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**Transcriptional and epigenetic mechanisms underlying astrocyte identity**

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**Highlights**

- Astrocytes are crucial players in the developing and adult CNS in health and disease.
- Astrocytes play a key role in the balance of regenerative and degenerative functions.
- Differentiation, identity and functions of astrocytes are regulated through transcriptional and epigenetic programs.
- The capacity of astrocytes to become reactive or be reprogrammed provides promise for endogenous brain repair strategies.
- Deregulation of astrocytic transcriptional or epigenetic mechanisms may contribute to neurological disorders, such as neurodegenerative diseases and brain cancers.
- Molecular mechanisms underlying astrocytic identity may offer novel therapeutic opportunities.

**Abstract**

Astrocytes play a significant role in coordinating neural development and provide critical support for the function of the CNS. They possess important adaptation capacities that range from their transition towards reactive astrocytes to their ability to undergo reprogramming, thereby revealing their potential to retain latent features of neural progenitor cells. We propose that the

mechanisms underlying reactive astrogliosis or astrocyte reprogramming provide an opportunity for initiating neuronal regeneration, a process that is notably reduced in the mammalian nervous system throughout evolution. Conversely, this plasticity may also affect normal astrocytic functions resulting in pathologies ranging from neurodevelopmental disorders to neurodegenerative diseases and brain tumors. We postulate that epigenetic mechanisms linking extrinsic cues and intrinsic transcriptional programs are key factors to maintain astrocyte identity and function, and critically, to control the balance of regenerative and degenerative activity. Here, we will review the main evidences supporting this concept. We propose that unravelling the epigenetic and transcriptional mechanisms underlying the acquisition of astrocyte identity and plasticity, as well as understanding how these processes are modulated by the local microenvironment under specific threatening or pathological conditions, may pave the way to new therapeutic avenues for several neurological disorders including neurodegenerative diseases and brain tumors of astrocytic lineage.

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**Abbreviations**

5hmC= 5-hydroxymethylcytosine

5mC= 5-methylcytosine

A $\beta$ = amyloid  $\beta$

AD= Alzheimer's disease

ASCL1= Achaete-Scute Family BHLH Transcription Factor 1

BBB= blood-brain barrier

BDNF= brain-derived neurotrophic factor

BHLH= basic helix-loop-helix

BMP= bone morphogenetic protein

CSCs= cancer stem-like cells

CNS= central nervous system

CNTF= ciliary neurotrophic factor

Coup-tfI and Coup-tfII= Chicken ovalbumin upstream promoter-transcription factors I and II

DNMT1= DNA methyltransferase 1

E= embryonic day

EZH2= Enhancer of zeste homolog 2 gene

ESET= ERG-associated protein with SET domain

FGF2= fibroblast growth factor

GASC1= gene amplified in squamous cell carcinoma 1

GBM= Glioblastoma

G-CIMP= glioma-CpG island methylator phenotype

GFAP= glial fibrillary acidic protein

GSC= GBM cancer stem-like cells

H1= Histone 1

H2A= Histone 2A

H2B= Histone 2B

H3= Histone 3

H4= Histone 4

H3K4me= methylation of Lysine 4 of Histone 3

H3K9me= methylation of Lysine 9 of Histone 3

H3K4me<sub>2</sub>= dimethylation of Lysine 4 of Histone 3

H3K9me<sub>2</sub>= dimethylation of Lysine 9 of Histone 3

H3K9me<sub>3</sub>= trimethylation of Lysine 9 of Histone 3

H3K27me<sub>3</sub>= trimethylation of Lysine 27 of Histone 3

H3K36me<sub>3</sub>= trimethylation of Lysine 36 of Histone 3

H4K20me= methylation of Lysine 20 of Histone 4

HDACs= histone deacetylases

HDAC3= histone deacetylase 3

hESCs= human embryonic stem cells

HMT= histone methyltransferase

IDH1= isocitrate dehydrogenase 1

IDH2= isocitrate dehydrogenase 2

IFN- $\gamma$ = interferon-gamma

IL-6= interleukin 6

JAK= Janus kinase

JNK= c-Jun N-terminal kinase

KDM5A= lysine-specific demethylase 5A

LCN2= lipocalin 2

LIF= leukemia inhibitory factor

lncRNA= long non-coding RNA

MAPK= Ras/mitogen-activated protein kinase

MECP2= methyl CpG binding protein 2

MGMT= DNA repair enzyme O<sup>6</sup>-methylguanine-DNA methyltransferase

miRNAs= microRNAs

MPTP= 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NADPH= nicotinamide adenine dinucleotide phosphate

ncRNA= non-coding RNA

NFIA= nuclear factor I A-type

NFIB= nuclear factor I B-type

NF- $\kappa$ B= nuclear factor kappa-light-chain-enhancer of activated B cells

NICD= Notch intracellular domain

NSCs= neural stem cells

RA= retinoic acid

RARs= retinoic acid receptors

RXR= retinoid X receptors

RBP-J= recombining binding protein suppressor of hairless

SCI= spinal cord injury

SHH= Sonic hedgehog

STAT3= signal transducer and activator of transcription 3

STAT/CBP= signal transducer and activator of transcription/CRE binding protein

SVZ= subventricular zone

TBI= traumatic brain injury

TET= ten-eleven translocation family of enzymes



TGF- $\beta$ = transforming growth factor-beta

TNF= tumor necrosis factor-alpha

PD= Parkinson's disease

WNT1= wingless-type MMTV integration site1

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## 1. Introduction

Astrocytes represent the most abundant cell population in the adult brain. As such, they are involved in several activities, ranging from regulating ion and fluid homeostasis, establishing and maintaining the blood-brain barrier (BBB) (Oberheim *et al.*, 2006; Zhang and Barres, 2010) to providing neurons with nutrients and metabolites (Escartin *et al.*, 2007; Pellerin *et al.*, 2007). Further, they are involved in regulating synaptic transmission through the uptake of the neurotransmitter glutamate (Parpura *et al.*, 1994), they interact with neurons as they actively participate in the formation and functioning of neuronal synapses (Barres, 2008) and they communicate with them through calcium signals (Nedergaard, 1994). The active communication between astrocytes and neurons is involved in sleep homeostasis, breathing, circadian regulation and memory (McIver Sally R. , 2013). Further, astrocytes promote the differentiation of oligodendrocyte progenitor cells into mature myelinating oligodendrocytes and support myelin maintenance (Domingues *et al.*, 2016).

Under CNS insults such as trauma, tumor, infection or neurodegeneration, astrocytes become activated, a process known as reactive astrogliosis, resulting in changes in their morphology and expression of their molecular repertoire (Pekny and Nilsson, 2005; Sofroniew, 2009). Reactive astrocytes release pro- and anti-inflammatory cytokines, thus implying both beneficial and detrimental effects in a context-dependent manner. Under specific conditions, the consequence of the neuroinflammatory response following brain injury is the formation of the glial scar, mainly constituted by reactive astrocytes, which acts as a physical insulator to contain the lesioned area. In mammals, intriguingly, the glial scar acts as a negative regulator of neurogenesis and neurite outgrowth, in comparison to lower vertebrates, which are able to recruit glial

progenitors that undergo reactive neurogenesis and replace the neurons lost upon injury (Kroehne *et al.*, 2011). Notably, reactive astrocytes exhibit endogenous NSC hallmarks such as self-renewal, multipotency and expression of immature markers. In the mouse cerebral cortex, only a subset of astrocytes are able to restore their proliferation capacity and enter a reprogramming mode, whereupon they are able to form neurospheres and generate neurons (with firing action potential), oligodendrocytes and astrocytes or be directly reprogrammed into neurons or neuroblasts *in vivo* (Bardehle *et al.*, 2013; Gascon *et al.*, 2017; Gotz *et al.*, 2015; Niu *et al.*, 2013). Could these terminally differentiated astrocytes be considered as immature or even as latent stem cells since they possess the capacity to re-enter a self-renewal process and, eventually, potentially a differentiation process and even acquire an alternate cell identity? It is possible that the mechanisms underlying reactive astrogliosis or astrocyte reprogramming may hold the secret of neuronal regeneration, a process that is lost during evolution of the mammalian CNS.

The onset of astrogenesis relies upon extracellular signaling and intrinsic epigenetic modifications, such as DNA methylation and histone modifications. Disruption in any of these mechanisms causes abnormal astrocyte differentiation and leads to neurodevelopmental disorders (Molofsky *et al.*, 2012; Sloan and Barres, 2014). The importance of astrocytes for neuronal integrity and survival is also prominent in the adult brain. Conditional ablation of astrocytes in adult mice results in neuronal loss and severe motor deficits (Schreiner *et al.*, 2015). On the other hand, their contribution to neuroinflammation and the loss of normal homeostatic functions, may corroborate their implication in the onset and progression of neurodegenerative diseases, including Parkinson's and Alzheimer's diseases (PD and AD) (Niranjan, 2014). Brain cancer is another neurological disease where astrocytes are currently implicated both as tumor initiating cells and modulators of the tumor microenvironment (Ahmed *et al.*, 2013). Supporting the notion that brain

tumors can arise from differentiated cells, transduction of murine astrocytes *in vivo* with oncogenic lentiviral vectors results in tumor formation. Mature astrocytes were shown to be able to dedifferentiate and gain expression of progenitor/stem cell markers or even transdifferentiate into neurons during tumorigenesis (Friedmann-Morvinski *et al.*, 2012). Transformed astrocytes can be reprogrammed to re-acquire stem cell features, but also give rise to more malignant phenotypes, thereby generating a heterogeneous cell population within the malignant tumor (Friedmann-Morvinski *et al.*, 2012; Friedmann-Morvinski and Verma, 2014).

Clearly, astrocytes are crucial players in the developing and adult CNS in both health and disease. Consequently, it is critical to elucidate the transcriptional and epigenetic mechanisms underlying acquisition of astrocyte identity and plasticity, as well as understanding how these processes are modulated under pathological conditions. The comprehension of these mechanisms will pave the way to novel therapeutic approaches aimed, for example, at restoring the homeostatic astrocytic functions in brain tumors or at enhancing their neurogenic properties in neurodegenerative diseases. This review focuses on those transcriptional and epigenetic mechanisms underlying the physiological differentiation and activation of astrocytes that contribute to our understanding of aberrant phenotypes identified under pathological conditions leading to neurological disorders.

## **2. Epigenetic modifications: basic mechanisms and their role in CNS development**

Epigenetics generally refers to any heritable alteration occurring in a cell that has an immediate effect on gene expression, without modifying the DNA sequence. This includes DNA methylation and histone modifications, which are able to alter DNA accessibility and chromatin

structure, and RNA-mediated epigenetic mechanisms that mainly act at the transcriptional and post-transcriptional level (Pavlou *et al.*, 2017).

## 2.1 DNA methylation

DNA methylation entails the transfer of a methyl group from S-adenosyl methionine to the 5' position of cytosines, resulting in the formation of 5-methylcytosine (5mC) (Mehler, 2008). The methylation process occurs throughout the entire genome, although it is enriched in CpG islands. These sites are usually found in promoter regions and, therefore, methylation generally leads to reduced gene transcription. This is mediated by two mechanisms: i) DNA methylation prevents the association of DNA binding factors, such as transcription factors, to their cognate DNA sequence or ii) the methylated CpGs can recruit proteins involved in gene repression, such as co-repressors (Figure 1) (Klose and Bird, 2006). 5mC can be converted to 5-hydroxymethylcytosine (5hmC) via an oxidation reaction catalyzed by the ten-eleven translocation family of enzymes (TET). Although its function still remains enigmatic, 5hmC was thought to exist both as an intermediate product in the process of active DNA demethylation and to represent an epigenetic modification regulating chromatin or transcriptional factors (Kriaucionis and Heintz, 2009; Tahiliani *et al.*, 2009). Total 5hmC levels increase during development of the human cerebellum and chromosomal regions positive for this mark are linked with neurodevelopmental genes (Wang *et al.*, 2012).

