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A Mathematical Model of Granule Cell Generation during Mouse Cerebellum Development

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Abstract

Determining the cellular basis of brain growth is an important problem in developmental neurobiology. In the mammalian brain, the cerebellum is particularly amenable to studies of growth because it contains only a few cell types, including the granule cells, which are the most numerous neuronal subtype. Furthermore, in the mouse cerebellum granule cells are generated from granule cell precursors (gcps) in the External Granule Layer (EGL), from one day before birth until about 2 weeks of age. The complexity of the underlying cellular processes (multiple cell behaviors, three spatial dimensions, time-dependent changes) requires a quantitative framework to be fully understood. In this paper a differential equation based model is presented, which can be used to estimate temporal changes in granule cell numbers in the EGL. The model includes the proliferation of gcps and their differentiation into granule cells, as well as the process by which granule cells leave the EGL. Parameters describing these biological processes were derived from fitting the model to histological data. This mathematical model should be useful for understanding altered gcp and granule cell behaviors in mouse mutants with abnormal cerebellar development and cerebellar cancers.

Introduction

Brain growth during fetal and postnatal development depends on maintaining a fine balance between neural cell proliferation and differentiation. The mammalian cerebellum provides a striking example of postnatal brain growth. In mouse, the cerebellum volume increases approximately 5-fold over the first 2 weeks after birth (Szulc *et al*, 2015), a period of marked molecular, cellular and large scale patterning changes (Sillitoe & Joyner, 2007; Sudarov & Joyner, 2007; Martinez *et al*, 2013). At the cellular level, the granule cell precursor (gcp)

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cells proliferate in the outer layer of the EGL in response to the mitogen Sonic Hedgehog (SHH), secreted by nearby Purkinje neurons (Wechsler-Reya & Scott, 1999; Dahmane & Ruiz I Altaba, 1999; Wallace, 1999). Following proliferation, the gcps differentiate to form granule cells, by first moving to the inner layer of the EGL where they extend their T-shaped axons, and then migrating towards and past the Purkinje cell layer to take up their final positions in the Internal Granule Layer (IGL). The granule cell axons, known as parallel fibers, contribute to the molecular layer (ML) between the EGL and Purkinje cell layer (Altman & Bayer, 1997). As the most numerous neuronal cell type in the mammalian brain, the granule cells contribute significantly to the growth of the cerebellum, adding volume to both the IGL and ML during the first 2 weeks of postnatal mouse development.

As a developmental model system, the mouse cerebellum provides a number of advantages. The number of cell types is relatively small, with a stereotyped layered cytoarchitecture throughout the cerebellum (Altman & Bayer, 1997; Sillitoe & Joyner, 2007). Furthermore, the folded morphology of the postnatal cerebellum develops similarly in all mammalian species, with 10 major lobules in the midline of mice that are further foliated in higher mammals, including humans. At the cellular level, the gcps and granule cells in mice have been studied extensively for close to 50 years (Fujita, 1967; Haddara & Nooreddin, 1966; Mares *et al*, 1970; Seil & Herndon, 1970; Hatten *et al*, 1982; Goldowitz *et al*, 1997), including recent reports using the most advanced genetic cell labeling and tracking tools, providing important new data from clonal analysis (Espinosa & Luo, 2008; Legué *et al*, 2015). Finally, a number of genetic factors have been identified, including SHH, that are involved in cerebellum growth and patterning, and many genetically engineered mouse models of abnormal cerebellum development have been produced and are available for *in vivo* studies (Joyner *et al*, 1991; Goldowitz *et al*, 1997; Goodrich *et al*, 1997; Corrales *et al*, 2006; Cheng *et al*, 2010).

Despite this wealth of biological data, it is still not clear how to predict cerebellum growth from the underlying neural cell behaviors. Mathematical models have the potential to make quantitative predictions of how changes in cell behaviors affect tissue growth. In the current study we developed and tested a model based on cellular kinetics, applying differential equations with rate constants derived from observed granule cell behaviors of proliferation and differentiation in the EGL. The model accurately predicted how granule cells are generated during mouse cerebellum development, including temporal changes of tissue layers within the EGL, and the average expected clone size of individual gcp cells. Simulated changes in cell parameters were shown to affect the numbers and distributions of gcps and granule cells within the EGL. This model should be useful for studying how changes in these cell properties give rise to abnormal tissue development in mouse models of human neuro-developmental diseases including cancer.

Experimental Methods

Animals, histological sections and staining

All mice used in this study were maintained under protocols approved by the Institutional Animal Care and Use Committees at New York University School of Medicine and Memorial Sloan-Kettering Cancer Center. Swiss-Webster mice were cardio-perfused with

4°C phosphate buffered saline (PBS) and 4°C paraformaldehyde (PFA) at P2, P6, P10 and P14 (day of birth is denoted P0) and their brains dissected, post-fixed overnight at 4°C in 4% PFA, cryoprotected in 30% sucrose in PBS and embedded/frozen in OCT (TissueTek) and cryosectioned (sagittal) at 12- μ m thickness.

