neurogenesis is accomplished and NSCs switch to undergoing gliogenesis, producing astrocytes and oligodendrocytes (Figure 1).

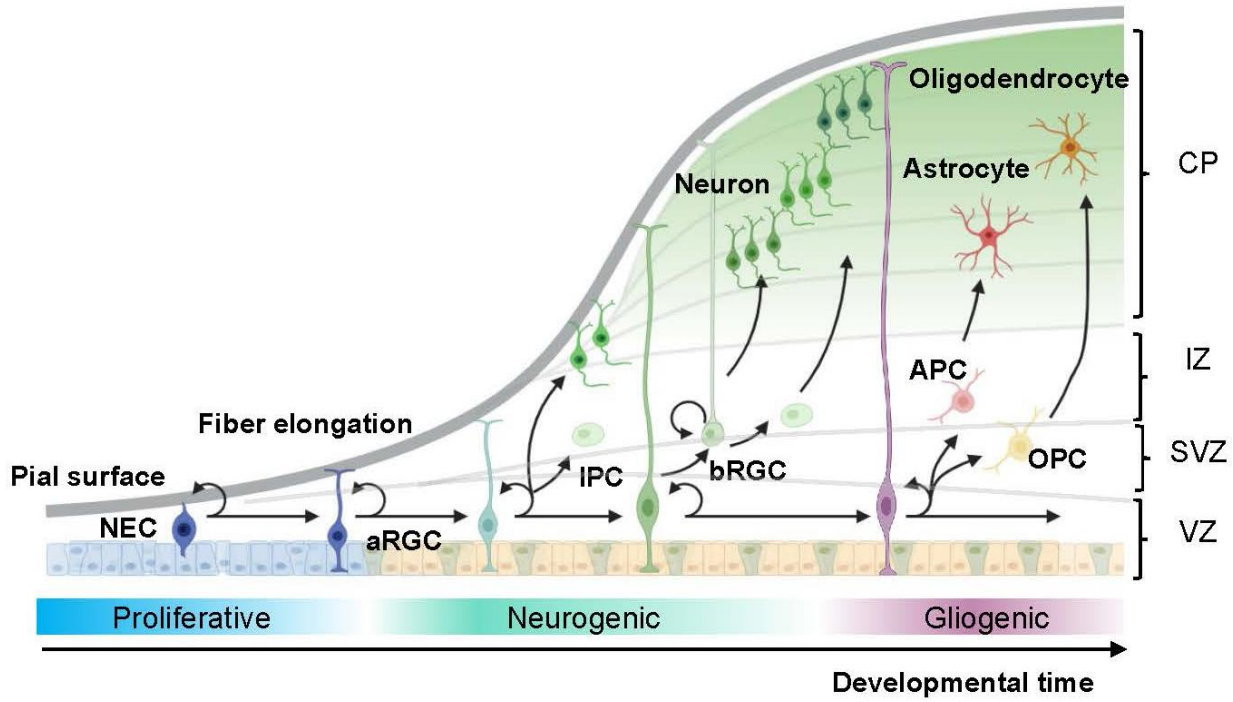

Figure 1. Spatiotemporal cortical development in mice.
During corticogenesis, neuroepithelial cells (NECs) first repeat symmetric divisions to expand their cell numbers (the proliferative phase in blue). Around the early-mid embryonic stage, NECs elongate their radial fibers and become apical radial glial cells (aRGCs). In the neurogenic phase (green), RGCs undergo asymmetric divisions and generate neurons or intermediate progenitor cells (IPCs). Basal RGCs (bRGCs) have their cell bodies located in more basal areas of the cortical wall. Newborn neurons migrate toward the cortical plate and form the six-layered cortex. Around the late embryonic stage, RGCs gradually lose their neurogenic potential and gain gliogenic potential. In the gliogenic stage (purple), RGCs start to generate astrocyte progenitor cells (APCs) or oligodendrocyte progenitor cells (OPCs).

As in any developing tissue, the timing of stem cell proliferation and differentiation must be tightly regulated for proper tissue growth and maturation. This is made possible by a genomic landscape inscribed with a variety of epigenetic modifications, including DNA methylation and histone modifications. These chemical modifications regulate the expression of transcription factors (TFs), and TFs also modulate the epigenetic landscape. As such, TFs and epigenomic statuses influence each other and are deeply implicated in cell fate decisions.[3] Moreover, specialized

intracellular organelles control many stem cell functions and stem cell destiny, about which we summarize recent findings in a later chapter.

2.Transcription factors that direct neural stem cell fate choice

Transcription factors are the proteins that bind to specific regions on genomic DNA and regulate gene expression by interacting with other TFs to form complexes and/or induce changes of epigenetic modifications. The expression patterns of TFs differ depending on the cell types, resulting in different gene expression profiles in each cell type to perform distinct functions. Therefore, the regulation of TF expressions is crucial for defining cell fate during development. In this context, the Sox (SRY-related high-mobility group box) family member genes and the basic helix-loop-helix (bHLH) member TFs are known to be particularly important for the fate determination of NSCs and have been well studied, as summarized here.

In mammals, 20 Sox genes have been identified, and classified into 8 subgroups (SoxA to SoxH) based on the phylogenetic analysis of their functional domains.[4] It is widely believed that Sox proteins cooperate with other TFs, including other Sox family members, to perform multiple essential functions. Furthermore, the expression of each Sox subgroup member overlaps slightly during cortical development,[5] and consequently NSCs gradually change their properties according to the expressed Sox members and differentiate into cells that have specific functions (Figure 2 left).