In the mammalian genome, DNA methylation is mediated by DNA methyltransferases 1, 3a and 3b (DNMT1, 3a, 3b). However, only DNMT3a and DNMT3b are able to exert *de novo* methylation, while DNMT1 is responsible for the maintenance of DNA methylation after replication (Klose and Bird, 2006). DNMTs are essential for embryonic development, and their

loss interferes with tissue homeostasis (Bird, 2002). DNMT3a and DNMT3b are expressed in NSCs and have a crucial function in neurogenesis and neuronal function (Okano *et al.*, 1999; Wu *et al.*, 2010).

## 2.2 Histone modifications

DNA methylation and demethylation are critical regulatory mechanisms underlying CNS development. Nevertheless, the ability of various regulatory factors to access their target genes is also influenced by chromatin modifications occurring at the histones level. Histones are essential proteins enabling the packaging of DNA into chromatin. They are responsible for the first level of chromosome condensation, the nucleosome. This protein-DNA complex is comprised of an octamer of core histone proteins and a 147bp long DNA segment wrapped around the histones (Figure 1). Every nucleosome core is composed of two molecules of each histone 2A (H2A), histone 2B (H2B), histone 3 (H3) and histone 4 (H4), and is separated from the next by a ~80bp DNA sequence, known as linker DNA. It is in this region that histone 1 (H1) binds, enabling the stabilization of the structure. All histones are subjected to post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination or sumoylation that primarily occur at the N-terminal tail of the proteins (Figure 1). The tagging of histones with specific modifications shapes the condensation of chromatin in different manners, modulating the ability of DNA to associate with the transcriptional machinery and thereby regulating gene expression (Graff and Mansuy, 2008; Kouzarides, 2007; Tessarz and Kouzarides, 2014). Histone acetylation is catalyzed by histone acetyl transferases (HATs) and is, in principle, linked to transcriptional activation. On the other hand, histone deacetylation is catalyzed by histone deacetylases (HDACs) and is

associated with transcriptional repression. Histone methylation is related to both transcriptional activation and repression, depending on the amino acid residue that is modified. For instance, lysine methylation of histone tails is associated with both activation and repression of gene expression; hence the effect of histone methylation on gene expression differs according to the position in the histone tail and the number of methylation in the lysine residues. For example, H3 methylation at lysine 4 (K4me), K36, and K79 leads to transcriptional activation, whereas H3 methylation at K9 and K27 is associated with transcriptional silencing. Several of these histone marks are essential during CNS development as they enable the sequential activation and deactivation of neurogenic and gliogenic gene promoters, resulting in the production of neuronal and glial cells at the appropriate developmental stages (Murao *et al.*, 2016).

### 2.3 Non-coding RNAs

Non-coding RNA (ncRNA) molecules are transcribed from DNA, but are not translated into proteins. Among these, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) play a variety of roles in fine-tuning gene expression by transcriptional and post-transcriptional regulation. Many studies have shown that, in addition to histone modifications and DNA methylation, ncRNAs also participate in the mechanisms that ensure the sequential production of distinct neural cell types from NSCs during development and, generally, in multiple aspects of brain development and connectivity (Follert *et al.*, 2014). MiRNAs represent one of the best characterized groups of ncRNAs. They are approximately 21-23 nucleotides long and are known for binding to the 3' untranslated region (UTR) of their mRNA targets and either degrading them or inhibiting protein translation, thus contributing to post-transcriptional repression of gene

expression (Figure 1) (Mehler, 2008). Certain miRNAs have been described to be restricted in a particular organ suggesting a tissue-specific function. Likewise, several miRNAs are brain specific and play an important role in neurogenesis and in the development of glial cells (Fiore *et al.*, 2011), with some being preferentially expressed in neurons or in astrocytes and others being equally distributed (Smirnova *et al.*, 2005). These small ncRNAs seem to be regulated upon brain development. For example, a group of brain-expressed miRNAs was reported to be upregulated during neuronal differentiation, suggesting a potential contribution in controlling the timing of neuronal fate specification (Sempere *et al.*, 2004). Another study documented a sequential increase of specific miRNA clusters at different developmental stages of the murine brain (Miska *et al.*, 2004).

Taken together, it is evident that epigenetic modifiers, such as DNA methylation, histone modifications and RNA-mediated processes, accurately drive the development of the brain influencing lineage commitment of CNS cells, including neurons, oligodendrocytes and astrocytes.

### **3. Differentiation of NSCs into astrocytes**

NSCs have the ability to self-renew and to differentiate into neurons, oligodendrocytes and astrocytes. Throughout mammalian brain development, the process of differentiation towards a neurogenic or gliogenic fate is finely timed and regulated. During the fetal expansion phase, NSCs self-replicate via symmetric cell division, whilst later, during mid-gestation, they undergo asymmetric cell division and receive cues to differentiate into neurons. Lastly, in late-gestation to



perinatal periods they enter the gliogenic phase and differentiate into astrocytes and oligodendrocytes (Hirabayashi and Gotoh, 2005; Takouda *et al.*, 2017).

### 3.1 Key extracellular signals and transcriptional activators

#### 3.1.1 The Notch pathway

The choice of NSCs between a neuronal and a glial cell fate is tightly regulated by environmental and intrinsic factors. Numerous studies have shown that specific signals, such as the neurogenic basic helix-loop-helix (bHLH) transcription factors, are involved in fate determination of the three neural lineages. They can, for example, promote the production of neurons whilst concurrently inhibiting the acquisition of glial fate. For instance, the pro-neural genes *Achaete-Scute Family BHLH Transcription Factor 1* (*ASCL1*, *MASH1*), *Neurogenin 1* (*NEUROG1*), or *Neurogenin 2* (*NEUROG2*) all promote neuronal fate determination while suppressing expression of astrocytic genes (Nieto *et al.*, 2001; Sun *et al.*, 2001). Inhibition of neurogenesis induced by pro-neural genes requires expression of transcriptional repressors such as HES1 and HES5, which are the downstream targets and effectors of the Notch pathway. The Notch signaling is highly conserved (for review see: (Louvi and Artavanis-Tsakonas, 2006)) and comprises four transmembrane Notch receptors (Notch1-4) and several ligands (Jagged1-2 and Delta-like1-4). Binding of the ligands to Notch receptors induces a proteolytic cleavage of the receptor resulting in the release of the Notch intracellular domain (NICD). Once released, the NICD fragment is translocated to the nucleus where it acts as a transcriptional coactivator. NICD cannot bind directly to DNA, but instead interacts with the DNA binding protein, suppressor of

hairless (RBP-J) to initiate transcription of Notch target genes, such as the *HES* gene family (Andersson *et al.*, 2011; Borggreffe and Oswald, 2009). Notch signaling is required to maintain a balance between the progenitor cell pool and its differentiating progeny (Borggreffe and Oswald, 2009; Imayoshi *et al.*, 2010). In addition, Notch signaling promotes a glial cell fate while neuronal identity is repressed (Gaiano *et al.*, 2000; Louvi and Artavanis-Tsakonas, 2006; Taylor *et al.*, 2007). Later, Notch inhibits generation of both neurons and oligodendrocytes and promotes the differentiation of astrocytes (Grandbarbe *et al.*, 2003).

### 3.1.2 The JAK-STAT signaling pathway

Following astrocyte specification, the first step of astrocytic differentiation is loss of neurogenic competence and expression of astrocytic genes. Members of the interleukin (IL)-6 family of cytokines, including leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and cardiotrophin-1, bind to their cognate receptors inducing their dimerization. This enables the receptor-associated Janus kinase (JAK) to autophosphorylate and become activated. JAKs in turn phosphorylate and activate signal transducer and activator of transcription 3 (STAT3) enabling formation of homodimers. STAT3 homodimers subsequently translocate to the nucleus where they bind to the promoters of astrocytic genes, including *GFAP*, permitting its expression resulting in astrocytic differentiation (Bonni *et al.*, 1997). The JAK-STAT signaling pathway regulates several distinct cellular events including stem cell self-renewal and pluripotency capacity (Niwa *et al.*, 1998; Raz *et al.*, 1999), proliferation in *Drosophila* germ (Tulina and Matunis, 2001) and intestinal lines (Beebe *et al.*, 2010). In all these cases, STAT3 governs expression of genes that determine cell identity. It might be that this common pathway essential for astrocyte differentiation and stem

cell self-renewal reinforces the similarities between these cells and reflects a potentially evolutionary remnant of plasticity.

### 3.1.1 The BMP pathway

The bone morphogenetic protein (BMP) pathway also plays a role in astrocyte differentiation. BMP signaling activates the transcription factors SMAD1, 5 and 8 to form a complex which translocates to the nucleus to activate its target genes. BMPs have an important role in NSCs fate determination. Notably, SMADs inhibit oligodendrocyte development, but promote both neurogenesis and astroglialogenesis as well as astrocytic differentiation and maturation (Mabie *et al.*, 1999; Setoguchi *et al.*, 2004; Xiao *et al.*, 2010). For example, BMP signaling promotes astrocytes maturation through phosphorylation of SMAD1/5/8 pathway leading to acquisition of a process-bearing morphology and expression of the most common astrocytic markers *GFAP*, *Aquaporin 4 (AQP4)*, and *S100B* (Scholze *et al.*, 2014). Moreover, *in vivo*, in a BMP receptor double knockout mouse (*Bmpr1a* and *Bmpr1b*), the number of astrocytes was decreased between 25-40 % (See *et al.*, 2007). Astrocyte number is also reduced *in vitro*, following inhibition of BMP by noggin, while the oligodendrocyte number is increased (Cate *et al.*, 2010). Notably, both STAT3 and SMAD1 form a complex that is bridged by the coactivator p300 leading to induction of astrocytic genes in a synergistic manner (Nakashima *et al.*, 1999).

Both the levels and timing of expression of pro-astrogenic genes is critical for successful orchestration of initiation, maintenance and termination of differentiation. However, some of these components are also involved in the maintenance of stem cell identity, thus emphasizing their fine-

tuned regulation of NSCs and astrocyte uniqueness. This duality must be accommodated in any forward translational strategy.

The signaling pathways responsible for the astrocytic differentiation represent the essential players in physiological astrogenesis, but these mechanisms are not sufficient to define the spectrum of gene activity that occurs upon astrocytic differentiation.

### **3.2 Importance of DNA methylation in the acquisition of the astrocytic fate**

In the CNS, cell intrinsic factors and extracellular cues influence the onset of astrogliogenesis. Concomitantly, dramatic changes in DNA methylation of astrocyte-specific promoters contribute to the neurogenic to gliogenic switch. This process explains why the responsiveness of NSCs to the astrogenic signaling pathways varies according to developmental stages. For example, early-derived cortical precursor cells are not able to undergo astrocytic differentiation in the presence of LIF, even though they express functional LIF receptors (Molne *et al.*, 2000). In contrast, cultures of murine neuroepithelial cells derived from embryonic day (E) 14 differentiate readily into astrocytes upon LIF stimulation. However, *GFAP* is not expressed in cultures from E11.5 neuroepithelial cells even when the STAT3 pathway is induced (Takizawa *et al.*, 2001). These studies reveal that the well-coordinated switch from neurogenic to astrogenic fate is not solely dependent on the presence of extracellular factors, but also requires intrinsic determinants.

