Sonic Hedgehog Signaling Switches the Mode of Division in the Developing Nervous System

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SUMMARY

The different modes of stem cell division are tightly regulated to balance growth and differentiation during organ development and homeostasis, and these regulatory processes are subverted in tumor formation. Here, we developed markers that provided the single-cell resolution necessary to quantify the three modes of division taking place in the developing nervous system in vivo: self-expanding, PP; self-replacing, PN; and self-consuming, NN. Using these markers and a mathematical model that predicts the dynamics of motor neuron progenitor division, we identify a role for the morphogen Sonic hedgehog in the maintenance of stem cell identity in the developing spinal cord. Moreover, our study provides insight into the process linking lineage commitment to neurogenesis with changes in cell-cycle parameters. As a result, we propose a challenging model in which the external Sonic hedgehog signal dictates stem cell identity, reflected in the consequent readjustment of cell-cycle parameters.

INTRODUCTION

Stem cells are found in all multicellular organisms, and they are characterized by their capacity to self-renew. Such populations are maintained in two ways: through asymmetric divisions that generate one stem cell daughter with a developmental potential indistinguishable from that of the parental cell and a cell with a more restricted potential or through symmetric proliferative divisions that generate two stem cells and that serve to expand the stem cell pool. Alternatively, stem cells can undergo symmetric divisions that generate two cells that enter the differentiation pathway and exhaust the stem cell pool. The tight regulation of these different modes of division is crucial to balance growth and differentiation during organ development and homeostasis, and the subversion of these regulatory processes may lead to tumor formation. Nevertheless, the mechanisms controlling such events are far from being well understood.

The embryonic vertebrate nervous system represents an ideal model to study these processes, because these three modes of divisions occur early in the developing neuroepithelium. Neuroepithelial cells form a single-cell thick pseudostratified epithelium, with cell division occurring at the apical face (Sauer, 1935). The intense research over recent years has defined some of the intrinsic mechanisms that govern the mode of division in the developing nervous system. In Drosophila neuroblasts, particular attention has been paid to the contribution of centrosome asymmetry, spindle orientation, and the inheritance of apical membrane domains (Gonzalez, 2007; Rebollo et al., 2007; Yu et al., 2006). Although not fully understood, the picture emerging suggests that some of the intrinsic mechanisms controlling the mode of division in the developing vertebrate nervous system may reflect similar features (Das and Storey, 2012; Ghosh et al., 2008; Lesage et al., 2010; Marthiens and ffrançois-Constant, 2009; Morin et al., 2007; Shitamukai et al., 2011; Wang et al., 2009). However, although these decisions are likely to be primarily dictated by extrinsic signals, the nature of these factors remains unknown.

Signaling through growth factors is associated with both development and cancer, and factors like Wnts and Hedgehog are believed to increase stem cell number by stimulating stem cell proliferation. However, the maintenance of stem cell identity is believed to depend on direct cell-to-cell communication and intrinsic cell behavior (Beachy et al., 2004; Pierfelice et al., 2011). Sonic Hedgehog (Shh) is a growth factor that augments proliferation in stem cell niches within the adult telencephalon (Lai et al., 2003; Machold et al., 2003). Moreover, Shh signaling promotes the proliferation and survival of neural progenitors in the developing nervous system (Cayuso et al., 2006), in addition to its main role in patterning (Briscoe, 2009).

In this study, we have analyzed the possibility that Shh signaling contributes to neural stem cell identity. We combined in vivo analysis with a mathematical model to quantify the dynamics of cell division in the motor neuron (MN) progenitor domain of the chick spinal cord. In this way, we unveil a switch in the mode of division, and, indeed, the model predicts this switch to occur in synchrony with the loss of Shh activity in the ventral spinal cord. Experimental data showed that maintaining Shh signaling artificially high is sufficient to prevent this developmental switch and to maintain self-expanding divisions. In
addition, this study provides insight into the process linking lineage commitment to neurogenesis and changes in cell-cycle parameters, allowing us to propose a model in which Shh signaling dictates stem cell identity and the cell-cycle parameters rearrange as a consequence.

