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Developmental control of cortico-cerebral astrogenesis

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ABSTRACT A remarkable body of research over the last 15 years has been aimed at disentangling the cellular and molecular mechanisms which regulate murine cortico-cerebral astrogenesis. This research effort has allowed the reconstruction of the actual sizing of this process, as well as a better definition of its temporal, spatial and clonal articulation. Moreover, these investigations have shed substantial light on the cardinal molecular mechanisms governing the transition from pallial neuronogenesis to astrogenesis, as well as subsequent progress of the latter. It has turned out that proper temporal articulation of astrogenesis relies on a plethora of tightly interlaced mechanisms, which synergistically dampen astrogenesis prior to birth and promote it during peri- and postnatal life. The aim of this review is to provide a comprehensive and organic synthesis of these mechanisms, as well as a critical evaluation of their specific relevance to proper articulation of cerebral cortex astrogenesis in time and space.

KEY WORDS: *cerebral cortex*, *astrocyte*, *development*, *molecular mechanism*, *chromatin*

Introduction

Albeit less numerous than reported in old literature (Hilgetag and Barbas, 2009), astrocytes form a remarkable fraction of the cortico-cerebral neural complement. Non-neuronal cells numbers are an approximately linear function of brain mass, which conversely correlates with neuronal numbers according to a power law (the scale factor, depending on the CNS district and the genus the animal belongs to, equals 1.7 and 1.1 circa, in rodents and primates cerebral cortex, respectively) (Herculano-Houzel, 2012). Thus, non-neuronal cells are 47% and 79% of total cortico-cerebral cells, in mice and humans, respectively (Azevedo *et al*., 2009; Herculano-Houzel *et al*., 2011), resulting less abundant in grey compared to white matter (in humans 58% vs 94%, respectively (Azevedo *et al*., 2009)). Astrocytes - as documented in the rodent neocortex (Irintchev, 2004) - amount to slightly less than half of these non-neuronal cells.

Astrocytes play a large variety of roles, in cortico-cerebral development, physiology and pathology (Wang and Bordey, 2008). They shape the morphology of neuronal dendrites (Ballas *et al*., 2009; Jacobs *et al*., 2010) and assist migration of some neuronal progenitors (Kaneko *et al*., 2010). They contribute to genesis and function of the blood-brain barrier (Tao-Cheng *et al*., 1987; Abbott *et al*., 2006; Alvarez *et al*., 2011; Bozoyan *et al*., 2012), provide structural and metabolic support to neurons (Allen and Barres, 2009; Bélanger *et al*., 2011; Prebil *et al*., 2011), and modulate synaptic transmission and information processing (Nedergaard *et al*., 2003; Eroglu and Barres, 2010; Sasaki *et al*., 2011; Min and Nevian, 2012). Finally, they react to pathological conditions, by upregulating specific gene products (intermediate filament glial librillary acidic protein (Gfap) and inhibitory extracellular matrix (ECM) proteins among them), and demarcate the damaged site from surrounding tissue with an ECM-rich scar, poorly permissive for axonal regeneration (Silver and Miller, 2004; Schachtrup *et al*., 2010, 2011).

Spatio-temporal and clonal architecture of the cortico-cerebral astrogenic compartment

Like elsewhere in CNS, even within the developing cerebral cortex astrocytes are mainly generated after neurons and before oligodendrocytes. Classical birthdating experiments showed that mouse astrogenesis initiates in the last third of the prenatal neuronogenic window, at around embryonic day 15 (E15). Astrocyte birthrates arise abruptly after neuronogenesis completion, peaking around postnatal day 3 (P3) (Ge *et al*., 2012). Then, they smoothly decrease. Consistently, Gfap$^+$ astroglial elements, just detectable around E16 and still rare at birth (< 2.0%), double every 3-4 days in the first postnatal week (Qian *et al*., 2000), and reach their ab-

Abbreviations used in this paper: BS, binding site; ChIP, chromatin immunoprecipitation; CpG, CG dinucleotide; GOF, gain of function; LOF, loss of function; RE, responsive element; NSC, neural stem cell; TF, transcription factor; TSS, transcriptional start site.

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solute plateau around P21 (Bandeira *et al.*, 2009).

Regional articulation of cortico-cerebral astrogenic matrices is relatively simple, as compared to neuronogenic and oligodendrogenic ones. Cortical neurons are generated in as many as seven tangentially distinct structures: pallium, generating the majority of glutamatergic neurons; cortical hem, cortical antihem and septum, generating pioneer glutamatergic neurons of Cajal-Retzius; medial-, lateral- and caudal ganglionic eminences (MGE, LGE and CGE), generating murine gabaergic interneurons (Guillemot, 2005). Cortico-cerebral oligodendrocytes are also born in distinct birthplaces: MGE, LGE, pallium and, apparently, thalamus (Kessaris *et al.*, 2006). Conversely, all cortico-cerebral astrocytes exclusively derive from pallial precursors, as robustly demonstrated by cre/loxP-mediated labelling, driven by the *Emx1* promoter (Gorski *et al.*, 2002; Tsai *et al.*, 2012). Moreover, clonally-related astrocytes share their specific areal location. They may be found clustered along abventricular processes of remants of radial glia from which they presumably originated (Tsai *et al.*, 2012) or adjacent to apical (i.e. abventricular) dendrites of isoclonal projection neurons (Magavi *et al.*, 2012). That was demonstrated by injecting little amounts of cre-encoding adenoviruses into P1 Z/EG reporter mice and immunoprofiling them at P4-P28 (Tsai *et al.*, 2012), or examining cortical development in transgenic mice in which a random and sparse subset of neural progenitors undergoes CRE/lox recombination, so permanently labeling their progeny (Magavi *et al.*, 2012).

Concerning clonal topology of the astrogenic compartment, it was addressed in vivo, at a variety of developmental stages, by labelling periventricular neural precursors with diluted tracer-viruses (retroviruses or retroviral libraries, both selectively infecting intermitotic elements) and immunotyping the resulting clones. Depending on cases, clones were singled out on the basis of geometrical vicinity among their components (case tracer-retroviruses) or based on sharing of the same molecular tag, regardless of distance (that is the case of libraries). Precursors with mixed neuronogenic and gliogenic potencies resulted to be frequent in the early preneuronogenic forebrain (McCarthy *et al.*, 2001), more rare (circa 10%) in the rat E15 cortex (Walsh and Cepko, 1992, 1993; Reid *et al.*, 1995). Glial clones containing both astrocytes and oligodendrocytes were found upon retroviral infection of the neonatal rat SVZ (at frequency of 15%) (Levison and Goldman, 1993), but even in mouse E9.5 forebrains (albeit at frequency of only 5%) (McCarthy *et al.*, 2001). Finally, purely neuronal, astrocytic or oligodendrocytic clones were detected at high frequency when retroviruses were injected into the rat E15-16 cortex (Grove *et al.*, 1993; Luskin *et al.*, 1993) or the neonatal cortex (Luskin and McDermott, 1994). These data are consistent with a model characterized by a progressive restriction of histogenetic potencies displayed by proliferating precursors, from mixed neuronal-glial, through glial, to purely astrogenic or oligogenic. However, such progression seems to be not tightly synchronized among clones. In particular, the appearence of committed elements with restricted potencies may predate quite a lot the birth of their ultimate postmitotic progenies. Conversely, some precursors may retain their tripotency until relatively late developmental stages.

Concerning immunological identification of distinct astrogenic precursors, specific molecular markers are available, largely used in developmental studies. The A2B5+PSANCAM- antigenic profile has been specifically associated to bipotent glial progenitors, both rhombo-spinal and telencephalic, endowed with mixed astrogenic and oligogenic properties (Rao *et al.*, 1998; Han *et al.*, 2004; Strathmann *et al.*, 2007). NG2$^+$ cells may act as progenitors of oligodendrocytes and paleocortical grey matter astrocytes (Zhu *et al.*, 2007). Cluster of differentiation 44 antigen (CD44) was described in unipotent astrogenic progenitors (Liu *et al.*, 2004). Aldolase C (AldoC; Bachoo *et al.,* 2004) and aldehyde dehydrogenase 1 family, member L1 (Aldh1L1; Cahoy *et al.*, 2008) label all astrocytes, Gfap and S100b are preferentially associated to protoplasmatic and fibrous astrocytes of grey and white matter, respectively (see ref (Magavi *et al.*, 2012)).

Concerning radial location of the different types of astrogenic precursors, the scenario is quite complex. About 13 year ago, Goetz and coll. unexpectedly found that radial glial cells, previously

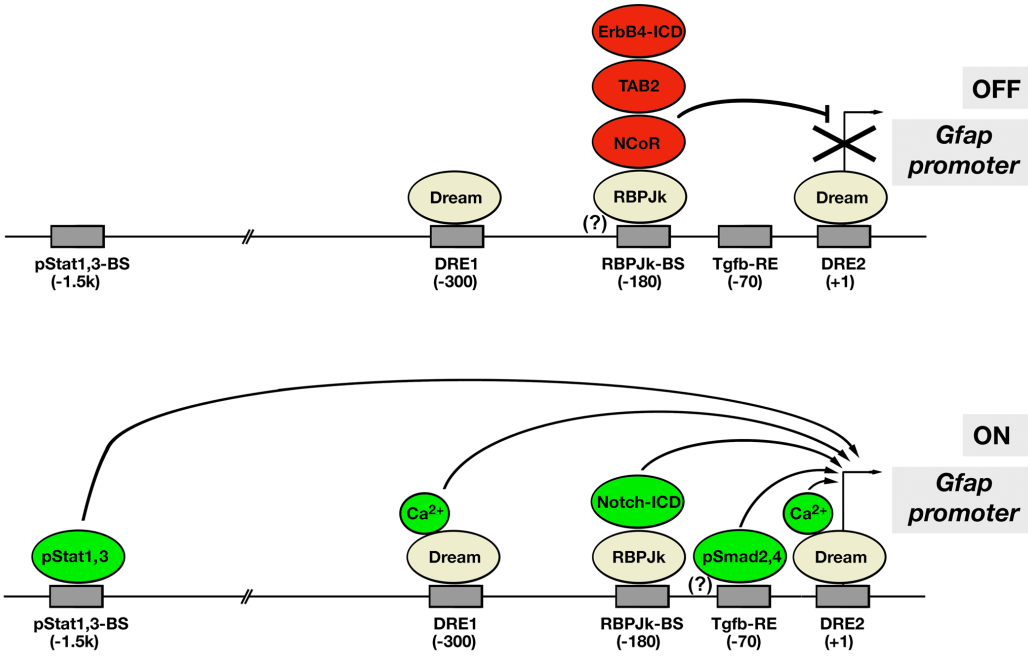

Fig. 1. Transmodulators interacting with the *Gfap* promoter. *Shown are presumptive* Gfap *promoter configurations in OFF and ON states. Approximate positions of cis-active modules within the murine* Gfap *promoter refer to the transcriptional start site. They have been compiled on the basis of pooled mouse and rat data cited in the text, taking into account mouse/rat sequence homologies, as detected by Blat software at http://genome.ucsc.edu. Note that physical interaction between pSmad2,4 and the Tgfβ1-RE has not yet been experimentally proven. Moreover, the quaternary complex sitting on the silent promoter around -180 was simply inferred on the basis of NCoR affinity for both RBPJk and ErbB4-ICD, the latter mediated by a Tab2 bridge (see Text).*

considered as part of the glial lineage and commonly known as guiding cables for migrating neurons, also are neuronal precursors. Moreover, they showed that, only upon neurogenesis completion, radial glia shifts towards generation of astrocytes (Malatesta *et al*., 2000, 2003). These new concepts took quite a long time to be fully metabolized by the scientific community (Malatesta and Götz, 2013). Nowadays they have been widely accepted and early multipotent elements generating both neurons and glia are commonly supposed to coincide with a subset of prenatal radial glia (Kriegstein and Alvarez-Buylla, 2009). Moreover, it has been shown that, after completion of prenatal neuronogenesis, many radial glial cells lose their contract with the ventricular cavity and get straightly transformed in astrocytes (Noctor *et al*., 2004). In addition, radial glia is further believed to generate subventricular precursors (Rakic, 1988) committed to gliogenesis, bipotent or unipotent (Marshall *et al*., 2003), which populate the perinatal and neonatal subventricular zone and supply cerebral cortex with a substantial part of its astrocytic and oligodendrocytic complements (Zerlin *et al*., 1995; Kakita and Goldman, 1999; Kakita *et al*., 2003; Kriegstein and Alvarez-Buylla, 2009). More recently, however, it has been shown that neocortical astrocytes are actually born in different places, depending on their ultimate radial location. When EGFP-encoding plasmids were transfected into SVZ/radial glial cells of P0-P2 mice, by intraventricular DNA injection and electroporation, 16-20 days later, 75% and 22% of labelled astrocytes were found in SVZ/white matter and layers VI/V, respectively, and only 3% in layers IV-I. This suggests that, at P0-P2, progenitors of deeper and more superficial astrocytes are near and far from the ventricle, respectively. On the other hand, injection of high titer EGFP-encoding gammaretroviruses into the cortex of P0-P2 mice was followed, 7-10 days later, by high frequency labelling of astrocytes (almost 50%), regardless of their radial location. This means that, albeit far from the ventricle, the almost totality of layer IV-I astrocytes is generated by local proliferating progenitors (Ge *et al*., 2012). Actually, it was found that they derive from precursors which, still intermitotic, had left periventricular layers and undergone an almost complete astroglial differentiation (astrocyte-like dividing cells, astrolike-D cells). These cells constitute a substantial (and progressively decreasing) fraction of cortico-cerebral astrocytes (from 19% at P3, to 0.3% at P50). They give rise to repeated, symmetric divisions and supply superficial layers of grey matter with the almost totality of their astrocytic complement (Ge *et al*., 2012; Magavi *et al*., 2012).

