Int. J. Dev. Biol. 53: 895-908 (2009)
doi: 10.1387/ijdb.082721ml

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Cell cycle control of Notch signaling and the functional regionalization of the neuroepithelium during vertebrate neurogenesis

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ABSTRACT A critical feature of vertebrate neural precursors is the to-and-fro displacement of their nuclei as cell cycle progresses, thus giving rise to a pseudostratified epithelium. This nuclear behavior, referred to as interkinetic nuclear migration (INM), is translated into the disposition of the cell somas at different orthogonal levels depending on the cell cycle stage they are. The finding that important regulators of neurogenesis, such as the proneural and neurogenic genes, undergo cyclic changes of expression and function in coordination with the cell cycle and the INM, and that the neurogenic process correlates with a particular window of the cell cycle, in coincidence with the apical localization in the neuroepithelium of neural precursors, is a novel concept that facilitates our understanding of the neurogenic process in vertebrates. As such, recent data support the notion that the three-dimensional structure of the neuroepithelium is crucial for proper neuronal production. In this review, we describe current knowledge of the molecular mechanisms involved in the differential expression and function of the proneural and neurogenic gene products along the cell cycle, and we discuss important consequences for vertebrate neurogenesis derived from this observation.

KEY WORDS: *proneural gene, neurogenic gene, lateral inhibition, mRNA stability*

During the initial stages of development, the vertebrate embryo undergoes a dorsal invagination of the neuroectoderm to form the neural tube. This structure, which subsequently will generate the brain and spinal cord as well as the neural crest derivatives, is initially a monostratified epithelium with its apical side forming the lumenal surface. As development proceeds neural precursors divide vigorously in an unsynchronized manner, increasing dramatically its cellular density and acquiring a highly packed, pseudostratified disposition characterized by the presence of their nuclei at different levels depending on the cell cycle stage they are (Fig. 1). A hallmark of the neural precursors is therefore the to-and-fro displacement of the nucleus during the cell cycle, a process that is referred to as interkinetic nuclear migration (INM) (Sauer, 1935; Sauer and Walker, 1959; Sidman *et al.*, 1959; Fujita, 1962; Takahashi *et al.*, 1993; Hayes and Nowakowski, 2000). This nuclear movement spans the entire apical-basal axis of the cell, with the nucleus migrating to the basal side during the first gap (G1) phase of the cell cycle, staying at the basal side during the DNA synthesis phase (S-phase), migrating back to the apical side during the second gap (G2) phase, and undergoing mitosis (M) at the apical side. This cellular behavior, which has been conserved throughout evolution as it can be observed in some invertebrate neuroepithelia such as the eye imaginal disc of

Abbreviations used in this paper: Adcyap, adenylate cyclase-activating polypeptide; APC, anaphase-promoting complex; ARE, AU-rich element; asc, achaete scute; ascl, asc-like; ato, atonal; atoh, ato homolog; bHLH, basic helix-loop-helix; CDK, cyclin-dependent kinase; CKI, CDK inhibitor; csnk, casein kinase; Dll1, Delta-like 1; ELAV, embryonic lethal abnormal vision; elavl, ELAV-like; emc, extra macrochaetae; E(spl), enhancer of split; Esr, enhancer of split related; Hes, hairy and enhancer of split; Her, hairy and enhancer of split related; HLH, helix-loop-helix; Id, inhibitor of differentiation; INM, interkinetic nuclear migration; Lfng, lunatic fringe; miRNA, microRNA; neurog, neurogenin; NICD, notch intracellular domain; RBP, RNA-binding protein; Su(H), suppressor of Hairless; Shh, sonic hedgehog; Tacc, transforming acidic coiled-coil.

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Accepted: 10 October 2008. *Published online:* 22 May 2009.

ISSN: Online 1696-3547, Print 0214-6282

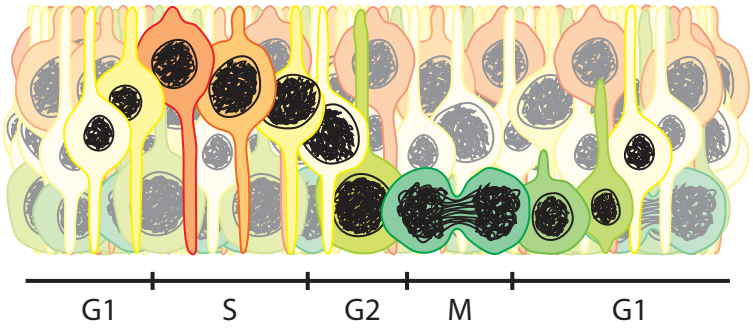

Fig. 1. Scheme of the interkinetic nuclear migration observed in vertebrate neuroepithelia. *Neural precursors displace their nuclei as they progress through the cell cycle. During G1, nuclei are displaced to the basal surface (top), where they undergo DNA replication (S). Once S-phase is finished, nuclei move back to the apical portion of the neuroepithelium (bottom) as they go through G2, and then they divide to give rise to two daughter cells (M). G1, first gap phase; G2, second gap phase; M, mitosis; S, DNA synthesis phase.*

Drosophila (Tomlinson, 1988), likely results from the epithelial nature of this tissue characterized by the attachment of neural precursors to each other by apically-located adherens junctions. The adherens junctions are belt-like junctions composed of cadherins, which are linked to a ring-like cytoskeleton of actin microfilaments just above the centrosome (Chenn *et al.*, 1998). This tight interaction among neural precursors forces them to displace their nuclei to the apical side in order to acquire a round morphology during M, and to the basal side during interphase in order to make space for other neural precursors disposed to undergo M.

So far, a few reviews have been published dealing either directly (Frade, 2002; Baye and Link, 2008; Miyata, 2008) or indirectly (Gotz and Huttner, 2005) with INM. In this review we will focus on the interplay among INM, cell cycle, and neurogenesis, discussing on molecular mechanisms coordinating the neurogenic process with the cell cycle and how the three-dimensional structure of the neuroepithelium is crucial for the proper neurogenic process.

Molecular and cellular mechanisms regulating INM

Nuclear positioning in eukaryotic cells depends on active mechanisms which move nuclei within the cytoplasm and maintain them in the correct cellular location (Reinsch and Gonczy, 1998). In many cells, nuclear positioning is a microtubule-dependent process with relative participation of the actin cytoskeleton. This seems to be the case for INM in the vertebrate neuroepithelium since a number of pharmacological studies carried out in this tissue indicate that INM is dependent on the integrity of both actin and microtubule cytoskeleton (Karfunkel, 1972; Messier and Auclair, 1977; Webster and Langman, 1978; Murciano *et al.*, 2002).

