REVIEW

Transcriptional regulation of adult neural stem/progenitor cells: tales from the subventricular zone

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Abstract

In rodents, well characterized neurogenic niches of the adult brain, such as the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampus, support the maintenance of neural/stem progenitor cells (NSPCs) and the production of new neurons throughout the lifespan. The adult neurogenic process is dependent on the intrinsic gene expression signatures of NSPCs that make them competent for self-renewal and neuronal differentiation. At the same time, it is receptive to regulation by various extracellular signals that allow the modulation of neuronal production and integration into brain circuitries by various physiological stimuli. A drawback of this plasticity is the sensitivity of adult neurogenesis to alterations of the niche environment that can occur due to aging, injury or disease. At the core of the molecular mechanisms regulating neurogenesis, several transcription factors have been identified that maintain NSPC identity and mediate NSPC response to extrinsic cues. Here, we focus on REST, Egr1 and Dbx2 and their roles in adult neurogenesis, especially in the subventricular zone. We review recent work from our and other laboratories implicating these transcription factors in the control of NSPC proliferation and differentiation and in the response of NSPCs to extrinsic influences from the niche. We also discuss how their altered regulation may affect the neurogenic process in the aged and in the diseased brain. Finally, we highlight key open questions that need to be addressed to foster our understanding of the transcriptional mechanisms controlling adult neurogenesis.

Key Words: *adult neurogenesis; aging; extracellular signaling; gene regulation; neural stem/progenitor cells; transcription factors*

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Introduction

Contrary to the long held "no new neurons after birth" dogma, neurogenesis occurs in adult rodents and possibly other mammals, sustained by pools of neural stem/progenitor cells (NSPCs) that reside in specialized brain niches (Ming and Song, 2011).

In mice, the subventricular zone (SVZ), adjacent to the walls of the lateral ventricles, is the adult neurogenic niche containing the largest number of NSPCs. Specialized astrocytes of the SVZ that contact the lateral ventricle with an apical process extending through the ependymal layer, known as type B cells, have been identified as the neurogenic stem cells of this niche (Fuentealba et al., 2015). These cells can exist in a quiescent or in an activated state. In the latter case, they proliferate to self-renew and/or generate transit amplifying progenitor cells (type C cells or TAPs). Type C cells give rise to neuroblasts (type A cells), which migrate to the olfactory bulb, where they terminally differentiate into distinct types of interneurons that are important for odour discrimination (Kohwi et al., 2007; Brill et al., 2009). Another well described niche is the subgranular zone (SGZ) of the hippocampal dentate gyrus, where NSPCs continuously produce neurons that provide additional plasticity to the adult brain and support several hippocampus-dependent cognitive functions, such as spatial learning and contextual memory capabilities (Gonçalves et al., 2016).

Physical exercise, learning, odour discrimination and enriched environment, among others, are physiological stimuli regulating NSPCs and their progeny in a niche-specific manner. External stimuli might affect neurogenesis by changing the levels of several extracellular molecules (e.g., neurotransmitters, morphogens, growth factors, cytokines and adhesion/repulsion molecules) in the niche milieu. For example, the use of optogenetics and chemogenetics to activate or inhibit medial septum γ-aminobutyric acid-ergic neurons projecting to the dentate gyrus has indicated that medial septum neuron activity maintains neural stem cell quiescence and controls hippocampal neurogenesis through γ-aminobutyric acid release (Bao et al., 2017). Furthermore, it has been proposed that physical exercise-mediated release of acetylcholine by the septal cholinergic system increases NSPC proliferation in mice, suggesting that acetylcholine may act as an extrinsic signal mediating the positive effect of voluntary running on neurogenesis (Itou et al., 2011).

In rodents, aging also has a major impact on the adult neurogenic process, leading to a reduction of the proliferating NSPC pool and of new neuron production (Lupo et al., 2019). The depletion of proliferating NSPCs in the aged niches may be caused by the progressive consumption of the NSPC reservoir due to terminal differentiation (Encinas et al., 2011), by a decreased ability of quiescent stem cells to undergo activation (Kalamakis et al., 2019), and by age-related changes in different NSPC features, such as their pattern of cell division (symmetric *versus* asymmetric), cell cycle length or survival (Lupo et al., 2019). At the molecular level, the negative regulation of neurogenesis in the aged brain crucially involves age-associated alterations in the niche environment impinging on NSPCs, including the decreased ac-

tivation of signaling pathways promoting NSPC proliferation and neuronal differentiation, such as the epidermal growth factor (EGF) and Wnt pathways, and the increased stimulation of pathways inhibiting neurogenesis, such as transforming growth factor β (TGF-β) signaling and inflammatory pathways (Pineda et al., 2013; Dulken et al., 2019; Kalamakis et al., 2019; Kase et al., 2019). In turn, the extracellular niche modifications due to physiological stimuli or aging induce changes in the gene expression programs controlling NSPC proliferation and differentiation. In this context, transcription factors (TFs) serve as key intracellular hubs in NSPCs, integrating the extrinsic pathways acting in the niche and regulating the intrinsic transcriptional networks driving the neurogenic process.

A number of TFs involved in NSPC regulation, neuronal differentiation and maturation, and synaptic integration have been identified. Several of these TFs are implicated in the control of both adult and embryonic neurogenesis, although their mode of action and expression levels may change at different developmental stages (Götz et al., 2016). In this review, we highlight the regulation of adult neurogenesis by three TFs that we have recently investigated, namely the repressor element 1-silencing transcription factor (REST), Egr1 and Dbx2. We discuss their function in controlling NSPC proliferation and differentiation and the transcriptional networks that they regulate during neurogenesis, with particular focus on the adult SVZ niche. Furthermore, we describe how different extrinsic influences can modulate the activity of REST, Egr1 and Dbx2 in NSPCs or differentiating neurons, suggesting that these TFs might act as regulatory nodes in the molecular pathways controlling neurogenesis in physiological conditions, aging or disease.

