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# Beyond boundaries—Eph:ephrin signaling in neurogenesis

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Eph:ephrin signaling plays an important role in embryonic development as well as tissue homeostasis in the adult. At the cellular level, this transduction pathway is best known for its role in the control of cell adhesion and repulsion, cell migration and morphogenesis. Yet, a number of publications have also implicated Eph:ephrin signaling in the control of adult and embryonic neurogenesis. As is the case for other biological processes, these studies have reported conflicting and sometimes opposite roles for Eph:ephrin signaling in neurogenesis. Herein, we review these studies and we discuss existing mathematical models of stem cell dynamics and neurogenesis that provide a coherent framework and may help reconcile conflicting results.

## Introduction

### Eph:ephrin signaling

Eph receptors and ephrins are cell surface proteins that mediate local cell-to-cell communication. Typically, upon cell-cell contact, Eph receptors and ephrins expressed on neighboring cells interact with each other, thus triggering a signaling cascade. The best characterized biological outcome of Eph:ephrin signaling—its canonical function—is the regulation of cell adhesion, either positively or negatively, depending on the cellular context.<sup>1</sup> Based on this important activity, and on the broad expression of its members, it does not come as a surprise that Eph:ephrin signaling has been implicated in numerous developmental, physiological and pathological processes.<sup>2–4</sup>

Eph receptors and ephrins are subdivided in 2 classes, A and B, based on sequence homologies and binding affinity preferences. There is a high degree of intraclass promiscuity (all Ephs of one class bind to all ephrins of the same class, albeit with different affinities) and a small number of interclass interactions have been reported.<sup>5</sup> A special feature of Eph:ephrin signaling is that both

partners in the receptor:ligand pair are in theory capable of activating simultaneous downstream signal transduction cascades upon interaction. Signaling cascades downstream of Eph receptors and downstream of ephrins are referred to as forward and reverse, respectively. Over the years, a wealth of publications has identified downstream effectors of Eph receptor tyrosine kinases, such as small GTPases, GAPs, GEFs, cytoplasmic kinases and phosphatases.<sup>1, 4</sup> On the contrary, the molecular characterization of reverse signaling has progressed at a slower pace, perhaps because the biological outputs of reverse signaling are in general less robust than those of forward signaling. The majority of molecular effectors of Eph:ephrin signaling identified to date relate to the adhesive function of the pathway and to local regulation of the cell's cytoskeleton.<sup>1, 4</sup>

Over the years, the role of Eph:ephrin signaling has been extensively studied in the context of the developing mammalian nervous system where it has been involved in topographic mapping, neurulation, axon guidance, axon fasciculation, dendritic pruning, neuronal migration and synapse formation (for recent reviews, see refs<sup>6, 7</sup>). A number of studies have also reported a role for Eph:ephrin signaling in neurogenesis, both in the embryonic and adult brain. These latter studies are the basis of this review.

### Neurogenesis

Neurogenesis refers to the process by which neurons are generated from neural stem cells throughout the nervous system. Herein we will discuss only neurogenesis in the embryonic and adult brain. In mammals, the bulk of neurons present in the adult brain are born during fetal life, yet it is now clearly established that active neurogenesis remains present throughout adult life in at least 2 specific regions of the adult brain, the subventricular zone lining lateral ventricles and the subgranular zone of the hippocampus.

While embryonic and adult neurogenesis share common principles and key molecular players, there are also substantial differences between both processes. The first difference between embryonic and adult neurogenesis is that neural stem cells in the embryo (henceforth called neural progenitors or NP) are actively dividing cells while adult neural stem cells (NSC), as is the case for other adult stem cells, are quiescent cells that rarely divide. A second difference is in the repertoire of neuronal subtypes produced. Indeed, NP give rise to a large array of neuronal subtypes, while adult NSC give rise only to a limited set of neuronal subtypes in physiological conditions, indicating that adult NSC have a restricted potential

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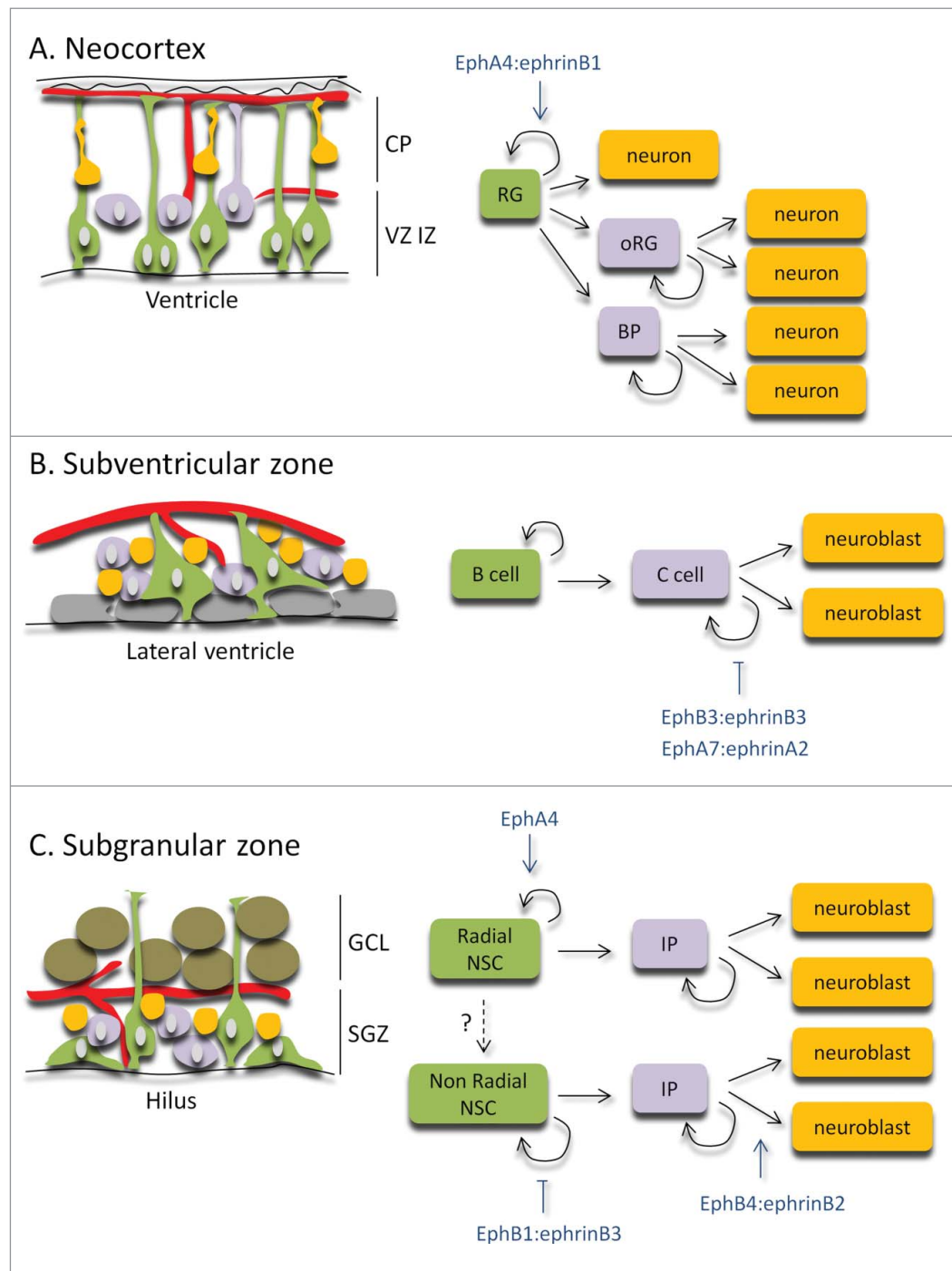
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compared to NP. Lastly, embryonic neurogenesis is a final-state system, in which a large population of neurons is produced from a transient pool of NP in a finite time window whereas adult neurogenesis is a steady-state system in which the pool of NSC must be continuously maintained over time.

