Emerging pharmacological approaches to promote neurogenesis from endogenous glial cells

Abstract

Neurodegenerative disorders are emerging as leading contributors to the global disease burden. While some drug-based approaches have been designed to limit or prevent neuronal loss following acute damage or chronic neurodegeneration, regeneration of functional neurons in the adult Central Nervous System (CNS) still remains an unmet need. In this context, the exploitation of endogenous cell sources has recently gained an unprecedented attention, thanks to the demonstration that, in some CNS regions or under specific circumstances, glial cells can activate spontaneous neurogenesis or can be instructed to produce neurons in the adult mammalian CNS parenchyma. This field of research has greatly advanced in the last years and identified interesting molecular and cellular mechanisms guiding the neurogenic activation/conversion of glia. In this review, we summarize the evolution of the research devoted to understand how resident glia can be directed to produce neurons. We paid particular attention to pharmacologically-relevant approaches exploiting the modulation of niche-associated factors and the application of selected small molecules.

Abbreviations (alphabetical order)

+, positive; AMPK, AMP-activated protein kinase; Ascl1, Achaete-scute homolog 1; Atoh7, Atonal basic-helix-loop-helix transcription factor 7; β-cat, beta-catenin; BDNF, brain-derived neurotrophic factor; BLBP, brain lipid-binding protein; BMP, Bone Morphogenetic Protein; Brn2, murine brain-2 transcription factor; cAMP, cyclic adenosine monophosphate; CNS, Central Nervous System; Creb1, cAMP responsive element binding protein 1; Crx, cone-rod homeobox gene; DAPT, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester; DCX, doublecortin; Dll, delta-like; Dlx, related to the Drosophila distal-less homeobox transcription factor; DNA, deoxyribonucleic acid; Dnmt3b, DNA methyltransferase 3b; EGF, epidermal growth factor; FGF, fibroblast growth factor; GABA, gamma-aminobutyric acid; GF, growth factor; GLAST, Glutamate aspartate transporter; GSK3β, glycogen synthase kinase 3 beta; HB-EGF, heparin-binding EGF-like growth factor; HDAC, histone deacetylase; Hes, hairy and enhancer of split; HK2, hexokinase; Hmga2, high mobility group AT-hook 2; IFN-γ, interferon gamma; IGF1, insulin growth factor 1; IL-1β, interleukin 1 beta; iPSC, induced pluripotent stem cell; Jag1, Jagged 1; Jak/Stat, Janus kinase/signal transducer and activator of transcription; JNK, c-Jun N-terminal kinase; Klf4, Kruppel-like factor 4; LDHA, lactate dehydrogenase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Math3, mouse Ath3 (Atonal basic-helix-loop-helix Transcription Factor3); Mbd1, methyl-CpG binding domain protein 1; MEF2, myocyte enhancer factor-2; MG, Müller glia; miRNA, microRNA; MNU, N-methyl-N-nitrosourea; Myc, myelocytomatosis oncogene; Myt1L, Myelin Transcription Factor 1 Like; NeuN, neuronal nuclear antigen; NeuroD1, Neurogenic differentiation 1; NG2, neural/glial antigen 2; Ngn2, Neurogenin2; NICD, Notch intracellular domain; NMDA, N-Methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NSC, neural stem cell; NT3, Neurotrophin-3; Oct4, octamer-binding transcription factor 4; Olig2, Oligodendrocyte Lineage Transcription Factor 2; P, postnatal day; p16, cyclin-dependent kinase inhibitor

2A; p21, cyclin-dependent kinase inhibitor 1; p53, Tumor protein p53; Pax6, Paired Box 6; PDGFRα, Platelet-derived growth factor receptor alpha; PGE2, Prostaglandin E2; PKA, protein kinase A; Plp, proteolipid protein; QA, quinolininc acid; RA, retinoic acid; Rbpj, recombining binding protein suppressor of hairless; RC2, Radial Glial Cell Marker-2; REST, Repressor element 1 (RE1)-silencing transcription factor; RNA, ribonucleic acid; ROCK, Rho-associated protein kinase; ROS, reactive oxygen species; Shh, Sonic hedgehog; SGZ, subgranular zone; SIRT1, Sirtuin 1; Smo, Smoothened; Sox2, SRY (sex determining region Y)-box 2; Sox4, SRY (sex determining region Y)-box 4; Sox9, SRY (sex determining region Y)-box 9; SVZ, subventricular zone; TF, transcription factor; TGFβ, transforming growth factor; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; VPA, valproic acid; Wnt, wingless-type MMTV integration site family

1. New neurons in the mature central nervous system: the dream of a new brain

Neurodegeneration after injury or disease is a chronic and incurable condition whose disabling effects may continue for years or even decades. While the contribution of neurodegenerative pathologies including stroke, Alzheimer's and Parkinson's diseases to the global disease burden is growing fast, regeneration of functional neurons still remains an unmet need.

Strategies to replace lost neurons can rely on either transplantation of exogenous cells or the exploitation of endogenous sources. The field of cell transplantation has developed over a long time now, and progressed enormously, to the extent that it appears to be very close to proposing for clinical trials authentic human neurons derived from human embryonic stem cells [1]. However, the use of human stem cells faces both ethical issues and the challenge to overcome immunorejection. Induced pluripotent stem cells (iPSCs) can represent an excellent alternative for autologous applications. Still, the field needs further advancement in differentiation protocols and solutions to manage risks of introduction of genetically manipulated material. In this evolutionary landscape, further complicated by the costs of stem cell therapies based on good manufacturing practices and delicate surgical procedures, exploitation of endogenous neural cells has recently gained an unprecedented attention. Today this field of research has become very active despite initial disappointments due to the failure to obtain replacement of neurons after damage by endogenous neural stem cells (NSCs) of the adult germinative niches [2,3]. Crucial to attract researchers' interest were the clear demonstrations that the central nervous system (CNS) can activate spontaneous neurogenesis, and that endogenous glia can be instructed to produce neurons by reprogramming (see below).

Targeting local glia comprising astrocytes and neural/glial antigen 2 (NG2)-expressing glia (NG2 cells) appears particularly desirable in view of neuronal replacement because of their

abundance and ubiquitous distribution in the CNS. Moreover, these glial cells set up a complex reaction to injury that partly increases their similarity to neural stem cells and can include a cytogenic response leading to some degree of amplification, thereby allowing to direct some elements toward neurogenesis while avoiding glial cell depletion [4].

In this review we will revise the current status of research devoted to understand if and how resident glia can be directed to produce neurons, with specific attention to in vivo data. We will discuss mechanisms and factors, either intrinsic or environmental, which may be of relevance for potential pharmacological approaches aimed at boosting the production of new neurons from endogenous sources. Our focus will be mostly on studies on the mammalian brain, spinal cord and retina, which, due to its peculiar inherent regenerative properties, has been intensely investigated with outcomes possibly exploitable also for other systems.

2. Parenchymal neurogenesis: who, when, where

2.1 Spontaneous parenchymal neurogenesis

Adult neurogenesis in the constitutive germinal niches of the subventricular zone (SVZ) and hippocampal subgranular layer (SGZ) is highly conserved in different mammalian species. Whether other CNS regions can be neurogenic has been the subject of a long debate that is still partly unresolved. Initial studies referred to the rest of the CNS parenchyma as non-neurogenic. This concept was mainly derived from the observation that when heterotopically transplanted outside the constitutively active neurogenic niches, NSCs differentiated almost exclusively into glial cells and not in neurons [5-7]. These observations were consistent with the absence of neurogenesis in the mature healthy CNS parenchyma in rodents, as reported after initial controversial evidence for the spinal cord, cortex and striatum by numerous studies [8-13].

By contrast, comparative analyses indicated that in some mammalian species low-level neurogenesis can occur also outside the two canonical niches. Neuroblasts were observed in the striatum and neocortex of rats, rabbits, guinea pigs and primates and in the amygdala, piriform cortex and adjoining perirhinal cortex of primates (see [14-15]). Furthermore, striatal neurogenesis has now been suggested also in humans [16]. The observation of parenchymal neurogenic processes in intact animals may suggest their participation in homeostatic functions and normal brain activity. However, no data are currently available that support this idea. Further, the timing and the transient nature of neurogenic events observed in some of these cases (e.g. transient activation of neurogenesis in the guinea pig at weaning age [15]) rather favors their interpretation as events related to temporary forms of plasticity.

Of note, injury can induce neurogenic events also in regions that are normally non-neurogenic. Newly generated neurons were observed after acute degeneration both in the striatum (experimental stroke, [17]; quinolinic acid (QA)-induced excitotoxic lesion, [18]) and the neocortex (transient ischemia, [19]; focal apoptosis, [10, 13]) as well as in a genetic model of progressive striatal neurodegeneration [20].

Despite the SVZ can contribute neuroblasts to the injured parenchyma [2,21], several studies provided initial evidence that neurogenic events in non-neurogenic regions were a local SVZ-independent phenomenon. In both rabbits under physiologic conditions and in mice during striatal progressive neurodegeneration, tracing of SVZ derivatives and tridimensional reconstructions of striata and adjacent SVZs showed that chains of striatal neuroblasts were separated in space and did not derive from the niche [20,22]. Further evidence came from ex vivo approaches showing neuroblast generation in striatal explants [22], and by in vivo observations of the association between neuroblasts and parenchymal clusters of proliferative cells with the features of the intermediate progenitors typical of NSC lineages (proliferative cells positive for the brain lipid-binding protein – BLBP, SRY

(sex determining region Y)-box 2 - Sox2, SRY (sex determining region Y)-box 9 - Sox9, epidermal growth factor – EGF - receptor, and distal-less homeobox transcription factor – Dlx) [20, 22]. Local generation of new neurons has been also reported in the cerebral cortex, where layer I progenitors traced by retrovirus-mediated labeling were shown to produce neurons [19]. Taken together, these findings strongly indicated that new neurons can be generated locally in the brain parenchyma, at least in specific areas, under both physiological and pathological condition.

However, these investigations did not clarify the cellular source of the neo-generated neuroblasts. Tight lineage relationships as well as phenotypic and functional similarities between germinative NSCs and neuroglia ~~-specifically astrocytes-~~ suggested that these cells could harbor a neural progenitor potential (revised in [4]). Subsequent studies therefore investigated astrocytes and NG2 cells as the most likely suspects of local neurogenesis.

NG2 cells, also known as oligodendrocyte precursor cells, comprise a population of glial cells widely distributed throughout the adult brain parenchyma. In the adult healthy and injured brain, these cells account for the vast majority of proliferating cells outside the stem cell niches and constantly divide to generate differentiated, myelinating oligodendrocytes, as well as further NG2 glia [23]). While in vitro studies showed that NG2 cells can differentiate into neurons [24-27], clear evidence that NG2 cells contribute to parenchymal neurogenesis in vivo is missing. By inducing genetic recombination in adult intact PDGFRα (Platelet-derived growth factor receptor α) -CreERT2 mice (see Figure 1), Rivers and colleagues [28] detected some labeled neurons in the piriform cortex. Based on the expression of PDGFRα by NG2 glia, these finding were interpreted as indicating neurogenesis from NG2 cells. Similar results were shown by Guo et al., 2010 [29] in a mouse line where proteolipid protein (Plp) promoter activity (also occurring in NG2 cells) drives the expression of a tamoxifen-inducible Cre transgene. However, these findings

could not be reproduced in subsequent studies by the same authors [30] . Even other labs exploiting the same PDGFRα-CreERT2 mouse line [31] or other oligodendroglia-specific inducible lines [32-34] could not replicate these results. Thus, evidence collected so far does not support a neuronal differentiation potential of NG2 cells in physiological conditions. Nevertheless, Honsa et al. [35] reported the generation of neuroblasts from NG2 cells in the late phases after focal cerebral ischemia in adult NG2CreBAC:ZEG mice. Nonetheless, these findings were not confirmed by other fate mapping studies with the same Cre-inducible line, where the generation of neuroblasts from NG2 cells after lesion was clearly excluded [17,18]. Taken together, these data collectively do not provide evidence for NG2 glia as a parenchymal source of new neurons.

Unlike NG2 cells, astrocytes outside of the germinal niches do not divide in the heathy brain. However, in injury conditions, they acquire NSC features as shown by the upregulation of NSC markers (Nestin, vimentin, BLBP), activation of proliferation, and expression of self-renewal and multipotency ex vivo [36,37]. Of note, these features are not acquired by reactive NG2 glia [36]. Astrocytes, therefore, are the most likely player in parenchymal neurogenesis. In accordance with this view are data on physiological neurogenesis in the guinea pig external capsule and lateral striatum [15]. Here, neurogenesis is absent at birth but newly generated neuroblasts transiently appear between postnatal day 7 (P7) and P18 (weaning age). They are produced in the lateral striatum, concomitantly with a time window of intense proliferation of local astroglia. Along this line, the direct demonstration that astrocytes act as neuronal progenitors was recently offered by two independent studies where fate mapping strategies demonstrated that striatal resident astrocytes produced new neurons after stroke [17] or QA-induced neurodegeneration [18]. In these studies astrocytes were tagged before lesion in mice obtained by crossing a reporter line and mutants expressing the tamoxifen inducible recombinase CreERT2 under the control of distinct astrocyte genes (Connexin-30 in the

stroke model; Glutamate Aspartate Transporter – GLAST- in QA experiments; See Figure 1 for inducible Cre-based models). The progenies of the tagged astroglia included proliferative intermediate progenitors and neuroblasts. Nato et al. [18] also detailed that the response of GLAST-positive (+) striatal astrocytes to injury included the upregulation of nestin and the subsequent generation of intermediate progenitors positive for Achaete-scute homolog 1 (Ascl1, [38]) or Sox9 ([39]). In turn, these progenitors gave rise to neuroblasts. Moreover, in both studies the conclusive proof of neurogenesis from local astrocytes came from virus-based fate mapping of astrocytes transduced before QA or stroke. The same approach excluded a major contribution of the SVZ to QA-induced striatal neurogenesis [18]. Interestingly, astroglial neurogenic activation appeared to persist for several months, prompting the question on the mechanisms activating and sustaining this neurogenic switch. In agreement with other pathologic and physiologic models of striatal neurogenesis [15, 20, 22], induced neurons displayed a short life span (our unpublished observations) and did not differentiate into striatal neuronal types. The only exception were few calretinin or neuronal nitric oxide synthase (nNOS) expressing interneurons ([17]; our unpublished observations). The phenotype of these new neurons, the extent of their integration in the host circuits, mechanisms of cells death remain to be established. These transient neurons might sustain some forms of post-lesion compensatory plasticity [18] and may exert protective roles for neurons that have lost their targets. Thus, striatal astrocytes comprise quiescent neuronal progenitors that become activated after lesion. Of note, the process leading to this form of spontaneous neurogenesis took several weeks to start. This finding suggests that astrocytes transit through a multi-step activation, possibly influenced by changing environmental signals. They first become reactive but not yet neurogenic, then competent for neurogenesis and ultimately actively neurogenic.

Another paradigmatic example of parenchymal glia endowed with a latent neurogenic potential is the retinal Müller glia (MG). MG account for the 4-5% of all retinal cells, span the retinal epithelium and perform typical astroglia supportive functions [40]. In fish, birds and rodents, following a retinal injury a subset of normally quiescent MG resumes proliferation, gives rise to multipotent Paired Box 6 (Pax6)+/nestin+ retinal progenitors that further amplify and can generate new retinal neurons [41-44]. This response is particularly efficient in fish, also at post-developmental stages, while its shows clear limitations in birds and mammals [45]. Intriguingly, such process recapitulates some aspects of retinal development, including interkinetic nuclear migration and asymmetric division in activated MG, and neuroblast migration along radial processes retained by MG (Figure 2). Further, the layer distribution and phenotype of newly-generated neurons mainly corresponds to those of the lost elements and, when sufficient number of neurons are produced, adult neurogenesis even results in positive functional effects ([43, 44] and references therein). Nevertheless, in rodents, MG regenerative response is rather modest, both in quantity (i.e. few MG entering cell cycle and yielding differentiated neurons after injury), and types of neurons generated (mostly photoreceptors, bipolar or amacrine cells). Further, most newly-generated neurons display a limited life-span [42]. In humans, this phenomenon is even more restricted because MG can re-enter the cell cycle, but there is no de novo neurogenesis in adults in disease or after injury ([46] and references therein). However, the in vivo manipulation of intrinsic and extrinsic signals can ameliorate the final neurogenic outcome in the mammalian retina (see below), and, when exposed to proper environmental signals, even human MG can unleash a latent neurogenic potential in vitro [45,47]. Interestingly, on the molecular level, mouse MG show many similarities with retinal progenitor cells, suggesting the idea that MG may represent a form of late stage progenitor cell persisting in the adult tissue ([45] and references therein). Consistent with this idea, the epigenetic landscape of the promoters of pluripotency factors in mouse MG is rather

similar to that found in progenitor cells (i.e. their hypomethylation allows their chromatin to assume a more “open” state that is permissive for gene expression). This likely prompts MG to rapidly re-enter the cell cycle upon injury and facilitates their dedifferentiation [48]. It is still unknown whether MG is an exception among astrocyte populations or also extra-retinal astrocyte subsets with a neurogenic potential share such molecular/epigenetic features.

In summary, when reacting to injury parenchymal astrocytes and MG in the retina have the potential not only to activate a stem cell response, but also to express a neurogenic program in specific conditions. In doing so, astrocytes and MG appear to undergo phases typical of NSCs of the adult neurogenic niches: after activation they generate amplifying intermediate elements, which in turn give rise to neurons. These findings suggest that if properly activated, the ubiquitary parenchymal glia might ultimately become able to sustain an effective brain cell replacement upon damage.

2.2 Unlocking glial neurogenic potential via modulation of cell intrinsic factors: induced in vivo cell reprogramming

Evidence of spontaneous neurogenic activation of parenchymal glia strongly prompted the view that, upon appropriated stimulation, ubiquitary glia could undergo a neurogenic activation at all CNS sites. This suggests the possibility of becoming able to sustain an effective neuronal replacement upon damage. First hypotheses for strategies to foster neurogenesis in glia emerged from comparative and developmental studies, showing that cell-intrinsic determinants necessary for providing the neurogenic competence to reactive MG in non-mammalian vertebrates or to direct the generation of specific neural classes in embryonic neural progenitors are not (re-)expressed in mature glial cells upon injury in mammals (e.g. [49–51]). In particular, comparative investigations on the retina (thoroughly revised in [44,45]) highlighted a number of molecular players (e.g. Ascl1, Neurogenic

differentiation 1- NeuroD, Notch signaling) subsequently targeted to foster neurogenesis in mammalian glia.

First attempts to overexpress proneurogenic determinants in proliferative reactive glia led to a limited production of transient neuroblasts in the lesioned brain (e.g. [51]). However, the field evolved very rapidly, and offered clear in vitro evidence of successful derivation of functional neurons from early postnatal astrocytes or NG2 cells through forced expression of Pax6, Neurogenin2 (Ngn2), or Ascl1 [52–54]. Concomitant breakthroughs established the innovative concept of cell reprogramming. This consists in changing the phenotype of somatic cells such as fibroblasts (or glia) into that of another cell type including iPSCs (e.g. [55,56]), NSCs [57] or neurons [58] through the overexpression of specific transcription factors (TFs), in combination or alone. This evidence prompted further research on glia into neuron conversion strategies that successfully transferred in vitro evidence in vivo. Studies on in vivo reprogramming of glial cells in the CNS have been already discussed in detail in several recent reviews [1,59,60] and are here summarized in Table 1. Importantly, not all TFs shown to efficiently reprogram glial cells in vitro were successful in vivo (e.g. Ngn2, Ascl1, see Table 1), highlighting the importance of the cellular milieu in cell fate specification and reprogramming. When induced, in most cases obtained neurons were electrophysiologically active (see Table 1), although evidence for the implication of induced neurons in some degree of functional repair is still starting to emerge [61]. Moreover, in contrast to spontaneous neurogenic activation, which is fully proved only for astrocytes so far, both astroglia and NG2 cells were efficiently converted into neuronal cells by ectopic expression of specific TFs.

Neurogenic actions of transcription factors

It is interesting to note that neuronal induction in the brain or spinal cord occurs either through a process that appears to recapitulate the features of the activation of

spontaneous neurogenesis from glia (i.e. with a transition through a neural progenitor stage including amplifying Ascl1 expressing cells and subsequent generation of doublecortin (DCX)+ neuroblasts) or by direct conversion of glial cells into neurons.

