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Glial cells in non-germinal territories: insights into their stem/progenitor properties in the intact and injured nervous tissue

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ABSTRACT

In the adult murine central nervous system (CNS), the germinal astroglia residing in the subependymal zone of the lateral ventricles and in the subgranular layer of the hippocampal dentate gyrus behaves as neural stem cells actively undergoing neurogenesis and gliogenesis. Although neurogenesis does not normally occur outside the germinal niches, two types of parenchymal glial cells, namely astrocytes and NG2-expressing cells, display distinct progenitor activities in the intact brain or upon injury. Importantly, in defined experimental conditions both cell types reveal the potential to behave as multipotent stem cells suggesting that the mature CNS parenchyma may retain a latent stem cell potential, normally inhibited in vivo that, if properly evoked, might be exploited in situ for endogenous cell replacement following injury. In this review we scrutinise recent studies focussing on (i) the molecular and functional relationships between precursors in germinal niches and non-germinal areas; (ii) the ability of adult parenchymal glia to undergo lineage transgressions and neurogenesis in the intact brain and upon CNS injury. We also compare evidence for lineage plasticity in astrocytes or NG2+ cells, and discuss possible approaches for the implementation of stem/progenitor cell capabilities in non-germinal glial cells.

Key words
Astrocytes • Neurogenesis • NG2 • De-differentiation • Lineage transitions

Abbreviations
BMP, bone morphogenic protein; CNS, central nervous system; EGF, epidermal growth factor;
FGF, Fibroblast growth factor; GFAP, glial fibrillary acidic protein;
PDGFRa, plateled-derived growth factor alpha; SEZ, subependymal zone

1. Introduction

Adult neural stem cells actively undergoing neurogenesis and gliogenesis are retained only in two regions of the murine central nervous system (CNS): the subependymal zone of the lateral wall of the lateral ventricles (SEZ), and the subgranular layer of the hippocampal dentate gyrus (SGL) (Doetsch et al., 1999a,b; Seri et al., 2001; Kriegstein and Alvarez-Buylla, 2009). As it also occurs during development, defined glial subsets constitute the primary progenitors at these germinal sites (see below). Outside these "typical" germinal niches no constitutive neurogenesis takes place under normal conditions in the mature parenchyma with few exceptions amongst mammals (see Ponti et al., in this number). However, it is now known that the mature nervous parenchyma retains some active proliferation and glial turnover, sustained by persisting glial progenitors. Furthermore, in defined culturing conditions parenchymal cells (see for review Buffo et al., 2007 and below) show properties similar to neural pro-

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genitors: they self-renew and generate cells of all the three neural lineages, namely astroglia, oligodendroglia and neurons. On these bases, the idea emerged that the non-germinal CNS may be endowed with a latent stem cell potential, normally repressed *in vivo*, that, if properly evoked, might be exploited *in situ* for reparative purposes.

In this paper we illustrate the features of neural stem/progenitor cells in typical germinal niches and at parenchymal non-germinal sites. Furthermore, we provide an overview on recent studies on the differentiation potential of parenchymal precursors focussing on their capability to undergo lineage transgressions and neurogenesis at specific sites or in defined experimental conditions ("atypical germinal locations"). We also summarise data on the relationship between precursors in germinal niches and non-germinal areas and scrutinise the molecular mechanisms possibly regulating stem cell behaviours in the nervous parenchyma in view to propose targets for the development of novel therapeutic approaches.

2. Glial cells, stem cells and progenitors: functions and phenotypes

Glial cells are essential cellular components of the nervous tissue during both development and adulthood. Cells with radial morphology and glial identity ("radial glia") are ubiquitous scaffolds of the forming neuroepithelium (revised in: Mori et al., 2005; Pinto and Götz, 2007) while in the mature CNS glial elements of the astroglial and oligodendroglial lineages (Nishiyama et al., 2005) make up about 90% of all cellular components. For a long time, glia cells have been viewed as purely passive constituents of the nervous system, simply absolving supportive functions for neurons. More recently, a variety of unexpected roles have been recognised for glia, including an active contribution to information processing (Kettenmann and Verkhratsky, 2008; Bakiri et al., 2009) and, surprisingly, neural stem/progenitor cell functions (Kriegstein and Alvarez-Buylla, 2009). As described in details below, at germinal sites astroglial subsets display the hallmarks of stem cells: i) capability to perpetuate themselves throughout the life of the animal, and ii) multipotency, i.e. capability to give rise to multiple cell lineages (Weissman, 2000). Accordingly, they are the at the source of a progeny including other proliferating progenitors, presumably endowed with a more restricted fate and capable of dividing for a limited number of times, and postmitotic end products that proceed to differentiation. In basal conditions, another glial type identified on the basis of the expression of the NG2 chrondroitin sulphate proteoglycan comprises the major population of actively cycling progenitors outside the germinal niches in the adult CNS (Horner et al., 2000; Dawson et al., 2003; Buffo et al., 2005). These cells are also called polydendrocytes to highlight their stellate morphology (Nishiyama et al., 2002), or synantocytes (Butt et al., 2002) for their contiguity to neurons, or oligodendrocyte progenitor cells because found capable of generating myelinating oligodendrocytes (Stallcup and Beasley, 1987; Horner et al., 2000; Reynolds et al., 2002).