Intrinsic and extrinsic mechanisms controlling astrocyte fate choice

Two seminal studies published about 12 year ago formally demonstrated that the switch from neuronogenesis to astrogenesis taking place in the rodent cerebral cortex around birth is the result of two key concurrent factors: the advancement of an intrinsic "developmental clock" hardwired in neural multipotent precursors (as proposed by Martin Raff and coll. in mid 80s', for optic nerve oligodendrogenesis (Raff *et al*., 1985; Temple and Raff, 1986)), and the activity of paracrine regulatory signals, impinging on these precursors from their surroundings.

The first study, performed by the Temple group (Qian *et al*., 2000), showed that isolated E10-E11 mouse cortico-cerebral stem cells, grown in Fgf2-containing basal medium, give rise to large clonal trees including both neurons and astrocytes, the latter ones generated 2- 5 generations past the former ones. That means that early cortical stem cells, even in the absence of extrinsic cues, are intrinsically programmed to switch from early neuronogenesis to late astrogliogenesis, according to a biologically plausible timetable.

The second study, performed by the Ghosh group (Morrow *et al*., 2001), conversely showed that proliferating neural precursors obtained by dissociation of mouse E15 cortico-cerebral tissue, give preferentially rise to neurons or astrocytes, depending on the substrate on which they are cultivated, rat E18 and P15 cortical slices, respectively. That was not due to differential survival or expansion of the committed subclones originating from these elements. Rather it reflected a differential commitment of these proliferating precursors to distinct histogenetic pathways. This commitment was induced by the surrounding micro-environment and diffusible substances were apparently responsible for it. That was suggested by the replication of these results in the presence of semipermeable barrier, interposed between the inducer tissue and the induced precursors. However, when P5 mouse precursors were assayed by this test, these cells always differentiated as astrocytes, regardless of the developmental age of the substrate supporting them. This means that, albeit the behaviour of cortical precursors is influenced by the surrounding environment, however their plasticity is limited and their histogenetic properties change irreversibly as development proceeds.

Molecular mechanisms by which intrinsic and extrinsic control of astrogenesis are implemented have been subject of an articulated dissection, still in progress. This dissection has been largely focused on factors and constraints regulating transcription of select genes, such as *Gfap* and *S100β*, chosen for historical and objective reasons as models of astroglia-specific transcription. Consistently with the classical findings of Temple and Ghosh, it emerged that astrocyte-specific gene expression is regulated by specific extracellular ligands secreted by surrounding cells, acting on multipotent neural precursors and modulating their histogenetic properties. Information carried by these ligands is generally conveyed to the nucleus of neural precursors via dedicated receptors and transducers. This ultimately results in differential nuclear availability of transcription factors, which, interacting with chromatin of astroglial genes, may modulate its epigenetic state and regulate its current transcription rate. Collectively, expression levels of these receptors and transducers as well as the epigenetic state of this chromatin dictate precursors ability to respond to astrogenic stimuli, namely the other key determinant of astrogenesis progression.

Transactive pathways modulating astrogenesis

It has been shown that at least 5 main trans-active pathways regulate transcription from astrocytic promoters (*Gfap* and *S100β* the best characterized ones): (1) cardiotrophin 1 (Ct1)/janus kinase 2 (Jak2)/Signal transducer and activator of transcription 1 and 3 (Stat1 and Stat3), (2) Delta/Notch, (3) Neuregulin 1 (Nrg1)/v-erb-a Erythroblastic leukemia viral oncogene homolog B4 (ErbB4), (4) Transforming growth factor β1 (Tgfβ1)/Tgfβ-receptors I and II (TgfβRI and TgfRII)/Small mothers against decapentaplegic homologs 2 and 4 (Smad2 and Smad4), and (5) Pituitary adenylate cyclase-activating polypeptide (Pacap)/Pituitary adenylate cyclase (Pac)/Downstream regulatory element antagonist modulator (Dream) (Fig. 1). These pathways apparently impact astrocytic promoters in direct ways.

Some of them are further provided of "auxiliary branches", finely tuning their activity. The majority of them are functionally interconnected, according to an intricate topology.

Cardiotrophin 1-type ligands released by neurons and astrocytes, signalling via the Jak2/Stat3 axis, are the main promoters of astroglial commitment and astrocytic differentiation (He *et al*., 2005). Nuclear translocation of Notch-IntraCytoplasmic Domain (Notch-ICD), induced by Delta-type signals (from the neuronal lineage), promotes astroglial specification as well (Ge *et al*., 2002). Nuclear translocation of ErbB4-IntraCytoplasmic Domain (ErbB4-ICD), induced by Nrg1 (from the neuronal lineage), conversely antagonizes it (Sardi *et al*., 2006). Finally, further positive inputs to astrogenesis promotion come from firing of the pSmad2,3 pathway, triggered by Tgfβs (from both neuronal and astrocytic lineages) (Stipursky and Gomes, 2007), as well as from Pac/Dream signalling, stimulated by Pacap ligands available within the perinatal periventricular zone (Cebolla *et al*., 2008). An overview of these pathways follows.

The Ct1 /Jak2/Stat3 cardinal pro-astrogenic pathway

A number of IL6-related ligands, often referred to as "astrogenic cytokines" and including Cardiotrophin 1 (Ct1), Leukemia Inhibiting Factor (Lif), Ciliary Neurotrophic Factor (Cntf), Neuropoietin (Np) and Cardiotrophin-like Cytokine (Clc), are able to strongly promote expression of astroglial genes, by acting on cortico-cerebral NSCs and astrogenic progenitors which express the corresponding receptor complex (Bonni *et al*., 1997; Rajan and McKay, 1998; Nakashima *et al*., 1999a; Ochiai *et al*., 2001; Uemura *et al*., 2002; Derouet *et al*., 2004; Barnabé-Heider *et al*., 2005). These astrogenic cytokines bind to their α-coreceptors (including the plasmamembrane-bound ciliary neurotrophic factor receptor α, CntfRα, and its secreted variant sCntfR α) and trigger the heterodimerization of the two β-subunits of their main receptor, glycoprotein 130 (gp130) and Lif-receptor β (LifR β)". Such β-subunits contain one, plasmamembrane-proximal suppressor of high-copy PP1 protein 2 (Shp2)-binding site (YxxV) as well as several, membrane-distal Signal transducer and activator of transcription 1 and 3 (Stat1/3)-binding sites (YxxQ). Ligand-induced heterodimerization of β-subunits is followed by their multiple Y-phosphorylation as well as by further Y-phosphorylation of the gp130/LifRb-associated signalling mediators Stat1,3 and Shp2 (reviewed in ref (Ernst and Jenkins, 2004)). All these Y-phosphorylations are catalyzed by Janus tyrosine kinases 1 and 2 (Jak1,2), which are constitutively associated to gp130/LifRb and are phosphorylated upon gp130/LifRb stimulation. Y-phosphorylations are followed by the detachment of pStat1,3 and pShp2 from cytokine receptors (reviewed in ref (Ernst and Jenkins, 2004)). pStat1 and pStat3 homo- and heterodimers translocate into the nucleus, where they interact with chromatin (Ernst and Jenkins, 2004) and transactivate astrocyte-specific genes, such as *Gfap*, *S100β*, *aquaporin*, etc (He *et al*., 2005). pShp2 conversely stimulates the mitogen-activated Erk kinase (Mek)/extracellular signal-regulated kinase (Erk) and the Akt cascades (Ernst and Jenkins, 2004), not directly implicated in transactivation of astroglial genes (Barnabé-Heider *et al*., 2005), and is involved in self-inhibition of the main astrogenic axis (Lehmann *et al*., 2003).

The primary role of the Ct1/Jak2/Stat3 axis in astrogenesis promotion has been thoroughly documented. mRNAs of *CntfRα* (Derouet *et al*., 2004), *gp130*, *LifRβ*, *Jak1*, *Stat1* and *Stat3* (He *et al*., 2005) were found in the pallial neuroepithelium as early as at E11-12, albeit at low level. Moreover, all these genes resulted to be subsequently upregulated, peaking around birth and afterwards (Derouet *et al*., 2004; He *et al*., 2005). Knock-out of *gp130* almost abolished *Gfap* expression in Lif- or Cntf-treated E14.5 telencephalic precursors, as well as in the E18.5 brain (except fimbria and hippocampus) (Nakashima *et al*., 1999b). A similar *in vivo* suppression of *Gfap* expression was elicited by inactivation of LifRβ (Koblar *et al*., 1998). Moreover, pharmacological inhibition of Jak by AG490 (but not inhibition of Mek by PD98059) reduced the gliogenic effect elicited by CNTF (Barnabé-Heider *et al*., 2005). Finally, as for Stat3, its positive implication in stimulation of astroglial genes was proven by a variety of approaches. It was observed upon transduction of rodent telencephalic precursors with artificial alleles of *Stat3*, encoding for a gain-of-function version of this protein (Stat3c, constitutively dimerizing, however still requiring Lif stimulation for its transactivating properties), or encoding for dominant-negative versions of it (Stat3f, unable to get Y-phosphorylated, and Stat3d, unable to bind to DNA) (He *et al*., 2005)(Gu *et al*., 2005). Moreover, astrogenic properties of pStat3 were evident upon cre/loxP-mediated ablation of *Stat3* in murine E14.5 cortico-cerebral NSCs (Cao *et al*., 2010), as well as after knock-down of *Stat3* mediated by RNAi (Aberg *et al*., 2001; Barnabé-Heider *et al*., 2005; He *et al*., 2005). Interestingly, reduced astrogenesis caused by *Stat3*-LOF manipulations was often associated to an excess of neuronal differentiation (Gu *et al*., 2005; Cao *et al*., 2010).

Concerning real ligand(s) triggering the astrogenic cascade in the developing cerebral cortex, the situation is as follows. *Cntf* (Derouet *et al*., 2004) and *Lif* (Barnabé-Heider *et al*., 2005) are not expressed in the embryonic CNS. *Np*-mRNA is detectable throughout murine embryonic neuroepithelia since E11-12, disappearing around birth (Derouet *et al*., 2004). *Clc*- and *Ct1*-mRNAs, quite scarce at E12-14, are readily detectable by E17.5 (Uemura *et al*., 2002; Barnabé-Heider *et al*., 2005). Moreover Ct1 is specifically expressed by cortical neurons and the medium conditioned by these neurons can promote *Gfap* upregulation. Remarkably, this upregulation can be prevented by the addition of a neutralizing antibody against Ct1 as well as by genetic ablation of *Ct1*. No prevention effects are conversely obtained by an anti-Lif neutralizing antibody or via *Lif* knock-out. Consistently, the Gfap$^+$ astroglial complement, as evaluated at P3, is reduced by 50-75% in *Ct1-/-* mutants and almost unaffected in *Lif* $^{-/-}$ mutants (Barnabé-Heider *et al*., 2005) (except a 30% decrease in the dentate area (Koblar *et al*., 1998)). All that suggests that perinatal cortical astrogenesis is mainly triggered by Ct1 released by previously born neurons, possibly with the help of Lif in some regions. Later, astrogenesis might be further promoted by Cntf released by astrocytes (Lillien *et al*., 1988), so self-sustaining its advancement.