A major mechanism for nuclear positioning in eukaryotic cells is based on a tight association of the nucleus with a microtubule organizing center such as the centrosome (Reinsch and Gonczy, 1998). Recent evidence demonstrates that centrosome and microtubule-associated proteins play a major role in regulating INM (Xie *et al.*, 2007) but, unlike other examples where nuclei follow the centrosome during migration, in neural precursors the centrosome remains apically located (Hinds and Ruffett, 1971; Astrom and Webster, 1991; Chenn *et al.*, 1998). Therefore, the length of the microtubule network coupling the nucleus with the centrosome must be dynamically regulated. In this regard, Xie *et al.* (2007) have shown that Cep120, a centrosomal protein preferentially expressed in neural precursors, can interact with transforming acidic coiled-coil protein (Tacc)3, a microtubule-associated protein involved in microtubule growth, and recruit it to the centrosome. Both Cep120 and Tacc3 are crucial for coupling the centrosome and the nucleus, and Tacc3 could regulate the changes in the distance between these organelles that is observed during INM (Xie *et al.*, 2007). Indirect evidence also suggests that the minus end-directed motor dynein participates in the nuclear movement in neural precursors, as occurs in several other examples of nuclear positioning (Reinsch and Gonczy, 1998). As such, mutations in the dynein-regulating protein Lis1 (Wynshaw-Boris and Gambello, 2001) are known to deregulate INM (Gambello *et al.*, 2003; Tsai *et al.*, 2005; Xie *et al.*, 2007). Furthermore, truncating mutations in the dynein-interacting protein dynactin are responsible for *glued* phenotypes in *Drosophila* (Schroer, 2004). In these mutant flies, the nuclei of photoreceptor cells fail to move apically toward the minus-end of microtubules and instead remain at more basal locations, a phenotype that can be suppressed by mutations in subunits of kinesin, the plus-end directed microtubule motor (Whited *et al.*, 2004). INM is also affected in zebrafish *dynactin-1* mutants. In the developing retina of these mutants interkinetic nuclei migrate faster to the basal surface and further basally, take longer to return, and often enter M before they have reached the apical domain (Del Bene *et al.*, 2008).

Another regulator of INM in vertebrates is casein kinase (Csnk)2, a Ser/Thr kinase able to phosphorylate both molecular motors and cytoskeletal components (Canton and Litchfield, 2006). Csnk2 is expressed by retinal precursors and pharmacological inhibition of this kinase blocked INM in a dose-dependent way (Carneiro *et al.*, 2008), but the molecular mechanism behind the function of Csnk2 in INM is currently unknown. The transcription factor Pax6 has also been shown to participate in nuclear displacement to the apical surface during INM in cortical precursors, likely due to its participation in the stabilization of the centrosome at the apical side of the neuroepithelium (Tamai *et al.*, 2007).

In specific areas of the developing vertebrate nervous system, such as the retina, external factors to the neuroepithelium have also been shown to modulate INM. The developing retina is surrounded by the prospective pigment epithelium, a monostratified epithelial structure which will give rise to a layer of pigmented cells in the adult eye, and that can modulate the development of the neural retina (Frade *et al.*, 1999). The prospective pigment epithelium has been shown to be coupled with the developing retina by gap junction-dependent Ca^{2+} activity (Pearson *et al.*, 2004). INM has been associated with Ca^{2+} transients, and gap-junctional communication is an important requirement for the maintenance of normal INM in retinal precursor cells (Pearson *et al.*, 2005). The actual mechanism by which gap junctions modulate INM is currently unknown.

Molecular and cellular mechanisms regulating cell cycle progression

The cell cycle is a highly conserved mechanism by which eukaryotic cells proliferate. This process is typically divided into

four phases: G1, S, G2, and M (Murray and Hunt, 1993) (Fig. 2). The G1, S, and G2 stages are globally referred to as interphase, in contrast with M, the phase when the cell generates two daughter cells with equal DNA content and chromosome number. Progression through these phases is regulated by the sequential expression, activation, and inhibition of cyclin-dependent kinase (CDK) complexes and their activating partners, the cyclins, as well as CDK inhibitors (CKIs) (Lees, 1995). Cycling cells usually exit the cell cycle after having completed M and remain in a quiescent G0 state.

In proliferating cells, progress through the cell cycle is tightly controlled by checkpoints. These checkpoints function as molecular switches that ensure the completion of critical events in one phase of the cell cycle before entering of the next phase, thereby coordinating cell growth with cell proliferation. Two major check points have been described, the G1/S check point, that allows a cell to initiate replication of its DNA; and the G2/M check point, that controls whether DNA replication has been correctly performed before the cell divide.

In mammals, seven CDKs and eight cyclins have been described to date, of which the best studied will be briefly introduced here (for review, see Murray and Hunt, 1993; Grana and Reddy, 1995; Lees, 1995; Morgan, 1997). Following mitogenic stimulation, D-type cyclins are synthesized and bind and activate CDK4 and CDK6, thereby allowing cells to induce cyclin E expression and leave the G1 phase (Sherr *et al.*, 1994). Cyclin E associates with CDK2 (Sherr, 1994). This cyclin E/CDK2 complex is necessary for transition into S phase. DNA synthesis is associated with an active cyclin A/CDK2 complex (Grana and Reddy, 1995). When DNA replication is completed, cyclin A forms a complex with CDK1, also termed CDC2, and drives the cell through G2 phase (Sherr, 1994). At the G2/M transition, cyclin A is degraded and CDK1 associates with newly synthesized cyclin B (Grana and Reddy, 1995). The cyclin B/CDK1 complex is required for progression through M (Grana and Reddy, 1995). In late M phase, cyclin B/CDK1 is inactivated due to the degradation of cyclin B by the anaphase-promoting complex (APC), an E3-ubiquitin ligase (Harper, 2002; Peters, 2002), and the cell complete M followed by entry into G1 (or G0 if the daughter cell is programmed to remain quiescent).

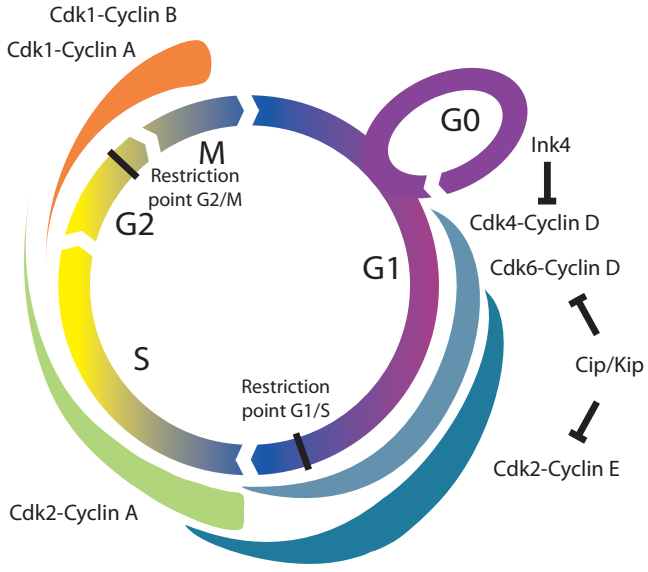

Fig. 2. Schematic diagram of cell cycle and its main regulators. *Progression through the cell cycle is regulated by cyclin/CDK complexes and their inhibitors (Ink4 family and Kip/Cip family). Two main restriction points have been described, that control whether cells enter a new round of DNA replication (G1/S check point), and whether DNA replication has been correctly performed before the cell divide (G2/M check point). G0, quiescent state; G1, first gap phase; G2: second gap phase; M, mitosis; S, DNA synthesis phase.*

Two families of proteins inhibit the activity of cyclin-CDK complexes (Lees, 1995; Sherr and Roberts, 1999). Members of the Kip/Cip family of proteins ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) regulate the activity of all the G1 cyclin-CDK complexes and, to a lesser extent, cyclin B/CDK1, while members of the Ink4 family ($p16^{Ink4}$, $p15^{Ink4}$, $p19^{Ink4}$ and $p18^{Ink4}$) specifically inhibit CDK4 and CDK6 (Sherr and Roberts, 1999). As it occurs to CDKs and cyclins, the activity of CKIs is tightly regulated by transcription, translation, and ubiquitin-mediated proteolysis (Sherr and Roberts, 1999).