During neuronal differentiation, astrocyte-specific gene promoters are methylated and transcription is silenced. However, in late-gestation many astrocytic genes become demethylated enabling NSCs to acquire gliogenic competences (Hatada *et al.*, 2008). Recently, directly isolated

murine neural stem and progenitor cells from different developmental stages (E11.5, 14.5 and 18.5) were used to determine the DNA methylome of each stage, revealing distinct and successive waves of global DNA methylation/demethylation that regulate the sequential generation of neurons, astrocytes and oligodendrocytes in the developing brain (Sanosaka *et al.*, 2017). Notch ligands expressed in neuroblasts and immature neurons during mid-gestation activate their neighboring NSCs by inducing expression of the transcription factor nuclear factor I A (*NFIA*). *NFIA* in turn performs a dual act: it induces demethylation of the *GFAP* promoter and promotes dissociation of DNMT1 from the *GFAP* promoter, unveiling its STAT binding site (Namihira *et al.*, 2009) (Figure 2). Deletion of the *Dnmt1* gene results in demethylation of astrocytic genes and of genes involved in the JAK-STAT signaling pathway. Consequently, increased STAT activity and expression of astrocyte-related genes leads to precocious astroglial differentiation (Fan *et al.*, 2005). Further, specific deletion of *Dnmt1* in NSCs increased the generation of astrocytes in the dentate gyrus (Noguchi *et al.*, 2016b). Ablation of *Dnmt1* during late embryonic development did not impact astrogliogenesis *per se*, but *Gfap* expression was upregulated in the existing astrocytes of adult mice (Noguchi *et al.*, 2016a). In addition, DNA methylation has an important role in the control of expression of the immature astrocytic marker *S100B*. Methyl CpG binding protein 2 (MECP2), a protein linked to gene silencing which recruits histone deacetylases (HDACs) and corepressors, is bound on the promoter region of *S100b* inactivating gene expression. However, at E14 a specific cytosine residue of a CpG site within the promoter region is demethylated and MECP2 can no longer bind to this site leading to the initiation of murine *S100b* expression (Namihira *et al.*, 2004) (Figure 2).

Overall, it appears that demethylation of astrocyte-specific gene promoters is crucial for the timely activation of gene expression and therefore for the regulation of astrocyte differentiation in the developing brain.

### 3.3 Histone modifications associated to astrocytic differentiation

During neural development, both neurogenic and gliogenic gene promoters undergo various histone modifications, which ensure the sequential production of each cell type. In the neural tube at E14, exposure of neuroepithelial cells to the astrocyte inducing cytokine BMP2 resulted in a significant increase of histone H3 acetylation in the *SI00B* gene promoter (Namihiro *et al.*, 2004). During astrocyte differentiation, LIF acts synergistically with retinoic acid (RA) to induce an open chromatin conformation through histone H3 acetylation, resulting in activation of the *GFAP* promoter in NSCs (Asano *et al.*, 2009). RA receptors (RARs) form complexes with retinoid X receptors (RXRs) and bind to RA response elements in the promoter regions of target genes. When the RA ligand is absent, RAR/RXR associates with transcriptional repressors leading to gene silencing by recruitment of HDACs (Figure 2). Conversely, binding of RA enables the release of HDACs from the RAR/RXR complex and the recruitment of histone acetyltransferase (HAT) co-activators. Thus, RA-bound RAR/RXR associates with the *GFAP* promoter allowing the chromatin to adopt an open conformation through histone H3 acetylation, specifically in the STAT site-containing region. This facilitates efficient binding of STAT3 to the promoter, therefore inducing *GFAP* expression (Asano *et al.*, 2009) (Figure 2). In addition, activated STAT3 is able to recruit the transcriptional co-activators CBP/p300, resulting in acetylation of H3K9 and H3K14 at the *GFAP* promoter (Figure 2). These histone modifications further lead to enhanced H3K4me3

and recruitment of RNA polymerase II and activation of gene transcription (Cheng *et al.*, 2011). Intriguingly, histone deacetylase 3 (HDAC3) was reported to regulate the switch between oligodendrocyte and astrocyte fate; deletion of HDAC3 induces robust astrocyte differentiation with concomitant loss of oligodendrocytes (Zhang *et al.*, 2016). Similarly, inhibition of HDACs either with trichostatin A or sodium butyrate reduced *GFAP* expression in human astrocytes and reorganized the intermediate filament network of the cells (Kanski *et al.*, 2014). Further histone modifications are for the expression of *GFAP*. Fibroblast growth factor 2 (FGF2), which regulates the competence of rodent cortical progenitors to differentiate into astrocytes in response to CNTF, facilitates access of the signal transducer and activator of transcription/CRE binding protein (STAT/CBP) to the *GFAP* promoter by strongly increasing H3K4me while blocking H3K9me around STAT binding site of the *GFAP* promoter (Figure 2). Since H3K4me and H3K9me are linked to transcriptional activation and repression respectively, FGF2 alters H3 methylation in a way that favors the activation of the *Gfap* promoter (Song and Ghosh, 2004).

Deposition of repressive histone marks is also evident during astrocyte differentiation. ERG-associated protein with SET domain (ESET) protein, a H3K9 histone methyltransferase (HMT), is highly expressed during the early stages of brain development, but it is strongly downregulated during the transition from neurogenesis to astrogenesis. The *GFAP* promoter is a direct target of ESET and its expression at early stages is repressed via elevated H3K9me3 marks (Tan *et al.*, 2012). At later stages of brain development where ESET levels are reduced, H3K9me3 is decreased and *GFAP* is activated (Figure 2). Similarly, the histone demethylase known as gene amplified in squamous cell carcinoma 1 (*GASC1*) is important for the developmental stage-dependent differentiation of astrocytes. *GASC1* hypomorphic mutant mice exhibit an increased amount of GFAP<sup>+</sup> cells in the forebrain together with abnormal behaviors and synaptic activity

(Sudo *et al.*, 2016). The roles of astrocytes on synaptic plasticity may partially explain why the mutant mice present such phenotypes and further help to understand the abnormal behaviors. Moreover, deletion of *Enhancer of zeste homolog 2* gene (*EZH2*), encoding a polycomb group protein which is a H3K27 HMT, on cortical progenitor cells leads to accelerated astrocyte differentiation (Pereira *et al.*, 2010).

On the other hand, the H3K4, lysine-specific demethylase 5A (*KDM5A*) is pivotal for the repression of astrocyte differentiation in NSCs. Knockdown of *KDM5A* in NSCs increases the generation of astrocytes, while *KDM5A* overexpression reduces transcriptional activity of the *GFAP* promoter. Induction of astrocytic differentiation reduced recruitment of *KDM5A* to the *GFAP* promoter and increased H3K4me, thus maintaining NSCs in an undifferentiated state by suppression of astrogliogenesis (Kong *et al.*, 2017).

### 3.4 Essential miRNAs for the generation of astrocytes

Several studies unveiled the important regulatory role that miRNAs play in astrocyte differentiation. During glial specification, miR-153 targets *NFIA* and *NFIB* mRNAs, an essential step for the initiation of astrocyte differentiation (Figure 2). Overexpression of miR-153 delayed the onset of astrogenesis favoring an undifferentiated state, while inhibition of miR-153 induced premature gliogenesis (Tsuyama *et al.*, 2015). In early developmental stages of the forebrain, miR-153 is highly expressed. As CNS development progresses, the levels of miR-153 are reduced followed by increased *NFIA/B* expression, revealing its role in astrocyte fate specification.

MiR-31 was shown to be necessary for the specification and differentiation of astrocytes. In NSCs, miR-31 is suppressed by multiple stem cell factors, such as LIN28, C-Myc, SOX2 and



OCT4. However, upon astrogliogenesis, miR-31 is upregulated via the STAT3 and SMAD1/5/8 signaling pathways contributing to the promotion of astrocyte differentiation, in part by targeting and reducing *Lin28* mRNA levels. Indeed, in the absence of miR-31, astrocytes fail to properly differentiate, while overexpression of miR-31 is able to partially induce astrocyte differentiation (Meares *et al.*, 2018).

Investigations into the role of *Chicken ovalbumin upstream promoter-transcription factors I and II (Coup-tfI and Coup-tfII)* during CNS development have identified miR17/106 as another important regulator of the neurogenic to gliogenic switch. COUP-TFI/II is transiently expressed in the ventricular zone of early embryonic CNS. Double knockdown of *Coup-tfI/II* in NSCs resulted in greater silencing of the STAT3-binding site of the *Gfap* promoter due to reduction of acetylated histone H3 and dimethylated H3K4 (H3K4me2) and increase of H3K9me2. In the developing mouse forebrain, double knockdown of the transcription factors inhibited the initiation of astrogenesis (Naka *et al.*, 2008). Later on, the same group identified miR17/106 as a downstream regulator of COUP-TFI/II. The mRNA of *p38*, which is pivotal for the physiological process of astrogenesis, was found to be a direct target of miR17/106 (Naka-Kaneda *et al.*, 2014). Therefore, it seems that miR17/106 is an important regulator of the neurogenic to gliogenic switch.

#### **4. Spinal cord/traumatic brain injuries and neurodegenerative disorders**

Apart from their importance and multifunctionality in the healthy CNS, astrocytes respond to brain damage, infection or disease through a process known as reactive astrogliosis. The first description of astrocyte reactivity was documented by Virchow, showing that the spinal cord tissue was more fibrillary in neurosyphilis patients than in healthy individuals (Barcia *et al.*, 2016). The

concept of astrocyte reactivity emerged with the discovery of the intermediate filament GFAP (Eng *et al.*, 1971) and strong GFAP expression in astrocytes became a sign of reactivity (A. and DORIS, 1976). Another distinctive attribute of reactive astrocytes reported by early neuropathologists was hypertrophy as evidenced by enlarged cell body and processes (Wilhelmsson *et al.*, 2006). During astrogliosis, the glial scar formation is accompanied with the appearance of newly proliferated astrocytes. Human specimens showed evidence of astrocytic proliferation in response to a variety of insults, such as infection and acute demyelinating lesions (Colodner *et al.*, 2005). However, if these newly divided astrocytes have been originated either from mature astrocytes that re-entered the cell cycle or from local progenitor cells is still a matter of investigation (Buffo *et al.*, 2008; Carlen *et al.*, 2009).

Astrogliosis is primarily associated with neuroprotection. For example, during ischemia, reactive astrocytes (i) protect neurons from oxidative stress through a glutathione dependent mechanism (Chen *et al.*, 2001; Iwata-Ichikawa *et al.*, 1999), (ii) offer protection from  $\text{NH}_4^+$  toxicity (Rao *et al.*, 2005), (iii) contribute to BBB repair (Tian *et al.*, 2011), (iv) participate in the water brain homeostasis (Zador *et al.*, 2009) and (v) are involved in the clearance of excitotoxic glutamate (Brown, 1999; Rothstein *et al.*, 1996). The subsequent formation of the glial scar acts as a physical insulator to contain the lesioned area. Selective ablation of reactive astrocytes in adult mice expressing a herpes simplex virus-thymidine kinase from the *Gfap* promoter by treatment with ganciclovir, led to pronounced inflammatory responses, leukocyte infiltration, extensive tissue disruption, increased demyelination, neuronal degeneration, and failure of the BBB repair (Faulkner *et al.*, 2004; Myer *et al.*, 2006; Voskuhl *et al.*, 2009).

#### 4.1 The molecular repertoire of reactive astrocytes

At the molecular and functional levels, astrogliosis is highly diverse (Anderson *et al.*, 2014); even in the same region of the mouse cerebral cortex, astrocytes respond heterogeneously to stab wound injury. In fact, although it was shown that almost all astrocytes become hypertrophic and overexpress GFAP following injury, some had their processes polarized towards the lesion, others proliferated and others remained static (Bardehle *et al.*, 2013). In addition, activated astrocytes acquire NSC properties and modify not only their phenotypic profile, but they also undergo major gene expression changes. The transition from a normal to a reactive astrocytic phenotype is induced by extra- and intra-cellular signaling and epigenetic mechanisms that triggers, resolves or maintains astroglial reactivity. The machinery that will promote the “conversion” of astrocytes depends on the specific injury or degeneration event. Several epigenetic factors and signaling pathways have been described to play a role in astrogliosis, including the JAK/STAT pathway, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) pathway, and the MAPK pathway (Ben Haim *et al.*, 2015).

