Cell Output, Cell Cycle Duration and Neuronal Specification: a Model of Integrated Mechanisms of the Neocortical Proliferative Process

The neurons of the neocortex are generated over a 6 day neuronogenetic interval that comprises 11 cell cycles. During these 11 cell cycles, the length of cell cycle increases and the proportion of cells that exits (Q) versus re-enters (P) the cell cycle changes systematically. At the same time, the fate of the neurons produced at each of the 11 cell cycles appears to be specified at least in terms of their laminar destination. As a first step towards determining the causal interrelationships of the proliferative process with the process of laminar specification, we present a two-pronged approach. This consists of (i) a mathematical model that integrates the output of the proliferative process with the laminar fate of the output and predicts the effects of induced changes in Q and P during the neuronogenetic interval on the developing and mature cortex and (ii) an experimental system that allows the manipulation of Q and P in vivo. Here we show that the predictions of the model and the results of the experiments agree. The results indicate that events affecting the output of the proliferative population affect both the number of neurons produced and their specification with regard to their laminar fate.

Introduction

The majority of neocortical neurons arises from a proliferative pseudostratified epithelium (PVE) at the margin of the embryonic cerebral ventricles [for reviews see (Sidman and Rakic, 1982; Nowakowski et al., 2002)]. The operation of the PVE is regulated with precision in its time and neuronal output domains (Caviness et al., 2000b). By time domain we refer to the durations of successive cell cycles (Tc) and those of the G1, S, G2 and M (Tc1, Tc2, Tc3 and Tc4) phases that constitute the respective cycles. We also refer to the total number of cell cycles executed over the interval through which neurons arise from the epithelium. This last parameter determines the overall length of the neuronogenetic interval. By output domain we refer to the fraction of postmitotic cells that either exits the cell cycle (Q or Q fraction) or persists (P or P fraction) to replenish the proliferative pool after each cell cycle. We also refer to the total number of cells that exits with each cycle and the total number of cells formed in the course of the neuronogenetic interval. In mouse, the majority of neocortical neurons is formed during a 6 day embryonic interval E11–E17 (neuronogenetic interval). Tc increases from 8 to 18 h, corresponding to a succession of 11 integer cell cycles. This change reflects entirely an increase in Tc1 of 4-fold, and no change in Tc2 and Tc3. Concurrently with cell cycle succession, Q advances with complementary decline in P. The progression of Q and Tc1 are approximately parallel with the most rapid rate of change occurring in the cell cycle 4–8 succession.

Neurons arising from the PVE do so in overlapping layer VI-II/III succession (Angevine and Sidman, 1961; Rakic, 1974; Takahashi et al., 1999) and appear at terminal mitosis to be specified with respect to the classes that form these layers (McConnell, 1989; Schwartz et al., 1991; McConnell, 1995). The majority are glutamatergic pyramidal or projection neurons with layer-specific cytological features and patterns of connectivity (Parnavelas et al., 1991; Anderson et al., 2002). It is not certain if the PVE output also includes some GABAergic interneurons, many of which are known to arise from basal forebrain sources rather than the neocortical PVE (Anderson et al., 1997; Parnavelas et al., 2000; Letinic and Rakic, 2001; Anderson et al., 2002; Letinic et al., 2002). The processes of laminar specification are coordinated with mechanisms that regulate the proliferative process in its time and output domains. Thus, for the most part, the neurons of layers VI–Vb are formed in the initial three cycles, of the midcortical layers Va–Iib in cycles 4–8 and of Ila–II during the terminal three cycles (Takahashi et al., 1999). To be sure, however, there is significant overlap of the production of neurons for each layer and the complement of cells produced at any given cell cycle can reside in more one layer (Takahashi et al., 1999).