**RESULTS**

**The Cell-Cycle Parameters Rearrange at the Time of Motor Neuron Differentiation**

To study the dynamics of MN generation, we used specific markers that identify progenitors (Olig2) and newly differentiated MNs (Islet1: Figure 1A). We quantified the total number of Olig2+ and Islet1+ cells that emerge over a 100 hr period of development. Olig2+ cells first appear at ~48 hr post fertilization (hpf; Hamburger-Hamilton [HH] stage 12, Hamburger and Hamilton, 1992), and there is a rapid expansion of these cells that peaks at ~70/80 hpf (HH stage 25/26). Islet1+ MNs first appear at ~60/70 hpf (HH stage 17/18), and their number increased before reaching a plateau at ~120 hpf (HH stage 26: Figure 1B). The rate at which Olig2+ cells become incorporated into the system depends on the balance between self-expansion and the cell loss mediated by cell death.
However, experimental data reveal that apoptotic events can be disregarded during this developmental period (Cayuso et al., 2006). Thus, to study the rate of cell incorporation, we determined the cell-cycle parameters of Olig2+ cells by assessing the accumulation of 5-ethyl-2-deoxyuridine (EdU), both at the time of progenitor expansion (HH14, 55 hpf) and at the time of progenitor consumption (HH24, 96 hpf, Figure 1B). The growth fraction decreased over the 100 hpf period analyzed (from 0.93 ± 0.02 during the expansion phase to 0.60 ± 0.02 in the reduction phase) (Figures 1C and 1D; Nowakowski et al., 1989). The length of G2+M+G1 was comparable in the early pMNs (8 hr) and in the late pMNs (7 hr). However, when the length of the S phase (TS) and the total cell cycle (Tc) was calculated, these parameters were longer for early (8 ± 1 and 16 ± 2 hr) than for late (3.0 ± 0.5 and 10 ± 1 hr) pMNs, indicating that cells in the neurogenic phase have a shorter cell cycle.

To study the duration of the G2 and M phases, we combined EdU labeling with staining for phospho-Histone H3 (pH3). The appearance of EdU in mitotic figures revealed that there were fewer pH3/EdU-positive cells among the early pMNs than the late pMNs. Indeed, the average G2 length (Tg2) calculated from the curve was 1.40 ± 0.05 hr in the early phase, and it decreases to 0.8 ± 0.1 hr in late pMNs (Figures 1E and 1F). With regards to the M phase, we first determined the proportions of Olig2+ cells that were in M phase in function of the pH3 immunolabeling, and we then calculated the length of the M phase (TM) from the respective Tg2 and growth fractions (Figure 1G). Accordingly, the Tg2 appeared to be comparable in early (1.5 ± 0.4 hr) and in late pMNs (1.2 ± 0.3 hr; Figure 1H). These data revealed a global acceleration of division during the neurogenic phase due to the alteration of several cell-cycle parameters (Figure 1I), including the shortening of G2, as reported in the spinal cord (Peco et al., 2012), and a striking shortening of the S phase, as reported during the neurogenic phase in the cerebral cortex (Arai et al., 2011). Additionally, the proportion of the cycle occupied by the G1 phase was bigger in the late stages, as reported in the cerebral cortex (Arai et al., 2011; Lange et al., 2009; Pilaz et al., 2009).

Self-Expanding Proliferative Divisions Are Extinguished by the Time of Motor Neuron Generation

Although the rate of cell division accelerates, the number of MN progenitors decreases, suggesting that the mode of division might change over time. Each Olig2+ cell can expand the pool of progenitor by generating two progenitors (PP). Alternatively, Olig2+ cells can generate one (PN) or two Isl1+ cells (NN: Figure 2A), although these events cannot be determined with the currently available molecular markers. Thus, to obtain the single-cell resolution necessary for such analysis, we generated molecular tools that unequivocally identify the three types of divisions in the chick NT and followed their behavior in vivo (Figure 2B).

We first took advantage of the Sox2p enhancer element that drives expression in the NT (Uchikawa et al., 2003) to track proliferative progenitors. The Sox2p-GFP construct proved to be a reliable reporter to selectively label divisions that will generate at least one progenitor (PP/PN: Figure S1; Movie S1). Next, we assessed the expression of the antiproliferative gene Tis21 (PC3, BTG2) (Iacopetti et al., 1999), whose expression in the mammalian neuroepithelium is restricted to neurogenic progenitors (Haubensak et al., 2004), and we demonstrated that the Tis21p-RFP construct identifies neurogenic divisions (PN/NN) after electroporation into the chick NT (Figure S1).