At early developmental stages, NP are organized into a pseudo-stratified neuroepithelium. Around the time the first neurons are born, NP acquire molecular and morphological characteristics of radial glial (RG) cells. In the neocortex, RG cells self-renew and give rise to newborn neurons as well as to a second class of intermediate progenitors called basal progenitors (BP) that undergo a

few rounds of proliferative divisions before experiencing terminal neuronal differentiation (Fig. 1A). Other types of intermediate progenitors have been described recently, the most famous of which being outer radial glia cells (oRG) that were first discovered in primates and ferrets<sup>8,9</sup> but have also been identified in rodents albeit in a much lower proportion.<sup>10</sup> RG cells, oRG cells and BP may be identified based on their differential expression of transcription factors but also based on their distinct morphologies. Indeed, RG cells are attached both apically and basally to the ventricular and pial surfaces of the neocortex, respectively. In contrast, oRG cells have only a basal attachment site while basal progenitors have neither apical nor basal attachment site.<sup>11</sup> RG cells, oRG cells and BPs are compartmentalized in specific regions of the cortical anlage (ventricular and subventricular zones) that may be compared to stem cell niches as they provide environmental conditions necessary to maintain the progenitor fate (Fig. 1A).



**Figure 1.** Neurogenic niches and Eph:ephrin signaling. (A) In the developing neocortex, radial glial (RG) cells are self-renewing neural progenitors. They may self-renew or give rise directly to neurons or to intermediate progenitors (oRG cells and BP). Bidirectional EphA4:ephrinB1 signaling is required to maintain self-renewal of RG cells. (B) Neural stem cells in the subventricular zone (B cells) are in contact with the ventricle and blood vessels (in red). They give rise to intermediate progenitors (C cells) which differentiate into neuroblasts. Several members of the Eph:ephrin pathway have been shown to limit proliferation of C cells. (C) Two types of neural stem cells (NSC) are present in the subgranular zone (radial NSC and non radial NSC). Both give rise to intermediate progenitors. Eph:ephrin family members play distinct roles along that neurogenesis pathway. BP: basal progenitors; CP: cortical plate; GCL: granule cell layer; IP: intermediate progenitors; IZ: intermediate zone; SGZ: subgranular zone; VZ: ventricular zone. Adapted from Miller and Gauthier-Fisher.

As mentioned above, neurogenesis in the adult brain is restricted to 2 discrete regions, the subventricular zone (SVZ) lining lateral ventricles and the subgranular zone (SGZ) of the hippocampus (for a comprehensive review, see<sup>12</sup>). Both regions, or niches, are different in terms of cellular composition, structural organization and in the types of neurons they produce (Fig. 1B and C). In the SVZ, the quiescent NSC, called B cells, are apposed to—and sometimes intercalated within—a monolayer of ependymal cells lining the ventricle. Upon activation, B cells give rise to transient amplifying cells or intermediate progenitors (C cells) that divide more rapidly and in turn give rise to neuroblasts (A cells) expressing markers of newborn neurons (Fig. 1B). Neuroblasts migrate to the olfactory bulb where they differentiate into interneurons and integrate into existing neuronal circuits. In the SGZ, two populations of NSC have been described, one that divides rarely, called radial NSC, and one that divides more frequently called non-radial NSC. Radial and non-radial NSC are morphologically distinct with radial NSC extending a long radial process while non-radial NSC possess a short process (Fig. 1C). Both types of NSC apparently have different embryonic origin.<sup>13</sup> Both will give rise to intermediate progenitors that will expand and differentiate into newborn neurons. Upon differentiation, newborn neurons migrate into the adjacent granule layer where they integrate into neuronal networks.

One very important feature of stem cell niches is that they are vascularized and neural stem cell niches, both in the adult and in the embryo, are no exceptions. The role of the vasculature in controlling adult neurogenesis is well established, in fact, it has been shown in the SVZ that NSC are in close contact with endothelial cells allowing for control of neurogenesis by environmental factors transported through blood (nutrients, hormones, etc. . .), but also by growth factors secreted by endothelial cells. Similarly, there are strong indications that extrinsic cues provided by blood vessels control embryonic neurogenesis.<sup>14,15</sup>