Search Strategy and Selection Criteria

Studies cited in this review were found on the PubMed database, between September and November 2019, using the search terms: adult neurogenesis, neural stem cells, neural progenitors, subventricular zone, transcription factors, REST, Egr1, Dbx2, and various combinations of the above terms.

The Repressor Element 1-Silencing Transcription Factor Supports Adult Neurogenesis with Cell Type-Specific and Context-Dependent Mechanisms

REST is a zinc finger protein that binds a highly conserved 21–23 bp DNA sequence (REST binding site; RE1 site) and regulates neurogenesis by repressing the transcription of neuronal specific genes (Chong et al., 1995; Schoenherr and Anderson, 1995). Reducing REST activity reconfigures chromatin accessibility, allowing neuronal gene expression and the direct conversion of non-neuronal cells (e.g., astrocytes or fibroblasts) into neurons (Masserdotti et al., 2015; Drouin-Ouellet et al., 2017). With the advent of genome-wide analyses, several thousand RE1 sites linked to genes that encode both neuronal and non-neuronal proteins have been identified (Bruce et al., 2004; Mortazavi et al., 2006; Johnson et al., 2008). Binding of REST at the RE1 sites allows the recruitment of CoREST and mSin3A, and the assembly of a repressor complex including histone deacetylases, histone methyltransferases and the histone demethylase LSD1. As part of this complex, REST controls gene expression through an epigenetic remodelling mechanism (Ballas et al., 2005).

REST functions as an intrinsic factor controlling neural development. Its levels are elevated in neural progenitors, where REST represses neuronal genes, and decrease when neural progenitors differentiate into neurons (Chen et al., 1998; Ballas et al., 2005; Mandel et al., 2011; Yang et al., 2012; Nechiporuk et al., 2016). Transient overexpression of REST during the neurogenic phase of brain development blocked progenitor migration and neuronal differentiation, indicating that REST downregulation is crucial to allow neurogenesis (Mandel et al., 2011). In addition, REST keeps neuronal genes protected from DNA damage in apical cortical neural progenitors during the S phase of the cell cycle, avoiding accumulation of chromosomal abnormalities that would either commit cells to apoptosis and result in microcephaly or, in combination with the loss of p53, trigger the formation of brain tumors (Nechiporuk et al., 2016). These observations highlight the importance of properly regulating the timing and the levels of REST activity during brain development.

REST is also expressed in adult NSPCs and its inactivation affects neurogenesis, leading to NSPC depletion and decreased granule neuron generation in the adult hippocampus (Kuwabara et al., 2004; Gao et al., 2011; Mukherjee et al., 2016). To better dissect its role in specific NSPC subpopulations, REST was deleted in quiescent hippocampal neural stem cells (qNSC) by injections of a Cre-p2A-mCherry lentivirus expressed under the control of the human glial fibrillary acidic protein promoter into REST fl/fl mice, finding that REST prevented qNSC activation. The conditional inactivation of REST in the TAP population caused its decrease and the activation of the neuronal differentiation program. These findings indicate that REST acts cell autonomously to support the maintenance of both the qNSC and the TAP pools by preventing their progression along the neurogenic lineage (Mukherjee et al., 2016).

Genome-wide ChIP-seq analysis of REST binding sites, combined with RNA-seq profiling of genes deregulated by REST knockdown, showed that the majority of the REST targets were neuronal genes, suggesting that their REST-dependent repression may be crucial to preserve the qNSC and TAP populations (Mukherjee et al., 2016). It would be interesting to overexpress neuronal REST target genes in qNSCs or TAPs to verify whether this is sufficient to promote their lineage progression. Notably, several of the REST-controlled neuronal genes were differentially expressed in qNSCs and TAPs, indicating that the transcriptional response of REST target genes is cell type-dependent and stage-dependent, as previously proposed (Bruce et al., 2004; Hohl and Thiel, 2005; Johnson et al., 2008; Noh et al., 2012; Lu et al., 2014; McClelland et al., 2014; Mukherjee et al., 2016). Since REST levels are similar in qNSCs and TAPs, the differential regu-

lation of neuronal target genes in these cell populations may involve context-dependent interactions of REST with other TFs (Mukherjee et al., 2016).

This analysis also identified unique REST targets involved in ribosome biogenesis (e.g., the genes coding for nucleophosmin 1 (*Npm1*) and ribosomal protein L4 (*Rpl4*)) and cell cycle regulation (e.g., *Mms22l*), whose overexpression either alone or in combination stimulated proliferation in quiescent cells. These data suggest that REST-regulated genes implicated in ribosome biogenesis and cell cycle are important players in controlling the transition between quiescent and activated stem cells (Mukherjee et al., 2016). They also strengthen the idea that REST is a multifaceted protein with context-dependent and cell type-specific roles, highlighting the importance of studying REST in different cell types, and contexts, to fully unveil its function in adult neurogenesis.

Along with other groups, we have investigated the role of REST in the SVZ by means of *in vitro* NSPC cultures. SVZ-derived NSPCs in which REST function was impaired generated a higher percentage of neurons when induced to differentiate. Notably, these cells showed reduced neurosphere formation and increased neuronal differentiation in comparison with control cultures even when maintained in culture media supplemented with growth factors, a condition suitable for NSPC expansion and the maintenance of multipotency (Gao et al., 2011; Soldati et al., 2015). Altogether, these data indicate that REST/NRSF is required for the self-renewal of adult SVZ NSPCs and that REST directly controls the neurogenic lineage program *in vitro* (**Figure 1**). This conclusion is supported by the observation that NSPCs isolated from the SVZ of 3 week-old REST/NRSF^{loxP} mice and then infected with an adenovirus expressing Cre/GFP showed activation of REST target genes and reduced binding of REST at the RE1 sequence of proneural genes, such as *Ascl1* and *NeuroD1*. *NeuroD1* upregulation following REST inactivation correlated with increased histone H4 acetylation, increased H3K4me2 and decreased H3K27me3. This is in accordance with the role of REST in the recruitment of histone methylases/demethylases and REST-dependent acetylation/deacetylation (Gao et al., 2011; Mukherjee et al., 2016).