Auto-regulation of the Ct1/Jak2/Stat3 axis

The Ct1/Jak2/Stat3 axis is provided of positive (Fig. 2A) and negative (Fig. 2B) regulatory loops, which finely tune its capability to sense Ct1 and Ct1-like signals and transduce them to the nucleus. These loops are crucial to proper timing of astrogenesis and appropriate regulation of the astrogenic-to-neuronogenic balance. They may be classified as follows:

Modulating gp130, Jak1, Stat1 and Stat3 expression levels

gp130, *Jak1*, *Stat1* and *Stat3* expression levels are very low in E11 cortico-cerebral precursors, they arise more and more during neuronogenesis progression and finally peak around birth. This

upregulation is promoted by the astrogenic cytokines, through the Ct1/Jak2/Stat3 axis. This was proven, by transducing cortico-cerebral precursors with mutated alleles of *Stat3*, encoding for gain-of-function or dominant-negative versions of it (He *et al.*, 2005). Remarkably, such upregulation is direct. In fact, evolutionarily conserved, pStat-binding sites are in the promoters af all four genes and - as proven by ChIP - they are specifically enriched in pStat3 upon Lif stimulation (He *et al.*, 2005).

Modulating Jak2 phosporylation

Two molecular devices, triggered by Ct1 stimulation and encoded by *suppressor of cytokine signalling gene 3 (Socs3)* and *Shp2*, provide a negative feedback, limiting levels of phospho-Jak2 upon cytokine stimulation and so concurring to proper balance between astrogenesis and neuronogenesis.

Socs3, upregulated by Jak/Stat signalling, binds to a single, membrane-proximal Y residue of LifRβ or gp130 (Y757 of mouse gp130), upon phosphorylation of this residue which follows cytokine-dependent stimulation of the receptor complex (Schmitz *et al.*, 2000). In this way, Socs3 recruits the proteasome to the ligand-occupied receptor complex and triggers degradation of its components as well as inhibition of Jak2 phosphorylation (Krebs and Hilton, 2001). Relevance of Socs3 to proper tuning of astrogenic rates was demonstrated by adenoviral transduction of *Socs3* to rat E15-17 striatal precursors, followed by exposition of these cells to Lif or Lif/Fgf2 (Cao *et al.*, 2006). It was found that *Socs3* downregulates the astroglial output elicited by cytokine stimulation, in as little as 1 day. This was associated to early upregulation of NSC markers and delayed increase of the percentage of microtubule associated protein 2-expressing (Map2$^+$) elements originating from the culture. That reasonably reflected an inhibition of glial commitment of NSCs, which, even in the presence of gliogenic citokines, retain their identity and are consquently available for alternative histogenetic pathways (Cao *et al.*, 2006).

The phosphorylation-dependent Shp2 tyrosine phosphatase also binds to the LifRβ/gp130 phospho-tyrosine residues which interact with Socs3, upon cytokine stimulation (Schmitz *et al.*, 2000). Following that, Shp2 dephosphorylates the adjacent Jak2, so attenuating Jak/Stat signalling (Lehmann *et al.*, 2003). In humans, mutations leading to constitutive activation of SHP2 cause the Noonan Syndrome (NS), which includes learning disabilities and mental retardation. In the mouse, knockdown of Shp2 in cultured cortical precursors or in the developing embryonic cortex inhibits neuronogenesis, anticipates astrogenesis and enlarges the astrocytic complement. Conversely, expression of an constitutively active Shp2 mutant causes an opposite phenotype, like it also happens in a mouse model of human NS. Thus, in normal corticogenesis, Shp2 channels early neural precursors to make neurons and not astrocytes, so contributing to postposition of astrogenesis to late gestational and early postnatal ages (Gauthier *et al.*, 2007).

Hetero-regulation of the Ct1/Jak2/Stat3 axis, by orthogonal modulatory plugins

Several orthogonal regulatory branches converge onto the Ct1/Jak2/Stat3 axis, finely tuning its firing rates (Fig. 3). Based on position of their entry points on this axis, these branches may be classified as follows.

Regulating levels of the Ct1-receptor (gp130)

Two heterologous players regulate gp130 expression levels, the proneural machinery and the Mek/Erk signalling machinery (Fig. 3A).

As shown by both gain- and loss-of-function experiments, Neu-

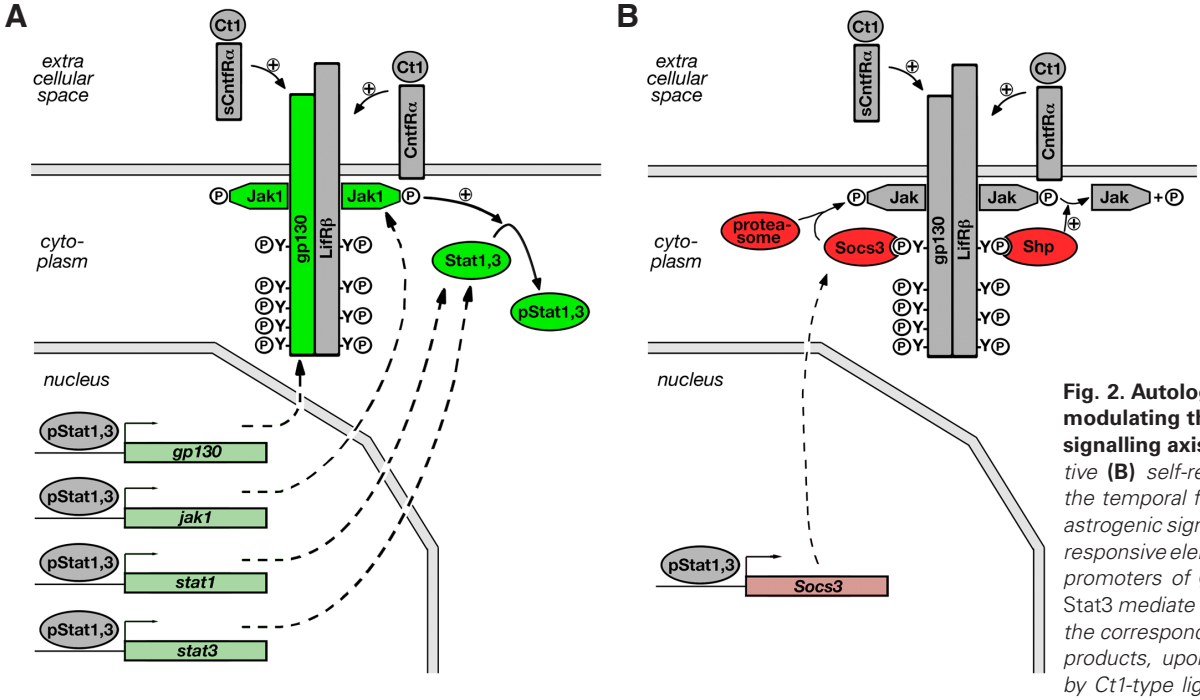

Fig. 2. Autologous regulatory loops modulating the astrogenic cytokine signalling axis. *Positive* **(A)** *and negative* **(B)** *self-regulating loops shaping the temporal firing profile of the main astrogenic signalling axis.* **(A)** *pStat1,3-responsive elements located within the promoters of* gp130, Jak1, Stat1 *and* Stat3 *mediate the progressive surge of the corresponding mRNAs and protein products, upon activation of this axis by Ct1-type ligands.* **(B)** *pStat1,3 also directly promote transcription of* Socs3, *whose protein product binds the most plasma membrane-proximal tyrosine residue of gp130/LifRb, phosphorylated upon Ct1 signalling. From this position, Socs3 recruits the proteasome to the gp130/LifRb-bound phosphorylated Jak, so paving the way to its degradation.* **(B)** *Finally, upon Ct1 signalling, the phosphorylated, most plasma membrane-proximal tyrosine residue of gp130/LifRb gets bound by the Shp phosphatase, which dephosphorylates the gp130/LifRβ-bound phospho-Jak and so prevents further phospho-Jak-dependent, Stat1,3 activation.*

rogenin1 (Neurog1) and Neurogenin2 (Neurog2)" do repress gp130 expression (He *et al.*, 2005). These two factors peak during the neurogenic period and so contribute to postponing astrogenesis to perinatal and postnatal phases.

Concerning the Mek/Erk axis, it has been recently shown that co-ablation of Mek1 and Mek2 in radial glial cells (RGCs), by Nestin-Cre, hGFAP-Cre or electroporated pCMV-Cre, inhibits the switch of RGCs from neuronogenesis to gliogenesis and prevents appearence of astrocyte- and oligodendrocyte-restricted progenitors. Consequently, surviving Mek1/2-deleted mice exhibit cortices almost devoid of astrocytes and oligodendroglia and undergo extensive neurodegeneration. A similar but less drastic phenotype follows co-ablation of Erk1 and 2. Conversely, electroporation of a constitutively active form of Mek1 (caMek1) leads to precocious activation of astrogenesis (Li *et al.*, 2012). Remarkably, the effects of the Mek/Erk machinery on astrogenesis are largely mediated by the Ets transcription family member Etv5/Erm, which - detectable in the VZ at E14.5-E18.5 - is necessary and sufficient for activation and progression of astrogenesis (Li *et al.*, 2012). Puzzlingly, however, extracellular signals triggering astrogenic firing of the rat sarcoma (Ras)/rapidly accelerated fibrosarcoma (Raf)/Mek/Erk axis have been poorly defined. For example, Fgf2, an established stimulator of this axis (Dorey and Amaya, 2010), promotes astroglial differentiation in the absence of any gp130 upregulation (Song and Ghosh, 2004), namely a major effect of Mek/Erk firing in this context. This suggests that Fgf2 impact on astrogenesis may be mediated by alternative pathways (Dorey and Amaya, 2010). On the other hand, Mek/Erk stimulation of astrogenesis is consistent with the capability of *Nf1* to limit gliogenesis (Dasgupta and Gutmann, 2005; Hegedus *et al.*, 2007; Wang *et al.*, 2012). In fact the product of *Nf1*, Neurofibromin1, is a Ras-GTPase that converts the GTP-bound active form of Ras to the inactive, GDP-bound form (Scheffzek *et al.*, 1997), so that *Nf1* inactivation leads to hyperactivation of the Ras/Raf/Mek/Erk pathway. *Nf1* was inactivated constitutively (Dasgupta and Gutmann, 2005), limited to E12.5 radial glia and its progenies (by Brain lipid binding protein gene-driven cre (Blbp-cre)"-cre and Gfap-cre deleters) (Hegedus *et al.*, 2007; Wang *et al.*, 2012), as well as in postnatal SVZ B cells and their progenies (by a Nestin-creET deleter and tamoxifen administration at P26-30) (Wang *et al.*, 2012). In all cases a dramatic increase of gliogenesis was reported. In constitutive and early embryonic *Nf1* -knock-out models that was associated to a remarkable enlargement of the subventricular zone and hyperthrophy of the corpus callosum (Dasgupta and Gutmann, 2005; Hegedus *et al.*, 2007). In postnatal knock-out models, NSC-restricted *Nf1* deletion resulted in increased gliogenesis at the expense of neurogenesis (Wang *et al.*, 2012). Remarkably, exaggerated glial commitment triggered by *Nf1* ablation could be phenocopied by constitutive overactivation of the Ras pathway (Hegedus *et al.*, 2007), and rescued by small inhibitors of Mek/Erk signalling (Wang *et al.*, 2012).