Co-electroporation of these two reporters revealed the coexistence of progenitors: (1) only expressing Sox2p-GFP (PP); (2) coexpressing Sox2p-GFP and Tis21p-RFP (PN); and (3) only expressing Tis21p-RFP (NN: Figures 2B and S2; Movie S2). Quantification of the three types of divisions in the pH3/Olig2+ cell population showed that at the time of pMN pool expansion (60 hpf), 82% ± 4% of progenitors undergo PP divisions, whereas the remaining 18% ± 4% undergo PN divisions (Figures 2C–2E). By contrast, when Olig2+ cell numbers reach the peak (~70 hpf) the PP mode of division is reduced to 37% ± 9% and replaced by 56% ± 6% PN and 7% ± 4% NN. Soon after the peak (~75 hpf) the PP mode of division accounts only for a 7% ± 6%, whereas 53% ± 7% PN and 40% ± 5% NN divisions are observed. At the time of pMN consumption (80–90 hpf), self-expanding progenitors are inexistent, whereas neurogenic progenitors are evenly distributed in this population (56% ± 3% PN and 44% ± 3% NN at 80 hpf, and 42% ± 6% PN and 58% ± 6% NN at 88 hpf: Figures 2C–2E). These data show that PP divisions are most abundant at the time of pMN growth, although this mode of division is rapidly replaced by PN and NN divisions in similar proportions. Accordingly, Olig2+ progenitors are consumed by an accelerated rate of division and a change in the mode of division.

Mathematical Modeling Reveals a Developmental Switch for Motor Neuron Generation

To elucidate how the transition between the different modes of division is achieved as the cell cycle becomes shorter, we devised a mathematical model (Figures 3A and 3B). Our model is based on chemical kinetics formalism, and it assumes three irreversible reactions that account for the different modes of division observed: PP, PN, and NN. This set of reactions can be translated into a pair of ordinary differential equations that characterize the production of progenitor cells (P) and MNs (N) as a function of the rate of division (Supplemental Information). Thus, a change in the number of progenitors indicates a phase in which the rate of proliferative division is higher/lower than the rate of symmetric neurogenic divisions. Note that the rate of division is inversely proportional to the length of the cell cycle, which, according to our data, decreases as development progresses. We can reconcile these facts and reproduce the dynamics of progenitor and MN generation by invoking a sharp switch or transition in the mode of division (Figure 3A): before a given developmental time, t*, only PP divisions occur, whereas only PN/NN divisions occur after t*. This basic model already qualitatively reproduces the experimental observation in terms of the number of progenitors (P) and differentiated cells (N) and the proportion of divisions (Figure 3B). Moreover, it allows us to predict the number of MNs generated in the long-term Nmax as a function of the maximum number of progenitors, Pmax:

$$N_{\text{max}} = 3P_{\text{max}}$$

Using this model, we can estimate the time of switching as ~80 hpf (HH stage 18). Importantly, we must stress that the fundamental mechanism driving this dynamic situation is...
not the difference in the cell-cycle duration (neither that between modes of cell division nor that at different developmental stages) but, rather, on the switch between the modes of division.

Further quantitative insight can be obtained by modifying the model in order to incorporate cell-cell/embryo-embryo variability, and a less restricted set of parameters in terms of the switch. Importantly, in this modified version of the model we included a fourth reaction \( b \) that accounts for the rate at which Olig2+ cells are incorporated into the system de novo due to Shh-driven Olig2 expression (Balaskas et al., 2012), a number that cannot be resolved on the basis of experimental data. The model parameters were then estimated by minimizing the total error with respect to the experimental data (number of P and N cells) without any further constraints other than the cell-cycle duration at 55 hpf (16 ± 2 hr) and 96 hpf (10 ± 1 hr), and the initial cell number: \( P_0 = 12, N_0 = 0 \). In terms of the dynamics of the number of P and N cells (Figure 3C), and the proportion of the type of divisions (Figure 3D; Movie S3), our results are consistent with the experimental data. Indeed, as shown in Figure 3D, the results confirm the existence of a switch in the mode of division at 73 hpf (HH stage 18) driven by a sudden drop in self-expanding proliferative divisions (see Experimental Procedures). Importantly, our model also suggests a sharp decrease in the incorporation of new Olig2+ cells (\( b \) reaction) at that time, as well as predicting an abrupt change in the length of the cell cycle at 83 hpf. The model shows that the maximum incorporation of Olig2+ cells due to Shh-mediated gene expression is around one cell per hour and this contributes as much as 30% of the population of progenitor cells up to the switching time (see Experimental Procedures).

Altogether, our mathematical analysis indicates the existence of a developmental switch at HH stage 18. The fact that the sharp reduction in the number of PP divisions is synchronized with a sudden loss in the Shh-mediated incorporation of Olig2+ cells suggests that Shh activity may act as an external signal controlling this process, a hypothesis that we tested experimentally.