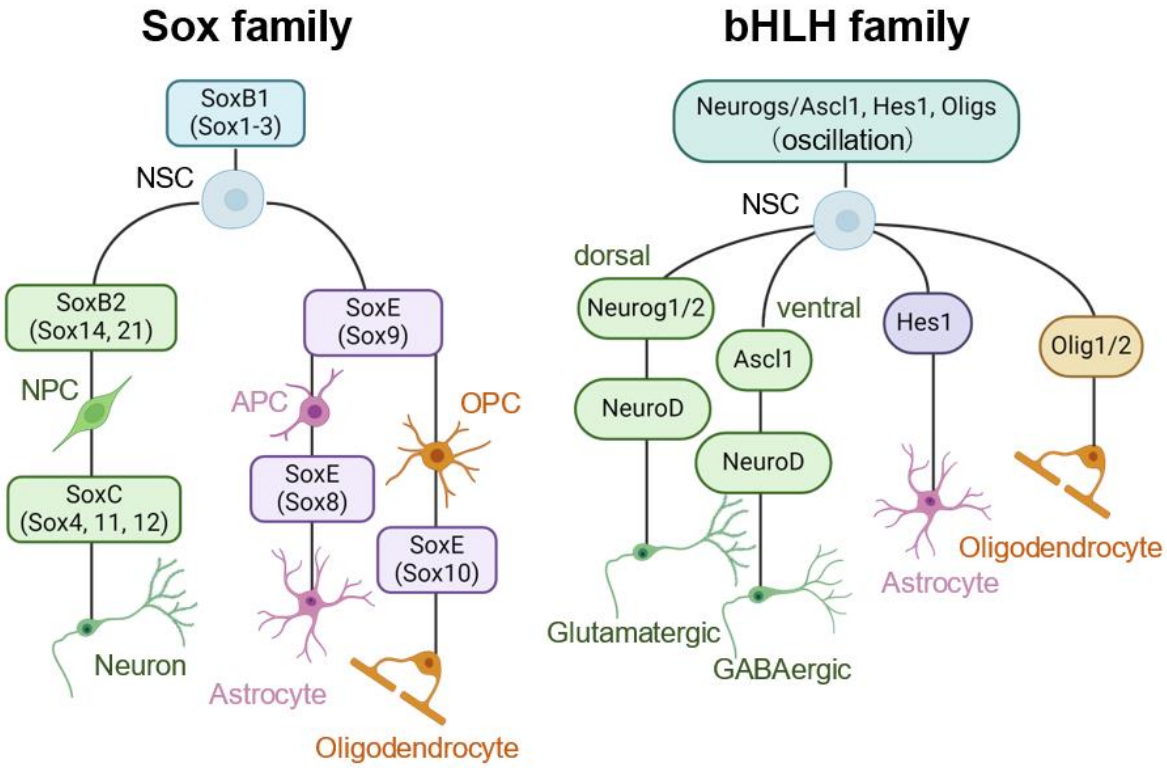

Figure 2. Neural lineage specification governed by Sox and bHLH TFs during development.
SoxB1 maintains NSC properties by activating self-renewal genes and repressing the expression of neuron-specific genes. During neurogenesis, SoxB2 interferes with SoxB1, and then SoxC further promotes cell maturation by activating a subset of neuronal genes. Glia-related genes are initially targeted by Sox9, and then specific genes are activated by Sox8 or Sox10, respectively, generating astrocytes or oligodendrocytes (left). Oscillatory expression of Neurogs/Ascl1 (in the dorsal/ventral telencephalon), Hes1, and Oligs maintains NSCs, but sustained expression directs neuronal (glutamagic neurons/GABAergic neurons), astrocytic, and oligodendrocytic differentiation, respectively.

The bHLH TF family is one of the largest TF gene families in mammals, as about 130 members have been found in humans and 117 in mice.[5] These bHLH TFs regulate the fate of NSCs temporally and region-specifically according to their expression pattern. Furthermore, some of these bHLH TFs are known to oscillate in expression every few hours, and the exit from these oscillation cycles triggers the differentiation of NSCs. Some examples of this are *achaete-scute homolog-1* (*Ascl1*), *hairy and enhancer of split-1* (*Hes1*), and *oligodendrocyte transcription factor-2* (*Olig2*), whose sustained expression directs

NSCs to neuronal, astrocytic, and oligodendrocytic differentiation, respectively as we discuss detail in below (Figure 2 right).

2.1 EXPANSION OF NEURAL STEM CELLS

Despite NSCs being defined as multipotent and self-renewable cells, they cannot differentiate into neurons, astrocytes, and oligodendrocytes in the proliferation phase, which is the earliest phase of development, as mentioned earlier.[6] In the proliferation phase, NSCs actively proliferate and expand the stem cell pools to supply sufficient numbers of cells for the formation of proper brain structure.[1]

From the early neuroectoderm stage, SoxB1 family members (Sox1–3) are expressed in a strongly overlapping manner and continue to be expressed in self-renewing NSCs through the entire CNS development.[7] Gain- and loss-of-function studies have demonstrated their relevance in regulating the fundamental functions of NSCs, such as their maintenance and proliferation.[8] Key to their negative effect on neuronal differentiation is the ability of SoxB1 proteins to counteract co-expressed proneural proteins. As reported by Wegner et al., SoxB1 members possess a strong ability to sequester proneural proteins, whereby proneural proteins lose the ability to induce neuronal differentiation and cell-cycle exit-associated gene expression.[9]

A real-time imaging analysis using luciferase reporters revealed that the expression of bHLH factor *Hes1*, which is important for astrocytic differentiation, is dynamically oscillating in NSCs of the mouse telencephalon.[10] A knockout (KO) study of *Hes1* showed its clear function in NSC maintenance, as the KO mouse exhibits accelerated neuronal differentiation, resulting in microcephaly.[11] Hes1 is a transcriptional repressor and downregulates the expression of other proneural bHLH factors, such as *Ascl1* and *Neurogenin2 (Neurog2)*, and thereby controls the proper timing of neurogenesis. Furthermore, Hes1 represses its own expression by directly binding to its promoter, hence forming a negative feedback loop.[12] Since *Hes1* oscillation periodically represses other bHLH genes that induce cell differentiation, *Ascl1*, *Neurog2*, and *Olig2* expression oscillates in NSCs, which allows NSCs to maintain their stem cell properties.

