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Review article

# Genetic regulation of proliferation/differentiation characteristics of neural progenitor cells in the developing neocortex

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**Abstract**

Brain size variation among different mammals is tightly associated with different levels of cerebral function. Mechanisms that regulate the number of neurons and hence the size of the brain must be at least partially embedded within the very early phase of neocortical development, that is, embedded in proliferation/differentiation characteristics of the neural progenitor cells (NPCs) of the neocortex. Here we review a sequence of critical events through which the neocortex is formed in the embryonic forebrain, with particular emphasis on cell cycle kinetics of the NPCs that produce non-GABAergic projection neurons, the majority of neurons in the neocortex. In general, the critical parameters that determine the total number of cells produced by a given progenitor population through a sequence of cell cycles are (1) the number of cell cycles that constitute the production period and (2) the probability of cell cycle exit (Q fraction or Q) of progenitor cells for each of the cell cycles. We will also review molecular mechanisms that modulate the critical parameters above, with a special reference to the cell cycle regulatory protein $p27^{Kip1}$, inhibitor of G1 phase progression of the cell cycle. Finally the neocortical dysgenesis caused by genetic modification in mice where $p27^{Kip1}$ is either deleted or overexpressed is presented as examples of neuron number changes and resultant neocortical dysgenesis by Q fraction alteration.
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*Keywords:* Neocortex; Development; CDKI; Cell cycle kinetics

## 1. Introduction

Size of the brain differs greatly among different mammals. Even among primates, the brain size variation is significant; human has average weight of 1350 g and macaque monkeys 100 g. Moreover, the size of the brain differs significantly even within a species; brain-size variance in human is as much as 1000–1500 g.

Is this brain size variation associated with different levels of cerebral function? That is, the bigger brain, the better function? The answer could be quite straightforward as far as brain size variation and functional evolution across different species are concerned. That is, in view of phylogeny or evolution, the brain size or neuron number is the major determinant of brain functions. Number of neurons is by no means the only parameter which determines the brain size. Nonetheless, it takes bigger brain to accommodate more neurons: it takes more neurons to foster higher cortical function that we human being enjoy.

However, when it comes to the issue of brain size variation within a single species, the issue is not that simple. We know that those children such as Sotos syndrome with large brain size suffer from impaired higher cortical function. We also know that those with abnormally small brain such as those with cell cycle related gene mutation also suffers from impaired higher cortical functions.

Thus, within a single species it seems that the number of neurons and size of the brain are under strict

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biological constraint: producing an appropriate number of neurons for the right size of the brain seems to be a prerequisite to ensure normal brain functions.

The mechanisms that regulate such an important strategy must be at least partially embedded within the very early stage of neocortical development. Reviewed in this article is a sequence of cellular and molecular events that occur during the neocortical development *in utero* with particular emphasis on the proliferation/differentiation characteristics of neural progenitor cells (NPCs).

## 2. Three steps of neocortical histogenesis

The cerebral cortex of mammals assumes six-layered structure, composed by excitatory non-GABAergic projection neurons, inhibitory GABAergic interneurons and glial cells. Among those, projection neurons, the major component (approximately 80%) of neocortical neurons, are produced from NPCs within the ventricular zone (VZ) surrounding the lateral ventricles of the embryonic cerebral wall [1]. The initial step of neuron production is cell divisions of NPCs within the VZ (Fig. 1, “neuronogenesis”). Then, the postmitotic young neurons produced by NPCs migrate radially toward the pial surface (Fig. 1, “migration”) [2]. Finally, within the six-layered neocortex, further maturation of the neural network occurs through synaptogenesis, myelination and apoptosis (Fig. 1, “maturation”) [3]. During the same period of neocortical histogenesis, inhibitory GABAergic interneurons are being generated within the embryonic striatum and migrate tangentially into the cerebral cortex [4].