2.1. Glial stem/progenitor cells in typical germinal niches

During CNS development, radial glial cells were found to undergo active proliferation (reviewed in Pinto and Götz, 2007) and to serve as primary multipotent progenitors leading to the initiation of lineages forming both neurons and glial cells (Malatesta et al., 2003; Anthony et al., 2004; Costa et al., 2009). They self-renew in the embryo, produce intermediate progenitors and terminally differentiated derivatives until they transform into parenchymal astrocytes, ependymal cells, or continue their stem activity originating adult stem cells in the SEZ and SGL (see below, Bonfanti and Peretto, 2007; Kriegstein and Alvarez-Buylla, 2009). In physiological conditions, adult primary progenitors exhibiting structural and biological markers of astrocytes (see Table I, Doetsch et al., 1999a,b; Seri et al., 2001, 2004; Garcia et al., 2004) proliferate slowly, self-renew throughout life, and generate actively dividing cell intermediates that function as transit amplifying progenitors. The latter ones have distinct features in ventricular and hippocampal germinative areas (Kriegstein and Alvarez-Buylla, 2009). They produce immature neuroblasts migrating in chains to the olfactory bulb in the SEZ (Doetsch et al., 1999b), while in the hippocampus generate predominantly dentate gyrus granule neurons (Seri et al., 2004). SEZ intermediates also generate myelinating oligodendrocytes, NG2-positive (+) parenchymal glia (Menn et al., 2006) and possibly non-germinal astrocytes (Hack et al., 2005). Although

Table I. Marker expression in adult germinal and parenchymal glia *in vivo*.

	GERMINAL GLIA		NON-GERMINAL GLIA					
			Astrocytes			NG2+ cells		
Marker	Astrocytes	NG2+ germinal intermediates	GM	WM	LESION	GM	WM	LESION
GFAP	+	–	+/-	+	++	–	–	+/–
vimentin	+	nd	+/–	+	+	–	–	+/–
Tenascin C	+	– *Kazanis et al., 2007*	+/–	+/–	++	– *Czopka et al., 2009*	– *Zhao et al., 2009a*	– *Zhao et al., 2009a*
BLBP	+	nd	+/–	+/–	+	– *Schmid et al., 2006*	– *Schmid et al., 2006*	nd
S100b	–	–	+	+	+	+/–	+/–	+/– *Sellers et al., 2009*
GS	+	nd	+	+	+	+/–	nd	nd
GLAST	+	–	+	+	+	–	–	nd
GLT1	+	nd	+	+	+	nd	nd	nd
EGFR	+	+/–	–	–	+	–	+/–	nd
FGFR	+	nd	+	+	++	+	+	+
NG2	–	+	–	–	–	+	+	++
PDGFRa	+/–	+	–	–	– *Chen, et al., 2008*	+	+	+/–
Sox2	+	+	+	+	nd	–	–	nd
Sox9	+	+	+	+	nd	–	–	nd
Musashi	+	– (but espressed in some intermediates)	+	+	+	–	–	–

nd, not described; +, present in most cells; ++, upregulated; –, absent; +/–, present in a minor fraction of cells.

Abbreviations: GFAP, glial fibrillary acidic protein; BLBP, brain lipid-binding protein; GS, glutamine synthetase; GLAST, glutamate aspartate transporter; GLT1, glutamate transporter 1; EGFR, epidermal growth factor receptor; FGFR fibroblast growth factor receptor; PDGFRa, plateled-derived growth factor alpha; GM, grey matter; WM, white matter.

References not included in the table can be found in the text.

it is not known whether all adult neural stem cells generate both neurons and glia *in vivo*, or distinct neuronal and glial subsets exist, as a group germinal astrocytes display multipotency and show a high degree of lineage and cell plasticity upon appropriate instruction (Hack et al., 2005; Colak et al., 2008; Brill et al., 2008, Jessberg and Gage, 2008). Germinal niches do not harbour only glia with astrocytic traits: NG2+ cells also populate the SEZ (see Table I; Aguirre et al., 2004a) and were proposed to belong to intermediate progenitors capable to originate neurons in the postnatal olfactory bulb and in germinal niches upon transplantation (Aguirre et al., 2004a,b; see also below). These findings built up the view of parenchymal NG2+ cells as intermediate progenitors located at non-germinal sites.

The finding that glia exerts active stem/progenitor functions at germinal sites (germinal glia) triggers several key questions on the biology of glia cells in other territories (parenchymal/non-germinal glia), with broad implications for possible reparative interventions. Namely, which are the similarities between germinal and non-germinal glia, and is there any condition in which also parenchyma glia can reveal stem/progenitor functions?

2.2. Glial cells at non-germinal sites in the intact nervous tissue

Are glia cells at non-germinal sites terminally differentiated end products, or are they endowed with active or potential stem/progenitor properties? Astrocytes house-keeping functions are well established, ranging

from metabolic support and modulation of electrical activity to orchestration of the nervous tissue healing after injuries (Kettenmann and Verkhratsky, 2008; Buffo et al., 2010). While all these functions point to a remarkable adaptive properties and, possibly, heterogeneity (see Table I), parenchymal astroglia constitutes only a small proportion amongst the actively dividing cells of the intact adult CNS (Horner et al., 2000; Buffo et al., 2005, 2008), showing that quiescent non-germinal astrocytes lack one of the key feature of progenitors, namely the capability to divide. Thus, parenchymal astroglia appears engaged in fully postmitotic functions, just as mature oligodendrocytes and neurons. On the contrary, NG2+ cells exhibit a significant proliferative activity (Horner et al., 2000; Dawson et al., 2003; Buffo et al., 2005) despite also participating in a bidirectional interplay with neurons as indicated by neurotransmitter receptor expression, synaptic associations with axons and, to some extent, generation of active responses (reviewed in Bakiri et al., 2009; Nishiyama et al., 2009). Thus, in the mature CNS, astrocytes and NG2+ cells have dissimilar progenitor behaviours. One way to gain insights into the possible mechanisms subserving this diversity is to look at their lineage and morphochemical contiguity to germinal glia.