For antibody staining against p27, an antigen retrieval step was performed: Slides were equilibrated first in PBS for 5 minutes, then in 10mM Na⁺ citrate buffer, 0.05% Tween20, pH6 for 10 minutes at room temperature (RT). Slides were then transferred into pre-heated 10mM Na⁺ Citrate buffer, 0.05% Tween20, pH6 and maintained at 95–100°C for 30 minutes. Slides were transferred back into RT Na⁺ citrate buffer, 0.05% Tween20, pH6 and let cool down for 10 minutes, then rinsed in PBS 3 times before proceeding with the vendor-specified antibody staining protocol (primary mouse anti-p27; 1:20,000, BD Biosciences 610241/secondary biotinylated goat anti-mouse; 1:500, ABC kit, Vector laboratories) using nickel-enhanced DAB revelation (DAB kit, Vector Laboratories). Slides were counterstained with Strong Nuclear Fast Red before being coverslipped and imaged.

Image acquisition and analysis

Images of the stained sagittal sections were captured on a digital whole slide scanner (Leica SCN400F). These scans were viewed and images of selected mid-sagittal sections were output to ImageJ (Fiji) using SlidePath Digital Image Hub (Leica Biosystems). Contours were drawn manually in ImageJ to measure the areas of the outer (oEGL) and inner (iEGL) EGL layers in lobule III. These area data were then stored in Excel files (Microsoft) for statistical analyses and plotting. Selected data were also transferred to Matlab (MathWorks) for fitting model parameters and to make comparisons between the experimental data and model results.

Mathematical Model

A balance between gcp proliferation and differentiation is required to generate the required number of granule cells in the mature cerebellum. To describe this process mathematically, we took into consideration the cellular behaviors observed in the EGL (Fig. 1). Gcps undergo symmetric division in the EGL during cerebellum development (Espinosa & Luo, 2008; Legué *et al*, 2015). Proliferation occurs in the outer EGL (oEGL), where a gcp generates two daughter gcp cells with each division. Eventually, each gcp divides terminally to generate two differentiating granule cells that move into the inner EGL (iEGL). Finally, after an axon is extended, each differentiated granule cell exits the iEGL, migrating through the ML to take up its final position in the IGL of the mature cerebellum. To summarize, proliferation leads to the growth of the EGL (in the proliferative oEGL layer), while differentiation initially increases the size of the iEGL, but ultimately leads to depletion of the entire EGL as cells leave the cell cycle and exit this transient tissue layer.

Model for granule cell generation in the EGL

To model the cellular behaviors described above, we need to describe mathematically the changes in the number of proliferating gcps, N_o , located in the oEGL, and the number of differentiated granule cells, N_i , located in the iEGL (Fig. 1). We introduce the parameter α_P

the rate constant for the division of gcps, and δ , the probability that a gcp divides terminally to generate two differentiated granule cells. Since cell divisions in the EGL are assumed to be symmetric, $(1-\delta)$ is the probability that a gcp divides to generate two gcps. An additional rate constant, α_E , is introduced to account for the exit of granule cells from the EGL. With these three parameters, we can write a pair of coupled ordinary differential equations (ODEs) for N_o and N_i :

$$\frac{dN_o}{dt} = \overbrace{\alpha_P(1-\delta)N_o}^{\text{Proliferation}} - \overbrace{\alpha_P\delta N_o}^{\text{Differentiation}} \quad (1a)$$

$$\frac{dN_i}{dt} = \overbrace{2\alpha_P\delta N_o}^{\text{Differentiation}} - \overbrace{\alpha_E N_i}^{\text{Exit}} \quad (1b)$$

Note that the different components of equations (1a) and (1b) have natural and intuitive interpretations in terms of the addition or subtraction of cells in the oEGL and iEGL layers due to proliferation, differentiation and exit from the EGL (see annotations included with the equations).

Need for a time-dependent probability function, $\delta(t)$

In the case that the probability δ is a constant, equations (1a) and (1b) have analytical solutions. Specifically, the solution to equation (1a) is particularly simple:

$$N_o(t) = N_o(0) e^{(1-2\delta)\alpha_P t} \quad (2)$$

where $N_o(0)$ is the initial value of N_o at time $t=0$. Since δ must have a value between 0 and 1, it is easy to determine the behavior of N_o for different values of δ :

- i. For $0 < \delta < 1/2$, $N_o(t)$ is an increasing exponential function of time;
- ii. For $\delta = 1/2$, $N_o(t)$ is a constant ($=N_o(0)$);
- iii. For $1/2 < \delta < 1$, $N_o(t)$ is a decreasing exponential function of time.

None of these behaviors are consistent with observations (see Results section), which indicate that the number of oEGL cells initially increases and then decreases to zero during development. We therefore conclude that δ must be a time-dependent function. For this case, the solution of equation (1a) is more complicated than equation (2), but can still be written analytically:

$$N_o(t) = N_o(0) e^{\alpha_P \int_0^t (1-2\delta(\tau)) d\tau} \quad (3)$$

To be consistent with observed oEGL behavior, the $\delta(t)$ probability function must be less than $\frac{1}{2}$ initially, and then greater than $\frac{1}{2}$ after some time. Since $t = 0$ is the time just before gcps begin to differentiate into granule cells, we also assumed that $\delta(0) = 0$. We investigated three single-parameter (a) functions, chosen to meet these conditions:

a. Linear Function

$$\delta(t) = \begin{cases} at, & 0 \leq t < T = 1/a \\ 1, & t \geq T \end{cases} \quad (4a)$$

where a is the slope (in units of h^{-1}) and $T = 1/a$ is the time (in h) when δ becomes 1.