We identified REST target genes in SVZ NSPCs by combining a transcriptomic analysis of REST deficient cells together with a ChIP-seq profiling of REST binding sites throughout the genome. These analyses provided a comprehensive REST-regulated gene expression resource and indicated that REST direct target genes were significantly enriched for pathways associated with nervous system development/function and disease (Soldati et al., 2015). Of note, a few genes identified as REST targets in our study (e.g., *Bcl11B*, *Cels 3*, *BMP6*) had been previously annotated as stem cell enriched genes by a transcriptome analysis performed on NSPCs freshly isolated from the SVZ of adult mice by means of FACS-based purification (Beckervordersandforth et al., 2010). Among the genes directly regulated by REST, we found that the expression of a bone morphogenetic protein (BMP) family member, *BMP6*, was increased both at the mRNA and protein level upon REST knockdown. BMPs are important regulators of neurogenesis and gliogenesis (Panchision and McKay, 2002). In addition, BMP pathway activation is implicated in the control of neural stem cell quiescence in the adult hippocampus (Mira et al., 2010). We showed that administration of exogenous BMP6, at a concentration comparable to that released by NSPCs upon REST knockdown, elicited an anti-proliferative and pro-differentiative effect on NSPCs, suggesting that REST-mediated control of BMP signaling may regulate NSPC properties in the SVZ (Soldati et al., 2015).

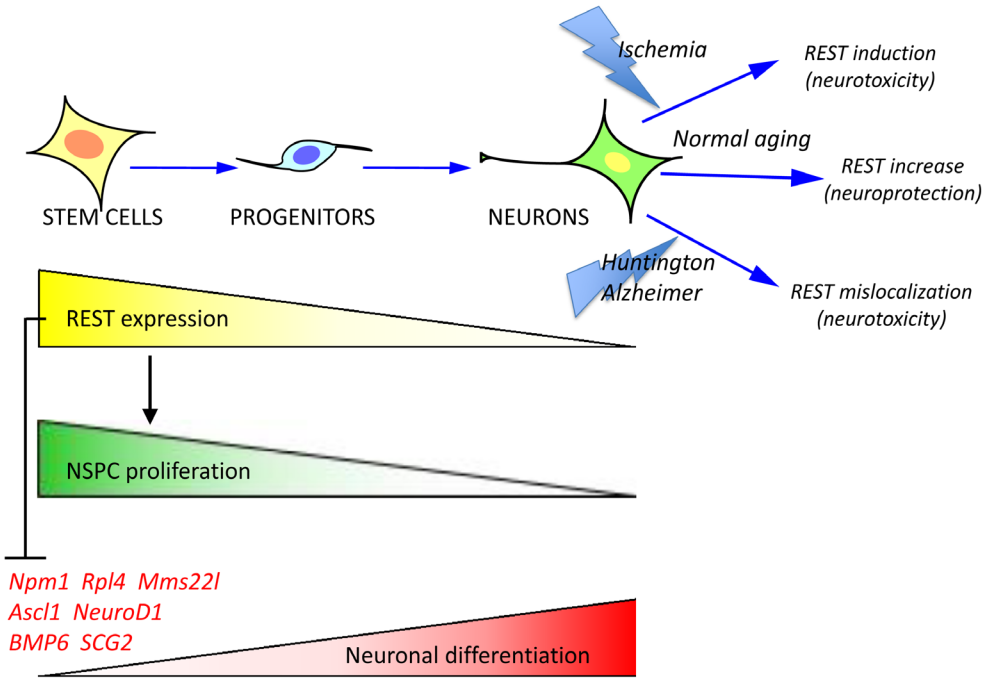

Figure 1 Proposed model of the REST-dependent control of NSPC proliferation and neuron differentiation/survival.
Decreased levels of REST activity in NSPCs derepress the transcription of genes associated with the inhibition of NSPC proliferation and the stimulation of differentiation, thus promoting NSPC progression along the neurogenic lineage. In differentiated neurons, REST levels and/or cellular localization change as a result of different physiological or pathological events. These alterations of REST activity may exert neurotoxic or neuroprotective effects, with context-dependent mechanisms. NSPCs: Neural stem/progenitor cells; REST: repressor element 1-silencing transcription factor.

In other studies, secretory genes have been identified as a class of REST target genes (Kim et al., 2015). Among them, SCG2 encodes for Secretogranin II, which has been proposed to act as an extrinsic molecule mediating the effects of REST knockdown in promoting neuronal differentiation and enhanced neuritic outgrowth in hippocampal NSPCs. Notably, these effects were observed both in the cells transfected with a shREST-mCherry plasmid and in the non-transfected cells within the same cultures, suggesting that REST controls neuronal differentiation in a paracrine manner. Gain and loss of function experiments allowed the authors to identify SCG2 as part of a non-cell autonomous signaling pathway, downstream of REST, which is necessary for neuronal differentiation in hippocampal NSPCs. In future work, it will be interesting to explore whether similar signals also act in the adult SVZ both *in vitro* and *in vivo*.