Synchrony of the INM and the cell cycle

One hallmark of INM is the coordination of the nuclear movement with the cell cycle. This coordination has been studied pharmacologically. When nucleokinesis is blocked by drugs inhibiting microtubule (Karfunkel, 1972) or actin (Karfunkel, 1972; Messier and Auclair, 1977; Webster and Langman, 1978; Murciano *et al.*, 2002) polymerization, cell cycle still can progress, thus suggesting that, although INM and cell cycle progression are tightly coordinated in the vertebrate neuroepithelium, both processes can actually be molecularly dissociated. A similar conclusion can be raised from the work by Tamai *et al.* (2007) and Del Bene *et al.* (2008). The former authors found that in the ventricular zone of the Pax6-deficient cerebral wall, coordination between the cell cycle and INM is disrupted as neural precursor cells lacking Pax6 often exhibit premature displacement of their nuclei to the apical side during S-phase. Likewise, Del Bene *et al.,* 2008) described the presence of alterations of INM and ectopic mitoses in the retina of zebrafish *dynactin-1* mutant embryos. In accordance with this conclusion, inhibition of cell cycle progression in S-phase with hydroxyurea, a reversible ribonucleoside reductase inhibitor that blocks deoxynucleotide synthesis, inhibits DNA replication, and induces synchronization in S-phase, has been shown not to interfere with the nuclear movement of neural precursors to the apical side of the neuroepithelium (Murciano *et al.*, 2002). In contrast with these results, prevention of cell cycle progression in M by colchicine inhibits nuclear displacement to the basal neuroepithelium (Waterson *et al.*, 1956). This lack of basally-oriented nuclear migration induced by colchicine is likely due to the inhibition of the apical-basal microtubule network required for the basal displacement of the nuclei (see above). These studies have been recently confirmed by Ueno *et al.* (2006) who used 5-azacytidine to induce G2/M-phase arrest, also resulting in the inhibition of basally-oriented nuclear migration in the neuroepithelium. The latter authors also used cyclophosphamide to induce S-phase arrest, and in contrast with previous findings, they found that this drug was able to prevent apically-oriented nuclear migration in neural precursors. Whether the effects of cyclophosphamide on INM directly derive from S-phase arrest or

additional side effects induced by this drug could account for this observation remains currently unknown.

Molecular mechanisms regulating neurogenesis in vertebrates

The process of neurogenesis implies the production of different neuron types in the adequate number and position. As all neurons in the vertebrate central nervous system (CNS) derive from the neural tube, CNS correct formation requires the precise regulation of all the events that take place during its development. The proneural genes and the Delta-Notch signaling pathway actively participate and determine the accuracy of the neurogenesis process, and coordinate neurogenesis with the cell cycle (see below).

Proneural genes

Genetic studies in *Drosophila* and vertebrates have proved that a limited number of genes coding for transcription factors of the basic helix-loop-helix (bHLH) family, known as proneural genes, are necessary for the selection of neural precursors and for their commitment to differentiate along specific neural fates (Bertrand *et al.*, 2002). There are two families of proneural genes characterized by their homology to *achaete-scute* (*asc*) complex and *atonal* (*ato*) in *Drosophila*. In vertebrates, *asc* family has four members: the *asc-like* gene (*ascl*), which is present in all species analyzed to date (*ascl1* in zebrafish, *Xenopus*, chick and mouse), and three other genes, each one of them has been found in one single vertebrate specie (*ascl3* in *Xenopus*, *ascl4* in chick and *ascl2* in mammals). Only *ascl1* in mouse and *ascl3/ascl4* have been shown to be implicated in nervous system development (Bertrand *et al.*, 2002). As well, the genes belonging to the *ato* family in vertebrates can be divided in three subfamilies attending at differences in their basic domain: *neurogenin* (*neurog*), *ato* homologues (*atoh*) and *NeuroD* (Hassan and Bellen, 2000).

In *Drosophila*, proneural genes are first expressed in groups of quiescent ectodermal cells (i.e. “proneural clusters”) that have both epidermal and neuronal potential, and that determine the region where the CNS or the peripheral nervous system will develop (Campuzano and Modolell, 1992). Proneural activity results in the commitment of precursor cells to a neural fate, but these cells remain multipotent, and divide giving rise to both neurons and glia. In contrast, vertebrate proneural genes are first expressed in precursor cells that are already specified for a neural fate and are self-renewing. In this case, proneural activity results in the generation of cells that have limited mitotic potential, giving rise to postmitotic neurons in many instances (Bertrand *et al.*, 2002).

Proneural genes are sufficient and necessary to promote neural precursor cells formation from the *Drosophila* ectoderm (Jimenez and Modolell, 1993); and lack of these genes in vertebrates implies the loss of neurons, while their ectopic expression in regions where they are not normally expressed induces the development of ectopic neurons. Thus, the proneural gene *neurog1* has been shown to induce ectopic neurogenesis and ectopic expression of *NeuroD* mRNA in *Xenopus* embryos, indicating that the properties of neurog are analogous to those of the *Drosophila* proneural genes (Ma *et al.*, 1996). Gene disruption studies indicate that mouse ascl1 function is required for the proper development of the autonomic nervous system and olfactory neurons (Guillemot *et al.*, 1993). When ascl3 is expressed at high levels in *Xenopus* embryos the neural tube expands, and low levels of this gene generates ectopic differentiated neurons in this model system (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994). Mouse NeuroD3 has also been shown to induce ectopic neuronal formation when injected into *Xenopus* embryos (McCormick *et al.*, 1996).

Proneural proteins bind DNA as heterodimeric complexes with E proteins, a type of bHLH proteins expressed ubiquitously. Since heterodimerization is required for these proteins to bind DNA, the factors that interfere with this heterodimerization act as passive repressors of proneural genes activity. Hence, the gene products belonging to the helix-loop-helix (HLH) type of factors, *extra macrochaetae* (*emc*) in *Drosophila* and *inhibitor of differentiation* (*Id*) in vertebrates, compete with proneural proteins since they bind with high affinity to E proteins. Since these proteins lack the DNA binding domain, the resulting heterodimers with the repressor proteins cannot bind to DNA (Massari and Murre, 2000). Furthermore, the *Enhancer of split* (*E(spl)*) factors in *Drosophila* or the *Hes/Her/Esr* family in vertebrates are inhibitors of proneural genes which act as transcriptional repressors when bound to DNA, although it is also possible that they act by interfering in the formation of proneural proteins and E proteins complexes (Davis and Turner, 2001).

Delta-Notch signaling

The Notch receptor and its ligands, Delta and Serrate (known as Jagged in mammals), are transmembrane proteins with large extracellular domains, encoded by the so-called neurogenic genes. Ligand binding promotes two proteolytic cleavages which result in release of the Notch intracellular domain (NICD) and its translocation to the nucleus (Weinmaster, 2000). Then, NICD cooperates with the DNA-binding protein CSL (named after CBF1, Su(H) and LAG-1) and its coactivator Mastermind to promote transcription of its major targets, *E(spl)* or the *Hes/Her/Esr* family members in vertebrates. Therefore, Notch activation by its ligand lead to transcriptional repression of proneural genes (Bray, 2006). Glycosyltransferases like Fringe alter the capability of ligands to activate Notch. Thus, modifications of extracellular domain of Notch by Lunatic fringe (Lfng), the mammalian homologue of Fringe, potentiates Delta binding and promotes Notch activity (Haines and Irvine, 2003).