##### 4.1.1 Reactive astrocytes in spinal cord and traumatic brain injuries

Following CNS injury, activated microglia react rapidly and recruit astrocytes by secreting several pro-inflammatory mediators including tumor necrosis factor-alpha (TNF), IL-1 $\beta$ , IL-6, and interferon-gamma (IFN- $\gamma$ ) (Sofroniew and Vinters, 2010). Reactive astrocytes can proliferate, become hypertrophic and migrate to the site of the injury leading to the formation of a physical glial barrier that impedes axonal regeneration in chronic spinal cord injury (SCI). Nevertheless, ablation of reactive astrocytes or interference with their activation mechanism upon injury, results

in exacerbated tissue degeneration and spread of inflammatory cells indicating that the glial scar has a potential function (Karimi-Abdolrezaee and Billakanti, 2012). The transcription factor NF- $\kappa$ B is responsible for triggering gene expression of several inflammatory mediators during inflammation (Figure 3). Astrocyte-specific inactivation of NF- $\kappa$ B resulted in improved functional recovery following SCI (Brambilla *et al.*, 2005). Notably, NF- $\kappa$ B activation of astrocytic cultures by TNF was unable to induce a reactive phenotype, but seemed to convert astrocytes into neural progenitor-like cells (Gabel *et al.*, 2016). After SCI, JAK-STAT signaling is also activated (Figure 3). Reactive astrocytes in STAT3 conditional knockout mice showed limited migration to the lesion site, failure of astrocyte hypertrophy and *GFAP* upregulation, widespread leukocyte infiltration, neural disruption and demyelination (Herrmann *et al.*, 2008; Okada *et al.*, 2006). Furthermore, the investigation of astrocyte-related signaling responses resulting from diverse neurotoxic insults, revealed the implication of STAT3 pathway as a broadly triggered signaling pathway for astrogliosis (O'Callaghan *et al.*, 2014).

It is evident that in response to different injury or inflammatory stimuli, distinct pathways are stimulated, all leading to astrocytic activation. Fibrinogen, a blood protein that leaks into the CNS following BBB disruption, was discovered to activate transforming growth factor-beta (TGF- $\beta$ ) signaling pathway after traumatic CNS injury. Activation of TGF- $\beta$  results in neurite outgrowth inhibition, while inhibition of TGF- $\beta$  signaling attenuated glial scar formation (Schachtrup *et al.*, 2010). Toxin-induced reactive gliosis in the corpus callosum showed that endothelin-1 induces astrocyte proliferation and *GFAP* expression through activation of ERK- and c-Jun N-terminal kinase (JNK)-dependent pathways (Gadea *et al.*, 2008) (Figure 3). Similarly, traumatic scratch injury in astrocytes triggered a calcium influx from the extracellular compartment and activated the JNK pathway to activate *GFAP* expression (Gao *et al.*, 2013).

Transcriptomic analysis of reactive astrogliosis showed that gliosis consists of a rapid induction of gene expression after insult and identified lipocalin 2 (*LCN2*) and *Serpina3* as robust markers of reactive astrocytes (Zamanian *et al.*, 2012). Initially described as iron-trafficking protein involved in multiple processes such as apoptosis, innate immunity and renal development, *LCN2* is secreted in several brain injury conditions such as inflammation (Jin *et al.*, 2014) and stroke (Elneihoum *et al.*, 1996), or in response to neurodegeneration (Bi *et al.*, 2013). It also promotes apoptosis, morphological changes, and migration of astrocytes both *in vitro* and *in vivo*. In all cases, it has been detected in high amounts predominantly in astrocytes and to be selectively toxic to neurons. In fact, *LCN2* deficiency reduced astroglial-induced neurotoxicity *in vitro* (Jin *et al.*, 2014) and resulted in a significant attenuation of hippocampal neuronal loss, white matter damage, BBB permeability and cognitive decline in a mouse model of cerebral ischemia (Kim *et al.*, 2017). NF- $\kappa$ B and STAT3 are able to regulate the expression of *LCN2* (Figure 3). Intriguingly, *LCN2* is capable of initiating the activation of JAK2/STAT3 and NF- $\kappa$ B signaling pathways to induce the production of chemokines and astrocytic cell migration (Lee *et al.*, 2011) (Figure 3), thus establishing a positive feedback loop which maintains astrocytes in a reactive state.

Interestingly, a recent study using a SCI mouse model defined three distinct astrocytic populations based on their marker genes: naive, reactive and scar-forming astrocytes (Hara *et al.*, 2017). When reactive astrocytes were transplanted into naive or injured spinal cord models, they were converted to naive astrocytes or formed astrocytic scars respectively, unveiling their environment-dependent plasticity. Upregulation of genes associated with type I collagen in the lesioned area following SCI is responsible for the conversion of reactive into scar-forming astrocytes. Pharmacological blockage of reactive astrocyte–type I collagen interaction was

sufficient to prevent scar formation strengthening the beneficial effect of reactive, but not scar-forming astrocytes (Hara *et al.*, 2017).

Traumatic brain injury (TBI) is an acute injury of the brain resulting in a direct neuronal loss followed by a neuroinflammatory phase. In the acute stage, the neuroinflammatory “cascade” attempts to respond against brain damage. However, in the chronic stage, this reaction leads to neurodegenerative-like symptoms, including diminished or altered state of consciousness, impaired motor and cognitive skills (Lozano *et al.*, 2015). In an effort to treat TBI, the hematopoietic growth factor granulocyte colony-stimulating factor was used in a TBI mouse model resulting in behavioral recovery associated with increased astrogliosis and hippocampal neurogenesis. Activated astrocytes participated together with microglia in the release of neurotrophic factors to mediate repair and enhance survival of injured neurons (Song *et al.*, 2016). In a TBI rat model, the increase of GFAP<sup>+</sup> cells under brain injury revealed the possible involvement of astrocytes in perivascular caspase-3-mediated apoptosis (Glushakova *et al.*, 2018).

#### **4.1.2 Reactive astrocytes in neurodegenerative diseases**

Given the multitude of vital roles that astrocytes play in the regulation of brain physiology, it is not surprising that reactive astrocytes are involved in the initiation and progression of age-related neurodegenerative diseases including AD, PD, and amyotrophic lateral sclerosis.

The most prevalent neurodegenerative disease is AD, which is accompanied by a progressive accumulation of senile plaques, mainly composed of amyloid  $\beta$  (A $\beta$ ), and neurofibrillary tangles composed of tau protein. Tissue samples from AD patients present an overabundance of reactive astrocytes that are closely associated with amyloid plaques in the

cerebral cortex. A remarkable and stereotypical spatial organization of glial cells around A $\beta$  plaques has been recently characterized, with an inner shell of amoeboid/activated microglia and an outer shell of reactive and polarized astrocytes, a structure described as “reactive glial net” (Bouvier *et al.*, 2016). Reactive astrocytes endocytose A $\beta$  peptides released from degenerating neurons, resulting in the toxic accumulation of A $\beta$ , followed by astrocytic death and subsequent release of A $\beta$  (John Lin and Deneen, 2013; Maragakis and Rothstein, 2006).

Extracellular cues play important regulatory roles of the expression of astrocyte-specific genes during astroglial development and it is possible that similar mechanisms are responsible for controlling gene expression in neurodegenerative diseases. Activation of JAK2/STAT3 pathway precedes the onset of astrogliosis in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD (Sriram *et al.*, 2004). On the other hand, deletion of astrocytic STAT3 in the MPTP model markedly reduced the number of reactive astrocytes (O'Callaghan *et al.*, 2014). These observations clearly identify a role for activated STAT3 in astrocyte damage, induction of astrogliosis and possibly *GFAP* upregulation. In the MPTP mouse model, increased expression of wingless-type MMTV integration site1 (*WNT1*) was attributed to reactive astrocytes. It was shown that Wnt1, together with other factors derived by these cells, promoted the generation of dopaminergic neurons from adult NSCs *in vitro*, and that pharmacological activation of Wnt/ $\beta$ -catenin pathway *in vivo* enabled the recovery of dopaminergic neurons, revealing a neuroprotective effect of Wnt signaling and reactive astrocytes in PD (L'Episcopo *et al.*, 2011). Wnt signaling regulates the development of midbrain dopaminergic neurons and is required for their differentiation (Arenas, 2014). In addition, Wnt proteins are abundantly present in the adult brain maintaining and protecting neuronal functions, including the dopaminergic neurons of the *substantia nigra* (Inestrosa and Arenas, 2010). On the other hand, canonical Wnt signaling is

upregulated in PD and proteins encoded by *PARK* genes, involved in familial PD, have been shown to modify Wnt signaling by regulating  $\beta$ -catenin levels or by interacting with key Wnt signaling components (Berwick and Harvey, 2014). Further exploration of the role of the Wnt pathway in astrocytic function and their subsequent beneficial effect on dopaminergic neurons might be of significant importance in order to fully understand the contribution of reactive astrocytes to both neurodegeneration and neuroprotection during ageing.

#### 4.1.3 Heterogeneity of reactive astrocytes

As it was discussed, in response to threatening events, astrocytes could adopt protective or deleterious relationships towards neural tissue. In fact, gene expression analysis of reactive astrocytes derived from stroke or inflammatory mouse models revealed the existence of two distinct context-dependent populations with distinct phenotypes that relied on the type of the injury (Zamanian *et al.*, 2012). While reactive astrocytes from the different models shared several deregulated genes, at least 50% of the altered genes were unique for each condition. Reactive astrocytes in ischemia (called A2 astrocytes) seem to possess a molecular identity that may be protective, while the ones activated by LPS (namely A1 astrocytes) have a negative effect. This type of astrocyte was found abundantly in several neurodegenerative diseases, including PD, AD and Huntington's disease (Liddelow *et al.*, 2017). Recently, it was shown that aged astrocytes acquired an A1-like identity that depends on the brain region. It was suggested that this type of astrocyte might contribute to cognitive decline in normal aging, while increasing the vulnerability of the aged brain during injury (Clarke *et al.*, 2018). These studies highlight the heterogeneity of astrogliosis, where astrocyte activity depends on the type of inducing injury and constitute an additional hint that epigenetic mechanisms may participate in this astrocytic response since



different genes must be activated each time in response to specific stimulations. Accurate coordination of gene expression depending on the type of injury is necessary and it appears that this expression is additionally brain region- and age-specific. It is possible that the same molecular pathways are used in CNS insults, leading to different expression patterns at each time. In this sense, this diverse responsive ability of astrocytes unveils their highly plastic potential and offers an opportunity for this to be exploited in therapeutic strategies.

#### **4.2 MiRNAs' functioning in the process of astrocyte reactivity**

Similarly to astrocyte differentiation, epigenetic regulation plays a role in reactive astrogliosis as well. For example, multiple miRNAs have been reported to be modulated upon reactive astrogliosis. MiR-145, enriched in rat spinal neurons and astrocytes, was found to be a negative regulator of astrogliosis. It was downregulated in SCI and astrocyte-specific overexpression of miR-145 decreased the size of astrocytes and their proliferative and migratory abilities. *GFAP* and *MYC* mRNAs were suggested as potential targets of miR-145 (Wang *et al.*, 2015) (Figure 3). MiR-145 has also been detected at low levels in human embryonic stem cells (hESCs) and increasing levels during differentiation suppress the pluripotency genes OCT4, SOX2 and KLF-4 (Figure 3) (Xu *et al.*, 2009). An important aspect would be to identify whether miR-145 reduction in SCI is able to activate the neural stem cell genes in astrocytes, enabling them to obtain an immature identity and if miR-145 downregulation contributes to the acquisition of a stem cell-like fate in an attempt to restore the brain damage and regenerate.

In contrast to the down-regulation of miR-145, miR-21 was upregulated in a time-dependent manner after SCI in mouse. Inhibition of miR-21 abrogated the increased expression of reactive markers such as GFAP (Figure 3), augmented astrocytes' hypertrophic response and

increased axon density in the site of the lesion (Bhalala *et al.*, 2012), depicting the detrimental effects of increased miR-21 levels upon injury. This study revealed a novel role of miR-21 in regulation of astrocyte hypertrophy and glial scar progression. Interestingly, BMP signaling has been described to modulate miR-21 levels in cultured astrocytes (Sahni *et al.*, 2010), and a miR-21 regulatory sequence contains two STAT3-binding sites, providing the possibility that astrocytic miR-21 levels may also be regulated by JAK-STAT signaling (Loffler *et al.*, 2007).