Among the different tested factors, the first mode of induced neurogenesis was mimicked only by overexpression of Sox2, a TF factor involved in stem cells maintenance in both SVZ and hippocampal SGZ [62], used to induce pluripotent stem cells together with other TFs (Oct4, Myc, Klf4; [56]). Such a NSC-like induction mode was particularly patent when astrocytes in the intact striatum were targeted [63,64]. Similar results were obtained in the injured spinal cord [65]. Differently, an injury was instead required to prompt glial cells, mostly comprised of NG2 cells, to respond to Sox2 in the cerebral cortex [66]. These data suggest that distinct CNS areas may be differently conducive to reprogramming. This is likely due to the interplay between distinct cell-intrinsic properties and environmental factors, and in some instances injury-related signals may promote the response to the genetic manipulations.

The other tested transcriptional regulators, which are proneural genes, were instead essentially reported to promote direct conversion mechanisms, although with distinct efficiency and outcomes in terms of obtained neuronal phenotypes (see Table 1). ~~Neurogenic differentiation 1 (~~NeuroD1~~)~~, a TF essential for adult neurogenesis [67,68] and for terminal neuronal maturation [69], exerted a particularly strong reprogramming action on astrocytes and NG2 cells in the cerebral cortex of both stab-injured and Alzheimer's disease model mice [70]. Interestingly, while astrocytes were mainly reprogrammed into glutamatergic neurons, NG2 cells were converted into both glutamatergic and gamma-aminobutyric acid (GABA)-ergic neurons [70].

Conversely, the efficiency of the proneural gene Ascl1, shown to be sufficient to induce neurons from fibroblasts [71], remains controversial in inducing glia reprogramming in the brain and spinal cord. Several studies reported that the overexpression of Ascl1 in striatal

astrocytes [64], adult injured cortex [66] and injured spinal cord [65] was not sufficient to induce glia-to-neurons conversion. Instead, another study reported that Ascl1 converts astrocytes into functional neurons in the dorsal midbrain, striatum, and somatosensory cortex [72]. These different results may depend on the different viral vector used to promote Ascl1 overexpression. Such vectors could induce distinct levels of expression, and/or different levels of immune responses.

It is worth mentioning one recent example of astrocytic reprogramming toward dopaminergic neurons. In a mouse model of Parkinson's disease, overexpression in striatal astrocytes of a combination of NeuroD1, Ascl1, LMX1a and miR218, formerly proved to be effective in vitro, triggered the induction of neurons with a dopaminergic phenotype. Most interestingly, induced dopaminergic neurons appeared to promote recovery of deficits in spontaneous motor function relevant for Parkinsonism [61].

Also in the retina forced expression of stemness inducers (i.e. β-catenin – β-cat, Lin28) and/or proneural genes (Ascl1, Atonal basic-helix-loop-helix transcription factor 7 - Atoh7, NeuroD) has been used to foster/expand the intrinsic MG neurogenic potential. In vivo gene transfer of β-cat in young adult mouse retinas was shown to activate proliferation, interkinetic nuclear movement and expression of amacrine cell markers in MG even without retinal injury [73]. Similarly, MG reprogramming via cell fusion with transplanted hematopoietic progenitor cells with activated wingless-type MMTV integration site family (Wnt) pathway resulted in the generation of photoreceptors and functional amelioration in a mouse model of inherited retinitis pigmentosa [74]. Forced expression of the RNA-binding protein Lin28B in MG stimulated its proliferation, de-differentiation, and promoted its neuronal commitment in a rat model of retinitis pigmentosa [75]. The same was observed even in the uninjured mouse retina [73]. In explants of adult mouse retina, Ascl1 overexpression was sufficient to activate a neurogenic program in MG [76]. Further, in vivo transgenic expression of Ascl1 in MG resulted in the generation of amacrine and bipolar

cells and photoreceptors in the injured retina of young mice [49]. The in vivo forced expression of the neurogenic factor Atoh7 promoted the differentiation of MG-derived stem/progenitor cells into retinal ganglion cells when transplanted in a rat model of glaucoma [77]. NeuroD has also been successfully employed in the injured rat retina ex vivo, where it induced the appearance of newly generated amacrine cells. The production of this neuron type could be further implemented by several combination with Pax6 and mouse atonal basic-helix-loop-helix transcription factor 3 (Math3). On the contrary, the co-expression of Crx (cone-rod homeobox gene) with NeuroD promoted the generation of photoreceptors [41]. Notably, evidence collected so far showed that in vivo MG reprogramming via forced expression of the above-cited factors essentially recapitulated the phases of spontaneous adult neurogenesis, implying a certain degree of MG proliferation, appearance of multipotent Pax6+/nestin+ intermediate progenitors and a final step of neuronal differentiation (Figure 2). When appropriately fostered upon injury (see also some examples in section 3) the neurogenic attempt succeeded to replace, at least in part, the lost elements by producing neurons that acquired the appropriate layering and phenotype, and, in few cases, provided a functional rescue [74,75]. Thus, in vivo gene-transfer manipulations appear a relatively advanced and promising strategy for the cure of retinal pathologies in humans.

Neurogenic actions of epigenetic factors

The transcriptional regulators employed in reprogramming are understood to exert their action by recruiting other transcriptional activators/repressors and inducing/suppressing specific target gene programs. In this regard, for instance, factors such as Ngn2 or Ascl1 are considered as pioneer factors with access to chromatin closed in the targeted regions and ability to recruit of other TFs inducing complex neuronal gene transcription [78,79]. However, the reprogramming factors cited above also influence the epigenetic landscape

of the cells. A clear example is Sox2, which in NSCs binds to bivalently marked promoters of poised proneural genes (i.e. Ngn2 and NeuroD1), where it maintains the bivalent chromatin state by reducing polycomb repressive complex 2 activity [80]. Indeed, an interesting feature of chromatin in stem cells and iPSCs is the presence of bivalent domains that harbor histones with both active and repressive modifications [81]. These indicate a transcriptionally poised state that can rapidly change and thus enhance cellular potency. Loss of Sox2 shifts the state of proneural gene chromatin toward a more repressed configuration, impairing their activation even in the presence of differentiating cues [80]. It is likely that a Sox2-dependent re-installation/maintenance of a permissive epigenetic state similar to that of NSCs contributes to Sox2-induced reprogramming of parenchymal astrocytes and to the recapitulation of a NSC-like multistep neurogenic program. Other TFs may act on gene programs more downstream in the neurogenic process. For instance, the action of NeuroD1 is not limited to the promotion of a complex transcriptional program driving neuronal differentiation. It also converts heterochromatin to euchromatin and marks epigenetically neuronal fate genes so to maintain them active [82]. These observations raised the idea that changes in the epigenetic setting and chromatin organization in adult glia may be crucial factors for their activation and neuronal conversion. Although our understanding of the regulation of the epigenetic landscape in neurogenically activated glia is still very limited, changes of expression of epigenetic modification enzymes, variations in DNA methylation, histone modifications and chromatin accessibility were shown to occurr in glial cells upon spontaneous neurogenic activation or reprogramming. Such information may allow identifying target mechanisms and windows of opportunities to possibly manipulate the system pharmacologically to promote local neurogenesis or reprogramming.

Intriguingly, some chromatin remodeling factors (i.e. high mobility group AT-hook 2 - Hmga2, and methyl-CpG binding domain protein 1 - Mbd1) were shown to be differentially

expressed in MG of mouse strains exhibiting different degrees of spontaneous damage-induced MG proliferation and de-differentiation (i.e. acquisition of progenitor markers [83]). Further, in quiescent MG chromatin accessibility at the regulatory regions of neural progenitor genes (e.g. Notch targets and ligands, Lin28, Ngn2, Ascl1, oligodendrocyte lineage transcription factor 2 - Olig2) is significantly lower in adult mice compared to young animals. This correlates well with their differential ability to be effectively reprogrammed [49].

Initial studies in zebrafish retina [48] proposed the idea that early DNA demethylation (which reflects chromatin accessibility for gene expression) may allow the transcription of genes associated with glia neurogenic activation and reprogramming. Consistently, a recent study [84] showed an early and transient decrease in the DNA methyltransferase 3b (Dnmt3b) expression after N-Methyl-D-aspartate (NMDA)-induced retinal injury in mice. Dnmt3b expression returned to basal levels after 24 hours. Such dynamics well corresponded to the rapid and temporary decrease of methylation of the promoter of the pluripotency gene Oct4 and with its expression level (initially upregulated and then immediately suppressed) in MG. The in vivo administration of the DNA-methyltransferase inhibitor SGI-1027 induced a sustained upregulation of Oct4 expression in mouse MG, thus increasing their potential to acquire progenitor features [84]. Further, in vitro neuronal reprogramming of human astrocytes by forced expression of the pluripotency genes Oct4/Sox2/Nanog was associated by significant decrease in DNA methylation of genes involved in developmental process and neuronal differentiation [85]. Thus, methylation-mediated silencing of pluripotency and neuronal genes may be hypothesized as an epigenetic barrier preventing spontaneous glia neurogenic activation in the mammalian adult parenchyma.

Histone modifications were also proved to be implicated in glial response to reprogramming agents. Notably, forced overexpression of Ascl1 in mouse MG modified

histone acetylation and methylation, and chromatin remodeling at specific promoters (i.e. Hes5, Hes6, Dll1, Dll3, Insm1a*)* and converted them from a repressive to an active configuration [75]. Such Ascl1-mediated mechanisms have been reported also during fibroblasts-to-neurons reprogramming [79] and neurogenesis from NSCs [86]. Interestingly, Ascl1 and β-cat also acted at the post-transcriptional level to modulate gene expression in glia during reprogramming. Such factors promoted the upregulation of the RNA-binding protein Lin28 that in turn reduced the biogenesis of the let-7 family of microRNAs (miRNAs). Lin28 and let-7 can regulate the mRNA levels of a variety of factors, including Ascl1 and Wnt-targets. For this reason they are important players in glia proliferation and reprogramming, and in the differentiation of glia derivatives [44,73]. Sox2 was reported to exert a similar function at Lin28 gene promoter in developmental neural progenitors, suggesting that this miRNA-mediated mechanism could also occur during Sox2-dependent reprogramming [87]. Along this line, miR124 and miR9-9* were able to reprogram fibroblasts to neurons [88] and promoted neuronal differentiation while inhibiting glial genes when expressed in NSCs [39,89]. More recently, Wohl and colleagues [90] showed that lentiviral expression of these miRNAs in MG induced the expression of Ascl1 and reprogrammed MG to retinal neurons in vitro. Such effects were mediated by the direct targeting of components of the Repressor element 1 (RE1)-silencing transcription factor (REST) complex, known to repress neuronal gene expression in non-neuronal cells [91] and to block neuronal reprogramming in cortical astrocytes [92].

In summary, in vivo reprogramming into functional neurons shows exciting perspectives. However, it still faces many challenges such as direction toward desired neuronal phenotypes, demonstration of functional recovery, control of safety of exogenous genetic material and optimization of in vivo delivery for most applications.

3. Pharmacology of adult parenchymal neurogenesis

Evidence provided so far indicate that, depending on the CNS area, adult parenchymal neurogenesis may be attained by two modes: (i) a niche-like process, where the spontaneous or induced activation/dedifferentiation of glia leads to the production of neurons through the generation of intermediate amplifying progenitors (i.e. post-lesional striatal neurogenesis, retinal regeneration, Sox2 forced expression in striatal and spinal cord astroglia; see above); (ii) the direct conversion of glial cells into neurons without any progenitor phase (i.e. upon most reprogramming approaches tested so far in the brain or spinal cord). Pharmacological treatments could be tailored to reproduce or complement either of the two modes in view of fostering neurogenesis.

Remarkably, in most cases, spontaneous or induced parenchymal neurogenesis occurring according to the first mode requires the presence of a lesion. This suggests that signals provided by the injured microenvironment are supportive of the neurogenic process. The injured parenchyma may partly acquire features of a neurogenic niche, as a consequence of gliosis, degenerative events or thanks to the supply of peripheral components after blood brain barrier breakdown. Since the “learning from nature” strategy has been repeatedly and successfully applied in the history of pharmacology, a niche-based approach may be a good option to design effective treatments to evoke or implement developmental-like neurogenic processes. In this context, comparative studies have also helped in identifying niche-associated candidate signals. Although injury-induced parenchymal neurogenesis closely resembles the regenerative processes occurring in non-mammalian vertebrates, it is well known that the extent and efficiency of the production of new neurons in adult individuals is remarkably reduced in mammals compared to cold-blooded animals [93]. Of note, injuries lead to a certain degree of astroglia/MG reactivity and proliferation across species, suggesting shared or convergent

mechanisms regulating these steps. Divergent pathways across species are instead mainly related to the processes of glia dedifferentiation (with mammalian cells showing intrinsic constraints limiting the acquisition of a stem cell-like phenotype; see above), neuronal differentiation, integration and survival. Namely, comparative studies have shown that anti-neurogenic signals (e.g. the sustained activation of the Notch-mediated signaling cascade; see below) supported by the parenchymal environment of mammals operate by maintaining newly generated cells along the glial lineage ([46,93]; and references therein). Further, the different quality, duration and intensity of inflammatory cytokine release after injury were suggested to differently modulate glial cell reactivity and potency as well as the survival of their derivatives in different species ([93]; see below).

Here, we summarize what injury-related signals have been so far implicated in the activation of parenchymal neurogenesis and in the differentiation of the generated neurons. Much of the actual knowledge derives from the study of retinal regeneration. Thanks to its peculiar features (i.e. a common architecture across species; the presence of few types of well characterized neurons; the accessibility for manipulation and imaging; the presence of a functional readout- i.e. vision- that can be tested in vivo), the retina has been proven as an excellent system to study the pharmacology of adult parenchymal neurogenesis. Furthermore, it offered a number of candidate mechanisms possibly exploitable in other CNS systems.

3.1 Toward a niche-based approach

3.1.1 Role of morphogens and growth factors

Sonic hedgehog – stem cells response in reactive glia

Sonic hedgehog (Shh) is a soluble signaling protein playing pleiotropic functions during CNS development, including regulation of progenitor proliferation, specification, and axonal targeting. In the adult CNS, Shh is expressed in the canonical neurogenic niches, where it modulates NSC self-renewal and specification [94]. Interestingly, this morphogen is highly upregulated in lesions where astrocytes activate a significant degree of proliferation and a stem cell response, as shown by the generation multipotent neurospheres ex vivo (i.e. cortical stab wounding or cerebral ischemia; [37]). Cortical astrocytes express the Shh transducer Smoothened (smo), whose selective deletion impairs astrocyte proliferation in vivo, and their potential to form neurospheres in vitro. Notably, addition of Shh or the Smo agonist SAG in the culture medium is sufficient to elicit neurosphere formation from cells of the cerebral cortex even in absence of any injury [37]. Chromatin immunoprecipitation experiments showed that Shh gene is a direct target of Sox2 [62], thus suggesting that these two factors may act in concert in the activation of the parenchymal astroglial neurogenic potential. Again in line with a critical role of endogenous Shh in eliciting glia activation and possibly broaden their neurogenic competence, the intraocular injection of cyclopamine, a Smo inhibitor, greatly reduces MG proliferative response to N-methyl-N-nitrosourea (MNU)-induced retinal injury in rats [95]. Conversely, daily injections of Shh for 3-7 days consecutive days after retinal lesion boosts MG proliferation and promotes the differentiation of MG-derived progenitors in rod photoreceptors in vivo [95].

Notch signaling – repressor of the glial neurogenic switch

The Notch pathway is one of the most evolutionarily ancient and well-conserved signaling cascade in animals. By mediating juxtacrine cell-to-cell communication, Notch signaling plays fundamental and pleiotropic roles during development and adult life of multicellular organisms, including regulation of cell self-renewal, differentiation, death and metabolism.

Four Notch receptors (i.e. Notch1, Notch2, Notch3 and Notch4) and many Notch ligands (e.g. Jagged 1 - Jag1 - and Delta-like - Dll - proteins) are known in mammals. Both Notch receptors and ligands are transmembrane proteins. Notch receptors include an intracellular domain (Notch intracellular domain - NICD) that, following ligand binding, is released into the cytosol through a γ-secretase-dependent cleavage. In the canonical Notch pathway, NICD is targeted to the nucleus, where it interacts with the recombining binding protein suppressor of hairless (Rbpj) complex and converts it from a transcriptional inhibitor to an activator. Such cascade leads to the transcription of a plethora of Notch target genes, including the the hairy and enhancer of split (Hes)-related genes ([96] and references therein).

In the adult canonical neurogenic niches, Notch signaling negatively modulates NSC cell-cycle entry. At the same time, it balances NSC maintenance with production of their derivatives, thereby preventing premature depletion of the niche ([96] and references therein). Notch-mediated juxtacrine signals in active NSCs directly control the quiescence of the neighboring NSCs [97]. Further, activation of the canonical Notch pathway in SVZ NSCs promotes gliogenesis at the expense of neurogenesis [98]. Thus, on the whole, Notch signaling operates as a negative regulator of adult neurogenesis in the germinative niches.

Notch signaling is also implicated in astrogliosis, although its contribution to both reactivity and acquisition of a neurogenic competence in parenchymal astrocytes may appear somehow counterintuitive. By using a Notch/Rbpj signaling reporter mice, Marumo and colleagues [99] showed that the canonical Notch/Rbpj pathway is activated in reactive astrocytes, suggesting that Notch signaling takes part in their post-injury response. In line with this idea, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT, a γ-secretase inhibitor) treatment or Notch1 conditional ablation in astroglia reduces the number of proliferative and radial glial cell marker (RC2)+/nestin+ astrocytes after an

ischemic stroke [99,100]. It seems therefore that Notch signaling may sustain an initial stem cell response in astrocytes. However, inhibition of the Notch canonical pathway was shown to promote neurogenesis from astrocytes of the striatum in the absence of an injury [17]. The same authors also reported that the spontaneous emergence of neuroblasts and clusters of proliferative cells in the striatum after stroke is accompanied by a significant reduction of both Notch and NICD. How Notch inhibition can unleash the neurogenic potential of the resident astroglia is still obscure. Previous studies indicated that in vivo DAPT treatment inhibits the nuclear-translocation of Olig2 [99], which is a repressor of neurogenesis in cells reacting to brain injury [51] and is indispensable for the acquisition of a gliogenic fate of reactive astrocytes [99]. However, in Magnusson's study [17] astrogliosis does not take place, prompting the question of whether astrogliosis – and which type of astrogliosis (see also [101]) - is a pre-requisite for the acquisition of a neurogenic competence. Collectively, data accumulated so far essentially point to Notch-activated mechanisms in reactive astrocytes as repressors of the neurogenic switch in the adult brain.

In the retina, although the extent of Notch contribution to each step of retinal regeneration is far from being completely understood, Notch signaling appears to exert distinct roles in MG activation and neurogenic competence. An early activation of the Notch pathway – as well as of a Notch-induced proliferative response – appears essential to trigger the acquisition of progenitor-like features in MG in the avian and murine retina [42,102,103]. However, when Notch signaling is blocked at later stages, the number of newly generated neurons significantly increases, suggesting that Notch signaling hampers the expression of a neurogenic competence [102-104]. That Notch signaling mediates a variety of effects is no surprise, based on its well-known role of "integrator" of multiple signaling cascades, with distinct Notch receptors tuning distinct downstream targets, and cell-type specific

Notch partners as well as distinct epigenetic states at the level of the Notch target genes modulating the final outcome of Notch signaling activation ([96] and references therein).

Wnt/β-catenin signaling – regulator of proliferation and neurogenic competence of glia

The canonical Wnt/β-catenin (cat) pathway is one of the most conserved signaling cascades operating in NSCs during brain development and in the adult neurogenic niches, where it contributes to the regulation of cell self-renewal, expansion, asymmetric cell division, maturation and differentiation [105]. A direct evidence of Wnt/β-cat involvement in parenchymal adult neurogenesis in the mammalian brain is still lacking. However, Wnt/β-cat signaling is upregulated in astrocytes upon damage [106,107]. Of note, in vitro wound healing experiments in adult astrocyte monolayers showed that Wnt/ β-cat signaling contributes to the initiation of the post-injury activation of astrocytes [108]. These findings suggest that Wnt may initiate and sustain astrocyte activation, and possibly facilitate their transition toward a multipotent progenitor phenotype. This possibility is corroborated by the fact that β-cat expression sustains the upregulation of stem cell markers during the activation of astrocytes in vitro [108], and, in turn, Wnt3a expression depends on Sox2 [62]. This suggests a Wnt/β-cat-Sox2 positive loop in activated astrocytes.