Neural precursor selection by lateral inhibition

An essential role of proneural proteins is to restrict their own activity to single neural precursor cells. Proneural genes inhibit their own expression in adjacent cells, thereby preventing these cells from differentiating. This is achieved through activation of the Notch signaling pathway, in a process termed “lateral inhibition”, which is initiated by the induction of a Notch ligand (Fig. 3). In vertebrates, ascl1 and neurogs can induce direct and dose-dependent expression of *Delta-like 1* (*Dll1*) (Castro *et al.*, 2006). Expression of the Delta ligand in the precursors activates the Notch signaling cascade in neighboring cells, resulting in the expression of repressors, *E(spl)* genes in *Drosophila* and *Hes/Her/Esr* in vertebrates that, in turn, directly downregulate proneural gene expression (Bertrand *et al.*, 2002). Through lateral inhibition, therefore, differences between neighbors caused by

stochastic events are stabilized and amplified. Eventually, the initial pattern is refined and proneural gene expression is restricted to single cells that enter a neural differentiation pathway (Artavanis-Tsakonas *et al.*, 1999)

Therefore, neural precursor fate control involves two phases. First, proneural genes are expressed or activated at low levels and a reversible selection of neural precursors takes place. Then, proneural genes reach high levels of expression or activity and neural precursors undergo an irreversible commitment to the neural fate (Culi and Modolell, 1998; Bertrand *et al.*, 2002; Kintner, 2002).

Molecular and cellular mechanism coordinating cell cycle and INM with neurogenesis

Neurogenesis in the CNS involves proliferation and subsequent differentiation of neural precursor cells located at the apical portion of the neuroepithelium. Coordination between proliferation and differentiation is required for the correct formation of CNS since alterations in the balance between both processes are translated into dramatic changes in the net production of neurons as well as the growth of the neuroepithelium (Murciano *et al.*, 2002). Furthermore, any disturbance of the three-dimensional structure of the neuroepithelium results in a higher rate of neuronal differentiation and the depletion of the neural precursor pool (see below; Murciano *et al.*, 2002; Xie *et al.*, 2007; Del Bene *et al.*, 2008).

In recent years, a large body of evidence has demonstrated that some of the key factors that regulate progression through the cell cycle have an effect on neural cell fate, whereas several determination and differentiation factors have a role in cell cycle regulation (Ohnuma *et al.*, 2001). Nevertheless, only a few studies have focused on the specific expression and function of the regulators of neurogenesis at particular cell cycle stages and their differential localization within the neuroepithelium. In the following lines we will summarize what is known on all these important aspects in CNS neurogenesis.

Cell cycle elements regulating determination/differentiation

Many of the G1 phase components affect neural determination. A key cell cycle restriction point is located at the end of G1 phase. If cells pass this point, they will almost invariably complete the cell cycle. In order to differentiate, neural precursor cells need to exit the cell cycle in G1 and enter G0 without passing the cell cycle restriction point.

During development, downregulation of CDKs and cyclins, as well as induction of CKIs lead to cell cycle exit (Gui *et al.*, 2007). In particular, $p19^{Ink4}$ and $p27^{Kip2}$ are required to maintain the quiescent state of neurons in the cortex and hippocampus (Zindy *et al.*, 1999). Numerous studies have also shown that factors whose function is inhibiting cell cycle are able to activate determination pathways. In the *Xenopus* retina, arrest in G1 phase by CKI $p27^{Xic1}$, a Kip/Cip family homolog, potentiates proneural gene *Xenopus* atoh7. Conversely, activation of cell cycle by cyclin E1, essential for entry into S-phase, reduces its activity (Ohnuma *et al.*, 2002). Data suggest that $p27^{Xic1}$ may enhance neurogenesis by stabilizing the bHLH protein neurogenin (Vernon *et al.*, 2003). On the other hand, elements mediating progression through the cell cycle could have a role on differentiation process. Thus, cyclin D1 represses *NeuroD* transcription in mammalian cells culture and this is independent from its effects on the cell cycle (Ratineau *et al.*, 2002). The APC, whose role as mediator of M and G1 transitions has been well established, has, along with its co-activator Cdh1, effects upon the nervous system ranging from regulation of axon growth and patterning to development of synapses and neural survival (Kim and Bonni, 2007).

Geminin is a dual function molecule with roles in regulating both DNA replication and neural cell fate during embryonic development. This protein prevents reinitiation of replication within a single cell cycle to maintain chromosomal integrity and euploidy. Its levels rise during S-phase and it is degraded during M enabling a new round of DNA replication to be initiated in the subsequent S-phase. On the other hand, this protein controls the transition from proliferating neural precursor to differentiated post-mitotic neuron by modulating interactions between SWI/SNF chromatin-remodeling complex and bHLH transcription factors that are critical for neurogenesis or through interactions with Six3 and Hox transcriptions factors and Polycomb group proteins (Seo and Kroll, 2006).

Determination/differentiation factors regulating cell cycle progression

Some of the transcription factors that control patterning of CNS at early stages of development mediate cell cycle progression within the regions where they are expressed. For instance, mammalian factor Emx2 promotes expansion of cortical precursors regulating symmetric cell divisions in the developing cortex (Heins *et al.*, 2001). Over-expression of eye-specific transcription factors Optix2/Six6 (Zuber *et al.*, 1999), Six3 (Kobayashi *et al.*, 2001) and Rx1 (Andreazzoli *et al.*, 1999) in *Xenopus* and zebrafish embryos results in giant eyes. All these factors bind to groucho, whose over-expression also leads to increased eye size (Lopez-Rios *et al.*, 2003). Eye-specific factors may work by repressing

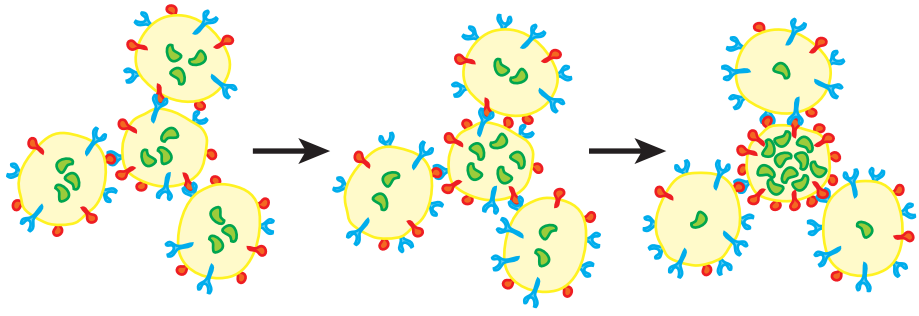

Fig. 3. Lateral inhibition with feed-back. *Proneural genes (green) are initially expressed at similar levels in a cluster of equivalent cells. These genes encode bHLH transcription factors responsible for neuronal differentiation as well as for Delta ligand (red) expression. Delta ligand activates Notch (blue) in adjacent cells, thus resulting in the inhibition of proneural gene expression in these cells. A stochastic increase of Delta ligand levels in a particular cell is translated into reduced expression of Delta and proneural genes in the adjacent cells, thus facilitating the upregulation of the latter genes in the cell, which becomes differentiated.*

cell cycle inhibitors. In fact, knockouts of *Optix2*/*Six6* show upregulation of $p27^{Kip1}$ and $p19^{Ink4}$ (Li *et al.*, 2002).

Furthermore, various determination factors facilitate differentiation. For instance, neurog2 overexpression in the chick embryo spinal cord results in cell cycle withdrawal and early differentiation of neural precursors (Farah *et al.*, 2000). Also, other proneural genes act at G1 cell cycle phase allowing cells to exit the cell cycle to G0 upregulating CKIs like $p16^{Ink4}$, $p21^{Cip1}$ and $p27^{Kip1}$. Thus, transitory activation of NeuroD2, mouse ascl1 and neurog1 can convert mouse P19 embryonic carcinoma cells into differentiated neurons by induction of $p27^{Kip1}$ and arrest of cell cycle in G1 (Farah *et al.*, 2000). Likewise, proneural gene *NeuroD* causes $p21^{Cip1}$ direct activation in HeLa cells (Farah *et al.*, 2000).