MiR-140 was shown to suppress normal astrocytic cell proliferation by binding to the 3' UTR of brain-derived neurotrophic factor (*BDNF*) mRNA inhibiting its translation (Figure 3). LPS-induced inflammation resulted in increased production of BDNF, IL-6 and TNF, which were restored with ectopic miR-140 expression (Tu *et al.*, 2017). Glioma patients also present low amount of miR-140, which has been shown to restrict tumor growth and metastasis. The anti-proliferative effect of miR-140 may be used to restrict the detrimental effects of reactive astrogliosis on the damaged tissue and as a potential therapeutic strategy for cancer patients.

The family of miR-181 was reported to be present in the mature CNS but not early in brain development, showing a high expression in astrocytes compared to neurons (Hutchison *et al.*, 2013). Induction of inflammation by treating mice with LPS leads to significant decrease of all miR-181 family members (Figure 3). Overexpression of miR-181c in astrocytic cultures exposed to LPS increased cell death and changes in the levels of miR-181 resulting in modified expression of several inflammatory cytokines. Interestingly, mRNAs encoding MeCP2 and X-linked inhibitor of apoptosis were identified as miR-181 targets. Notably, in the hematopoietic system, miR-181 represses LIN28, a well-described pluripotent marker, mediating stem cell differentiation (Li *et al.*, 2012). Further investigation of the possible role of miR-181 towards cell fate specification during brain development would be of great value. These findings unveil the important role of

miRNAs in the molecular responses of astrocytes under inflammatory conditions and illustrate how they alter the astrocytic transcriptome under reactive astrogliosis.

Although it has been recently shown that a TNF treatment of murine astrocytes differentiated from neural precursor cells *in vitro* resulted in the modifications in the levels of H3K4me3 and H3K27me3 at the promoters of specific genes (Michelucci *et al.*, 2016), evidences on the extents of DNA methylation and histone modifications in reactive astrocytes are still lacking. Identifying additional epigenetic modifications that influence the process of astrocytic activation will be crucial not only to further understand the underlying mechanisms, but also to intervene and potentially attain functional recovery after a CNS injury or disease. However, it has to be borne in mind that reactive astrogliosis *per se* offers several protective roles to the CNS, such as uptake of potentially excitotoxic glutamate, repair of the BBB and limitation in the spreading of inflammatory cells or infectious elements from the injured or diseased tissue to the healthy parts. Interestingly, it is possible that some clinically approved drugs may act predominantly on astrocytes or on the astrocytic network, although originally designed for other targets (Pekny and Pekna, 2014). In this sense, it is possible that already available drugs are able to act on molecules selective to astrocytes or on epigenetic mechanisms related to reactive astrogliosis offering at least the possibility of adjusting the damage-recovery equilibrium within the CNS.

Overall, the ability of astrocytes to become reactive, undergoing dramatic morphological and molecular changes, highlights their plasticity. Under specific environmental and intrinsic cues, this plasticity may also confer astrocytes the capacity to dedifferentiate, thus re-expressing features of NSCs, or even to be reprogrammed into a different cell lineage.

## 5. Astrocyte plasticity: dedifferentiation and reprogramming

Cellular plasticity refers to the ability of cells to change their fate identity in response to microenvironmental stimuli. Astrocytes exhibit a high degree of morphological and functional plasticity. Recent reports have shown that astrocytes can be converted into functional neurons both *in vitro* (Berninger *et al.*, 2007; Heinrich *et al.*, 2010) and *in vivo* (Liu *et al.*, 2015; Torper *et al.*, 2013). In a mouse model of AD, reactive astrocytes were directly reprogrammed into functional neurons in response to NeuroD1 (Guo *et al.*, 2014). Under physiological conditions, astrocytes generally remain in a quiescent state, but retain their capacity to resume proliferation. Fate mapping of astrocytes in the adult mouse cerebral cortex revealed that, after injury, a subset of mature astrocytes acquired stem cell properties and recapitulated features of early developmental stages (Buffo *et al.*, 2008). This process was originally coined by the authors as “dedifferentiation”. Intriguingly, these cells were able to form neurospheres, to self-renew and to differentiate into neurons and oligodendrocytes *in vitro*. This was later confirmed in models of traumatic or ischemic brain injury (Shimada *et al.*, 2012; Sirko *et al.*, 2013). Furthermore, *in vivo* imaging of astrocytes following acute injury identified a subpopulation of proliferating reactive astrocytes enriched along blood vessels (Bardehle *et al.*, 2013). Notch signaling is crucial for controlling neurosphere formation in the cultures of reactive astrocytes (Shimada *et al.*, 2012). Finding routes to bypass the anti-neurogenic environment in the adult brain, due for example to BMP and Notch signaling, would potentially allow exploitation of the latent multipotency of reactive astrocytes to elicit neuronal repair.

### 5.1 Influence of external stimuli

Different factors have been identified to induce the conversion of mature astrocytes into neural progenitors. *In vitro*, transforming growth factor alpha promotes sequential conversion of mature astrocytes into neural progenitors and stem cells (Sharif *et al.*, 2007). This study described a novel population of mature astrocytes capable, in response to a single growth factor, to regress progressively into a neural stem-like cell stage via an intermediate glial progenitor stage. *In vivo*, Sonic hedgehog (SHH) signaling is a key mediator enabling astrocytes to retain latent stem cell-like properties. Inducible deletion of SHH's transducer Smoothed followed by stab injury in adult mice resulted in a significant reduction of proliferation in reactive astrocytes and to a decreased ability of these cells to form neurospheres *in vitro* (Sirko *et al.*, 2013). Another study identified elevated SHH levels in astrocytes upon mechanical injury. *In vitro* incubation of astrocytes with conditioned medium derived from injured astrocytes enabled cells to acquire neural progenitor cell characteristics, including self-renewal, multipotency, downregulation of *GFAP* and *S100B* and upregulation of *NES*, *SOX2* and *CD133* (Yang *et al.*, 2012). Notably, in the mouse forebrain, SHH acts as a mitogen in neural progenitor cells of the SVZ enabling them to proliferate and produce new interneurons in the olfactory bulb (Palma *et al.*, 2005). Therefore, SHH is a crucial pathway during neurogenesis and astrocytes dedifferentiation, thus mirroring another important pathway shared by both NSCs and astrocytes.

Further, the role of inflammatory mediators to induce astrocytes dedifferentiation has also been a matter of recent investigation. Exposure to the pro-inflammatory factor TNF of primary astrocytes prepared from newborn mice induces expression of several genes associated with the NF- $\kappa$ B pathway (Birck *et al.*, 2016). More precisely, administration of TNF initiated loss of *Gfap* expression and the decrease of expression of genes related to glycogen metabolism. Moreover, a subset of these cells gained expression of stemness markers, including Oct4, CD44 and Musashi-

1 as well as were able to form neurospheres giving rise to neural progenitors and neurons (Gabel *et al.*, 2016). Notably, *in vitro* treatment with TNF or the BMP inhibitor noggin of murine astrocytes differentiated from neural precursor cells resulted in the acquisition of NSC-like properties accompanied by alterations in both the epigenome and transcriptome. Dedifferentiation of astrocytes was associated with modifications in the levels of H3K4me3 and H3K27me3 at the promoters of genes related to cell cycle, stemness or neuronal fate thereby permitting the re-activation of neural progenitor markers (Michelucci *et al.*, 2016).

## 5.2 Effects of genetic manipulation

As mentioned above, EZH2 is a HMT involved in NSC renewal and maintenance by inducing gene silencing via histone methylation and deacetylation. Forced expression of *Ezh2* in postnatal mouse astrocytes resulted in partial, but not complete dedifferentiation of these cells accompanied with the downregulation of *Gfap* and *S100B* and the upregulation of NSC markers, including *Nes* and *Sox2* (Sher *et al.*, 2011), revealing the role of *Ezh2* in retaining cells in the NSC state and even reverting differentiated cells into a more immature state.

Additional studies suggested that reactive astrocytes share common characteristics with NSCs. *Dicer* is an indispensable enzyme of the miRNA machinery, responsible for the maturation of miRNAs. Selective deletion of *Dicer* in astrocytes resulted in transgenic mice that exhibited ataxia, profound neuronal degeneration of the cerebellum, seizures, and premature death. Prior to the onset of any neurological symptom, the transcriptome of *Dicer*-deficient astrocytes was modified in a way that resembled an immature molecular signature. Specifically, hallmark genes of immature/reactive astrocytes were upregulated, while astroglial genes related to mature functions were downregulated contributing to excitotoxicity and a failure to support normal mature

brain circuits (Howng *et al.*, 2015; Tao *et al.*, 2011). Collectively, Dicer and subsequently miRNA production are critically involved in the maintenance of astrocyte identity and function.

These studies reveal that, besides the two neurogenic niches of the adult brain, subpopulations of mature astrocytes in other brain regions maintain their ability to dedifferentiate and acquire NSC-like properties under specific conditions. Nevertheless, the origin of these plastic cells is still a matter of controversy, at least under stroke conditions where it was described that NSCs from the SVZ give rise to a subpopulation of reactive astrocytes in the cortex which contributes to astrogliosis and scar formation (Faiz *et al.*, 2015). This is especially important in cell replacement therapies in order to obtain a desired cell fate and potentially regenerate damaged tissue. Forced expression of the transcription factor *ASCL1* in astrocytes elicits GABAergic neurons, while expression of *NEUROG2* induces glutamatergic neurons, and concomitant expression of *CEND1* and *NEUROG2* induces generation of a mixed GABAergic and dopaminergic neuronal population *in vitro* (Aravantinou-Fatorou *et al.*, 2015; Berninger *et al.*, 2007; Heinrich *et al.*, 2010). In 2013, several research groups managed to reprogram astrocytes into functional neurons *in vivo*. Overexpression of the single transcription factor *SOX2* (Niu *et al.*, 2013) or *NeuroD1* (Guo *et al.*, 2014) or a combination of *ASCL1*, *BRN2A*, and *MYT1L* (Torper *et al.*, 2013), was sufficient to directly reprogram astrocytes into neurons. Furthermore, it was shown that striatal astrocytes of stroke-induced mice entered a neurogenic program and generated new neurons (Magnusson *et al.*, 2014). In a mouse model of Huntington's disease, some activated striatal astrocytes acted as neural progenitors and gave rise to neurons *in vivo* (Nato *et al.*, 2015). Activated astrocytes directly generated dopaminergic neurons in a Parkinson's disease mouse model (Rivetti di Val Cervo *et al.*, 2017). *In vivo* reprogramming of adult astroglial cells has emerged as a potential therapeutic approach that would avoid cell transplantation and possibly

immunosuppression. However, reprogramming is a time consuming process characterized by low efficiency. According to a population shift view of cellular reprogramming, a small population of cells already resides in energetically favorable trajectories that will allow them to respond more readily to reprogramming signals. Consequently, if expression of reprogramming signal is retained over time, stochastic fluctuations in the transcriptome and epigenome will permit other cells to enter this primed state, thereby progressively increasing the efficiency of reprogramming (Del Sol and Buckley, 2014). Unlocking the molecular mechanisms that control cell fate switch may lead to improvements in reprogramming efficiency and provide a regenerative medicine strategy for mitigating neuronal loss in degeneration or trauma.

Taken together, the capacity of astrocytes to become reactive or be reprogrammed provides promise for endogenous brain repair strategies. However, these approaches have to be taken with caution as this may have unwanted side effects, such as tumorigenesis.