To date, there are few published *in vivo* studies addressing REST function in the SVZ. One of them performed a conditional knockout of REST in NSPCs using Sox1-Cre/+; $Rest^{2lox/2lox}$ mice (Aoki et al., 2012). The authors showed that neurogenesis was affected neither in the adult SVZ, nor in the dentate gyrus, at variance with a previous study reporting altered neurogenesis after the conditional deletion of REST in the adult hippocampus of Nestin-CreERT2/R26R-YFP mice injected with tamoxifen (Gao et al., 2011). This discrepancy might be due to the different strategies used to delete REST *in vivo*. REST knockout in Sox1-Cre/+; $Rest^{2lox/2lox}$ adult mice starts at an early embryonic stage, since Sox1 expression begins at embryonic day 7.5–8.5 in the neural tube, whereas abrogation of REST function in Nestin-CreERT2/R26R-YFP mice was induced in the adult brain. Compensatory mechanisms activated by the early embryonic deletion of REST may mask its role in the adult brain and explain the lack of an obvious neurogenic phenotype. Notably, it has been reported that REST knockout animals carrying a deletion of exon 2 or 4 still produce the C-terminal or the N-terminal REST repressor domains (Gao et al., 2011; Aoki et al., 2012; Mukherjee et al., 2016), which might retain repressive functions (Andrés et al., 1999; Naruse et al., 1999; Grimes et al., 2000; Roopra et al., 2000; Ballas et al., 2001), questioning the conclusion that REST function is dispensable for adult neurogenesis (Aoki et al., 2012). The use of classical knockout REST models, complemented with mice containing a conditional gene trap cassette in an intron of the REST gene that terminates transcription upstream of the initiator codon (Nechiporuk et al., 2016), might help to unravel the role of REST in adult neurogenesis.

It will also be important to shed light on the molecular mechanisms regulating REST expression and function under both physiological and pathological conditions. REST is also expressed, although at low levels, in specific neuronal subpopulations at embryonic stages (Nechiporuk et al., 2016), during postnatal development (Rodenas-Ruano et al., 2012), and in the adult brain (Kuwabara et al., 2004; Gao et al., 2011), suggesting that REST function may be required even in differentiated neurons (Yeo et al., 2009; Rodenas-Ruano et al., 2012). Accordingly, mice conditionally overexpressing REST in cells expressing dopamine receptor 2, including striatal neurons, showed impaired spontaneous locomotion and displayed some of the alteration associated with human Huntington's disease (Lu et al., 2018). Importantly, the expression of REST and of its target genes is altered in post-mitotic neuronal subpopulations in pathological conditions, such as ischemia (Calderone et al., 2003; Noh et al., 2012; Kaneko et al., 2014), seizures (Palm et al., 1998), Huntington's disease (Zuccato et al., 2003), and Alzheimer's disease (AD) (Lu et al., 2014) (**Figure 1**). Elevated REST levels have been associated with striatal neurotoxicity in Huntington's disease. Huntingtin mutations destroy REST interactions and retention in the cytoplasm, favouring REST aberrant accumulation in the nucleus and the repression of specific target genes, such as the one coding for brain-derived neurotrophic factor, a neurotrophin essential for striatal neurons survival (Zuccato et al., 2003). *Vice versa*, loss of nuclear REST occurs in AD patients and AD animal models. In this context, cognitive impairment and neurodegeneration have been correlated with the derepression of REST target genes involved in cell death (e.g., *BAX* and *DAXX*) and AD (e.g., *Presenilin2*). Compared to control neurons, REST-deficient neurons treated with $A\beta_{42}$ showed increased degeneration and cell death, which could be rescued by lentiviral transduction of REST. Moreover, REST overexpression in the SH-SY5Y human neural cell line decreased cell death upon oxidative stress. These observations suggest that, in differentiated neurons, REST may exert a neuroprotective function (Lu et al., 2014).

Although an impairment of adult neurogenesis has been associated with several neurodegenerative diseases (Steiner et al., 2006), there are very few studies addressing the effects of changes in REST function on the response of NSPCs to brain insults. In a recent *in vitro* study on NSPCs derived from sporadic AD patients or in pluripotent stem cells carrying an AD-associated *APO4* allele, reduced nuclear REST localization and decreased REST-RE1 binding have been described (Meyer et al., 2019). The transcriptomic analysis of NSPC models of AD showed modifications in REST regulated gene networks, loss of self-renewal capability, premature neuronal differentiation, accelerated synapse formation and increased electrical excitability, as compared with control cells, indicating that REST dysfunction emerges in NSPCs and persists in neurons (Meyer et al., 2019). Since REST knockout triggers a depletion of the NSPC pool (Gao et al., 2011; Mukherjee et al., 2016), it is tempting to speculate that an impaired neurogenesis due to an altered REST function may contribute to the cognitive decline associated with AD.

Finally, high levels of REST are associated with enhanced proliferation in several neural cell tumours. In particular, elevated REST expression has been detected in medulloblastoma, neuroblastoma and glioblastoma and its inhibition promotes cell cycle exit and differentiation of cancer cells (Su et al., 2006; Singh et al., 2011; Conti et al., 2012). These data suggest that REST plays a key role in the self-renewal of both normal NSPCs and malignant neural cancer cells, thus representing a candidate target for therapeutic interventions in

multiple neuropathological contexts.

Egr1 and Epidermal Growth Factor Signaling in the Regulation of Adult Neural Stem/Progenitor Cell Proliferation and Differentiation

Egr1 is an immediate early gene defined as an "early growth response gene" (Sukhatme et al., 1988), also known under other names and acronyms, such as *Krox24*, *Zif268*, *Tis8* and *NGFI-A*. It encodes for a zinc finger TF that binds to the DNA sequence GCG(G/T)GGGCG (Crosby et al., 1991), which is present at the promoter of a large number of genes involved in a wide variety of physiological and pathological cell processes.