It is a further complexity that the operations of time, output and specification do not proceed concurrently across the entire face of the PVE. The sequence is initiated on E10 (Miyama et al., 1997) at the rostro-lateral margin of the neocortical PVE and progresses caudomedially with initiation in dorsomedial cortex on E11 (Miyama et al., 1997). This corresponds to the ‘neurogenetic gradient’ (Bayer and Altman, 1991; Caviness et al., 2000b). Thus, the same classes of neurons will arise at different times with progression along the caudomedial axis. As a corollary, different classes of neurons will be formed at the same time in positions arrayed along this axis. Two critical issues must be stressed. First, regardless of the position along the gradient axis, the same number of neurons are produced by the PVE over a 11 cell cycle neuronogenetic interval (Miyama et al., 1997). Secondly, regardless of the position along the gradient axis and independently of when cells of a given class arise, their origin will be associated with the identical cell cycle in an 11 cycle sequence having the same time and output parameters (Takahashi et al., 1999).

The close coordination of time and output parameters with the regulation of neuron number dictates that any modification of the proliferative process at any time during the neuronogenetic interval will result in a change in both the output at that cell cycle and in the number of proliferating cells. The change in the number of proliferating cells will lead to a change in the number of neurons produced at all of the cell cycles that follow the modification, and in the total number of neurons produced over the neuronogenetic interval. Moreover, the close coordination of the time and output parameters with the processes of neuronal class specification invites the hypothesis that regulation of the time domain, the output domains and neuronal specification depend upon integrated or even shared
mechanisms. Alternately, there might be three largely distinct sets of mechanisms, largely independent of each other, that regulate the time and the output domains and specification of cell class. Necessarily there follows the challenge to distinguish these opposing hypotheses experimentally in that the implications of the distinction are substantial. At an obvious and practical level the adaptive capacities of the proliferative system in response to cell external influences might most directly drive or inhibit only one of the sets of mechanisms. At a more fundamental level is the matter of the efficient design of regulatory strategies necessary to assure a favorable course of the cellular populations of developing organs as they proceed through complex molecular and morphological transformations and to buffer from external influences.

The experimental examination of these issues requires that one of the three sets of mechanisms be modulated selectively where this effect and its consequences for the other two may be interrogated. This ideal condition may be approximated by molecular genetic manipulations of the operation of mechanisms that regulate the output domain. Specifically, we consider here the impact upon the output and time domains and upon specification resulting from either up-regulation or down-regulation of a critical molecular switch, the cycle inhibitor p27 that modulates the probability that cells will exit the cell cycle after a mitotic division (Sherr and Roberts, 1999; Mitsuhashi et al., 2001).

**Methods and Results**

At the outset, it is necessary to develop a quantitative framework by which changes in the major output parameters, i.e. in $Q$ and its complement $P$, could affect both cell number and cell class. To achieve this, we will consider the predictions of a theoretical model of output operations (Nowakowski et al., 2002) and how the predictions would be modified for selectively induced up-regulation or down-regulation. Here we have posited conditions that, because they are general and schematic, are expected to reveal qualitative aspects of up and down-regulation of $Q$. They are not intended to predict entirely the range of cellular responses following the actual genetic manipulations in the intact animal.

We assume the normal murine 11 cycle neuronogenetic interval, and derive output and changes in the PVE produced by driving $Q$ downward or upward temporarily for a succession of three cycles before $Q$ is allowed to return to its normal trajectory (Fig. 1A). Such changes in $Q$ were evaluated at early (cycles 2–4), intermediate (cycles 5–7) and late (cycles 7–10) phases of neuronogenesis, but the results of the calculations are shown only for intermediate changes (Fig. 1B–F). Using previously described (Nowakowski et al., 2002) principles and equations, we estimated for each condition and cell cycle the predicted output at each cell cycle (Fig. 1B), the size of the proliferative pool of the PVE (Fig. 1C) and the cumulative contribution to post-proliferative cells (Fig. 1D). From these calculations we also estimated the cortical thickness in the mature animal (Fig. 1E). In addition, assuming (as a null hypothesis) that the perturbation in $Q$ will impair the operation of post-proliferative histogenetic and migratory mechanisms leading to neocortical pattern formation (Takahashi et al., 1999), we calculated the laminar fate of the neurons produced (Fig. 1F).