### Eph:ephrin signaling in adult neurogenesis

#### *EphB:ephrinB*

The first study reporting a role for Eph:ephrin signaling in adult neurogenesis was performed by the group of Alvarez-Bulleya in 2000. The authors showed that EphB1-B3 and ephrinB1-B3 were expressed in the adult SVZ and demonstrated that EphB:ephrinB signaling was involved both in chain migration of neuroblasts, as well as in the control of proliferation of NSC in the SVZ.<sup>16</sup> EphB:ephrinB signaling was compromised by infusing recombinant proteins into the lateral ventricle of wild type adult mice. These recombinant proteins, composed of the extracellular domain of Ephs or ephrins fused to the Fc fragment of human IgG (EphB2-Fc and ephrinB2-Fc, respectively for this study), have been used extensively both *in vivo* and *in vitro* to activate or inhibit forward and/or reverse signaling, depending on their degree of clustering. The caveat of this approach, especially when used *in vivo*, is that it is often difficult to discriminate between an activating and an inhibiting effect of the recombinant proteins with respect to endogenous Eph:ephrin signaling. Thus, from the data presented in this paper, it was not possible to conclude on

the exact role of endogenous Eph:ephrin signaling in NSC. However, a direct control of NSC proliferation by EphB:ephrinB signaling was confirmed a few years later, using *in vitro* cultures of adult NSC isolated from the SVZ. Katakowski et al. showed that stimulation of NSC with EphB2-Fc increased BrdU incorporation and promoted the neuronal fate.<sup>17</sup> This data suggested that ephrinB reverse signaling promoted neurogenesis. The use of recombinant proteins does not allow the discrimination between members of EphB or ephrinB families due to promiscuity in binding. Expression studies have shown that all three members of the ephrinB families are expressed in or around the SVZ, albeit with different patterns: EphrinB1 and ephrinB2 are expressed in NSC but not in neuroblasts, with ephrinB2 also expressed in astrocytes. EphrinB3 on the other hand is expressed outside of the SVZ (Table 1).

The role of ephrinB3 in adult neurogenesis was addressed genetically by studying *EfnB3*<sup>-/-</sup> animals. While neuroblasts migrated normally in absence of ephrinB3, the authors noted an increased proliferation in the SVZ of *EfnB3*<sup>-/-</sup> animals.<sup>18</sup> Interestingly, no change in proliferation was observed in animals carrying a mutant form of ephrinB3 in which reverse signaling is abolished (*EfnB3*<sup>LacZ/LacZ</sup>). Infusion of pre-clustered ephrinB3-Fc decreased the number of BrdU-positive cells in *EfnB3*<sup>-/-</sup> SVZ. Furthermore, primary NSC isolated from the SVZ of *EfnB3*<sup>-/-</sup> animals generated more neurospheres than control cells. Altogether, this study suggested that ephrinB3, expressed outside the SVZ, is required to activate forward signaling in NSC, thus limiting their proliferation. Interestingly, EphB3 which is a cognate receptor for ephrinB3, has been shown recently to play an anti-proliferative role in early postnatal SVZ progenitors.<sup>19</sup> Such a non-autonomous role for ephrinB3 in limiting NSC proliferation has also been observed in the SGZ.

EphB1 is expressed in slow cycling stem cells and in transient amplifying cells in the SGZ. In *EphB1*<sup>-/-</sup> adult brain, an increased number of proliferating cells as well as abnormal positioning and polarity of progenitors in the SGZ was observed.<sup>20</sup> Some of those phenotypes were also observed in *EfnB3*<sup>-/-</sup> mutant brains, but not in *EfnB3*<sup>LacZ/LacZ</sup> mutants, suggesting that ephrinB3, which is expressed in neurons of the granule layer, serves as a ligand to activate EphB1 forward signaling in NSC. There was no evidence of a reduction in neuron numbers in *EphB1*<sup>-/-</sup> adult brain, yet compound *EphB1*<sup>-/-</sup>;*EphB2*<sup>-/-</sup> double mutants exhibited a marked reduction in the volume of the dentate gyrus, indicating that EphB2 also plays an important role in the formation and/or maintenance of this structure.<sup>20</sup>

In a later study, the same group thus analyzed the role of EphB2 in the dentate gyrus and showed that EphB2 forward signaling is vital for the early migration of SGZ progenitors.<sup>21</sup> In a recent study, a non-autonomous function for ephrinB2 in instructing neuronal differentiation of SGZ progenitors has been described.<sup>22</sup> Ashton et al. demonstrated that ephrinB2 expressed on astrocytes of the SGZ stimulates EphB4 expressed on progenitors thus increasing the number of cells committed to the neuronal fate. Using a combination of *in vivo* and *in vitro* studies, including the use of shRNAs and infusion of recombinant proteins, the authors demonstrated

**Table 1.** Expression patterns of Eph:ephrin family members in neurogenic niches.

Structure	Cell type	Family members		References
		Receptor	Ligand	
Neocortex	NPC	Eph A2	ephrin B1 ephrin B2 ephrin B3	(Aoki et al. 2004; <sup>31</sup> Del Valle et al. 2011 <sup>19</sup> )
		Eph A4		
		Eph B1		
		Eph B2		
		Eph B3		
		Eph B4		
	CP	Eph A2	ephrin A2	(North et al. 2009; <sup>30</sup> Aoki et al. 2004 <sup>31</sup> )
		Eph A3	ephrin A3	
		Eph A4	ephrin A4	
		Eph A5	ephrin A5	
		Eph A7	ephrin B1 ephrin B2 ephrin B3	
		Eph B1		
	IZ	Eph A3	ephrin A1	(North et al. 2009 <sup>30</sup> )
		Eph A4	ephrin A2	
		Eph A7	ephrin A3	
VZ	Eph B1	ephrin A4	(Stuckmann et al. 2001; <sup>28</sup> Qiu et al. 2008; <sup>29</sup> North et al. 2009; <sup>30</sup> Murai et al. 2010; <sup>37</sup> Arvanitis et al. 2013 <sup>44</sup> )	
	Eph B2	ephrin A5		
	Eph B3	ephrin B1		
	Eph B6	ephrin B2		
	Eph A7	ephrin A2		
SVZ	NSC	EphA4	ephrin B1	(Aoki et al. 2004; <sup>31</sup> Ricard et al. 2006; <sup>18</sup> Holmberg et al. 2005; <sup>23</sup> Kodosecitch et al. 2011; <sup>25</sup> Conover et al. 2000 <sup>16</sup> )
		Eph A7	ephrin B1 ephrin B2	
	Transient amplifying cells astrocytes SVZ	Eph A4	ephrin A2 ephrin B2/B3 ephrin A2 ephrin A3 ephrin A5	(Holmberg et al. 2005; <sup>23</sup> Conover et al. 2000 <sup>16</sup> ) (Conover et al. 2000; <sup>16</sup> Holmberg et al. 2005 <sup>23</sup> ) (Conover et al. 2000; <sup>16</sup> Ricard et al. 2006; <sup>18</sup> Jing et al. 2012 <sup>47</sup> )
		Eph B2		
		Eph A7		
		Eph A1		
		Eph A2		
		Eph A3		
		EphA4		
		Eph A5		
		Eph A6		
		Eph A7		
	lateral ventricle	Eph A8		(Conover et al. 2000 <sup>16</sup> )
		Eph B1		
	border SVZ NSC	Eph B2	ephrin B3 ephrin B1 ephrin A5	(Ricard et al. 2005 <sup>18</sup> ) (Ashton et al. 2012 <sup>22</sup> ) (Kodosecitch et al. 2011 <sup>25</sup> ) (Chumley et al. 2007 <sup>20</sup> ) (Hara et al. 2010 <sup>26</sup> ) (Catchpole et al. 2011 <sup>21</sup> )
		Eph B4		
	Dentate Gyrus Astrocytes	Eph A4	ephrin B1 ephrin A2 ephrin A3 ephrin B2 ephrin A5	(Jiao et al. 2008; <sup>24</sup> Ashton et al. 2012; <sup>22</sup> Kodosecitch et al. 2011 <sup>25</sup> )
Mature granule neurons			(Hara et al. 2010 <sup>26</sup> )	

that the main function of ephrinB2>EphB4 forward signaling is to instruct neuronal differentiation without affecting proliferation of NSC.<sup>22</sup>