It has been long established that early-born projection neurons are distributed in the deep layers of the neocortex (i.e., layers V–VI), while the later-born projection neurons migrate into the superficial layers (i.e., layers II–IV) (Fig. 1, curved lines). Analysis in mice has revealed that there is a strict correlation between the layer position of the projection neurons within the neocortex and their cell cycle of origin [5] (Fig. 2). Such correlation is preserved across different regions of the cerebral cortex, which implies that regulatory mechanisms for the projection-neuron phenotype, represented here by their layer distribution, are indeed embedded in the process of proliferation/differentiation of the NPCs.

## 3. Determinants of neuron numbers

There are only two parameters that determine the total number of cells to be produced by a given progenitor population through a sequence of cell cycles: (1) the number of cell cycles that constitute the production period and (2) the probability of cell cycle exit (Q fraction) of progenitor cells for each of those cell cycles [9]. In case of neocortical histogenesis, the first of the two parameters is the number of cell division cycles that NPCs in the VZ execute during the period of neuronogenesis (Fig. 1).

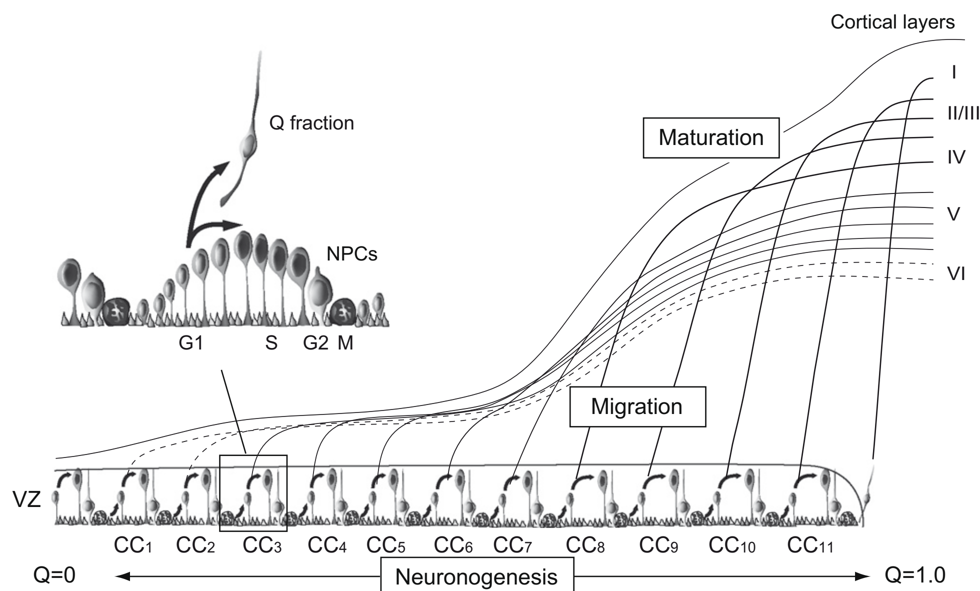


Fig. 1. Sequence of events of neocortical histogenesis. The initial step of neocortical histogenesis is neuronogenesis which occurs within the ventricular zone (VZ). Nucleus of NPCs undergo so-called interkinetic nuclear migration through G1, S, G2, M phases of the cell cycle (upper left panel). Over the course of neuronogenesis, NPCs execute 11 cell cycles ($CC_1$–$CC_{11}$, abscissa). The young projection neurons (upper left, Q fraction) produced by NPCs then migrate radially toward the pial surface (series of curved lines). Finally, within the six-layered neocortex (layers II/III–VI), further maturation through synaptogenesis, myelination and apoptosis occurs. The early-born neurons are destined for the deepest cortical layers (dotted curved lines), whereas the later-born neurons are destined for more superficial layers (solid curved lines). Adapted from Takahashi et al. [5].