## **6. Brain tumors of astrocytic lineage**

Gliomas represent the most common primary tumors of the CNS with an incidence rate of 6.6 per 100.000 individuals in the USA (Ostrom *et al.*, 2016). Adult diffuse gliomas are classified based on histopathological and molecular features in oligodendroglioma, astrocytoma, and Glioblastoma (GBM) (Louis *et al.*, 2016). A fundamental feature of glioma is their cellular diversity, often reflecting pathological analogues of the normal tissue. In this cellular heterogeneity, the question of the cellular origin of gliomas remains largely unresolved. Current evidence indicates that NSCs (Lathia *et al.*, 2015) as well as differentiated cells, such as astrocytes and even neurons, can give rise to gliomas (Friedmann-Morvinski *et al.*, 2012). For the latter, it has been demonstrated that differentiated astrocytes transduced with oncogenic lentiviral vectors



are able to give rise to malignant gliomas that match a GBM subtype. Under these conditions, astrocytes enter a reprogramming mode, dedifferentiate and generate tumors that present a progressive loss of *GFAP* expression and are positive for several progenitor/stem cell markers including nestin and SOX2 (Friedmann-Morvinski *et al.*, 2012). This is reminiscent of the high plasticity of reactive astrocytes under CNS insults. Likewise, following particular cues from the local microenvironment or genetic alteration, mature astrocytes dedifferentiate and obtain characteristics of neural precursor cells which can maintain their pluripotency and initiate tumorigenesis. A second study reported that the transduction of primary human astrocytes with lentiviral vectors expressing four defined genetic factors (Myc, Oct-4, p53DD and Ras) induced efficient generation of malignant cells with powerful tumor-initiating capabilities (Li *et al.*, 2016). Indeed, when transplanted into immunodeficient mice, these transduced cells were sufficient to induce tumor formation, showed unlimited self-renewal and expressed typical glioma stem-like cell markers. *In vitro* cultivation of the transformed astrocytes revealed their potential to form spheres and differentiate into neuron-, astrocyte- and oligodendrocyte-like cells. The observation that reactive astrocytes are able to share common progenitor markers might explain why, under specific conditions, astrocytes may encompass the starting point of brain tumor formation, further supporting the concept that a fine line separate the dedifferentiated astrocytic fate towards reprogramming or tumorigenesis.

In line with the loss of *GFAP* expression by dedifferentiated astrocytes, *GFAP* expression is usually silenced in a progressive manner with increasing grade of astrocytoma (Restrepo *et al.*, 2011). The fact that no *GFAP* mutations or major DNA rearrangements or deletions are detected in human glioma, prompted investigation of the role of epigenetic mechanisms in the loss of *GFAP* expression. Aberrant DNA methylation was detected in the promoter region of *GFAP* leading to

its downregulation (Restrepo *et al.*, 2011). In contrast to *GFAP* silencing, *DNMT1* and *DNMT3B* were found to be overexpressed in GBM. Specifically, the *DNMT1* promoter was enriched with active chromatin marks, including acetylated histones H3, H4 and H3K4me2, in GBM patients and cell lines. Conversely, normal brain tissue exhibited enrichment of the repressive histone modifications H3K9me2 and H3K27me3. Furthermore, the *DNMT3B* promoter was hypomethylated in the same tissue and overexpression of these DNMTs led to inactivation of the tumor suppressor genes *p21* and *PTEN* (Rajendran *et al.*, 2011). Promoters of additional tumor suppressor genes including *p16/CDKN2A* (Costello *et al.*, 1996), *RBI* (Nakamura *et al.*, 2001), *p14(ARF)* (Yin *et al.*, 2002), *PTEN* (Baeza *et al.*, 2003), *MGMT* (Blanc *et al.*, 2004), *RASSF1A* (Gao *et al.*, 2004), *p73* (Yu *et al.*, 2004) were all shown to be hypermethylated and silenced in gliomas, thus resulting in the deregulation of multiple signaling pathways responsible for cell growth and apoptosis.

### 6.1 The importance of *IDH* genes

Integrated genomic analysis of human GBM samples revealed recurrent mutations in the active site of isocitrate dehydrogenase 1 gene (*IDH1*) in a small number of GBM patients (Parsons *et al.*, 2008), that later turned out to largely represent secondary GBM. Mutations in *IDH2* gene have also been identified in gliomas (Yan *et al.*, 2009). Under normal conditions, these enzymes catalyze the oxidative carboxylation of isocitrate to  $\alpha$ -ketoglutarate, resulting in the production of NADPH. Glioma bearing mutated *IDH1* or *IDH2* display a glioma-CpG island methylator phenotype (G-CIMP) and produce high levels of D-2-hydroxyglutarate, proposed to inhibit several histone and DNA (de)methylases, including the TET enzymes (Chowdhury *et al.*, 2011; Xu *et al.*, 2011). Almost all mutations in *IDH1* in glioma occur at the amino acid residue 132 or the

analogous residue 172 in *IDH2*, with the vast majority presenting a substitution of arginine with histidine (R132H) (Balss *et al.*, 2008). Introduction of this single point mutation in immortalized human astrocytes was sufficient for the alteration of specific histone marks and for inducing DNA hypermethylation, influencing the expression of approximately 600 genes (Turcan *et al.*, 2012), although hypermethylation alone was not sufficient to induce tumorigenesis. Recently, the same research group identified enrichment of the active histone mark H3K4me3, the repressive histone marks H3K9me3, H4K20me3 and H3K36me3, which prevent intragenic cryptic transcript initiation in these cells. One gene that showed increased H3K4me3 was *PDGFRA*, already linked to gliomagenesis (Turcan *et al.*, 2018). Taken together, these results show that *IDH1* R132H mutation is able to reshape the chromatin state and transcriptome, however how this contributes to gliomagenesis remains to be determined.

## 6.2 The methylation phenotype of brain tumors

As indicated above, the IDH mutation is associated with a collective hypermethylation at a large number of loci signifying a G-CIMP (Noushmehr *et al.*, 2010).

Another critical epigenetic mark in glioma is the DNA repair enzyme O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT), which repairs the naturally occurring mutagenic O<sup>6</sup>-methylguanine to guanine, thereby protecting against mutagenesis and malignant transformation. The *MGMT* promoter is frequently methylated in GBM patients, leading to gene silencing, which has been related with increased sensitivity to alkylating agents used for cancer therapy in patients with GBM (Weller *et al.*, 2010). A meta-analysis study conducted in GBM patients showed that MGMT promoter methylation was associated with better progression-free survival and overall

survival in these patients regardless of the fact of having received any therapeutic treatment and linked to longer overall survival in GBM patients treated with alkylating agents (Zhang *et al.*, 2013). Therefore, the prognostic and predictive value of MGMT promoter methylation in GBM patients is highly relevant.

Analysis of diffuse glioma samples using different omics technologies including DNA methylation profiling, identified six distinct methylation clusters allowing the most robust and relevant distinction (Ceccarelli *et al.*, 2016). A recent study suggests a new DNA methylation-based classification of CNS tumors, offering a novel approach to tumor classification and diagnosis precision, further strengthening the importance of comprehending and assessing the cancer methylome and thus the epigenetic mechanisms that contribute to tumorigenesis (Capper *et al.*, 2018). Generally, global DNA hypomethylation is common in cancer, including gliomas, and it is estimated to influence 10 million CpG dinucleotides per haploid tumor genome (Cadieux *et al.*, 2006). Demethylation was described to mainly occur in satellite sequences and pericentromeric regions promoting genomic instability and reactivation of transposable elements, thereby leading to tumorigenesis through activation of oncogenes (Cadieux *et al.*, 2006).

### **6.3 Histone changes linked to Glioblastoma**

Pediatric GBM as well as a subtype of adult GBM are characterized by the presence of somatic histone mutations. Two single point mutations have been described in the genes encoding the H3.3 (*H3F3A*) and H3.1 (*HIST1H3B*, *HIST1H3C*) histone variants. These mutations result in the substitution of lysine-to-methionine at position 27 (K27M) and glycine-to-arginine or -valine at position 34 (G34R/V) (Jones *et al.*, 2017; Williams *et al.*, 2017). It was reported that aberrant binding of mutant K27M to EZH2 inhibits the enzymatic activity of Polycomb repressive complex

2 which is needed to maintain gene repression. In addition, K27M mutation leads to a global reduction of H3K27me3 levels and to global DNA hypomethylation, events that drive gene expression, including those involved in neuronal differentiation and gliomagenesis (Bender *et al.*, 2013; Lewis *et al.*, 2013; Venneti *et al.*, 2013). However, a substantial gain of H3K27me3 and EZH2 at specific gene loci has also been reported, which can be advantageous for tumor cells if the silenced gene is a tumor suppressor as has been described for *p16/CDKN2A* (Chan *et al.*, 2013).

Comparative analysis of the chromatin state in GBM stem-like cells (GSCs) versus more differentiated tumor cells and nonmalignant neural cells revealed a prevalence of gene activation. Repressed promoters in human astrocytes lose the H3K27me3 mark in GSCs and become activated. Among the upregulated genes, a large set of developmental transcription factors (TFs) was activated in GSCs. In particular, ASCL1, a member of the bHLH family and known to play a role in neuronal commitment and differentiation, was found to directly activate Wnt signaling and to be necessary for GSCs maintenance and their tumorigenicity *in vivo* (Rheinbay *et al.*, 2013). The Wnt pathway has long been associated with oncogenesis (Polakis, 2007) and is also essential for maintaining NSCs in a self-renewing state (Kalani *et al.*, 2008). Notably, ASCL1 is important for Müller glia activation, cells essential for retina regeneration in fish. LIN28, a downstream ASCL1 effector, decreases let-7 miRNA levels enabling expression of the pluripotency genes, *KLF-4*, *OCT4* and *MYC* (Alunni and Bally-Cuif, 2016). The same LIN28/let-7 regulatory loop seems to be active in cancer stem cells, regulating their properties and contributing to oncogenesis (Chien *et al.*, 2015; Sun *et al.*, 2016), revealing how some molecular mechanisms are shared during development and tumorigenesis.

#### 6.4 MiRNAs associated with Glioblastoma

A systematic review reported that 253 miRNAs were found to be significantly upregulated and 95 miRNAs downregulated in GBM (Moller *et al.*, 2013). Target genes of these miRNAs are implicated in many cancer-associated processes, such as cell proliferation, cell cycle regulation, apoptosis, autophagy, drug resistance, angiogenesis, invasion and metastasis (Banelli *et al.*, 2017; Luo *et al.*, 2015).

For example, a significant decrease in the levels of miR-145 is not only observed in reactive astrocytes (see above), but also in astrocytic tumors, and it is correlated with poor prognosis in GBM patients (Lee *et al.*, 2013a) and also inhibits glioma cell migration and self-renewal (Lee *et al.*, 2013a; Lee *et al.*, 2013b). The latter is consistent with experiments in hESCs where increased levels of miR-145 inhibit self-renewal (Xu *et al.*, 2009). In addition, *OCT4*, a miR-145 target, is expressed in human gliomas and OCT4 protein levels correlate with increasing glioma grade (Du *et al.*, 2009).

In addition to its role in regulating astrocytic responses following SCI (see above), elevated levels of miR-21 were found in various types of cancer, including GBM (Akers *et al.*, 2013). MiR-21 promotion of cancer growth (Chung *et al.*, 2013; Yang *et al.*, 2014) depends on STAT3 (Loffler *et al.*, 2007). Could miR-21 represent a link in the transition from reactive astrogliosis to brain tumorigenesis? Reactive astrocytes might upregulate miR-21, thereby acquiring persistent stem cell characteristics leading to uncontrolled cell proliferation, growth and ultimately resulting in malignancy. The identification of molecular pathways which contribute to the transition from reactive astrocytes to neoplastic cells may pave the way to the development of anti-tumorigenic therapies.