The modulation of its activity levels depends on several extracellular signals, such as tumour necrosis factor α, TGF-β, EGF and platelet-derived growth factor A and B (Khachigian et al., 1995; Liu et al., 1996; Yao et al., 1997; Kaufmann and Thiel, 2001). Intracellular Ca^{2+}-dependent pathways also regulate Egr1 expression and its biological functions (Thiel et al., 2010). Egr1 function is also modulated by intracellular co-activators, such as CREB-binding protein and p300 (Silverman et al., 1998), and co-repressors, such as NGFI-A binding protein 1 and 2, which bind to a specific Egr1 domain and block the activation of its target genes (Thiel et al., 2000). In turn, Egr1 regulates several tumor suppressors, such as TGF-β1, PTEN, p53 and fibronectin (Baron et al., 2006). Egr1 protein levels, when detectable, are generally low and depend on the analyzed cell type and developmental stage; they may rapidly increase or decrease in physiological and pathological or stressful states, possibly as a consequence of a rapid turnover. Egr1 activity is also dependent on its phosphorylation state, which is regulated through the phosphorylation of Egr1 Ser^{26} by the extracellular signal-regulated kinase-1 (ERK) (Gregg and Fraizer, 2011; Santiago et al., 2019). In particular, a role for EGF and its receptor (EGFR) in controlling Egr1 phosphorylation via the ERK pathway has been well characterized (Alagappan et al., 2013).

In the nervous system, Egr1 expression and activity has been demonstrated in the adult hippocampus, where it is involved in synaptic plasticity, learning, long term potentiation and memory consolidation (Jones et al., 2001; Cheval et al., 2012). Egr1 role in the control of cell proliferation has been described in several cell types, including astrocytes (Biesiada et al., 1996). As demonstrated in PC12 pheochromocytoma cells (Levkovitz and Baraban, 2001), and in a mouse neuroblastoma cell line (N18TG2) (Salani et al., 2009), Egr1 also regulates neuronal differentiation. Several *Egr1* transcriptional targets have been identified in PC12 cells, used as an *in vitro* model of neuronal differentiation (Adams et al., 2017). Nerve growth factor (NGF) signaling via the NGF receptor TrkA promotes PC12 differentiation (Segal and Greenberg, 1996), whereas EGF stimulates PC12 proliferation (Huff et al., 1981). Both NGF and EGF activate the ERK signaling pathway, but a short (30–60 minutes) EGF stimulation promotes proliferation, whereas NGF treatment induces a longer lasting stimulation (4–6 hours) of ERK signaling, that ultimately leads to differentiation. In this context, *Egr1* cooperates with CREB and AP-1 to activate genes involved in the NGF-dependent differentiation of PC12 cells. This cohort of *Egr1* targets includes several genes implicated in neural development, such as the gene encoding for p35, which is necessary for Cdk5 activation and neurite outgrowth in response to NGF; *Vgf* and *Hbegf*, which encode for neurotrophic factors; *Kdm6b*/*Jmjd3* and *Kctd11*, which contribute to the differentiation of stem cells to neurons; *Sema6*a, which is involved in axon guidance; and *Arc*, which plays a role in synaptic plasticity, learning and memory (Adams et al., 2017). It will be important to investigate whether these genes are also regulated by *Egr1* in differentiating NSPCs in the *in vivo* neurogenic niches.

We have studied the role of Egr1 in controlling the proliferation and differentiation of adult mouse SVZ NSPCs using *in vitro* culture systems, in which SVZ NSPCs can be maintained in a proliferative state by treatment with basic fibroblast growth factor and EGF. In these conditions, *Egr1* expression is upregulated in NSPCs and supports their proliferation. The presence of EGF is specifically required to maintain elevated *Egr1* transcription levels in NSPCs and sustain their proliferation via EGF receptor and ERK pathway activation. In contrast, EGF removal from the culture medium promotes Egr1 downregulation in NSPCs, as well as their cell cycle exit and differentiation towards neuronal and glial fates. We found that EGF removal causes the downregulation of the genes coding for cyclin D1, D2 and D3, and the upregulation of p27, coding for a cell cycle inhibitor. *Egr1* overexpression in NSPCs has the opposite effect, causing an increase in the expression levels of cell cycle regulators and a decrease in those of cell cycle inhibitors. Of note, *Egr1* overexpression rescues the cell proliferation decrease observed after EGF removal, suggesting that this TF may play a significant role in supporting the proliferation of adult SVZ NSPCs downstream of the EGF-EGFR signaling pathway (Cera et al., 2018; **Figure 2**).

Other studies have investigated the role played by *Egr1* in adult SVZ NSPCs *in vivo*. Using a mouse model of recovery from an ischemic injury, it was found that *Egr1* expression is upregulated, and its nuclear levels increase, in SVZ NSPCs of mice recovering after a treatment that simulates hypoxic conditions. Moreover, upon hypoxia/ischemia, *EGFR* mRNA and protein levels increase, promoting the recruitment of NSPCs in the cell cycle, a reduction in their transition time through the cell cycle and an expansion of the NSPC pool (Alagappan et al., 2013). It was therefore important to determine whether there is a causal relationship between the concomitant increase in the expression levels of both *Egr1* and EGFR and the expansion of the SVZ NSPC population following ischemia. In support of this hypothesis, Egr1 binding to the EGFR promoter region has been detected following ischemic injury, suggesting that *Egr1* can stimulate NSPC proliferation in the SVZ through the positive regulation of EGFR expression, at least in this experimental paradigm (Alagappan et al., 2013). Furthermore, it has been demonstrated