As expected, when $Q$ is decreased output for the affected cell cycles is decreased (Fig. 1B), but concomitant with this there is an increase in $P$ (Fig. 1A). The increase in $P$ causes an increase in the rate of expansion of the PVE (Fig. 1C). The net result is a brief decline in output (Fig. 1B), but after $Q$ resumes its normal path of ascent the PVE volume is increased (Fig. 1C) so that in the subsequent cell cycles there is a marked increase in output (Fig. 1B). The effect over the whole neuronogenetic interval is an increase in the total output (Fig. 1D). Qualitatively similar changes are predicted when $Q$ is raised during the early, middle or late portions of the neuronogenetic period but are progressively reduced with later changes in $Q$, and, of course, depend on the magnitude of change in $Q$ (not illustrated).

The immediate decrease in output per cell cycle but longer term increase in total output over the neuronogenetic interval would have additional predictable consequences for the assembly of the neocortex. In particular, the additional expansion of the volume of the PVE (Fig. 1C) would produce an increase in the tangential expansion of the PVE because PVE thickness is a relative constant during neocortical development (Caviness et al., 1995). We exploited this fact to estimate the thickness of the cerebral cortex of the mature cortex (Fig. 1E) by dividing the final cumulative output (Fig. 1D) by the square root of the maximum PVE volume (Fig. 1C). As it turns out, the predicted cumulative output is greater than the predicted tangential expansion of the PVE so the mature cortex should be slightly thicker than normal (Fig. 1E). Decreases in $Q$ at either earlier or later stages of the neuronogenetic period are also predicted to produce changes in cortical thickness (data not shown). We note, however, that if $Q$ is not changed until cell cycle 7 or afterwards there is relatively little effect on the expansion of the PVE. This is because at cycle 7 ascending $Q$ comes to equal descending $P$ at the ‘steady state’ value of 0.5. Thus, the timing of the decrease in $Q$ is an important consideration.

Finally, based upon (i) the assumption that there is a programmed fixation of cell class specification to cell cycle number and that this specification follows the distribution per cell cycle, as determined experimentally (Takahashi et al., 1999), and (ii) on the calculated number of neurons produced at each cell cycle (Fig. 1B), we calculated the changes in the proportional representation of each of the layers of the cortex (Fig. 1F). These calculations predict that an induced decrease in $Q$ would produce a reallocation of cell representation with the cells of the infragranular layers proportionally over represented with respect to supragranular layers (Fig. 1F).

The predicted effects of increases in $Q$ are opposite in effect (Fig. 1). In particular, the increase in $Q$ would produce a short burst of additional output per cell cycle followed by a marked decrease in output (Fig. 1B) due to a long-term reduction in the amount of PVE expansion (Fig. 1C). The net result would be a marked decrease in the number of neurons produced (Fig. 1D). In addition, cortical thickness would be decreased (Fig. 1E), and the cells of the supragranular layers would be proportionally over represented at the expense of the infragranular layers (Fig. 1F).

With respect to the time domain, we have observed in the normal animal that $T_{G1}$ increases with $Q$ with cell cycle successions in the PVE (Caviness et al., 1995; Nowakowski et al., 2002). To the extent that regulation of output and time domains are integrated in this proliferative system (Takahashi et al., 2001), we anticipate that the time and output parameters will covary in some way with the modulation of one of them. At present, however, there is no theoretical basis from which to offer a companion model as to how the time domain might be modulated if its operations were closely interdependent with those governing output. (As the experimental observations of manipulations of $Q$ and $P$ become more elaborate, we shall develop appropriate models.)
Genetic Manipulation of p27: a Possible Test of Regulation Hypothesis

The cell cycle inhibitor p27kip1 (p27) operates at the G1 phase restriction point to increase the likelihood that cells will revert to the Q fraction (Sherr and Roberts, 1999). If p27 is underexpressed, it is to be expected that Q will be relatively reduced during some portion of the neuronogenetic interval. On the other hand, if p27 is overexpressed, it is expected that Q will be relatively increased during some period of the neuronogenetic interval. We have, therefore, used two different genetic manipulations of p27 to produce either underexpression or overexpression of p27 in vivo and to serve as experimental