2.2 NEURONAL DIFFERENTIATION

The Neurog family, comprising Neurog1–3, is a class of proneural bHLH TFs expressed in the dorsal telencephalon and essential for the specification of glutamatergic neurons.[13,14] The Neurog family plays major roles in the commitment of progenitors to neurons by (1) inducing a cascade of pan-neuronal gene expression including *neurogenic differentiation-1 (NeuroD1)*,[15] (2) inhibiting glial fate,[16] and (3) promoting cell cycle exit.[17] Another proneural bHLH TF, Ascl1, is weakly expressed in the dorsal cortical progenitors and strongly expressed in the ventral telencephalon, which is the source of GABAergic inhibitory neurons.[18] Although Neurogs and Ascl1 show distinct expression patterns in the telencephalon, they both induce NeuroD family members, a class of differentiation bHLH TFs.[19] NeuroD family members are expressed and function later in committed neuronal precursors and play important roles in neuronal maturation.[20]

Proneural Sox family proteins are known to reciprocally suppress the expression and activity of SoxB1 proteins in neural precursors. Sox21 and Sox14 together make up the SoxB2 group, which is closely related to the SoxB1 group, and therefore these 2 groups of proteins bind to similar target sequences, but the SoxB2 group possesses a repression domain instead of a C-terminal transcriptional activation domain.[21] They specifically interfere with SoxB1 and consequently promote neuronal differentiation. Bergsland et al. demonstrated that neural lineage-specific genes are first bound by SoxB1 in NSCs, and later bound and activated by Sox11.[22] Sox11 is a member of the SoxC subgroup, together with Sox4 and Sox12, and known to be upregulated as NSCs transit to neural precursor cells (NPCs), and its expression is maintained until neuronal maturation.[9,23]

2.3 GLIAL DIFFERENTIATION

More recently, it has been demonstrated that glial-specific gene sets are also preselected in NSCs through the binding of Sox3.[24] During the subsequent lineage-restriction of glial precursors, astrocyte-specific genes become additionally targeted and activated by Sox9, while oligodendrocyte-specific genes are prebound by Sox9 only and later on they are targeted and activated by Sox10 during oligodendrocyte maturation.[24] Compared with Sox9, Sox8 is expressed in lower amounts at a later developmental stage,[25] and these gliogenic Sox proteins (Sox8–10) make up the SoxE group in vertebrates. Since *Sox8* knockout mice show no obvious abnormalities other than weight loss,[26] partial redundancy has been postulated between *Sox8* and the other two SoxE group genes in mice. A link between SoxE genes and human nervous system pathology has been reported, as *Sox8* has been identified as a genetic risk for multiple sclerosis.[27] Multiple sclerosis is an autoimmune neuroinflammatory disease, in which astrocytes are

increasingly recognized as cells that critically contribute to pathogenic development. Also, the epigenetic status of *Sox10* correlates with its downregulation, and oligodendrocyte dysfunction in schizophrenia has been reported.[28] Taking all these findings together, alteration of the SoxE gene expression changes the functional properties of glial cells, leading to abnormal activation of astrocytes or aberrant myelination of neurons by oligodendrocytes. In both neurogenic and gliogenic phases, Sox proteins preselect gene programs, and later, other members of the Sox family bind and activate the genes. Thus, the Sox family functions to coordinate lineage selection and maintenance of NSCs from early to late stages of neural development (Figure 2 left).

The Olig gene family is composed of Olig1–3, which belong to the bHLH TFs family and are expressed in both the developing and mature CNS. Olig1 and Olig2 have been identified as important factors in the fate choice of NSCs to become oligodendrocytes, and strictly regulate the differentiation, maturation, and myelination processes.[29] On the other hand, Olig3 is weakly expressed in the CNS, but highly expressed in skeletal muscle, testis, and submaxillary gland.[30] It is likely that Olig2 is an earlier stage factor for oligodendrocyte differentiation, while Olig1 appears to play a critical role in the later stage of oligodendrocyte maturation and myelin formation,[31] as it physically associates with Sox10 to activate myelin basic protein expression.[32] A long-term lineage tracing approach for cells that had expressed Olig2 revealed that Olig2 is widely expressed in NSCs of the ventral telencephalon, induces differentiation of NSCs into GABAergic neurons first, and later induces formation of oligodendrocytes.[33] Therefore, Olig2 sequentially controls neuron-to-glial cell fate determination in the ventral telencephalon, whereas Olig1 accomplishes oligodendrocyte differentiation.

2.4 DIRECT REPROGRAMMING

The groundbreaking discovery in 2006 that forced expression of only four TF genes was sufficient to reprogram fibroblasts into a pluripotent embryonic stem cell-like state established the exciting field of direct reprogramming. Neurons were first demonstrated to be directly produced from mouse fibroblasts by forced expression of three neural lineage-specific TFs: *Ascl1*, *Brn2*, and *Myt1l*,[34] and soon after, the addition of *NeuroD1* was reported to further enhance the efficiency of conversion of human fibroblasts to neurons.[35] These direct reprogramming events clearly reveal that TFs have powerful directive force to reprogram cell fate. Since then, many studies have reported successful direct conversion of various cell types to desired cell types, and it has now been shown that direct conversion of astrocytes to neurons is possible with the expression of only a single TF, such as *Neurog2*, *NeuroD1*, or *Ascl1*.[36–39] Similarly, we have shown the conversion of microglia, a major class of immune cells in the CNS, into neurons by NeuroD1,[40] suggesting that NeuroD1 has powerful functions as a pioneer factor that penetrates neuronal gene regions in many cell types. Indeed, exogenous NeuroD1 bound to closed chromatin associated with distinct histone modifications was demonstrated,[40] and thus NeuroD1 can increase the accessibility of DNA, and remodel the epigenetic landscape.

3.Epigenetic regulation that directs neural stem cell fate choice

As we summarized in the previous section, TF expression levels are important determinants of cell behavior, but epigenetic modifications also play critical roles as gatekeepers of cell fate. This is because, except for pioneer TFs, most TFs can only bind to accessible chromatin loci and cannot override epigenetic barriers to transform cells into other lineages. This was evident in our previous work showing that forced expression of the astrocytic TF Sox8 in neurogenic NSCs prepared from mid-gestational cortices did not cause generation of astrocytes in response to differentiation stimuli, whereas astrocytes were thus produced from NSCs prepared at late-gestation.[41] We showed that the chromatin accessibility of TFs dramatically changes genome-widely in NSCs during developmental progression, and consequently stage-dependent daughter cells are generated in response to the same differentiation cue, such as bone morphogenic protein.[42] At least three epigenetic mechanisms allow regulation of chromatin accessibility, namely, DNA methylation, histone modifications, and chromatin remodeling.