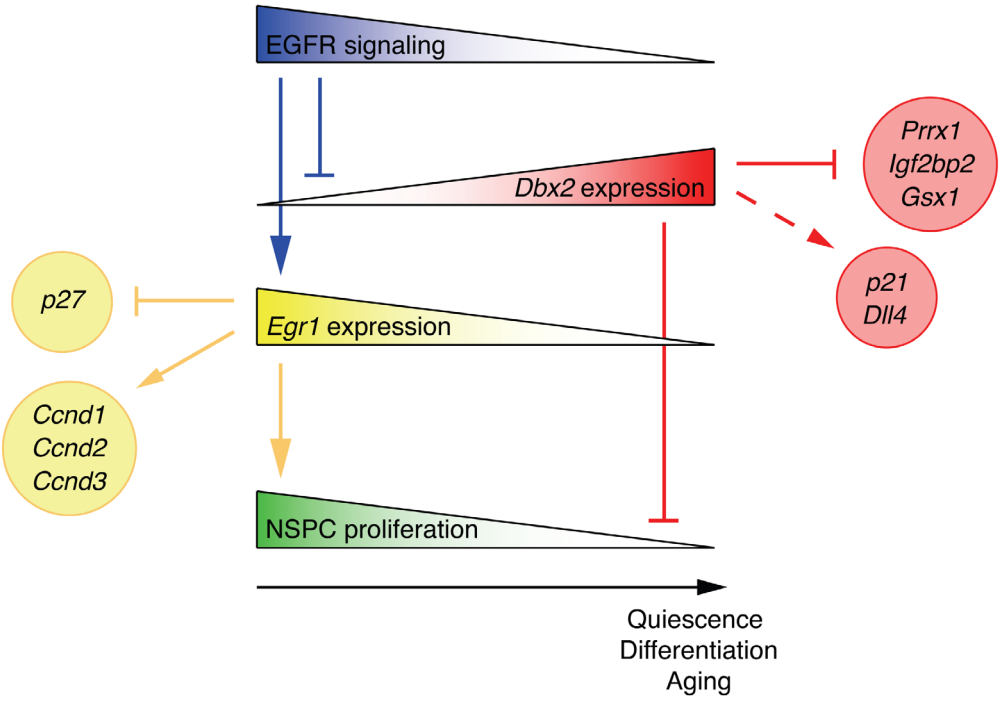

Figure 2 Proposed model of the regulation of SVZ NSPC proliferation by the antagonistic activities of *Dbx2* and *Egr1*.
EGFR signaling may promote NSPC proliferation by repressing *Dbx2* and by activating *Egr1* expression. In turn, these TFs may exert opposite effects on the expression of genes coding for cell cycle activators and inhibitors and for other regulators of NSPC proliferation. According to this model, changes in the levels of EGFR signaling may be instrumental, acting via *Dbx2* and *Egr1*, to the decrease in NSPC proliferation associated with the transition towards quiescent or differentiating states or with aging. EGFR: Epidermal growth factor receptor; NSPCs: neural stem/progenitor cells; SVZ: subventricular zone; TFs: transcription factors.

that *Egr1* overexpression in several cancer cell lines causes *Egr1* binding to the EGFR promoter and enhances EGFR expression (Nishi et al., 2002).

Another study recently reported the ability of quercetin, a flavonoid with a strong antioxidant activity that stimulates adult hippocampal neurogenesis (Tchantchou et al., 2009), to increase the number of proliferating NSPCs and *Egr1* mRNA levels in the adult dentate gyrus of a rat model of AD (Karimipour et al., 2019). This suggests the interesting possibility that Egr1 may be involved in mediating the positive effects of quercetin on hippocampal neurogenesis, and, more in general, regulate the neurogenic process also in the SGZ niche.

In the developing brain, *Egr1* expression pattern has been mapped and its mRNA has been detected in neural progenitors and neurons both in the embryonic and in the neonatal rat brain, but not in radial glial cells or in mature glial cell types (Wells et al., 2011). This result is in agreement with above mentioned studies (Alagappan et al., 2013), which found that Egr1 upregulation in the adult SVZ following ischemic injury takes place in the neural progenitor population, but not in astrocytes or in astrocyte-like cells. Although it seems unlikely that Egr1 role changes drastically when comparing the neurogenic process in the developing or in the adult brain, it remains to be investigated whether there are specific functions of this TF that are peculiar to either the developing or the adult stages.

The *Dbx2* Homeobox Gene in the Regulation of Adult Neural Stem/Progenitor Cell Proliferation during Aging

Over the last 3 years, several studies have employed next generation sequencing methodologies to investigate the genome-wide molecular changes that take place during SVZ aging, especially in NSPCs. To this aim, different laboratories have compared the transcriptomic signatures of single SVZ cells (Shi et al., 2018; Dulken et al., 2019; Kalamakis et al., 2019), sorted SVZ NSPC bulk populations (Leeman et al., 2018; Kalamakis et al., 2019), SVZ NSPC *in vitro* cultures (Lupo et al., 2018; Shi et al., 2018), or dissected SVZ tissues (Apostolopoulou et al., 2017; Shi et al., 2018) obtained from mice at different ages between 2 and 24 months old. In one of these studies, we have described the genome-wide profiling of mRNA, DNA methylation and histone H3 lysine 4/27 trimethylation levels in NSPCs of the 3 and the 18 months old mouse SVZ that were expanded for two passages in non-adherent culture conditions (Lupo et al., 2018). Among the genes displaying age-associated transcriptional and/or epigenetic changes in this analysis, we focused on the homeobox gene *Dbx2*, given the pivotal roles that homeodomain TFs play in neural development (Lupo et al., 2000; Wigle and Eisenstat, 2008). *Dbx2*, in particular, is involved in the specification of interneuron fates in the developing spinal cord together with its close homolog *Dbx1* (Pierani et al., 1999). When compared with young adult samples, aged NSPC cultures showed enhanced *Dbx2* transcript levels, which were also detected in freshly sorted NSPCs from the SVZ of adult and aged mice (Lupo et al., 2018). These results are supported by independent transcriptomic datasets of freshly sorted SVZ NSPCs, or freshly dissected SVZ tissues, from young adult and aged mice, reporting increased *Dbx2* mRNA levels in aged samples (Apostolopoulou et al., 2017; Kalamakis et al., 2019).

