Research Paper

Proliferation rate and differentiation potential are independent during the transition from neurogenesis to gliogenesis in the mouse embryonic spinal cord

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A B S T R A C T

Neural stem cells (NSC) restrict their differentiation potential as the central nervous system develops. Experimental evidence suggests that the mechanisms governing the transition from the neurogenic to the gliogenic phase irreversibly affect the ability of NSC to generate neurons. Cell cycle regulation has been associated with cell fate in different models. In this work, we assessed the temporal correlation between the loss of the neurogenic potential and cell cycle lengthening of NSC obtained from embryonic mouse spinal cords, during the transition of the neurogenic to the gliogenic phase, using neurospheres. We also used the cell cycle inhibitor Olomoucine to increase cell cycle length by decreasing the proliferation rate. Our results show that neurospheres obtained from a neurogenic stage give rise mostly to neurons, whereas those obtained from later stages produce preferentially glial cells. During the transition from neurogenesis to gliogenesis, the proliferation rate dropped, and the cell cycle length increased 1.5 folds, as monitored by DNA BrdU incorporation. Interestingly, Olomoucine-treated neurogenic-neurospheres display a reduced proliferation rate and preserve their neurogenic potential. Our results suggest that the mechanisms that restrict the differentiation potential of NSC are independent of the proliferation control.

1. Introduction

During prenatal development, Neural Stem Cells (NSC) undergo a series of symmetrical divisions that expand, asynchronously, NSC pools throughout distinct regions of the central nervous system (CNS). Once the NSC expansion phase ends in each region, asymmetric divisions follow and originate, during several rounds, neural progenitors committed to produce neuroblasts. By the end of neurogenesis, neural progenitors become compromised to generate glioblasts (Qian et al., 2000; Temple, 2006). In most of the developing central nervous system, this transition ends with the ability of NSC to produce neurons (Miller and Gauthier, 2007). Hence, the transition from neuron to glia production may hold clues on the cellular processes underlying the restriction of the NSC neurogenic potential. In this regard, it is well known that this transition is modulated by the interplay of extracellular signals and transcription factors that regulate the expression of specific cell-lineage genes (Christie et al., 2013). Nevertheless, the manipulation of these signaling cascades does not lead to the full recovery of NSC neurogenic potential (He et al., 2009; Martens et al., 2002; Seaberg et al., 2005; Whittemore et al., 1999). Understanding the mechanisms that regulate neurogenesis in vivo, can lead to the design of new therapies to promote neural repair after injury (as reviewed by Ruddy and Morshhead (2018)) or treatment for pathologies that impair the functioning of NSC during development, like parasitic infections (Bottari et al., 2019; Fracasso et al., 2019). Many of these studies have focus on the telencephalic NSC, in this work we focus on the differentiation potential of the spinal cord because of the biomedical relevance of this tissue.

A mechanistic possibility to explain the loss of NSC neurogenic...
potential comes from studies on the regulation of cell cycle. The lengthening of cell cycle (particularly the G1 phase) by inhibiting cyclin-dependent kinases (CDK) 1 and 2, has been shown to induce a premature onset of neurogenesis in the ventral mesencephalon and rostral telencephalon of whole embryo cultures (Calegari and Huttner, 2003). Conversely, if the lengthening of G1 is inhibited, the neurogenic phase gets delayed and the expansion of NSC increases (Lange et al., 2009). It is known that the cell cycle length is variable, depending on the position within the neural tube and the age of the embryo. A thorough characterization of the neural tube growth and specification shows that the proliferation rate decreases, and the cell cycle lengthens, once the neuronal differentiation starts in mouse and chicken embryos (Richeva et al., 2014). Given that cell cycle lengthening participates on the transition of NSC from the expansion phase to neurogenesis, it is possible that a similar process takes place between neurogenesis and gliogenesis, when the neurogenic potential is lost throughout the developing CNS.