b. Rational Function

$$\delta(t) = \frac{at}{1+at} \quad (4b)$$

c. Exponential Function

$$\delta(t) = 1 - e^{-at} \quad (4c)$$

Calculation of granule cell clone size

If we ignore the spatial location of the cells, equations (1) can be re-written to describe the numbers of proliferating gcps, denoted N_o (as before), and differentiated granule cells (whether they are in the EGL, IGL or in between), denoted N_g :

$$\frac{dN_o}{dt} = \alpha_p (1 - 2\delta) N_o \quad (5a)$$

$$\frac{dN_g}{dt} = 2\alpha_p \delta N_o \quad (5b)$$

These equations are equivalent to equations (1), the only difference being that the term describing exit from the EGL in equation (1b) is no longer included. Furthermore, equations (5a) and (5b) can be added together to yield:

$$\frac{d}{dt}(N_o + N_g) = \alpha_p N_o \quad (6)$$

which is independent of δ . This makes sense, since the right side of equation (6) is the rate at which gcp cell divisions occur (regardless of outcome) and every cell division increases the

total number of cells by 1. Integrating equation (6) from 0 to ∞ , and using the fact that $N_g(0) = 0$ and $N_o(\infty) = 0$, then the total number of granule cells generated can be written as:

$$N_g(\infty) = N_o(0) + \alpha_P \int_0^\infty N_o(t) dt \quad (7)$$

The expected clone size of each original gcp is the ratio $N_g(\infty)/N_o(0)$, since $N_g(\infty)$ is the total number of granule cells generated by the $N_o(0)$ gcp cells present at $t = 0$. This ratio can be calculated by solving equation (7) numerically, after substituting equation (3) for $N_o(t)$. Note that for the linear function $\delta(t)$, given by equation (4a), there is an analytical solution for the clone size (see Appendix):

$$\frac{N_g(\infty)}{N_o(0)} = 2 + \sqrt{(\alpha_P T) \pi} e^{\left(\frac{\alpha_P T}{4}\right)} \operatorname{erf}\left(\frac{\sqrt{\alpha_P T}}{2}\right) \quad (8)$$

where $\operatorname{erf}(x)$ is the error function defined as (Abramowitz & Stegun, 1964):

$$\operatorname{erf}(x) = \frac{2}{\sqrt{\pi}} \int_0^x e^{-v^2} dv \quad (9)$$

Note on time scales and conversions from cell numbers to tissue layer areas

The time scale in the equations described above is defined in terms of an initial time ($t = 0$) just before differentiation begins. At $t=0$ there are no differentiated granule cells ($N_i(0) = N_g(0) = 0$), but there are gcp cells ($N_o(0) > 0$). The time $t=0$ was assumed to be one day before birth (Legué *et al*, 2015). The experimental data were measured in postnatal days (P2, P6, P10 and P14), so $t=0$ in the model is actually “P-1”.

The model equations deal with numbers of cells (N_o , N_i , N_g), while the experimental data were measured from histological sections to determine areas of the oEGL (A_o) and iEGL (A_i) (measured in μm^2). Conversion between cell numbers and tissue areas was made using the following simple formula:

$$N_{o,i} = \frac{A_{o,i} \times L}{v_c} \quad (10)$$

where v_c is the volume of a granule cell, assumed to be $300\text{-}\mu\text{m}^3$ (Mares *et al*, 1970; Seil & Herndon, 1970; Altman & Bayer, 1997), and L is the medial-lateral width (measured in μm) of the vermis (central cerebellum), containing lobule III from which we measured the data. Both histological and MRI studies have shown that L is relatively constant over the early postnatal developmental period, with most of the growth of the cerebellum occurring in the anterior-posterior direction, along the length of each of the lobules (Legué *et al*, 2015, Szulc *et al*, 2015). From MRI data (Szulc *et al*, 2015), we estimated L (measured along the medial-lateral outer contour of the vermis) to be $1775\text{-}\mu\text{m}$ ($\pm 20\%$) between P2 and P14.

Matlab implementation of the model

To solve the ODEs in equations (1), we used Euler's method, implemented in Matlab (MathWorks). Parameter optimization was performed using *fminsearch*, which is Matlab's implementation of the Nelder-Mead optimization algorithm (Nelder & Mead, 1965). For this, we first defined an objective function, which was a root-mean-square error estimate to be minimized:

$$\text{Objective function} = \sqrt{\frac{1}{8} \left\{ \sum_{n=1}^4 [A_o^m(n) - A_o^d(n)]^2 + \sum_{n=1}^4 [A_i^m(n) - A_i^d(n)]^2 \right\}} \quad (11)$$

where $A_{o,i}^m$ denotes the model predictions and $A_{o,i}^d$ the measured data values of the oEGL and iEGL areas (indicated by subscripts o and i, respectively). The index counter, n is used to denote the postnatal stage of each measurement: $n=1$, P2; 2, P6; 3, P10; 4, P14. The *fminsearch* function was called with 4 parameters: α_P , α_E , a (the slope of the function $\delta(t)$ in equation (4)), and $A_o(0)$, the area equivalent to $N_o(0)$ (equation (10)). *fminsearch* was used to find the optimal values of these 4 parameters, which minimized the value of the objective function.