More direct evidence is available on the role of the canonical Wnt/β-cat in adult retinal regeneration. By using a Wnt/β-cat reporter mice, in 2007 Osakada and colleagues [109] showed nuclear accumulation of β-cat in dividing MG responding to a NMDA-induced retinal injury in mice. Treatment with Dickkopf-related protein 1, a negative modulator of Wnt signaling, greatly reduces MG proliferative response and the number of retinal progenitors in explants of adult mouse retina. In the canonical pathway, Wnt signaling leads to inhibition of glycogen synthase kinase 3β (GSK3β), which subsequently targets β-cat to the nucleus, where it promotes the transcription of target genes associated with cell cycle entry, such as cyclin D1 [110]. Treatment with inhibitors of GSK3β markedly

increases cell proliferation across the retinal layers and amplified the number of dividing Pax6+ progenitors in explants of the adult mouse retina [109]. Such effect is reminiscent of that obtained by the treatment with Wnt2b/3a in vivo and ex-vivo [104,109] and point to the implication of the canonical Wnt signaling in MG proliferation and de-differentiation upon injury. In vivo gene-transfer of β-cat or deletion of GSK3β in adult mouse MG markedly increase MG proliferation even in absence of injury. This effect is mediated by the upregulation of Lin28 and subsequent repression of the let-7 miRNA expression. In this injury-free experimental paradigm, a fraction of Wnt-activated MG-derived progenitors eventually differentiate in amacrine cells [73].

In vitro experiments suggest that Wnt is also implicated in a cell-autonomous/autocrine manner in the neuronal differentiation of MG-derivatives: exposure of human MG with a cocktail of factors that induce their differentiation in photoreceptors markedly upregulates the expression of components of the canonical Wnt pathway and its secretion. Further, inhibitors of the canonical Wnt signaling prevents MG conversion in photoreceptor [47]. Taken together, these findings provide compelling evidence that the canonical Wnt signaling, along with its downstream targets, constitute a central signaling axis in regulating proliferation and the neurogenic potential of MG in the adult mammalian retina.

Bone Morphogenetic Proteins – promoter of gliogenic fates

Bone morphogenetic proteins (BMP) are a group of secreted signaling molecules that belong to the transforming growth factor β (TGFβ) superfamily. Despite their involvement in retinal and brain development [111-113] and adult neurogenesis in the canonical niches [114], few data are currently available about their contribution to post-injury astrocyte/MG activation and neurogenic response. Interestingly, both BMP ligands and receptors are increased following CNS injury in astrocytes [115]. Studies on adult mouse retinal explants showed that EGF-induced proliferation in MG requires the activation of the BMP pathway

[116]. These data suggest an early implication of the BMP signaling in post-injury glia activation and proliferation. However, BMP are also well known gliogenic factors participating in the neurogenesis-to-gliogenesis switch occurring in the late embryonic phases of CNS development [112,113]. In addition, BMP has a role and in astroglia lineage commitment of SVZ-derived cells [114]. Thus, to favor a neurogenic outcome, after an early activation, BMP signaling cascades should be repressed (see The small molecule approach section).

Growth factors – promoter of glia activation, neurogenic switch and neuronal survival

Upon injury, reactive microglia and astrocytes upregulate the expression of a broad array of growth factors (GFs), including basic fibroblast growth factor (FGF2), EGF, insulin growth factor 1 (IGF1) and vascular endothelial growth factor (VEGF) [101]. All of these factors can activate intracellular signal cascades via Janus kinase/signal transducer and activator of transcription (Jak/Stat) and/or mitogen-activated protein kinase (MAPK) pathways. Hypothetically, GFs may promote glia proliferation and acquisition of progenitor-like traits [117]. Of note, in vitro GFs are involved in the acquisition of progenitor features in reactive astrocytes, including expression of NSC markers (nestin and CD133) and multipotency [118,119]. While GF infusion did not *per se* elicits an appreciable neurogenic response from parenchymal reactive glia in vivo [120], in vitro and in vivo reprogramming approaches have been designed by combining forced expression of inducers of stemness/proneural genes and administration of GFs [85,120]. After a stab wound in the adult rat brain, the local administration of FGF2 and EGF potentiates the reprogramming effect of the retrovirally-mediated expression of Ngn2, increasing the number of newly-generated DCX+/ neuronal nuclear antigen (NeuN)+ neurons in the striatal and cortical parenchyma. Of note, GFs do not operate by simply inducing the expansion of genetically modified cells, but likely facilitate neuronal reprogramming and sustain cell survival [120].

Similar to what described upon brain injury, retinal lesions stimulate the upregulation of GF receptors in MG and the release of a variety of factors, including EGF, FGF1, FGF2 and IGF-1 ([117,121] and refs therein), suggesting their implication in MG response. Interestingly, differently from what observed in the cortex and striatum, post-injury intraocular injection of GFs, either alone or in various combinations, can enhance injury-induced MG proliferation and neurogenesis in adult rodents [117]. Namely, the acute in vivo treatment with EGF, FGF1 or FGF1+insulin after NMDA-induced lesion increased the number of dividing MG and of MG acquiring progenitor markers (i.e. Pax6) in the mouse [42]. This was accompanied by the appearance of some newly-generated amacrine cells. However, to obtain a significant number of these new neurons FGF1/insulin had to be injected once a day for 4 consecutive days. Although most of the MG progeny obtained by these manipulations died within the first week of their production, a subset of these cells survived at least 1 month and appeared stably integrated in the tissue [42]. Similarly, the in vivo injection of heparin-binding EGF-like growth factor (HB-EGF) in the mouse retina stimulated the proliferation of MG following NMDA-induced damage [122]. Thus, in the injured mammalian retina, the in vivo administration of GFs potentiates spontaneous parenchymal neurogenesis by boosting both MG activation and differentiation of MG derivatives.

3.1.2 Potential contribution of circulating multipotent cells

In the damaged parenchyma, infiltrating circulating progenitor cells of bone marrow origin, might support the neurogenic conversion of local astroglia through the local release of morphogens, mitogens and instructive signals [123-125]. Moreover, fusion events between bone marrow-derived stem cells and parenchymal cells could influence glia responses.

Cell fusion can occur between bone marrow-derived stem cells and neural cells, specifically neurons. Although these events appear to occur at a very low frequency in physiology [126,127] and have been shown to be limited to specific neuronal types [128,129] and phagocytic glial cells (e.g. MG in the retina), inflammation promotes both migration and infiltration of bone marrow-derived cells to sites of brain injury [123-125] and fusion events [129,130]. It might therefore occur that in the lesioned parenchyma, blood-derived elements fuse with parenchymal astroglia thereby fostering the activation of the neurogenic program. Indeed, this strategy was successfully exploited to transfer exogenous reprogramming factor to retinal MG [74]. However, no evidence in support of the occurrence of blood-derived cells-astroglia fusion is currently available outside the retina.

3.1.3 Role of inflammation-related signals

Upon injury, pro-inflammatory cytokines (e.g. tumor necrosis factor (TNF)-α, interferon gamma (IFN)-γ, interleukin (IL)-1β) are released by reactive astrocytes and, in even larger amounts, by activated microglia [101]. Intriguingly, when cultivated in presence of TNF-α, astrocytes are induced to acquire stem/progenitor properties, associated with transcriptomic and epigenetic changes consistent with a neurogenic-like state [131]. Mechanisms mediating such effects are still obscure. In vivo, inflammatory factors entering the brain after injury could contribute to upregulate Shh expression and activation of Shh signaling in reactive astrocytes [132]. Further, TNFα potentiates glutamate release by astroglia [119]. In principle, both Shh- and neutrotransmitter-induced signaling may contribute to astrocyte activation and acquisition of a neurogenic competence. In line with this idea, in a certain concentration range, pro-inflammatory molecules positively modulate

NSC proliferation and neuroblast production in the canonical niches in the adult rodent brain [97,133]. In the SVZ and SGZ, the main cellular source of these mediators is local microglia. Of note, microglia/macrophage ablation significantly reduces Shh expression in the cortex upon injury [132]. Thus, it is likely that microglia/astrocyte crosstalk may contribute to astroglia activation and acquisition of a neurogenic potential after brain damage.

Notably, when macrophage/microglia ablation or immunosuppression (i.e. activation of the glucocorticoid signaling) are induced before the onset of a retinal degeneration, MG proliferative response and photoreceptor replacement are significantly delayed even in fish, where a prompt and efficient regenerative response is normally accomplished upon injury/pathology [134]. Thus, a certain degree of microglia/macrophages activation/release of pro-inflammatory molecules appears required to induce MG activation and, possibly, a neurogenic response.

Nevertheless, pro-inflammatory signals and immune-mediated mechanisms may also be detrimental for the neurogenic outcome. It is well known that high levels of inflammation suppress the neurogenic functions of the canonical niches ([133] and references therein). Such effect may be due, at least in part, to a negative interference on the Wnt/β-cat pathway (i.e. β-cat downregulation and phospho-GSK3β increase [135]. In line with this idea, parenchymal neurogenesis appeared compatible only with moderate levels of inflammation and transient microglia activation. Examples include mild ischemia [19], experimental cell ablation in specific cortical layers [13] or of selected neuronal populations (e.g. light or NMDA-induced retinal damage inducing cell death in photoreceptors or amacrine and ganglion cells, respectively [41,49,73]; experimental models of retinitis pigmentosa, with a selective degeneration of photoreceptors [74,75]), when neurodegeneration is slow and progressive [20], or several weeks after an excitotoxic insult or stroke [17,19].

Indeed, it is hypothesized that one factor promoting parenchymal neurogenesis and tissue repair in fish is the short duration of the post-injury inflammatory burden [136]. Consistently, immunosuppression at post-acute stages after neurodegeneration onset further accelerates the kynetics of neuronal replacement in zebrafish retina [134]. Moreover, in case of severe and extensive damage with persistent accumulation of immune system cells at the site of injury, regeneration of the lost elements does not occur properly even in fish [45,132].

Indeed, some immune-related molecules may limit MG proliferation and differentiation along the neuronal lineage. TGFβ1, is considered as a key player maintaining MG quiescence in the adult mammalian retina [137]. Further, it is able to suppress the neuronal differentiation of human MG induced by a cocktail of factors including FGF2, IGF-1, taurine and retinoic acid (RA). Such TGFβ1 effect is associated to the reduction of the expression of components of the canonical Wnt signaling pathway [47]. Similarly, IFN-γ hampers FGF2-mediated astroglial dedifferentiation into neurogenic NSCs in vitro [138].

In conclusion, evidence collected so far indicate the importance of a correct tuning of inflammatory/immune-mediated mechanisms for the promotion of parenchymal neurogenesis. As for other forms of post-lesion repair, a bifacet model of pharmacological intervention should be implemented to limit the detrimental effects of a persistent/overt inflammation, while maintaining/boosting the contribution of inflammatory/microglia-mediated signals on astroglia/MG activation.

3.1.4 Role of Neurotransmitters

Upon injury, damaged and dying neurons as well as reactive astrocytes may release significant amounts of neurotransmitters, including glutamate, GABA, purines, etc.

[101,119]. Neurotransmitters are well known modulators of several aspects of developmental neurogenesis, including proliferation, migration and differentiation in various CNS areas, such as the telencephalon, ventral midbrain and retina. Recent studies have also shown their implication in adult neurogenesis in the canonical niches [139].

Besides its broad actions during developmental neurogenesis and in neurogenic niches [140], dopamine signaling is particularly noteworthy because it was proposed to provide a negative feedback to progenitors generating dopaminergic neurons. This has been demonstrated in aquatic salamanders where dopamine negatively controls the production of dopamine neurons both during homeostasis and regeneration of the dopaminergic system at adult stages [140,141,142]. Studies in rodents showed that ventral cells expressing markers of dopaminergic progenitors (Lmx1a, Nestin, Sox2, Sox3 and prominin) persists, although in limited numbers, beyond embryogenesis into adulthood in the midbrain aqueductal zone. These cells express dopamine receptors and are exposed to a dense meshwork of midbrain dopamine fibres. Of note, their proliferation can be stimulated by antagonizing dopamine receptors ultimately leading to increased neurogenesis in vivo beyond the normal period of embryonic dopamine neurogenesis [143]. It remains to be tested whether at adult stages these cells are maintained quiescent by dopamine and if blockade of dopaminergic signalling can induce their reactivation, possibly providing the mechanistic substrate for de novo neurogenesis of dopaminergic neurons, as shown in some reports [144].

To our knowledge, no data are available on the contribution of other neurotransmitters in the modulation of the neurogenic competence in the brain or spinal cord parenchyma. Conversely, interesting data have been obtained in the retina, where NMDA- and nicotinic-receptor mediated signaling foster the spontaneous neurogenic processes.

In a pioneer study, Sahel and colleagues [145] observed that the proliferative response induced in adult rat MG by NMDA, kainic acid, domoic acid and oubain could be reduced

by ketamine anesthesia. Since ketamine is an antagonist of NMDA-receptor, this finding suggests that post-lesion MG proliferation is not only triggered by injury-related signals, but also by a direct effect of excitatory amino acids. More recently, by subretinal injection of various concentrations of glutamate and alpha-aminoadipate (a glutamate analogue that specifically binds MG) Takeda and colleagues [146] showed that sub-toxic levels of glutamate directly stimulate MG to re-enter the cell cycle and induce neurogenesis in vivo in adult mice. Alpha-aminoadipate also stimulates MG differentiation into photoreceptors in vivo. These data clash with the negative effect of NMDA-receptor agonists on neural progenitor proliferation reported in the intact hippocampal SGZ. However, they are in line with data on hippocampal neurogenesis in the ischemic and epileptic brains [139]. Thus, the effect of NMDA-agonists on neural progenitors is largely context-dependent. These results suggest that glutamate signaling is not a pivotal determinant of progenitor behavior, but rather acts as a modulator of other signaling cascades.

Cholinergic signaling has also been implicated in the de-novo generation of retinal neurons. The α7 nicotinic acetylcholine receptor agonist PNU-282987 activates neurogenesis in the adult rat retina even in absence of injury or administration of exogenous GFs [147]. After treatment with PNU-282987, MG increase proliferation and MG-derived progenitors differentiate and migrate to both the photoreceptor and retinal ganglion cell layers. Agonization of α7 nicotinic receptors expressed by parenchymal glia has recently obtained attention as a way to modulate post-lesion inflammation [148,149]. It is also reported to increase expression levels of neuroblast markers in the mouse brain [150], but this action requires to be better understood.

Purinergic signals are well-established modulators of astroglia/MG reactivity upon injury [101,151]. In vivo studies also show their involvement in the regulation of the progenitor functions in the adult SVZ [152]. However, so far no evidence has been provided about purine contribution in favoring/eliciting astroglia/MG neurogenic competence.

Nevertheless, their implication may be somehow expected since GF receptors (i.e. FGF2 and EGF receptors) can be transactivated by P2Y receptor activation through different intra-/extra-cellular mechanisms ([101], and references therein). Further studies are needed to assess whether this can be relevant for astroglia/MG activation and/or differentiation toward neuronal phenotypes.

3.1.5 Role of cholesterol metabolites

Cholesterol derivatives also received attentions as pharmacological targets to effectively modulate neural stem cell functions and promote neurogenesis. Liver X receptors (LXRs), are members of the nuclear receptor superfamily that heterodimerize with retinoid X receptors and are activated by specific oxidized cholesterol natural metabolites, oxysterols, that function as endogenous ligands. In vivo and in vitro evidence in animal models showed that LXRs and their ligands are potent regulators of ventral midbrain neurogenesis, and dopaminergic neuron development [153]. Moreover, oxysterols were shown to drive dopaminergic neurogenesis from human embryonic stem cells [154], suggesting that they could improve current reprogramming protocols (see below). Similar effects were recently found for dendrogenin A and B, which also belong to the family of oxysterols andshowed potent mitogenic effects on mouse neural stem cells in vitro as well as moderate differentiative actions along the neuronal lineage [155].

The known actions of LXR agonists in reducing neuroinflammation and amyloid aggregates [154], further increase the interest for studies assessing their efficacy in neural replacement in neurodegeneration. Notably, treatment with the LXR agonist GW3965 increased the number of proliferating neural progenitors in the SGZ of Alzheimer's Disease mouse models [156]. This effect appears at least in part mediated by epigenetic

mechanisms (i.e. changes in the DNA methylation state of neurogenesis-related genes; [157]). It would be interesting to examine whether these signals take part and could improve spontaneous parenchymal neurogenesis and/or reprogramming strategies.

3.2 Combinatorial approaches to foster the production of neurons from glia

As mentioned before, after the initiation of neurogenesis, most of the neuroblasts generated in the adult CNS parenchyma do not stably integrate in the pre-existing circuitries and die [17,20,42]. Further, reprogramming often occurs with a limited efficiency (e.g. [65]). Moreover, after either spontaneous regenerative events or reprogramming, new neurons undergo a limited maturation and may belong to restricted number of neuronal subtypes (see Table 1) [17,18]. Thus, the efficient production of fully differentiated neurons or the generation of a larger repertoire of neuronal phenotypes may require additional manipulations, including the exposure to factors that boost neurogenesis, promote survival or full differentiation, or trigger the acquisition of a specific identity in newborn elements. Until now, several attempts were directed toward the implementation of reprogramming efficiency and neuron maturation.

Among the factors that affect neuron generation from glia or other somatic cells, metabolic states have recently gained significant attention. Single-cell transcriptomic analyses showed that the activation of NSCs in the canonical niches is accompanied by the downregulation of genes associated with the glycolytic metabolism [97], which is the way by which astroglia and fibroblasts meet energy demands [158,159]. A similar transition from glycolysis to oxidative phosphorylation has been found during neuronal differentiation of human iPSCs derived from reprogrammed fibroblasts. In these cells, the forced constitutive expression of the glycolitic enzymes hexokinase (HK2) and lactate

dehydrogenase (LDHA) during differentiation leads to cell death, indicating that the shut-off of glycolysis is essential for reprogrammed cell survival [160]. Notably, such metabolic transition was shown to be a prerequisite also for astroglia conversion in neurons, which is associated with a peak in oxidative stress eventually leading to cell death in most reprogrammed cells [161](Table 1). Notably, by apoptosis-independent mechanisms involving reduction of reactive oxygen species (ROS) and lipid peroxidation occurring during fate conversion, inhibitors of ferroptosis, antioxidants, and forced expression of Bcl-2 greatly improve the resolution of this critical point and promote glial-to-neuron conversion after traumatic brain injury in vivo [161].

Another intrinsic metabolic constrain to neuron generation from glia and other cell types appears to be cell senescence. Indeed, it has been demonstrated that p53/p21- or p16-induced cell-cycle exit of Sox2-reprogrammed glia or reprogrammed iPSCs largely reduces their neurogenic outcome and constitutes a critical checkpoint for cell reprogramming (Table 1; [162,163] and references therein). These results suggest that blocking cell senescence pathways may enhance glia cell fate conversion and adult parenchymal neurogenesis.

Other adjuvant treatments to promote neuronal survival and/or differentiation included supplementation with neurotrophins (i.e. brain derived neurotrophic factor, BDNF), histone deacetylase inhibitors (i.e. valproic acid, VPA) and BMP signaling antagonists [63–65,162](see also Table 1). RA has also been used to stimulate neuronal differentiation of human MG and MG-cell lines in vitro [47,164]. Consistently, when injected in the adult rat retina, RA does not alter post-injury MG activation, but rather enhance the fraction of MG-derived cells acquiring the phenotype of bipolar neurons [41]. Further, when preceded by Wnt3a treatment, RA induces the differentiation of MG-derived progenitors in mature photoreceptors in adult retinal explants [109]. Further studies are needed to assess the

exact contribution of these and other factors in the final steps of the adult parenchymal neurogenesis in vivo.

3.3 The small molecule approach

Spontaneous activation of neurogenic programs in the CNS parenchyma is a rare phenomenon, with clear limitations in terms of neuronal output. Similarly, in vivo reprogramming into functional neurons, although showing exciting perspectives, still faces many challenges as for efficiency and safety (see section 2). As an innovative and promising solution to the potential risks of introducing exogenous genetic material and altering the genome, small molecules have been employed to implement reprogramming and even to completely replace ectopic transgenes in a variety of cell systems [165]. Small molecules are low molecular weight (<900 daltons; around 1nm in size) organic compounds, including include lipids, monosaccharides, second messengers, natural metabolites as well as drugs and xenobiotics. Small molecules can rapidly diffuse across cell membranes so to reach intracellular sites of action and can generally be easily synthesized. For reprogramming strategies, annotated libraries have been scrutinized to select compounds i) recognized to act on pathways and target proteins known to be involved in cell maturation, growth, survival [166], or ii) best synergizing with reprogramming master TFs [167]. Alternatively, agents formerly proved to be efficacious to convert somatic cells into induced NSCs or iPSCs were directly applied to obtain neurons [78,168,169] (see below). In view of achieving the specification of desired neuronal phenotypes, recent studies have also adopted a bioinfomatic approach based on computational matching of identified pathways specific of diverse neuronal cell types and the related modulating drugs [170]. After screening, dose escalation is normally performed

to optimize synergies or additive actions and avoid toxicity, with subsequent analysis of the effect of removal or replacement of single compounds to identify the components most critical for the desired effects. Thus, as other chemical approaches, the small molecule strategy has the additional advantage to be transient, finely tunable according to the desired effects and amenable to scaling up. Thus, it may potentially lead to the development of drug therapies to stimulate the patients' endogenous cells to repair and regenerate in vivo.