The key mechanism mediating astrogenic properties of the Ras/Raf/Mek/Erk axis is Mek1,2-dependent upregulation of gp130, encoding for one of the two subunits of the cardiotrophin 1-receptor. This upregulation, in fact, considerably sensitizes neural precursors to Ct1-family astrogenic cytokines. However, additional mechanisms concur to astrogenic properties of the Ras/Raf/Mek/Erk axis. First, Mek1,2 contribute to glial commitment of neural precursors by upregulating two hubs mastering this process. These are Chicken ovalbumin upstream promoter-transcription factor 1 (Couptf1), which triggers key epigenetic changes propedeutic to astrogenesis, and Epidermal growth factor receptor (EgfR), which promotes expression of the key Ct-1 transducer Stat3 and makes neural precursors more responsive

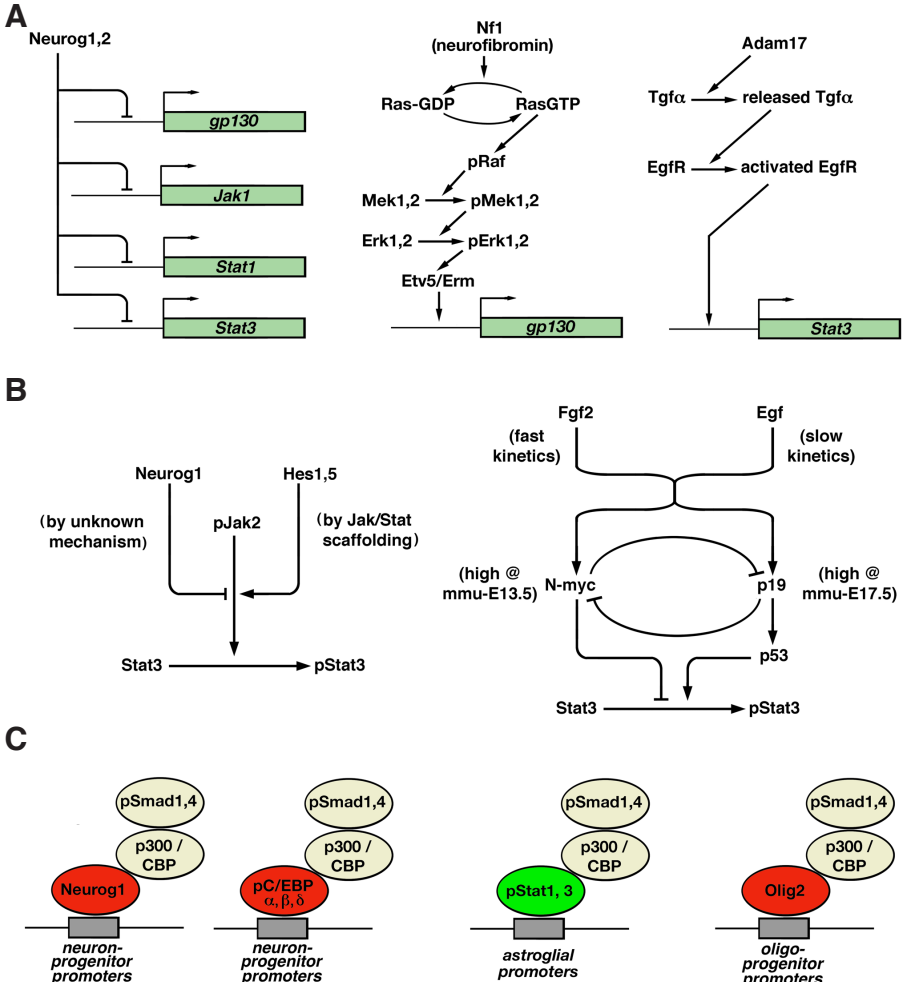

Fig. 3. Heterologous regulatory plugins modulating the astrogenic cytokine signalling axis. **(A)** *Transcriptional regulation of genes encoding for key members of the signalling axis. Neurog1 and 2 inhibit* gp130, Jak1, Stat1 *and* Stat3*; the Mek/Erk cascade upregulates* gp130*; Egf signalling promotes* Stat3. **(B)** *Modulation of Stat3 phosphorylation, by Neurog1, Hes1,5, N-myc and p19.* **(C)** *Competition among pro-neuronogenic Neurog1 and pC/EBPαuβuδ, pro-astrogenic pStat1,3, and pro-oligodendrogenic Olig2 for limited amounts of p300/CBP-pSmad1,4 heterodimeric cofactors available in neural precursors.*

to its active pStat3 form (see below). Second, firing of this axis stimulates proliferation and selective expansion of astroglial lineage elements. This happens upon defective Nf1-dependent Ras inihibition in embryonic NSCs, resulting in increased self-renewal of these elements and more frequent late-born Olig2$^+$ glial progenitors originating from them (Hegedus *et al*., 2007). In a similar way, sustained Mek1,2 signalling forces astrocytes to keep proliferating for longer times after their generation, so further amplifying the glial output of the system *in vivo* (Li *et al*., 2012).

Regulating Jak1 levels

During the early neurogenic period, Jak1 protein levels are kept low by proneural genes (Fig. 3A), which contributes to delayed activation of astrogenesis (He *et al*., 2005).

Modulating Stat1 and Stat3 levels

Two main players modulate expression levels of Stat proteins, namely the proneural machinery and the Egf transduction machinery (Fig. 3A).

Downregulation of Stat1 and Stat3 by Neurog1 and Neurog2 was demonstrated by both gain- and loss-of-function approaches (He *et al*., 2005). It is a key mechanism preventing precocious activation of astrogenesis.

Conversely, signalling through EgfR (aka ErbB1) promotes cortico-cerebral astrogenesis. EgfR is expressed at low level in the ventricular zone of the neuronogenic pallium and, at higher and higher levels in basal proliferative layers of the developing cortex (Caric *et al*., 2001). Already at the end of 90's, it was reported that mice lacking EgfR suffer of delayed astrocyte development (Kornblum *et al*., 1998; Sibilia *et al*., 1998). In 1997 and 2003, two seminal papers from the Lilien group, clarified cellular and molecular modalities of EgfR-dependent regulation of astrogenesis (Burrows *et al*., 1997; Viti *et al*., 2003). In the former study, the authors found that retrovirus-mediated overexpression of EgfR, in the E15 rat brain or in E12/E15 organotypic cultures of rat cortex, upregulates their astrocytic output, whereas delivery of the two main EgfR ligands, Egf and Tgfa, to EgfR-wt brains of the same developmental ages does not alter astrogenic rates. These data suggest that, at both E12 and E15, EgfR levels, rather than ligand levels, limit firing of the Egf transduction pathway and, therefore, astrogenesis rates. Only starting from E18, endogenous EgfRs levels would become sufficiently high to be saturating for astrocytes generation. Further dissection of this phenotype by clonal analysis showed that the increased astrogenic output elicited by EgfR/Egf manipulations actually has a dual origin. It reflects an enhanced astroglial commitment of multipotent precursors and it is amplified by more pronounced proliferation of committed elements. Remarkably, whereas combined EgfR/Egf manipulation was required to increase astroglial commitment at E12 and E15, simple delivery of exogenous ligand was sufficient for that at E18. This suggests that minimal firing of the Egf transduction pathway necessary for astroglial commitment may be higher than that required for promotion of proliferation (Burrows *et al*., 1997). Consistently with results of EgfR-GOF studies, cortical explants from E17 EgfR$^{-/-}$ mutant mice did not react to exogenous Lif administration. They differred from wt controls, which conversely responded with a quite enhanced astrogenic output (Viti *et al*., 2003).

Concerning molecular mechanisms underlying EgfR promotion of astrogenesis, Egf signalling upregulates astrogenesis rates, mainly by facilitating transmission of the Ct1 signal through the Jak2/Stat3 axis via EgfR-dependent upregulation of Stat3 expression. In fact, retroviral transduction of EgfR into murine, wild type cortico-cerebral precursors of different developmental ages considerably increases the frequency of Stat3^{high} cells (ten-fold at E11, two-fold at E16), whereas EgfR knock-out in E16 precursors grown *in vitro* for 4 days reduces this frequency by about 5/6-fold (Viti *et al*., 2003). On the other hand, following retroviral transduction of Stat3 into E11.5 precursors and in the only presence of endogenous astrogenic cytokines, frequencies of pStat3$^+$ elements and S100β^+ derivatives reach plateau values (not further upregulatable by exogenous Lif stimulation), which may be elicited in Stat3-wt cells of similar age by simple administration of exogenous Lif. In other words, the upregulation of Stat3 triggered by EgfR sensitizes embryonic neural precursors to the astrogenic cytokines, sufficiently to achieve a pronounced astrogenic response, even in front of low levels of these ligands (Viti *et al*., 2003).

However, pro-astrocytogenic activities of EgfR signalling cannot be fully accounted for in the light of Stat3 upregulation. In fact, experimental overexpression of EgfR in early cortical precursors (E11-13) upregulates frequencies of both S100β^+ and Gfap$^+$ astrocytes, whereas straight Stat3 overexpression in the same cells increases S100β^+ cells, but not Gfap$^+$ ones. That suggests that an Egf-dependent mechanism different from Stat3 upregulation may be selectively required to activate *Gfap*. This mechanism cannot be Stat3 phosphorylation, as Lif stimulation elicits similar levels of pStat3 in both EgfR-GOF and Stat3-GOF early cortical precursors (E11-13). It could be something else, working in pa rallel to pStat3 or downstream of it (Viti *et al*., 2003). On the other side, the expansion of the Gfap$^+$ compartment triggered by EgfR stimulation might partially have nothing to do with *Gfap* activation, being alternatively due to increased proliferation of astroglially-committed progenitors (Gadient *et al*., 1998).

Concerning the ligand triggering the EgfR-mediated astrogenic response in vivo, it is possible that this function is early shared by Egf and transforming growth factor α (Tgfα) and subsequently played by Tgfα only. In fact, both ligands are expressed in the neuronogenic cortical primordium (the former throughout it, the latter confined to its lateral border) (Assimacopoulos *et al*., 2003), but only Tgfα (and no Egf at all) is detectable in postnatal periventricular precursors (Romero-Grimaldi *et al*., 2011). Consistently with this prediction, mice expressing reduced Tgfα levels have a decreased number of astrocytes (Weickert and Blum, 1995). Remarkably, the activity of A disintegrin and metallopeptidase domain (Adam) "sheddases" is required to make Egf/Tgfα ligands available in the extracellular space (Blobel, 2005), so enabling astrogenesis. Both Adam10 and Adam17 are co-expressed in the VZ of the mid-neuronogenic cortical primordium (Diez-Roux *et al*., 2011) as well as in periventricular precursors of postnatal cortex (Romero-Grimaldi *et al*., 2011). However only Adam17 seems implicated in astrogenesis promotion. In fact, Adam17 knock-down impairs astrogenesis and its expression levels go up in damaged brain regions undergoing astrogliosis. That, conversely, does not apply to Adam10 (Romero-Grimaldi *et al*., 2011).

Modulating Stat3 phosphorylation

Mechanisms mediating astrogenic properties of Notch include promotion by the Delta/Notch/Hes axis of signalling through the Jak/Stat pathway. That is achieved by the Notch-effectors hairy

enhancer of split homologs 1 and 5 (Hes1 and Hes5), which act as bridges between Jak2 and Stat3 and facilitate phosphorylation of the latter by the former (Fig. 3B) (Kamakura *et al*., 2004). As a consequence of that, overactivation of the Notch signalling in murine E12 telencephalic precursors, followed by their *in vitro* expansion for 9 days, leads to a considerable enhancement of their astroglial differentiation. Consistently, such enhancement is fully suppressed, when a dominant-negative form of Stat3 is introduced in these cells (Kamakura *et al*., 2004).

Among mechanisms by which proneural genes inhibit astrogenesis, there is Neurog1 inhibition of Lif/Ct1-induced Stat1,3 phosphorylation (Fig. 3B). Molecular details of this inhibition are poorly known. It has been suggested that such inhibition contributes to diversify the responses exhibited by both E13.5 and E17.5 murine cortical precursors to Lif/Ct1. Both precursor types undergo Jak2 phosphorylation, only the latter phosphorylates Stat3 (Sun *et al*., 2001). As a consequence of that, E13.5 are only neuronogenic, E17.5 are mainly astrogenic. Consistently, the cortex of mutants double knock-out for *Neurog2* and *Mouse achaete scute homolog 1 (Mash1)* has increased numbers of glial progenitors and activates astrogenesis earlier (Nieto *et al*., 2001).

