**Subtype specification of cerebral cortical neurons in their immature stages**

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**ABSTRACT**

The diversification of neuronal subtypes during corticogenesis is fundamental to the establishment of the complex cortical structure. Although subtype specification has been assumed to occur in neural progenitor cells, increasing evidence has begun to reveal the plasticity of subtype determination in immature neurons. Here, we summarize recent findings regarding the regulation of subtype specification during later periods of neuronal differentiation, such as the post-mitotic and post-migratory stages. We also discuss thalamocortical axons as an extra-cortical cue that provides information on the subtype determination of immature cortical neurons.

**INTRODUCTION**

The mammalian isocortex or neocortex, which forms a six-layered structure histologically, comprises hundreds of different neuronal ‘subtypes’ that differ in cell morphology, axonal/dendritic projection pattern, gene expression profile and others [1-3]. This diversification of neuronal subtypes is the basis for the execution of higher brain functions. However, the full picture of how and to what extent neurons become diversified during corticogenesis is yet to be determined.

Almost all excitatory neocortical neurons arise from common neural progenitor cells (NPCs) in the pallium, while other neuronal populations (e.g., Cajal-Retzius neurons and inhibitory interneurons) arise from different brain regions [4-6]. A diverse repertoire of cortical subtypes is generated from NPCs in a sequential manner; the subtypes that eventually locate in the deepest layer are generated at the earliest stage, followed by the production of subtypes that locate above the deepest layer. This process is repeated until the six-layered structure is formed, resulting in a so-called inside-out pattern of neuronal layering [7].

Because each layer in the neocortex is occupied by the neurons generated at around the same time [8,9], a model whereby NPCs change their potential over time, thereby determining the eventual subtype of newly generated neurons, has been widely accepted (Figure 1A) [10]. In fact, this type of mechanism is utilized in some cases of subtype specification in the *Drosophila* nervous system [11]. In mammals, seminal studies from McConnell’s group involving heterochronic transplantation experiments have demonstrated that cortical NPCs undergo a progressive restriction of subtype-producing potential during cortical development; early NPCs, which produce layer 6 neurons, can differentiate into superficial/upper layer neurons when transplanted into the late VZ, while late NPCs, which produce layer 2/3 neurons, transplanted into the early VZ do not differentiate into deep layer neurons [12,13]. However, only a few fate-determining factors with temporally controlled expression profiles in NPCs have been identified in the mammalian nervous system [14-17]; thus, the molecular mechanisms that regulate the temporal changes of NPCs remain largely unknown.

In addition to the aforementioned model, it is also conceivable that cortical lamination or appropriate cell positioning in the cortical plate play a role in the full differentiation or maturation of the neurons. According to this model, the positioning of immature neurons is primarily regulated by a mechanism utilizing information corresponding to their birthdate, followed by the determination of eventual subtypes via surrounding environmental signals (Figure 1B). This hypothesis has not attracted much attention because the neocortex of *reeler* mice (*reelin* deficient mice), which exhibits a highly-disorganized layer structure [18], possesses an almost normal set of neuronal subtypes [19,20]. However, the *reeler* neocortex still has inverted or mirror-imaged layer structures [21], thereby potentially retaining laminar information, leaving it an open question as to whether all laminar fates are determined intrinsically in NPCs or whether they can be modulated during later stages. Here, we will summarize progress that has begun to reveal the regulation of subtype specification of cortical neurons during later periods, such as the post-mitotic and post-migratory stages of immature neurons.

**Identification of subtype-determining genes**

Recent progress towards understanding the mechanisms of cortical subtype specification involves the identification of a dozen subtype-determining genes, most of which are transcription factors. For example, Macklis’ group identified several subtype determinants using an elegant system, revealing gene expression profiles in different cortical subtypes that were separately collected based on different axonal projection patterns [22,23]. Our group reported that two types of transcription factors, Rorb and Brn1/2, regulate the specification of layer 4 and layer 2/3 neurons, respectively [24] (see below).Other factors were also identified and have been reviewed elsewhere [1-3].