#### *EphA:ephrinA*

The role of EphA:ephrinA signaling in adult neurogenesis has also been addressed genetically. Specifically, the roles of EphA7 which is expressed in NSC, astrocytes and ependymal cells and its cognate ligand ephrinA2, expressed in transient amplifying cells, have been analyzed.<sup>23</sup> As an example of the difficulty of interpreting effects of recombinant proteins, in this study, both clustered and unclustered EphA7-Fc protein in the lateral ventricle led to an increase in BrdU incorporation in the lateral ventricle wall. Similarly, *EfnA2*<sup>-/-</sup> and *EphA7*<sup>-/-</sup> mutants exhibited increased proliferation of transient amplifying progenitors in the SVZ and increased number of newborn neurons in the olfactory bulb, without obvious migration defects or changes in apoptosis. This data suggested that recombinant proteins acted as inhibitors of endogenous Eph:ephrin signaling. Using a combination of *in vitro* and *in vivo* analyses, the authors proceeded to demonstrate that ephrin-A2 acts cell-autonomously to control cell cycle length of C type progenitors. Thus EphA7>ephrinA2 reverse signaling is required to inhibit SVZ neurogenesis. Intriguingly, a more recent study has shown that ephrinA2 and ephrinA3 expressed on astrocytes in non-neurogenic regions of the adult brain are also necessary to inhibit neurogenesis in these regions. In this instance, however, ephrinA2 and ephrinA3 act as ligands, activating EphA7-mediated forward signaling, presumably in progenitor cells.<sup>24</sup>

Khodosevich et al. have analyzed the role of EphA4 in adult SVZ neurogenesis using a knock-down approach.<sup>25</sup> EphA4 is expressed in slow cycling progenitors in the SVZ but not in transient amplifying cells or in neuroblasts. Knocking down EphA4 expression in primary NSC *in vitro* led to a decrease in BrdU incorporation and an increase in glial and neuronal differentiation. Similarly, knocking down EphA4 expression in the SVZ of p6 and adult mice induced a reduction in BrdU incorporation as well as a reduction in neuroblasts numbers in the rostral migratory stream, suggesting an exhaustion of the stem cell pool. No

evidence of apoptosis was reported. Moreover, the authors showed that EphA4 acts cell autonomously in B type cells to maintain the stem cell fate and that this function involves its kinase domain.<sup>25</sup> The ligand for EphA4 in the SVZ has not been identified.

In the dentate gyrus, ephrinA5 has been shown to control proliferation of SGZ progenitors and survival of newborn neurons.<sup>26</sup> EphrinA5 is expressed in mature granule neurons, in astrocytes and in progenitors of the adult SGZ. Genetic ablation of ephrinA5 led to a decreased proliferation of progenitors and to an overall reduction in BrdU-positive neurons suggestive of decreased survival. Importantly, the authors observed that the SGZ vasculature was disrupted in *EfnA5*<sup>-/-</sup> mutant, which could indirectly affect neurogenesis.

In conclusion, both reverse and forward signaling via A-class and B-class Eph:ephrin have been shown to control adult neurogenesis (Table 2 and Table 3). As with other biological processes, it seems that the biological outcome—promotion or inhibition of neurogenesis—is specific for each Eph and ephrin. Yet, the general trend is an anti-proliferative function for ephrin>Eph forward signaling in adult neurogenesis. It is important to note that in the majority of cited studies, the rate of neuronal differentiation was also analyzed and was found unaffected by Eph:ephrin signaling, indicating that the main function of Eph:ephrin signaling is to modulate the proliferative status of adult NSC. Excitingly, a strong anti-proliferative function for EphA:ephrinA signaling has also been reported in retinal stem cells.<sup>27</sup> Thus, it would be interesting to test whether this is a general function of Eph:ephrin signaling in other adult stem cells outside the nervous system.

On a cautionary note, one must keep in mind that Eph:ephrin signaling plays an important role in angiogenesis, and that members of the family are expressed in endothelial cells, thus non-targeted manipulations of Eph:ephrin signaling either with infusion of recombinant proteins and/or genetic excision of Eph or ephrins could indirectly perturb neurogenesis by interfering with endothelial-mediated signals. Moreover, many Eph receptors and ephrins are expressed in the developing brain and could potentially play a role in normal development of the structures analyzed at adult stages (see below). It is thus possible that some of

**Table 2.** EphB:ephrinB signaling in adult neurogenesis

Family member	Function	Structure	In vivo Approach	Forward vs. Reverse	References
EphB	Control of proliferation and migration	SVZ	Infusion of recombinant proteins	Not possible to conclude	(Conover et al. 2000 <sup>16</sup> )
EphB1	Anti-proliferative, organization of DG	SGZ	Genetic	Forward	(Chumley et al. 2007 <sup>20</sup> )
EphB2	Progenitor migration to DG	SGZ	Genetic	Forward	(Catchpole and Henkemeyer. 2011 <sup>21</sup> )
EphB3	Anti-proliferative	SVZ	Genetic	Not addressed	(del Valle et al. 2011 <sup>19</sup> )
EphB4	Commitment to neuronal fate	SGZ	shRNA; Infusion of recombinant proteins	Forward	(Ashton et al. 2012 <sup>22</sup> )
ephrinB3	Anti-proliferative	SVZ	Genetic	Forward	(Ricard et al. 2006 <sup>18</sup> )
ephrinB3	Anti-proliferative	SGZ	Genetic	Forward	(Chumley et al. 2007 <sup>20</sup> )
ephrinB2	Commitment to neuronal fate	SGZ	shRNA; Infusion of recombinant proteins	Forward	(Ashton et al. 2012 <sup>22</sup> )

SVZ: subventricular zone; SGZ: subgranular zone.