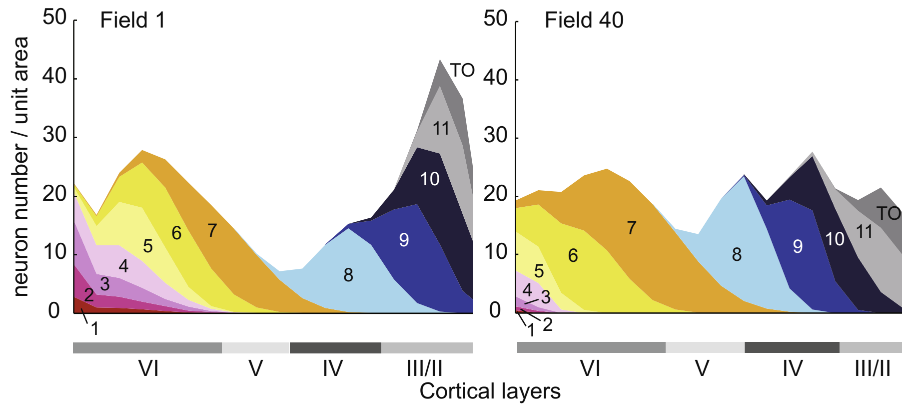


Fig. 2. Layer distribution of projection neurons and cell cycle of origin. The output from each of the 11 cell cycles of NPCs is color-coded and numbered. TO: the terminal output after cell cycle 11 at the end of the neuronogenesis. Layer distributions of cohorts of neurons are mapped at postnatal day 22 with respect to neocortical layer in both field 1 and field 40 in the mouse neocortex. Note that correlation is preserved across different regions of the cerebral cortex. Adapted from Takahashi et al. [5].

To estimate the number of cell division cycles of NPCs, the outset and the termination of neuronogenesis must be first defined. The outset of the neuronogenesis is defined as the time point when a small group of NPCs exits the cell cycle for the first time during neuronogenesis to become mitotically quiescent (or Q): the termination of neuronogenesis is defined as the time point when the last group of NPCs leaves the cycle (Fig. 1) [7]. In the mouse primary somatosensory cortex, the neuronogenetic period has been determined to be 6 days or 144 h in length, occurring from embryonic day (E) 11 through E17 [7].

Next the total cell cycle length must be determined for each of the cycles that constitute neuronogenetic period to estimate the number of cell cycles during the defined neuronogenetic period. The total cell cycle length can be experimentally measured by cumulative labeling analysis with a single S-phase tracer, bromodeoxyuridine (BrdU) [6]. It revealed that the number of cell cycles constituting neuronogenetic interval is about 11 in mice [8].

The value of Q is the fraction of NPC population that exits the cell cycle at a given point of neuronogenesis. It follows that before neuronogenesis begins, Q of the NPC population must be zero and that Q must ascend to become 1.0 at the completion of neuronogenesis (Fig. 1).

Q can be experimentally determined by two-S-phase-tracer protocol with BrdU and tritiated thymidine or iododeoxyuridine [7]. Normal ascending pattern of Q in mice shows slow increase during the early phase of neuronogenesis until Q becomes 0.5 at around the 8th of 11 cell cycles on E14 (Fig. 3A). At the point when Q is 0.5, the half of the NPC population remains in the proliferative cell cycle while the other half leaves the cycle. At this moment, the population of the NPCs reaches its maximum size and starts diminishing towards extinction at the end of neuronogenesis.