Results

The mouse cerebellum grows significantly over the first 2 weeks after birth

The mouse cerebellum undergoes tremendous growth over the first two weeks of postnatal life (Fig. 2), when the majority of granule cells are generated (Sudarov & Joyner, 2007; Legué *et al*, 2015; Szulc *et al*, 2015). Histological analysis clearly revealed the increase in cerebellum size and complexity of foliation (Fig. 2A), and closer examination of individual lobules showed that much of this increase was localized within the IGL and ML, which include contributions from the gcps (generated by the EGL) in the form of the granule cell bodies and dendrites (IGL) and their parallel fibers (ML). We focused our analysis on lobule III (Fig. 2B), which has a relatively simple shape that could be identified and measured from the earliest developmental stages analyzed.

The thickness of the oEGL and iEGL changes between P2 and P14

At the tissue level, histological analyses demonstrated changes in the thickness of the cell layers in the EGL during postnatal development (Fig. 3). Immunostaining for the early differentiation marker, p27, enabled segmentation of the oEGL (p27⁻) and iEGL (p27⁺) layers. Between P2 and P6, we observed relatively constant thicknesses of the 2 layers. Both the oEGL and iEGL layers showed significantly reduced thickness at P10, and were depleted of granule cells by P14, with only a few scattered p27⁺ cells present at the outer edge of the ML at this time point.

Areas of the oEGL and iEGL were measured to quantify cellular changes

The recent demonstration that gcps do not cross the borders between lobules during postnatal development (Legué *et al*, 2015) justified a single lobule analysis, since each

lobule represents a separate developmental unit where granule cells are generated and maintained (Fig. 4). To quantify the growth characteristics of the oEGL and iEGL layers, contours were drawn in mid-sagittal sections, covering the entire extent of lobule III at each stage (P2, P6, P10 and P14) (Fig. 4A; N=6 sections from 3 animals at each stage). The resulting data indicated relatively small variability between sections and mice, and that the numbers of cells and resulting tissue area measurements in the oEGL (Fig. 4B) and iEGL (Fig. 4C) initially increased (from P2 to P6) and then decreased to near zero (from P10 to P14). We also observed a phase/time delay, which was expected, between the oEGL (larger at early time points) where gcps and granule cells are generated, and iEGL (larger at later time points), where the differentiated granule cells reside before exiting the EGL. These data were used to test the proposed mathematical model of granule cell generation.

Determining the best model type and parameter values

The mathematical model contained several parameters, some of which have been estimated previously but others that are unknown or not known with precision. For example, the rate constant for cell division, α_P is related to the gcp doubling time T_d through the formula:

$$\alpha_P = \frac{\ln 2}{T_d} \quad (12)$$

Previous studies have indicated that T_d is ~19h at P10-11 (Fujita, 1967), which provides an initial estimate of $\alpha_P \approx 0.036\text{h}^{-1}$. Also, the time required for differentiating granule cells to transit the EGL was estimated previously to be $T_E \sim 28\text{h}$ (Fujita, 1967), corresponding to an

exit rate, $\alpha_E (= \frac{1}{T_E}) \approx 0.036\text{h}^{-1}$. However, the value of $A_o(0)$ (the area equivalent to the initial number of gcps $N_o(0)$) has not been reported in the literature, and the value of a (the initial slope of $\delta(t)$), or even the most appropriate functional form of $\delta(t)$, is not known at all. We therefore used an optimization algorithm to fit the 4 model parameters (α_P , α_E , a , $A_o(0)$) to the measured data, minimizing an objective function defined by equation (11).

Three versions of the $\delta(t)$ function were implemented for optimization: linear, equation (4a); rational, equation (4b); and exponential, equation (4c). In each case, the *fminsearch* function converged within 60–70 iterations, resulting in a significant improvement in the fit compared to the initial guess values (Fig. 5). The final values of the objective function were slightly lower (~10%) for the optimal rational and exponential functions compared to the optimal linear function. The optimized parameter values (α_P^{opt} , α_E^{opt} , a^{opt} , $A_o(0)^{opt}$), as well as the derived times T_d and T_E determined by *fminsearch* for each $\delta(t)$ function are listed in Table 1. The optimal linear function reached the value 1 (100% likelihood that a gcp will differentiate into 2 granule cells) close to P13.5, approximately half a day before P14, when the EGL was observed to be depleted. In comparison, the optimal rational and exponential functions only achieved values between 0.6–0.75 by P14 (Fig. 6). Interestingly, the three different $\delta(t)$ functions each reached the value of 0.5 (when N_o and A_o are maximized) at approximately the same time (~P6).

Using the optimal parameters (Table 1), model predictions were compared to measured data (Fig. 7) for each of the $\delta(t)$ functions. Each model provided a good fit to the data acquired from oEGL (Fig. 7A) and iEGL (Fig. 7B), showing a delay of approximately 2 days between the peak number of proliferating oEGL cells (~P6) and the peak number of differentiating iEGL cells (~P8). There were some common discrepancies between the experimental data and model predictions, including small underestimation at early stages and overestimation at later stages in the numbers of iEGL cells (Fig. 6C), which also led to an overestimation of the time when the EGL was depleted of granule cells (experimental ~P14 vs model ~P16.5), which was slightly less for the linear model compared to the two alternatives.