A number of studies have shown that addition of small molecules during reprogramming into pluripotency [171–173] or multipotency [174,175] increased the efficiency of the conversion and in some cases even replaced individual reprogramming determinants [176-178] or the need for transgene expression [179–181]. Transfer of these approaches to direct reprogramming into neurons resulted in a 6-fold increase in direct conversion of human fibroblasts transduced with Ascl1, Brn2, Myt1L through more efficient downregulation of fibroblast programs and upregulation of neuron-specific genes and regulatory networks [166]. This result was achieved by the combinational treatment with Kenpaullone, Prostaglandin E2 (PGE2), Forskolin, BML210, Amonoresveratrolsulfat, and PP2 (see Table 2 for specific molecular actions here and below). A former study demonstrated that Ngn2, normally sufficient to reprogram neural cells but incapable to convert fibroblasts, in the presence of Forskolin and Dorsomorphin promoted the generation of cholinergic neurons from human fetal fibroblasts [182]. In a subsequent report the same research group shed light on how the employed small molecules initiates the acquisition of neuronal phenotypes [78]. They found that the compounds simultaneously activated Ngn2 and co-transcription of the prosurvival factor Creb1 (cyclic adenosine monophosphate - cAMP -responsive element binding protein 1), induced Sox4 expression while enhancing both Sox4-dependent and independent epigenetic changes and chromatin remodeling [78]. In turn, Ngn2 and Sox4 synergized to enhance

the expression of diverse pro-neural TFs including NeuroD1 and NeuroD4. These data show that the applied compounds essentially act by i) targeting master regulators of both reprogramming and neuronal induction and 2) modifying epigenetic barriers opposing cell fate changes.

Two recent studies further showed that appropriate cocktails of small molecules can completely replace genetic strategies to convert fibroblasts into functional neurons. Li et al. employed four small molecules (Forskolin, ISX9, CHIR99021, and I-BET151) to reprogram mouse fibroblasts into neurons with very high efficiency [183]. The authors suggested that I-BET151 suppressed the fibroblast-specific program, and ISX9 activated the expression of the endogenous neurogenic TFs NeuroD1 and Ngn2, which synergistically promote neuronal conversion [183]. In a companion paper Hu et al. demonstrated the generation of neurons from human fibroblasts using a small molecule cocktail (VPA; histone deacetylase (HDAC) inhibitor, CHIR99021, Repsox, Forskolin, SP600125, GO6983, Y-27631, and Dorsomorphin) [167]. Furthermore, via this chemical cocktail, fibroblasts from familial Alzheimer's disease patients could also be reprogrammed to neurons now available for in vitro disease modeling and drug screenings [167]. In this case while VPA and Repsox were suggested to inhibit fibroblast genes, SP600125, GO6983, Y-27631 were proposed to promote neuronal genes. In both cases, neuronal conversion occurred without transiting through an intermediate neural progenitor state, and yielded more glutamatergic than GABAergic neurons.

In regard to manipulation of glia, in the retina small molecules have been so far employed to promote neuroprotection [184,185]. On the contrary, successful conversion of brain astrocytes into neurons has been already achieved. Cheng and collaborators [168] reported that three compounds (VPA, CHIR99021, Repsox) converted both postnatal and adult mouse astrocytes into neurons with various neuronal phenotypes. In this study VPA was shown to be the most active molecule and essentially responsible of the upregulation

of NeuroD1. Conversely, Repsox and CHIR99021 had a minor or minimal impact on neuronal induction. Of note, no transition through a Sox2+ stage or upregulation of proneuronal TFs was observed. Interestingly, when the VPA, CHIR99021, and Repsox mix was also employed to treat human adult astrocytes, it failed to convert the cells [169]. This outcome suggests remarkable differences in the astroglial barrier to reprogramming depending on species and age. The authors therefore added Forskolin, ISX9, CHIR99021, and I-BET151 to the cocktail and obtained functional neurons with predominant glutamatergic phenotypes that could survive and became electrophysiologically active even after trasplantation in the postnatal rodent brain. Of note, while NeuroD1, Ngn2 and Ascl1 resulted upregulated during conversion, markers of neural progenitor cells (proliferation, Sox2, Pax6, Nestin) were not altered, confirming a direct conversion. Here, ISX9 confirmed its action as activator of neuronal genes while I-BET151 was proposed to down-modulate astroglial gene programs. Moreover, as the authors refer, in line with [168] VPA functioned to activate neuronal genes and Forskolin promoted morphological changes. However, removal of CHIR99021 and Repsox completely abrogated neuron induction, indicating their essential, although not sufficient roles.

Zhang et al. instead reported a protocol based on i) the sequential inhibition of factors promoting glial fates and exposure to compounds activating neuronal signalling pathways, and ii) exposure to neural patterning factors (LDN193189; SB431542; Thiazovivin; CHIR99021, VPA, DAPT, the Smo agonist SAG and Purmorphamine, TTNPB) [186]. BDNF, neurotrophin-3 (NT3) and IGF1 were also applied by the end of the defined sequential treatment to promote neuronal maturation. In this case, human fetal astrocytes from the cerebral cortex, midbrain and spinal cord were analyzed. Cortical and midbrain astroglia efficiently converted into glutamatergic neurons with deep layer/hippocampal phenotypes. This was achieved through upregulation of NeuroD1, Ascl1 and Ngn2 with no transition through a neural progenitor stage. Conversely, spinal cord neurons did not

respond to these manipulations, highlighting a high degree of heterogeneity in the conduciveness to reprogramming of distinct astroglial subsets. Mechanistically, the small molecules acted through both epigenetic and transcriptional modulation. VPA and activation of the Shh pathway essentially implemented reprogramming efficiency, while Thiazovivin and TTNBP removal had no effect. Inhibition of Notch, GSK3β, BMP and TGFβ were instead indispensable for reprogramming. Remarkably, human induced neurons were functional and could survive more than 5 months under cell culture conditions and upon transplantation into the mouse brain.

On the whole, these studies show that astrocytes are amenable to be efficiently converted in neurons by small molecules in vitro and suggest that specific combinations of compounds have to be designed depending on the target astroglial types and the desired neuronal output. It is tempting to speculate that commonalities among different protocols, such as inhibition of GSK3β with the resulting activation of Wnt signaling and of TGFβ, may act by recapitulating neural development programs and/or modulate some level of astrocyte activation contrasting gliogenic mechanisms (Figure 3). However, what pathways are implicated in this chemical-based induction protocols remains to be understood. In addition, it is interesting to note that RA signaling, which is crucial for NSC functions, appeared dispensable for astrocyte conversion [186]. Moreover, Shh treatment, shown to be critical to activate stem cell properties in astrocytes [37], turned out to only moderately increase reprogramming efficiency [186]. These results clarified that the tested combination of compounds, like those of the other listed studies, act through direct conversion and do not recapitulate the mechanisms of spontaneous activation of the neurogenic potential of parenchymal astrocytes.

Thus, small molecules constitute a concrete perspective to replace the worries of unwanted actions of master TFs in vivo by promoting neuron generation from glia either directly or through a neural progenitor stage. They hold an immediate application for the in

vitro derivation of transplantable, safe, desired cell types; however, their in vivo exploitation still requires a long way of fundamental research and technological development. Applications to discrete sites of damage (e.g. retinal damage, localized injury to the CNS) are feasible (just as direct injections of viral particles for gene therapy) and avoid the risks of genetic manipulations. However, treatments must minimize unwanted side effects on non-target cells in the tissue and solve the complications of potential sequential combinations.

Small molecules could even be more interesting for application to broad CNS areas affected by neurodegeneration. In such instances, systemic treatments would be most appropriate. Efficient passage of the blood brain barrier should then be ensured and issues related to pharmacokinetics and pharmacodynamics of each compound, as well as of their combinations solved. Yet, alternative routes as intranasal delivery can be considered [170]. On top of this, for systemic treatments it is mandatory to minimize side effects or toxicity on non-target organs. The development of devices or strategies for targeted delivery and controlled release of compounds will greatly benefit the field. However, first in vivo applications of a related approach based on the administration of a single compound to foster and direct the activity of mouse SVZ are encouraging [170], and stimulate further in vivo pioneer studies.

4. Open issues and Concluding remarks

Regeneration of functional neurons remains an unmet need in CNS repair. In this review we have summarized the enormous advancement obtained in the last years toward the generation of functional neurons from endogenous glial cells. This field of research is still in its infancy and big efforts are still needed both to fully understand the molecular and cellular mechanisms guiding the neurogenic activation of glia, and to find the right

combination of genetic and pharmacological approaches to eliminate cell intrinsic and environmental constraints. Moreover, it will be essential to understand which pharmacological approaches increase the efficiency of neuronal induction and direct neuroblast differentiation toward specific desired phenotypes. So far, differentiation of new neurons is modest and a limited number of neuronal subtypes has been generated. More research is needed to obtain distinct neuronal subtypes, although some types of neurons might turn out to be difficult or impossible to produce by reprogramming approaches.

Importantly, while evidence for functional recovery operated by induced neurons has been provided in the retina, the achievement of functional repair by spontaneous neurogenesis or reprogramming remains to be demonstrated for the rest of the CNS.

The possibility to employ small molecules as an alternative to reprogramming approaches based on forced expression of exogenous genes appears very attractive because it bypasses the risks of genetic manipulations in cell types that may be prone to generate tumors. However, the efficacy of the small molecule approach has still to be proved in vivo. Nevertheless, the availability of these new compounds, as well as a more profound understanding of niche-associated factors, indicate that we are on the way to reach an era of “pharmacological plausibility”. These drug-base strategies could not only prevent neurodegeneration, but they could also impact on tissue regeneration.

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Figure and Table legends

Figure 1. Schematic representation of the inducible Cre-based lineage tracing approach. The inducible Cre lox system allows to trace the lineage of the cells based on the activity of specific promoters at the stage of tamoxifen administration. Mice expressing the tamoxifen-inducible recombinase CreERT2 under the control of a cell type specific promoter (P) are crossed with ROSA26-loxP-STOP-loxp-YFP reporter mice. Cre-ERT2 is a fusion protein derived from Cre recombinase and mutated estrogen receptor (ERT2). In the absence of tamoxifen Cre-ERT2 localizes in the cytoplasm and does not reach the DNA to operate recombination. When tamoxifen binds to the ERT2 domain, activated Cre-ERT2 enters the nucleus, recombines the loxP sites removing the stop codon and leads to the expression of YFP. The stop codon removal is not reversible. Thus, these cells will remain YFP-positive even if the P is switched off. YFP expression is inherited to the progeny of the recombined mother cell, therefore allowing fate mapping studies.

Figure 2. Schematic representation of MG-derived neurogenic events occurring in the retina upon injury or after transgenic reprogramming. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; MG, Müller glia; NP, neural progenitor; HC, horizontal cell; BC, bipolar cell; AC, amacrine cell; RGC, retinal ganglion cell.

Figure 3. Schematic representation of protocols for astroglial reprogramming by small molecule. Ngn2, neurogenin2; for acronyms see Table 2.

Table 1. In vivo and ex vivo (retina) reprogramming factors. TF, transcription factor; AAV, adeno-associated virus; ABM, Ascl1, Brn2a, Mytl1; AD, Alzheimer disease; ALN, Ascl1,

Lmx1a, Nurr1; AV, adenovirus; BDNF, brain-derived neurotrophic factor; DCX, doublecortin expressing neuroblasts; EAN, electrophysiologically active neurons; ERGR, electroretinographic response; GABAergic, gamma-aminobutyric acid neurons; GFs, fibroblast growth factor (FGF) + epidermal growth factor (EGF); HUC/D, postmitotic neurons; LV, lentivirus; MG, Müller glia; NeAL218, NeuroD1, Ascl1, Lmx1a, miR218; NMDA, N-methyl-D-aspartate receptor; MNU, N-methyl-N-nitroso urea; NeuN, neuronal nuclear antigen, mature neurons; Neurogenin2, Ngn2; NOG, noggin; 6-OHDA, 6-hydroxydopamine; RV, retrovirus; TH, Tyrosine hydroxylase; Tuj1, beta III tubulin expressing neuroblasts; VPA, valproic acid; na, not assessed.

Table 2. List of compounds employed for in vitro reprogramming of fibroblasts and astrocytes. References can be found in the text. AMPK, AMP-activated protein kinase; BLM 210: N-(2-aminophenyl)-N'-phenyloctanediamide; BMP, bone morphogenetic protein; cAMP, *cyclic* adenosine monophosphate; CHIR99021: 6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile; DAPT, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester; GO6983, 3-[1-[3-(Dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione; GSK3β, glycogen synthase kinase 3 β; HDAC, histone deacetylase; I-BET151, BET family bromodomain inhibitor 151; ISX9, isoxazole 9; LDN193189, 4-[6-[4-(1-Piperazinyl)phenyl]pyrazolo[1,5-a]pyrimidin-3-yl]-quinoline; JNK, c-Jun N-terminal kinase; MEF2, *myocyte enhancer factor-2;* PGE2: Prostaglandin E2; *PKA,* protein kinase A; *PKC,* protein kinase C; *PP2, 4-Amino-3-(4-chlorophenyl)*-1-(t-butyl)-1H-pyrazolo[3,4-d]pyrimidine; RA, retinoic acid; Repsox, E-616452; 2-[3-(6-Methyl-2-pyridinyl)-1H-pyrazol-4-yl]-1,5-naphthyridine; ROCK, Rho-associated protein kinase; SAG, Smoothened agonist; SB431542, 4-(5-Benzol[1,3]dioxol-5-yl-4-pyrldin-2-yl-1H-imidazol-2-yl)-benzamide; SP600125, 1,9-Pyrazoloanthrone; Shh: Sonic hedgehog, SIRT1, Sirtuin 1; TGFβ,

transforming growth factor β; TTNPB, Selective agonists of the RAR; VPA, valproic acid; Wnt, wingless-type MMTV integration site family.

Marked Table 1

TF	CNS region	Cell type	Lesion	Combinatorial treatment	Final cell type	Functionality	Administration	Species	Ref
Pax6	Cortex	Proliferating cells	Stab wound		DCX	na	RV	Mouse	[51]
NeuroD1	Cortex	Astrocytes	Brain injury/ AD model		Glutamatergic	EAN	RV	Mouse	[70]
		NG2 cells			Glutamatergic, GABAergic				
Sox2 w/o Ascl1	Cortex	NG2 cells	Stab wound		DCX, NeuN	EAN	RV	Mouse	[66]
Ascl1					no neurons				
Sox2	Striatum	Astrocytes		BMP, NOG, VPA	DCX, NeuN, Calretinin	EAN	LV	Mouse	[63,64]
Ascl1					No neurons				
ABM	Striatum	Astrocytes			NeuN	na	LV	Mouse	[187]
NeAL218	Striatum	Astrocyte	6-OHDA		DCX, TH, SLC6A3	EAN, behavioral study	LV/AAV	Mouse	[61]
ALN	Striatum	NG2 cells			NeuN, MAP2	EAN, integrated into host neural circuits (rabies-virus-based tracing)	AAV	Mouse	[188]
		Astrocytes			Glutamatergic, GABAergic				
Ascl1	Midbrain	Astrocytes			Glutamatergic, GABAergic	EAN	AAV	Mouse	[72]
	Striatum				NeuN				
	Cortex				NeuN				
	Cortex	Proliferating cells	Stab wound	GFs	no neurons		RV	Rat	[120]
	Striatum				DCX, NeuN	na			
Ngn2	Cortex Striatum	Proliferating cells	Stab wound	w/o GFs	DCX, NeuN	na			
PAX6	Cortex/ Striatum	Proliferating cells	Stab wound	w/o GFs	DCX, NeuN	na			
Ascl1	Cortex/ Striatum	Proliferating cells	Stab wound	w/o GFs	no neurons				
Ngn2, Bcl2	Cortex				DCX, NeuN	na	RV	Mouse	[161]
	Spinal cord	Proliferating cells: majority Olig2+/NG2 cells	Laminectomy	GFs	HuC/D, TuJ1	na	RV	Rat	[189]
Ngn2				GFs	HuC/D, NeuN				
				BDNF	NeuN				
Ascl1					no neurons				
Sox2	Spinal cord	Astrocytes	w/o hemisection	VPA	DCX, MAP2, NeuN, glutamatergic, GABAergic	na	LV	Mouse	[65]
Sox2, p53-p21 removal	Spinal cord	Astrocytes	w/o traumatic injury	BDNF, NOG	DCX, glutamatergic	na	LV	Mouse	[162]

Ascl1	Retina (juvenile)	MG/astroglia	NMDA-injection/ light damage	Bipolar cells, Amacrine cells and Rod photoreceptors	na (expression of ribbon synapse markers)	Transgenic mouse	Mouse	[49]
Atoh7	Retina	MG	Model of glaucoma	Retinal ganglion cells	na	LV	Rat	[77]
β-catenin	Retina	MG/astroglia	No	Amacrine, retinal ganglion cells	na	AAV	Mouse	[73]
GSK3β deletion				Expression of the pluripotency factor Lin28 in MG	na	Transgenic mouse		
Lin28				Pax6 MG-derived progenitors, amacrine and retinal ganglion cells	na	AAV		
Lin28	Retina	MG/astroglia	Model of retinitis pigmentosa	Photoreceptors and bipolar cells	ERGR	AV	Mouse	[75]
Inhibition of GSK3β	Retina	MG/astroglia	MNU-injection	Photoreceptors	ERGR	In vivo cell fusion	Mouse	[74]
NeuroD	Retina (adult retinal explant)	Proliferating cells	NMDA-injury	Amacrine cells	na	RV	Rat	[41]
Math3				Amacrine cells	na			
Pax6, NeuroD				Increased production of amacrine cells	na			
Pax6, Math3				Increased production of amacrine cells	na			
Crx, NeuroD				Photoreceptors	na			

Emerging pharmacological approaches to promote neurogenesis from endogenous glial cells

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Abstract

Neurodegenerative disorders are emerging as leading contributors to the global disease burden. While some drug-based approaches have been designed to limit or prevent neuronal loss following acute damage or chronic neurodegeneration, regeneration of functional neurons in the adult Central Nervous System (CNS) still remains an unmet need. In this context, the exploitation of endogenous cell sources has recently gained an unprecedented attention, thanks to the demonstration that, in some CNS regions or under specific circumstances, glial cells can activate spontaneous neurogenesis or can be instructed to produce neurons in the adult mammalian CNS parenchyma. This field of research has greatly advanced in the last years and identified interesting molecular and cellular mechanisms guiding the neurogenic activation/conversion of glia. In this review, we summarize the evolution of the research devoted to understand how resident glia can be directed to produce neurons. We paid particular attention to pharmacologically-relevant approaches exploiting the modulation of niche-associated factors and the application of selected small molecules.