**Sonic Hedgehog Controls the Mode of Division in the Neural Tube**

We first determined the temporal and spatial dynamics of intracellular Shh signaling in the chick NT, by electroporating either a 8×3’Gli-BS-RFP or a 8×3’Gli-BS-Luc reporter. Shh-responding...
cells were restricted to the ventral NT, largely within the pMN domain (Olig2+ cells; 26% ± 6% RFP+ cells at 45–50 hpf, Figures 4A and 4D). Endogenous Shh activity peaks at ~60–66 hpf (49% ± 4% RFP+ cells), following this peak, the amplitude of Gli activity declines progressively (30% ± 1% RFP+ cells at ~75–82 hpf; 14% ± 2% RFP+ cells at ~90–98 hpf), similar to what was recently reported in a transgenic mouse (Balaskas et al., 2012). By luciferase assay, a similar profile representing Shh activity in the NT has been obtained (Figures 4C and 4D). Interestingly, the high activity of Shh signaling coincides with the expansion phase of the Olig2 domain followed by a progressive decrease (70/80 hpf) concomitant with appearance of the first neurogenic divisions. Later on, Gli activity is barely detectable in the ventral NT from ~90 hpf onward, when PP divisions are extinguished (Figures 4A–4D). All these observations suggest a role for Shh signaling in the expansion of progenitors in the system.

To test the potential role of Shh in controlling the mode of division, we artificially maintained Shh activity high by introducing a dominant active form of the Hh receptor Smoothened (SmoM2) (Hynes et al., 2000) (Figure 5A). HH12 embryos coelectroporated with SmoM2 together with the Sox2p /Tis21p reporters were analyzed at 24 hour post exposure (hpe; 75 hpf), in which the rate of PP divisions (in pH3+ cells) increased significantly (from 29% ± 6% in controls to 58% ± 7% in SmoM2 embryos). The increase in PP divisions takes place at the expense of NN divisions (20% ± 5% in the control as opposed to 6% ± 1% in SmoM2 embryos), together with a reduction, although not statistically significant in the proportion of PN divisions (51% ± 7% in the control to 36% ± 7% in SmoM2 embryos (Figures 5B and 5C). The same phenotype has been obtained by introducing another activator of Shh signaling, a dominant-negative form of PKA (dnPKA) (Figures S3).

These data indicate that active Shh signaling maintains PP divisions and prevents the switch to neurogenic divisions. These changes in the mode of division would not only result in the overgrowth reported previously (Cayuso et al., 2006) but, in addition,
in an unbalanced generation of neurons. As such, embryos analyzed 48 hpe of Shh signaling activation had more progenitors and fewer neurons (Figure S3). Moreover, when we interrogate our mathematical model for the mode of division, in response to a 24 hr delay in $t^*$ (see Experimental Procedures), we observed a result that is in agreement with our experimental data (Figure 5C).

The complementary experiments were performed by prematurely reducing Shh activity through the expression of a dominant active form of the Hh receptor, Patched1 (mPtc1$^{D\text{loop}2}$) (Briscoe et al., 2001). Dampening Shh activity reduced the rate of PP divisions from 25% ± 7% in the control embryos to 8% ± 1% in the mPtc1$^{D\text{loop}2}$-EP embryos. This reduction in PP divisions took place at the expense of NN divisions, which increased from 18% ± 2% in the control to 42% ± 8% in mPtc1$^{D\text{loop}2}$ embryos, whereas PN divisions remained largely unaffected (57% ± 8% in the control and 50% ± 7% in mPtc1$^{D\text{loop}2}$ embryos: Figures 5D and 5E). Again, the in silico counterpart provides similar results when we anticipate $t^*$ by 16 hr (Figure 5E). In summary, these data indicate that active Shh signaling favors the self-expansion mode of division and that a reduction in Shh activity is required to switch the mode of division to that which generates neurons.

Shh Signaling Instructs Stem Cell Commitment, which Is Reflected in the Readjustment of Cell-Cycle Parameters