**Table 3.** EphA:ephrinA signaling in adult neurogenesis.

Family member	Function	Structure	In vivo Approach	Forward vs. Reverse	References
EphA4	Maintenance of stem cell fate	SVZ	shRNA	Forward	(Khodosevich et al. 2011 <sup>25</sup> )
EphA7	Anti-proliferative	SVZ	Infusion of recombinant proteins; genetic	Reverse	(Holmberg et al. 2005 <sup>23</sup> )
ephrinA2	Anti-proliferative	SVZ	Infusion of recombinant proteins; Genetic	Reverse	(Holmberg et al. 2005 <sup>23</sup> )
ephrinA2	Anti-proliferative	Non-neurogenic regions	Genetic	Forward	(Jiao et al. 2008 <sup>24</sup> )
ephrinA3	Anti-proliferative	Non-neurogenic regions	Genetic	Forward	(Jiao et al. 2008 <sup>24</sup> )
ephrinA3	Anti-proliferative	CE	Genetic	Forward	(Fang et al. 2013 <sup>27</sup> )
ephrinA5	Pro-proliferative; pro-survival	SGZ	Genetic	Not addressed	(Hara et al. 2010 <sup>26</sup> )

CE: ciliary epithelium; SVZ: subventricular zone; SGZ: subgranular zone.

the phenotypes observed in constitutive mutants could be consequences of earlier developmental defects.

### Eph:ephrin signaling in embryonic neurogenesis

EphrinB1 is highly expressed in NP of the developing neocortex and its expression is switched off in neurons<sup>28-30</sup> (Table 1). In a collaborative study with Qiang Lu's team, we showed that expression of ephrinB1 in NP is necessary to maintain the progenitor fate. Targeted knock down of ephrinB1 in cortical NP led to precocious differentiation. In contrast, forced expression of ephrinB1 in cells poised to differentiate partially blocked neuronal differentiation. Interestingly, using a mutant form of ephrinB1 that is unable to bind PDZ-RGS3, Qiu et al. showed that ephrinB1 acts cell-autonomously in NP to block their differentiation. Lastly, genetic ablation of *EfnB1* led to a larger proportion of NP exiting the cell cycle, and to a decrease in the mitotic index in the neocortex.<sup>29</sup> Altogether, these results indicate that Eph>ephrinB1 reverse signaling is required to maintain the neural progenitor fate.

Neural progenitors in the developing neocortex express high levels of EphA4 while EphA3, EphA7, EphB1 and EphB2 are expressed at more modest levels throughout the cortical wall.<sup>30,31</sup> North et al. have analyzed the role of EphA4 in cortical development using genetic ablation of *EphA4* as well as targeted loss of function approaches. Analysis of *EphA4*<sup>-/-</sup> embryos revealed that the thickness of the cortical wall was reduced, as was the number of cells incorporating BrdU. Knock down of EphA4 expression in NP led to a reduction in electroporated cells after 3 days, as well as to a decreased proportion of proliferative electroporated cells. Based on their survey of ephrin expression in the developing neocortex and on the fact that ephrinB1 has been implicated in embryonic neurogenesis,<sup>29</sup> the authors postulated that ephrinB1 could be the main ligand for EphA4 in cortical progenitors. Targeted gain of function experiments indeed showed that overexpression of ephrinB1 induced an increased number of mitotic progenitors in the neocortex, in a non-autonomous manner. Altogether this study showed that ephrinB1>EphA4 forward signaling promotes cortical progenitors proliferation and reduces their differentiation.<sup>30</sup>

As mentioned above, EphA7 is expressed at low levels in the developing neocortex. Analysis of *EphA7*<sup>-/-</sup> embryos revealed that proliferation of NP was unchanged and that differentiation

proceeded normally in absence of EphA7.<sup>32</sup> Similarly, analysis of *EfnA2;EfnA3;EfnA5* triple knock out embryos (at a later stage of corticogenesis) showed no change in the proliferation of NP.<sup>33</sup> However, the use of cortical slices infused with recombinant proteins EphA2-Fc proteins to block EphA:ephrinA signaling led to a decreased number of neurons which was attributed to decreased differentiation, yet, apoptosis was not investigated in the slice cultures.<sup>31</sup> This point is of import as a number of studies have reported that EphA:ephrinA signaling plays a prominent role in programmed cell death in the developing neocortex. Indeed, a two-fold decrease in NP apoptosis was observed in *EphA7*<sup>-/-</sup> embryos, suggesting that EphA7 promotes cell death<sup>32</sup> and two other studies have since reported a critical role for EphA:ephrinA signaling in promoting apoptosis during early brain development.<sup>34,35</sup> (Table 4).

In conclusion, the main players in embryonic neurogenesis are the EphA4 receptor and its ligand ephrinB1 (Table 4). Unlike in the adult stem cell niches where Eph and ephrins are often expressed in distinct cell types or complementary patterns, in the neocortex both EphA4 and ephrinB1 are co-expressed in NP. Thus, EphA4:ephrinB1 bi-directional signaling between neighboring NP is required for proper maintenance of the progenitor fate. EphA:ephrinA have been implicated prominently in programmed cell death during corticogenesis. Intriguingly, both their pro-apoptotic role during embryonic neurogenesis and their strong anti-proliferative role during adult neurogenesis serves to limit the pool of NSC.

### Molecular Mechanisms

The vast majority of molecular effectors identified thus far downstream of forward or reverse signaling converge on the regulation of cell adhesion, cell migration and cell morphology.<sup>1,4</sup> So, how does Eph:ephrin signaling control maintenance, proliferation and/or differentiation of NP at the molecular level? More pointedly, does Eph:ephrin signaling directly control NSC proliferation and/or maintenance or is it an indirect control secondary to NSC positioning in their niche? Primary cultures of NP are excellent models to address this question, since position in the niche may be disregarded and molecular changes can be analyzed in specific time windows thus discriminating direct and indirect