Finally, a reciprocal regulatory loop, including *Neural myelocytomatosis prooncogene (N-myc)* and *INK4a p19 protein/alternate reading frame of the INK4a/ARF locus (p19(Arf))*, regulates Stat3 phosphorylation, thus channelling Jak/Stat signalling machinery to distinct OFF and ON states (Fig. 3B). *N-Myc and p19(Arf)* are expressed by NSCs prevalently during the embryonic and the perinatal/postnatal phases of cortico-cerebral histogenesis, respectively. The former promotes NSC self-renewal and neuronogenesis, the latter sustains astrogenesis. Both are induced by Fgf2 and Egf, along a fast and a slow kinetics, respectively. The latter is further promoted by CNTF-dependent pStat3. N-Myc and p19(Arf) reciprocally inhibit their expression. N-Myc counteracts CTNF-dependent phosphorylation of Stat3 at Y705, p19(Arf) facilitates this phosphorylation, via p53. In this way, the N-Myc/p19(Arf) loop concurs to neatly define the functional state of the Ct1/Jak2/Stat3 axis, OFF or ON, during the embryonic and the post-natal life, respectively (Nagao *et al*., 2008).

Acting downstream of or in parallel with pStat3

It was shown that *in vitro* cultured telencephalic precursors from E14.5 mouse embryos activate *Gfap* in as little as 2 days under combined Lif/Bmp2 stimulation, taking at least 2 days more in the presence of either cytokine (Nakashima *et al*., 1999c). Remarkably, such capability of Lif and bone morphogenetic factor 2 (Bmp2) to synergically promote astrogenesis was confirmed in E16.5 cortico-cerebral precursors kept under thyroid hormones (Adachi *et al*., 2005). Looking for molecular mechanisms underlying this phenomenon, it was found that no direct interaction occurs between the two key transducers of Lif and Bmp signalling, Stat3 and Smad1, respectively. Conversely, they are bridged by the transcriptional coactivator p300/CBP, interacting with Stat3 at its amino terminus (even in the absence of Lif/Ct1 stimulation) and with Smad1 at its carboxyl terminus (upon Bmp stimulation). This leads to the formation of a pStat3-p300/CBP-pSmad1 ternary complex, which is recruited to the *Gfap* promoter and mediates cooperative promotion of its transcription by Lif and Bmp2 (Fig. 3C). More generally, it accelerates induction of astrocytes from neural progenitors and amplifies the astrogenic output (Nakashima *et al*., 1999a). Intriguingly, addition of Bmp2 alone to gp130-/- neural cultures, insensitive to Lif, elicits a moderate but reproducible activation of the *Gfap* promoter, suggesting that pSmads might also directly transactivate such promoter, independently of pStat3 (Nakashima *et al*., 1999c).

As reported above, the proneural factor Neurog1 inhibits astrogenesis by counteracting Stat3 phosphorylation. However, it also inhibits pStat3 activity, by competing with pStat3 for binding to p300/CBP-pSmad1 (Fig. 3C) and so diverting such heterodimer to neuron-specific promoters (Sun *et al*., 2001). Actually, it was shown that in cultures of rat E18 cortico-cerebral precursors, Ngn1 downregulates Gfap promoter-driven transcription, even upon administration of Cntf or Bmp. On the other hand, overexpression of either p300/CBP or Smad1 significatively rescues glial differentiation from Ngn1 suppression. Moreover, both CBP/p300 and Smad1 potentiate the transcription of a Ngn1-responsive promoter (Sun *et al*., 2001). Interestingly, in extracts from rat E14 cerebral cortex, p300/CBP is associated with both Neurog1 and Smad1, but not with Stat3 (which - nevertheless - is expressed at high levels in these extracts). In contrast, in extracts of P3 cortico-cerebral SVZ, rich of astroglial precursors and not expressing Neurog1 anymore, Stat3 is associated to p300/CBP (Sun *et al*., 2001). The most parsimonoius explanation of these data is that the p300/CBP-pSmad1 heterodimer may potentiate both neuron- and astrocyte-specific transcriptions, by complexing Neurog1 and pStat3, respectively. However, p300/CBP-pSmad1 is available only to a limited extent, so that a competition takes place between its interactors for binding to it. This competition is intrinsically biased in favor of Neurog1. Therefore, until proneural genes are abundantly expressed, it is won by their products, so resulting in competitive silencing of astroglial transcriptions. Only after neuronogenesis completion, proneural factors disappear, so allowing astrocyte-specific transcriptions to rise (Sun *et al*., 2001). This capability of Ngns to antagonize astroglial transcriptions might be the main mechanism underlying exaggerated generation of astrocytes, which has been recently found in cultures of pallial precursors knock-out for *Pax6* (Sakayori *et al*., 2012), namely a direct transactivator of *Neurog1* and *Neurog2* promoters (Scardigli, 2003; Blader, 2004).

An additional transcription factor set limiting pStat3 activity includes the leucin-zipper family transcription factors CCAAT/enhancer binding protein (C/EBP)-α, -β, and -δ. These are expressed by E12-18 cortico-cerebral precursors, are activated by Mek/Erk-dependent phosphorylation, and are necessary and sufficient to promote neuronogenesis. That was shown in vitro, transfecting E12 precursors with plasmids encoding for two distinct, dominant-negative variants of *C/EBP*-β (counteracting all *C/EBP* paralogs) or a constitutively active, phosphomimetic variant of it, and scoring their progenies 3-4 days later. This was confirmed in vivo, electroporating E15 brains with these constructs and analyzing them 3 days later (Ménard *et al*., 2002; Paquin *et al*., 2005). Remarkably, in addition to impairment of neuronogenesis, functional knock-down of C/EBPs also promotes Cntf-dependent astroglial differentation, suggesting that C/EBPs may normally contribute to prevent precocious activation of this process. However mechanisms mediating these phenomena are still matter of debate. The increased astroglial output obtained upon *C/EBPs* knock-down might simply reflect the higher fraction of neuronogenic precursors which retain the stem state upon this manipulation, so resulting available to subsequent glial differentiation (Ménard *et al*., 2002; Paquin *et al*., 2005). However, overexpression of a phospho-dead variant of C/EBP-β,

while repressing neuronogenesis, did not affect astrogenesis at all (Paquin *et al*., 2005), suggesting that mechanisms mediating C/EBP-dependent regulation of astrogenesis should at least partially differ from those impacting neuronogenesis. It was shown that C/EBP-β is able to bind CBP/p300 (Mink *et al*., 1997). As such, similar to Neurogenins and Olig2 (Sun *et al*., 2001; Fukuda *et al*., 2004), it might sequester the p300/CBP-pSmad1 heterodimer (Fig. 3C), making it not available to bind pStat3 and so preventing transcription of glial genes.

Finally, it has been shown that the basic, helix-loop-helix (bHLH) factor Olig2, implicated in cortico-cerebral gliogenesis, may also counteract the astrogliogenic activity of pStat3, in a *Neurog1*-like way (Fig. 3C) (Fukuda *et al*., 2004). *Olig2* is widely expressed in the Gaba-ergic neuronal lineage as well as by oligodendroglial cells at different stages of their maturation (Cai *et al*., 2007). Within the astrocytic lineage, it gives rise to a transient perinatal activation wave (Cai *et al*., 2007; Ono *et al*., 2008), subsequently declines and fully disappears by P21 (Cai *et al*., 2007). Such *Olig2* downregulation in differentiating astrocytes is fully replicated in vitro, in 4 days-old cultures of murine E14,5 telencephalic precursors, kept under Lif (Fukuda *et al*., 2004). Remarkably, *Olig2*, if overexpressed in perinatal cortical precursors, competes with pStat3 for binding to the p300/CBP-pSmad1 dimer, so antagonizing astrocytic differentiation (Fukuda *et al*., 2004). This mechanism might be crucial to restrict full activation of the *Gfap* promoter to mature astrocytes and avoid any leakage of it in the oligodendrocytic lineage. Moreover, it could help explaining some traits of the complex phenotype displayed by *Olig2-/-* mutants, such as reversion of Gfap and S100β relative expression levels in layers II-IV of neocortex (Cai *et al*., 2007).

The anti-astrogenic Nrg1/ErbB4 pathway

A major role in setting up the proper onset of astrogenesis is played by the ligand Neuregulin1 (Nrg1), expressed by neurons and neural precursors, and its ErbB4 receptor, transducing its signal within cortico-cerebral precursors. This was demonstrated by Sardi *et al.,* (Sardi *et al*., 2006), co-manipulating the Nrg1/ErbB4 and the Ct1/Jak2/Stat3 pathways in telencephalic precursors and assaying consequences of that on the activation of astroglial genes.

Transduction of the Nrg1 signal along the "canonical" pathway starts with Nrg1 binding to ErbB receptors, inducing their hetero- or homodimerization. That results into activation of their intracellular tyrosine kinase activity and generation of docking sites for adaptor proteins, in turn activating the Raf/Mek/Erk and the phosphoinositide-3-kinase (PI3K) machineries (Mei and Xiong, 2008). Remarkably, this canonical pathway is not sufficient to control astrogenesis. Nrg1 control of astrogenesis is conversely exerted via an alternative transduction pathway, called "non canonical". In this case, binding of the Nrg1 ligand to the juxta-membrane α (jMα) isoform of ErbB4 is followed by ErbB4 cleavage by Tumor necrosis factor α (TNFα)-converting enzyme (TACE), releasing a soluble extracellular peptide that contains the NRG1 binding site (ecto-ErbB4). The residual membrane-anchored fragment (ErbB4-CTF) is further cleaved in its transmembrane domain by presenilin-dependent γ-secretase, releasing the ErbB4 intracellular domain (ErbB4-ICD). As shown by two-hybrid and co-immunoprecipitation assays, ErbB4-ICD specifically binds to nuclear receptor corepressor (NCoR), via a Tgfβ-activated protein kinase 1 (Tak1)-associated binding protein 2 (Tab2) bridge. Then ErbB4-ICD, being provided with a NLS, conveys the resulting trimeric complex ErbB4-ICD/Tab2/NCoR into the nucleus, where each component of it can be found associated to the chromatin of *Gfap* and *S100β* (Sardi *et al*., 2006). Interestingly, the ErbB4-ICD capability to drive cytoplasm-to-nucleus NCoR translocation is dominant over the opposite ability of the firing Jak/Stat axis to extrude this cofactor from nucleus. Actually, binding sites of the ternary ErbB4-ICD/Tab2/NCoR complex within *Gfap* and *S100β* promoters were not mapped. It was proposed that this complex would be further connected to recombination signal binding protein for for immunoglobulin kappa J region (RBPJk), for which NCoR has a very high affinity, and, in such a way, it might bind to the *Gfap* promoter in correspondence of its RBPJk binding site (Miller and Gauthier, 2007). Remarkably, ErbB4 cleavage, its Tab2-mediated interaction with NCoR, and nuclear translocation of the ErbB4-ICD/Tab2/NCoR complex, all do require binding of Nrg1 to the jMa isoform of ErbB4. The last two steps further rely on the first. Interestingly, when the "non canonical" ErbB4 pathway is overactivated in rat E14.5 telencephalic precursors in vitro, this suppresses the increase of *Gfap*- and *S100β*-promoter-driven transcription, otherwise observable after subsequent Cntf stimulation. Conversely, no effect is exerted by ErbB4 pathway overactivation on basal *Gfap*- and *S100β*- transcription levels detectable in the absence of Ctnf (Sardi *et al*., 2006). Consistent data were obtained after experimental manipulation of the non canonical Nrg1/ErbB4 axis in vivo. Gfap expression levels were dramatically upregulated in the E17.5 cerebral cortex of mutant mice with CNS-restricted inactivation of ErbB4. Electroporation of human jMα-ErbB4 into the brain of these mice, at E13.5, specifically rescued this phenotype.