2.3. Lineage relationships and molecular similarities between germinal and non-germinal glia

Anatomical and fate mapping studies together with visual inspection of living cells demonstrated that perinatally radial glia cells directly transform into adult germinal (Merkle et al., 2004, 2007; Ventura and Goldman, 2007; Bonfanti and Peretto, 2007) and non germinal astrocytes (Voigt, 1989; Noctor et al., 2004; Barry and McDermott, 2005) and ependymocytes (Spassky et al., 2005). At some sites parenchymal astrocytes with radial morphology persist (e.g. Bergmann glia and retinal Muller cells), although the maintenance of a radial shape does not per se imply the persistence of progenitor activity. Sources other than direct transformation of radial glia are also known for parenchymal astrocytes, including ventricular (Noctor et al., 2004; Burns et al., 2009) and cortical (Costa et al., 2007) intermediate glial precursors, likely derived from pallial and subpallial radial glia. The heterogeneity of location and specification mechanisms (Marshall et al., 2003; Cai et al., 2007; Hochstim et al., 2008) displayed by astrocyte precursors highlights the concept of parenchymal astroglia diversity and prompts the questions of its functional relevance.

At difference with astrocytes, no direct conversion of radial glia cells into NG2+ cells has so far been demonstrated. Ventricular progenitors are never found to express NG2 that rather appears during the maturation of intermediate committed progenitors of the oligodendroglial lineage (Zhu et al., 2008; Nishiyama et al., 1996). The ventricular sources of such NG2+ intermediates likely comprise radial glial subsets at late developmental stages (Malatesta et al., 2003; Ventura and Goldman, 2007). In the adult brain, germinal astroglia contributes callosal NG2+ cells in normal and demyelinated states (Menn et al., 2006), and parenchymal proliferation of NG2+ cells likely participates in the maintenance of the NG2+ pool. As for astroglia, whether distinct sources of NG2+ cells produce functionally equivalent progenies remains to be determined. In summary, while for parenchymal astrocytes a direct origin from primary radial glia progenitors by cellular morphological conversion is assessed, NG2+ cells are generated through intermediate progenitors, indicating for these cells a broader divergence from the astroglial lineage containing neural stem cells.

The close lineage contiguity of germinal and non-germinal astroglia is reflected by their molecular kinship, as summarised in Table I. Adult germinal astrocytes exhibit an immunophenotypical profile resembling that of radial glial cells and maintained in non-germinal astrocytes at immature stages (for reviews see: Mori et al., 2005; Pinto and Gotz, 2007; Wang and Bordey, 2008). However, during maturation some precursor/immature molecular features (e.g. Tenascin C, vimentin, see Table I) undergo downregulation and become induced again only during the gliotic reaction to injury (see section 4.1). Conversely, non-germinal astrocyte maturation coincides with increasing levels of the beta subunit of the calcium binding protein S100beta (S100b), which is found in radial glia, but undergoes downregulation at adult germinal sites (Raponi et al., 2007). No expression of germinal markers is found in NG2+ glia, whereas minor subsets of these cells co-express some astroglial proteins (see Table I). However, a fraction of germinal astrocytes in the SEZ expresses the alpha receptor for the Plateled

Derived Growth Factor (PDGFRa) (Jackson et al., 2006), which is one of the most typical features of NG2+ cells (Table I) mediating their survival and proliferation (Nishiyama et al., 2009). Notably, PDGFRa+ germinal astrocytes are capable to give rise to both neurons and oligodendrocytes, suggesting that PDGFRa signalling might regulate the balance between neurogenesis and oligodendrogliogenesis (Jackson et al., 2006). Conversely, parenchymal NG2+ cells may resemble the fraction of NG2+ germinal cells identified by enhanced Green fluorescent protein (eGFP) expression under the *2',3'-cyclic nucleotide 3'-phosphodiesterase* (*cnp*) promoter activity that was reported to produce both neurons and glia (Aguirre et al., 2004a,b). This finding might suggest that some neurogenic potential is also retained in non-germinal NG2+ glia. However, it is actually controversial whether this SEZ NG2+ fraction truly belongs to intermediate progenitors, given their lower proliferation rate compared to the other SEZ intermediates, and their lack of neurogenic activity when fate-mapped on the basis of *ng2* gene activity (see also Table III, cfr. Aguirre et al., 2004a; Zhu et al., 2008; Komitova et al., 2009; Platel et al., 2009). Thus, these cells may just be parenchymal NG2+ glia dispersed amongst germinal cells. Overall, this marker survey underlines that parenchymal astrocytes display a level of similarity to germinal astroglia that is higher compared to NG2+ cells. Nevertheless, this evidence is based on structural similarities and remains poorly informative on stem/progenitor functions.

More relevant it can be the analysis of markers and signals related to germinal functions, such as stem cell maintenance or specification (see Table I and II). As transductors active at germinal sites (Doetsch et al., 2002; Pastrana et al., 2009), Epidermal Growth Factor receptors (EGFRs) are similarly absent in both astrocytes and NG2+ cells (Aguirre et al., 2005; Liu et al, 2006), whereas basic Fibroblast growth factor receptors (FGFRs) are expressed by the two types of non-germinal glia (see Table I, Redwine et al., 1997; Chadashvili and Peterson, 2006). On the contrary, stem/progenitor-related transcription factors and regulatory molecules (Sox2, Sox9, Musashi) are similarly co-expressed by germinal and parenchymal astroglia, but are absent in NG2+ cells (see Table I Sakakibara and Okano, 1997; Pevny and Nikolis, 2010; Lyssiotis al., 2007; Komitova and Eriksson 2004; Wagner and Stolt, 2005; Cheng et al., 2009). Other germinal signals and morphogens (see Table II) appear instead equally inactive in both quiescent astrocytes and NG2+ cells. On the whole, despite the absence of active germinal signalling, marker-based analysis confirms that in intact conditions non-germinal astrocytes may harbour a neural progenitor potential broader than NG2+ cells.

2.4. Functional similarities between germinal and non-germinal glia in the intact nervous tissue

The development of approaches to disclose stem cell potentialities in niche-like *in vitro* conditions (i.e. the neurosphere assay, see Box 1), together with the advances of mouse genetics allowing permanent tagging of defined cell populations (see Box 2) have led to better define the stem/progenitor functions of non-germinal glia.