Lineage commitment of stem cells and cell-cycle progression are two tightly linked events, although it is still unclear whether changes in cell-cycle parameters are the cause or the consequence of differentiation. Our experimental data revealed an accelerated rate of division in the neurogenic phase, although the mathematical model does not attribute particular relevance to the speeding up of the cell cycle when defining the parameters underlying differentiation. Thus, we tested whether stem cell maintenance mediated by Shh involves the regulation of cell-cycle parameters. We examined this by coelectroporating the two reporters (Sox2$^p$/Tis21$^p$) together with control or Shh activator (GOF), and, at 24 hpe, single-cell suspensions were sorted via fluorescence-activated cell sorting (FACS), and the proportions of PP, PN, and NN cells were assessed. The results confirmed the histological data obtained, because activation of Shh signaling increased the proportion of PP divisions from 37% ± 4% in the controls to 52% ± 2% in the Shh-GOF embryos. This increase occurred at the expense...
of NN divisions (23% ± 1% in the control as opposed to 11% ± 2% in Shh-GOF), with no significant changes in the proportion of PN divisions (41% ± 4% in the control to 37% ± 1% in Shh-GOF: Figure 6A). The DNA content was assessed in such samples by flow cytometry (following Hoechst staining). In a population of asynchronous cycling cells, the fraction of cells in a given phase of the cell cycle (G1/G0 identified by 2n content and G2/M identified by 4n) is proportional to the length of that phase, and this is relative to the total length of the cell cycle. The DNA content profiles obtained for PP and PN cells were very similar in control and Shh-overexpressing embryos (PP, 60% ± 2% of 2n and 11% ± 2% of 4n in controls as opposed to 62% ± 3% of 2n and 9% ± 2% of 4n in Shh-GOF; PN, 50% ± 2% of 2n and 17% ± 2% of 4n in controls as opposed to 47% ± 5% of 2n and 16% ± 4% of 4n in Shh-GOF: Figure 6B). Hence, whereas activation of the Shh pathway increased the proportion of PP divisions, cell-cycle parameters were not affected. By contrast, assessing the DNA content of the NN cell population revealed a very different profile, with numerous aneuploid cells and a nonnegligible proportion of hypoploid cells (<2n: Figure 6B). Interestingly, this particular DNA-content profile was comparable in NN divisions from control and Shh-GOF electroporated embryos: 26% ± 2% of 2n, 15% ± 3% of 4n in controls as opposed to 22% ± 3% of 2n, 16% ± 6% of 4n in Shh-GOF embryos (Figure 6B). Accordingly, this feature would appear to be associated with neurogenesis and not with morphogen activity.

**DISCUSSION**

Using newly developed markers that identify the three modes of division in vivo (self-expanding, PP; self-renewing, PN; and self-consuming, NN) and a mathematical model that predicts their dynamics, our study reveals a role for the morphogen Sonic hedgehog in the maintenance of stem cell identity in the developing ventral spinal cord.
This study takes advantage of the unequivocal identification of MN progenitors by Olig2 expression and newly differentiated MNs by Islet1 expression. We define a phase of progenitor expansion followed by a phase of reduction in which MN progenitors are consumed. We used cumulative EdU labeling to provide a breakdown of the cell-cycle parameters in these two phases, defining the TC in the expansion phase to be very similar in length to that determined by live imaging of the chick spinal cord in organotypic slice culture at an equivalent stage (Wilcock et al., 2007). However, the first striking observation is the significant shortening of the TC in the neurogenic phase; the length of the early G1 phase is maintained constant, even though it now represents \( \frac{3}{4} \) of the whole cycle.

Previous analyses of cell-cycle kinetics during neurogenesis considered neural progenitors as a uniform population. However, a recent study in the developing mammalian telencephalon discriminated four subpopulations of progenitors considering proliferative versus neurogenic divisions in both apical and basal progenitor populations (Arai et al., 2011). These analyses demonstrate that the variations of two cell-cycle phases, G1 and S, are correlated to distinct behaviors. A G1 lengthening is associated with the transition from apical to basal progenitors and thereby represents a direct relation with a restriction in lineage potential. However, S phase shortening is correlated to terminal division and commitment to neuronal differentiation, both in apical and basal progenitors. Because the duration gained by G1 lengthening is longer than the duration lost due to S phase shortening, the transition from apical to basal progenitors is accompanied by a global increase in cell-cycle duration. Thus, this study confirms the recent evidence that neuron production is associated with short cell cycle (due to S phase shortening) and provides additional observations by dissecting the relationships between cell-cycle parameters and lineage restriction and commitment.

Our results show that the TC is shortened by curtailing the late S and G2 phases of the cycle. The striking shortening of the S phase is reminiscent of the data obtained in terminal committed progenitors in the developing mammalian cerebral cortex (Arai et al., 2011), and it raises the intriguing possibility that the duration of the S phase is a key factor in the maintenance of the proliferative capacity of neural stem cells. Indeed, self-expanding progenitors should be more dependent on DNA replication fidelity and repair, as errors would be inherited by their progeny. Moreover, progenitors committed to differentiation in the mammalian cerebral cortex upregulate genes implicated in shortening the S phase (Arai et al., 2011).

We sorted the three populations and analyzed their DNA content by flow cytometry using the newly developed reporters to identify the three progenitor identities (PP, PN, and NN). The profiles of DNA content obtained for PP and PN populations were comparable, with a similar proportion of 2n-containing cells (\( \approx 50\% / 60\% \)) representing cells within the G1 phase, and a similar proportion of 4n-containing cells (\( \approx 10\% / 16\% \)) representing cells in the G2/M phases. In this study, cell-cycle parameters have been analyzed separately in each type of progenitor.
indicating that the switch from divisions driving cell expansion to that of self-renewal takes place without significant rearrangement of cell-cycle parameters.