Figure 1. Graphs showing (A) the normal changes of Q and P (solid lines) during the 11 cell cycle neuronogenetic interval (NI) and the three cell cycle period (cycles 4, 5 and 6) during which Q was lowered (long dashes) or raised (short dashes) for the calculations shown in B–F. (B–D) The predicted output per cell cycle, the predicted volume of PVE expansion and the predicted cumulative output, respectively, for the normal neuronogenetic interval (solid line) and for neuronogenetic intervals that have Q lowered (long dashes) or Q raised (short dashes). (E, F) The predicted relative change in the thickness of the neocortical wall and the predicted changes in the laminar proportions, respectively, for the normal, lowered Q and raised Q scenarios as shown in A.
approaches to test the predictions outlined above. The changes produced by genetic manipulations obviously may differ from our theoretical models in that the cell cycles of the neuronogenetic period during which p27 expression will be modified will not correspond precisely to those changed in the model (Fig. 1A). However, the time of expression of p27 in the normal developing neocortical PVE is limited to the middle period of the neuronogenetic interval (Delalle et al., 1999) so that the theoretical predictions may, in fact, be rather apt.

**p27 Underexpression: the p27 KO Mouse**

Our experimental model for underexpression is the p27 knockout (KO) mouse. This manipulation leads to the animal being some 30% increased in size in comparison to normal litter mates (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). This reflects an increase in cell numbers in most organs including the neocortex. Our analyses to date have been undertaken with reference to the general anatomy and cytoarchitecture of the mature cortex at postnatal day 21 (P21), and also the developmental parameters at E14, i.e. the embryonic date when the neuronogenetic gradient is steepest and the advances of both T_{G1} and Q with cell cycle succession are greatest (Goto et al., 2000, 2001, 2002) (T. Goto et al., unpublished data). At E14 the cerebral wall in the p27 KO is already increased in width by 23% (Fig. 2A). This finding is not predicted by the model when it is based on a brief decrease in Q after the neuronogenetic interval has already been initiated. We interpret this disparity to indicate that in the P27 KO there are reductions in cell cycle duration that accompany the decrease in Q. This would provide for cell output over more cell cycles in the p27 KO than in the wildtype. This possibility has not yet been examined experimentally, but it is, nevertheless, a plausible explanation of this apparent early augmentation in the rate of cell production in the p27 KO (Goto et al., 2002) (T. Goto et al., unpublished data). On E14, the size of the Q fraction cohort is 5% less than that of the normal littermate (Goto et al., 2001) (T. Goto et al., unpublished data). This reduction in Q confirms the expectation that p27 underexpression affects cell cycle re-entry in the developing PVE. This reduction in the Q fraction reflected exclusively a 22% reduction in a subpopulation of Q fraction cells which exits the ventricular zone (VZ) rapidly after mitosis (Goto et al., 2001) (T. Goto et al., unpublished data); the size of a slowly exiting fraction (Takahashi et al., 1996) is not affected. In addition, the fraction of cells in the VZ not undergoing S-G2-M phases is reduced from 0.5 to 0.25 (Goto et al., 2002). This suggests that the reduction of Q may also be associated with a reduction in T_{G1}, although the extent of the change in T_{G1} cannot, at present, be estimated because T_C is not known.

The height of the cortex at P21 (Fig. 2B) is increased 8%, corresponding entirely to a 23% increase in width of supragranular layers, that is, of the late formed cells (Goto et al., 2000) (T. Goto et al., unpublished data). Somatic size and packing density appear to be the same in WT and KO. This relative increase in the population of the outer layers also reflects an

![Figure 2](https://academic.oup.com/cercor/article-abstract/13/6/592/360927/595)

**Figure 2.** The effects of underexpression of p27 in a knockout mouse on the developing (E14) and mature (P21) neocortex. (A) Photographs of the forebrain from wildtype (left) and p27kip1 knockout mouse (right) at E14; the area of the dorsomedial cortex at which all analyses was performed is enclosed in the rectangle and is shown at higher magnification below. (B) High power photographs of the mature dorsomedial neocortex at P21 showing the relatively thicker cortex in the p27kip1 knockout mouse (right) and the increase in the thickness of the supragranular layers (II-IV).
-17% increase in the relative proportion of projection neurons. GABAergic interneurons were not over represented at any level of the p27 KO cortex.