Abbreviations (alphabetical order)

+, positive; AMPK, AMP-activated protein kinase; Ascl1, Achaete-scute homolog 1; Atoh7, Atonal basic-helix-loop-helix transcription factor 7; β-cat, beta-catenin; BDNF, brain-derived neurotrophic factor; BLBP, brain lipid-binding protein; BMP, Bone Morphogenetic Protein; Brn2, murine brain-2 transcription factor; cAMP, cyclic adenosine monophosphate; CNS, Central Nervous System; Creb1, cAMP responsive element binding protein 1; Crx, cone-rod homeobox gene; DAPT, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester; DCX, doublecortin; Dll, delta-like; Dlx, related to the Drosophila distal-less homeobox transcription factor; DNA, deoxyribonucleic acid; Dnmt3b, DNA methyltransferase 3b; EGF, epidermal growth factor; FGF, fibroblast growth factor; GABA, gamma-aminobutyric acid; GF, growth factor; GLAST, Glutamate aspartate transporter; GSK3β, glycogen synthase kinase 3 beta; HB-EGF, heparin-binding EGF-like growth factor; HDAC, histone deacetylase; Hes, hairy and enhancer of split; HK2, hexokinase; Hmga2, high mobility group AT-hook 2; IFN-γ, interferon gamma; IGF1, insulin growth factor 1; IL-1β, interleukin 1 beta; iPSC, induced pluripotent stem cell; Jag1, Jagged 1; Jak/Stat, Janus kinase/signal transducer and activator of transcription; JNK, c-Jun N-terminal kinase; Klf4, Kruppel-like factor 4; LDHA, lactate dehydrogenase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Math3, mouse Ath3 (Atonal basic-helix-loop-helix Transcription Factor3); Mbd1, methyl-CpG binding domain protein 1; MEF2, myocyte enhancer factor-2; MG, Müller glia; miRNA, microRNA; MNU, N-methyl-N-nitrosourea; Myc, myelocytomatosis oncogene; Myt1L, Myelin Transcription Factor 1 Like; NeuN, neuronal nuclear antigen; NeuroD1, Neurogenic differentiation 1; NG2, neural/glial antigen 2; Ngn2, Neurogenin2; NICD, Notch intracellular domain; NMDA, N-Methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NSC, neural stem cell; NT3, Neurotrophin-3; Oct4, octamer-binding transcription factor 4; Olig2, Oligodendrocyte Lineage Transcription Factor 2; P, postnatal day; p16, cyclin-dependent kinase inhibitor

2A; p21, cyclin-dependent kinase inhibitor 1; p53, Tumor protein p53; Pax6, Paired Box 6; PDGFRα, Platelet-derived growth factor receptor alpha; PGE2, Prostaglandin E2; PKA, protein kinase A; Plp, proteolipid protein; QA, quinolininc acid; RA, retinoic acid; Rbpj, recombining binding protein suppressor of hairless; RC2, Radial Glial Cell Marker-2; REST, Repressor element 1 (RE1)-silencing transcription factor; RNA, ribonucleic acid; ROCK, Rho-associated protein kinase; ROS, reactive oxygen species; Shh, Sonic hedgehog; SGZ, subgranular zone; SIRT1, Sirtuin 1; Smo, Smoothened; Sox2, SRY (sex determining region Y)-box 2; Sox4, SRY (sex determining region Y)-box 4; Sox9, SRY (sex determining region Y)-box 9; SVZ, subventricular zone; TF, transcription factor; TGFβ, transforming growth factor; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; VPA, valproic acid; Wnt, wingless-type MMTV integration site family

1. New neurons in the mature central nervous system: the dream of a new brain

Neurodegeneration after injury or disease is a chronic and incurable condition whose disabling effects may continue for years or even decades. While the contribution of neurodegenerative pathologies including stroke, Alzheimer's and Parkinson's diseases to the global disease burden is growing fast, regeneration of functional neurons still remains an unmet need.

Strategies to replace lost neurons can rely on either transplantation of exogenous cells or the exploitation of endogenous sources. The field of cell transplantation has developed over a long time now, and progressed enormously, to the extent that it appears to be very close to proposing for clinical trials authentic human neurons derived from human embryonic stem cells [1]. However, the use of human stem cells faces both ethical issues and the challenge to overcome immunorejection. Induced pluripotent stem cells (iPSCs) can represent an excellent alternative for autologous applications. Still, the field needs further advancement in differentiation protocols and solutions to manage risks of introduction of genetically manipulated material. In this evolutionary landscape, further complicated by the costs of stem cell therapies based on good manufacturing practices and delicate surgical procedures, exploitation of endogenous neural cells has recently gained an unprecedented attention. Today this field of research has become very active despite initial disappointments due to the failure to obtain replacement of neurons after damage by endogenous neural stem cells (NSCs) of the adult germinative niches [2,3]. Crucial to attract researchers' interest were the clear demonstrations that the central nervous system (CNS) can activate spontaneous neurogenesis, and that endogenous glia can be instructed to produce neurons by reprogramming (see below).

Targeting local glia comprising astrocytes and neural/glial antigen 2 (NG2)-expressing glia (NG2 cells) appears particularly desirable in view of neuronal replacement because of their

abundance and ubiquitous distribution in the CNS. Moreover, these glial cells set up a complex reaction to injury that partly increases their similarity to neural stem cells and can include a cytogenic response leading to some degree of amplification, thereby allowing to direct some elements toward neurogenesis while avoiding glial cell depletion [4].

In this review we will revise the current status of research devoted to understand if and how resident glia can be directed to produce neurons, with specific attention to in vivo data. We will discuss mechanisms and factors, either intrinsic or environmental, which may be of relevance for potential pharmacological approaches aimed at boosting the production of new neurons from endogenous sources. Our focus will be mostly on studies on the mammalian brain, spinal cord and retina, which, due to its peculiar inherent regenerative properties, has been intensely investigated with outcomes possibly exploitable also for other systems.

2. Parenchymal neurogenesis: who, when, where

2.1 Spontaneous parenchymal neurogenesis

Adult neurogenesis in the constitutive germinal niches of the subventricular zone (SVZ) and hippocampal subgranular layer (SGZ) is highly conserved in different mammalian species. Whether other CNS regions can be neurogenic has been the subject of a long debate that is still partly unresolved. Initial studies referred to the rest of the CNS parenchyma as non-neurogenic. This concept was mainly derived from the observation that when heterotopically transplanted outside the constitutively active neurogenic niches, NSCs differentiated almost exclusively into glial cells and not in neurons [5-7]. These observations were consistent with the absence of neurogenesis in the mature healthy CNS parenchyma in rodents, as reported after initial controversial evidence for the spinal cord, cortex and striatum by numerous studies [8-13].

By contrast, comparative analyses indicated that in some mammalian species low-level neurogenesis can occur also outside the two canonical niches. Neuroblasts were observed in the striatum and neocortex of rats, rabbits, guinea pigs and primates and in the amygdala, piriform cortex and adjoining perirhinal cortex of primates (see [14-15]). Furthermore, striatal neurogenesis has now been suggested also in humans [16]. The observation of parenchymal neurogenic processes in intact animals may suggest their participation in homeostatic functions and normal brain activity. However, no data are currently available that support this idea. Further, the timing and the transient nature of neurogenic events observed in some of these cases (e.g. transient activation of neurogenesis in the guinea pig at weaning age [15]) rather favors their interpretation as events related to temporary forms of plasticity.

Of note, injury can induce neurogenic events also in regions that are normally non-neurogenic. Newly generated neurons were observed after acute degeneration both in the striatum (experimental stroke, [17]; quinolinic acid (QA)-induced excitotoxic lesion, [18]) and the neocortex (transient ischemia, [19]; focal apoptosis, [10, 13]) as well as in a genetic model of progressive striatal neurodegeneration [20].

Despite the SVZ can contribute neuroblasts to the injured parenchyma [2,21], several studies provided initial evidence that neurogenic events in non-neurogenic regions were a local SVZ-independent phenomenon. In both rabbits under physiologic conditions and in mice during striatal progressive neurodegeneration, tracing of SVZ derivatives and tridimensional reconstructions of striata and adjacent SVZs showed that chains of striatal neuroblasts were separated in space and did not derive from the niche [20,22]. Further evidence came from ex vivo approaches showing neuroblast generation in striatal explants [22], and by in vivo observations of the association between neuroblasts and parenchymal clusters of proliferative cells with the features of the intermediate progenitors typical of NSC lineages (proliferative cells positive for the brain lipid-binding protein – BLBP, SRY

(sex determining region Y)-box 2 - Sox2, SRY (sex determining region Y)-box 9 - Sox9, epidermal growth factor – EGF - receptor, and distal-less homeobox transcription factor – Dlx) [20, 22]. Local generation of new neurons has been also reported in the cerebral cortex, where layer I progenitors traced by retrovirus-mediated labeling were shown to produce neurons [19]. Taken together, these findings strongly indicated that new neurons can be generated locally in the brain parenchyma, at least in specific areas, under both physiological and pathological condition.

However, these investigations did not clarify the cellular source of the neo-generated neuroblasts. Tight lineage relationships as well as phenotypic and functional similarities between germinative NSCs and neuroglia suggested that these cells could harbor a neural progenitor potential (revised in [4]). Subsequent studies therefore investigated astrocytes and NG2 cells as the most likely suspects of local neurogenesis.

NG2 cells, also known as oligodendrocyte precursor cells, comprise a population of glial cells widely distributed throughout the adult brain parenchyma. In the adult healthy and injured brain, these cells account for the vast majority of proliferating cells outside the stem cell niches and constantly divide to generate differentiated, myelinating oligodendrocytes, as well as further NG2 glia [23]). While in vitro studies showed that NG2 cells can differentiate into neurons [24-27], clear evidence that NG2 cells contribute to parenchymal neurogenesis in vivo is missing. By inducing genetic recombination in adult intact PDGFRα (Platelet-derived growth factor receptor α) -CreERT2 mice (see Figure 1), Rivers and colleagues [28] detected some labeled neurons in the piriform cortex. Based on the expression of PDGFRα by NG2 glia, these finding were interpreted as indicating neurogenesis from NG2 cells. Similar results were shown by Guo et al., 2010 [29] in a mouse line where proteolipid protein (Plp) promoter activity (also occurring in NG2 cells) drives the expression of a tamoxifen-inducible Cre transgene. However, these findings could not be reproduced in subsequent studies by the same authors [30] . Even other labs

exploiting the same PDGFRα-CreERT2 mouse line [31] or other oligodendroglia-specific inducible lines [32-34] could not replicate these results. Thus, evidence collected so far does not support a neuronal differentiation potential of NG2 cells in physiological conditions. Nevertheless, Honsa et al. [35] reported the generation of neuroblasts from NG2 cells in the late phases after focal cerebral ischemia in adult NG2CreBAC:ZEG mice. Nonetheless, these findings were not confirmed by other fate mapping studies with the same Cre-inducible line, where the generation of neuroblasts from NG2 cells after lesion was clearly excluded [17,18]. Taken together, these data collectively do not provide evidence for NG2 glia as a parenchymal source of new neurons.

Unlike NG2 cells, astrocytes outside of the germinal niches do not divide in the heathy brain. However, in injury conditions, they acquire NSC features as shown by the upregulation of NSC markers (Nestin, vimentin, BLBP), activation of proliferation, and expression of self-renewal and multipotency ex vivo [36,37]. Of note, these features are not acquired by reactive NG2 glia [36]. Astrocytes, therefore, are the most likely player in parenchymal neurogenesis. In accordance with this view are data on physiological neurogenesis in the guinea pig external capsule and lateral striatum [15]. Here, neurogenesis is absent at birth but newly generated neuroblasts transiently appear between postnatal day 7 (P7) and P18 (weaning age). They are produced in the lateral striatum, concomitantly with a time window of intense proliferation of local astroglia. Along this line, the direct demonstration that astrocytes act as neuronal progenitors was recently offered by two independent studies where fate mapping strategies demonstrated that striatal resident astrocytes produced new neurons after stroke [17] or QA-induced neurodegeneration [18]. In these studies astrocytes were tagged before lesion in mice obtained by crossing a reporter line and mutants expressing the tamoxifen inducible recombinase CreERT2 under the control of distinct astrocyte genes (Connexin-30 in the stroke model; Glutamate Aspartate Transporter – GLAST- in QA experiments; See Figure

1 for inducible Cre-based models). The progenies of the tagged astroglia included proliferative intermediate progenitors and neuroblasts. Nato et al. [18] also detailed that the response of GLAST-positive (+) striatal astrocytes to injury included the upregulation of nestin and the subsequent generation of intermediate progenitors positive for Achaete-scute homolog 1 (Ascl1, [38]) or Sox9 ([39]). In turn, these progenitors gave rise to neuroblasts. Moreover, in both studies the conclusive proof of neurogenesis from local astrocytes came from virus-based fate mapping of astrocytes transduced before QA or stroke. The same approach excluded a major contribution of the SVZ to QA-induced striatal neurogenesis [18]. Interestingly, astroglial neurogenic activation appeared to persist for several months, prompting the question on the mechanisms activating and sustaining this neurogenic switch. In agreement with other pathologic and physiologic models of striatal neurogenesis [15, 20, 22], induced neurons displayed a short life span (our unpublished observations) and did not differentiate into striatal neuronal types. The only exception were few calretinin or neuronal nitric oxide synthase (nNOS) expressing interneurons ([17]; our unpublished observations). The phenotype of these new neurons, the extent of their integration in the host circuits, mechanisms of cells death remain to be established. These transient neurons might sustain some forms of post-lesion compensatory plasticity [18] and may exert protective roles for neurons that have lost their targets. Thus, striatal astrocytes comprise quiescent neuronal progenitors that become activated after lesion. Of note, the process leading to this form of spontaneous neurogenesis took several weeks to start. This finding suggests that astrocytes transit through a multi-step activation, possibly influenced by changing environmental signals. They first become reactive but not yet neurogenic, then competent for neurogenesis and ultimately actively neurogenic.

Another paradigmatic example of parenchymal glia endowed with a latent neurogenic potential is the retinal Müller glia (MG). MG account for the 4-5% of all retinal cells, span

the retinal epithelium and perform typical astroglia supportive functions [40]. In fish, birds and rodents, following a retinal injury a subset of normally quiescent MG resumes proliferation, gives rise to multipotent Paired Box 6 (Pax6)+/nestin+ retinal progenitors that further amplify and can generate new retinal neurons [41-44]. This response is particularly efficient in fish, also at post-developmental stages, while its shows clear limitations in birds and mammals [45]. Intriguingly, such process recapitulates some aspects of retinal development, including interkinetic nuclear migration and asymmetric division in activated MG, and neuroblast migration along radial processes retained by MG (Figure 2). Further, the layer distribution and phenotype of newly-generated neurons mainly corresponds to those of the lost elements and, when sufficient number of neurons are produced, adult neurogenesis even results in positive functional effects ([43, 44] and references therein). Nevertheless, in rodents, MG regenerative response is rather modest, both in quantity (i.e. few MG entering cell cycle and yielding differentiated neurons after injury), and types of neurons generated (mostly photoreceptors, bipolar or amacrine cells). Further, most newly-generated neurons display a limited life-span [42]. In humans, this phenomenon is even more restricted because MG can re-enter the cell cycle, but there is no de novo neurogenesis in adults in disease or after injury ([46] and references therein). However, the in vivo manipulation of intrinsic and extrinsic signals can ameliorate the final neurogenic outcome in the mammalian retina (see below), and, when exposed to proper environmental signals, even human MG can unleash a latent neurogenic potential in vitro [45,47]. Interestingly, on the molecular level, mouse MG show many similarities with retinal progenitor cells, suggesting the idea that MG may represent a form of late stage progenitor cell persisting in the adult tissue ([45] and references therein). Consistent with this idea, the epigenetic landscape of the promoters of pluripotency factors in mouse MG is rather similar to that found in progenitor cells (i.e. their hypomethylation allows their chromatin to assume a more “open” state that is permissive for gene expression). This likely prompts

MG to rapidly re-enter the cell cycle upon injury and facilitates their dedifferentiation [48]. It is still unknown whether MG is an exception among astrocyte populations or also extra-retinal astrocyte subsets with a neurogenic potential share such molecular/epigenetic features.

In summary, when reacting to injury parenchymal astrocytes and MG in the retina have the potential not only to activate a stem cell response, but also to express a neurogenic program in specific conditions. In doing so, astrocytes and MG appear to undergo phases typical of NSCs of the adult neurogenic niches: after activation they generate amplifying intermediate elements, which in turn give rise to neurons. These findings suggest that if properly activated, the ubiquitary parenchymal glia might ultimately become able to sustain an effective brain cell replacement upon damage.

2.2 Unlocking glial neurogenic potential via modulation of cell intrinsic factors: induced in vivo cell reprogramming

Evidence of spontaneous neurogenic activation of parenchymal glia strongly prompted the view that, upon appropriated stimulation, ubiquitary glia could undergo a neurogenic activation at all CNS sites. This suggests the possibility of becoming able to sustain an effective neuronal replacement upon damage. First hypotheses for strategies to foster neurogenesis in glia emerged from comparative and developmental studies, showing that cell-intrinsic determinants necessary for providing the neurogenic competence to reactive MG in non-mammalian vertebrates or to direct the generation of specific neural classes in embryonic neural progenitors are not (re-)expressed in mature glial cells upon injury in mammals (e.g. [49–51]). In particular, comparative investigations on the retina (thoroughly revised in [44,45]) highlighted a number of molecular players (e.g. Ascl1, Neurogenic differentiation 1- NeuroD, Notch signaling) subsequently targeted to foster neurogenesis in mammalian glia.

First attempts to overexpress proneurogenic determinants in proliferative reactive glia led to a limited production of transient neuroblasts in the lesioned brain (e.g. [51]). However, the field evolved very rapidly, and offered clear in vitro evidence of successful derivation of functional neurons from early postnatal astrocytes or NG2 cells through forced expression of Pax6, Neurogenin2 (Ngn2), or Ascl1 [52–54]. Concomitant breakthroughs established the innovative concept of cell reprogramming. This consists in changing the phenotype of somatic cells such as fibroblasts (or glia) into that of another cell type including iPSCs (e.g. [55,56]), NSCs [57] or neurons [58] through the overexpression of specific transcription factors (TFs), in combination or alone. This evidence prompted further research on glia into neuron conversion strategies that successfully transferred in vitro evidence in vivo. Studies on in vivo reprogramming of glial cells in the CNS have been already discussed in detail in several recent reviews [1,59,60] and are here summarized in Table 1. Importantly, not all TFs shown to efficiently reprogram glial cells in vitro were successful in vivo (e.g. Ngn2, Ascl1, see Table 1), highlighting the importance of the cellular milieu in cell fate specification and reprogramming. When induced, in most cases obtained neurons were electrophysiologically active (see Table 1), although evidence for the implication of induced neurons in some degree of functional repair is still starting to emerge [61]. Moreover, in contrast to spontaneous neurogenic activation, which is fully proved only for astrocytes so far, both astroglia and NG2 cells were efficiently converted into neuronal cells by ectopic expression of specific TFs.

Neurogenic actions of transcription factors

It is interesting to note that neuronal induction in the brain or spinal cord occurs either through a process that appears to recapitulate the features of the activation of spontaneous neurogenesis from glia (i.e. with a transition through a neural progenitor

stage including amplifying Ascl1 expressing cells and subsequent generation of doublecortin (DCX)+ neuroblasts) or by direct conversion of glial cells into neurons.

Among the different tested factors, the first mode of induced neurogenesis was mimicked only by overexpression of Sox2, a TF factor involved in stem cells maintenance in both SVZ and hippocampal SGZ [62], used to induce pluripotent stem cells together with other TFs (Oct4, Myc, Klf4; [56]). Such a NSC-like induction mode was particularly patent when astrocytes in the intact striatum were targeted [63,64]. Similar results were obtained in the injured spinal cord [65]. Differently, an injury was instead required to prompt glial cells, mostly comprised of NG2 cells, to respond to Sox2 in the cerebral cortex [66]. These data suggest that distinct CNS areas may be differently conducive to reprogramming. This is likely due to the interplay between distinct cell-intrinsic properties and environmental factors, and in some instances injury-related signals may promote the response to the genetic manipulations.

The other tested transcriptional regulators, which are proneural genes, were instead essentially reported to promote direct conversion mechanisms, although with distinct efficiency and outcomes in terms of obtained neuronal phenotypes (see Table 1). NeuroD1, a TF essential for adult neurogenesis [67,68] and for terminal neuronal maturation [69], exerted a particularly strong reprogramming action on astrocytes and NG2 cells in the cerebral cortex of both stab-injured and Alzheimer's disease model mice [70]. Interestingly, while astrocytes were mainly reprogrammed into glutamatergic neurons, NG2 cells were converted into both glutamatergic and gamma-aminobutyric acid (GABA)-ergic neurons [70].

Conversely, the efficiency of the proneural gene Ascl1, shown to be sufficient to induce neurons from fibroblasts [71], remains controversial in inducing glia reprogramming in the brain and spinal cord. Several studies reported that the overexpression of Ascl1 in striatal astrocytes [64], adult injured cortex [66] and injured spinal cord [65] was not sufficient to

induce glia-to-neurons conversion. Instead, another study reported that Ascl1 converts astrocytes into functional neurons in the dorsal midbrain, striatum, and somatosensory cortex [72]. These different results may depend on the different viral vector used to promote Ascl1 overexpression. Such vectors could induce distinct levels of expression, and/or different levels of immune responses.

It is worth mentioning one recent example of astrocytic reprogramming toward dopaminergic neurons. In a mouse model of Parkinson's disease, overexpression in striatal astrocytes of a combination of NeuroD1, Ascl1, LMX1a and miR218, formerly proved to be effective in vitro, triggered the induction of neurons with a dopaminergic phenotype. Most interestingly, induced dopaminergic neurons appeared to promote recovery of deficits in spontaneous motor function relevant for Parkinsonism [61].