2.4.1. *Ex vivo* stem cell properties and neurogenic activity

Typical of stem/progenitor cells, postnatal parenchymal astrocytes actively divide (Burns et al., 2009) and display a considerable degree of lineage plasticity, as demonstrated *in vitro* by the possibility to reprogram them into spiking neurons by viral-mediated overexpression of neurogenic transcription factors (Heins et al., 2002; Berninger et al., 2007). Further, both germinative and immature parenchymal astroglia reveal the two "key-features of stem cells", namely self-renewal and multipotency *in vitro* in the neurosphere assay (Laywell et al., 2000; Wang and Bordey, 2008; see Table III). Notably, mouse parenchymal astrocytes give rise to neurospheres up to the second postnatal week but by large lack this property at mature stages (Laywell et al., 2000; Buffo et al., 2008; Jiao and Chen, 2008), indicating that they transiently maintain a neural stem cell potential.

Although no data are so far available on overexpression of neurogenic factors in NG2+ glia, *in vitro* studies indicate a broad differentiation potential for postnatal NG2+ cells (Table III). Originally, these cells were shown to differentiate into mature oligodendrocytes *in vitro* (Stallcup and Beasley, 1987). However, in postnatal optic nerve cultures a fraction of NG2+ cells coexpressing glial fibrillary acidic protein – GFAP- (O2A cells, Raff et al., 1983) behaves as a bipotent source for oligodendro-

Table II. - Signalling pathways at germinal niches and in quiescent and reactive glial cells in the adult brain or spinal cord. The table illustrates *in vivo* data on the activity of distinct signalling pathways and the expression of their molecular components (ligands and receptors).

	GERMINAL GLIA		NON-GERMINAL GLIA					
			Astrocytes			NG2+cells		
Signalling Pathways	Astrocytes	NG2+ germinal intermediates	GM	WM	LESION	GM	WM	LESION
Shh	**active**, Shh+ *Palma et al., 2005; Ahn and Joyner 2005; Han et al., 2008*	Nd (active in some germinal intermediates) *Palma et al., 2005; Ahn and Joyner 2005*	**inactive**, Shh– *Amankulor et al., 2009*	**inactive**, Shh– *Amankulor et al., 2009*	partly **active**, Shh+ (likely active in Olig2+ astrocytes) *Amankulor et al., 2009*	**inactive**, Shh– *Amankulor et al., 2009*	**inactive**, Shh– *Amankulor et al., 2009*	**active**, Shh– *Amankulor et al., 2009*
Wnt	**active**, Wnt+ *Shimogori et al., 2004; Adachi et al., 2007 (β-cat activity); White et al., 2010 (β-cat activity)*	nd	**active** in a minor fraction, Wnt– *Shimogori et al., 2004; White et al., 2010*	**active** in a minor fraction, Wnt– *Shimogori et al., 2004; White et al., 2010*	partly **active**, Wnt nd *White et al., 2010*	**active** in a minor fraction, Wnt– *Shimogori et al., 2004; White et al., 2010*	**active** in a minor fraction, Wnt– *Shimogori et al., 2004; White et al., 2010*	partly **active**, Wnt nd *White et al.,2010*
Notch	**active**, NotchR+ *Givogri et al., 2006*	nd	**inactive**, NotchR– *Chen et al., 2005*	**inactive**, NotchR– *Chen et al., 2005*	activity nd, NotchR+/-, Notch+ *Stidworthy et al.,2004; Chen et al., 2005*	**inactive**, NotchR– *Chen et al., 2005*	**inactive**, NotchR– *Chen et al.,2005*	activity nd, NotchR+ *Stidworthy et al., 2004;* NotchR– *Chen et al., 2005*
BMPs	**active**, BMP+ *Colak et al., 2008*	nd (active in some germinal intermediates)	**inactive**, BMP nd *Colak et al., 2008*	**inactive**, BMP nd *Colak et al., 2008*	**active**, BMP+ *Chen et al.,2004*	nd	nd	**inactive**, BMP nd *Hampton et al., 2007;* BMP+ *Sabo et al., 2009*

nd, not described or proven *in vivo*; active, active pathway, inactive, inactive pathway; R, receptor; +, expressed; –, not expressed; Shh, Sonic Hedgehog Homolog; Wnt, Wingless mammalian homolog; β-cat, β-catenin; BMPs, Bone Morphogenic Proteins.

cytes and astrocytes, depending on the culturing conditions. In further studies, postnatal NG2+ cells could be reprogrammed *in vitro* to neural stem-like cells differentiating into neurons, astrocytes and oligodendrocytes after undergoing an astrocytic stage upon exposure to Bone Morphogenic Proteins (BMPs) and PDGFa (Kondo and Raff, 2000). Cells isolated from the early postnatal forebrain that displayed *cnp* promoter activity and expressed NG2 were also shown to generate multipotent neurospheres *in vitro* (Table III, Belachew et al., 2003). Similar cells from the adult human subcortical white matter displayed the same behaviour (Nunes et al., 2003). These data were interpreted as the demonstration that NG2+ cells at postnatal ages or at specific sites in the CNS have a stem cell potential and generate neurons (see Table III). However, in other studies early postnatal NG2+ cells selected by NG2-driven reporter gene expression were found exclusively gliogenic in conditions that normally disclose a stem cell potential (Zhu et al., 2008). Further, NG2+/PDFRa+ cells from the adult intact grey matter did not ensue multipotent neurospheres (Buffo et al., 2008), or were found to be predominantly oligodendrogliogenic (Jiao and Chen, 2008). These conflicting findings may be explained by selection of diverse populations, possibly including only in some cases NG2+ cells co-expressing the neuroblast marker doublecortin (Aguirre et al., 2004a; Tamura et al., 2007), known to retain some ability to form multipotent neurospheres (Walker et al., 2007). Despite both astrocytes and NG2+ cells appear endowed with a stem cell potential at defined ages or locations, these data collectively point to its progressive restriction during maturation and may indicate a predominant gliogenic potential for NG2+ glia.