The second striking observation is that the DNA content of progenitors committed to a terminal NN neurogenic division displays a very unusual profile, with numerous aneuploidies and a nonnegligible proportion of hypoploid cells (<2n). Somatic aneuploidies are at least partially generated by chromosome missegregation during mitosis, and they might account for the origins of neuronal diversity in the normal nervous system (Muotri and Gage, 2006). Aneuploidies in the embryonic cerebral cortex account for ~30% of the total pool of neural progenitor cells (Rehen et al., 2001), and they are also present in the developing retina (Morillo et al., 2010). This report of aneuploidies in the spinal cord provides evidence that this phenomenon may be a general mechanism operating throughout the developing CNS, whereby shortening of the S phase in terminal neurogenic divisions would expose cells to more chromosome missegregation, and the resulting aneuploid cells may contribute to neuronal diversity. However, these data indicate that the rearrangements in cell-cycle parameters are the consequence and not the cause of cell-fate choices in neurogenesis.

The Contribution of Quantitative Biology
Here, we present a mathematical model that highlights a straightforward mechanism, a division mode switch, that couples extrinsic Shh signaling to the maintenance of stemness and consequently, with the dynamics of proliferation and the generation of appropriate numbers of MNs. Our proposal transcends the customary definition of “model” in biology because it provides a quantitative mathematical description of the mechanism that not only explains the observed phenomenology, but also allows predictions to be made. Indeed, our approach can produce a long-term prediction of the number of MNs as a function of the maximum number of stem cells, which was consistent with the experimental observations. Moreover, we benefited from this model to elucidate the relative contribution of a regulatory process during proliferative expansion, namely, the Olig2 expression driven by Shh, which would have been impossible to resolve on the basis of molecular markers. In this regard, we estimated that delayed Shh-driven Olig2 expression contributes as much as 30% to the final number of stem cells, that symmetric NN neurogenic divisions do not consume proliferative cells between stages HH14-HH18, and that most of the increase in the stem cell pool during that period is due to self-expanding proliferative divisions. As a matter of discussion, we note that the developmental switch, although sharp and validating our proposal, is somehow smoother in experiments than in the modeling prediction. In this regard, we point out that the timing of the experimental events (embryonic stage in hours) cannot possibly be assessed with total precision and the modeling cannot capture this fact. The latter leads to a source of unpredictability that we interpret as the origin of this quantitative, but not qualitative, disagreement. In any case, the modeling correctly captures and predicts the mechanism underlying the cellular kinetics and has been shown to be a valid and predictive framework for tissue homeostasis due to proliferation and differentiation in the developing neural tube.

The Contribution of Sonic Hedgehog to Stem Cell Fate
During normal growth of the nervous system, Shh signaling plays an important role in maintaining neural progenitor proliferation by directly controlling the expression of cell-cycle regulators (Alvarez-Medina et al., 2009; Bénazéraf et al., 2006; Cayuso et al., 2006; Kenney and Rowitch, 2000; Lobjois et al., 2004; Peco et al., 2012). However, we propose here a challenging model in which Shh signaling drives cell-fate choices upstream of the rearrangement of cell-cycle parameters. Thus, the oncogenic capacity of Hh signaling (Beachy et al., 2004) might depend not only on the expression of cell-cycle regulators, but also on the maintenance of stem cell identity. The responses to Shh are mediated by two transmembrane proteins, Smoothened (Smo) and Patched (Ptc), as well as by downstream transcription factors of the Gli family (Ingham et al., 2011; Jiang and Hui, 2008). Moreover, Hedgehog signaling in vertebrates is strongly connected to the primary cilium (Huangfu and Anderson, 2005). The cilia of neuroepithelial cells are located apically, the membrane domain in which the proteins required for Shh activity localize, including the Shh ligand (Chamberlain et al., 2008) and components of the signal transduction machinery (Ingham et al., 2011; Jiang and Hui, 2008). The ciliary basal body, formed from the mother centriole, serves as a docking area for a large number of pericentriolar proteins, including a pool of PKA that participates in the transduction of Shh signals (Barzi et al., 2010). Because a functional link between centriole maturation and the maintenance of stem cell identity was recently demonstrated in the developing mammalian neocortex (Wang et al., 2009), it is tempting to speculate that the daughter cell that inherits the mother centriole projects a cilium before its sister cell, and thus it continues to receive Shh signals. Additional intrinsic mechanisms related to maintenance of stem cell identity in the developing vertebrate nervous system, such as the control of spindle orientation and the inheritance of apical membrane domains (Lesage et al., 2010), might also be directly regulated by the reception and or activation of Shh, possibilities that open an attractive field for future research.