During brain development, the p19ARF-p53 signaling pathway regulates the transition from neurogenesis to gliogenesis in telencephalic NSC. Indeed, p19ARF overexpression decreases neurogenesis and promotes glial cell generation by increasing the NSC responsiveness to gliogenic signals (e.g., CNTF); downstream p19ARF, p53 decreases NSC self-renewal by inhibiting Myc activity, thus allowing the progression of gliogenesis (Nagao et al., 2008). In addition, downstream p53, some cyclin-dependent kinases inhibitors (CDK) might be upregulated, lengthening the cell cycle and allowing the neurogenesis to gliogenesis transition to occur. This last possibility has not been explored in the spinal cord. The likelihood of it, however, is supported by evidence showing that, during spinal cord development, the three members of the Cip/Kip family of CKI might be expressed during neurogenesis; the expression of at least one of them regulates the timing of the cell cycle exit in differentiating neurons (Gui et al., 2007). Hence, this work was set out to address whether the onset of gliogenesis and the loss of NSC neurogenic potential are associated with the lengthening of the NSC cycle in response to the accumulation of cell cycle inhibitors in the spinal cord of mouse embryos.

2. Materials and Methods

2.1. Animals

CD1 mouse embryos and newborn male and female mice were used to conduct the experiments described below. Embryos were obtained through caesarean sections performed to timed pregnant females at embryonic days 11 and 14 (E11 and E14). Newborns were sacrificed within the first 24-36 h at postnatal day 1 (P1) of having been born. Pregnant or parturient females were all kept under regular housing conditions (12/12-dark/light cycles, controlled temperature and humidity) at the animal facility of the Instituto de Investigaciones Biomédicas (IIB), Universidad Nacional Autónoma de México (UNAM). Animal handling and experimental protocols were revised and approved by the Animal Rights Committee at IIB, UNAM.

2.2. Neurosphere cultures

Neurospheres were produced from the cervical segment of the spinal cord dissected from E11, E14 and P1 mice. Cervical spinal cords were collected in pools (n = 5) and incubated 20 min at 37°C in embryonic digestion solution (0.1% trypsin diluted in versene) or postnatal digestion solution (trypsin (1.33 mg/mL), hyaluronidase (0.67 mg/mL), kynurenic acid (0.2 mg/mL) and DNAse (0.02 mg/mL) diluted in artificial cerebrospinal fluid); the embryonic tissue digestion was inactivated with 10% Fetal Bovine Serum (FBS) diluted in versene and the postnatal tissue digestion with 0.7 mg/mL of trypsin inhibitor. Then, the samples were mechanically dissociated. The resultant cell suspensions were centrifuged at 3000 rpm for 5 min at room temperature and the pellet was then re-suspended in culture media (Dulbecco’s modified Eagle’s medium (DMEM) and F12 (1:1 v/v supplemented with 25 μg/mL insulin, 100 μg/mL transferring, 20 ng/mL progesterone, 60 μM putrescine, 30 nM sodium selenite, 1 μg/mL heparin and 1 × Glutamax supplement). Cells were plated at a density of 250,000 cells/mL and maintained at 37°C, 5% CO2. The culture was supplemented with 20 ng/mL FGF2 and 20 ng/mL EGF on days 1, 3, 5 and 7. Secondary neurospheres were obtained from 7 days old, primary neurospheres disaggregated with a solution containing trypsin (0.025%) diluted in versene for 10 min at 37°C; the disaggregation was inactivated with 10% FBS. Secondary neurospheres were kept in culture for 7 days following the protocol described previously, except for the initial plating density (50,000 cells/mL).

2.3. Neurosphere staining and measurement

To induce cell adhesion and differentiation, neurospheres were harvested and seeded onto poly-L-lysine treated coverslips for 48 h at 37°C, 5% CO2 and fixed with buffered paraformaldehyde (2%) for 30 min at room temperature. After a thorough wash with phosphate buffered saline (PBS; 0.1 M, pH 7.4), neurospheres were treated with 0.2% Triton X-100, 1% glycine and 10% horse serum diluted in PBS for 20 min at room temperature, and then incubated with a mixture of primary polycional antibodies raised in rabbit against MAP2 (1:500, AB5622, Millipore) and raise in chicken against GFAP (1:500, AB5541, Millipore) during 2 h at room temperature. After three PBS rinses, neurospheres were incubated with a mixture of secondary polycional antibodies raised in donkey against chicken (biotin conjugated) and against rabbit (Alexa 594 conjugated) for 90 min; finally, they were incubated with fluorescent avidin for 60 min at room temperature. Neurospheres were counterstained with DAPI and the coverslips carrying them mounted on microscope glass slides using anti-fading mounting medium (Dako). These preparations were used to acquire digital images on a Nikon confocal laser scanning microscope or an Olympus disc spinning unit microscope. The diameter (or the short axis in non-spherical neurospheres) was measured using ImageJ software (NIH) at the middle focal plane.