## 7. Conclusions and perspectives

Astrocytes are implicated in the orchestration of neural development and constitute essential regulators of brain functions both in health and disease. Coordinated astrocyte differentiation is critical during development, and dysfunction of astrocytes can lead to several brain disorders. In the presence of a CNS insult, astrocytes respond by altering their morphology and molecular profile. Notably, they are able to be reprogrammed to a stem cell-like state permitting their eventual transdifferentiation into neurons or oligodendrocytes, which might be harnessed to restore brain damage. These processes rely on widespread transcriptional and epigenetic modifications that act in a highly coordinated manner leading to a fine balance between a healthy and pathological state.

So far, the (de)differentiation potential of reactive astrocytes has been mainly characterized *in vitro*, based on experimental tractability and the ease of monitoring the role of specific growth factors favoring commitment to specific lineages, a task that is much more difficult within the complex cellular milieu of the brain. However, the capacity of astrocytes *per se* to dedifferentiate holds significant promise in developing endogenous brain repair strategies. Studying astrocyte plasticity and exploring the means that will allow such a switch of cellular fate *in vivo*, will provide new opportunities to implement novel endogenous therapeutic approaches. The ease of manipulation of 2D astrocyte cultures has provided the first vital mechanistic insight into their plasticity, but more complex cell systems that better recapitulate the cellular complexity of the brain will be necessary to fully understand the intricate relationship between signaling, transcriptome and epigenome that ultimately drive their phenotypic response *in vivo*. For example, 3D cell culture using microfluidic devices or brain organoids may represent a valuable compromise

between *in vitro* and *in vivo* approaches, which will allow us to study the effect of other CNS cell types on astrocyte programs. Using these systems, it will be possible to study transcriptional and epigenetic changes in astrocytes to analyze, for example, the effect of familial AD or PD mutations. Screening compounds in a 3D system that show a specificity for differentially expressed epigenetic modulators in astrocytes may allow us to identify promising therapeutic treatments.

Apart from the identification of epigenetic drugs which usually shows a lack of selectivity, another arising and promising approach is the targeted epigenome editing that enables a precise alteration in the epigenome. For example, designer methyltransferases can be constructed to achieve targeted methylation of a gene promoter (Siddique *et al.*, 2013). Furthermore, by using the deactivated Cas9 (dCas9) nuclease in combination with the catalytic domain of a DNMT, it is possible to enable targeted CpG methylation of single or even multiple CpG sites, with the DNA methylation activity being heritable across mitotic divisions (Vojta *et al.*, 2016). On the other hand, the dCas9 system can be used for targeted DNA demethylation of specific genomic loci (Xu *et al.*, 2016). In this way, the induced methylation/demethylation silences or activates, respectively, the expression of the target gene and may represent a potential locus-specific therapeutic strategy of the epigenome. For example, dCas9:Tet1 was recently used to target demethylation of a specific STAT3-binding site upstream of the *Gfap* promoter resulting in decreased methylation of the promoter and flanking region accompanied by increased *Gfap* expression (Morita *et al.*, 2016).

Another critical, and often underappreciated feature of astrocytes is their heterogeneity. Due to their multiple roles in the CNS it would not be surprising to uncover the existence of several astrocytic subpopulations, each displaying specific properties and functions. To fully understand the extent, studies including transcriptomics combined with chromatin accessibility and proteomics analyses at single cell resolution will be crucial. In fact, methods that utilize average



responses of a cell population are not able to detect subtle differences across individual cells. Applying single-cell techniques, for example in homeostatic and reactive astrocytes, will provide pivotal information on their cellular state under specific conditions. In addition, single-cell analyses will highlight any heterogeneity within the different brain regions and reveal the existence of possible astrocytic subpopulations. Such data will allow to infer the functional states of the identified cellular diversity and to understand the fate of specific subpopulation of astrocytes under homeostatic and pathological conditions. Such analyses are beginning to emerge and, for example, single-cell transcriptomic analysis of the adult NSC lineage revealed their existence on a continuum through the processes of activation and differentiation (Dulken *et al.*, 2017).

In the last few years, more consideration has been given to the roles and potential therapeutic contribution of non-coding RNAs. For example, the long non-coding RNA (lncRNA) MALAT1, secreted by transplanted adipose-derived stem cells, has been shown to exert neuroprotective effects and ameliorate motor and cognitive impairments in a TBI mouse model. In the absence of MALAT1, both motor and cognitive improvements were prevented and neuronal loss was not reduced (Tajiri *et al.*, 2014). Transcriptional analyses of the brain tissue in a TBI rat model revealed that inflammatory, cell cycle, cell death, and regenerative molecular pathways are dependent on MALAT1 modulation, reinforcing the therapeutic potential of this lncRNA (Patel *et al.*, 2018). Similar studies conducted in NSCs or astrocytes may reveal important aspects of the lncRNAs in these cells unveiling potential therapeutic applications of these molecules.

There are clearly many parallels between the signaling, transcriptional and epigenetic mechanisms guiding astrocyte development and those that regulate astrocyte pathology. It is this commonality of mechanism that underwrites the possibility of identifying therapeutic mechanisms in neurodegeneration and tumorigenesis. For the latter, this approach would result particularly

relevant, where the identification of novel developmental factors influencing the molecular and cellular astrocytic identities would enable adopting new approaches in the context of the differentiation therapy (Laug *et al.*, 2018), as attempts towards this direction have not been prosperous so far. Efforts to induce cell cycle arrest and differentiation of GSCs conducted *in vitro*, revealed that only a subset of GSCs responded the desired way. The cells still remained vulnerable to re-enter cell cycle and dedifferentiate as the differentiation-accompanied epigenetic modifications were not complete (Caren *et al.*, 2015). Therefore, caution must be exerted since these overtly differentiated cells still retain the potential to re-acquire their old identity and become once more tumorigenic. Single cell genetic fate mapping of the differentiated GSCs will allow to identify which of these cells truly altered their epigenome and gene expression profile towards a non-proliferating and mature cell type. In this way, it may be possible to select specific subpopulations for further usage in therapeutic applications. From a different perspective, genetically engineered human NSCs have been successfully transplanted into mice bearing brain metastases from lung cancer where they were able to target and inhibit tumor growth. In this study, the NSCs were essentially acting as transient drug delivery vehicles, by converting a pro-drug into its cytotoxic form that preferentially targeted dividing cells and then undergoing programmed cell death (Hong *et al.*, 2013). Thus, NSC strategies employed to arrest aberrant cell proliferation could also be considered in brain tumor therapies.

Although an increasing number of studies implicate specific epigenetic events with gliomagenesis, further research is needed to fully comprehend their role in tumor initiation, progression and recurrence. In this way, biomarkers of diagnostic and prognostic value can be identified together with derivation of new tools to manipulate the epigenome of the patients potentially opening new avenues for GBM treatment. Pharmacological intervention on DNA

methylation and histone acetylation has been broadly exploited in tumor biology with some compounds showing promise as tools to combat cancer. However, only few are approved by the US FDA and they have serious limitations. For example, HDAC inhibitors target only a small percentage of all acetylation sites, indicating that there is still room for improvement and large-scale screenings and trials will be needed to identify novel lead compounds. The construction of target specific drugs, i.e. intervening with a unique acetylation, methylation event or selective inhibition of specific HDACs and DNMTs is a highly demanding task but, if successful, it will reduce the side effects that occur with global inhibition of these epigenetic modulators. Identification of the genome-wide DNA methylation profile of patients with glioma tumors is becoming relatively straightforward (Capper *et al.*, 2018). Characterizing the methylome of these patients may enable physicians to utilize personalized medicine approaches and make informed choices on the potential efficacy of therapeutic agents that target DNA methylation. Pharmacoeugenomics is an emerging field that makes use of epigenetic processes that underpin CNS diseases. The ability to use individuals' epigenetic profiles in order to identify sensitivity or resistance to existing drugs, represents a valuable tool in the emerging field of personalized medicine. In addition, these advances allow a theranostic strategy whereby changes in the epigenome can be monitored during the course of treatment to provide a dynamic biomarker of drug efficiency and disease progression.

Taken together, it is evident that, in astrocytes, epigenetic mechanisms, such as DNA methylation and histone modifications, are extremely complex and their causative role in pathological conditions is still a matter of investigation. However, it is clear that a deep molecular understanding of their mechanism of action will bridge the gap between extrinsic and intrinsic factors that drive change of astrocytes identity as well as to shed light on their involvement in brain

disease. Understanding how these various mechanisms eventually alter gene expression and define or re-define astrocytic cell fate is critically important. Not only will it unveil neurodevelopmental mechanisms, but it will also provide the knowledge base to exploit these processes to manipulate astrocytes *in vivo*, thereby paving the way to novel therapies for multiple CNS disorders.

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**Figure legends**

**Figure 1: Depiction of key epigenetic mechanisms.** DNA methylation conducted by DNMTs results in the inability of transcription factors to bind to gene promoter regions or in the recruitment of repressor proteins. Both events contribute to gene repression. Conversely, DNA demethylation catalyzed by demethylases allows gene transcription. The major histone modifications include methylation (Me), acetylation (Ac), phosphorylation (P), ubiquitination (Ub) and sumoylation (S), influencing chromatin condensation and gene transcription both positively and negatively depending on the histone marks. Targeted mRNAs are recognized by miRNAs via sequence complementarity. The final result of this interaction involves the degradation of the mRNA target or the repression of its translation.

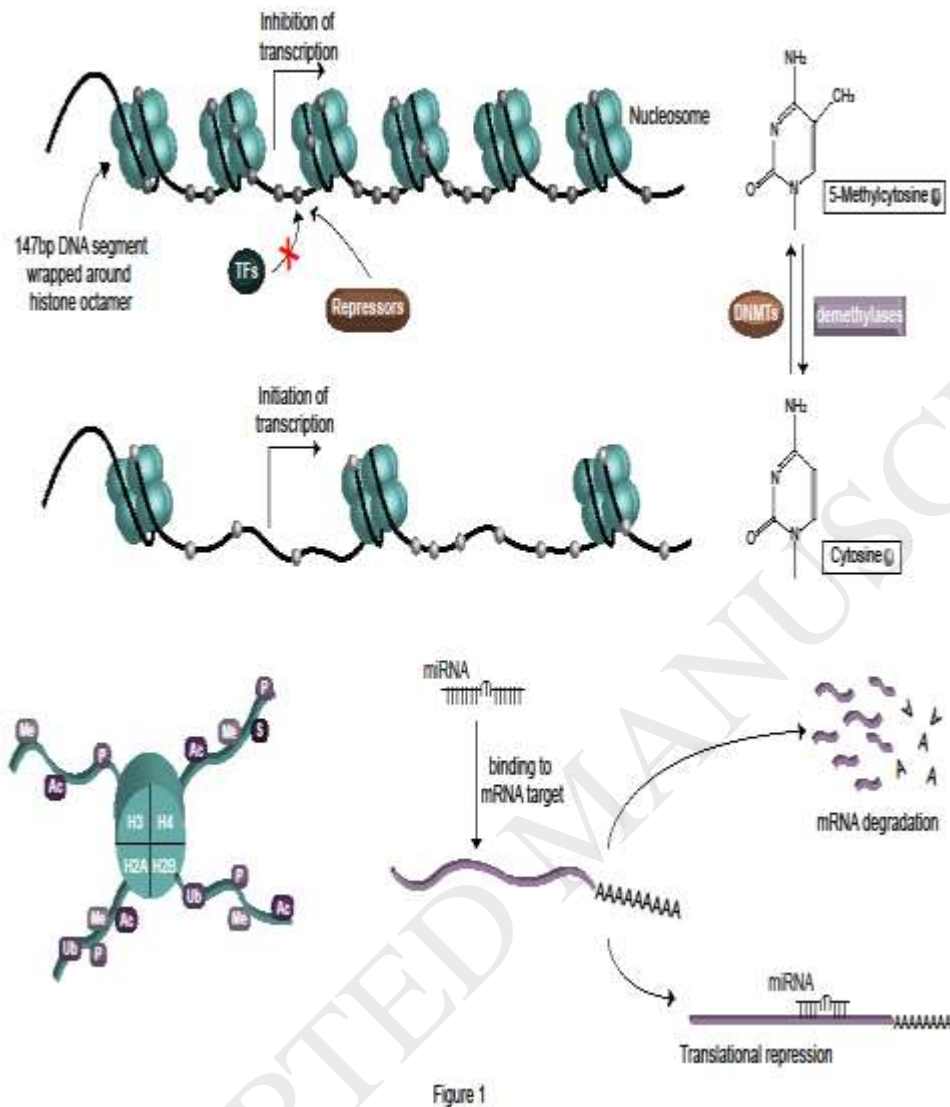


Figure 1

**Figure 2: Examples of major transcriptional and epigenetic events driving gene expression of two main astrocytic markers, *GFAP* and *S100b*.** In NSCs, *GFAP* promoter is highly methylated due to DNMT1 activity. In addition, ESET protein is responsible for the elevated trimethylation marks of histone H3K9. Also, the dimer RAR/RXR forms a complex with various co-repressors recruiting HDACs which maintain a closed chromatin structure, thus leading to the repression of *GFAP* expression. During late-gestation, Notch ligands expressed in cells determined