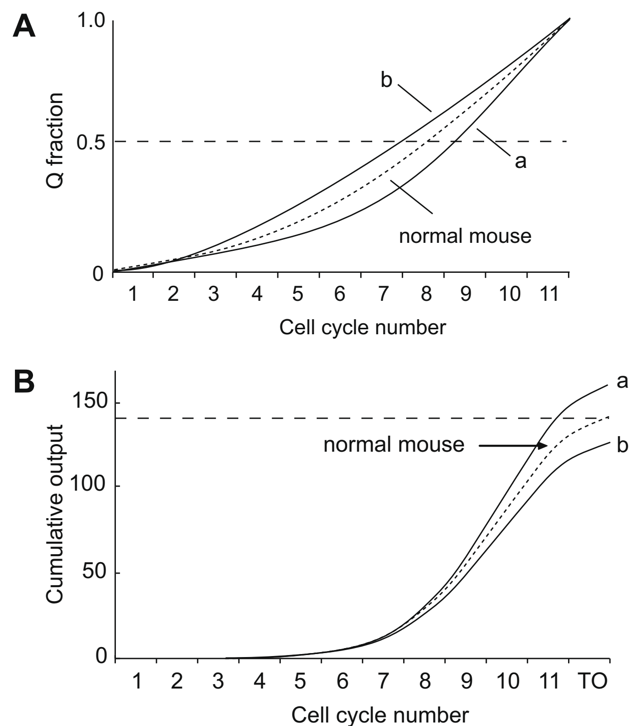


Fig. 3. Q ascent pattern determines the overall neuron production through a complete set of cell cycles. Dotted line: pattern observed in normal mouse. Solid lines a and b: altered patterns of Q ascent and its consequence in cumulative output. See text for details. Adapted from Takahashi et al. [9].

Once the number of cell cycles that NPCs execute and the ascending pattern of Q from 0 to 1.0 are both determined, the total number of projection neurons to be produced from a single NPC over the course of neuronogenesis is mathematically estimated (Fig. 3B) [9]. According to the experimental data in mice, the total number of projection neurons estimated such is about 140 cells (Fig. 3B, ”normal mouse”) [9].

If a pattern of Q ascent is to be altered with the cell cycle number being unchanged (Fig. 3A), the total number of projection neurons would be significantly modified (Fig. 3B). For example, if the Q-ascent curve were only slightly bended downward, that is, if the value of Q were only slightly decreased in the course of neuronogenesis, the total number of projection neurons would be substantially increased. On the contrary, if Q-ascent were shifted upward, the total number of projection neurons would be much decreased.

## 4. Molecular mechanisms of G1 phase progression and decision making characteristics of NPCs

Cell cycle lengths of the mouse neocortical NPCs estimated by cumulative S-phase labeling with BrdU gradually increases from 8 to 18 h over the neuronogenetic interval (Fig. 4) [8]. This systematic increment is exclusively due to prolongation of G1 phase from 4 to 12 h: the lengths of other cell cycle phases (G2, M and S) remain relatively stable (Fig. 4) [8]. This observation strongly suggests that G1 phase of the cell cycle is critically involved in the orderly progress of neocortical histogenesis. In fact, G1 phase is considered to be critical phase in terms of fate decision making of NPCs, that is, whether to remain in the cell cycle as a part of the proliferative pool or to leave the cycle as a young migrating neuron (as a part of Q fraction) [10].

Progression of the cell cycle through each of the cell cycle phases is precisely controlled by a set of proteins called cyclins and cyclin dependent kinases (CDKs) [10]. As far as molecular mechanisms of G1 phase progression are concerned, cyclin Ds/CDK4/6 complex and cyclin E/CDK2 complex are the critical sets of molecules: they hyperphsophorylate retinoblastoma protein (Rb): hyperphsophorylated Rb in turn is essential for S-phase progression [10].

There is a group of CDK inhibitors (CDKIs), which serves as negative regulators of cell cycle progression. As is the case with cyclins/CDKs, it is only upon specific pairs of cyclins/CDKs that each of CDKIs assumes specific inhibitory activity and functions as a decelerator of cell cycle progression: p27$^{Kip1}$, one of CDKIs, specifically inhibits cyclin E/CDK2 kinase activity and inhibits the entry into S phase [11].

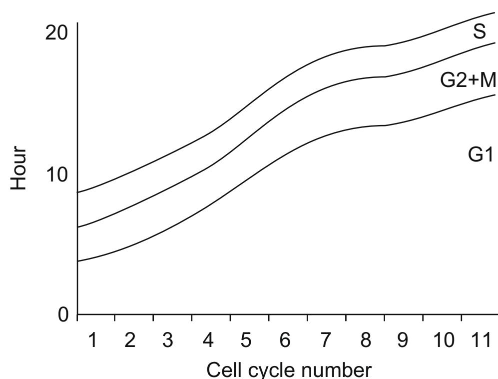


Fig. 4. Lengths of cell cycle phases in mouse NPCs. Adapted from Takahashi et al. [8].