**Table 4.** Eph:ephrin signaling in embryonic neurogenesis

Family member	Function	Structure	In vivo Approach	Forward vs. Reverse	References
EphA3	Neuronal differentiation	neocortex	Slice cultures; recombinant proteins	Forward	(Aoki et al. 2004 <sup>31</sup> )
EphA4	Maintenance of progenitor fate	neocortex	Genetic; shRNA	Forward	(North et al. 2009 <sup>30</sup> )
EphA7	Pro-apoptotic	neocortex	Genetic	Forward	(Depaepe et al. 2005 <sup>32</sup> )
ephrinB1	Maintenance of progenitor fate	neocortex	Targeted gain of function	Forward	(North et al. 2009 <sup>30</sup> )
ephrinB1	Maintenance of progenitor fate	Neocortex	shRNA; Targeted gain of function; Genetic	Reverse	(Murai et al. 2010; <sup>37</sup> Qiu et al. 2008; <sup>29</sup> Wu et al. 2009; <sup>40</sup> Arvanitis, 2010 <sup>41</sup> #180)
ephrinA5	Pro-apoptotic	Neocortex	Genetic	Forward	(Park et al. 2013 <sup>34</sup> )
ephrinA5	Pro-apoptotic	Neocortex	Genetic	Reverse	(Kim et al. 2013 <sup>35</sup> )

consequences of Eph:ephrin activation. In the adult, only a handful of studies have attempted to identify the molecular mechanisms downstream of Eph:ephrin signaling. For instance, a transient decrease in *Notch1* and *Zic1* mRNA levels was correlated with the pro-neurogenic function of EphB2>ephrin reverse signaling in cultured NSC isolated from the SVZ.<sup>17</sup> The caveat of this experiment is that mRNA levels were analyzed after 24h of treatment with EphB2-Fc which is too late to determine whether the expression of *Notch1* and *Zic1* is directly modulated by reverse signaling. More recently, it has been shown that the pro-neurogenic function of ephrinB2>EphB4 forward signaling in the SGZ requires activation of  $\beta$ -catenin independently of Wnt.<sup>22</sup> Interestingly, activation of  $\beta$ -catenin was observed as early as 4 h following ephrinB2-Fc treatment in cultured NSC, providing strong evidence that it could be a direct regulation.

The strongest evidences for a direct role of Eph:ephrin signaling independently of niche positioning come from studies on embryonic neurogenesis. For instance, stimulation of primary NP with ephrinA-Fc increased phosphorylation of ERK1/2 within 5 minutes and active ERK1/2 is required for EphA3-induced neuronal differentiation.<sup>31</sup> The best characterized transduction cascade to date is reverse signaling downstream of ephrinB1 which is required to maintain the progenitor fate. A pioneer collaborative study between the group of Qiang Lu and our group led to the identification of PDZ-RGS3 as an essential effector of ephrin-reverse signaling in NP maintenance.<sup>29</sup> Since RGS proteins are known regulators of G protein signaling,<sup>36</sup> in a subsequent study, Murai et al. investigated the role of the G $\alpha$  subunit downstream of ephrinB1. Using a combination of *in utero* electroporation and genetic gain-of-function approaches, the authors demonstrated that ephrinB1/PDZ-RGS3 reverse signaling modulates G $\alpha$  subunit activity in NP thus controlling the balance between self-renewal and differentiation and, conversely, that G $\alpha$  activation counteracts the RGS-mediated function of ephrinB1.<sup>37</sup> The same group identified the transcriptional repressor ZHX2 as a binding partner for the intracellular domain of ephrinB1 (ephrinB1-ICD). Intramembrane cleavage of ephrinBs by  $\gamma$ -secretase has been shown to release ICD in response to Eph binding.<sup>38,39</sup> The authors showed that transcriptional repression mediated by ZHX2 inhibits neuronal differentiation and that ephrinB1-ICD potentiates transcriptional repression mediated by ZHX2 and thus promotes NP self-renewal.<sup>40</sup> As another

example of regulation of gene expression downstream of reverse signaling, we have identified the pro-neurogenic miRNA miR-124 as a downstream target of ephrinB1 reverse signaling in NP.<sup>41</sup> Stimulation of reverse signaling in cultured NP led to a decrease in miR-124 levels as early as 4 h and miR-124 levels were elevated in *EfnB1*<sup>-/-</sup> developing neocortex and in *EfnB1*<sup>-/-</sup> NP. Changes in miR-124 levels correlated with inverse changes in expression levels of some of its targets, including *Sox9* and *EfnB1* itself. Indeed, we demonstrated that ephrinB1 and miR-124 are locked in a double negative feedback loop which is necessary to control the balance between self-renewal and differentiation of NP.<sup>41</sup>

Another potential transcriptional mechanism by which ephrinB1-reverse signaling may modulate NP self-renewal is via the recruitment of STAT3 and potentiation of its transcriptional activity. STAT3 is required for NP self-renewal<sup>42</sup> and stimulation of NP with EphB2-Fc led to increased phosphorylation, nuclear translocation and transcriptional activity of STAT3.<sup>43</sup> An attractive hypothesis that has not been tested so far would be that STAT3 controls the expression of miR-124 downstream of ephrinB1. Altogether, these studies provide strong evidence that ephrinB1 reverse signaling directly controls maintenance of the progenitor fate independently of cell positioning. Yet, we have shown recently, using a combination of genetic, *ex vivo* and *in vitro* approaches, that ephrinB1 also exerts an important structural role in maintaining the apical adhesion of NP to the ventricular surface.<sup>44</sup> We showed with *ex vivo* electroporation and slice cultures that the short term consequence of a loss of ephrinB1 in NP is detachment of the apical membrane and scattering of the cells in the cortical wall. We further showed that this was a cell-autonomous function of ephrinB1 implicating reverse signaling, yet, puzzlingly, the ephrinB1-ICD was not required. Lastly, we showed that ephrinB1 reverse signaling inhibited ARF6 activity and modulated integrin- $\beta$ 1 apical localization in NP.<sup>44</sup> Interestingly, EphB2 but not EphA4 was the cognate receptor modulating apical adhesion together with ephrinB1. In addition to Eph-dependent functions, it cannot be excluded that ephrinB1 also has Eph-independent functions in embryonic neurogenesis, for instance via G-protein signaling or via FGF signaling.<sup>37,43</sup> Thus, these studies demonstrate that ephrinB1 controls self-renewal vs. differentiation of neocortical NP by complex and varied molecular mechanisms.