Also in the retina forced expression of stemness inducers (i.e. β-catenin – β-cat, Lin28) and/or proneural genes (Ascl1, Atonal basic-helix-loop-helix transcription factor 7 - Atoh7, NeuroD) has been used to foster/expand the intrinsic MG neurogenic potential. In vivo gene transfer of β-cat in young adult mouse retinas was shown to activate proliferation, interkinetic nuclear movement and expression of amacrine cell markers in MG even without retinal injury [73]. Similarly, MG reprogramming via cell fusion with transplanted hematopoietic progenitor cells with activated wingless-type MMTV integration site family (Wnt) pathway resulted in the generation of photoreceptors and functional amelioration in a mouse model of inherited retinitis pigmentosa [74]. Forced expression of the RNA-binding protein Lin28B in MG stimulated its proliferation, de-differentiation, and promoted its neuronal commitment in a rat model of retinitis pigmentosa [75]. The same was observed even in the uninjured mouse retina [73]. In explants of adult mouse retina, Ascl1 overexpression was sufficient to activate a neurogenic program in MG [76]. Further, in vivo transgenic expression of Ascl1 in MG resulted in the generation of amacrine and bipolar cells and photoreceptors in the injured retina of young mice [49]. The in vivo forced

expression of the neurogenic factor Atoh7 promoted the differentiation of MG-derived stem/progenitor cells into retinal ganglion cells when transplanted in a rat model of glaucoma [77]. NeuroD has also been successfully employed in the injured rat retina ex vivo, where it induced the appearance of newly generated amacrine cells. The production of this neuron type could be further implemented by several combination with Pax6 and mouse atonal basic-helix-loop-helix transcription factor 3 (Math3). On the contrary, the co-expression of Crx (cone-rod homeobox gene) with NeuroD promoted the generation of photoreceptors [41]. Notably, evidence collected so far showed that in vivo MG reprogramming via forced expression of the above-cited factors essentially recapitulated the phases of spontaneous adult neurogenesis, implying a certain degree of MG proliferation, appearance of multipotent Pax6+/nestin+ intermediate progenitors and a final step of neuronal differentiation (Figure 2). When appropriately fostered upon injury (see also some examples in section 3) the neurogenic attempt succeeded to replace, at least in part, the lost elements by producing neurons that acquired the appropriate layering and phenotype, and, in few cases, provided a functional rescue [74,75]. Thus, in vivo gene-transfer manipulations appear a relatively advanced and promising strategy for the cure of retinal pathologies in humans.

Neurogenic actions of epigenetic factors

The transcriptional regulators employed in reprogramming are understood to exert their action by recruiting other transcriptional activators/repressors and inducing/suppressing specific target gene programs. In this regard, for instance, factors such as Ngn2 or Ascl1 are considered as pioneer factors with access to chromatin closed in the targeted regions and ability to recruit of other TFs inducing complex neuronal gene transcription [78,79]. However, the reprogramming factors cited above also influence the epigenetic landscape of the cells. A clear example is Sox2, which in NSCs binds to bivalently marked promoters

of poised proneural genes (i.e. Ngn2 and NeuroD1), where it maintains the bivalent chromatin state by reducing polycomb repressive complex 2 activity [80]. Indeed, an interesting feature of chromatin in stem cells and iPSCs is the presence of bivalent domains that harbor histones with both active and repressive modifications [81]. These indicate a transcriptionally poised state that can rapidly change and thus enhance cellular potency. Loss of Sox2 shifts the state of proneural gene chromatin toward a more repressed configuration, impairing their activation even in the presence of differentiating cues [80]. It is likely that a Sox2-dependent re-installation/maintenance of a permissive epigenetic state similar to that of NSCs contributes to Sox2-induced reprogramming of parenchymal astrocytes and to the recapitulation of a NSC-like multistep neurogenic program. Other TFs may act on gene programs more downstream in the neurogenic process. For instance, the action of NeuroD1 is not limited to the promotion of a complex transcriptional program driving neuronal differentiation. It also converts heterochromatin to euchromatin and marks epigenetically neuronal fate genes so to maintain them active [82]. These observations raised the idea that changes in the epigenetic setting and chromatin organization in adult glia may be crucial factors for their activation and neuronal conversion. Although our understanding of the regulation of the epigenetic landscape in neurogenically activated glia is still very limited, changes of expression of epigenetic modification enzymes, variations in DNA methylation, histone modifications and chromatin accessibility were shown to occurr in glial cells upon spontaneous neurogenic activation or reprogramming. Such information may allow identifying target mechanisms and windows of opportunities to possibly manipulate the system pharmacologically to promote local neurogenesis or reprogramming.

Intriguingly, some chromatin remodeling factors (i.e. high mobility group AT-hook 2 - Hmga2, and methyl-CpG binding domain protein 1 - Mbd1) were shown to be differentially expressed in MG of mouse strains exhibiting different degrees of spontaneous damage-

induced MG proliferation and de-differentiation (i.e. acquisition of progenitor markers [83]). Further, in quiescent MG chromatin accessibility at the regulatory regions of neural progenitor genes (e.g. Notch targets and ligands, Lin28, Ngn2, Ascl1, oligodendrocyte lineage transcription factor 2 - Olig2) is significantly lower in adult mice compared to young animals. This correlates well with their differential ability to be effectively reprogrammed [49].

Initial studies in zebrafish retina [48] proposed the idea that early DNA demethylation (which reflects chromatin accessibility for gene expression) may allow the transcription of genes associated with glia neurogenic activation and reprogramming. Consistently, a recent study [84] showed an early and transient decrease in the DNA methyltransferase 3b (Dnmt3b) expression after N-Methyl-D-aspartate (NMDA)-induced retinal injury in mice. Dnmt3b expression returned to basal levels after 24 hours. Such dynamics well corresponded to the rapid and temporary decrease of methylation of the promoter of the pluripotency gene Oct4 and with its expression level (initially upregulated and then immediately suppressed) in MG. The in vivo administration of the DNA-methyltransferase inhibitor SGI-1027 induced a sustained upregulation of Oct4 expression in mouse MG, thus increasing their potential to acquire progenitor features [84]. Further, in vitro neuronal reprogramming of human astrocytes by forced expression of the pluripotency genes Oct4/Sox2/Nanog was associated by significant decrease in DNA methylation of genes involved in developmental process and neuronal differentiation [85]. Thus, methylation-mediated silencing of pluripotency and neuronal genes may be hypothesized as an epigenetic barrier preventing spontaneous glia neurogenic activation in the mammalian adult parenchyma.

Histone modifications were also proved to be implicated in glial response to reprogramming agents. Notably, forced overexpression of Ascl1 in mouse MG modified histone acetylation and methylation, and chromatin remodeling at specific promoters (i.e.

Hes5, Hes6, Dll1, Dll3, Insm1a*)* and converted them from a repressive to an active configuration [75]. Such Ascl1-mediated mechanisms have been reported also during fibroblasts-to-neurons reprogramming [79] and neurogenesis from NSCs [86]. Interestingly, Ascl1 and β-cat also acted at the post-transcriptional level to modulate gene expression in glia during reprogramming. Such factors promoted the upregulation of the RNA-binding protein Lin28 that in turn reduced the biogenesis of the let-7 family of microRNAs (miRNAs). Lin28 and let-7 can regulate the mRNA levels of a variety of factors, including Ascl1 and Wnt-targets. For this reason they are important players in glia proliferation and reprogramming, and in the differentiation of glia derivatives [44,73]. Sox2 was reported to exert a similar function at Lin28 gene promoter in developmental neural progenitors, suggesting that this miRNA-mediated mechanism could also occur during Sox2-dependent reprogramming [87]. Along this line, miR124 and miR9-9* were able to reprogram fibroblasts to neurons [88] and promoted neuronal differentiation while inhibiting glial genes when expressed in NSCs [39,89]. More recently, Wohl and colleagues [90] showed that lentiviral expression of these miRNAs in MG induced the expression of Ascl1 and reprogrammed MG to retinal neurons in vitro. Such effects were mediated by the direct targeting of components of the Repressor element 1 (RE1)-silencing transcription factor (REST) complex, known to repress neuronal gene expression in non-neuronal cells [91] and to block neuronal reprogramming in cortical astrocytes [92].

In summary, in vivo reprogramming into functional neurons shows exciting perspectives. However, it still faces many challenges such as direction toward desired neuronal phenotypes, demonstration of functional recovery, control of safety of exogenous genetic material and optimization of in vivo delivery for most applications.

3. Pharmacology of adult parenchymal neurogenesis

Evidence provided so far indicate that, depending on the CNS area, adult parenchymal neurogenesis may be attained by two modes: (i) a niche-like process, where the spontaneous or induced activation/dedifferentiation of glia leads to the production of neurons through the generation of intermediate amplifying progenitors (i.e. post-lesional striatal neurogenesis, retinal regeneration, Sox2 forced expression in striatal and spinal cord astroglia; see above); (ii) the direct conversion of glial cells into neurons without any progenitor phase (i.e. upon most reprogramming approaches tested so far in the brain or spinal cord). Pharmacological treatments could be tailored to reproduce or complement either of the two modes in view of fostering neurogenesis.

Remarkably, in most cases, spontaneous or induced parenchymal neurogenesis occurring according to the first mode requires the presence of a lesion. This suggests that signals provided by the injured microenvironment are supportive of the neurogenic process. The injured parenchyma may partly acquire features of a neurogenic niche, as a consequence of gliosis, degenerative events or thanks to the supply of peripheral components after blood brain barrier breakdown. Since the “learning from nature” strategy has been repeatedly and successfully applied in the history of pharmacology, a niche-based approach may be a good option to design effective treatments to evoke or implement developmental-like neurogenic processes. In this context, comparative studies have also helped in identifying niche-associated candidate signals. Although injury-induced parenchymal neurogenesis closely resembles the regenerative processes occurring in non-mammalian vertebrates, it is well known that the extent and efficiency of the production of new neurons in adult individuals is remarkably reduced in mammals compared to cold-blooded animals [93]. Of note, injuries lead to a certain degree of astroglia/MG reactivity and proliferation across species, suggesting shared or convergent mechanisms regulating these steps. Divergent pathways across species are instead

mainly related to the processes of glia dedifferentiation (with mammalian cells showing intrinsic constraints limiting the acquisition of a stem cell-like phenotype; see above), neuronal differentiation, integration and survival. Namely, comparative studies have shown that anti-neurogenic signals (e.g. the sustained activation of the Notch-mediated signaling cascade; see below) supported by the parenchymal environment of mammals operate by maintaining newly generated cells along the glial lineage ([46,93]; and references therein). Further, the different quality, duration and intensity of inflammatory cytokine release after injury were suggested to differently modulate glial cell reactivity and potency as well as the survival of their derivatives in different species ([93]; see below).

Here, we summarize what injury-related signals have been so far implicated in the activation of parenchymal neurogenesis and in the differentiation of the generated neurons. Much of the actual knowledge derives from the study of retinal regeneration. Thanks to its peculiar features (i.e. a common architecture across species; the presence of few types of well characterized neurons; the accessibility for manipulation and imaging; the presence of a functional readout- i.e. vision- that can be tested in vivo), the retina has been proven as an excellent system to study the pharmacology of adult parenchymal neurogenesis. Furthermore, it offered a number of candidate mechanisms possibly exploitable in other CNS systems.

3.1 Toward a niche-based approach

3.1.1 Role of morphogens and growth factors

Sonic hedgehog – stem cells response in reactive glia

Sonic hedgehog (Shh) is a soluble signaling protein playing pleiotropic functions during CNS development, including regulation of progenitor proliferation, specification, and

axonal targeting. In the adult CNS, Shh is expressed in the canonical neurogenic niches, where it modulates NSC self-renewal and specification [94]. Interestingly, this morphogen is highly upregulated in lesions where astrocytes activate a significant degree of proliferation and a stem cell response, as shown by the generation multipotent neurospheres ex vivo (i.e. cortical stab wounding or cerebral ischemia; [37]). Cortical astrocytes express the Shh transducer Smoothened (smo), whose selective deletion impairs astrocyte proliferation in vivo, and their potential to form neurospheres in vitro. Notably, addition of Shh or the Smo agonist SAG in the culture medium is sufficient to elicit neurosphere formation from cells of the cerebral cortex even in absence of any injury [37]. Chromatin immunoprecipitation experiments showed that Shh gene is a direct target of Sox2 [62], thus suggesting that these two factors may act in concert in the activation of the parenchymal astroglial neurogenic potential. Again in line with a critical role of endogenous Shh in eliciting glia activation and possibly broaden their neurogenic competence, the intraocular injection of cyclopamine, a Smo inhibitor, greatly reduces MG proliferative response to N-methyl-N-nitrosourea (MNU)-induced retinal injury in rats [95]. Conversely, daily injections of Shh for 3-7 days consecutive days after retinal lesion boosts MG proliferation and promotes the differentiation of MG-derived progenitors in rod photoreceptors in vivo [95].

Notch signaling – repressor of the glial neurogenic switch

The Notch pathway is one of the most evolutionarily ancient and well-conserved signaling cascade in animals. By mediating juxtacrine cell-to-cell communication, Notch signaling plays fundamental and pleiotropic roles during development and adult life of multicellular organisms, including regulation of cell self-renewal, differentiation, death and metabolism. Four Notch receptors (i.e. Notch1, Notch2, Notch3 and Notch4) and many Notch ligands (e.g. Jagged 1 - Jag1 - and Delta-like - Dll - proteins) are known in mammals. Both Notch

receptors and ligands are transmembrane proteins. Notch receptors include an intracellular domain (Notch intracellular domain - NICD) that, following ligand binding, is released into the cytosol through a γ-secretase-dependent cleavage. In the canonical Notch pathway, NICD is targeted to the nucleus, where it interacts with the recombining binding protein suppressor of hairless (Rbpj) complex and converts it from a transcriptional inhibitor to an activator. Such cascade leads to the transcription of a plethora of Notch target genes, including the the hairy and enhancer of split (Hes)-related genes ([96] and references therein).

In the adult canonical neurogenic niches, Notch signaling negatively modulates NSC cell-cycle entry. At the same time, it balances NSC maintenance with production of their derivatives, thereby preventing premature depletion of the niche ([96] and references therein). Notch-mediated juxtacrine signals in active NSCs directly control the quiescence of the neighboring NSCs [97]. Further, activation of the canonical Notch pathway in SVZ NSCs promotes gliogenesis at the expense of neurogenesis [98]. Thus, on the whole, Notch signaling operates as a negative regulator of adult neurogenesis in the germinative niches.

Notch signaling is also implicated in astrogliosis, although its contribution to both reactivity and acquisition of a neurogenic competence in parenchymal astrocytes may appear somehow counterintuitive. By using a Notch/Rbpj signaling reporter mice, Marumo and colleagues [99] showed that the canonical Notch/Rbpj pathway is activated in reactive astrocytes, suggesting that Notch signaling takes part in their post-injury response. In line with this idea, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT, a γ-secretase inhibitor) treatment or Notch1 conditional ablation in astroglia reduces the number of proliferative and radial glial cell marker (RC2)+/nestin+ astrocytes after an ischemic stroke [99,100]. It seems therefore that Notch signaling may sustain an initial stem cell response in astrocytes. However, inhibition of the Notch canonical pathway was

shown to promote neurogenesis from astrocytes of the striatum in the absence of an injury [17]. The same authors also reported that the spontaneous emergence of neuroblasts and clusters of proliferative cells in the striatum after stroke is accompanied by a significant reduction of both Notch and NICD. How Notch inhibition can unleash the neurogenic potential of the resident astroglia is still obscure. Previous studies indicated that in vivo DAPT treatment inhibits the nuclear-translocation of Olig2 [99], which is a repressor of neurogenesis in cells reacting to brain injury [51] and is indispensable for the acquisition of a gliogenic fate of reactive astrocytes [99]. However, in Magnusson's study [17] astrogliosis does not take place, prompting the question of whether astrogliosis – and which type of astrogliosis (see also [101]) - is a pre-requisite for the acquisition of a neurogenic competence. Collectively, data accumulated so far essentially point to Notch-activated mechanisms in reactive astrocytes as repressors of the neurogenic switch in the adult brain.

In the retina, although the extent of Notch contribution to each step of retinal regeneration is far from being completely understood, Notch signaling appears to exert distinct roles in MG activation and neurogenic competence. An early activation of the Notch pathway – as well as of a Notch-induced proliferative response – appears essential to trigger the acquisition of progenitor-like features in MG in the avian and murine retina [42,102,103]. However, when Notch signaling is blocked at later stages, the number of newly generated neurons significantly increases, suggesting that Notch signaling hampers the expression of a neurogenic competence [102-104]. That Notch signaling mediates a variety of effects is no surprise, based on its well-known role of "integrator" of multiple signaling cascades, with distinct Notch receptors tuning distinct downstream targets, and cell-type specific Notch partners as well as distinct epigenetic states at the level of the Notch target genes modulating the final outcome of Notch signaling activation ([96] and references therein).

Wnt/β-catenin signaling – regulator of proliferation and neurogenic competence of glia

The canonical Wnt/β-catenin (cat) pathway is one of the most conserved signaling cascades operating in NSCs during brain development and in the adult neurogenic niches, where it contributes to the regulation of cell self-renewal, expansion, asymmetric cell division, maturation and differentiation [105]. A direct evidence of Wnt/β-cat involvement in parenchymal adult neurogenesis in the mammalian brain is still lacking. However, Wnt/β-cat signaling is upregulated in astrocytes upon damage [106,107]. Of note, in vitro wound healing experiments in adult astrocyte monolayers showed that Wnt/ β-cat signaling contributes to the initiation of the post-injury activation of astrocytes [108]. These findings suggest that Wnt may initiate and sustain astrocyte activation, and possibly facilitate their transition toward a multipotent progenitor phenotype. This possibility is corroborated by the fact that β-cat expression sustains the upregulation of stem cell markers during the activation of astrocytes in vitro [108], and, in turn, Wnt3a expression depends on Sox2 [62]. This suggests a Wnt/β-cat-Sox2 positive loop in activated astrocytes.

More direct evidence is available on the role of the canonical Wnt/β-cat in adult retinal regeneration. By using a Wnt/β-cat reporter mice, in 2007 Osakada and colleagues [109] showed nuclear accumulation of β-cat in dividing MG responding to a NMDA-induced retinal injury in mice. Treatment with Dickkopf-related protein 1, a negative modulator of Wnt signaling, greatly reduces MG proliferative response and the number of retinal progenitors in explants of adult mouse retina. In the canonical pathway, Wnt signaling leads to inhibition of glycogen synthase kinase 3β (GSK3β), which subsequently targets β-cat to the nucleus, where it promotes the transcription of target genes associated with cell cycle entry, such as cyclin D1 [110]. Treatment with inhibitors of GSK3β markedly increases cell proliferation across the retinal layers and amplified the number of dividing Pax6+ progenitors in explants of the adult mouse retina [109]. Such effect is reminiscent of that obtained by the treatment with Wnt2b/3a in vivo and ex-vivo [104,109] and point to the

implication of the canonical Wnt signaling in MG proliferation and de-differentiation upon injury. In vivo gene-transfer of β-cat or deletion of GSK3β in adult mouse MG markedly increase MG proliferation even in absence of injury. This effect is mediated by the upregulation of Lin28 and subsequent repression of the let-7 miRNA expression. In this injury-free experimental paradigm, a fraction of Wnt-activated MG-derived progenitors eventually differentiate in amacrine cells [73].

In vitro experiments suggest that Wnt is also implicated in a cell-autonomous/autocrine manner in the neuronal differentiation of MG-derivatives: exposure of human MG with a cocktail of factors that induce their differentiation in photoreceptors markedly upregulates the expression of components of the canonical Wnt pathway and its secretion. Further, inhibitors of the canonical Wnt signaling prevents MG conversion in photoreceptor [47]. Taken together, these findings provide compelling evidence that the canonical Wnt signaling, along with its downstream targets, constitute a central signaling axis in regulating proliferation and the neurogenic potential of MG in the adult mammalian retina.

Bone Morphogenetic Proteins – promoter of gliogenic fates

Bone morphogenetic proteins (BMP) are a group of secreted signaling molecules that belong to the transforming growth factor β (TGFβ) superfamily. Despite their involvement in retinal and brain development [111-113] and adult neurogenesis in the canonical niches [114], few data are currently available about their contribution to post-injury astrocyte/MG activation and neurogenic response. Interestingly, both BMP ligands and receptors are increased following CNS injury in astrocytes [115]. Studies on adult mouse retinal explants showed that EGF-induced proliferation in MG requires the activation of the BMP pathway [116]. These data suggest an early implication of the BMP signaling in post-injury glia activation and proliferation. However, BMP are also well known gliogenic factors participating in the neurogenesis-to-gliogenesis switch occurring in the late embryonic

phases of CNS development [112,113]. In addition, BMP has a role and in astroglia lineage commitment of SVZ-derived cells [114]. Thus, to favor a neurogenic outcome, after an early activation, BMP signaling cascades should be repressed (see The small molecule approach section).