2.4. Cumulative BrdU incorporation and cell cycle length estimate

Secondary neurosphere cultures (n = 3 per exposure time) were prepared as described above and BrdU (5 μM) was added during the last 1, 3, 6, 12, or 24 h before being collected (i.e. all exposure times were collected at the same time, on day seven of culture). After BrdU exposure, neurospheres were harvested and disaggregated to a single cell suspension that was seeded on poly-L-lysine treated coverslips at a density of 1000 cell/μL. After a 2 h incubation at 37°C, 5% CO2, cells were then fixed with 2% paraformaldehyde for 30 min, as described above. For BrdU immunodetection, fixed cells were sequentially treated

### Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| CNS          | Central nervous system |
| NSC          | Neural stem cells |
| MAP2         | Microtubule associated protein 2 |
| GFAP         | Gial fibrillary acidic protein |
| DAPI         | 4′,6-diamidino-2-phenylindole |
| E11          | Embryonic day 11 |
| E14          | Embryonic day 14 |
| P1           | Postnatal day 1 |
| CDK          | Cyclin dependent kinases |
| CKI          | CDK inhibitors |
| ARF          | Alternative reading frame |
| INK4         | Inhibitor of CDK4 |
| Cip          | CDK interacting protein |
| Kip          | Kinase inhibitor protein |

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with 0.5 N HCl for 20 min at 37 °C, 0.1 M Sodium Borate for 10 min, PBS for 10 min and 0.2% Triton X-100 for 20 min at room temperature. Then, they were incubated with the primary polyclonal antibody raised in mouse against BrdU (1:500, Roche Applied Science) for 2 h at room temperature. After three PBS rinses, cells were incubated with a secondary polyclonal antibody raised in donkey against mouse IgGs conjugated to Alexa 488 for 90 min at room temperature. Finally, cells were counter-stained with DAPI and the coverslips were mounted as described above. These preparations were imaged with an Olympus disc spinning unit microscope. Ten sites per exposure time from separate cultures were imaged using the Systematic Random Sampling tool of the Stereo Investigator software (MBF Bioscience). The proportion of BrdU and DAPI stained nuclei was determined to estimate the cell cycle length using a regression analysis of the active portion of the labeling curve of each age, as described before (Kippin et al., 2005).

2.5. CKI mRNA expression levels

Seven-day-old cultured, secondary neurospheres from four different cultures per age, were harvested and disaggregated to a single cell suspension as previously described. One million cells per condition were rapidly frozen and stored at −80 until used. When thawed for extraction, the cells were re-suspended in Trizol and the RNA was extracted following protocol recommended by the supplier (Invitrogen). RNA concentration was determined using a NanoDrop and cDNA was synthesized using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The following sense and antisense primers were used for RT-PCR: p19\textsuperscript{ARF}, TGAAGTTCTGTCGATC CCCGG and TGAAGGAGAC-GAAGTCGTAG (160 bp), p16\textsuperscript{INK4D}, TCCCCATCATCTGGCGGATA and ATGGCCTGTGCTGTAGGAG (400 bp), p16\textsuperscript{INK4A}, TCA-CACGACTGGGGCAGTT and AGCTCTGCGTCGGTTGG (79 bp), p21\textsuperscript{βCIP}, GGAACATCTCACGGCGAAA and CTAGGATCACCCC-GAGTGA (519 bp), p27\textsuperscript{KIP}, AGATACAGTGGCGAGGAGGTT and GTTGTACGCTTCGGCGTGA (381 bp), p53 ATTCAGCCCTCATCTTCCT and GAAGCCTAGTGGCCTGGTT. Actin was used as an internal control. Amplification was accomplished by incubating 100 ng of the cDNA in a 20 μl reaction volume by using 20 μmol of specific sense and antisense primers and KapaTaq Ready mix DNA polymerase (KAPA BIOSYSTEMS) using following conditions: 2 min at 94 °C; 25 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C; and 5 min at 72 °C. PCR products were separated by electrophoresis in 2% agarose gels. We quantify the expression levels of each mRNA as the ratio of the Mean Gray Value determinations of the band that each of them displayed on the gel, relative to the loading control. The Mean Gray Value determinations were made with ImageJ software (NIH).