Box 1. The neurosphere assay

This assay (Reynolds and Weiss, 1992) is commonly acknowledged to reveal the presence of cells with a stem cell potential. Cells are cultured at clonal densities in the presence of mitogens such as EGF and bFGF. Under these conditions responsive cells divide and generate clonal aggregates called neurospheres whose differentiation capabilities can be assessed upon mitogen withdrawal. If neurons, astrocytes and oligodendrocytes are produced from a single neurosphere, this offers evidence for multipotency of the neurosphere-founding cells. Likewise, when a neurosphere can give rise to secondary neurospheres after dissociation, this is taken as a proof of the self-renewing capacity of the cell originating the primary neurosphere.

2.4.2. *In vivo* progenies of non-germinal glia

Consistent with *in vitro* data (see section 2.4.1), some capacity for neurogenesis and oligodendrogliogenesis from immature postnatal astrocytes has been suggested *in vivo* in fate mapping analyses, where cells with active human *gfap* promoter have been genetically and permanently tagged and their progeny followed over time (hGFAPCreER^{T2} mice, Ganat et al., 2006; Silbereis et al., 2009). In the cerebral cortex at postnatal day 5 (P5) astrocytes generate other astrocytes, some oligodendrocytes and a small fraction of neurons (Ganat et al., 2006). Consistently, astrocytic cells resident in the perinatal cerebellar white matter are the source of GABAergic interneurons (see Leto et al. in this number) and glia cells until P12, when they switch to exclusive astrogliogenesis. Although these results may be indicative of multipotency *in vivo*, they do not provide a clear proof for it because the first study does not exclude the contribution of germinal astroglia to the observed lineages and the early cerebellar white matter is indeed a germinative territory, where niche factors may transiently preserve a specific germinal astroglia phenotype. At more mature ages, consistent with entrance in a post-mitotic phase, no significant changes were observed over time in the number and phenotype of astrocytes in the adult intact cortical grey matter after genetic tagging based on expression of the glutamate aspartate transporter GLAST or viral infection (Buffo et al., 2008). Local environmental signals promoting progenitor progression toward fully differentiated astroglial phenotypes as well intrinsic changes of competence including epigenetic modifications (Hirabayashi et al., 2009 and references therein) are likely to take part in the progressive demise of active stem/progenitor traits in parenchymal astroglia.

The bi/multipotent behaviour of NG2+ cells *in vitro* (section 4.2.1) together with their active prolifera-

Box 2

Transgenic or knock-in mouse lines were generated that express the bacteriophage Cre recombinase driven by various promoters active in NG2 cells. When these mouse lines are crossed to strains where reporter gene expression is regulated by a stop-cassette floxed by LoxP sites, Cre recombinase acts by excising the stop cassette thereby permanently allowing expression of the reporter in Cre+ cells that also inherit this expression to their progeny. In some lines, Cre recombinase is fused to the mutated ligand-binding domain of the oestrogen receptor engineered to bind Tamoxifen (ERT) with a higher affinity than endogenous estradiol. Notably, this receptor fused to Cre is able to translocate to the nucleus thereby carrying Cre to its target for recombination only upon Tamoxifen binding. These lines where Cre nuclear action is only triggered upon administration of Tamoxifen to the animals are termed “inducible”.

Table III. - Stem/precursor properties of germinal and non-germinal glia in the adult CNS.

	GERMINAL GLIA		NON GERMINAL GLIA					
			Astrocytes			NG2+ cells		
	Astrocytes	NG2+ germinal intermediates	GM	WM	LESION	GM	WM	LESION
Proliferation	+ *(expression data)*	++ *(expression data)* – *(expression data)*	– *(expression data and fate-mapping)*	– *(expression data and fate-mapping)*	+/– *(fate-mapping)*	+* *(expression data and fate-mapping)*	++* *(expression data and fate-mapping)*	++* *(expression data and fate-mapping)*
Neurogenesis	+ *(fate-mapping)*	+ *(expression data)* – *(fate-mapping)*	– *(fate-mapping)*	– *(fate-mapping)*	– *(fate-mapping)*	+ *(expression data; CX, STR)* – *(fate-mapping; CX, SC, CRB)*	+ *(expression data CX)* – *(fate-mapping)*	– *(fate-mapping, CX)*
Oligodendrogenesis (generation of myelinating oligodendrocytes)	+ *(fate-mapping)*	+ *(expression data)*	– *(fate-mapping)*	– *(fate-mapping)*	– *(fate-mapping)*	– *(fate-mapping; CX)* + *(fate-mapping; SC, CRB)*	+ *(fate-mapping)*	+/– *(expression data; WM CX, SC)* – *(fate-mapping; GM CX)*
Astrogliogenesis	+ *(fate-mapping)*	nd	– *(fate-mapping)*	– *(fate-mapping)*	+ *(fate-mapping)*	– *(fate-mapping; CX)* + *(fate-mapping; VL TEL and SC)*	– *(fate-mapping)*	+ *(expression data; CX)*
Capability to form multipotent neurospheres *in vitro*	+ adult and postnatal	+ adult and postnatal	– adult	nd adult	+ adult	– adult	+ adult	– adult
			+ postnatal		nd postnatal	+ *postnatal (EGFP+ from Cnp-EGFP mice)* – *postnatal (DsRED+ from NG2DsRedBAC mice)*		nd postnatal

CX, cerebral cortex; STR, striatum; SC, spinal cord; CRB, cerebellum; VL TEL, ventrolateral telencephalon; GM, gray matter; WM, white matter; nd, not described; +, present; –, absent.