But, cell cycle phases other than G1 are regulated by determination/differentiation factors as well. *Drosophila* Tramtrack protein inhibits cell cycle progression throughout cyclin E inhibition (Badenhorst, 2001). In *Drosophila* wing development, asc activity induces transient G2 phase arrest by downregulation of the M-inducing phosphatase *string* (*cdc25*) (Johnston and Edgar, 1998).

In the same manner, external determination factors and proliferating signals act throughout cell cycle components present in G1 phase. Wnt regulates cell cycle by modulation of cyclins D1 and D2, as well as Myc (Kioussi *et al.*, 2002; Baek *et al.*, 2003), whereas Sonic hedgehog (Shh) modulates *cyclin D1* and *Mycn* transcription (Kenney *et al.*, 2003; Oliver *et al.*, 2003; Lobjois *et al.*, 2004). Pituitary adenylate cyclase-activating polypeptide (Adcyap) is a neuropeptide expressed in growth zones of the brain. Via the Shh glycoprotein, the small GTPase Rho or the CKI $p57^{Kip2}$, Adcyap1b inhibits the cyclin E-CDK2 complex. This signaling may elicit cortical precursor withdrawal from the cell cycle, antagonizing mitogenic stimulators, and promote neuronal differentiation (Meyer, 2006).

Cell-cycle dependent expression and function of regulators of neurogenesis in the neuroepithelium

The expression of particular CNS determinants has been shown to be associated to specific cell cycle stages. Prox1, for example, a homeodomain protein involved in horizontal retina cells determination, is predominantly expressed during G2 phase (Dyer *et al.*, 2003). Likewise, Dyrk1a, a protein required for proliferation, is expressed by neural precursors during M and G1 phase, and it is asymmetrically inherited, probably, in order to define proliferation-differentiation transition (Hammerle *et al.*, 2002). In the same manner, glial cells determinant Tramtrack is upregulated during S-phase in *Drosophila* (Badenhorst, 2001).

The expression levels of neurogenic and proneural genes have also been shown to oscillate as the neural precursors proceed through the cell cycle. During vertebrate development, *Notch1* expression increases at the neuroepithelium apical region (Lindsell *et al.*, 1996; Myat *et al.*, 1996; Henrique *et al.*, 1997). *Dll1* can also be detected in this region, expressed by neural precursors committed to neuronal fate once they have completed their final S-phase (Henrique *et al.*, 1995; Myat *et al.*, 1996; Murciano *et al.*, 2002). Therefore, the expression of both *Notch1* and its ligand *Dll1* in neural precursor cells is restricted to the region of the neuroepithelium where the neural precursors going through G2/M/G1 are located (Murciano *et al.*, 2002; Cisneros *et al.*, 2008). In contrast with this notion, Del Bene *et al.* (2008) found that in the embryonic zebrafish retina *dlb* and *dlc* mRNAs were mainly expressed at the basal neuroepithelium. Surprisingly, these authors found evidence of Notch-specific activity only at the apical neuroepithelium, suggesting that other Delta ligands could activate Notch in that region.

The capacity to differentially express *Notch1* and *Dll1* in a cell-cycle dependent manner is an intrinsic characteristic of the neural precursors. Indeed, *in vitro* isolated neural precursors from chicken (E4) and mouse (E11) brain neuroepithelia, chemically synchronized either in M or S-phase, showed reduced expression levels of *Notch1* and *Delta1* mRNAs when synchronized in the latter stage, as observed *in vivo* (Cisneros *et al.*, 2008). Accordingly, freshly dissociated neural precursors in G2/M were observed to have higher levels of *Notch1* than those in S-phase (Cisneros *et al.*, 2008). *Id2* expression levels were diminished when neural precursors were synchronized in M, but not when synchronized in S-phase, proving that the reduced mRNAs levels for *Notch1* and *Delta1* during S-phase is specific and not a consequence of the neural precursors isolation and culture, and posterior chemical treatment for synchronization.

The glycosyltransferase Lfng modifies Notch extracellular domain and facilitates Notch activation by Dll1 (Haines and Irvine, 2003). Interestingly, its mRNA is also expressed in the apical region of the neuroepithelium (Cisneros *et al.*, 2008), showing the same expression pattern as *Notch1*. This suggests that Notch activity is enhanced in neural precursors undergoing M. The cell cycle-dependent changing expression of *Lfng* may also reflect Notch1 activity, since *Lfng* promoter responds to Notch activation in the presomitic mesoderm (Morales *et al.*, 2002), although other contributions to its regulation cannot be discounted.

Co-localization of *Notch1* receptor, *Dll1* ligand and *Lfng* mRNAs is not enough to prove activation of Notch signaling cascade in the apical region of the neuroepithelium. This notion was proved to be true as the expression of the downstream effectors of Notch, *Hes1* and *Hes5* (Davis and Turner, 2001; Fior and Henrique, 2005; Bray, 2006; Nelson *et al.*, 2006), was also observed to be cell cycle dependent. *Hes5* expression in the apical region of the neuroepithelium (Nelson *et al.*, 2006; Cisneros *et al.*, 2008), as well as the increment of its mRNA levels consequently to the high density of the cultured neural precursors synchronized in M but not in S-phase (Cisneros *et al.*, 2008), imply that Notch activity in neural precursors is maximal during M. *Hes1* expression is also increased in high-cell density cultured neural precursors synchronized in M, but not in S-phase, although at a lesser degree when compared to *Hes5* expression (Cisneros *et al.*, 2008), likely as the result of *Hes1* expression regulation being less dependent on Notch activity (de la Pompa *et al.*, 1997). In accordance with these studies, the downstream effector of Notch *her4* has been shown to increase its transcription in neural precursors from zebrafish embryonic retina as they move from the basal to the apical end of the neuroepithelim (Del Bene *et al.*, 2008), further indicating that Notch signaling is activated during G2/M. Tokunaga *et al.* (2004) have shown that the presence of NICD, an evidence for Notch activity, can be detected in a minority of nuclei undergoing S-phase (i.e. incorporating BrdU), and that the cells expressing active Notch1 were located closer to the ventricular surface than those expressing the Notch1 receptor, suggesting that Notch1-signaling is activated in a cell cycle phase-dependent manner. Nevertheless, in this study NICD was not detected in mouse neural precursors undergoing M-phase (Tokunaga *et al.*, 2004).

Del Bene *et al.* (2008) have also shown NICD-specific immunoreactivity in the nuclei of neural precursors located at the ventricular surface of the mouse embryonic retina. In another study, immunoreactivity for NICD has been described throughout the CNS neuroepithelium closely resembling that of BrdU staining (Del Monte *et al.*, 2007), therefore indicating that Notch1 is active in proliferating cells. Nevertheless, co-localization of NICD with BrdU (i.e. in neural precursors undergoing S-phase) or lack of this co-localization was not showed by these authors. Further and more detailed studies are necessary in order to precisely determine the activation and function of Notch1 along cell cycle. Expression of Notch1 effectors such as *Hes1* and *Hes5* may be subject to autonomous regulation in a cell-cycle dependent manner, just as it has been described for *Nocth1* and *Dll1*, and we have to take into account the delay existing between RNA presence in the cell and its translation, if any, into functional proteins. All these facts may account for the existing divergences among the mentioned studies.