2.6. Olomoucine treatment

Olomoucine is a specific inhibitor of G1 cyclin-dependent kinases that lengthens the cell cycle of neuroepithelial cells. It was added to the culture medium of secondary neurospheres from embryonic day 11 to a final concentration of 50 μM (from a 50 mM stock in DMSO). The same volume of DMSO was added to the control cultures. The neurospheres were collected at day seven of culture, fixed, stained and measured as described above.

2.7. Statistics

Data analyses were carried out with GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). Data are given as means plus/minus standard errors. Statistical comparisons of neurosphere diameter between ages and treatments were done with one-way ANOVA test followed by a Tukey’s post hoc analysis. The Kruskal–Wallis test was used to compare CKI mRNA expression levels.

3. Results

3.1. The timing of the neurogenesis to gliogenesis transition of spinal cord NSC is preserved in vitro

Neurospheres were used to evaluate the temporal association between cell cycle lengthening and the loss of the NSC neurogenic potential. In the cervical spinal cord, neurogenesis progresses through days 10–14 of embryonic development (Nornes and Carry, 1978). We then compared the neurogenic/gliogenic potential of neurospheres from a neurogenic stage, embryonic day 11 (E11), a transition stage, embryonic day 14 (E14) and a gliogenic stage, postnatal day 1 (P1). The differentiation potential was assessed by double immunostaining with MAP2, a neuron marker, and GFAP, a marker of astrocytes. Given that each cellular aggregate is the result of clonal divisions (Suslov et al., 2002), quantifying the proportion of neurospheres that were formed mostly by neurons, mostly by astrocytes or by a mixed population of neurons and astrocytes, allows the assessment of the heterogenic differentiation potential of the parental cells that were obtained from each stage. As shown in Fig. 1, almost 60% of neurospheres obtained from E11 displayed MAP2 immunoreactivity. A similar proportion of E14 neurospheres gave rise to either MAP2 or GFAP positive cells. Lastly, P1 neurospheres preferentially originated GFAP positive cells. The neurospheres illustrated on Fig. 1A represent the most abundant profile of each stage of development.

3.2. Neurogenic NSC divide faster and display shorter cell cycle duration

At first glance, neurospheres reduced their sizes from E11 to P1 (Figs. 1 and 2A). Reduction in neurosphere size has been associated to a decrease in the self-renewal potential of NSCs that is not associated with an increase of cell death, but to a reduced proliferation (Molofsky et al., 2003); thus we decided to test if proliferation decreases, and the cell cycle length increases, as the developmental time progresses. Cumulative BrdU labeling allows the assessment of changes in NSC proliferation rate; we used this technique to estimate changes in the duration of the cell cycle during development. The number of BrdU labeled cells was significantly higher in E11 neurospheres than in E14 and P1 neurospheres (Fig. 2B). The regression analysis conducted to estimate cell cycle length based on BrdU cumulative data revealed that E11 NSC cycle lasts 34.7 h, whereas that for E14 and PD1 NSC spans 51.4 and 52.8 h respectively. Hence, the neurogenesis to gliogenesis transition encompasses a lengthening of NSC cell cycle and a decay of the proliferation rate.