Given the similarity in fits between models using different $\delta(t)$ functions, we used comparison of model predictions to previous estimates of the rate constants a_P and a_E to choose the best model. Using the linear $\delta(t)$ function, a_P^{opt} was determined to be 0.0348h^{-1} (Table 1), corresponding to a doubling time $T_d \approx 20\text{h}$ using equation (12), which is very close to Fujita's estimate of 19h (Fujita, 1967). Similarly, the exit rate constant a_E^{opt} was 0.0387h^{-1} (Table 1), indicating that $T_E (=1/a_E^{opt})$ was approximately 26h. This value is also close to Fujita's estimate of 28h (Fujita, 1967). In contrast, the optimal a_P and a_E rate constants and their associated times (T_d , T_E) were significantly different than Fujita's estimates when the rational and exponential $\delta(t)$ functions were used (Table 1). For example, the doubling times were $T_d \approx 12\text{h}$ and 15h , while the exit times were $T_E \approx 17\text{h}$ and 21h for the rational and exponential functions, respectively, which were considered to be unrealistically small. Based on these considerations, we decided that the model with the linear $\delta(t)$ function provided the best parameter estimates and fit to measured data.

The model provides predictions of initial number of gcps and gcp clone size

Using the model with the linear $\delta(t)$ function, $A_\alpha(0)^{opt}$ was found to be $1994\text{-}\mu\text{m}^2$ (Table 1). Using equation (10), this implies that the initial number of gcp cells ($N_\alpha(0)$) in lobule III is close to 12,000. Using equation (8) with the optimal (linear model) values of the parameters a_P and $T = 1/a$ (Table 1), we can predict that the average clone size of each initial gcp in lobule III is ~124. Therefore, the model presented in this paper provides quantitative predictions about the granule cell properties and behaviors that can be compared to past and future data acquired from the developing mouse cerebellum.

Model simulations provide insights into developmental disorders

We used the model to simulate several cases of abnormal granule cell behaviors that might be expected to arise in the initial stages of medulloblastoma, a common pediatric brain tumor of the cerebellum (Fig. 8). Previous studies of mouse medulloblastoma models have demonstrated EGL thickening and persistence of gcp proliferation at stages after the EGL would normally be depleted (Goodrich *et al*, 1997; Kim *et al*, 2003; Matsuo *et al*, 2013; Oliver *et al*, 2005; Suero-Abreu *et al*, 2014). The optimal model parameters (Fig. 8A) were modified to examine the effects of an increase in the proliferation rate (Fig. 8B), a decrease in the differentiation rate (Fig. 8C), and a delay in the exit of granule cells from the EGL (Fig. 8D–F). Each of these simulations resulted in an increase in the numbers of cells in the oEGL and/or iEGL, and a persistence of granule cells in the EGL beyond the normal stage of depletion, similar to what is observed in early stages of medulloblastoma.

These altered parameters can also effect the predicted clone sizes: compared to the normal cerebellum (clone size = 124), our simulation of increased proliferation (Fig. 8B, $\alpha_P = 1.5 \times \alpha_P^{opt}$) resulted in an increased clone size of 677, while decreased differentiation (Fig. 8C, $\alpha = 0.75 \times \alpha^{opt}$) resulted in a clone size of 387. Our simulations of delayed exit from the EGL (Fig. 8D, $\alpha_E = 0.5 \times \alpha_E^{opt}$; Fig. 8E, $\alpha_E = 0.05 \times \alpha_E^{opt}$; Fig. 8F, $\alpha_E = 0$) had no effect on the clone size, which makes sense since equation (8) does not depend on α_E . Even a complete failure of all granule cells to exit (Fig. 8F) does not change the final number of differentiated granule cells, but it does maintain them in the EGL. While these simulations are able to recapitulate some of the early aspects of eduloblastoma formation, modeling the progression to overt tumors would require altering the underlying assumption in our model that $\delta(t)$ increases and ultimately becomes larger than 0.5. Indeed, the probability function $\delta(t)$ would need to remain less than 0.5 (as discussed in the Mathematical Model section) in order to model sustained gcp proliferation and indefinite tumor growth.

Discussion

In this study, we started from first principles, using observed behaviors of gcps in the EGL—symmetric cell division; proliferation in the oEGL; differentiation in the iEGL; ultimate exit from the EGL—to write a pair of coupled ODEs that model the time-dependent generation of granule cells during early postnatal mouse cerebellum development. This model incorporates intuitive parameters related to the observed cell behaviors—differentiation probability function, $\delta(t)$; proliferation rate constant, α_P ; EGL exit rate constant, α_E —that were subsequently fit to experimental data derived from histological sections. After fitting, the model showed excellent agreement to the experimental data from the oEGL and iEGL. We further showed how the model could be extended to compute the number of progeny (clone size) generated by each gcp cell. Finally, we showed that the model provides a quantitative framework to analyze how differences in cell behaviors can affect the generation of cells within the EGL and the resulting granule cell clone size and/or tissue growth in developmental disorders of the cerebellum. Overall, experimental data has suggested that proliferation, differentiation and exit are important for EGL growth, but our model makes quantitative a plausible description of cerebellum growth control: the rate of gcp proliferation controls the early increase in the volume of the EGL, the transition from proliferation to differentiation is responsible for slowing the growth, and the exit of differentiated granule cells is responsible for the decline in volume and eventual disappearance of the EGL.