3.1 DNA METHYLATION

DNA methylation is an epigenetic modification that generally repressively regulates gene expression and chromatin accessibility. In the initiation of development, inherited DNA methylation patterns in specific genomic regions, called genomic imprinting, are important and abnormalities in these patterns can cause a variety of developmental disorders.[43] For instance, the survival rate of cloned animals generated by somatic cell nuclear transfer (SCNT) is low, and such cloned animals sometimes show abnormalities after birth. Ohgane et al. indicated that because SCNT uses somatic cells with different DNA methylation profiles, the low survival rate of cloned animals results from developmental defects caused by genomic imprinting abnormalities.[44,45]

DNA methylation typically occurs in the cytosine nucleotide of consecutive CpG dinucleotides, and CpG islands are defined as regions where CpG dinucleotides are highly enriched compared to other regions. CpG islands are differentially methylated in a tissue-specific manner, and their methylation levels correlate with gene expression levels.[46]

During cortical development, the global DNA methylation profile of NSCs dynamically changes according to the type of cells that will be produced. We previously demonstrated that the genome-wide DNA methylation patterns in NSCs prepared from mid- and late-developmental stages are different, since Pearson correlation based on the DNA methylation profiles revealed that embryonic day (E)11 and E14 NSCs were clustered near neurons, whereas later-stage (E18) NSCs were clustered near astrocytes.[47] In contrast, hierarchical clustering of global gene expression showed that NSCs at different developmental stages (E11, E14, E18) all clustered together and formed a separate branch from terminally differentiated cells such as neurons and astrocytes. Ultimately, the epigenetic landscape of NSCs at different stages is somewhat different from their respective gene expression patterns and reflects more the differentiation potential of the cells. We also showed that DNA methylation levels in glial-specific genes decrease as the development progresses[47–49], and motif analysis of those regions indicated that the nuclear factor 1 (Nfi) binding motif was remarkably enriched.[47] Since it was previously shown that Nfia binds to glial gene promoters and induces DNA demethylation of those regions,[49] it is conceivable that Nfia can also induce global DNA demethylation around astrocyte-associated genes. Thus, Nfia pays an important role in NSCs for acquiring gliogenic potential.[49]

3.2 HISTONE MODIFICATIONS

Nucleosomes comprise ~147 base pairs of DNA wrapped around histone octamers, which include four core histones (H2A, H2B, H3, and H4). Post-transcriptional modifications, such as methylation, acetylation, phosphorylation, and ubiquitination, can occur on specific residues of each histone core.[50] Proteins known as “writers” insert these modifications, while “reader” proteins recognize them, mediating signals for proper cellular functions.[51] Another set of proteins, termed “erasers”, are responsible for removing these modifications, and thereby epigenetic states can be reversibly altered. These dynamic changes of histone modifications play crucial roles in cell type- and developmental stage-dependent gene expression.[52–54] In this section, we will delve into histone methylation, one of the most extensively studied modifications that exhibit diverse functions dependent on the location of each methyl group in a histone, which occur primarily on lysine and arginine residues.

Trimethylation of Histone H3 lysine 4 (H3K4me3) is commonly recognized as a marker linked to actively transcribed genes.[55] This modification is inserted by the Su(var)3-9/enhancer of zeste/trithorax (SET) domain-containing KMTs (histone lysine methyltransferases) and erased by KDMs (histone lysine demethylases). Reader proteins specific to H3K4me3, such as chromodomain helicase DNA-binding 1 (CHD1), recognize the methylation of lysine residues, leading to the activation of gene expression.[56] Conversely, demethylation of H3K4me3 is associated with gene repression. In the context of neural development, the essential role of KDM1A/(also known as LSD1) in demethylating H3K4me3 for NSC proliferation has been highlighted.[57] Moreover, KDM5A/Jarid1A suppresses astrocytic genes in NPCs, ensuring proper neuronal differentiation.[58] Notably, forced expression of KDM5A/Jarid1A induces reduced promoter activity of *Gfap*, a typical astrocytic marker gene. In addition, knockdown of KDM5A/Jarid1A significantly increased the number of Gfap-positive astrocytes. These data suggest that KDM5A/Jarid1A demethylates H3K4me3 to prevent aberrant astrocytic differentiation of NPCs in the early developmental stage.[58]

Another notable histone modification, H3K9me3, is predominantly localized around pericentric heterochromatin and transposon-inserted regions, supporting its reputation as a long-term gene repression mark. For instance, during the transition from pluripotent embryonic stem cells to the neural lineage, H3K9me3 signals are intensified around genes linked to pluripotency or those unrelated to neural functions.[59] Furthermore, demethylation of H3K9me3 plays a role in neurogenesis. The previously highlighted proneural gene Neurog2, associated with the initiation of neuronal differentiation, is reported to interact with KDM3A/Jmjd1a, leading to the demethylation of H3K9me3 around Neurog2 target genes such as *NeuroD1*.[60] Additionally, the knockdown of KDM3A/Jmjd1a resulted in defective primary neurogenesis, indicating that demethylation of H3K9me3 has a critical role in early neural development.[60]

KMT6B/Ezh1 or KMT6A/Ezh2 serves as a component within the Polycomb repressive complex 2 (PRC2) and facilitates H3K27 methylation.[61,62] H3K27me3 produced by these enzymes is

recognized by PRC1, which in turn, interacts with RNA polymerase II to impede transcriptional elongation, establishing H3K27me3 as a repressive mark.[63] Despite both KMT6B/Ezh1 and KMT6A/Ezh2 being components of PRC2, their behavior diverges according to developmental processes. In early developmental stages, KMT6A/Ezh2 is expressed in NSCs, with its expression gradually diminishing as neural development advances, while KMT6B/Ezh1 is expressed in adult brains.[64] Consequently, KMT6A/Ezh2 primarily regulates the methylation level of H3K27 during ontogenesis, especially in the shift from neurogenic to astrogenic potential in NSCs. Previous findings indicated that during this transition, there was an increase in H3K27me3 within the *Neurog1* and *Neurog2* promoters, resulting in diminished potential for neurogenesis.[65] The importance of KMT6A/Ezh2 is underscored by observations in its deficient mice, which showed delayed astrogenesis.[65] These findings highlight the crucial role of KMT6A/Ezh2 in orchestrating both neurogenesis and astrogenesis for the proper development of the neural system.