3.3. NSC expression of CKI increase during the transition to the gliogenic phase

CKI participate in the control of cell proliferation and cell cycle length, as well as in the modulation of the cell cycle length during the onset of the gliogenic phase in telencephalic structures (Nagao et al., 2008). Then, mRNA expression levels of p27\textsuperscript{KIP}, p21\textsuperscript{KIP}, p19\textsuperscript{ARF}, p16\textsuperscript{INK4A} and p19\textsuperscript{ARF}, prominent members of the Cip/Kip and INK4 CKI families, respectively, were estimated in E11, E14 and P1 spinal cord neurospheres. As seen in Fig. 3, there seemed to be an increased expression of p21\textsuperscript{KIP} mRNA on E14 and P1 neurospheres, but not of p27\textsuperscript{KIP}, nor p19\textsuperscript{ARF}; nevertheless, this was not a statistically significant increase (p > 0.05). p16\textsuperscript{INK4A} mRNA expression was not consistently detected. A significant increased expression was observed for p19\textsuperscript{ARF} and because of it, we reasoned p53 could be involved in the regulation of the NSC proliferation and cell cycle length during the transition from the neurogenic to the gliogenic phase. Even though high levels of the p53 mRNA expression were observed at all time points, its levels were comparable among E11, E14 and P1 neurospheres (Fig. 3).
Fig. 1. Embryonic spinal cord neurospheres recapitulate timely the neurogenesis to gliogenesis transition reported in vivo. A) Fluorescence photomicrographs showing one secondary neurosphere from E11, E14 and P1 cultures, stained for MAP2 and GFAP, and counterstained with DAPI. Scale bar: 100 µm. B) Quantification of the proportion of neurospheres that were formed mostly by neurons, mostly by astrocytes or by a mixed population of neurons and astrocytes on the secondary neurosphere cultures from each stage of development.
3.4. Olomoucine affects the proliferation rate but not the differentiation potential of E11 neurospheres

So far, our results support the idea that the loss of the neurogenic potential of spinal cord NSC during the neurogenesis to gliogenesis transition is associated with increments of the expression of key CKI that might reduce NSC proliferation rate by lengthening the cell cycle. This notion was further evaluated by exposing E11 neurogenic neurospheres to Olomoucine, a specific inhibitor of CDK that lengthen the G1 phase progression. Accordingly, the size of Olomoucine-treated, E11 neurospheres was significantly smaller than that documented for untreated E11 neurospheres (Fig. 4A). We quantified again the proportion of neurospheres that were formed mostly by neurons, mostly by astrocytes or by a mixed population of neurons and astrocytes, to assess the effect of the olomoucine treatment on the differentiation potential of the cells obtained from the E11 spinal cord. DMSO treated cultures gave rise to neurospheres formed mostly by neurons and formed by neurons and astrocytes; the percentage difference with the non-treated culture (Fig. 1B) was not significant (according to a chi-square test). Despite the effect on size, the proportion of neurospheres MAP2 positive cells did not change with the Olomoucine treatment (Fig. 4B and C), suggesting that the intracellular mechanisms that regulate the gliogenesis onset are independent of the cell cycle progression. The neurospheres illustrated on Fig. 4C represent the most abundant profile of each treatment; it was surprising to find small neurospheres mostly formed by neurons because the similar size neurospheres from P1 cultures (Fig. 1A) were mostly formed by astrocytes.

4. Discussion

The mechanisms underlying the restriction of the neurogenic potential of embryonic NSC remain unclear. The fact that this constraint coincides with the onset of glial cell generation suggests that the molecular processes governing the transition from the neurogenic to the gliogenic phase might be involved. Accordingly, it has been established that the imbalance of pro-neuronal/anti-glial and/or anti-neuronal/pro-glial transcription factors and of their associated second messenger signaling cascades influence NSC commitment to one or the other phenotype (Christie et al., 2013). Multiple efforts have been made to understand lineage determination in the CNS, there is a clear biomedical need to gain control over cell fate and to be able of directing neuronal differentiation (Grade and Götz, 2017; Ninkovic and Götz, 2013); unfortunately these studies have focused mainly on NSC from the brain and we believe the spinal cord population should also be characterized. Data generated in this study shows that mechanisms that had been described for telencephalic NSC might be different in the spinal cord.