Although the global transcriptional changes taking place during NSPC aging have been partially elucidated by the above described studies, the molecular drivers of these changes remain unclear. Several extracellular signaling

pathways, such as the EGFR, TGF-β, mammalian target of rapamycin and Wnt pathways, are known to be differentially modulated in the young adult and in the aged SVZ neurogenic niche and they could potentially determine the age-related gene expression changes detected in NSPCs (Paliouras et al., 2012; Pineda et al., 2013; Zhu et al., 2014; Lupo et al., 2019). We found that *Dbx2* mRNA levels increased when young adult SVZ NSPCs were cultured for 24 hours without EGF, suggesting that EGFR signaling might negatively regulate *Dbx2* expression (Lupo et al., 2018). Since the levels of EGFR, of its TGF-α ligand and of its intracellular mediator ERK have been shown to be downregulated in the aged SVZ (Enwere et al., 2004; Kase et al., 2019), there might be a causative link between the decrease of EGFR signaling levels and the enhanced *Dbx2* mRNA levels detected in the aged SVZ. Further experiments specifically addressing the epistatic relationships between EGFR signaling and *Dbx2* need to be performed. Moreover, the age-related increase of *Dbx2* expression levels may result from an increase in its transcription and/or its transcript stability, with the latter potentially involving microRNAs. Since microRNAs are implicated in NSPC regulation (Cacci et al., 2017) and brain aging (Danka Mohammed et al., 2017), it would be worth to address their potential role in regulating *Dbx2* expression in NSPCs.

To investigate whether *Dbx2* upregulation in aged NSPCs may be involved in promoting the phenotypic changes associated with NSPC aging, we overexpressed this gene in young adult NSPCs by producing transgenic NSPC cultures with constitutive, or doxycycline-inducible expression of a *Dbx2* transgene. These assays showed a reduction in NSPC culture growth upon constitutive or acute overexpression of *Dbx2*, as exemplified by the smaller size of neurospheres obtained by non-adherent culture of NSPCs overexpressing *Dbx2* in comparison with control NSPCs. Elevated *Dbx2* expression levels did not cause major effects on cell viability or cell differentiation in young adult NSPCs cultured in proliferating conditions (Lupo et al., 2018). This suggests that the growth phenotype of *Dbx2* overexpressing cultures may result from altered NSPC proliferation dynamics, which might be related to the previously reported changes in the transition between activated and quiescent states and/or in the cell cycle length of aged NSPCs (Daynac et al., 2016; Apostolopoulou et al., 2017; Bast et al., 2018; Kalamakis et al., 2019). Further experiments are required to pinpoint the alterations of NSPC proliferation caused by *Dbx2* overexpression, and to investigate whether the inhibition of *Dbx2* function may improve the proliferative capacity of aged NSPCs.

Since *Dbx2* encodes for a TF, the phenotypic effects observed in NSPCs undergoing elevated expression of this gene are expected to result from the dysregulation of *Dbx2*-dependent transcriptional networks. By performing gene expression analyses in NSPCs overexpressing *Dbx2*, we have identified a few putative targets of its transcriptional activity (Lupo et al., 2018; **Figure 2**). In particular, *Dbx2* may negatively regulate the homeobox genes *Prrx1* and *Gsx1*, since they were both downregulated in SVZ NSPCs overexpressing *Dbx2*. *In vivo*, Prrx1 is co-expressed in NSPCs of the SVZ and the SGZ together with the Sox2 TF, and these proteins may bind each other according to biochemical assays. When retroviral vectors where employed *in vivo* to constitutively express *Prrx1* in NSPCs of the adult mouse SGZ, NSPCs were forced to remain in an undifferentiated state and their progression to neuronal fates was impaired. Conversely, *Prrx1* knockdown in NSPC cultures from the whole mouse forebrain caused a depletion of proliferating cells (Shimozaki et al., 2013). These results suggest a functional link between the downregulation of *Prrx1* and the growth phenotype caused by *Dbx2* overexpression, although the specific role of *Prrx1* in the regulation of the SVZ niche needs to be investigated. Expression of *Gsx1* has been described in the mouse embryonic ventral telencephalon, at the level of NSPCs transiting between the ventricular Zone and the SVZ during a period of active neurogenesis at midgestation (Pei et al., 2011). This spatio-temporal pattern suggests a potential role for *Gsx1* in the regulation of embryonic NSPC proliferation and differentiation, which appears to be supported by the analysis of transgenic or mutant mouse embryos with *Gsx1* gain or loss of function. *Gsx1* activity, however, is largely redundant with that of the closely related homolog *Gsx2*, and the individual roles of these TFs remain unclear (Pei et al., 2011; Chapman et al., 2018). Furthermore, *Gsx2* is also implicated in the adult mouse SVZ niche, where it modulates NSPC progression towards neuronal differentiation in physiological conditions and in response to injury (López-Juárez et al., 2013), but the role of *Gsx1* in adult neurogenesis is still unknown. *Igf2bp2*, coding for a RNA binding protein, is another gene downregulated in NSPCs overexpressing *Dbx2*. Similar to *Gsx1/2*, *Igf2bp2* role during embryonic neurogenesis overlaps with that of the close homolog *Igf2bp1*. Both of them are involved in regulating NSPC proliferation and differentiation in the developing cerebral cortex, but *Igf2bp1* expression declines postnatally and is undetectable in the young adult SVZ (Fujii et al., 2013; Nishino et al., 2013). There is no available report to date on the function of *Igf2bp1* in adult neurogenesis.