EXPERIMENTAL PROCEDURES
DNA Constructs
The Sox2p-GFP reporter corresponds to the chicken genomic fragments that cover the 7.6–14 kb Sox2 locus (Uchikawa et al., 2003), and it was cloned into the ptk:EGFP plasmid. The Tis21 promoter (nucleotides −442 to +65) was amplified by PCR from the mouse genome using the following primers:

5′-GGGATGAGTGGCAGAGATGT-3′
5′-GGGATGAGTGGCAGAGATGT-3′

and then cloned into the ptk:RFP plasmid (Uchikawa et al., 2003). The DNAs inserted into the pCIG or pCS2 expression plasmid (with or without −H2B:GFP or −H2B:RFP) were a mutant form of Patched1 (mPtc1popb) (Briscoe et al., 2001), a mutant version of Smoothened (SmooM2) (Hynes et al., 2000), and a mutant version of PKA-R1 (dnPKA) (Epstein et al., 1996).

Endogenous Shh activity was tested by electroporation of a Gli-BS-RFP reporter construct containing synthetic 8×3′Gli binding sites (Sasaki et al., 1997).

Chick Embryo In Ovo Electroporation
White-Leghorn chick embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1992) and electroporated with
Clontech Laboratories purified plasmid DNA (1–2 μg/ml) in H2O with Fast Green (50 ng/ml). Transfected embryos were allowed to develop to the specific stages and then dissected out and processed as indicated.

In Situ Hybridization, Immunohistochemistry, and Cumulative EdU Incorporation

For in situ hybridization, embryos were fixed overnight at 4°C in 4% paraformaldehyde diluted in PBS, rinsed, and processed for whole-mount RNA in situ hybridization using probe to chickTis21 from the chicken EST project following standard procedures.

Immunofluorescence and EdU staining were performed on transverse sections (40 μm), after fixation in 4% paraformaldehyde for 2–4 hr at 4°C. EdU (1 mM) was injected into the lumen of the chick NT at ~2 hr intervals up to 8 hr and ~4 hr up to 16 hr before harvesting. EdU was detected in sections using the Click-IT EdU imaging kit (Invitrogen). The antibodies used were anti-Olig2 (Millipore), anti-islet1 (DSHB), anti-PH3 (Upstate), anti-Sox2 (Invitrogen), and anti-Tuj1 (Covance), and anti-RFP (kindly provided by Dr. S. Ponsi, IBB-CSIIC). The cells were counted in five sections from each of the six embryos in each experimental condition (n > 3).

Fluorescence-Activated Cell Sorting

Embryos were electroporated with dnPKA or pCIG in combination with Sox2p-GFP and Tis21p-RFP at the stages indicated, and 24 hpe a single-cell suspension was obtained after a 10–15 min digestion in Trypsin-EDTA (Sigma). At least three independent experiments (six embryos in each experimental condition) were analyzed by FACS. Hoechst and GFP/RFP fluorescence were determined by flow cytometry using a MoFlo flow cytometer (DakoCytomation), and the cellular DNA content was analyzed (Ploidy analysis) in single fluorescence histograms using Multicycle software (Phoenix Flow Systems).

In Vivo Luciferase Reporter Assay

Embryos were electroporated with the DNAs indicated together with a Sox2p-luciferase reporter, a NeuroDp-Luciferase reporter (Huang et al., 2006), and a Neurog2-Luciferase reporter (Huang et al., 2006), or with a 8 x 3′Gli-BS luciferase reporter construct containing synthetic Gli binding sites (Sasaki et al., 1997), and with a renilla construct (Promega) for normalization. GFP-positive NTs were dissected out at 48 hpe and homogenized in passive lysis buffer. Firefly- and renilla-luciferase activity was measured by the Dual Luciferase Reporter Assay System (Promega), and the data are represented as the mean ± SEM from 12 embryos per experimental condition (n > 3).

RT-Quantitative Real-Time PCR

Embryos were electroporated with the plasmids indicated and the NT was dissected out at 48 hpe. Single-cell suspensions were obtained after a 10–15 min digestion with Trypsin-EDTA (Sigma) and GFP+ cells were sorted by flow cytometry using a MoFlo flow cytometer (DakoCytomation). Total RNA was extracted following the Trizol protocol (Invitrogen), and reverse transcription and real-time PCR were performed according to manufacturer’s instructions (Roche) on a LC480 Lightcytcer (Roche). Specific primers for qPCR amplification of the Statmin2 gene were purchased (QuantiTec Primer Assays, Qiagen), whereas specific primers for the Sox2 gene were designed (5′-GGGACCAACTACATGAGCA-3′ and 5′-GGGTACTGGTTCCATCCCTGT-3′, Sigma). Primers specific for chick Gapdh were used for normalization. PCR amplifications were assessed from pools of electroporated NTs (15 embryos/pool), using three independent pools per experimental condition. The data represent standardized mean values ± SEM.