References can be found in the text. * The "+s" here underline that, at difference with astroglia, NG2+ cells comprise the majority of proliferative cells in the mature nervous tissue. However, the actively cycling cells are only a fraction of the NG2+ population (see text), suggesting heterogeneous proliferative capabilities.

tion *in vivo* has triggered intense investigation on the *in vivo* fate of NG2+ cells. While classical approaches including proliferation studies, expression analysis and grafting experiments (revised in Nishiyama et al., 2009) consolidated the view of NG2+ cells as endogenous reservoir of oligodendrocytes during development and adulthood, the issues of the capability of NG2+ cells to undergo astrogliogenesis or neurogenesis *in vivo* still remain open. Absence of marker co-expression in intact conditions built up to the notion that NG2+ cells and astrocytes are lineally segregated populations (see Table I, Nishiyama et al., 1996; Redwine et al., 1997). However, NG2+ cells can generate astrocytes when grafted in glia-depleted environments (Franklin and Bayley, 1995; Windrem et al., 2004), suggesting that they are inherently capable of astrogliogenesis but are prevented from this in the normal *in vivo* environment. Likewise, whereas *in vivo* correlative data reporting NG2 expression in adult newly generated/immature neurons (Dayer et al., 2005; Tamura et al., 2007) corroborated the view of NG2+ cells as neuronal parenchymal sources, other studies based on either expression analysis or NG2+ cell-specific reporter gene expression did not reproduce these findings (Zhu et al., 2008; Karram et al., 2008; Komitova et al., 2009).

A series of recent works addressed the issue of NG2+ cell differentiation *in vivo* by using the Cre-LoxP technology (see Box 2) to fate-map NG2+

cells. In studies including the analysis of NG2+ cells at early postnatal ages (NG2creBAC, Zhu et al., 2008; Plp-CreER^{T2}, Guo et al., 2009), the tagged cells were shown to generate other NG2+ cells, oligodendrocytes and a subpopulation of grey matter astrocytes in the ventral forebrain and spinal cord (Table III). Notably, no astrocytes were generated from NG2+ cells in the white matter under normal conditions, confirming heterogeneity of astroglia sources. One of the studies (Guo et al., 2009) also described the production of few neurons, suggesting some extent of neurogenesis from local NG2+ cells in the juvenile brain. Two other reports employed Cre-inducible mice to examined the fate of adult proliferative NG2+ glia expressing of the Olig2 transcription factor (Olig2::CreER^{TM,} Dimou et al 2008) or PRGFRa (Pdgfra-creER^{T2} Rivers et al., 2008). Both studies showed that NG2+ cells generated NG2+ glia or mature oligodendrocytes, but hardly any astrocytes. Notably, myelinating oligodendrocytes were produced only in the white matter, revealing distinct differentiation capabilities for grey and white matter NG2+ cells. In the progeny of recombined cells, Rivers and colleagues also detected a small number of projection neurons in the piriform cortex. However, the precise identity of these PDGFRa+ neuronal precursors and whether they express NG2+ remain to be assessed. Taken together, while all of these studies consistently support the oligodendrocyte fate of NG2+ cells, astrogliogenesis from NG2+ cells is confirmed at postnatal ages but it is not clearly proved during adulthood in intact conditions. Similarly, the data regarding neurogenesis are inconsistent. While these different results may be explained by differences in the specificity of Cre-targeting and in the efficacy of Cre induction, they may also reflect NG2+ cell functional heterogeneity, which is so far by large undisclosed.

3. The gliotic scenario

When vascular, traumatic, inflammatory or degenerative events occur in the CNS, while neurons and mature oligodendrocytes die or undergo regressive events, astrocytes and NG2+ cells set off a cytogenic and active response known as gliosis. In mammals the healing process produces signals largely restrictive or non-supportive for regenerative events such as axon re-growth or cell replacement (see Buffo et al., 2010). Nevertheless, in some injury conditions, regenerative attempts do occur. Amongst them, here we focus on de novo neuron generation and transitions between distinct glial lineages, whereas we leave aside remyelination attempts and failures, recently illustrated in several reviews (Franklin and ffrench-Costant, 2008; Nishiyama et al., 2009). After defined experimental lesions, low degrees of spontaneous neuron replacement have been attributed to local parenchymal reactions. For instance, upon discrete neocortical injury, few new neurons are generated concomitantly with the re-apperance of glial cells with radial progenitor traits, suggesting that injury-induced de-differentiatiation of resident astrocytes to a radial glia state may subserve local neurogenesis (Leavitt et al., 1999; Chen et al., 2004; Sirko et al., 2009). Also mild ischemic damage has been reported to trigger the production of a low number of GABAergic cortical interneurons from layer I cortical progenitors (Ohira et al., 2010). Further, indication for lineage transgressions after lesion comes from the detection of glia with astrocytic and NG2+ cell traits (Alonso et al., 2005; Zhao et al., 2009b). To possibly reveal spontaneous de-differentiation and multilineage fates, we will illustrate the modifications that injury induces in the phenotype and functions of astrocytes and NG2+ cells, and present data on their progenies and differentiation potential in pathological contexts.

3.1 Morphochemical changes, differentiation capability and stem cell potential in reactive glia

Although behaving as specialised post-mitotic cells under basal conditions, after injury parenchymal astrocytes undergo hypertrophy, hyperplasia, immunophenotypical and functional changes revealing the acquisition of traits of immature stages and stem cells (see Table I, II, Mori et al., 2005; Buffo et al., 2008; Wang and Bordey, 2008). Such changes include the upregulation of molecules contained in immature and germinal astroglia (see Table I, section 2.3 and Sun et al., 2005), such as EGFR (Liu et al., 2006; Codeluppi et al., 2009) and FGFRs (Messersmith et al., 2000; Chadashvili and Peterson, 2006). Importantly, fate-mapping analysis of quiescent astrocytes by astroglia-targeted viral vec-

tors and conditional activation of Cre in GLAST expressing cells (Buffo et al., 2008) has shown that they re-enter the cell cycle after a traumatic lesion, being thus able to resume the precursor function of cell proliferation. Despite partial de-differentiation to earlier phenotypes and acquisition of germinal traits, astrocytes reacting to injury produce only scar-forming astroglia and no cells of other lineages (Buffo et al., 2008). Nevertheless, when astrocytes tagged before injury were isolated from the lesioned grey matter and exposed to EGF and bFGF, they gave rise to self-renewing multipotent neurospheres generating astrocytes, oligodendrocytes and, most notably, neurons (Buffo et al., 2008, Table III). Of note, this response did not occur with cells isolated from the intact parenchyma. These data reveal that quiescent astrocytes responding to injury modify their biological status acquiring features and potential of neural stem cells. However, *in vivo* such potential is not expressed, possibly because of restrictive environmental signals (see below).