The scenario for the temporal specification of NPCs assumes that different fate determinants are expressed sequentially in NPCs, similar to the *Drosophila* nervous system [11]. However, many of the identified fate determinants, such as Tbr1, Satb2, Bhlhb5, and Zfpm2, begin to be expressed in post-mitotic migrating neurons or post-migratory neurons [25-28,23], albeit with a few exceptions (e.g., Fezf2 [17,29] and Brn factors [15]), implying that subtype determination generally takes place in post-mitotic immature neurons. An alternative explanation is that these expression profiles are caused by a ‘timer’-like mechanism, whereby the expression onset of fate determinants in immature neurons is already programmed in NPCs [30]. Further studies will be needed to reveal the exact timing of when neuronal subtypes are determined and the underlying molecular mechanisms.

**Plasticity of immature neurons in subtype specification**

Recent findings on cortical subtype determinants have opened the gate to understanding plasticity in NPCs and immature neurons. For example, ectopically expressed subtype determinants convert transfected cells to the subtypes that these factors normally control. The ectopic expression of the cortical deep layer determinant Fezf2 in ventral NPCs, which are normally fated to generate striatal neurons, is sufficient to instruct these cells to become cortical deep layer-like neurons [31]. Notably, this type of fate conversion was also observed in post-mitotic immature neurons. Ectopically expressed Fezf2 in post-mitotic superficial layer neurons reprograms the cells into deep layer-like neurons, including their molecular identify, morphology, physiology and functional input/output connectivity, indicating a high level of plasticity in post-mitotic cortical neurons [32]. Interestingly, this fate conversion does not occur in mature neurons (i.e., after postnatal day 10), suggesting a ‘critical period’ in this fate conversion process.

**Extrinsic control of subtype specification in immature neurons**

The aforementioned fate conversion occurs when a subtype determinant is forcedly expressed; thus, whether the fate determination of immature neurons is controlled by endogenous extracellular signals remains uncertain. To examine the events that occur in immature neurons and their possible involvement in subtype specification, our group screened for genes that are highly upregulated in immature neurons that have just finished radial migration beneath the marginal zone [33] or within the primitive cortical zone (PCZ) [34]. Among them, some genes were eventually expressed in a certain subtype of neurons [33]. Protocadherin20 (Pcdh20), which belongs to the cadherin superfamily, was identified as a gene that fulfills these criteria. Pcdh20 begins to be expressed in post-migratory neurons destined to become layer 4 neurons (‘future’ layer 4 neurons) and is expressed in layer 4 of the mature neocortex. Pcdh20 knockdown experiments revealed that a reduction in Pcdh20 expression causes the future layer 4 neurons to be located in layer 2/3, with barely, if any, effect on the proliferation of NPCs and the migratory behaviors of neurons. Surprisingly, these ectopically positioned neurons acquire the features of the surrounding neurons (i.e., layer 2/3 neurons) and lose their original characteristics (i.e., layer 4 neurons). Moreover, the disruption of neuronal positioning using another method recapitulates this fate conversion. These results indicate that the subtype determination of layer 4 neurons occurs during post-mitotic stages, at least in part, and can be influenced by extracellular signals [35]. On the other hand, another study reported that when future layer 2/3 neurons are forced to be placed into layer 5, they do not acquire layer 5 features [36]. These results suggest that a variety of extracellular signals may modify fate specification in a subtype-specific manner. In fact, we found that the maturation of layer 4 neurons is controlled by thalamocortical axons (TCAs), which mainly project to the layer 4 neurons in mature neuronal circuits (see below).

The occurrence of the fate conversion mentioned above raises the possibility that future layer 2/3 and layer 4 neurons have common characteristics in their immature stages. Indeed, both immature layer 2/3 and layer 4 neurons that have just finished radial migration express the transcription factor Brn2, which is eventually expressed in layer 2/3 but not in layer 4 [24]. In future layer 4 neurons, the expression of another transcription factor, Rorb, is sharply increased during maturation, while Brn2 is down-regulated. Further investigation of the regulatory mechanisms involving Brn2 and Rorb revealed a mutually repressive interaction, which could work as a positive feedback loop (e.g., an increase in Rorb expression causes a decrease in Brn1/2 expression, leading to the further upregulation of Rorb), and an essential role in the specification of layer 2/3 and layer 4 neurons. These results strongly support the notion that the eventual specification of superficial layer subtypes is modified in post-mitotic immature neurons [24]. Of special note, the onset of Rorb expression was observed in developing layer 4 neurons a few days after birth, while Rorb expression during embryonic stages was confined to future layer 5 neurons. Thus, Rorb cannot be used as a layer 4 marker in analyses of embryonic brains, as is often the case when analyzing mutant mice that show perinatal lethality.