Quantitatively, the model was in close agreement to data on granule cell behaviors available in the literature (e.g., Fujita, 1967), providing added confidence in the validity of our optimization procedure. Recent data from clonal analyses have shown considerable variability between clones, but reported mean clone sizes, when measured from embryonic day E17.5 (2 days before birth) to adulthood were 250 (Espinosa & Luo, 2008) and 450 (Legué *et al*, 2015), and were close to 120 when measured from P1 to adulthood (Legué *et al*, 2015). The model estimate of 124, measured from E18.5 (=“P-1”) to adulthood is close to these measurements, and certainly within the variability of the experimental data, especially considering that neither of the previous reports were focused only on lobule III. Our simulations with altered parameters (Fig. 7) might point to the cellular processes

responsible for the variability in the measured clone size; i.e., individual clones may have higher or lower rates of proliferation, differentiation and/or EGL exit.

As described above, the current model captures many key features that underlie the generation of granule cells that are responsible for much of the growth of the cerebellum. Furthermore, our approach of fitting the model parameters to histological data derived from a single lobule (III) was justified based on recent published results showing that each lobule represents a separate developmental unit, with little or no mixing of granule cells between different lobules (Legué *et al*, 2015). One limitation of our approach is that we did not attempt to take into account the stage-dependent differences in gcp and granule cell density in the EGL (Fig. 3). Estimation of the cell densities at each stage would provide a more accurate conversion between area measurements and cell numbers (equation (10)), but would require more accurate stereological methods of counting cells in histological sections. In addition, the current model does not incorporate any spatial information within or between lobules. Our choice of a constant medial-lateral vermis width, L , is a reasonable first approximation, since both histological and MRI studies have shown that the increases in L between P2 and P14 are small (Legué *et al*, 2015; Szulc *et al*, 2015). In future, it would be straightforward to incorporate a time-dependent function for L in the model, based on additional measured data. Another concern is the fact that known medial-lateral movements of gcp cells within the EGL are not incorporated into the model (Sgaier *et al*, 2005; Legué *et al*, 2015). Again, additional experimental data on the extent and rates of such medial-lateral migrations could be used to refine the model in future.

We argued intuitively that the probability of a gcp differentiating into a granule cell must be represented by a time-dependent function, $\delta(t)$. We further investigated several forms of this function, concluding that a linear $\delta(t)$ gave the best fit between known parameter values and experimental data. The linear function assumed in the current model enabled us to correctly predict the increase and decrease in the number of EGL cells observed experimentally during postnatal development. The fact that our optimal parameters resulted in an over-estimation of the time to depletion of the EGL suggests that the model might be improved further in future. It will be important in future to incorporate more of the unique properties of gcp proliferation and differentiation into the model. Previous studies have suggested that feedback regulation of cell lineages may be used to model the regenerative properties of some tissues (Lander *et al*, 2009), but these models will likely need revision to be applied to the EGL, where cell generation is achieved through symmetric cell divisions rather than the more usual asymmetric divisions of self-renewing stem cells. Another important area for future improvement should take into account the results of recent clonal analyses showing that the timing of cell differentiation within each gcp clone is not independent, as currently assumed in the model, but instead all the cells in a clone differentiate together within a short time period (Espinosa & Luo, 2008; Legué *et al*, 2015). When more data are available on the timing of this synchronized differentiation, this feature of granule cell behavior could be incorporated into the model.

In this paper, we showed that our mathematical model provided a good fit to the experimental data from a single cerebellar lobule, lobule III. In the future, it would be interesting to perform a similar analysis of other lobules in the developing mouse

cerebellum. We know from qualitative observations of histology (e.g., Fig. 2) and quantitative measurements from MRI (Szulc *et al*, 2015) that different lobules have different growth characteristics. Consistent with this observation, the average clone size is also larger in long vs short lobules (Legué *et al*, 2015). By fitting the model to other lobules, we could predict the proliferation and differentiation parameters that might be responsible for these differences, providing new insights into the factors controlling cerebellar growth. Overall, the model illustrates the relative balance of proliferation and differentiation processes to growth control in the normal EGL, and provides new insights into how changes in the granule cell exit rate, in addition to proliferation and differentiation, may contribute to the early thickening of the EGL observed in mouse medulloblastoma models. Beyond the cerebellum, similar mathematical models could be developed and applied to other tissues to study the balance between proliferation and differentiation during organogenesis. Taken together, the results described in this paper provide an illustration of how mathematical modeling can be used to provide insights into the cellular processes underlying tissue growth during brain and organ development.