Growth factors – promoter of glia activation, neurogenic switch and neuronal survival

Upon injury, reactive microglia and astrocytes upregulate the expression of a broad array of growth factors (GFs), including basic fibroblast growth factor (FGF2), EGF, insulin growth factor 1 (IGF1) and vascular endothelial growth factor (VEGF) [101]. All of these factors can activate intracellular signal cascades via Janus kinase/signal transducer and activator of transcription (Jak/Stat) and/or mitogen-activated protein kinase (MAPK) pathways. Hypothetically, GFs may promote glia proliferation and acquisition of progenitor-like traits [117]. Of note, in vitro GFs are involved in the acquisition of progenitor features in reactive astrocytes, including expression of NSC markers (nestin and CD133) and multipotency [118,119]. While GF infusion did not *per se* elicits an appreciable neurogenic response from parenchymal reactive glia in vivo [120], in vitro and in vivo reprogramming approaches have been designed by combining forced expression of inducers of stemness/proneural genes and administration of GFs [85,120]. After a stab wound in the adult rat brain, the local administration of FGF2 and EGF potentiates the reprogramming effect of the retrovirally-mediated expression of Ngn2, increasing the number of newly-generated DCX+/ neuronal nuclear antigen (NeuN)+ neurons in the striatal and cortical parenchyma. Of note, GFs do not operate by simply inducing the expansion of genetically modified cells, but likely facilitate neuronal reprogramming and sustain cell survival [120].

Similar to what described upon brain injury, retinal lesions stimulate the upregulation of GF receptors in MG and the release of a variety of factors, including EGF, FGF1, FGF2 and IGF-1 ([117,121] and refs therein), suggesting their implication in MG response.

Interestingly, differently from what observed in the cortex and striatum, post-injury intraocular injection of GFs, either alone or in various combinations, can enhance injury-induced MG proliferation and neurogenesis in adult rodents [117]. Namely, the acute in vivo treatment with EGF, FGF1 or FGF1+insulin after NMDA-induced lesion increased the number of dividing MG and of MG acquiring progenitor markers (i.e. Pax6) in the mouse [42]. This was accompanied by the appearance of some newly-generated amacrine cells. However, to obtain a significant number of these new neurons FGF1/insulin had to be injected once a day for 4 consecutive days. Although most of the MG progeny obtained by these manipulations died within the first week of their production, a subset of these cells survived at least 1 month and appeared stably integrated in the tissue [42]. Similarly, the in vivo injection of heparin-binding EGF-like growth factor (HB-EGF) in the mouse retina stimulated the proliferation of MG following NMDA-induced damage [122]. Thus, in the injured mammalian retina, the in vivo administration of GFs potentiates spontaneous parenchymal neurogenesis by boosting both MG activation and differentiation of MG derivatives.

3.1.2 Potential contribution of circulating multipotent cells

In the damaged parenchyma, infiltrating circulating progenitor cells of bone marrow origin, might support the neurogenic conversion of local astroglia through the local release of morphogens, mitogens and instructive signals [123-125]. Moreover, fusion events between bone marrow-derived stem cells and parenchymal cells could influence glia responses. Cell fusion can occur between bone marrow-derived stem cells and neural cells, specifically neurons. Although these events appear to occur at a very low frequency in physiology [126,127] and have been shown to be limited to specific neuronal types

[128,129] and phagocytic glial cells (e.g. MG in the retina), inflammation promotes both migration and infiltration of bone marrow-derived cells to sites of brain injury [123-125] and fusion events [129,130]. It might therefore occur that in the lesioned parenchyma, blood-derived elements fuse with parenchymal astroglia thereby fostering the activation of the neurogenic program. Indeed, this strategy was successfully exploited to transfer exogenous reprogramming factor to retinal MG [74]. However, no evidence in support of the occurrence of blood-derived cells-astroglia fusion is currently available outside the retina.

3.1.3 Role of inflammation-related signals

Upon injury, pro-inflammatory cytokines (e.g. tumor necrosis factor (TNF)-α, interferon gamma (IFN)-γ, interleukin (IL)-1β) are released by reactive astrocytes and, in even larger amounts, by activated microglia [101]. Intriguingly, when cultivated in presence of TNF-α, astrocytes are induced to acquire stem/progenitor properties, associated with transcriptomic and epigenetic changes consistent with a neurogenic-like state [131]. Mechanisms mediating such effects are still obscure. In vivo, inflammatory factors entering the brain after injury could contribute to upregulate Shh expression and activation of Shh signaling in reactive astrocytes [132]. Further, TNFα potentiates glutamate release by astroglia [119]. In principle, both Shh- and neutrotransmitter-induced signaling may contribute to astrocyte activation and acquisition of a neurogenic competence. In line with this idea, in a certain concentration range, pro-inflammatory molecules positively modulate NSC proliferation and neuroblast production in the canonical niches in the adult rodent brain [97,133]. In the SVZ and SGZ, the main cellular source of these mediators is local microglia. Of note, microglia/macrophage ablation significantly reduces Shh expression in

the cortex upon injury [132]. Thus, it is likely that microglia/astrocyte crosstalk may contribute to astroglia activation and acquisition of a neurogenic potential after brain damage.

Notably, when macrophage/microglia ablation or immunosuppression (i.e. activation of the glucocorticoid signaling) are induced before the onset of a retinal degeneration, MG proliferative response and photoreceptor replacement are significantly delayed even in fish, where a prompt and efficient regenerative response is normally accomplished upon injury/pathology [134]. Thus, a certain degree of microglia/macrophages activation/release of pro-inflammatory molecules appears required to induce MG activation and, possibly, a neurogenic response.

Nevertheless, pro-inflammatory signals and immune-mediated mechanisms may also be detrimental for the neurogenic outcome. It is well known that high levels of inflammation suppress the neurogenic functions of the canonical niches ([133] and references therein). Such effect may be due, at least in part, to a negative interference on the Wnt/β-cat pathway (i.e. β-cat downregulation and phospho-GSK3β increase [135]. In line with this idea, parenchymal neurogenesis appeared compatible only with moderate levels of inflammation and transient microglia activation. Examples include mild ischemia [19], experimental cell ablation in specific cortical layers [13] or of selected neuronal populations (e.g. light or NMDA-induced retinal damage inducing cell death in photoreceptors or amacrine and ganglion cells, respectively [41,49,73]; experimental models of retinitis pigmentosa, with a selective degeneration of photoreceptors [74,75]), when neurodegeneration is slow and progressive [20], or several weeks after an excitotoxic insult or stroke [17,19].

Indeed, it is hypothesized that one factor promoting parenchymal neurogenesis and tissue repair in fish is the short duration of the post-injury inflammatory burden [136]. Consistently, immunosuppression at post-acute stages after neurodegeneration onset

further accelerates the kynetics of neuronal replacement in zebrafish retina [134]. Moreover, in case of severe and extensive damage with persistent accumulation of immune system cells at the site of injury, regeneration of the lost elements does not occur properly even in fish [45,132].

Indeed, some immune-related molecules may limit MG proliferation and differentiation along the neuronal lineage. TGFβ1, is considered as a key player maintaining MG quiescence in the adult mammalian retina [137]. Further, it is able to suppress the neuronal differentiation of human MG induced by a cocktail of factors including FGF2, IGF-1, taurine and retinoic acid (RA). Such TGFβ1 effect is associated to the reduction of the expression of components of the canonical Wnt signaling pathway [47]. Similarly, IFN-γ hampers FGF2-mediated astroglial dedifferentiation into neurogenic NSCs in vitro [138].

In conclusion, evidence collected so far indicate the importance of a correct tuning of inflammatory/immune-mediated mechanisms for the promotion of parenchymal neurogenesis. As for other forms of post-lesion repair, a bifacet model of pharmacological intervention should be implemented to limit the detrimental effects of a persistent/overt inflammation, while maintaining/boosting the contribution of inflammatory/microglia-mediated signals on astroglia/MG activation.

3.1.4 Role of Neurotransmitters

Upon injury, damaged and dying neurons as well as reactive astrocytes may release significant amounts of neurotransmitters, including glutamate, GABA, purines, etc. [101,119]. Neurotransmitters are well known modulators of several aspects of developmental neurogenesis, including proliferation, migration and differentiation in

various CNS areas, such as the telencephalon, ventral midbrain and retina. Recent studies have also shown their implication in adult neurogenesis in the canonical niches [139].

Besides its broad actions during developmental neurogenesis and in neurogenic niches [140], dopamine signaling is particularly noteworthy because it was proposed to provide a negative feedback to progenitors generating dopaminergic neurons. This has been demonstrated in aquatic salamanders where dopamine negatively controls the production of dopamine neurons both during homeostasis and regeneration of the dopaminergic system at adult stages [140,141,142]. Studies in rodents showed that ventral cells expressing markers of dopaminergic progenitors (Lmx1a, Nestin, Sox2, Sox3 and prominin) persists, although in limited numbers, beyond embryogenesis into adulthood in the midbrain aqueductal zone. These cells express dopamine receptors and are exposed to a dense meshwork of midbrain dopamine fibres. Of note, their proliferation can be stimulated by antagonizing dopamine receptors ultimately leading to increased neurogenesis in vivo beyond the normal period of embryonic dopamine neurogenesis [143]. It remains to be tested whether at adult stages these cells are maintained quiescent by dopamine and if blockade of dopaminergic signalling can induce their reactivation, possibly providing the mechanistic substrate for de novo neurogenesis of dopaminergic neurons, as shown in some reports [144].

To our knowledge, no data are available on the contribution of other neurotransmitters in the modulation of the neurogenic competence in the brain or spinal cord parenchyma. Conversely, interesting data have been obtained in the retina, where NMDA- and nicotinic-receptor mediated signaling foster the spontaneous neurogenic processes.

In a pioneer study, Sahel and colleagues [145] observed that the proliferative response induced in adult rat MG by NMDA, kainic acid, domoic acid and oubain could be reduced by ketamine anesthesia. Since ketamine is an antagonist of NMDA-receptor, this finding suggests that post-lesion MG proliferation is not only triggered by injury-related signals,

but also by a direct effect of excitatory amino acids. More recently, by subretinal injection of various concentrations of glutamate and alpha-aminoadipate (a glutamate analogue that specifically binds MG) Takeda and colleagues [146] showed that sub-toxic levels of glutamate directly stimulate MG to re-enter the cell cycle and induce neurogenesis in vivo in adult mice. Alpha-aminoadipate also stimulates MG differentiation into photoreceptors in vivo. These data clash with the negative effect of NMDA-receptor agonists on neural progenitor proliferation reported in the intact hippocampal SGZ. However, they are in line with data on hippocampal neurogenesis in the ischemic and epileptic brains [139]. Thus, the effect of NMDA-agonists on neural progenitors is largely context-dependent. These results suggest that glutamate signaling is not a pivotal determinant of progenitor behavior, but rather acts as a modulator of other signaling cascades.

Cholinergic signaling has also been implicated in the de-novo generation of retinal neurons. The α7 nicotinic acetylcholine receptor agonist PNU-282987 activates neurogenesis in the adult rat retina even in absence of injury or administration of exogenous GFs [147]. After treatment with PNU-282987, MG increase proliferation and MG-derived progenitors differentiate and migrate to both the photoreceptor and retinal ganglion cell layers. Agonization of α7 nicotinic receptors expressed by parenchymal glia has recently obtained attention as a way to modulate post-lesion inflammation [148,149]. It is also reported to increase expression levels of neuroblast markers in the mouse brain [150], but this action requires to be better understood.

Purinergic signals are well-established modulators of astroglia/MG reactivity upon injury [101,151]. In vivo studies also show their involvement in the regulation of the progenitor functions in the adult SVZ [152]. However, so far no evidence has been provided about purine contribution in favoring/eliciting astroglia/MG neurogenic competence. Nevertheless, their implication may be somehow expected since GF receptors (i.e. FGF2 and EGF receptors) can be transactivated by P2Y receptor activation through different

intra-/extra-cellular mechanisms ([101], and references therein). Further studies are needed to assess whether this can be relevant for astroglia/MG activation and/or differentiation toward neuronal phenotypes.

3.1.5 Role of cholesterol metabolites

Cholesterol derivatives also received attentions as pharmacological targets to effectively modulate neural stem cell functions and promote neurogenesis. Liver X receptors (LXRs), are members of the nuclear receptor superfamily that heterodimerize with retinoid X receptors and are activated by specific oxidized cholesterol natural metabolites, oxysterols, that function as endogenous ligands. In vivo and in vitro evidence in animal models showed that LXRs and their ligands are potent regulators of ventral midbrain neurogenesis, and dopaminergic neuron development [153]. Moreover, oxysterols were shown to drive dopaminergic neurogenesis from human embryonic stem cells [154], suggesting that they could improve current reprogramming protocols (see below). Similar effects were recently found for dendrogenin A and B, which also belong to the family of oxysterols andshowed potent mitogenic effects on mouse neural stem cells in vitro as well as moderate differentiative actions along the neuronal lineage [155].

The known actions of LXR agonists in reducing neuroinflammation and amyloid aggregates [154], further increase the interest for studies assessing their efficacy in neural replacement in neurodegeneration. Notably, treatment with the LXR agonist GW3965 increased the number of proliferating neural progenitors in the SGZ of Alzheimer's Disease mouse models [156]. This effect appears at least in part mediated by epigenetic mechanisms (i.e. changes in the DNA methylation state of neurogenesis-related genes;

[157]). It would be interesting to examine whether these signals take part and could improve spontaneous parenchymal neurogenesis and/or reprogramming strategies.

3.2 Combinatorial approaches to foster the production of neurons from glia

As mentioned before, after the initiation of neurogenesis, most of the neuroblasts generated in the adult CNS parenchyma do not stably integrate in the pre-existing circuitries and die [17,20,42]. Further, reprogramming often occurs with a limited efficiency (e.g. [65]). Moreover, after either spontaneous regenerative events or reprogramming, new neurons undergo a limited maturation and may belong to restricted number of neuronal subtypes (see Table 1) [17,18]. Thus, the efficient production of fully differentiated neurons or the generation of a larger repertoire of neuronal phenotypes may require additional manipulations, including the exposure to factors that boost neurogenesis, promote survival or full differentiation, or trigger the acquisition of a specific identity in newborn elements. Until now, several attempts were directed toward the implementation of reprogramming efficiency and neuron maturation.

Among the factors that affect neuron generation from glia or other somatic cells, metabolic states have recently gained significant attention. Single-cell transcriptomic analyses showed that the activation of NSCs in the canonical niches is accompanied by the downregulation of genes associated with the glycolytic metabolism [97], which is the way by which astroglia and fibroblasts meet energy demands [158,159]. A similar transition from glycolysis to oxidative phosphorylation has been found during neuronal differentiation of human iPSCs derived from reprogrammed fibroblasts. In these cells, the forced constitutive expression of the glycolitic enzymes hexokinase (HK2) and lactate dehydrogenase (LDHA) during differentiation leads to cell death, indicating that the shut-

off of glycolysis is essential for reprogrammed cell survival [160]. Notably, such metabolic transition was shown to be a prerequisite also for astroglia conversion in neurons, which is associated with a peak in oxidative stress eventually leading to cell death in most reprogrammed cells [161](Table 1). Notably, by apoptosis-independent mechanisms involving reduction of reactive oxygen species (ROS) and lipid peroxidation occurring during fate conversion, inhibitors of ferroptosis, antioxidants, and forced expression of Bcl-2 greatly improve the resolution of this critical point and promote glial-to-neuron conversion after traumatic brain injury in vivo [161].

Another intrinsic metabolic constrain to neuron generation from glia and other cell types appears to be cell senescence. Indeed, it has been demonstrated that p53/p21- or p16-induced cell-cycle exit of Sox2-reprogrammed glia or reprogrammed iPSCs largely reduces their neurogenic outcome and constitutes a critical checkpoint for cell reprogramming (Table 1; [162,163] and references therein). These results suggest that blocking cell senescence pathways may enhance glia cell fate conversion and adult parenchymal neurogenesis.

Other adjuvant treatments to promote neuronal survival and/or differentiation included supplementation with neurotrophins (i.e. brain derived neurotrophic factor, BDNF), histone deacetylase inhibitors (i.e. valproic acid, VPA) and BMP signaling antagonists [63–65,162](see also Table 1). RA has also been used to stimulate neuronal differentiation of human MG and MG-cell lines in vitro [47,164]. Consistently, when injected in the adult rat retina, RA does not alter post-injury MG activation, but rather enhance the fraction of MG-derived cells acquiring the phenotype of bipolar neurons [41]. Further, when preceded by Wnt3a treatment, RA induces the differentiation of MG-derived progenitors in mature photoreceptors in adult retinal explants [109]. Further studies are needed to assess the exact contribution of these and other factors in the final steps of the adult parenchymal neurogenesis in vivo.

3.3 The small molecule approach

Spontaneous activation of neurogenic programs in the CNS parenchyma is a rare phenomenon, with clear limitations in terms of neuronal output. Similarly, in vivo reprogramming into functional neurons, although showing exciting perspectives, still faces many challenges as for efficiency and safety (see section 2). As an innovative and promising solution to the potential risks of introducing exogenous genetic material and altering the genome, small molecules have been employed to implement reprogramming and even to completely replace ectopic transgenes in a variety of cell systems [165]. Small molecules are low molecular weight (<900 daltons; around 1nm in size) organic compounds, including include lipids, monosaccharides, second messengers, natural metabolites as well as drugs and xenobiotics. Small molecules can rapidly diffuse across cell membranes so to reach intracellular sites of action and can generally be easily synthesized. For reprogramming strategies, annotated libraries have been scrutinized to select compounds i) recognized to act on pathways and target proteins known to be involved in cell maturation, growth, survival [166], or ii) best synergizing with reprogramming master TFs [167]. Alternatively, agents formerly proved to be efficacious to convert somatic cells into induced NSCs or iPSCs were directly applied to obtain neurons [78,168,169] (see below). In view of achieving the specification of desired neuronal phenotypes, recent studies have also adopted a bioinfomatic approach based on computational matching of identified pathways specific of diverse neuronal cell types and the related modulating drugs [170]. After screening, dose escalation is normally performed to optimize synergies or additive actions and avoid toxicity, with subsequent analysis of the effect of removal or replacement of single compounds to identify the components most

critical for the desired effects. Thus, as other chemical approaches, the small molecule strategy has the additional advantage to be transient, finely tunable according to the desired effects and amenable to scaling up. Thus, it may potentially lead to the development of drug therapies to stimulate the patients' endogenous cells to repair and regenerate in vivo.

A number of studies have shown that addition of small molecules during reprogramming into pluripotency [171–173] or multipotency [174,175] increased the efficiency of the conversion and in some cases even replaced individual reprogramming determinants [176-178] or the need for transgene expression [179–181]. Transfer of these approaches to direct reprogramming into neurons resulted in a 6-fold increase in direct conversion of human fibroblasts transduced with Ascl1, Brn2, Myt1L through more efficient downregulation of fibroblast programs and upregulation of neuron-specific genes and regulatory networks [166]. This result was achieved by the combinational treatment with Kenpaullone, Prostaglandin E2 (PGE2), Forskolin, BML210, Amonoresveratrolsulfat, and PP2 (see Table 2 for specific molecular actions here and below). A former study demonstrated that Ngn2, normally sufficient to reprogram neural cells but incapable to convert fibroblasts, in the presence of Forskolin and Dorsomorphin promoted the generation of cholinergic neurons from human fetal fibroblasts [182]. In a subsequent report the same research group shed light on how the employed small molecules initiates the acquisition of neuronal phenotypes [78]. They found that the compounds simultaneously activated Ngn2 and co-transcription of the prosurvival factor Creb1 (cyclic adenosine monophosphate - cAMP -responsive element binding protein 1), induced Sox4 expression while enhancing both Sox4-dependent and independent epigenetic changes and chromatin remodeling [78]. In turn, Ngn2 and Sox4 synergized to enhance the expression of diverse pro-neural TFs including NeuroD1 and NeuroD4. These data show that the applied compounds essentially act by i) targeting master regulators of both

reprogramming and neuronal induction and 2) modifying epigenetic barriers opposing cell fate changes.

Two recent studies further showed that appropriate cocktails of small molecules can completely replace genetic strategies to convert fibroblasts into functional neurons. Li et al. employed four small molecules (Forskolin, ISX9, CHIR99021, and I-BET151) to reprogram mouse fibroblasts into neurons with very high efficiency [183]. The authors suggested that I-BET151 suppressed the fibroblast-specific program, and ISX9 activated the expression of the endogenous neurogenic TFs NeuroD1 and Ngn2, which synergistically promote neuronal conversion [183]. In a companion paper Hu et al. demonstrated the generation of neurons from human fibroblasts using a small molecule cocktail (VPA; histone deacetylase (HDAC) inhibitor, CHIR99021, Repsox, Forskolin, SP600125, GO6983, Y-27631, and Dorsomorphin) [167]. Furthermore, via this chemical cocktail, fibroblasts from familial Alzheimer's disease patients could also be reprogrammed to neurons now available for in vitro disease modeling and drug screenings [167]. In this case while VPA and Repsox were suggested to inhibit fibroblast genes, SP600125, GO6983, Y-27631 were proposed to promote neuronal genes. In both cases, neuronal conversion occurred without transiting through an intermediate neural progenitor state, and yielded more glutamatergic than GABAergic neurons.