To review, in principle, the knockout of p27 should result in an increase in the probability that a daughter cell will re-enter S. That is, there should be a relative increase in $P$, and, concomitantly, a relative decrease in $Q$. Indeed, this is what was found at E14, i.e. a 5% decrease in $Q$. At P21 the cortex is slightly thicker than normal, and the relative number of neurons in the supragranular layers is increased relative to the number of neurons in the infragranular layers. Thus, the findings at p21 are entirely consistent with the changes predicted from lowering $Q$ (Fig. 1E,F) and not with raising $Q$.

**p27 Overexpression: the rtTA Conditional p27 Overexpression Mouse**

Our model for the overexpression of p27 is the rtTA conditional p27 overexpression mouse (Mitsuhashi et al., 2001). In a strain carrying a nestin intron II enhancer/promoter driven tet transactivator transgene, the transactivator expression is limited to the nestin expressing cells of the PVE but is constitutively expressed at a high level throughout the life of the PVE. When the transactivator and a reverse responsive operon driving a p27 transgene are included in the same genome by crossing the two lines, the level of p27 can be driven up by doxycycline, administered by oral gavage to the pregnant dam. In embryos hemizygotic for the two transcripts, levels of p27 expression are increased over native levels by two orders of magnitude (Mitsuhashi et al., 2001). Although this is associated with only a several fold increase in the level of the protein, this effect is realized within one or two cell cycles and may be sustained without apparent toxicity. As with the p27 KO model, the overexpression model is unassociated with modification of the post proliferative histogenetic processes of migration and pattern formation necessary to cortical lamination and the formation of neural systems.

In experiments undertaken thus far, doxycycline administered over the E12-14 interval induces overexpression of p27 from cycles 3–4 with reference to the relatively caudomedial region of the cerebral PVE (Tarui et al., 2001). The size of a labeling-specified cohort arising from this region of the PVE is increased as much as 25–30% with the implication that the output parameters were systematically up or down-regulated through early, intermediate or late cell cycles of the neuronogenetic interval. Based upon the mechanisms controlling proliferation and output (Nowakowski et al., 2002), the model predicts consequences for output, both immediate and long-lasting. Based upon the hypothesis of integration of mechanisms related to cell fate, the model also implies consequences for the rescaling of the laminar fates of the output.

First, we have presented results from a predictive model in which the output parameters were systematically up or down-regulated through early, intermediate or late cell cycles of the neuronogenetic interval. Based upon the mechanisms controlling proliferation and output (Nowakowski et al., 2002), the model predicts consequences for output, both immediate and long-lasting. Based upon the hypothesis of integration of mechanisms related to cell fate, the model also implies consequences for the rescaling of the laminar fates of the output.

Secondly, we have introduced yet incomplete experimental tests of this model based upon by either underexpression or overexpression of the cell cycle inhibitor p27 which, in principle, should lower and raise $Q$, respectively. Analysis still in progress shows that the manipulation of p27 had the predicted effect on $Q$ and did, in fact, lower and raise $Q$, respectively. Although confident interpretation must await actual quantitation of neuron numbers, the changes observed in the total output, as assessed by cortical thickness and, most notably, in the proportional representation of infragranular versus supragranular layers, agree remarkably well with the direction of the changes predicted by the model. Thus, the limited investigations performed thus far confirm the principles predicted by the model. However, at this time close quantitative agreement...
between the modeled and experimental conditions is not expected for two reasons. First, the timing, magnitude and duration of the changes in $Q$ that result from the manipulation of p27 are not yet known. Secondly, the concomitant effect on components of the time domain, i.e. cell cycle length, the length of G1, or indeed the number of cell cycles, are also not yet known. Both of these are the target of ongoing experiments.