The capacity of the developing chick neuroepithelium to express *Dll1*, *Neurog1* and *Neurog2* is reduced during S-phase *in vivo*, even though *Notch1* levels are also reduced in this cell-cycle phase (Murciano *et al.*, 2002) and, therefore, no inhibitory signals seem to be regulating the previously mentioned genes. Since *Dll1* is susceptible to regulation by the transcription factors codified by the proneural genes (Kunisch *et al.*, 1994; Heitzler *et al.*, 1996; Castro *et al.*, 2006), *Dll1* expression can be a measurement of the neurogenic capacity of neural precursors. As expected, *Dll1* mRNA levels are diminished under conditions where *Hes1* and *Hes5* levels are high due to high-density culture conditions and synchronization in M (Cisneros *et al.*, 2008). Nevertheless, the low level of expression of Notch effectors *Hes1* and *Hes5* during S-phase does not translate into an increase of *Dll1* expression, not even under high-density culture conditions nor when the neural precursors were free of Notch inhibitory signals at low-density culture conditions (Cisneros *et al.*, 2008). Therefore, data indicate the absence of neurogenic capacity during S-phase. The mechanism that prevents *Dll1*, *Neurog1* and *Neurog2* expression during S-phase is unknown at present. Hes1 presence may, in a Notch-independent manner, interfere with the mentioned genes during S-phase. Indeed, the observed oscillations of Hes1 protein levels (Shimojo *et al.*, 2008) could account for the downregulation of *Dll1*, *Neurog1* and *Neurog2* expression during S-phase. Low Notch1 expression levels during S-phase could also account for the reduced *Dll1*, *Neurog1* and *Neurog2* expression at this cell cycle stage. In this regard, Notch has been shown to present two sequential and opposite effects during neural development. Notch has the capacity to induce proneural gene expression in areas where it initially becomes expressed. This effect has been demonstrated in the *Drosophila* eye imaginal disc (Baker and Yu, 1997), the developing chick inner ear (Daudet and Lewis, 2005), and in embryonic stem cells (Lowell *et al.*, 2006). Later in development, Notch acts as a classical mediator of lateral inhibition. Therefore, the decrease of *Notch1* expression levels during S-phase could explain the observed diminished neurogenic capacity of neural precursors during this cell cycle phase.

Regulation of Notch1 and Dll1 levels along the cell cycle

Since *Notch1* and *Dll1* mRNA levels decrease during S-phase in vertebrate neuroepithelia, the different mechanisms involved in gene expression regulation, such as the transcription rate and mRNA stability, must take account for the changing expression levels of these genes. In order to determine the contribution of transcriptional regulation on the mRNAs levels, promoter studies were performed in the context of a mouse neural precursor line, b2T-H2 (Nardelli *et al.*, 2003), both in transient and stably transfections. Mouse promoter fragments for *Nocth1* and *Dll1*, and whose functionality had been already described (Beckers *et al.*, 2000; Yugawa *et al.*, 2007), do not differentially activate the expression of a reporter gene in cells synchronized either in M or S-phase (unpublished data). But far away located enhancers or other regulatory sequences situated within the untranslated regions of the gene, or even within the introns, could still be responsible for the observed changing mRNA levels. To clarify the participation of transcriptional regulatory factors in the expression of *Notch1* and *Dll1* genes, their transcription rate was determined in neural precursors synchronized in either M or S-phase by means of quantitative real-time RT-PCR of the levels for their heterogeneous nuclear RNAs (hnRNA). This RNA species stands for the population of newly transcribed RNAs in the nuclei before they get processed as they come out from the nuclei into the cytoplasm. The measurement of this RNA species reflects the transcription rate since no other regulatory factors, such as the ones involved in mRNA stability, have yet come into contact with the RNA (Elferink and Reiners, 1996). Analysis of the hnRNA levels has revealed that transcriptional regulation is not involved in the changing expression levels of endogenous *Notch1* and *Dll1* during cell cycle progression, either in chick or mouse neural precursors, since hnRNA levels remain the same during M or S-phase (Cisneros *et al.*, 2008).

The study of *Notch1* and *Dll1* mRNA stability in chick and mouse neural precursors by means of treatment with the transcription inhibitor actinomycin D has revealed that both genes mRNA present less stability in S-phase when compared to their stability during M (Cisneros *et al.*, 2008). This is not due to unspecific effects of the treatment, since the analysis of the stability of the *Id2* gene rendered no changes in mRNA stability for this gene. Regulation of mRNA stability constitutes an essential mechanism of gene expression control, which allows the rapid response of cells to internal and external stimuli. The expression of certain genes involved in cell cycle regulation, such as cyclins A, B1 or D1 (Maity *et al.*, 1995; Wang *et al.*, 2000; Lal *et al.*, 2004), or the CKI $p21^{Cip1}$ (Lal *et al.*, 2004) is regulated by means of differential stability of their transcripts. As well, variations in the activity of the bHLH transcription factors Myod1 and myogenin, both involved in muscle development, are controlled by stability of their mRNAs (Figueroa *et al.*, 2003). Surprisingly, the increased stability of *Notch1* and *Dll1* mRNAs during M contrasts with the general instability of transcripts observed during this cell-cycle phase, due to the hyperphosphorylation and the resultant reduction of the CDK1-dependent poly(A) polymerase activity (Colgan *et al.*, 1996).

The mechanisms that regulate *Notch1* and *Dll1* mRNA stability in neural precursors are, at present, unknown. Nevertheless, it has been described that in *Helobdella robusta* the activity of $p38^{MAPK}$ stabilizes the zygotic *Notch* transcript (Gonsalves and Weisblat, 2007). This kinase induces cell-cycle arrest in M under stress conditions (Takenaka *et al.*, 1998) and it is active during M in rat retinal cells, where it has been shown to be necessary for

normal cell cycle progression (Campos *et al.*, 2002). Yet, its role during normal SNC development has to be clearly characterized, being a candidate as a regulator of *Notch1* mRNA stability in neural precursors during M. Several elements located in mRNA sequences that promote the shortening of poly(A) tails, and the thereafter transcript degradation, have been described. AU-rich elements (AREs) are located within the 3' UTR region of mRNAs and are defined by the sequence AUUUA (Beelman and Parker, 1995). These AREs sequences are responsible for regulating *Notch* mRNA stability in *Helobdella robusta* and their presence has been detected by *in silico* analysis within *Notch* and *Delta* transcripts sequences, as well as within *Lfng*, in all species where these regions have been described (Cisneros *et al.*, 2008). Several RNA binding proteins interact with AREs sequences and regulate mRNA stability (Barreau *et al.*, 2005). RNA-binding proteins such as the Elavl1 proteins and other protein family members are candidates in regulating *Notch1* and *Dll1* stability in the neuroepithelium. Elavl1 belongs to the Embryonic Lethal Abnormal Vision (ELAV)-like family of RNA-binding proteins, which includes Elavl2, Elavl3 and Elavl4 proteins, and that can enhance *cyclins A*, *B1* and *D1* mRNA stability (Wang *et al.*, 2000; Lal *et al.*, 2004), as well as the mRNAs for Myod and myogenin transcription factors (Figueroa *et al.*, 2003). Although Elavl1 expression is ubiquitous, other type Elavl proteins are specifically expressed in neurons (Antic and Keene, 1997). In fact, the *elav* locus is essential for the development and maintenance of the *Drosophila* nervous system (Robinow *et al.*, 1988). Another potential candidate for the regulation of neurogenic genes expression is the HNRNPD protein, an AREs-binding protein that favors *cyclin D1* degradation (Lal *et al.*, 2004).