NG2+ cells also react to many pathological conditions by hypertrophy, NG2 upregulation and active proliferation (Keirstead et al., 1998; Reynolds et al., 2002; Buffo et al., 2005; Nishiyama et al., 2002). During their reaction, they maintain most of the traits typical of their quiescent state, including heterogeneity (see Table I and below). Some studies have described a limited degree of GFAP co-expression in proliferative NG2+ cells responding to injury (Alonso et al., 2005; Zhao et al., 2009b), suggesting the acquisition *in vivo* of a phenotype reminiscent of *in vitro* O2A cells (Raff et al., 1983), and the generation of scarring astrocytes. Yet, genetic tagging of NG2+ cells prior to a stab-wound demonstrated that their main progeny in the grey matter remained NG2+ glia, with no clear evidence for generation of astrocytes, no production of new neurons, and no effective remyelination (Dimou et al., 2008). However, Tatsumi and colleagues (2008) in the same conditional mouse line reported a significant production of scar-forming astrocytes from tagged cells after cryoinjury. This discrepancy can be due to distinct injury conditions implying that defined injury-related signals may instruct distinct glial fates in NG2+ cells, but also points again to the existence of various NG2+ subsets with specific differentiation capabilities (section 2.4.2). Therefore, it would be interesting to assess in other mutant lines (i.e. NG2creER™BAC, Trotter et al., 2010; Pdgfra-creER^{T2}, Rivers et al., 2008) whether diverse fates can be detected. Although there are no conclusive data on the bi/multipotent behaviour of NG2+ cells, evidence suggests that NG2+ cells possess a significant degree of plasticity between the oligodendroglial and astroglial lineages in the injured brain. Consistent with a gliogenic potential, when NG2+ cells were isolated from the injured neocortical grey matter on the basis of NG2 or PDGFRa expression, no multipotent neurospheres were obtained (Table III, Buffo et al., 2008). Astrocytes and NG2+ glia are therefore not equivalent in their reactive changes, as, according to these data, only astroglia lineage cells acquire multipotency.

Further support to the view that injury can trigger some glial de-differentiation into a plastic phenotype is the evidence that proliferating glia reacting to damage can be engaged in neurogenesis *in vivo* upon forced overexpression of determinants promoting neuronal fates (e.g. Pax6 or Neurogenin 2, Buffo et al., 2005; Ohori et al., 2006), while quiescent glia cells in the intact nervous tissue remain unresponsive (Rite et al., unpublished observations). However, the capacity of reactive glia to accomplish the instructive information is rather limited, as shown by the few responsive cells and their disappearance at long time points, due to possible restoration of the original glial fate or cell death. In the following sections we will discuss factors responsible for this limitation, and signals possibly participating in the plastic reversion of parenchymal glia to a stem/progenitor-like phenotype, or potentially implementing this de-differentiation as well as expression of multipotency.

3.2 Restriction and promotion of stem/progenitor properties in mature non-germinal glia

Restrictive factors for the re/activation of stem cell/progenitor properties and the expression of multipotency in adult glia may reside in both the intrinsic cell features and/or derive from the extracellular milieu. Recent data indicate that epigenetic modifications occurring during maturation may block neurogenic programs in both astroglia and NG2+ lineages (Hirabayashi et al., 2009; Lyssiotis et al., 2007; Liu et al., 2007). The maintenance of such silencing during gliosis may account for exclusive

gliogenesis and even counteract the effects of overexpression of neurogenic instructors *in vivo* (Buffo et al., 2005; Ohori et al., 2006). Genetic reprogramming might overcome these epigenetic restrictions in analogy with experiments on fibroblasts reverted to pluripotent stem cells (Takahashi and Yamanaka, 2006) or directly to excitatory neurons (Vierbuchen et al., 2010). Nevertheless, in the perspective of therapeutic applications, genetic manipulations are still inherently risky as inserting factors into the genome can potentially lead to gene misregulation. Thus, looking at the role of environmental factors in the modulation of glia plasticity is still of primary interest for the possibility to manipulate these targets by classical pharmacological treatments.

Indeed, the CNS milieu offers several challenges for the expression of stem cell potentials and complete differentiation along the neuronal lineage as shown by grafting approaches, where cultured neural stem cells or even SEZ-derived neuroblasts integrate and differentiate into neurons only within germinal regions. At other sites they become glia, demonstrating that the adult parenchyma permits only gliogenesis, particularly at sites of injury or neurodegeneration (Cao et al., 2002; Seidenfaden et al., 2006). Persistent inflammation or specific microglia activation states may be factors particularly hampering the acquisition of stem/progenitor traits and/or neuron differentiation and survival upon lesion. Indeed, anti-inflammatory treatments have been shown to promote young neuron maturation (Ekdahl et al., 2009 for review) while low-level of inflammation post-injury was associated with the appearance of radial glia phenotypes, spontaneous neurogenesis and long-term survival of newly generated neurons (Leavitt et al., 1999; Chen et al., 2004). However, inflammatory molecules as well as microglia phenotypes supportive for stem cells activity and neurogenesis constitutively participate in the endogenous germinal signalling (Ekdahl et al., 2009). The detailed knowledge of cytokine expression in defined lesion conditions and the appropriate balancing of inflammatory factors may thus be exploited to regulate the transition of adult glia into stem-like progenitors and sustain their appropriate differentiation for cell replacement.