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Glossary of terms

α_E	rate constant for granule cell exit from EGL (measured in h^{-1})
α_P	rate constant for gcp proliferation (measured in h^{-1})
$\delta(t)$	time-dependent probability that a gcp differentiates into a granule cell
v_c	volume of a gcp or differentiated granule cell (measured in μm^3)
a	initial slope of the probability function $\delta(t)$ (measured in h^{-1})
$A_i(t)$	area of the iEGL, a time-dependent function (measured in μm^2)
$A_o(t)$	area of the oEGL, a time-dependent function (measured in μm^2)
EGL	external granule layer of the cerebellum
gcp	granule cell precursor
iEGL	inner (differentiated) layer of the EGL
IGL	inner granule layer of the cerebellum
L	medial-lateral length of the cerebellar vermis, a constant (measured in μm)

ML	molecular layer of the cerebellum
MRI	magnetic resonance imaging
$N_i(t)$	number of cells in the iEGL, a time-dependent function
$N_o(t)$	number of cells in the oEGL, a time-dependent function
ODE	ordinary differential equation
oEGL	outer (proliferative) layer of the EGL
p27	molecular marker used to detect differentiated cells in the iEGL
P	postnatal day (e.g., P6 = postnatal day 6)
SHH	Sonic Hedgehog, a mitogen for gcp proliferation
T	$= 1/a$ (measured in h), the time that the linear $\delta(t)$ function becomes 1
T_d	doubling time of gcps (measured in h)
T_E	exit time of differentiated granule cells from the EGL (measured in h)

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Appendix: Derivation of the formula for average clone size for the linear $\delta(t)$ probability function

The total number of granule cells generated is given by:

$$N_g(\infty) = N_o(0) + \alpha_P \int_0^\infty N_o(t) dt \quad (\text{A1; same as equation (7) in main text})$$

where

$$N_o(t) = N_o(0) e^{\alpha_P \int_0^t (1 - 2\delta(\tau)) d\tau} \quad (\text{A2; same as equation (3) in main text})$$

and

$$\delta(\tau) = \begin{cases} \tau/T, & 0 \leq \tau < T \\ 1, & \tau \geq T \end{cases} \quad (\text{A3; same as equation (4a) in main text})$$

Substituting equations (A2) and (A3) into (A1), we have:

$$\frac{N_g(\infty)}{N_o(0)} = 1 + \alpha_P \int_0^T e^{\alpha_P \int_0^t (1 - \frac{2\tau}{T}) d\tau} dt + \alpha_P \int_T^\infty e^{-\alpha_P \left[\int_0^T (1 - \frac{2\tau}{T}) d\tau + \int_T^t d\tau \right]} dt \quad (\text{A4})$$

Note that $\int_0^T \left(1 - \frac{2\tau}{T}\right) d\tau = T - \frac{T^2}{T} = 0$, so equation (A4) becomes:

$$\begin{aligned} \frac{N_g(\infty)}{N_o(0)} &= 1 + \alpha_P \int_0^T e^{\alpha_P \left(t - \frac{t^2}{T}\right)} dt + \alpha_P \int_T^\infty e^{-\alpha_P (t-T)} dt \\ &= 2 + \alpha_P \int_0^T e^{\alpha_P \left(t - \frac{t^2}{T}\right)} dt \end{aligned} \quad (\text{A5})$$

By expressing

$$\left(t - \frac{t^2}{T}\right) = \frac{T}{4} - \frac{1}{T} \left(t^2 - tT + \frac{T^2}{4}\right) = \frac{T}{4} - \frac{1}{T} \left(t - \frac{T}{2}\right)^2,$$

equation (A5) can be rewritten as:

$$\frac{N_g(\infty)}{N_o(0)} = 2 + \alpha_P e^{\left(\frac{\alpha_P T}{4}\right)} \int_0^T e^{-\frac{\alpha_P}{T} \left(t - \frac{T}{2}\right)^2} dt \quad (\text{A6})$$

Now, we will define:

$$v = \sqrt{\frac{\alpha_P}{T}} \left(t - \frac{T}{2}\right), \text{ so that } dv = \sqrt{\frac{\alpha_P}{T}} dt \quad (\text{A7})$$

Then:

$$t=0 \Rightarrow v = -\frac{\sqrt{\alpha_P T}}{2}; \text{ and } t=T \Rightarrow v = \frac{\sqrt{\alpha_P T}}{2} \quad (\text{A8})$$

Substituting equations (A7) and (A8) into (A6), we have:

$$\begin{aligned} \frac{N_g(\infty)}{N_o(0)} &= 2 + \sqrt{\alpha_P T} e^{\left(\frac{\alpha_P T}{4}\right)} \int_{-\frac{\sqrt{\alpha_P T}}{2}}^{\frac{\sqrt{\alpha_P T}}{2}} e^{-v^2} dv \\ &= 2 + 2 \sqrt{\alpha_P T} e^{\left(\frac{\alpha_P T}{4}\right)} \int_0^{\frac{\sqrt{\alpha_P T}}{2}} e^{-v^2} dv \end{aligned}$$

Therefore the average clone size is:

$$\frac{N_g(\infty)}{N_o(0)} = 2 + \sqrt{(\alpha_P T) \pi} e^{\left(\frac{\alpha_P T}{4}\right)} \operatorname{erf}\left(\frac{\sqrt{\alpha_P T}}{2}\right) \quad (\text{A9, same as equation (8) in main text})$$

where $\operatorname{erf}(x)$ is the error function defined as (Abramowitz & Stegun, 1964):

$$\operatorname{erf}(x) = \frac{2}{\sqrt{\pi}} \int_0^x e^{-v^2} dv \quad (\text{A10, same as equation (9) in main text})$$

Figure 1. Granule cells are generated in the EGL during cerebellum development

Granule cell precursors (gcps; pink, denoted “o”) proliferate by symmetric division in the oEGL, after which they divide terminally to generate 2 differentiated granule cells (dark purple, denoted “i”) in the iEGL. The rate constant for proliferation is denoted α_p , while δ is the probability that a dividing gcp differentiates. α_E is the rate constant for the exit of differentiated granule cells from the iEGL to the molecular layer (ML).