Those so-called G1 cyclins and their partners are critically involved in decision making process of NPCs during G1 phases: Cyclins/CDKs decrease Q while CDKIs (i.e., p27$^{Kip1}$) increases Q.

p27$^{Kip1}$ mRNA expression within the VZ, in the course of neuronogenesis, analyzed by quantitative *in situ* hybridization, shows steep increase during the early to middle phase of neuronogenesis with the peak expression level at around the 8th of 11 cell cycles on E14 [12]. Moreover, mRNA expression of genes that promotes G1 phase progression and S-phase entry such as cyclin E is most robust in the early course of neuronogenesis when most of the NPCs remain proliferative and only a small fraction of cells leaves the cycle (i.e., Q being low). This pattern of p27$^{Kip1}$ expression in the course of neuronogenesis is actually in good concordance with the pattern of Q-ascent. Those facts taken together strongly imply that p27$^{Kip1}$ might be a critical molecule for proliferation/differentiation characteristics of NPCs.

## 5. Altered neuron production by altered p27$^{Kip1}$ expression

Those observation summarized in the previous sections led us to hypothesize that altered p27$^{Kip1}$ expression in the NPCs may perturb G1 phase regulation, specifically orderly increase of Q in the course of neuronogenesis, and eventually lead to abnormal neocortical histogenesis. We have tested our hypothesis using two sets of experiments where expression of p27$^{Kip1}$ is genetically altered: p27$^{Kip1}$ knockout mice (p27KO) [13] and transgenic mice where p27$^{Kip1}$ is overexpressed only among NPCs and only when doxycycline is administered [15].

We have shown increased thickness of superficial layers (II–IV) of the cerebral cortex in p27KO: thickening of those layers is due exclusively to increased number of non-GABAergic projection neurons in the respective layers [14]. In fact, the number of cells belonging to Q fraction was significantly decreased in those animals on E14, which means that loss of function of p27$^{Kip1}$ abnormally decreased Q fraction during the mid-term of neuronogenesis [14]. It follows that abnormally decreased Q increases the maximum number of NPCs as previously mentioned, which in turn results in overproduction of the late-produced neurons for the superficial layers (II–IV) during the terminal legs of neuronogenesis.

We have generated transgenic mice where p27$^{Kip1}$ overexpression is accomplished only in the NPCs only when doxycycline is administered [15]. Spatial expres-

sion was controlled under nestin intron II enhancer/promoter and temporal expression was accomplished by applying tetracycline inducible system [16–19]. When $p27^{Kip1}$ was overexpressed during the mid-phase of neuronogenesis (E12–E14), the number of Q fraction cells was increased as expected by the function of $p27^{Kip1}$ while the total cell cycle length remained unchanged [20]. As predicted, the thickness of the cerebral cortex was decreased due to decreased number of projection neurons in the superficial layers [20].

## 6. Future perspectives

It is to be determined whether those structural abnormalities of the cerebral cortex observed in genetically modified mice are actually associated with impaired cortical function. A series of batteries to access neurological impairment in mice is vital for the investigation on the higher cortical functions in those animals with abnormal neocortical histogenesis caused by aberrant molecular regulations of the proliferation/differentiation characteristics of the NPCs. For clinical applications of those experimental models of neocortical dysgenesis, tools that are capable of analyzing subtle changes in thickness and layer structures of the neocortex are indispensable but not yet available. In the near future, with the advent of diagnostic modalities with much higher resolution, not only a subtle change in neocortical thickness but also a relatively minor dysgenesis within the cortical layers resulting from abnormal neuronogenesis will come to be evaluated in human patients with a wide spectrum of impaired higher cortical function.

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