Regulation of mRNA stability can also be achieved by means of microRNAs (miRNAs). miRNAs are small non-coding RNAs of 18-25 nucleotides in length that regulate gene expression at a post-translational level by binding to complementary sites in the mRNA of target genes (Lee *et al.*, 1993; Wightman *et al.*, 1993). The major regulatory function of miRNAs is to prevent protein translation from the target mRNA, although a decrease in mRNA stability is a function of some miRNAs (Behm-Ansmant *et al.*, 2006). miRNAs provide a new level of gene expression regulation with an effect of fine-tuning tissue-specific gene expression. miRNAs are involved in multiple pathways in a variety of organisms, being the nervous system a rich source of miRNA expression (Krichevsky *et al.*, 2003; Miska *et al.*, 2004; Sempere *et al.*, 2004). In fact, most miRNA data relevant to nervous system have focused on development, participating in processes of patterning and cell specification, although miRNA function has lately been related to neuronal plasticity and CNS-related diseases (for reviews, see Kosik, 2006; Fiore *et al.*, 2008). Various miRNAs have already been proved to play a role in neurogenesis (Krichevsky *et al.*, 2006), such as the Mirn200 family controlling olfactory neurogenesis. In fact, one of the identified targets for the Mirn200 family members is the *Lfng* mRNA (Choi *et al.*, 2008). A large number of miRNA have been predicted to target genes involved in cell fate decisions in the developing nervous system, in particular several miRNA target genes within components of the Notch pathway in *Drosophila* (Enright *et al.*, 2003). As well, expression of many miRNAs has been detected during neural tube development in vertebrates (Darnell *et al.*, 2006; Cao *et al.*, 2007; Kapsimali *et al.*, 2007; Wulczyn *et al.*, 2007). Moreover, *Notch1* mRNA itself has been described as a target gene for various miRNAs in a human neuroblastoma cell line (Fukuda *et al.*, 2005), and *Delta* expression is regulated in *Drosophila* by mir-1 (Kwon *et al.*, 2005). Furthermore, *in silico* analysis has yielded many murine miRNAs whose target genes can be both *Notch1* and *Dll1*. Cell cycle dependant regulation of these genes may be mediated by miRNAs binding to their 3' UTR regions since it is already known that many important cell cycle molecules are target genes of various miRNAs (le Sage *et al.*, 2007; Linsley *et al.*, 2007; Ivanovska *et al.*, 2008; Schultz *et al.*, 2008). Moreover, miRNA regulation of translation is cell-cycle dependant, since it has been observed its strongest repressive potential in the S and S/G2 phases with minimal repression in the G1 phase (Vasudevan *et al.*, 2008). Furthermore, miRNA activity can be affected by RNA-binding proteins (RBP). For instance, the RNA-binding protein Elavl1 associates with the 3' UTR region of the Trpv6 mRNA after stress, counteracting the effect of Mirn122a. Hence, there is a crucial role for RBP/miRNA interplay on 3' UTRs of mRNAs in developmental decisions (Kedde and Agami, 2008). Both factors, RBPs and miRNAs, may be responsible for the observed change in *Notch1* and *Dll1* mRNA stability and both are being taken into account in present studies intended to determine the mechanisms that regulate *Notch1* and *Dll1* gene expression.

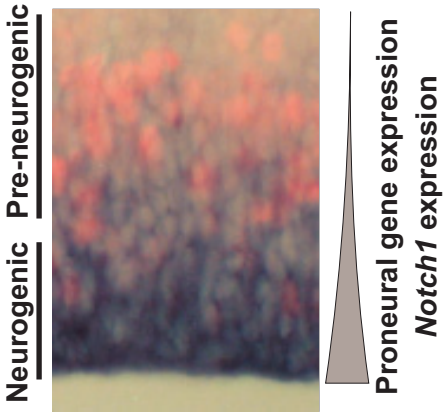

Fig. 4. Functional organization of the neuroepithelium in terms of its neurogenic potential. *The orthogonal arrangement of the cells moving their nuclei up and down as they progress through the cell cycle (see Fig. 1) is translated into the segregation of the neuroepithelium into two zones, each comprising the soma of neural precursors that are transiently involved in distinct functions. In the apical region (neurogenic zone), proliferating cells are able to express* Notch1 *(dark blue),* Dll1*, and the proneural determination gene* Neurog2*. In the basal epithelium (pre-neurogenic zone), neural precursors either cannot express these genes or express them at low levels, and they undergo S-phase as evidenced by a short pulse of BrdU (red). Apical surface down.*

Neurogenesis and INM: functional regionalization of the neuroepithelium in apical neurogenic zone and basal pre-neurogenic zone

In terms of neurogenesis, the differential expression through the cell cycle of the neurogenic genes *Dll1* and *Notch1*, and the proneural gene *Neurog2* suggests the segregation of the neuroepithelium into two zones. One region, correlating with neural precursors in S-phase, comprises a pre-neurogenic zone where the neural precursors present a diminished capacity to trigger

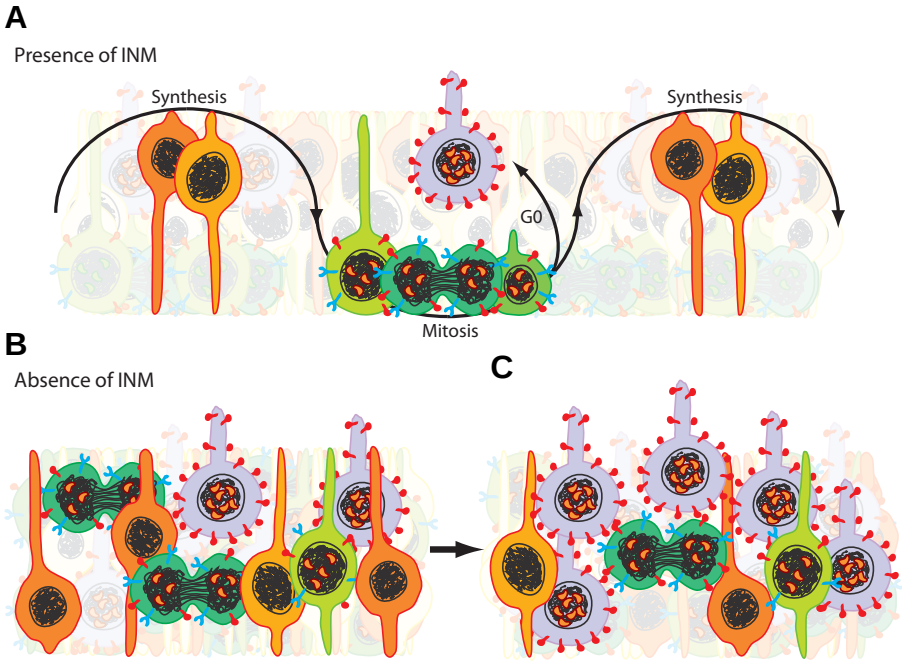

Fig. 5. Influence of INM in neurogenesis. (A) *Due to the INM, neural precursors in neurogenic state (green) are grouped close to each other, while neural precursors in pre-neurogenic state (red) are segregated from the former ones. Delta-Notch signaling is maximal in neurogenic precursors, thus resulting in the production of a relatively low number of neurons (blue).* **(B)** *In the absence of INM, neurogenic and pre-neurogenic precursors are intermingled and Delta-Notch signaling becomes reduced (see green cells surrounded by red cells in left panel). This translates into an increase of neurogenesis and a depletion of neural precursors in the neuroepithelium (right panel).*

inhibitory signals due to the lower expression levels of the previously mentioned genes. The second region, where proliferating neural precursors apically locate most of their soma during G2/M/early G1 phases, constitutes the neurogenic zone, which is characterized by a high expression level of neurogenic and proneural determination genes (Murciano *et al.*, 2002) (Fig. 4).