In synthesis, Nrg1/ErbB4 seems to mainly act as a brake, filtering the astrogenic outcome of the cardinal Ct1/Jak2/Stat3 pathway and dictating the temporal frame of its emergence. In particular, strong Nrg1 signalling in early (<E14.5) murine cortico-cerebral precursors would prevent premature activation of astrogenesis, despite of early firing of the Ct1/Jak2/Stat3 axis. Then, subsequent dampening of Nrg1 signalling, due to late ErbB4 downregulation (Kornblum *et al*., 2000; Fox and Kornblum, 2005), would lead to progressive derepression of astrogenesis, eventually becoming the prevalent histogenetic process, at E18.5 and later.

The pro-astrogenic Delta/Notch pathway

It has been shown that artificial overactivation of Notch signalling in mouse E11.5 or rat E13 cortico-cerebral precursors leads - 2-3 weeks later - to increased astrocytic outputs. Consistently, an opposite phenotype occurs upon blockade of Notch signalling by the γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Ge *et al*., 2002). As reported above, Notch signalling might sustain the astrogenic program thanks to the capability of Hes1 and Hes5 to physically connect Jak2 to Stat3, so facilitating the phosphorylation of the latter and the transduction of the cardinal astrogenic signal (Kamakura *et al*., 2004). Additionally, Notch effectors Hes1 and 5 might enhance astrogenesis by repressing transcription of proneural genes, endowed with antigliogenic properties (Kageyama *et al*., 2005). However, effectors of Notch signalling also directly interact with astroglial genes, so straightly regulating their transcription. One of these effectors is RBPJk, specifically binding to the *Gfap* promoter, circa 180 bps upstream of the TSS. Another is the (unknown) interactor of another Notch-RE, mapped within the same promoter between -100bp and the TSS (Ge *et al*., 2002). Remarkably, although Notch signalling is already active within pre-neuronogenic cortico-cerebral

precursors, astrogenesis initiates normally about 7 days later. This suggests that Notch signalling is not sufficient to trigger this process and addional conditions have to be fulfilled in order to unveil its pro-gliogenic power. Intriguingly, even artificial overstimulation of the Delta/Notch axis, while enhancing astrogenesis, does not anticipate it. Moreover, the astrogenic activity of Notch signalling requires Jak/Stat signalling, but not viceversa (Ge *et al.*, 2002). Key of these phenomena might be the transcriptional corepressor NCoR and temporal regulation of its subcellular distribution pattern. In fact, this molecule is able to bind RBPJk (Kao *et al.*, 1998) and is prevalently located within the nucleus of young neural precursors (Sardi *et al.*, 2006). Therefore, specifically within these precursors, it might convert RBPJk into a trans-repressor, silencing astroglial genes. Then, when firing of the Jak2/Stat3 axis translocates NCoR to the cytoplasm (Sardi *et al.*, 2006), RBPJk, freed from NCoR, would become prevalently complexed by Notch-IntraCytoplasmic-Domain (NICD) (Miller and Gauthier, 2007). As such, after E14.5, it might promote more and more transcription of astroglial genes. Consistently with this model, 70% of *NCoR* $^{-/-}$ embryos show prominent cortico-cerebral overexpression of *Gfap* as early as E14.5, a time at which wild type cortices barely express this marker (Hermanson *et al.*, 2002).

The pro-astrogenic Tgfβ1/ Tgfβ1RI,II pathway

It was originally reported that Tgfβ1 secreted by neurons and astrocytes induces the activation of the *Gfap* promoter in cultured astrocytes, so stimulating their maturation. In this respect, E14.5 neurons, expressing higher levels of Tgfβ1, resulted to be more efficient inducers than E18.5 neurons and late embryonic astrocytes were more responsive to Tgfβ1 than late post-natal ones. Moreover, this Tgfβ1-dependent upregulation of *Gfap* was peculiar to cerebral cortex. Midbrain and cerebellar astroglia, while able to respond to Tgfβ1, did not display any increase of Gfap levels (De Sampaio e Spohr *et al.*, 2002). More recently, it has been shown that Tgfβ1 signalling is not only implicated in late advancement of astrocyte differentiation, but may also help the commitment of pluripotent cortico-cerebral precursors to astroglial fates. In fact, the two Tgfβ1 receptors, TgfβRI and TgfβRII, and the Tgfβ1-signalling transducers, Smad2/3 and 4, are already expressed by the mouse E14.5 cortico-cerebral radial glia (RG). In this way, neuron-secreted Tgfβ1 may induce Smad2,3 translocation from RG cytoplasm to nucleus. This is followed by downregulation of the RG markers *nestin* and *Blbp*, and activation the astrocyte-lineage marker *Gfap*. (Stipursky and Gomes, 2007). However, two key aspects of progliogenic Tgfβ1 activity are still obscure. First, Tgfβ1 is abundantly expressed in intermitotic precursors and postmitotic neurons of the rat embryonic cortex as early as E16.5 (corresponding to mouse E14.5), and, at that time, astrogenesis is just going to be activated (Miller, 2003). Second, a Tgfβ1 response element has been detected within the *Gfap* promoter, at about 70 bases upstreatm the TSS, however binding of the intracellular Tgfβ1 effector pSmad2,3-BS to this element has not been yet demonstrated (Krohn *et al.*, 1999). As for the first issue, the TF Foxg1, strongly expressed within telencephalic proliferative layers, has been proposed to interfere with multiple Tgfβ1 responses, by associating with DNA binding proteins which function as Smad2,3 partners (Dou *et al.*, 2000). That could help to explain early hindering of the progliogenic Tgfβ1 activity. It might further suggest a possible mechanism for the reported *Forkhead box g1 (Foxg1)*-dependent inhibition of astrogenesis (Brancaccio *et al.*, 2010). As for the second issue, it is alternatively coinceivable that astrocytogenic results of Tgfβ signalling do not require binding of pSmad2,3 to the *Gfap* promoter. It was shown that, upon activation by Tgfβ, Tak1 interacts with Tab2 (Shibuya *et al.*, 1996), namely a key component of the ErbB4-ICD/Tab2/NCoR complex, which mediates early Nrg1-dependent prevention of astrocytic transcriptions (Sardi *et al.*, 2006). Therefore, Tgfβ1 might alternatively promote the astrogenic program, by sequestering - via the activated form of Tak1 - this essential cofactor of Nrg1 signalling (Stipursky and Gomes, 2007).

The pro-astrogenic Pacap/Pac/Dream pathway

A last trans-active pathway modulating cortico-cerebral astrogenesis is controlled by a ligand structurally related to Vasoactive Intestinal Peptide (Vip), termed Pituitary Adenylate Cyclase-Activating Polypeptide (Pacap) (Vallejo, 2009). Cortical precursors express predominantly the short isoform of the Pacap-specific receptor Pac1, which couples to adenylate cyclase. Remarkably, even a short exposure of cortical precursors to Pacap results in a dose-dependent increase in cyclic adenylic acid (cAMP) production, which is strictly necessary to achieve Pacap-dependent promotion of astrogenesis (Vallejo and Vallejo, 2002). Moreover, Pacap raises intracellular calcium concentration via a mechanism that relies on extracellular calcium and requires cAMP. Eventually, calcium is the last intracellular transducer that triggers enhanced transcription of astroglial genes, in response to Pacap (Cebolla *et al.*, 2008). Actually, it has been shown that, even in the absence of Pacap signalling, the transcriptional modulator Downstream Regulatory Element Antagonist Modulator (Dream) already sits near the *Gfap*-TSS, at two Dream-Responsive Elements, termed DRE1 and DRE2, located at -381/-359 and -70/-51, respectively. Then, the calcium wave triggered by Pacap induces Dream to undergo a profound conformational change, which makes it able to promote *Gfap* transcription. Cortical progenitors from *Dream*$^{-/-}$ mice fail to express *Gfap* in response to Pacap, whereas *Dream*$^{-/-}$ neural precursors, treated by Bdnf, generate about 2-fold more neurons. That suggests that the Pacap pathway contributes to the astrogenic-vs-neuronogenic histogenetic choice. Consistently, the neonatal cortex of *Dream*$^{-/-}$ mice exhibits a reduced content of astrocytes. However this is a transient phenomenon, as Gfap^{+} cells of these mutants, about 66% of controls at P1, bounce to 120% in as little as one week (Cebolla *et al.*, 2008). Interestingly, the Pacap pathway seems to act independently of Jak2/Stat3. In fact, *Dream* knock-out does not affect the responsivity of cortico-cerebral precursors to Lif/Ctnf (Cebolla *et al.*, 2008). Moreover, combined suboptimal stimulation of cortical precursors, by Pacap and Cntf, elicits an additive incremental effect on frequency of Gfap^{+} cells (Cebolla and Vallejo, 2006). However, more subtle parameters of astroglial differentiation, such as the Gfap content of treated cultures and the morphological complexity of astrocytes, display a more than additive upregulation by Pacap and Cntf, so implying some cryptic functional interaction among the corresponding transduction pathways (Cebolla and Vallejo, 2006).

Accessibility of chromatin to transcription

It has been shown that, in addition to *trans*-active machineries impinging on astroglial promoters, proper activation and adequate progression of cortico-cerebral astrogenesis requires an appropriate temporal regulation of chromatin accessibility to transcription.

Large-scale changes of chromatin structure

A first correlation has been described to occur between large scale chromatin configuration and the developmental choice taken by NSCs towards neuronal or astrocytic lineages. It turns out that the open configuration displayed by chromatin in young NSCs preferentially leads to self-renewal and neuronal differentiation, whereas its subsequent, progressive compaction biases stem cells to make astrocytes (Kishi *et al*., 2012). It is not clear how generalized changes of chromatin accessibility impact the histogenetic choice of NSCs. It is possible that such changes primarily reduce transcription rates of proneural genes and, because of the major trans-inhibitory role that proneural proteins exert on astrocyte-specific transcriptions (He *et al*., 2005), indirectly derepress astrogenesis. At least two gene sets have been implicated in modulating this epigenetic change.

High mobility group A (HmgA) proteins, 1 and 2, expressed at high level during early neuronogenesis, effectively compete with histone H1 for binding internucleosomal DNA linkers. In this way they keep chromatin accessible and promote the turnover of factors interacting with it, which is associated to prevalent neuronal differentiation. By the end of embryonic life, Hmga levels are reduced, which is possibly instrumental to upregulation of astrogenesis rates taking place at that time (Kishi *et al*., 2012). Consistently with this model, experimental manipulations of *Hmga1/2* expression levels, both *in vitro* and in vivo, affect neurono-to-astrogenic ratios, indicating that these genes are both necessary and sufficient to orientate NSCs to neuronogenesis and inhibit gliogenesis (Kishi *et al*., 2012).

N-myc, expressed at high level during early neuronogenesis as well, conversely upregulates the histone acetyl-transferase gene *GCN5*. In this way, it promotes the acetylation of histones H3 and H4 as well as a more open chromatin configuration (Knoepfler *et al*., 2006). That may contribute to the capability of *N-myc* to sustain neuronogenesis. It may help *N-myc* to counteract expression and activity of *p19(Arf)*, which is strongly expressed in late NSCs and channel them to astroglial differentiation (Nagao *et al*., 2008).

Epigenetic changes at proneural genes

A neat *trans*-regulatory effect of the epigenetic state of neuronal genes on astrogenesis rates has been rigorously documented in the case of the proneural gene *Ngn1*. It has been found that the repressive mark trimethyl-histone 3 lysine 27 (H3K27me3) becomes more and more frequent near the *Ngn1*-TSS, in mouse E11.5 cerebral precursors kept *in vitro* for 3, 6 and 9 days. That is consistent with the progressive downregulation of *Ngn1* occurring *in vivo,* around the end of the neuronogenic phase, and it is instrumental to late activation of astrogenesis (Hirabayashi *et al*., 2009). Actually, H3K27 trimethylation at the *Ngn1*-TSS depends on one of the two H3K27-methyltransferases included in the Polycomb Repressive Complex 2 (PRC2), enhancer of zeste homolog 2 (Ezh2). If *Ezh2* is conditionally knocked-out at E13.5-14.5, then H3K27me3 levels at the *Ngn1*-TSS persist low and no downregulation of *Ngn1* can be found around E17.5. Remarkably, this conditional knock-out is sufficient to shift the response of cortico-cerebral precursors to prodifferentiating agents *in vitro*, from from astrogenesis to neuronogenesis. *In vivo*, it dramatically reduces the frequency of S100β^+ cells, as evaluated in P1.5 cortices (Hirabayashi *et al*., 2009). Interestingly, a depression of astrogenesis may be also achieved by knocking down embryonic ectoderm development *(Eed)* (which encodes for another subunit of PRC2) or inactivating *ring finger protein 1 gene (Ring1b)* (which encodes for a subunit of the Polycomb Repressive Complex 1 (PRC1), a functional partner of PRC2) (Hirabayashi *et al*., 2009). Remarkably, the timing of these genomic manipulations is crucial to disclose the antigliogenic activity of PRC2, reasonably because of the involvement of this complex in additional decisional processes which precede the neurono-to-astrogenic switch. In fact, if *Ezh2* is disrupted *prior to* neuronogenesis, then neuronogenesis rates are exaggerated, at expenses of precursors self-renewal, and the activation of astrogenesis is paradoxically anticipated (Pereira *et al*., 2010).