**Regulation of subtype specification by thalamocortical axons**

The cerebral cortex comprises dozens of functional areas such as motor, somatosensory and visual areas [37]. Cortical areas differ in several aspects including histological organization, which mainly reflects differences in the thicknesses of layers, patterns of connectivity and molecular properties, indicating that the number and characteristics of the components (i.e., neuronal subtypes) vary in different areas. Therefore, the arealization process, especially when accompanied by changes in gene expression profiles, can be regarded as a diversification of neuronal subtype specification.

Two hypotheses have been postulated for the process of arealization of the neocortex. One is a proto-map model, in which regional differences are predetermined at early developmental stages [38]. This model is supported by the findings that the gradient expression of several fate determinants in NPCs along the rostrocaudal, mediolateral and dorsoventral axes play critical roles in arealization (e.g., expression patterns of Pax6 [39], Emx2 [40], Sp8 [41,42] and Coup-TF1 [43]). The other is a proto-cortex model, in which input/influence from outside of the neocortex regulates arealization while NPCs have equivalent potential [44]. This model requires the regulation of differentiation in immature neurons, which is of interest in this review.

TCAs have been postulated as an extra-cortical cue in the proto-cortex model because of their region-specific projection to the cortex from a given thalamic nucleus (e.g., projection to the primary somatosensory area from the ventral posterior medial nucleus [VPM] and to the primary visual area from the lateral geniculate nucleus [LGN]) [37]. This notion has been supported by several studies. Surgically induced ectopic axonal projection from the LGN (the axons of which normally project to the visual area) to the auditory area is sufficient to convert this area into a visual-like area [45]. Another study showed that the engraftment of visual areal tissues into the somatosensory area can result in the acquisition of some ‘somatosensory’ characteristics [46].

Recent genetic studies also support the notion of TCAs as an extra-cortical source for arealization. The genetic alteration of the LGN-visual area projection resulted in the dysregulation of the delineation of primary and high-order visual areas as well as areal size [47,48]. Moreover, Jabaudon’s group showed that the identity of layer 4 neurons is controlled by modality-specific thalamocortical signals [49]. Through the genetic disruption of the VPM, the primary somatosensory area, which receives input from the VPM, alternatively receives input from the posterior nucleus, a non-specific nucleus, which normally sends axons to layer 4 neurons in the secondary somatosensory area. This change instructs the primary somatosensory area to differentiate into the secondary somatosensory-like structure, which eventually responds to nociceptive stimuli, which normally activate the secondary somatosensory area [49].

Our finding that layer 4 subtype specification requires the correct positioning of immature neurons prompted us to examine whether TCAs provide a positional cue for the differentiation of superficial layer neurons. The analysis of mutant mice in which virtually all TCAs were absent in the neocortex revealed a decrease in layer 4 neurons and an increase in layer 2/3 neurons instead, suggesting that TCAs are essential for determining the number of neurons in superficial layers [35]. We speculated that TCAs induce the expression of Rorb or reduce that of Brn1/2 in future layer 4 neurons, unbalancing the mutual repressive interaction between these factors and leading to the establishment of Rorb-high and Brn1/2-low expression in layer 4 (Figure 2). The influences of TCAs on other layers have also been described [47,48,50], implying that TCAs may act on multiple layers to establish area-dependent features.