Notably, several signals known to crucially contribute to define germinal niches (BMPs, Notch signalling, Sonic Hedgehog Homologues-Shh, Wingless-Int glycoproteins – Wnt pathway –, extracellular nucleotides, EGF and bFGF) are to some extent activated upon lesion and mostly produced by reactive astroglia, confirming the acquisition of progenitor traits by these cells and suggesting that the injured parenchyma potentially represent an "atypical germinal location" (see Table II). Very little is known on the roles and interplay of these pathways upon injury. BMPs, directing adult germinal astroglia toward neurogenesis (Colak et al., 2008) and promoting multipotent astroglial phenotypes in NG2+ cells (Kondo and Raff, 2000), are indeed upregulated at the site of injury in both cortex and spinal cord (Hampton et al., 2007; Chen et al., 2005), though their action appears inhibited by the concomitant expression of the antagonist Noggin (Hampton et al., 2007). Interestingly, Noggin neutralisation *in vivo* promotes the appearance of NG2+/GFAP+ glial cells, reminiscent of the plastic phenotype induced in NG2+ cultures by BMP treatments (Kondo and Raff, 2000). Thus, BMP manipulation might be a potential route to influence cell fate and broaden glial differentiation potential following CNS trauma. Also Notch activity is found induced in response to CNS damage (Yamamoto et al., 2001; Chen et al., 2005, Arumugam et al., 2006). While Notch signalling in germinal progenitors (Givogri et al., 2006) represses gliogenic genes (Hermanson et al., 2002) and maintains a proliferative undifferentiated state (Androutsellis-Theotokis et al., 2006), in reactive glia the concomitant activation of janus kinase-signal transducer and activator of transcription pathway (JAK-STAT; Buffo et al., 2010) likely determines the execution of gliogenic transcriptional programs (Ge et al., 2002). Further, Notch blockade was reported to promote neurogenesis from grafted parenchymal precursors (Yamamoto et al., 2001). Hence, in injury conditions the Notch pathway may restrict glia multipotency suggesting that its neutralisation can implement a stem/progenitor behaviour. A similar restrictive action is suggested for the Wnt/β-catenin pathway, which, known to sustain adult germinal glia proliferation and neurogenesis (Adachi et al., 2007 and references therein), is found activated in glia after lesion (White et al., 2010) likely taking part to gliogenic committment of proliferative cells.

In addition to inhibitory signals, factors must operate after injury to elicit the de-differentiation

response. Hypothetically, their implementation may help in broadening glia potential and differentiation capability and possibly even circumvent epigenetic silencing. Furthermore, the enhancement of developmental/stem capabilities in non-germinal astrocytes may also allow a more effective healing including promotion of cell survival, axon re-growth and functional recovery, as it occurs during the immature gliotic response (Silver and Miller, 2004). Very early events at the time of damage may initiate the transition into a plastic phenotype. Interestingly, extracellular nucleotides released by damaged cells are known to trigger astroglia reactivity (Abbracchio and Ceruti, 2006) and regulate neural stem/precursor properties *in vitro* and at germinal sites (Neary and Zimmermann, 2009). Therefore, they are potential candidates for the acquisition of stem potentials in astroglia. However, such de-differentiation has to be sustained for a long period of time to allow expansion of the precursor pool and/or instruct derivative maturation. Amongst later players, EGF and bFGF are obvious candidates in the promotion of parenchymal progenitor expansion (Buffo et al., 2010). Shh production is as well upregulated in gliotic astrocytes *in vivo* and sustains the proliferation of reactive glia (Amankulor et al., 2009). Interestingly, Shh can induce the formation of neurospheres from astroglial cells dissociated from the adult intact CNS grey matter (Jiao and Chen, 2008), consistent with its function in adult stem cell maintenance and specification (Ahn and Joyner, 2005; Han et al., 2008; Palma et al., 2005). Thus, it may therefore take part in the injury-induced reactivation of stem cell like properties in astroglia after damage. In theory, a further step likely required for successful neuronal replacement is the promotion of survival and neuronal differentiation of reactive glia-derivatives. Factors supporting neurogenesis and neuronal differentiation such as BDNF may act helpful in sustaining neurogenesis in normally non-neurogenic brain regions (Ohori et al., 2006). Despite research in this field is in its infancy, overall these early findings indicate that the optimization of expression levels of these and other signalling components may have the potential of maximizing recovery following CNS injury.

4. Summary and Conclusions

In addition to the well-characterized adult neural stem cells residing in the germinal niches, NG2+ cells and astrocytes in the mature CNS parenchyma exhibit distinct stem/progenitor properties. In the intact brain and upon injury, NG2+ cells actively divide and generate some new myelinating oligodendrocytes. They also appear capable to produce astrocytes, thereby implementing or perhaps even vicariating in some cases the astrocyte cytogenic response to support scar formation. However, even in *in vitro* conditions favouring neurogenesis, their differentiation potential appears primarily gliogenic. Conversely, astrocytes do not display progenitor functions. Yet, upon injury they re/acquire immature/progenitor traits as indicated by activation of proliferation, acquisition of an immunophenotypical profile resembling that of immature and germinal astroglia, and production of signals, which are essential components of the germinal microenvironment. While *in vivo* they only give rise to other astrocytes, *in vitro* they disclose the capability to self renew and to generate neurons, indicating that multipotency is retained within the astroglia lineage and suggesting that astrocytes may be more amenable than NG2+ cells to neurogenic manipulations. To test whether the latent reparative potential retained in adult CNS parenchyma can be exploited for cell replacement after injury, promising approaches are *in vivo* pharmacological and/or biotechnological manipulations aimed at overcoming restriction to glial fates and implementing the acquisition of developmental/germinal functions. However, the envision of more defined therapeutic strategies may require further investigative efforts non only to gain a deeper knowledge of the factors operating upon injury, but also to unveil the specific molecular networks underpinning the identity of non-germinal glia.

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