Spinal Cord Slice Culture and Time-Lapse Analysis

Embryos were coelectroporated at HH14 or HH18 with the Tis21-RFP and Sox2-GFP reporters, and embryo slices (200 μm) were obtained 10–14 hpe with a Vibratome 800 series McIlwain Tissue Chopper. The tissue was embedded in rat tail collagen type I in glass bottom culture dishes (MatTek, coated with poly-L-lysine; Sigma) and cultured in 5% CO2 air at 37°C for 2 hr in DMEM-F12 (D6421; Sigma-Aldrich) supplemented with B27, GlutaMax (both from Gibco) and penicillin-streptomycin (Invitrogen). Slices cultures were imaged in an environmental chamber kept at 37.5°C and buffered with a 5% CO2/95% air mix using either a 25× (Plan Apochromat NA 0.75 water immersion) or a 40× lens (C-Apochromat NA 1.2 water immersion) on an inverted microscope (inverted Zeiss Axio Observer Z1) equipped with an Argon multiline gas laser at 488 nm and a DPSS laser at 561 nm. Images were acquired from 20–40 optical sections spaced 2 μm apart at 10 min intervals for up to 30 hr using ZEN software (Zeiss). The data were analyzed with Velocity (Improvision) or ImageJ software.

Mitotic EdU Labeling Index: Fitting Function

By means of an error minimization algorithm, we fit the mitotic labeling index data (see Figure 1F) to the following function:

\[
f(t) = \left(\frac{t - T_{G2}}{T_{G2}}\right)^n.
\]

In this function, there are two fitting parameters, \(T_{G2}\) and \(n\), that corresponds to the duration of the G2 phase and the sharpness of the mitotic response, respectively. Note that \(T / T_{G2} = 1 / 2\) regardless the value of \(n\).

Calculation of the Duration of the M Phase

At a given developmental stage, all cells of the ventral domain, \(N\), are identified by means of Olig2 labeling. Only a subset of these cells is cycling, \(\gamma N\), being the growth fraction calculated by the EdU labeling index (Figure 1D). In addition, only a percentage, \(\pi\), of the cells of the ventral domain are in the mitotic phase M as identified by the PH3 labeling (see Figures 1E and 1G). We estimate the duration of the M phase as follows. If the cell-cycle duration at a given developmental stage is \(T_C\), and assuming that the cycle progressions of cells are uncorrelated, then the probability density of finding a cell in a particular stage of its cell cycle is \(1 / T_C\). Thus, the probability of finding a cell in the M phase reads \(T_M / T_C\), where \(T_M\) is the duration of the M phase. Moreover, the number of cells in the M phase, \(N_M\), are \(N_M = \gamma T_M / T_C\). Consequently, the duration of the mitotic phase is \(T_M = T_C \pi^\gamma\).

Statistical Analysis of Experimental Data

Quantitative data are expressed as mean ± SEM. Statistical analysis was performed using the Statview software. Significance was assessed by performing ANOVA followed by the Student-Newman-Keuls test, except for experiments with Figures 1D, 1F, 5C, 5E, 6A, and S3 whose effects were examined using the Student’s t-test (\(p < 0.05\), ‘\(p < 0.01\), and ‘‘\(p < 0.001\)). The errors of the cell-cycle phases are estimated using the SE propagation technique using the corresponding formulas.

In Silico Shh Activity Maintenance/Reduction Experiments

In order to implement in silico Shh activity maintenance/reduction experiments that mimic their in vivo counterparts, we proceed as follows. In vivo maintenance of Shh activity is done by using a dominant active form of Smo (SmoM2). This sustains Shh activity levels at least for 24 hr (HH12 embryos electroporated with SmoM2 were analyzed at 24 hpe) (Figure 5A). Because Shh is responsible of the switch at a developmental time \(t^*\), we delay all \(t^*\)’s (see above) by 24 hr but keeping the rest of the parameters unchanged. As for the reduction of Shh activity, HH12 embryos electroporated with a dominant-negative regulator (mPtc1\(^{smo-}\)) were analyzed at 16 hpe (Figure 5A). Thus, we anticipate all \(t^*\)’s by 16 hr but, again, keeping the rest of the parameters unchanged. Importantly, note that the analyzed proportion of divisions, Equation 3, does not depend on either \(b\), the rate of incorporation of proliferative cells due to Shh activity that more likely changes due to these perturbation experiments, or the number of \(P\) and \(N\) cells in the primordium (the whole tube is analyzed in vivo and not only the ventral domain).

For additional materials and methods (Mathematical Modeling), see the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, three figures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.06.038.
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