3.3 CHROMATIN REMODELING

Packaging a DNA strand over one meter in length into ~20 μm of a nucleus was a challenge that was accomplished during evolution. Not only that, but the DNA still needed to be accessible to the TF complexes. Chromatin is a structure that meets these dual challenges of DNA packaging and efficient access to genetic information, and accordingly chromatin structure dynamically changes during development. The nucleosome is the basic structural unit of chromatin, and its localizations are controlled by chromatin remodeling factors.

Switch/Sucrose-Nonfermentable (SWI/SNF) complex is known to be one of the ATP-dependent chromatin remodelers, regulating chromatin structure by nucleosome unwrapping, translocation, and histone variants replacement using energy generated through ATPase activity in the complex. Brahma-associated factor (BAF) complex is classified as a SWI/SNF class remodeling factor, composed of more than 15 subunits including the ATPase subunit of either brahma or brahma-related gene1 (BRG1), and the scaffold subunit.[66] Those subunits are exchangeable depending on the cell type or developmental stage-specific gene expression profile, resulting in distinct gene targeting through differential interaction by various BAF components. During cortical development, the BAF complex is involved in cell fate specification, cell migration and maturation, and the formation of brain structure.[67] Especially in NSCs in the mid-developmental stage, the expression dynamics of Baf155 and Baf170 contribute to the switching of Baf components and are important for cell fate commitment. Around mid-gestation, Baf170 directly interacts with a repressor complex, Rest (restrictive element-1 silencing transcription factor) and represses *Tbr2* expression, which is important for the expansion of NSC pools, resulting in early neurogenesis from aRGCs.[68] After those neurogenic phases, Baf170 expression is decreased while Baf155 expression is augmented, leading to the switch of the BAF component. Since Baf155 lost its Rest-interacting region in the C-terminal domain, the BAF complex is no longer capable of *Tbr2* inhibition with Rest, and enhanced *Tbr2* expression induces the transition of aRGCs to intermediate progenitors (Figure 3A).[68]

The nucleosome remodeling and deacetylase (NuRD) is another chromatin remodeling complex, where the metastatic tumor antigen family (MTA) acts as the scaffold with its DNA binding motif. NuRD consists of ~10 proteins including histone deacetylase (HDAC), CHD protein, retinoblastoma binding protein (RBBP), and methyl CpG-binding domain protein (MDB).[69] The representative Chd family genes, Chd3, Chd4, and Chd5 are important components of the NuRD complex in the nervous system and exert non-redundant functions during cortical development,[70] as Chd4 is mostly expressed in neural progenitors, but Chd3 and Chd5 are expressed in differentiating neurons and regulate distinct gene sets. Chd4 is involved in the expansion of progenitor pools by binding to *Pax6*, *Sox2*, and *Tbr2* gene promoters to activate their transcriptions that are associated with the proliferation and undifferentiated state.[71,72] After the commitment to neuronal fate, Chd3 and Chd5 expression increases and Chd5 first promotes *Doublecortin* (*Dcx*) and *RhoA* expression for early neuronal migration but is not responsible for layer specification.[70] In contrast, Chd3 promotes late neural migration and regulates the expression of laminar-specific markers, since neurons lacking Chd3 were more likely to express deep layer markers and less likely to express upper layer markers such as *Brn2* and *Cux1* (Figure 3B). Taken together, these findings showed that alterations of the chromatin remodeler complex contribute to different cellular behaviors in each developmental stage.

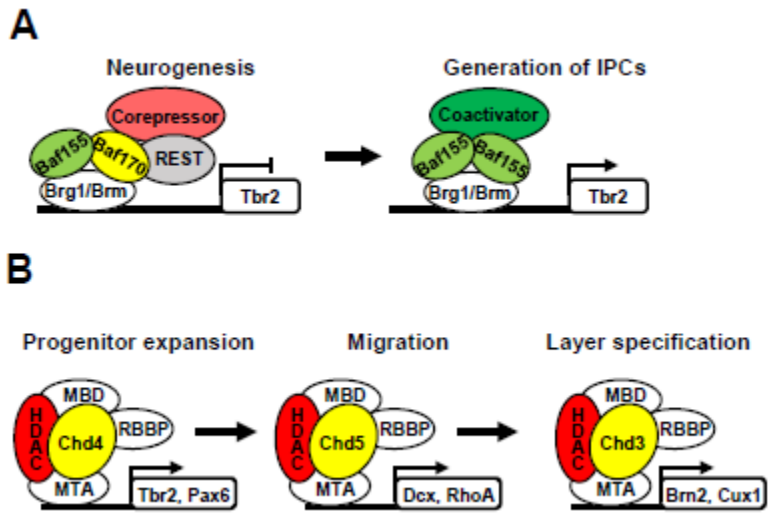

Figure 3. Types and compositions of neural chromatin remodeling complexes.
A. Switching of repressor to activator complex in BAF. Around mid-gestation, when neurogenesis is active, Baf170 interacts with the REST complex and inhibits Tbr2 expression to promote neurogenesis. After the early neurogenic phase, Baf170 expression is decreased and Baf155 is dominant in the BAF complex and activates Tbr2 expression. This switching of BAF components temporarily prohibits neurogenesis and induces expansion of the NPC pool. B. Switching of components in NuRD complex for the proper corticogenesis. In the NPCs, Chd4 promotes Tbr2 and Pax6 expressions associated with the cell division and inhibits neuron differentiation to expand NPC pools. After the commitment of NPCs to neurons, Chd5 starts to activate Dcx and RhoA expression for early neuronal migration. In the later neuronal migration, Chd3 enhances Brn2 and Cux1 expression for the cortical layer specification.

Overall, chromatin-regulating complexes cooperatively change the chromatin structure during neural development by changing its components depending on the developmental stages, and this switching is important for the differential behavior of other TFs that regulate gene expression appropriately.

3.4 CONCERTED CONTROL OF FATE BY THE INTERPLAY BETWEEN TRANSCRIPTION FACTORS AND EPIGENETIC MODIFICATIONS

As an integral example of how coordinated interactions between TFs and epigenetic modifications precisely regulate cell fate transition, we showed that Smads dramatically change their target genes during neural development.[42] Smads are TFs that are activated in the TGF-β signaling pathway and translocated into the nucleus to regulate gene expression.