to become neurons bind to Notch protein of their neighboring NSCs. This binding induces proteolytic cleavage and release of the NICD, which enters the nucleus and binds to RBP-JK. The NICD/RBP-JK complex induces the expression of *NFIA*. MiR-153 targets *NFIA* mRNA inhibiting its translation. *NFIA* is able to bind to the *GFAP* promoter resulting in the removal of methyl groups (CH<sub>3</sub>) and in the dissociation of DNMT1. Further, ESET protein levels are reduced leading to decreased H3K9me<sub>3</sub>. Lastly, binding of RA to RAR/RXR dimer attracts transcriptional co-activators, allowing the chromatin to obtain a relaxed structure through acetylation of histone H3. Upon these conformational changes, STAT3 is able to bind to the *GFAP* promoter recruiting the transcriptional co-activators CBP/p300. FGF2 additionally facilitates access of the STAT/CBP to the *GFAP* promoter, while also increases and decreases H3K4 methylation (H3K4met) and H3K9 methylation (H3K9met) respectively. As a result, *GFAP* expression is induced and astroglial differentiation commences. Similarly to *GFAP*, the expression *S100B* is also repressed in early brain development. This is due to promoter methylation and to the binding of MeCP2, which recruits HDACs and co-repressors to the promoter. In later stages, along astrocytic differentiation, promoter demethylation and decreased MeCP2 levels lead to the induction of *S100B* expression.

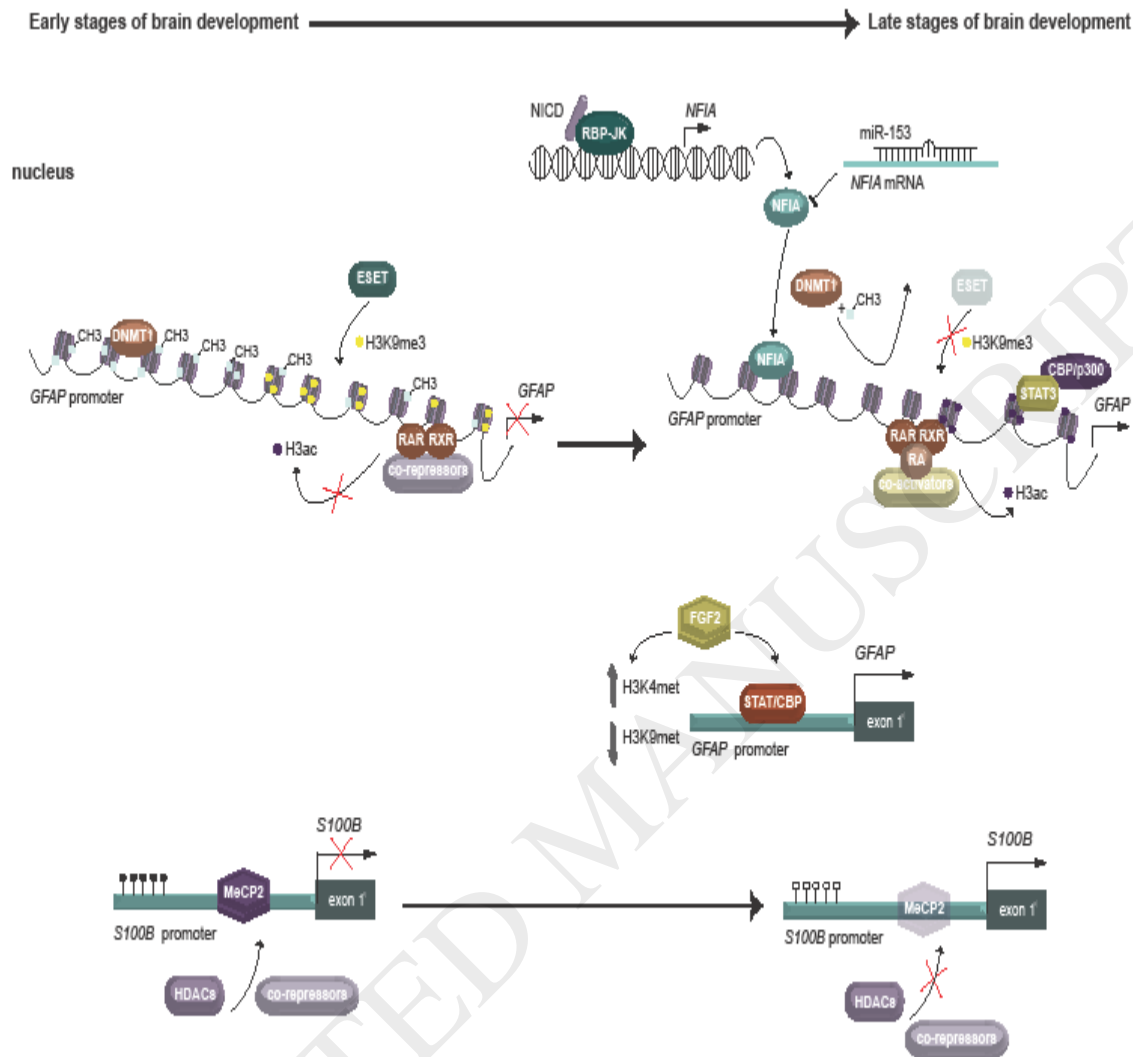


Figure 2

**Figure 3: Schematic illustration of specific transcriptional and epigenetic alterations during reactive astrogliosis.** Under inflammatory conditions or in the presence of a lesion, several signaling pathways are activated. Binding of TNF to the cognate receptor allows the dissociation and degradation of I $\kappa$ B from the I $\kappa$ B/NF- $\kappa$ B complex. NF- $\kappa$ B is then released and translocates into the nucleus, where it will activate the transcription of key reactive astrocytic markers, such as

*LCN2*. Activation of the JAK/STAT pathway enables the phosphorylation of STAT3 and its subsequent translocation to the nucleus, which will induce the transcription of *GFAP*, *LCN2* and potentially of *miR-21* as well. In turn, *LCN2* is able to further activate the JAK/STAT and NF- $\kappa$ B signaling pathways stimulating chemokine production and astrocyte migration. In addition, the JNK and MAPK/ERK pathways are activated and implicated in the induction of *GFAP* expression. MiR-21 levels are elevated in SCI and positively influence GFAP protein levels. In differentiated cells, the pluripotency factors OCT4, SOX2 and KLF-4 are not present due to miR-145 activity. However, reduced miR-145 levels in SCI may potentially reactivate the expression of these proteins, together with the translational activation of the suggested *GFAP* and *MYC* mRNA targets. In this way, reactive astrocytes obtain stem cell hallmarks. In addition, miR-181 is decreased upon inflammation and is no longer able to target and control the translation of *MECP2* mRNA. Under physiological conditions, miR-140 inhibits BDNF translation and cell proliferation participating in astrocyte differentiation. However, inflammation results in miR-140 decrease and is associated with cell proliferation as well as acquisition of NSC characteristics.



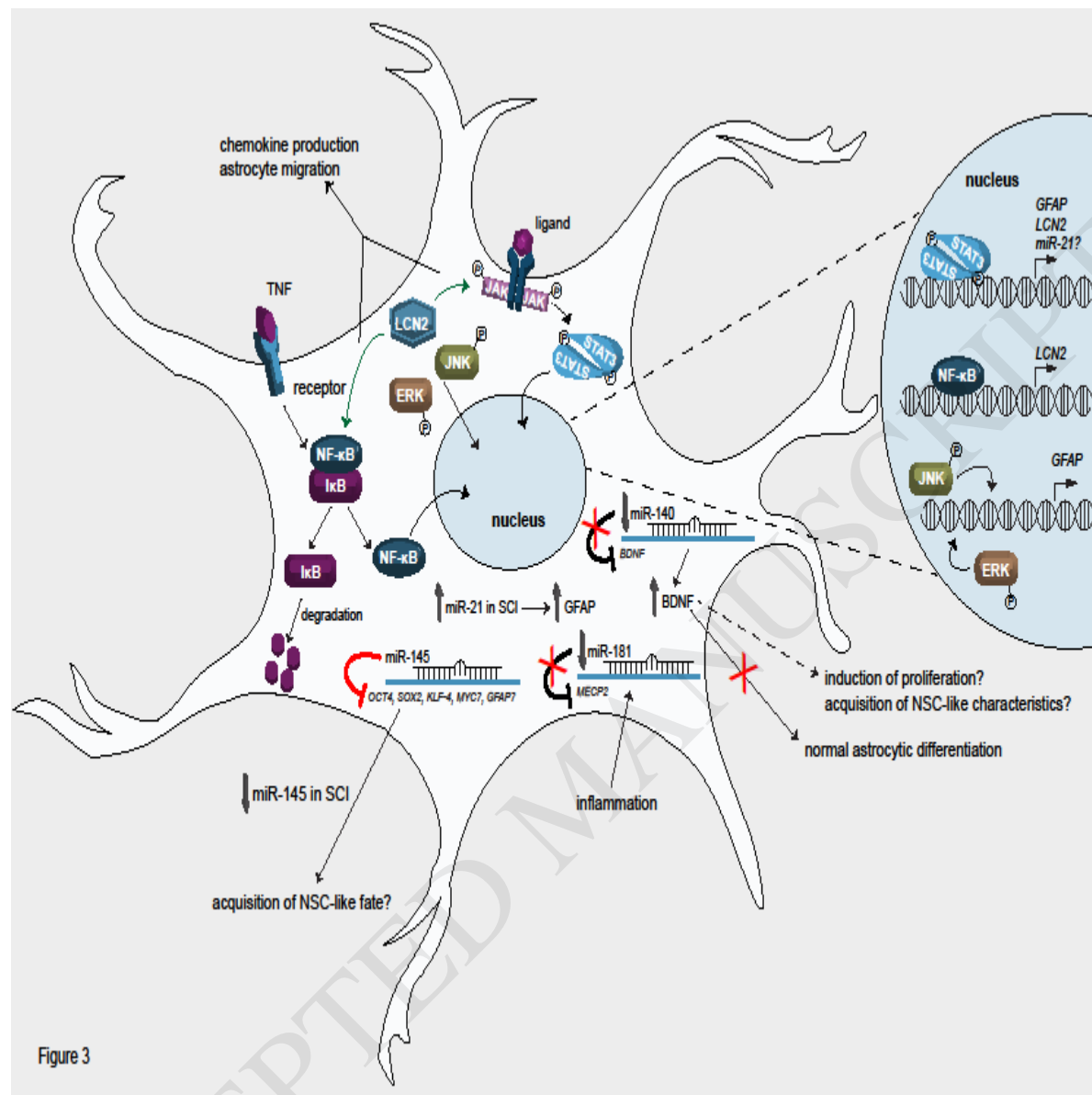


Figure 3