We also found genes that were upregulated upon Dbx2 overexpression, such as those coding for the cell cycle inhibitor p21 and for Dll4, a Notch ligand implicated in the modulation of NSPC proliferation and differentiation in the adult SVZ niche (Bicker et al., 2017; Lin et al., 2019), thus suggesting that Dbx2 may activate or repress the transcription of different genes. Other studies, however, indicate that Dbx2 functions as a transcriptional repressor. The N-terminal regions of Dbx1 and Dbx2 contain short aminoacidic stretches sharing sequence similarity with the transcriptional repressor domain of the Drosophila Engrailed homeoprotein (Muhr et al., 2001; Karaz et al., 2016). Both Dbx1 and Dbx2 could bind the Drosophila Groucho transcriptional co-repressor *in vitro* (Muhr et al., 2001), and exhibited transcriptional repressor activity in luciferase reporter assays performed in COS-7 or HEK293 cells (Ma et al., 2011; Karaz et al., 2016). Furthermore, misexpression of a chimaeric protein containing the Dbx2 homeodomain fused to the Engrailed repressor domain in the developing chick spinal cord could mimic some of the

effects of full-length Dbx2 misexpression, whereas a deletion mutant lacking the Engrailed repressor homologous sequence or a chimaeric protein containing the Dbx2 homeodomain fused to the transcriptional activator domain of VP16 were ineffective (Muhr et al., 2001). These observations raise the question of how Dbx2 overexpression leads to the increased transcript levels of certain genes, such as *p21* and *Dll4*. One possibility is that these genes are negatively regulated by a repressor, itself inhibited by *Dbx2*. Of note, Gsx1 and Gsx2 contain putative Engrailed homologous motifs, similar to Dbx proteins, suggesting that they too may function as transcriptional repressor (Muhr et al., 2001).

Taken together, the observations described in this section suggest the possible involvement of Dbx2 in the molecular mechanisms underlying the age-associated changes of NSPCs in the adult mouse SVZ. As schematized in **Figure 2**, decreased activation of EGFR signaling during SVZ aging may lead to an opposite increase in Dbx2 expression levels in NSPCs of the aged niche. In turn, as a result of its enhanced levels, Dbx2 may directly downregulate a subset of downstream genes including *Prrx1*, *Gsx1* and *Igf2bp2* by acting as a transcriptional repressor, whereas other genes such as *p21* and *Dll4* may be upregulated through an indirect mechanism (dashed lines in **Figure 2**). By modulating a cohort of genes implicated in the regulation of NSPC proliferation, elevated levels of Dbx2 in aged NSPCs may contribute to the decreased proliferative activity and the reduced neurogenic output of the aged SVZ niche. Bearing in mind that the aging process of the adult neurogenic niches entails several complex intrinsic and extrinsic mechanisms (Lupo et al., 2019), in future work it will be interesting to investigate Dbx2 function and regulation in SVZ NSPCs in more detail, including the genome-wide identification of its transcriptional targets and binding sites, the assessment of the effects of its functional inactivation and the elucidation of the upstream molecular pathways controlling its expression in the young adult and in the aged SVZ niche.

Conclusions and Future Perspectives

As discussed in this review, a substantial body of data has been collected on the role played by the TFs studied in our and other laboratories during adult neurogenesis. One major feature of the evidences presented here is that the activity of REST, Egr1 and Dbx2 in the adult neurogenic niches is modulated by extrinsic mechanisms associated with physiological stimuli, aging, injury or disease. Furthermore, these TFs and at least some of the extracellular pathways modulating their activity, such as EGF signaling, are able to affect NSPC proliferation and differentiation. Therefore, REST, Egr1 and Dbx2 may be involved both in the intrinsic molecular networks governing NSPC self-renewal and neurogenic potential and, crucially, also in NSPC responses to the extrinsic influences toning up or down neurogenesis in physiological or pathological conditions. Within this intriguing scenario, several unresolved issues remain to be addressed in follow up studies.

Among these TFs, the role of REST in adult neurogenesis has been investigated in much more detail, revealing its complex ability to regulate several events spanning the whole neurogenic process from qNSCs till neuronal maturation and activity. The molecular mechanisms by which this TF can perform distinct roles in different cell types and contexts is a major avenue for future investigations, along with a deeper characterization of the molecular pathways modulating its activity in response to various extrinsic stimuli. Moreover, although neuronal REST appears to have a protective role during physiological aging and the dysregulation of REST-dependent pathways in neurons may play a role in pathological aging (Lu et al., 2014), REST contribution to age-associated alterations in NSPCs remains surprisingly unaddressed. In the case of Egr1, there are several hints that it may be involved in normal or pathological brain aging processes, since changes in its expression levels have been detected in the hippocampus of aged AD human subjects (compared to adult and aged non-AD subjects) (Lanke et al., 2018), in the hippocampus of aged rats following spatial behaviour (compared to adult rats in the same behaviour conditions) (Penner et al., 2016), and in the cortex of aged mice (compared to young adult mice) (Rosa et al., 2018). As for REST, it is not known whether Egr1 may be implicated in aging processes in the adult neurogenic niches. Finally, our knowledge of the function of Dbx2 in adult neurogenesis remains very limited and much more work needs to be done to address it, especially using *in vivo* experimental models.

In future studies, it will also be important to map the possible interactions between REST, Egr1 and Dbx2 within the bigger picture of the global gene expression programs driving the neurogenic process. Based on their potential modulation by EGF signaling and their shared ability to affect NSPC proliferation, it is tempting to speculate that these TFs may functionally interact, directly or indirectly, in adult NSPCs. Supporting this hypothesis, we previously showed that Egr1 can regulate REST expression in neuroblastoma cells (Salani et al., 2009). The potential interplay among REST, Egr1 and Dbx2 will need to be investigated both by functional assays and by comparing the genome-wide transcriptional networks regulated by them. Finally, given the potential differences between adult neurogenesis in humans and rodents (Sanai et al., 2011; Sorrells et al., 2018), it would also be crucial to compare their expression profiles in the NSPC niches of these organisms and investigate their function using human *in vitro* models of the neurogenic process.

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