This model for the functional organization of the neuroepithelium due to the coordination between cell cycle and INM implies the local synchronization of neural precursors in relationship to their cell cycle stage and entails the grouping of cells with similar expression profiles of genes involved in neurogenesis. Hence, expression of *Notch1* receptor and its activity modulator *Lfng* in cells undergoing M, and located at the apical region, confers the capacity to respond to inhibitory signals from differentiating cells expressing Dll1 ligand present in that region, as shown by induced expression of Notch effectors *Hes1* and *Hes5* (Cisneros *et al.*, 2008). Indeed, an apical process has been shown to be connected with the ventricular side of the neuroepithelium in postmitotic neuroblasts as they migrate out from the neuroepithelium (Minaki *et al.*, 2005). This apical process is enriched in *Dll1* mRNA, further stressing that the lateral inhibitory signals take place at the apical neuroepithelium. In this same apical zone is where expression of *Neurog1* and *Neurog2* proneural genes greatly increases. Consequently to lateral inhibition, expression of proneural genes gets restricted to a few cells that enter the differentiation pathway and abandon the neuroepithelium. The cells that are not able to exit the cell cycle after the neurogenic stage, will keep cycling and will reduce the expression levels of neurogenic and proneural genes. Restricting the neurogenic capacity to a window prior to cell-cycle exit (G0) probably prevents the execution of the differentiation program during cell-cycle phases prior to M.

In different animal species, neurogenesis is linked to synchronization waves prior to neuronal differentiation. Hence, in *Drosophila* embryo, neuroblasts production from neuroectoderm is preceded by cell-cycle arrest in G2 (Hartenstein *et al.*, 1994). Similarly, in the eye imaginal disc of *Drosophila* has been observed synchronization in G1 of the photoreceptors precursors (Baker and Yu, 1997). Production of primary neurons in the anamniota neural plate, before it becomes a pseudostratified epithelium, is preceded by a M synchronization wave that runs along the neural plate, starting from the most lateral end and moving towards the medial region (Hartenstein, 1989). These strategies are characterized by the presence of long periods of time during which neural precursors stay in interphase and there is no neuronal production. The existence of INM in vertebrate neuroepithelium may allow the continuous production of neurons from neural precursors in G2/M/G1, which transiently undergo a neurogenic state that has facilitated the vertebrate evolution towards more complex brains with greater number and types of neurons.

Relevance of neuroepithelium division into two regions, one neurogenic and another pre-neurogenic, is evident when INM is prevented (Fig. 5). In the absence of INM, the possibility of interactions between cells expressing neurogenic and proneural genes, and cells that are not capable to express these genes increases, therefore lateral inhibition signals diminish. This translates into greater neurogenesis with the consequent depletion of neural precursors, as seen in experiments with pharmacologic blocking of INM or computer modeling simulations (Murciano *et al.*, 2002). Similarly, silencing Cep120 or Tacc3 in neural precursors during neocortical development impairs both INM and neural precursor cell proliferation, thus resulting in enhanced neuronal differentiation (Xie *et al.*, 2007). Another piece of evidence supporting that the integrity of the three-dimensional structure of the neuroepithelium is crucial for the process of neurogenesis comes from the work by Bel-Vialar *et al.* (2007). These authors demonstrated that turning off Pax6 function, known to disturb INM (Tamai *et al.*, 2007), provokes premature differentiation in the developing spinal cord. Alteration of INM in the retina of *dynactin-1* mutant zebrafish triggers enhanced retinal ganglion cell production (Del Bene *et al.*, 2008), as previously shown in the chick retina by Murciano *et al.* (2002). Importantly, NICD expression or *her4* disruption in the mutant zebrafish embryos suppressed neuronal overproduction (Del Bene *et al.*, 2008), further indicating that enhanced neurogenesis in the absence of INM is due to reduced Notch signaling.

One important question currently under debate regards to the

putative retraction of the basal process once a neural precursor becomes rounded during M. Indeed, retraction of this basal process would prevent mitotic cells from receiving lateral inhibitory signals from the Dll1-positive postmitotic neuroblasts that are located at the basal neuroepithelium as they migrate towards the differentiated zone. For quite long time it was believed that the basal process of each neural precursor is lost during cell division (Hinds and Ruffett, 1971; Berry and Johnson, 1975). However, other authors have challenged this view and presented evidence that a extremely thin basal process composed of membrane and lacking microtubules (Wilcock *et al.*, 2007) is retained by the neural precursors undergoing M and it is inherited by one of the daughter cells (Miyata *et al.*, 2001; Noctor *et al.*, 2002; Das *et al.*, 2003; Afonso and Henrique, 2006), but whether this is the case for all neural precursors is currently unclear. In this regard, a detailed analysis performed by Saito *et al.* (2003) in mouse retina slices demonstrated a reduced capacity to extend basal processes in those daughter cells that do not inherit these structures during M. These cells show short basal processes of approximately 20 μm, which do not reach the basal neuroepithelium. In line with these results, a recent study by Pearson *et al.* (2005) demonstrates the absence of basal process in some mitotic neural precursors. The reduced capacity of certain neural precursors to move their nuclei to the basal neuroepithelium has been also reported by Baye and Link (2007) in the zebrafish retina.

The proposed arrangement of the vertebrate neuroepithelium seems to be restricted to neural tube areas where neurons are been produced. In the caudal neural plate, there is a population of stem cells that give rise to the spinal cord. In this region, *Dll1* is expressed in cycling cells in a cell cycle independent manner and *Notch* is constantly active (Akai *et al.*, 2005). In this context, uniform expression of *Dll1* translates into mutual inhibition of all cells in the neural plate, preventing neuronal differentiation. When cells abandon this region, they initially maintain *Dll1* expression in neural precursors undergoing S-phase (Hammerle and Tejedor, 2007), and subsequently lateral inhibition gets activated and *Dll1* expression is restricted to cells undergoing neuronal differentiation (Mizuhara *et al.*, 2005). Finally, the cumulative data suggest that differential expression of neurogenic genes during cell cycle and its spatial localization along the apical-basal axis of the neuroepithelium are extremely important for the precise differentiation of neural precursors.

Response ability to environmental factors during cell cycle

In the nervous system of almost all species, there is a correlation between the phenotype a neuron adopts and its birth time (Caviness and Sidman, 1973). Different cell fates could be determined when cells are actively proliferating, near their final cell cycle, or post-mitotically. Studies in *Drosophila* (Isshiki *et al.*, 2001), zebrafish (Kay *et al.*, 2001) and *Xenopus* (Ohnuma *et al.*, 2002), indicate that distinct neural fates may be determined during the final cell cycle of neural precursors and, moreover, correct formation of nervous system depends on coordination between cell cycle regulation and cell fate determination.

Neural precursors response to extrinsic cues differs along cell cycle. Thus, neural precursors from mouse or ferret cortex (McConnell and Kaznowski, 1991; Fukumitsu *et al.*, 2006) or rat retina (Belliveau and Cepko, 1999) receive determination signals during S-phase but not during G2, M or G1. Motor neuron generation depends on two critical periods of Shh signaling: an early period during which naive neural plate cells are converted into ventralized neural precursors and a late period that extends well into S-phase of the final neural precursor cell division, during which Shh drives the differentiation of ventralized neural precursors into motor neurons (Ericson *et al.*, 1996). Regarding *Caenorhabditis elegans*, vulva precursor cells adopt different fates in response to lin-12 lateral signaling fate accordingly with their cell cycle phase. Coupling developmental decisions to cell cycle transitions may provide a mechanism for prioritizing or ordering choices of cell fates for multipotential cells (Ambros, 1999). Lack of neurogenic capacity in vertebrate neural precursors during S-phase could facilitate this process.

Acknowledgments

This work was supported by the Ministerio de Ciencia y Tecnología (BCM2003-03441/BFU2006-00805), Fundación "La Caixa" (BM05-71-0), and FUNDALUCE.

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