In early developmental stages, Smads interact with the neurogenic Sox family members (Sox4 and Sox11) and bind to target sites around neurogenic genes to promote neuronal differentiation. At this phase, astrocytic genes such as *Gfap* are inhibited by the epigenetic barrier, i.e., DNA methylation and H3K27me3.[47] Thus, TFs, including Smads, are not able to access those regions. However, in the late developmental stage, astrocytic genes gradually become more accessible through DNA demethylation and H3K27 demethylation, whereas neurogenic genes such as *Neurog1* and *Neurog2* acquire H3K27me3.[42] In this phase, the Sox11 expression level decreases notably while Sox8 and Sox9 expression becomes more dominant, indicating that switching of the Smads' interacting partners occurs. This shift in the partners interacting with Smads lead them to target a different set of genes, from neurogenic to astrocytic, and to promote astrocyte differentiation during later developmental stages (Figure 4A). Therefore, signal-inducible TFs, such as Smads, that are expressed in overall developmental stages facilitate sophisticated cell fate choice by reading the epigenetic landscape as well as switching of their interacting partners.

In addition, interactions between TFs contribute to strict control of the direction of differentiation. Thus, in the late developmental stage, Smads strongly induce the expression of Id (inhibitor of differentiation) and Hes family members, and these inhibit the transcriptional activity of neurogenic TFs such as Neurog1 and Ascl1, resulting in the inhibition of neuronal differentiation of NSCs[73] instead of inducing it as occurs at mid-gestation (Figure 4B). It is also well known that differentiation of NSCs is induced by the coordinated action of multiple external stimuli; for instance, we showed that leukemia inhibitory factor (LIF), one of the astrocyte inducing factors, and BMPs synergistically induce astrocyte differentiation of NSCs.[74] Signal transducer and activator of transcription 3 (Stat3), a TF activated downstream of LIF, interacts with BMP-activated Smads via transcriptional coactivator p300 to effectively induce astrocytic

genes.[74] Of note, not only Smads' binding sites but also DNA demethylation of the Stat3 binding site near astrocytic genes were evident during NSCs development (Figure 4C).[47–49] As such, NSC fate decisions are made by the exquisite interplay between TFs and epigenetic modifications.

4.Organelles that regulate the fate of neural stem cells

Universal organelles such as centrosomes, the Golgi, lysosomes, and mitochondria structurally and functionally change in NSCs during brain development, and dysfunction of organelle-specific proteins causes malformation of the cortex. Indeed, a variety of organelle-specific genes have been found to be associated with human brain developmental disorders. Furthermore, several species-specific genes that function specifically in each organelle have been identified, and it is becoming clear how organelle regulation has controlled cell fate (Figure 5) and contributed to the acquisition of highly developed brains such as those of humans.