Although several TCA-derived molecules that affect the dendritic development of cortical neurons have been identified [51], the overall molecular mechanisms of how TCAs regulate cortical subtypes remain to be determined. Because the timing of synapse formation between TCAs and layer 4 neurons corresponds to that of the maturation process of layer 4 neurons (e.g., the onset of Rorb expression and the morphological change into stellate cells), a neuronal activity-dependent mechanism has been assumed. Although several studies have shown that TCA input is critical for the formation of a barrel structure, a specific feature of the mature primary somatosensory area in rodents [52,53], the effect of synaptic TCA input on subtype specification has remained unclear. A recent study using conditional knockout mice that showed no synaptic transmission from TCAs to cortical neurons due to removal of all the glutamate transporters from thalamocortical neurons revealed that synaptic input is required for the maintenance of the cortical layer structure as well as that of subtype specific marker expressions [50]. Importantly, although barrel structures are missing in this mutant line similar to the observations made in previous studies, the specification of layer 4 neurons, as judged by the expression of molecular markers, occurred correctly until around one week after birth. Therefore, we speculate that the initial specification of layer 4 neurons does not require synaptic transmission but rather is instructed via molecular mechanisms evoked by direct cell-cell interactions or secreted molecules. Molecular interactions accompanied by synaptic transmission may also play a role. During the maturation stages of layer 4 neurons, synaptic input through TCAs would induce barrel structures [52,53] and then maintain the layer structure and neuronal subtypes after maturation [50]. More generally, this kind of mechanism can control subtype specification not only in the barrel structure, but also in the radially oriented columnar structure, which is a feature of the cerebral cortex [38,54].

**Concluding remarks**

One of the fundamental questions to be solved in cortical development is how the eventual ‘layer’, which is composed of several subtypes of neurons, is formed during development. A prevailing idea was that the formation of cortical layers is not regulated by precise mechanisms, but by a process whereby neurons finish radial migration and accumulate. However, some studies suggest that neurons are actively regulated so that they become located in their eventual positions irrespective of radial migration processes. An in vitro study has shown that cortical neurons acquire a birth-date-dependent segregation mechanism before they finish radial migration [55]. In Pcdh20-knockdown neurons in vivo, the process by which the post-migratory future layer 4 neurons begin to locate to the lower part of the superficial cortical plate is specifically disrupted [35]. Another study has reported that Robo1-knockdown neurons do not reach their final position correctly, mainly because of a defect in the process of cell positioning after radial migration [56]. The accumulation of cortical neurons in the PCZ, the outermost cell-dense region of the CP, has also been proposed to be involved in proper laminar formation via mechanisms such as cell sorting among the neurons [57,58].

Much attention has been paid to understanding the proliferation/differentiation of NPCs and the migration of neurons during corticogenesis. However, processes after radial migration, including subtype specification and cell positioning, also play important roles in establishing the highly organized cerebral cortex. What, then, is the advantage of forming a highly organized structure? Recently, Reelin, an essential extracellular matrix protein that regulates cortical lamination, has been reported to play a role in the formation of neuronal networks [59]. Although the paper also claimed that the inside-out positioning of neurons is essential for network formation, the *reeler* neocortex has a markedly disorganized cortical structure; thus, whether inside-out positioning is indeed involved in this process remains an unsolved question. Nevertheless, this result strongly suggests that an organized cortical structure is pivotal to the proper formation of neuronal networks, thereby enabling the execution of proper neuronal functions.

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**Figure legends**

**Figure 1. Models of subtype specification of cortical neurons.**

A. The differentiation potential of NPCs changes over time, resulting in the generation of different neuronal subtypes. The colors of the NPCs represent the different characteristics of the NPCs.

B. The neuronal subtypes are not determined during immature stages (left). After the positioning of the neurons in the cortical plate, they acquire their own characteristics by receiving extracellular signals, such as environmental cues (right).

**Figure 2. Subtype specification of post-migratory superficial layer neurons.**

Future layer 4 neurons are not yet specified when they reach the outer-most part of the cortical plate. In fact, they express Brn2, a layer 2/3 marker in the mature neocortex (upper left). After radial migration, future layer 4 neurons are positioned in the lower part of the cortical plate in a Pcdh20-dependent manner (upper middle). This positioning allows them to come in contact with thalamocortical axons (TCAs), thereby initiating programs to differentiate into layer 4 neurons. The programs include a mutually repressive interaction between Rorb and Brn1/2. Future layer 4 and layer 2/3 neurons finally begin to express Rorb and Brn1/2, respectively, with these factors playing critical roles in the differentiation of each neuronal subtype (upper right). If any of these steps is blocked, the future layer 4 neurons do not differentiate into layer 4, but instead acquire layer 2/3 characteristics (bottom).