Figure 2. The mouse cerebellum grows and forms lobules during postnatal development
 (A) Mid-sagittal sections of the mouse cerebellum from postnatal day (P)2 to P14 demonstrated the significant growth and foliation (lobules denoted by Roman numerals I to X) that occurs over these developmental stages. Scale bar = 200- μ m for each panel in (A). Sections were immunostained for p27 (dark purple, early differentiation marker) and counterstained with nuclear fast red (pink). (B) Magnified views of lobule III (arrows in A) show the developing layers (outer to inner): external granule layer, EGL; molecular layer, ML; inner granule layer, IGL. Note that the EGL is largely depleted in lobule III by P14. Scale bar = 100- μ m for each panel in (B).

Figure 3. The thickness of the oEGL and iEGL undergo changes during postnatal cerebellum development

Magnified views of the sections shown in Figure 2 revealed dynamic changes between P2-P14 in the thickness of the proliferating oEGL (pink, nuclear fast red) and differentiated iEGL (dark purple, p27 staining) layers (dotted lines show approximate layer borders), with the molecular layer (ML) below the iEGL at each stage. Scale bar = 50- μ m for each panel. Insert schematic (top) from Figure 1.

Figure 4. Areas of the oEGL and iEGL reflect developmental changes in the numbers of proliferating and differentiated granule cells in the EGL

(A) Areas of the oEGL and iEGL layers were measured in mid-sagittal sections of lobule III at P2, P6 and P10 (N=6 sections from 3 animals at each stage). Example contours measured at P6 are shown. Scale bar = 100- μm for each panel in (A). (B) Quantitative oEGL areas (mean $\pm$ standard deviation shown at each stage). (C) Quantitative iEGL areas. Note that the areas of both the oEGL and iEGL was observed to be zero at P14, so no contours/quantitation were generated at that stage.

Figure 5. A four-parameter optimization routine was used to fit the model to experimental data An objective function was defined in equation (11) to assess the (root-mean-square) error in the fit between model and data, and the Matlab *fminsearch* function was used determine the optimal fit, varying the parameters α_P , α_E , a , and $A_O(0)$ to find the minimum value of the objective function for each of the three $\delta(t)$ probability functions. The final optimal model parameters are given in Table 1.

Figure 6. Optimal forms of the $\delta(t)$ functions

The three time-dependent probability functions $\delta(t)$, (linear, equation (4a); rational, equation (4b); exponential, equation (4c)) were plotted between $t = 0$ (one day before birth, or “P-1”) and P20.

Figure 7. Model predictions with optimal parameters showed good agreement with measured data

Using the optimal parameters (Table 1), the model predictions were compared to the measured data. The model predictions for the oEGL (A) and iEGL (B) areas were in good agreement with the measured data (mean $\pm$ standard deviation at P2, P6 and P10; note that the areas of the oEGL and iEGL were both taken to be 0 at P14) for each of the optimized models.

Figure 8. Simulations of mouse mutants with developmental disorders

The model described in this paper can be used to simulate aberrant granule cell proliferation and differentiation relevant to a variety of mouse mutant models of developmental diseases, including medulloblastoma, a common pediatric brain tumor in which too many gcp-like cells are generated. To this end, model predictions of the growth dynamics of the oEGL (solid line) and iEGL (dashed line) were compared between normal EGL (A) and several cases of abnormal EGL which are often assumed to occur in the initial phases of medulloblastoma progression: increased proliferation (B, $\alpha_P = 1.5 \times \alpha_P^{PPL}$), decreased differentiation (C, $\alpha = 0.75 \times \alpha^{PPL}$) and delayed exit (D; $\alpha_E = 0.5 \times \alpha_E^{PPL}$; E, $\alpha_E = 0.05 \times \alpha_E^{PPL}$; F, $\alpha_E = 0$). In every case except when there is no exit (F), the oEGL and iEGL areas increase initially, and then decrease to zero (similar to the normal EGL), but the magnitude and temporal dynamics of the growth curves are significantly different between simulations. In the case of no exit (F), all the granule cells generated are maintained in the EGL.

Table 1
Optimal model parameters determined by fitting to measured data

Note that the parameters determined by *fminsearch* optimization were a^{opt} , $A_o(0)^{opt}$, α_P^{opt} and α_E^{opt} . The gcp doubling time T_d and the granule cell exit time T_E were calculated from α_P^{opt} and α_E^{opt} , respectively.

$\delta O/\text{function}$	$a^{opt} \text{ (h}^{-1}\text{)}$	$A_o(0)^{opt} \text{ (}\mu\text{m}^2\text{)}$	$\alpha_P^{opt} \text{ (h}^{-1}\text{)}$	$T_d \text{ (h)}$	$\alpha_E^{opt} \text{ (h}^{-1}\text{)}$	$T_E \text{ (h)}$
Linear	0.0029	1994	0.0348	19.9	0.0387	25.8
Rational	0.0059	1005	0.0558	12.4	0.0588	17.0
Exponential	0.0041	1411	0.0443	15.6	0.0474	21.1