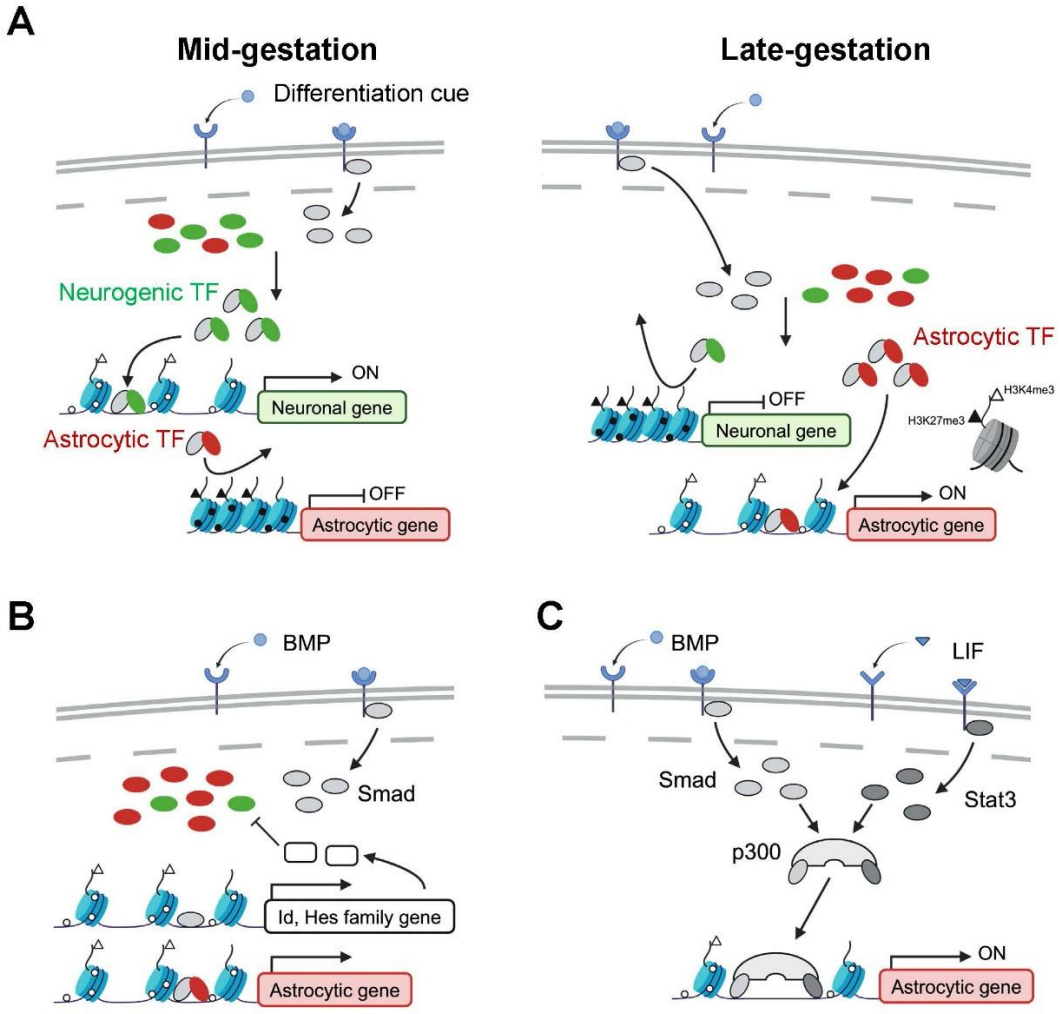

Figure 4. NSC fate decisions made by exquisite interplay between TFs and epigenetic modifications. A. When NSCs receive differentiation signals, the downstream signal-inducible TFs are activated and translocated to the nucleus. In mid-gestation, neuronal TFs are predominant, so signal-inducible TFs interact with them and bind to neuronal gene loci. Around this time, neurogenic gene regions are accessible with DNA hypomethylation and H3K4me3, leading neuronal TF complex bound to those regions to promote neuronal differentiation. Conversely, in late gestation, astrocytic TFs are predominant and form TF complexes that are recruited to astrocytic genes. Around the transition from mid to late gestation, the astrocytic gene loci become accessible with DNA demethylation and loss of H3K27me3, whereas neurogenic genes are repressed with the acquisition of H3K27me3. Thus, astrocytic TFs are able to engage in astrocyte differentiation. B. In late gestation, Smads induce the expression of Id and Hes family genes, and these inhibit the transcriptional activity of neurogenic TFs such as Neurog1 and Ascl1, resulting in inhibition of neuronal differentiation of NSCs. C. LIF and BMPs synergistically induce astrocyte differentiation of NSCs. Downstream TFs of Stat3 and Smads interact via transcriptional coactivator p300 to effectively induce astrocytic genes.

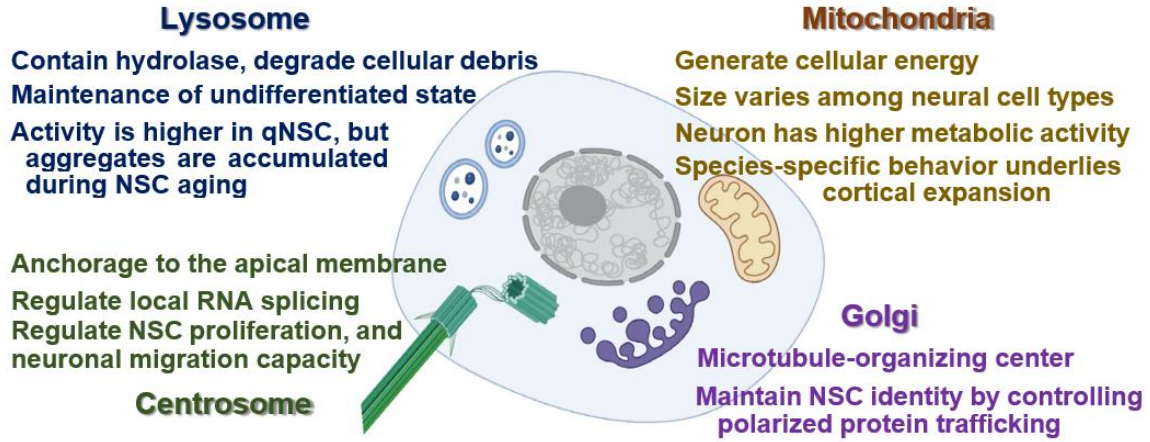

Figure 5. Schematic illustration of organelles that control NSCs' fate.
Specialized intracellular organelles control many functions and NSC fate choice.

4.1CENTROSOMES

In the developing mammalian cerebral cortex, RGCs are the major NSCs that generate neurons and glia, and their centrosome is positioned away from the nucleus at the apical surface of the ventricular zone, forming the primary cilium.[75] During cell division, the cilium retracts from the ventricular surface and the centriole duplicates in a semiconservative manner: the former basal body acts as a template for a new centriole and undergoes maturation to form the mother centriole of the primary cilium, while the daughter centriole undergoes huge rearrangement since newly born neurons require enormous microtubule organizing activity to migrate away from their birthplace. To date, many studies have shown that the centrosome controls the mitotic behavior and size of the cerebral cortex, as reviewed by Wilsch-Brauninger et al.[76] One such example is Centrosomal protein 83 (Cep83), which functions in proper anchorage of the centrosome to the apical membrane, so that the elimination of Cep83 causes disorganized microtubules, and stretches the apical membrane. This activates mechanosensitive YAP (Yes-associated protein), which promotes excessive RGC proliferation, resulting in cortical hypertrophy with abnormal folding.[77] In humans, biallelic mutations of *CEP83* have been found to cause infantile nephrosis and mental retardation,[78] highlighting the importance of CEP83 and centrosome positioning in controlling the development and function of the human brain.

More recently, O'Neill and their group analyzed the centrosome proteome of human iPSCs-derived NSCs and neurons and found that their composition differs between cell types and phases of maturation. They found that PRPF6 (pre-mRNA-processing factor 6), a ubiquitously expressed splicing protein, is enriched at the centrosome in NSCs but not in neurons, and overexpression of a mutant form of PRPF6 ($PRPF6^{R23W}$) found in patients with brain malformation periventricular heterotopia leads to a similar phenotype in mice.[79] It has been shown that certain mRNA transcripts localize to the centrosome, where their local protein translation is detected.[80] One such example is the microtubule-associated protein kinase Brsk2: co-expression of $PRPF6^{R23W}$ with a correctly spliced form of Brsk2, which lacks exon 19, rescued aberrant cell accumulation at the ventricle.[79] Thus, the centrosome of NSCs dynamically regulates RNA splicing locally, controlling the migration capacity of daughter cells.

4.2 GOLGI APPARATUS

Radial glial cells in the developing neocortex exhibit unique bipolar epithelial cell properties with apical projections to the ventricles and basal projections to the basement membrane, resulting in polar localization of various organelles. In RGCs, the Golgi apparatus is distributed in the apical process, between the apical plasma membrane and the nucleus, and no membrane structures identifiable as Golgi cisternae have been observed in the basal process.[81] As a result, membrane glycoproteins in RGCs of the basal process lack Golgi processing, and traffic to cell surface directly from the ER (endoplasmic reticulum). Notably, stimulus-dependent apical Golgi distribution in the RGC is important for maintaining stem cell identity, possibly by controlling polarized membrane protein trafficking during corticogenesis.[82] Each RGC contributes to the construction of the cortical layer by giving rise to daughter cells that maintain their properties as RGCs and detach from the apical surface toward the basement membrane. During this delamination, the Golgi undergoes basal translocation and starts to function as the microtubule-organizing center.[81] The proper functional rearrangement of the Golgi is particularly important, as mutations in Golgi proteins such as ARF1 (ADP-ribosylation factor) and