**Themes and Implications of Integrated Proliferative Regulatory Mechanisms**

The substantial agreement between the predictions of the model for output and specification and even for the time domain, and the observations based upon experimental manipulations of $Q$ confirm that the regulatory mechanisms for these three operative domains of the proliferative process are closely integrated. Moreover, the agreement encourages continued application of a coordinate theoretical and quantitative and cell biological approach to these operations. The full picture introduces three themes for ongoing work which we will designate as (i) the autonomy of the proliferative process, (ii) the stability of integrated proliferative mechanisms and (iii) the internal constraints within the mechanisms of neuronal production.

With respect to ‘autonomy’, we refer to the manifest independence of proliferative and post-proliferative histogenetic events. Specifically, we have shown that when output is either increased or decreased the neurons that are produced migrate normally and form a well-laminated cortex. Thus, even though the proliferative population ‘feeds’ the post-proliferative population, perturbations of the three cardinal mechanisms of neuron production do not perturb those mechanisms that follow and upon which migration, lamination and the formation of connections are dependent. This independence of proliferative and post-proliferative mechanisms is also consistent with observations based upon cortical malformations in man. Specifically, there is consistency with heterotopia and disorders of lamination that appear to be caused by cell biological mechanisms required in the immediately post-proliferative state but not required as proliferation proceeds (Walsh, 1999; Caviness et al., 2000a,c).

By ‘stability’ of integrated proliferative mechanisms we refer to the present observations that up- or down-regulation of $Q$ is matched by a corresponding up- or down-regulation of neuronal class specification and apparently also by an up or down-regulation of $T_{G1}$. That is, a deformation in the operation of one domain is met by a corresponding and integrated deformation in the other two domains. Thus, up- or down-regulation of $Q$ in a series of cycles is reflected in patterns of rescaling of the relative proportions of cell classes native to those cycles with respect to cycles to follow and in proportion to the number of cells produced at those cycles. For example, where the output of early cell cycles is increased at the expense of the proliferative pool, the relative number of layer VI/V neurons, native to early cycles, appears to be increased; the reduction in the size of the proliferative pool results in a deficit of cells produced at later cell cycle at the expense of the relative number of layer IV–II cells. Thus, both the observations and the model argue that cell class fate is stably determined by the cell cycle in which the neuron originates. $T_{G1}$ also appears to follow the direction of change in $Q$. However, one cannot at this juncture define the precision of correlation between the coordinately induced changes in the output and time domains.

By ‘internal constraints’ we note the observation that though deformable in an integrated way the mechanisms relating to regulation of the time and output domains and the specification of cell class appear to be limited in their capacities to stretch. In both the overexpression and underexpression models, $Q$ responds. However these responses are modest in amplitude even in the total absence of p27 in the KO model. Moreover, at some point, regardless of whether p27 is underexpressed or overexpressed, $Q$ apparently continues to observe an orderly ascending trajectory over the full succession of cell cycles such that the process is terminated with a terminal cycle with $Q$ of 1.0. This reflects, presumably, the restricted time of p27 expression during the neuronogenetic interval (Delalle et al., 1999) and the likely involvement of other participants in the mechanisms which govern the trajectory of $Q$. In addition, our limited evidence so far indicates that $T_{G1}$ in turn appears to be capped at ~13 h, corresponding to the normal $T_{G1}$ maximum. This cap seems to apply despite large and widely variable cell internal levels of p27 overexpression. Finally, from their appearances in general cell stains, it appears that when challenged with these perturbations the proliferative population in no sense alters its vocabulary, i.e. the type of cell classes comprising its output. Instead it adheres to the canonical sets, and rescales their relative proportions. Thus, the proliferative process appears to be constrained systematically in a cell cycle anchored way among a set of preprogrammed cell class options and does not conjure up new forms with new properties when the scheduling of origin is perturbed.

**Notes**

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