The relevance of gene expression for neurogenesis has been established by transcriptional profiling telencephalic NSC (Azim et al., 2018;
but some studies have also suggested the existence of an association between the restriction of the differentiation potential and the molecular machinery that controls the cell cycle progression and length. For instance, chromatin remodeling ongoing during cell cycle progression influences cell fate (Ma et al., 2015). Also, the shortening of the G1 phase and thus a faster proliferation rate, both restrict pluripotency of embryonic stem cells (Coronado et al., 2013). Our results in embryonic spinal cord NSC, however, suggest otherwise. We found that the loss of NSC neurogenic potential during the neurogenic to gliogenic transition phase is independent from the reduction of NSC proliferation rate, the latter being inferred by the observed increments of the expression of cell cycle regulators, known to decrease it, and the reduction of the BrdU cumulative labeling. Further support to our conclusion comes from the fact that Olomoucine treated-neurospheres showed reduced proliferation rates and sizes, without losing their neurogenic potential.

The proliferation kinetics of NSC isolated from the spinal cord are not easy to compare to the ones from telencephalic cells; cell cycle parameters that have been used for cortical cells (Takahashi et al., 1993) are not possible to calculate given that the quantification of BrdU accumulation does not reach the curve plateau in 24 h; this feature resemble the BrdU labeling of telencephalic neurospheres from 480-days old mice (Kippin et al., 2005); nevertheless, the analysis within the 24-h window is sufficient to show that neurogenic cells divide faster than those generating glia. We also found cell cycle regulators in our spinal cord model that have not been shown in telencephalic models; for example, in telencephalic NSC, p16INK4A has been described as effector of the Bmi1 transcription factor to regulate cell self-renewal (Molofsky et al., 2003);
we were not able to measure this molecule consistently and, instead, we found the other Bmi1 regulated molecule, p16INK4A, significantly increased in P1 spinal cord neospheres. All this strengthens the idea that it is necessary to go further on the spinal cord characterization.

Previous studies suggest that lengthening or shortening the cell cycle of telencephalic NSC delays or onwards neurogenesis (Calegari and Huttner, 2003; Lange et al., 2009). It is presumed that a lengthier cell cycle makes susceptible NSC to extracellular influences. In contrast, our results support that this might not be the case on NSC going through the neurogenic/gliogenic transition phase. Since Olomoucine did reduce NSC proliferation rates, thus shortening cell cycle length, without altering NSC neurogenic potential, our observations support that NSC enrolling in the neurogenic/gliogenic transition phase may be less susceptible to be modulated by extrinsic signals. This possibility agrees with data that highlights the relevance of intrinsic differences between subpopulations of NSC that affect their neurogenic potential (Seaberg et al., 2005).

The ultimate restriction of stem cells’ differentiation potential is commonly associated to cell cycle arrest and senescence, even though stem cells display a variety of mechanisms to evade them for some time (reviewed by Schultz and Sinclair (2016)). That stem cells senesce is clearly shown by a variety of indicators among of which the accumulation of cell cycle inhibitors as senescence markers, like p16INK4A, are paramount. The expression of cell cycle inhibitors and the lengthening of the cell cycle observed in embryonic spinal cord NSC in our experimental series suggest that senescence might also be part of the ontogeny of the nervous system, as suggested for other embryonic tissues (Muñoz-Espin et al., 2013; Storzer et al., 2013). If this was the case, the irreversibility of the changes in the cellular homeostasis associated with the senescence establishment, might be the explanation to the loss of the neurogenic potential of NSC. Estimating telomere length and beta-galactosidase activity during the transition from neurogenesis to gliogenesis could shed light into this important matter.

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Compliance with Ethical Standards

We further confirm that any aspect of the work covered in this manuscript has involved experimental animals has been conducted with the ethical approval of the Animal Rights Committee at Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México.

Author contributions

Conceptualization and design of the study: LOC, GGO. Acquisition of data: LOC, JRS. Analysis and interpretation of data: All authors. Drafting of the manuscript: LOC and GGO. Critical review of manuscript: All authors. Approval of final manuscript: All authors.

CRediT authorship contribution statement

Leonora Olivos-Cisneros: Conceptualization. Gabriel Gutiérrez-Ospina: Conceptualization.

Conflict of Interest

The authors declare that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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