ARFGEF2 (the guanine nucleotide exchange factor for ARF1) that cause cortical malformations in human have been reported.[83]

4.3LYSOSOMES

Lysosomes are membrane-enclosed acidic organelles that contain numerous acid hydrolases and degrade macromolecules and cellular debris. For a long time, lysosomes were considered as merely 'cellular incinerators' but now there is strong evidence that they have a broad range of cellular functions and even regulate cell fate.[84] Yuizumi et al. revealed the importance of lysosomes in the regulation of NSC differentiation.[85] They showed that knockdown of *Tfeb* and *Tef3*, master regulators of lysosomal biogenesis, cause premature differentiation of NSCs, whereas forced expression of an active form of Tfeb suppressed neuronal differentiation and promoted maintenance of the undifferentiated state of NSCs.[85] Thus, lysosomes function in the maintenance of the undifferentiated state of mouse NSCs during cortical development.

In mammals, most adult NSCs are in a quiescent state (qNSC), in which enlarged lysosomes were observed compared to those in active NSCs.[86] Not only do aged qNSCs accumulate more protein aggregates in lysosomes, but a decrease in the abundance of aggregates leads to the qNSCs' rejuvenation.[86] It has been reported that activated epidermal growth factor receptor (EGFR) is more rapidly degraded in qNSCs via lysosomes than in aNSCs.[87] Since EGF is a mitogen that enhances NSC proliferation and the activation of EGFR signal is sufficient to stimulate qNSCs to enter the cell cycle *in vivo* during early adulthood,[88] higher lysosomal activity of qNSC enables efficient and rapid degradation of activated EGFR to prevent the NSC exit from quiescence. It has also been demonstrated that conditional ablation of *Tfeb* increases NSC proliferation in the dentate gyrus of mice,[87] and thus lysosomal degradation capacity is an important regulator for the maintenance of NSCs.

4.4MITOCHONDRIA

Cells are consuming energy every moment for their survival, proliferation, and differentiation. This energy is supplied through metabolic processes such as glycolysis, the TCA cycle, and oxidative phosphorylation. Among those pathways, oxidative phosphorylation (OXPHOS) is known for its efficiency of ATP generation. Since OXPHOS is conducted in mitochondria, mitochondria are one of the most important organelles for metabolic activity in cells. Within the CNS, neurons have well-developed mitochondrial functions. This is because neurons consume more energy than other cell types as they need to maintain ion gradients across the cell membrane to transmit signals and this consumes large amounts of ATP.[89] Therefore, mitochondrial dysfunction can impair neuronal function.[89] Within the cell, mitochondria repeatedly fission and fuse to ensure the quality of their function. Mitochondria possess a unique circular genome that encodes genes associated with mitochondrial functions, including ATP synthesis and the electron transport system. However, these genes are more susceptible to stress-induced mutations compared to nuclear genomic DNA, and this may cause mitochondrial dysfunction. Through continuous fission and fusion, the cell controls the number of functioning mitochondria and discards damaged mitochondria through mitophagy.[90]

In addition to these basic functions, mitochondrial fission and fusion are involved in the cell fate decision. In 2020, Iwata et al. reported that mitochondrial size in daughter cells after mitosis is associated with the regulation of neural development.[91] They showed that when mitochondrial fusion occurs in the daughter cells after mitosis and mitochondria increase in size, they maintain NSC characteristics. On the other hand, when mitochondria fission occurs in the daughter cells after mitosis, the cells start to differentiate into NPCs and subsequently into neurons. These findings suggest that mitochondrial dynamics in NSCs after mitosis regulate the NSCs' cell fate decisions.[91] Interestingly, mitochondrial dynamics also contribute to neuronal development in a species-specific manner.[92] In general, mitochondria are small and sparse in newborn neurons, but gradually increase in size during maturation and exhibit higher metabolic activity.[91] For example, it is known that mitochondria in mouse newborn cortical neurons reach maximal levels of growth and size in 3 weeks. However, this takes several months or more in humans.[92] More recently, Iwata et al. demonstrated that enhancement of mitochondria oxidative metabolism by pharmacological and genetic manipulation of human developing cortical neurons accelerates neuronal maturation, as neurons exhibit more complex morphology and increased electrical excitability. Conversely, developmental rates of mouse neurons were reduced when mitochondria metabolism was inhibited.[92] These different timelines of mitochondrial maturation in newborn neurons contribute to the species-specific tempo of neural development.

Among the differences between mice and humans, the human-specific gene *ARHGAP11B* is involved in species-specific mitochondrial behavior.[93] ARHGAP11B, localized in mitochondria, inhibits mitochondrial degradation and cell death by preventing mitochondrial permeability transition pore (mPTP) opening.[93] The mPTP opening inhibits

glutaminolysis, resulting in the promotion of cell proliferation. And deficient glutaminolysis can cause microcephaly.[94] Thus, ARHGAP11B is involved in the expansion of neurogenic progenitor pools and contributes to the formation of larger brain sizes than those in other mammals. Taken together, the findings indicate that mitochondria regulate NSC proliferation and neuronal maturation by modulating metabolic activity during neural development. In addition, species-specific mitochondrial behavior is associated with different brain sizes as a result of regulating the pace of neural development.

5.Conclusions

Since cortical development is a dynamic process, our understanding of the diversity of gene expression associated with the formation of different neuronal subtypes and subsequent cortical layers is still limited. Furthermore, recent studies demonstrating not only heterogeneity of neurons but also functional heterogeneity of glial cells, including astrocytes, oligodendrocytes, and microglia, have been reported,[95–97] suggesting that gene expression and chromatin structural analyses in NSCs at single-cell resolution will be important for elucidating the mechanisms by which heterogeneity is established in these cells during development. An important mission in therapeutic applications is to efficiently lead NSCs to differentiate into appropriate specific cell types that are needed at that particular time. In this regard, not only transcriptional and epigenetic regulation but also the characterization of organelle function in each neural cell type will be of great importance. Since various intracellular organelles are highly developed for the performance of higher brain functions, such as in humans, we cannot afford to neglect these issues.

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