Epigenetic changes at astroglial genes

In addition to large scale chromatin dynamics and proper epigenetic tuning of proneural genes, it has been proven that a major pre-condition for the switch from neuronogenesis to astrogenesis is the progressive "opening" of astrocytic chromatin. This is a complex process which takes place while neuronogenesis is on, from E11.5 to E14.5 and beyond, and essentially consists in the acquisition by the astroglial genes of the capability to get bound by their specific transactivators (Hatada *et al*., 2008).

This capability depends first on the methylation state of DNA. Murine E11.5 cortico-cerebral precursors express the full set of molecular transducers of LiF-like signals (LiF-receptor subunits, Jaks and Stats among these). Exposed to LiF, they upregulate pStat3 to some extent. However, as it also happens in neurons and other non-neural cells, this does not result in *Gfap* transcription, because in these cells pStat3 cannot interact with its binding site, 1.5kb upstream of the *Gfap*-TSS (Takizawa *et al*., 2001). In normal conditions, in fact, such interaction starts to be allowed only from E14.5, just upon natural CpG demethylation at this site. Remarkably, if DNA methylation is inhibited by 5-azacytidine (5azaCdR) administration, then *Gfap* is activated already in E11.5 precursors, by only 4 days (Takizawa *et al*., 2001). An anticipation of astrogenesis also occurs upon knock-out of the "mainteinance" DNA methyl transferase gene, *Dnmt1*. In this case, E11.5 precursors, exposed to LIF, massively activate *Gfap* and *S100β* in as little as 2 days *in vitro*, whereas their wild type counterpart would take 7 more days for that (Fan *et al*., 2005). More recently, demethylation of astroglial genes just prior to glial differentiation of NSCs has been described in a variety of other cases (*AldoC*, *ATP-sensitive inward rectifier potassium channel 10 gene (Kcnj10)*, *serpin peptidase inhibitor b8 gene (Serpinb8)* and *SRY-box containing gene 8 (Sox8)* among them). Nowadays it is considered as a pervasive phenomenon, crucial to timely activation of the astrogenic program (Hatada *et al*., 2008).

However, the preparation of astrocytic genes to transcription is

TABLE 1

MAIN EFFECTORS MODULATING THE EPIGENETIC STATE OF THE *Gfap* PROMOTER

Effector	Effects on the Gfap promoter	References
Nf1a	5meC ↓	(Namihira et al., 2009)
Fgf2*	5meC ↓, H3K9-me2 ↓, H3K4-me2 ↑	(Song and Ghosh, 2004)
Coup-tf1,2	5meC ↓, H3K9-me2 ↓, H3K4-me2 ↑, H3-ac ↑	(Naka et al., 2008)
RA	H3-ac ↑	(Asano et al., 2009)
Eset	H3K9-me3 ↑, H3K9-ac ↓	(Tan et al., 2012)
Brg1	chrom remodeling	(Matsumoto et al., 2006)

* requires subsequent Ctnf stimulation to be effective

not limited to proper regulation of their DNA methylation levels. It encompasses also a fine modulation of the covalent modification profile of histones associated to them, as well a conformational rearrangement of chromatin (Matsumoto *et al.*, 2006). The former includes the replacement of the dimethyl-histone 3 lysine 9 mark (H3K9me2) by the dimethyl-histone 3 lysine 4 mark (H3K4me2), a phenomenon originally documented at the pStat3-BS of *Gfap*, within rat E15.5 cortico-cerebral precursors kept 2 days *in vitro* under Fgf2. Remarkably, this switch is associated to H3 hyperacetylation and was proven to be sufficient and necessary to make *Gfap* suitable to transcription (Song and Ghosh, 2004).

Complex molecular mechanisms dictate the epigenetic state of astroglial chromatin. At the moment, at least six main players have been shown to master this control: Notch (via Nuclear Factor Ia, NFIa), Fgf2, Couptf1&2, retinoic acid, RA (via RA-Receptors α, β and X, RARα, RARβ and RXR), the histone 3 lysine 9-methyltransferase (H3K9-MT) ERG-associated protein with SET domain (ESET) and one of the two ATPase subunits of the SWItch/Sucrose NonFermentable (SWI/SNF) chromatin remodeling complex, namely the product of *Brahma related gene 1, Brg1* (see Table 1 and Fig. 4).

It was early recognized that Notch signalling modulate cortico-cerebral histogenesis in a stepwise way, initially inhibiting neuronogenesis and subsequently promoting astrogenesis at expenses of oligogenesis (Grandbarbe *et al.*, 2003). Actually, the Notch receptor is expressed by apical precursors since E10.5-11.5. Its Delta-like ligands are early detectable on the plasma membrane of the same cells according to an early, dynamical salt-and-pepper blueprint (Shimojo *et al.*, 2008). Then, they are expressed, more and more, by basal precursors and newborn neurons (Yoon *et al.*, 2008). It was shown that artificial hyperstimulation of Notch signalling in E11.5 cortico-cerebral precursors makes these cells able to activate *Gfap* within 3 days *in vitro*, in response to Lif stimulation. Blockade of Notch signalling conversely suppresses this ability and inhibits astrogenesis *in vivo*. As for the underlying molecular mechanism, it was shown that canonical Notch signalling is sufficient and necessary to promote demethylation of the main pStat3-BS located in the *Gfap* promoter. That normally happens between E11.5 and E14.5 and it is a pre-condition for subsequent activation of this gene by Lif-like ligands (Namihira *et al.*, 2009). *Gfap* demethylation is associated to reduced binding of the "maintenance" Dnmt1 enzyme to the *Gfap* promoter, induced by Notch signalling, and requires precursors proliferation (possibly allowing the dilution of the previously established 5-methylcytosine (5meC) marks). However, notice that the Delta/Notch axis *does not repress Dnmt1* expression, conversely, it simply forces Dnmt1 *to detach* from the *Gfap* promoter. Actually, the Notch effector RBPJk binds to the *Nf1a* promoter, about 2.0 kb upstream of its TSS, and stimulates its transcription (Namihira *et al.*, 2009). In turn, Nf1a binds to the *Gfap* promoter (at least four Nf1a binding sites were found within the *Gfap* promoter (Cebolla and Vallejo, 2006; Piper *et al.*, 2010)). This is necessary and sufficient to detach Dnmt1 from this region and get it demethylated, so paving the way to the astrogenic program (Namihira *et al.*, 2009). That was assessed, by overexpressing Nf1a (or a dominant negative version of it) in E11.5 cortico-cerebral precursors, and assaying their behaviour in vitro, after 4 days of Lif stimulation. Thus, Nf1a mediates the pro-astrogenic function of the Delta/Notch axis, being transcribed in response to Notch signalling and possibly competing with Dnmt1 for binding to the *Gfap* promoter (Namihira *et al.*, 2009). Remarkably, overstimulation of the Delta/Notch/NfIa axis is *not sufficient per se* to enhance astrogenesis. The pro-astrogenic activity of this pathway only *emerges*, provided that the system is *co-stimulated*

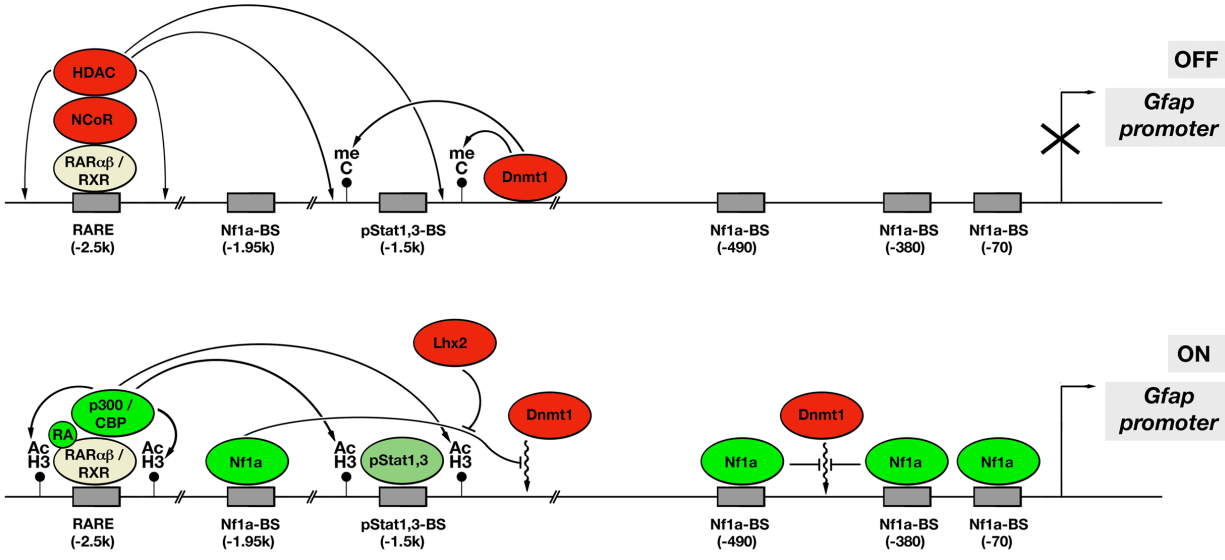

Fig. 4. Epigenetic regulation of the *Gfap* promoter by Nf1a and RA. (OFF state). *In the absence of Nf1a, Dnmt1 C5-methylates the surroundings of the pStat1,3-binding site, at -1.5kb, making such site not suitable to interact with its cognate transactivator. Meanwhile, in the absence of RA, the RARα,β/RXR receptor sits on the RARE at -2.5kb and, via an NCoR bridge, recruits histone deacetylases (HDACs). These ones deacetylate the* Gfap *promoter, around both -2.5kb and -1.5kb, so contributing to shut down transcription.* **(ON state)**. *Nf1a binds to the* Gfap *promoter, possibly at as many as four distinct locations, and inhibits Dnmt1 interaction with it, so allowing progressive lose of C5-methyl marks. Limited to the archicortex, Lhx2 counteracts this Nf1a-dependent inhibition of Dnmt1, so antagonizing astrogenesis arousal. On the other side, RA changes the conformational state of its RARE-bound RARα,β/RXR receptor, leading to replacement of NCoR-HDAC by p300/CBP. As a consequence of that, the* Gfap *promoter is richly acetylated and so made prone to transcription.*

by Lif (Namihira *et al*., 2009) and/or Pacap (Cebolla and Vallejo, 2006). Consistently, if the NF1a-binding site -79/-57 bps upstream the *Gfap*-TSS is dirsrupted, then the responsivity of the *Gfap* promoter to both Ctnf and Pacap is suppressed and even basal transcription of this gene collapses (Cebolla and Vallejo, 2006). It is worth mentioning that this mechanism seems to be not limited to *Gfap*. In fact, Nf1a-BSs can be found in the promoters of other astroglial genes (including *S100β*, *aquaporin4*, and *clusterin*), Nf1a binds to these sites upon activation of the Notch pathway, and *Nf1a* overexpression is sufficient to induce demethylation of the corresponding promoters (Namihira *et al*., 2009). More recently, it has been shown that electroporation of the LIM homeobox 2 TF gene *Lhx2* into the E15/17 ventricular zone reduces astrogenesis rates by almost 2/3-fold and prevents exaggerated astrogenesis induced by *Nf1a* overexpression. On the other side, disruption of *Lhx2* activity, by E15.5 electroporation of its dominant-negative cofactor gene *ClimΔDD*, magnifies astrogenesis at expenses of neuronogenesis, only provided that *Nf1a* is not ablated. In a few words, Lhx2 seems to inhibit transcription of astroglial genes, by preventing the activity of Nf1a on their promoters (Subramanian *et al*., 2011). However, these phenomena were documented exclusively in the archicortical anlage, where the late gestational drop of *Lhx2* expression levels may contribute to perinatal upregulation of astrogenesis. No gliogenic anomaly was conversely found upon manipulation of Lhx2 activity in *neocortical* precursors (Subramanian *et al*., 2011). Finally, in addition to promoting primary opening of *Gfap* chromatin, *Nf1a* might further sustain transcription of this chromatin at more advanced steps of astroglial differentiation, synergizing with its later activated paralogs, *Nf1b, Nf1c* and *Nf1x* (Wilczynska *et al*., 2009).