## Pathologies/Therapies

A number of studies have analyzed the role of Eph:ephrin signaling in injury—or pathology—induced neurogenesis, such as cerebral ischemia,<sup>45,46</sup> Parkinson disease<sup>47</sup> and traumatic brain injury.<sup>48</sup> Overall, these studies have shown that members of the family played similar roles under physiological and pathological conditions. Researchers have also started to harness the pro-neuronal potential of Eph:ephrin signaling for therapeutic purposes.<sup>49</sup>

## Models

As seen in the chapters above, the role of Eph:ephrin signaling in neurogenesis is complex and seems context dependent. For instance, its biological outcome appears to be different during embryonic neurogenesis and adult neurogenesis. It is thus difficult to clearly understand how Eph:ephrin signaling affect neurogenesis at the single cell level and more pointedly, how it may impact neurogenesis at the level of cell populations. In this chapter, we present examples of general models of stem cell maintenance vs. differentiation, and more specific models of neurogenesis that may be useful to our understanding of the role of Eph:ephrin in this process.

Models accounting for the growth dynamics of a population of cells undergoing proliferation and differentiation processes have been proposed as early as 1964<sup>50</sup> in the case of the highly proliferative hemopoietic tissue. With a simple model, the birth-and-death process (BDP, where death reflects differentiation), Till et al. demonstrated that control at the population level may be obtained if one considers that the fate of a dividing cell obeys a probabilistic choice, at each division, between a differentiated fate and an on-going proliferative fate. In this classic linear model, regulation by cell-cell interaction or by system-wide signal was not considered. On average, the output at the population level would be tightly controlled by the probability of differentiating because the behavior of cells would be stochastic. Variations on this classic linear model (Fig. 2A) have been widely used to model steady-state system such as adult stem cell dynamics but also final-state system such as embryonic neurogenesis (for a review see<sup>51</sup>).

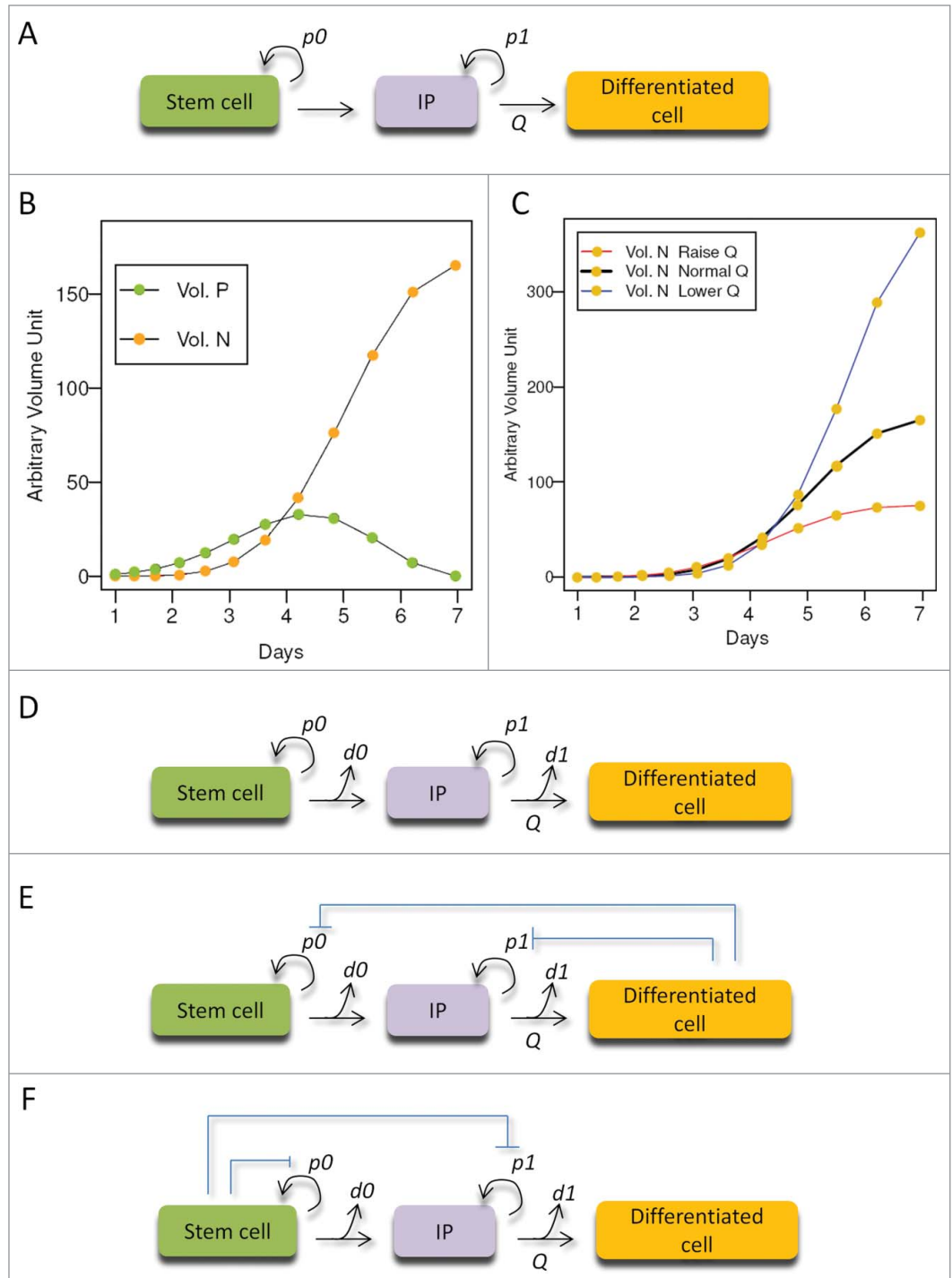
Additional studies have elaborated over this BDP with a particular focus on the NP proliferation / differentiation in the developing cortex<sup>52–55</sup> up to models integrating the fates and migration of newborn neurons.<sup>56</sup> This model is based on experimental data showing that one NP has a probability  $P$  ca 0.62–0.66 to reenter the NP state, and thus  $Q = 1 - P$ , ca 0.34–0.38 to exit the proliferative state and that  $P$  progresses along the neurogenic interval, from a highly proliferative population of NP at its beginning up to a complete differentiation in neurons by the end of it.<sup>53,54</sup> The basic feature of this model is the control of the final size of the neuronal population through the regulation of the ratio between proliferation and differentiation (Fig. 2B).<sup>55</sup> Experimental manipulation of  $Q$ , for instance using p27, an inhibitor of the cell cycle that modulates the probability that a

mitotic division will produce quiescent cells, revealed that modulation of  $Q$  has a strong impact on neuronal differentiation.<sup>56,57</sup> Interestingly, a similar impact on neuronal production is observed when modifying  $Q$  in the model. In addition, the predictive power of the model is that it allows to manipulate  $Q$  at specific times and to infer the resulting effect on the total neuronal output. For instance, the model shows that modulating  $Q$  as early as the first cell cycles induced an increase in total neuronal output which is detectable only after 5 d (Fig. 2C).

Importantly, the huge difference in final volume induced by a slight depression of  $Q$  indicates a high sensitivity of the final output to variance of  $Q$  (Fig. 2C). This suggests the existence of additional controls in the normal course of neurogenesis, either to finely tune the evolution of  $Q$  within a thin band of compatible regime, or to compensate for large  $Q$  deviations. In particular, McConnell et al. have put forward the need to refine this straightforward model by integrating cell apoptosis, which might have been long underestimated.<sup>58,59</sup> Their sensitivity analysis, which also integrates the neuronal production by progenitors outside the VZ, concluded that neural progenitor cell death could be as high as 50% during the last cell cycle and still give a full quantitative account of the neuronal production. As a matter of fact, McConnell et al. readily consider a temporal modulation of  $d$ , the probability of cell death, over the cycles, with a lower death risk in the earlier cycles, and a smooth progression parallel to the time profile of  $Q$  (Fig. 2D). Overall, this modeling line suggests that 2 main factors control the final neuronal output during embryonic neurogenesis: the time profile of  $Q$  and the time profile of  $d$ , 2 parameters that are modulated by Eph:ephrin signaling.

As mentioned previously, in the models described above, control at the population level is obtained without integrating regulatory mechanisms such as cell-cell interaction or system-wide feedback signals. In these models, cells obey stochastic processes of proliferation/death/differentiation governed by time-varying parameters (e.g.  $Q$  or  $d$  at a given cycle) which do not depend on the state of the system at any time (they are parameters, not variables). As a consequence, those systems are linear, meaning that the separate evolutions of 2 sub-populations would yield the same output if combined in the end than the evolution of the 2 sub-populations combined from the start. Linear systems are present even in the most refined models in which interactions between the state of the system and cells fates are not considered.<sup>60</sup> As a consequence, and due to the exponential nature of the proliferation process, the modeled dynamics are highly sensitive to perturbations (alterations of parameters), which is not compatible with the observed robustness of stem cell dynamics. Nonlinearity, in the form of feedback loops, has been used to refine the classical model of stem cell dynamics and has been shown to diminish sensitivity of the system to perturbations.<sup>61,62</sup> Specifically, Lander et al. have shown that negative feedback loops (in the form of diffusible molecules) originating from differentiated cells and modulating  $p$  (probability to self-renew) at the level of the stem cells and intermediate progenitors (Fig. 2E) ensures that the system is no longer sensitive to initial parameters and is more robust to perturbations.<sup>62</sup> This type of negative

**Figure 2.** Models of stem cell dynamics. **(A)** Classic model of stem cell dynamics with parameters for probability of self-renewal ( $p$ ) and probability of differentiation ( $Q$ ). **(B)** Evolution of progenitor population ( $P$ ) and neuron population ( $N$ ) over a time period corresponding to embryonic neurogenesis. The population dynamics and the final volume of  $N$  depend on the time profile of  $Q$ , which increases at each cycle. In the beginning,  $Q$  is lower than 0.5 so that cell division mainly supplies the population of  $P$ . As  $Q$  becomes greater than 0.5, the production of neurons  $N$  becomes greater at each cycle,  $N$  progresses faster and the pool of proliferating cells starts to decline. In the end, the few remaining proliferating cells  $P$  exhaustively differentiate into neurons and the process ends. Adapted from Nowakowski et al. **(C)** Impact of modified  $Q$  on neuronal production. Slight changes in  $Q$  time profiles can have a major impact upon the final volume  $N$ , which can vary by a factor 2. Upon up-regulation of  $Q$ , a larger proportion of progenitors exit the proliferative state earlier in the cycles, thus leading to faster exhaustion of  $P$ . Conversely, down-regulation of  $Q$  keeps a larger proportion of progenitors in proliferative state for longer, so that neurons from the late cycles become overly represented. **(D)** Modified version of the classic linear model incorporating a parameter for cell death ( $d$ ). **(E)** Non linear model in which  $p$  is modified by negative feedback regulation from differentiated cells. **(F)** Non linear model in which  $p$  is modified by the fate of neighboring cells.



feedback regulation may be applicable to the non-autonomous anti-proliferative role of ephrin-B3 during adult neurogenesis, with the restriction that unlike diffusible signal, Eph:ephrin signaling operates only between 2 cells which are in contact, which introduces a spatial constraint into the model. However, this type of model based on feedback regulation from the differentiated progeny does not seem appropriate to the observed role of EphA4:ephrin-B1 bi-directional signaling in embryonic

neurogenesis which operates between NP. Instead, the model described by Agur et al. focusing on local cell-cell interactions and how stem cell behavior is determined by the number of its stem cell neighbors (Fig. 2F) appears more pertinent.<sup>63</sup>

These few examples demonstrate the usefulness of implementing predictive models as they raise the importance of carefully considering the timing at which experimental manipulations and subsequent observations are made in order to draw definitive

conclusions when studying a dynamical system. Importantly, cell-cell communication depends at any given time upon the spatial organization of cells according to their types and states, thus to take into account local cell-cell interactions, models of stem cell dynamics should not only develop in time, but also in space.

## Conclusions

Since the first study implicating Eph:ephrin signaling in adult neurogenesis which was published almost 15 y ago, a relatively small number of studies have followed up on this topic. Quite telling for this lack of recognition is the fact that Eph:ephrin signaling is not (or barely) mentioned in reviews on neurogenesis.<sup>12,13</sup> Nevertheless, as described herein, there is strong evidence that Eph:ephrin signaling plays an important role in neurogenesis, both adult and embryonic, and in recent years, the use of sophisticated targeted approaches has allowed researchers to study this pathway in ever finer details. One important challenge for future studies will be to dissect the Eph:ephrin transduction cascade at the molecular level, from the plasma membrane to the nucleus and to identify the mechanisms responsible for the switch between self-renewal / proliferative and anti-proliferative functions of the pathway. A number of molecular effectors have already been identified in embryonic neurogenesis and future research will tell whether similar cascades are also at play during adult neurogenesis. In addition to this extremely valuable

reductionist approach, the use of systems biology, theoretical or computational approaches may help reconcile seemingly disparate results on Eph:ephrin signaling by modeling neurogenesis at multiple levels. Indeed, neurogenesis is governed by the concerted action of various intracellular, intercellular and tissue events which span different time domains. Additional quantitative analysis and experimental manipulation of Eph:ephrin signaling in neurogenesis will be useful to refine existing multiscale models.<sup>64,65</sup>

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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