As for Fgf2, it was reported that the exposure of E18 rat cortico-cerebral precursors to this GF for as little as 6 hours makes them responsive to a subsequent 24 hours Cntf stimulation. This results into a remarkable upregulation of *Gfap*, which, conversely, does not respond to the Cntf treatment alone. Consistently, these precursors, exposed to combined Fgf2 and Cntf for 6 days, give rise to 3-fold less neuronal clones and 2-fold more astrocytic clones. Concerning mechanisms of action of Fgf2, this factor does not influence expression or phosphorylation of Stat3. As proven by ChIP, it conversely triggers the replacement of H3K9me2 with H3K4me2 at the pStat3-BS of the *Gfap* promoter. However how does it happen, it is not known (Song and Ghosh, 2004). Moreover, Fgf2, stimulating proliferation, contributes to the "dilution" of the 5methylcitosines originally clustered at the *Gfap* and *S100β* promoters, which occurs upon the Notch-induced detachment of Dnmt1 from these promoters (Namihira *et al*., 2009). In these ways, Fgf2 allows pStat3 to bind to the astrocytic promoters and stimulate transcription (Song and Ghosh, 2004; Namihira *et al*., 2009).

Among agents promoting the opening of astrocyte genes chromatin, there are also the two TF genes *Coup-tf1* and *2*. *Coup-tf*s are widely expressed in the telencephalic ventricular zone, where they display a transient peak around E12.5. Their involvement in astrogenesis was recognized by an elegant shRNA screening, run in embryoid body- (EB)-derived neurospheres. It was found that shRNA-mediated silencing of *Coup-tfs* juvenilizes these cultures, increasing the neuronal output obtainable from tertiary neurospheres under Lif/Bmp stimulation, and reducing the astrocytic one. Consistently, lentivirus-mediated repression of *Coup-tf*s in cortico-cerebral precursors, from E12.5 onward, resulted - at P20 - in doubling of their neuronal progenies as well as in a dramatic shrinkage of their astrocytic output. Moreover, when *Coup-tf*s knock-down was anticipated to E10.5, then the switch from deep layer- to superficial layer-neurons production was defective too. This suggests that beyond astrogenesis activation, these two factors may play a more general role as promoters of pan-histogenetic progression. Interestingly, however, neither *Coup-tf1* not *Coup-tf2* are sufficient to anticipate the onset of astrogenesis, so pointing toward a permissive rather than instructive role. Remarkably, the two Coup-tfs exert only a moderate influence on the expression levels of key trans-active factors modulating astrogenesis. Their impact on gliogenesis is probably mediated by the epigenetic changes they evoke on the chromatin of astrocytic genes. That has been shown at the level of the pStat3-BS the *Gfap* promoter. Here, their knock-down upregulates H3K9me2 levels, at expenses of H3K4me2 and acetylated-histone 3 (H3ac), and leads to increased CpG methylation. However these effects are not direct. In fact, no straight interaction of Coup-tfs with the *Gfap* promoter was detected and both Coup-tfs were shown to play their pro-gliogenic role as transcriptional repressors (Naka *et al*., 2008).

Retinoic acid (RA), administered to murine E14.5 telencephalic precursors, synergizes with Lif in inducing the activation of the *Gfap* gene (within as little as 2 days). In the absence of RA, the RARαβ/RXR complex sits on a retinoic acid responsive element (RARE) located at circa 2.5kb upstream of the *Gfap* transcriptional start site, possibly recruiting specific corepressors (NCoR and/or silencing mediator for retinoid or thyroid-hormone receptors (SMRT)) to the chromatin. This makes chromatin hypoacetylated, both near the RARE and around the major STAT3 BS, at -1.5kb, and inhibits transcription. Upon binding of RA to RARα, the RARαβ/RXR complex releases the corepressor(s) and recruits the histone acetyl transferase (HAT) E1A binding protein p300/CRB binding protein (p300/CBP), so increasing histone acetylation at both -2.5kb and -1.5kb. This makes the pStat3-BS suitable to be bound by pStat3 and leads to LIF-dependent transcriptional activation (Asano *et al*., 2009).

ESET mRNA, encoding for the histone methyl transferase KMT1E, is expressed by the neuronogenic cortico-cerebral ventricular zone at progressively decreasing levels, almost disappearing prior to birth. Its experimental inactivation derepresses transcription of non-neural genes and endogenous retroviral (ERV) sequences and distorts temporal articulation of the cortical histogenetic schedule. *ESET* ablation anticipates both upper layer neurons generation and astrogenesis onset. Moreover, it hastens the subsequent increase of astrogenic rates. Conversely, in utero electroporation of *ESET* antagonizes astrogenesis, although not fully suppressing it. KMT1E binds to the *Gfap* promoter, it is necessary for its H3K9 trimethylation and prevents its H3K9 acetylation. As such, it contributes to the complex machinery which controls timed opening of astroglial genes chromatin and allows timely activation of astroglial programs (Tan *et al*., 2012). Based on the detection of a number of Hes1-binding sites in its promoter, it has been suggested that the *ESET* transcription unit might "sense" cell-cycle-locked Hes1 pulsation, integrate digital information encapsulated within such pulsation and convert such information into a cell-cycle number-dependent analog output, in charge of modulating the rates of the histogenic processes (Tan *et al*., 2012).

Finally, it is worth mentioning that a supportive role for astrogenesis is played by *Brg1*, encoding for one of the two catalytic

ATPase subunits of the SWI/SNF chromatin remodeling complex (Matsumoto *et al.*, 2006). *Brg1* is expressed by cortical neurons and is activated in the cortical ventricular zone after E13.5. Its early ablation impairs self-renewal of neural precursors and results into a depleted astrogenic pool, as proven by the dramatic downregulation of CD44, S100β and Gfap. Interestingly, if *Brg1* ablation is postponed (by infecting E14.5 *Brg1*$^{fl/fl}$ cortico-cerebral precursors with adenoviral cre-expressors, *in vitro*), then the proliferating pool is spared, however its astroglial differentiation is still very poor, suggesting a specific involvement of this gene in activation of the astrogenic program. Molecular details of this involvement are presently unknown (Matsumoto *et al.*, 2006).

Regulation of astrocyte committed progenitor proliferation

Our knowledge of such regulation is still very poor and only a few genes have been implicated in it. Fgf2 and Egf stimulate proliferation of neonatal rodent astroblasts (Mayer *et al.*, 2009; Riboni *et al.*, 2001). Fibroblast growth factor 9 (Fgf9) promotes a substantial expansion of the perinatal astrogenic proliferating pool (Seuntjens *et al.*, 2009) and delays terminal differentiation of mature astrocytes (Lum *et al.*, 2009). Smad interacting protein 1 (Sip1) TF limits astroblasts proliferation, possibly by inhibiting Fgf9 expression (Seuntjens *et al.*, 2009). A reduction of late astrocyte progenitors, compared to early, NSC-born ones, occurs upon overexpression of the TFs Foxg1 and Empty spiracle homolog 2 (Emx2) in NSCs, suggesting that astrocyte progenitors might undergo precocious terminal differentiation (Brancaccio *et al.*, 2010).

Finally, it has been shown that cortical ablation of the TF Olig2 leads to a dramatic upregulation of *Gfap* in the neocortical postnatal grey matter, pointing to a possible prolonged persistence of astrocytes in a proliferating state. However, kinetic analysis of *Olig2*-LOF mutants ruled out this hypothesis, suggesting that this phenotype might rather reflect defective *Gfap* silencing in S100β$^+$ cells of grey matter (Cai *et al.*, 2007). Conversely *Olig2*, upregulated in reactive adult astrocytes, is strictly necessary to sustain their proliferation and the formation of the glial scar (Chen *et al.*, 2008).

Concluding remarks

In synthesis, several interlaced molecular machineries regulate proper progression of cortico-cerebral astrogenesis.

Astrogenesis timing is firstly determined by temporally regulated accessibility of astrocytic promoters to transactive complexes stimulating their transcription. Undoubtedly, this is the main factor preventing precocious astrocyte generation within the early neuronogenic pallium. Distinct machineries, acting on both DNA and histones, contribute to such regulation. Three factors promote DNA demethylation at astrocytic promoters: the RBPJk target Nf1a removes the methylating Dnmt1 enzyme from these promoters; Fgf2 likely dilutes previously acquired C5-methyl marks; Couptf1,2 act according to still unknown mechanisms. As for histones, three factors promote a transcription-prone profile: Fgf2 and Couptf1,2 induce the replacement of H3K9-me2 by H3K4-me2; RA, secreted by telencephalic menginges, evokes H3 hyperacetylation. Conversely, progressive downregulation of the H3K9 trimethylating enzyme ESET possibly contributes to sharp astrogenesis activation in neural precursors which have completed neuronogenesis.

Moreover, temporal tuning of astrogenic rates is further refined by regulated firing of a specific transactive pathways impinging on astroglial promoters.

Astrocyte-specific transcriptions are mainly promoted by Ct1-family cytokines, via the intracellular Jak/Stat transducing axis. In this respect, both ligands availability and neural precursors responsivity to them are crucial to proper temporal progression of astrogenesis. Astrocyte-specific transcriptions are triggered by neuron-secreted Ct1, accumulating by the end of neuronogenesis, and they are later potentiated by Cntf, released by newborn astrocytes. Besides, sensitivity of neural precursor to these cytokines, very low around the onset of neuronogenesis, gets higher and higher, as neuronogenesis proceeds. This is due to progressive autoactivation of the Jak/Stat transduction axis, via pStat3-dependent transcription of *gp130*, *Jak1*, *Stat1* and *Stat3*, as well as to the effects of additional regulatory branches feeding this axis. These are: Mek/Erk and Egf/Tgfα signalling (arising during prenatal life), which upregulate *gp130* and *Stat3*, respectively; Hes1,5 (expressed by apical precursors in response to neuronal-progenitor Delta signalling), which ease pJak2-dependent Stat3 phosphorylation; and p19 (arising in the late pallium upon decrease of it early repressor N-myc), which also facilitates pJak2-dependent Stat3 phosphorylation.

On the other side, two additional transactive agents, expressed at higher level during early neuronogenesis and fading out around its completion, Neurogenins (Ngns) and ErbB4, antagonize astrogenesis, preventing its large scale activation around mid-neuronogenesis. Ngns inhibit transcription of four key effectors of the Ct1 transduction axis (*gp130*, *Jak1*, *Stat1* and *Stat3*), counteract pJak2-dependent Stat3 phosphorylation and further compete with pStat1,3 for limited amounts of the p300/CBP-pSmad2,4 complex, available upon Bmp signalling. The intracytoplasmic domain (ICD) of ErbB4, detaching from cell plasma membrane upon Nrg1 stimulation, promotes the translocation of the transcriptional corepressor NCoR into the nucleus, where it contributes to inhibit *Gfap* transcription, being likely conveyed to its promoter by an RBPJk bridge.

Finally, relatively little is known about mechanisms which, once macroglial differentiation has been initiated, modulate proliferation and differentiation rates of astrocyte-committed progenitors, so dictating the final astroglial output of the system. A few transcription factors (Sip1, Emx2 and Foxg1) as well a few secreted ligands (Fgf9 and Tgfα) have been implicated in this issue. However, molecular details of their action as well as their reciprocal epistatic relationships were poorly clarified. They will be likely subjects of future investigations.

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