In regard to manipulation of glia, in the retina small molecules have been so far employed to promote neuroprotection [184,185]. On the contrary, successful conversion of brain astrocytes into neurons has been already achieved. Cheng and collaborators [168] reported that three compounds (VPA, CHIR99021, Repsox) converted both postnatal and adult mouse astrocytes into neurons with various neuronal phenotypes. In this study VPA was shown to be the most active molecule and essentially responsible of the upregulation of NeuroD1. Conversely, Repsox and CHIR99021 had a minor or minimal impact on neuronal induction. Of note, no transition through a Sox2+ stage or upregulation of

proneuronal TFs was observed. Interestingly, when the VPA, CHIR99021, and Repsox mix was also employed to treat human adult astrocytes, it failed to convert the cells [169]. This outcome suggests remarkable differences in the astroglial barrier to reprogramming depending on species and age. The authors therefore added Forskolin, ISX9, CHIR99021, and I-BET151 to the cocktail and obtained functional neurons with predominant glutamatergic phenotypes that could survive and became electrophysiologically active even after trasplantation in the postnatal rodent brain. Of note, while NeuroD1, Ngn2 and Ascl1 resulted upregulated during conversion, markers of neural progenitor cells (proliferation, Sox2, Pax6, Nestin) were not altered, confirming a direct conversion. Here, ISX9 confirmed its action as activator of neuronal genes while I-BET151 was proposed to down-modulate astroglial gene programs. Moreover, as the authors refer, in line with [168] VPA functioned to activate neuronal genes and Forskolin promoted morphological changes. However, removal of CHIR99021 and Repsox completely abrogated neuron induction, indicating their essential, although not sufficient roles.

Zhang et al. instead reported a protocol based on i) the sequential inhibition of factors promoting glial fates and exposure to compounds activating neuronal signalling pathways, and ii) exposure to neural patterning factors (LDN193189; SB431542; Thiazovivin; CHIR99021, VPA, DAPT, the Smo agonist SAG and Purmorphamine, TTNPB) [186]. BDNF, neurotrophin-3 (NT3) and IGF1 were also applied by the end of the defined sequential treatment to promote neuronal maturation. In this case, human fetal astrocytes from the cerebral cortex, midbrain and spinal cord were analyzed. Cortical and midbrain astroglia efficiently converted into glutamatergic neurons with deep layer/hippocampal phenotypes. This was achieved through upregulation of NeuroD1, Ascl1 and Ngn2 with no transition through a neural progenitor stage. Conversely, spinal cord neurons did not respond to these manipulations, highlighting a high degree of heterogeneity in the conduciveness to reprogramming of distinct astroglial subsets. Mechanistically, the small

molecules acted through both epigenetic and transcriptional modulation. VPA and activation of the Shh pathway essentially implemented reprogramming efficiency, while Thiazovivin and TTNBP removal had no effect. Inhibition of Notch, GSK3β, BMP and TGFβ were instead indispensable for reprogramming. Remarkably, human induced neurons were functional and could survive more than 5 months under cell culture conditions and upon transplantation into the mouse brain.

On the whole, these studies show that astrocytes are amenable to be efficiently converted in neurons by small molecules in vitro and suggest that specific combinations of compounds have to be designed depending on the target astroglial types and the desired neuronal output. It is tempting to speculate that commonalities among different protocols, such as inhibition of GSK3β with the resulting activation of Wnt signaling and of TGFβ, may act by recapitulating neural development programs and/or modulate some level of astrocyte activation contrasting gliogenic mechanisms (Figure 3). However, what pathways are implicated in this chemical-based induction protocols remains to be understood. In addition, it is interesting to note that RA signaling, which is crucial for NSC functions, appeared dispensable for astrocyte conversion [186]. Moreover, Shh treatment, shown to be critical to activate stem cell properties in astrocytes [37], turned out to only moderately increase reprogramming efficiency [186]. These results clarified that the tested combination of compounds, like those of the other listed studies, act through direct conversion and do not recapitulate the mechanisms of spontaneous activation of the neurogenic potential of parenchymal astrocytes.

Thus, small molecules constitute a concrete perspective to replace the worries of unwanted actions of master TFs in vivo by promoting neuron generation from glia either directly or through a neural progenitor stage. They hold an immediate application for the in vitro derivation of transplantable, safe, desired cell types; however, their in vivo exploitation still requires a long way of fundamental research and technological

development. Applications to discrete sites of damage (e.g. retinal damage, localized injury to the CNS) are feasible (just as direct injections of viral particles for gene therapy) and avoid the risks of genetic manipulations. However, treatments must minimize unwanted side effects on non-target cells in the tissue and solve the complications of potential sequential combinations.

Small molecules could even be more interesting for application to broad CNS areas affected by neurodegeneration. In such instances, systemic treatments would be most appropriate. Efficient passage of the blood brain barrier should then be ensured and issues related to pharmacokinetics and pharmacodynamics of each compound, as well as of their combinations solved. Yet, alternative routes as intranasal delivery can be considered [170]. On top of this, for systemic treatments it is mandatory to minimize side effects or toxicity on non-target organs. The development of devices or strategies for targeted delivery and controlled release of compounds will greatly benefit the field. However, first in vivo applications of a related approach based on the administration of a single compound to foster and direct the activity of mouse SVZ are encouraging [170], and stimulate further in vivo pioneer studies.

4. Open issues and Concluding remarks

Regeneration of functional neurons remains an unmet need in CNS repair. In this review we have summarized the enormous advancement obtained in the last years toward the generation of functional neurons from endogenous glial cells. This field of research is still in its infancy and big efforts are still needed both to fully understand the molecular and cellular mechanisms guiding the neurogenic activation of glia, and to find the right combination of genetic and pharmacological approaches to eliminate cell intrinsic and environmental constraints. Moreover, it will be essential to understand which

pharmacological approaches increase the efficiency of neuronal induction and direct neuroblast differentiation toward specific desired phenotypes. So far, differentiation of new neurons is modest and a limited number of neuronal subtypes has been generated. More research is needed to obtain distinct neuronal subtypes, although some types of neurons might turn out to be difficult or impossible to produce by reprogramming approaches.

Importantly, while evidence for functional recovery operated by induced neurons has been provided in the retina, the achievement of functional repair by spontaneous neurogenesis or reprogramming remains to be demonstrated for the rest of the CNS.

The possibility to employ small molecules as an alternative to reprogramming approaches based on forced expression of exogenous genes appears very attractive because it bypasses the risks of genetic manipulations in cell types that may be prone to generate tumors. However, the efficacy of the small molecule approach has still to be proved in vivo. Nevertheless, the availability of these new compounds, as well as a more profound understanding of niche-associated factors, indicate that we are on the way to reach an era of “pharmacological plausibility”. These drug-base strategies could not only prevent neurodegeneration, but they could also impact on tissue regeneration.

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Figure and table legends

Figure 1. Schematic representation of the inducible Cre-based lineage tracing approach. The inducible Cre lox system allows to trace the lineage of the cells based on the activity of specific promoters at the stage of tamoxifen administration. Mice expressing the tamoxifen-inducible recombinase CreERT2 under the control of a cell type specific promoter (P) are crossed with ROSA26-loxP-STOP-loxP-YFP reporter mice. Cre-ERT2 is a fusion protein derived from Cre recombinase and mutated estrogen receptor (ERT2). In the absence of tamoxifen Cre-ERT2 localizes in the cytoplasm and does not reach the DNA to operate recombination. When tamoxifen binds to the ERT2 domain, activated Cre-ERT2 enters the nucleus, recombines the loxP sites removing the stop codon and leads to the expression of YFP. The stop codon removal is not reversible. Thus, these cells will remain YFP-positive even if the P is switched off. YFP expression is inherited to the progeny of the recombined mother cell, therefore allowing fate mapping studies.

Figure 2. Schematic representation of MG-derived neurogenic events occurring in the retina upon injury or after transgenic reprogramming. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; MG, Müller glia; NP, neural progenitor; HC, horizontal cell; BC, bipolar cell; AC, amacrine cell; RGC, retinal ganglion cell.

Figure 3. Schematic representation of protocols for astroglial reprogramming by small molecule. Ngn2, neurogenin2; for acronyms see Table 2.

Table 1. In vivo and ex vivo (retina) reprogramming factors. TF, transcription factor; AAV, adeno-associated virus; ABM, Ascl1, Brn2a, Mytl1; AD, Alzheimer disease; ALN, Ascl1,

Lmx1a, Nurr1; AV, adenovirus; BDNF, brain-derived neurotrophic factor; DCX, doublecortin expressing neuroblasts; EAN, electrophysiologically active neurons; ERGR, electroretinographic response; GABAergic, gamma-aminobutyric acid neurons; GFs, fibroblast growth factor (FGF) + epidermal growth factor (EGF); HUC/D, postmitotic neurons; LV, lentivirus; MG, Müller glia; NeAL218, NeuroD1, Ascl1, Lmx1a, miR218; NMDA, N-methyl-D-aspartate receptor; MNU, N-methyl-N-nitroso urea; NeuN, neuronal nuclear antigen, mature neurons; Neurogenin2, Ngn2; NOG, noggin; 6-OHDA, 6-hydroxydopamine; RV, retrovirus; TH, Tyrosine hydroxylase; Tuj1, beta III tubulin expressing neuroblasts; VPA, valproic acid; na, not assessed.

Table 2. List of compounds employed for in vitro reprogramming of fibroblasts and astrocytes. References can be found in the text. AMPK, AMP-activated protein kinase; BLM 210: N-(2-aminophenyl)-N'-phenyloctanediamide; BMP, bone morphogenetic protein; cAMP, *cyclic* adenosine monophosphate; CHIR99021: 6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile; DAPT, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester; GO6983, 3-[1-[3-(Dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione; GSK3β, glycogen synthase kinase 3 β; HDAC, histone deacetylase; I-BET151, BET family bromodomain inhibitor 151; ISX9, isoxazole 9; LDN193189, 4-[6-[4-(1-Piperazinyl)phenyl]pyrazolo[1,5-a]pyrimidin-3-yl]-quinoline; JNK, c-Jun N-terminal kinase; MEF2, *myocyte enhancer factor-2;* PGE2: Prostaglandin E2; *PKA,* protein kinase A; *PKC,* protein kinase C; *PP2, 4-Amino-3-(4-chlorophenyl)*-1-(t-butyl)-1H-pyrazolo[3,4-d]pyrimidine; RA, retinoic acid; Repsox, E-616452; 2-[3-(6-Methyl-2-pyridinyl)-1H-pyrazol-4-yl]-1,5-naphthyridine; ROCK, Rho-associated protein kinase; SAG, Smoothened agonist; SB431542, 4-(5-Benzol[1,3]dioxol-5-yl-4-pyrldin-2-yl-1H-imidazol-2-yl)-benzamide; SP600125, 1,9-Pyrazoloanthrone; Shh: Sonic hedgehog, SIRT1, Sirtuin 1; TGFβ,

transforming growth factor β; TTNPB, Selective agonists of the RAR; VPA, valproic acid; Wnt, wingless-type MMTV integration site family.

Table 1

TF	CNS region	Cell type	Lesion	Combinatorial treatment	Final cell type	Functionality	Administration	Species	Ref
Pax6	Cortex	Proliferating cells	Stab wound		DCX	na	RV	Mouse	[51]
NeuroD1	Cortex	Astrocytes	Brain injury/ AD model		Glutamatergic	EAN	RV	Mouse	[70]
		NG2 cells			Glutamatergic, GABAergic				
Sox2 w/o Ascl1	Cortex	NG2 cells	Stab wound		DCX, NeuN	EAN	RV	Mouse	[66]
Ascl1					no neurons				
Sox2	Striatum	Astrocytes		BMP, NOG, VPA	DCX, NeuN, Calretinin	EAN	LV	Mouse	[63,64]
Ascl1					No neurons				
ABM	Striatum	Astrocytes			NeuN	na	LV	Mouse	[187]
NeAL218	Striatum	Astrocyte	6-OHDA		DCX, TH, SLC6A3	EAN, behavioral study	LV/AAV	Mouse	[61]
ALN	Striatum	NG2 cells			NeuN, MAP2	EAN, integrated into host neural circuits (rabies-virus-based tracing)	AAV	Mouse	[188]
		Astrocytes			Glutamatergic, GABAergic				
Ascl1	Midbrain	Astrocytes			Glutamatergic, GABAergic	EAN	AAV	Mouse	[72]
	Striatum				NeuN				
	Cortex				NeuN				
	Cortex	Proliferating cells	Stab wound	GFs	no neurons		RV	Rat	[120]
	Striatum				DCX, NeuN	na			
Ngn2	Cortex Striatum	Proliferating cells	Stab wound	w/o GFs	DCX, NeuN	na			
PAX6	Cortex/ Striatum	Proliferating cells	Stab wound	w/o GFs	DCX, NeuN	na			
Ascl1	Cortex/ Striatum	Proliferating cells	Stab wound	w/o GFs	no neurons				
Ngn2, Bcl2	Cortex				DCX, NeuN	na	RV	Mouse	[161]
	Spinal cord	Proliferating cells: majority Olig2+/NG2 cells	Laminectomy	GFs	HuC/D, TuJ1	na	RV	Rat	[189]
Ngn2				GFs	HuC/D, NeuN				
				BDNF	NeuN				
Ascl1					no neurons				
Sox2	Spinal cord	Astrocytes	w/o hemisection	VPA	DCX, MAP2, NeuN, glutamatergic, GABAergic	na	LV	Mouse	[65]
Sox2, p53-p21 removal	Spinal cord	Astrocytes	w/o traumatic injury	BDNF, NOG	DCX, glutamatergic	na	LV	Mouse	[162]

Ascl1	Retina (juvenile)	MG/astroglia	NMDA-injection/ light damage	Bipolar cells, Amacrine cells and Rod photoreceptors	na (expression of ribbon synapse markers)	Transgenic mouse	Mouse	[49]
Atoh7	Retina	MG	Model of glaucoma	Retinal ganglion cells	na	LV	Rat	[77]
β-catenin	Retina	MG/astroglia	No	Amacrine, retinal ganglion cells	na	AAV	Mouse	[73]
GSK3β deletion				Expression of the pluripotency factor Lin28 in MG	na	Transgenic mouse		
Lin28				Pax6 MG-derived progenitors, amacrine and retinal ganglion cells	na	AAV		
Lin28	Retina	MG/astroglia	Model of retinitis pigmentosa	Photoreceptors and bipolar cells	ERGR	AV	Mouse	[75]
Inhibition of GSK3β	Retina	MG/astroglia	MNU-injection	Photoreceptors	ERGR	In vivo cell fusion	Mouse	[74]
NeuroD	Retina (adult retinal explant)	Proliferating cells	NMDA-injury	Amacrine cells	na	RV	Rat	[41]
Math3				Amacrine cells	na			
Pax6, NeuroD				Increased production of amacrine cells	na			
Pax6, Math3				Increased production of amacrine cells	na			
Crx, NeuroD				Photoreceptors	na			

Table 2

Targets	Compounds	Effect in neuronal reprogramming
Morphogens and gliogenesis/neurogenesis regulators		
RA	TTNPB, agonist	No effect on reprogramming from astrocytes
Shh	SAG, agonist	Increase of reprogramming efficiency from astroglia
Shh	Purmorphamine, agonist	Increase of reprogramming efficiency from astroglia
TGFβ	Repsox, inhibitor	Inhibition of fibroblast genes; increase of reprogramming efficiency from astroglia
BMP	LDN193189, inhibitor	Essential for neuronal reprogramming from astrocytes
TGFβ/activin	SB431542, inhibitor	Essential for neuronal reprogramming from astrocytes
Notch pathway	DAPT, inhibitor	Essential for neuronal reprogramming from astrocytes
Intracellular transduction pathways		
GSK3β (activation of Wnt pathway)	Kenpaullone, inhibitor	Essential for neuronal reprogramming from fibroblasts
GSK3β (activation of Wnt pathway)	CHIR99021, inhibitor	Essential for neuronal reprogramming from fibroblasts and astrocytes
cAMP/PKA	PGE2 modulator	Increased neuronal reprogramming from fibroblasts
JNK	SP600125, inhibitor, inhibitor	Promotion of neuronal gene expression
PKC	GO6983, inhibitor	Promotion of neuronal gene expression
AMPK (BMP receptor type I)	Dorsomorphin, inhibitor	Increased neuronal reprogramming from fibroblasts
Src kinase	PP2, inhibitor	Increased neuronal reprogramming from fibroblasts
ROCK	Thiazovivin, inhibitor	No effect on reprogramming from astrocytes
ROCK	Y-27631, inhibitor	Promotion of neuronal gene expression
Epigenetic and transcriptional regulators		
HDAC	VPA, inhibitor	Inhibition of fibroblast genes; increase of reprogramming efficiency from astroglia
HDAC	BML210, inhibitor	Increased neuronal reprogramming from fibroblasts
SIRT1	Amonoresveratrolsulfat, activator	Increased neuronal reprogramming from fibroblasts
Bromodomain protein	I-BET151, inhibitor	Disruption of fibroblast gene programs; downregulation of astrocyte gene programs
Second messengers		
MEF2-dependent calcium signalling	ISX9	Induction of neuronal genes
cAMP agonist	Forskolin	Induction of neuronal morphological differentiation; essential for neuronal reprogramming from fibroblasts *but also metabolic facilitator of neuronal induction [161]

Figure 1

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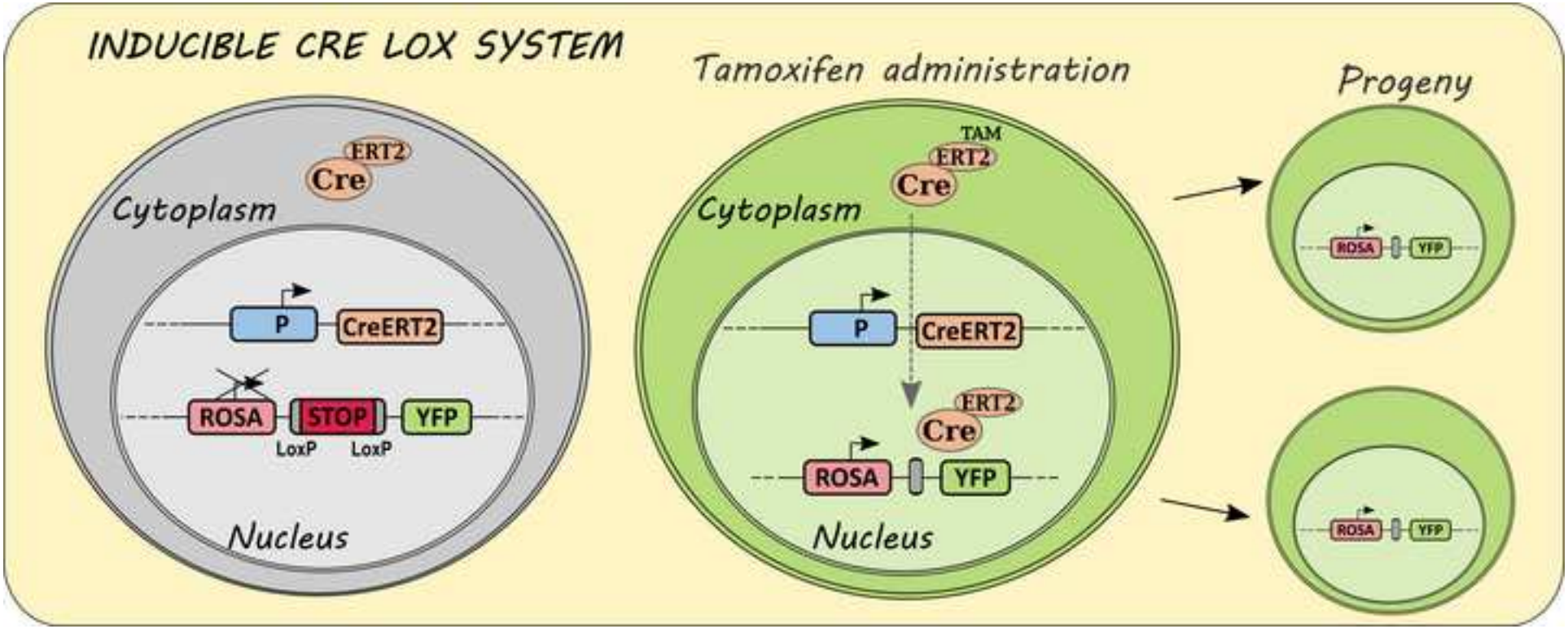

Figure 2

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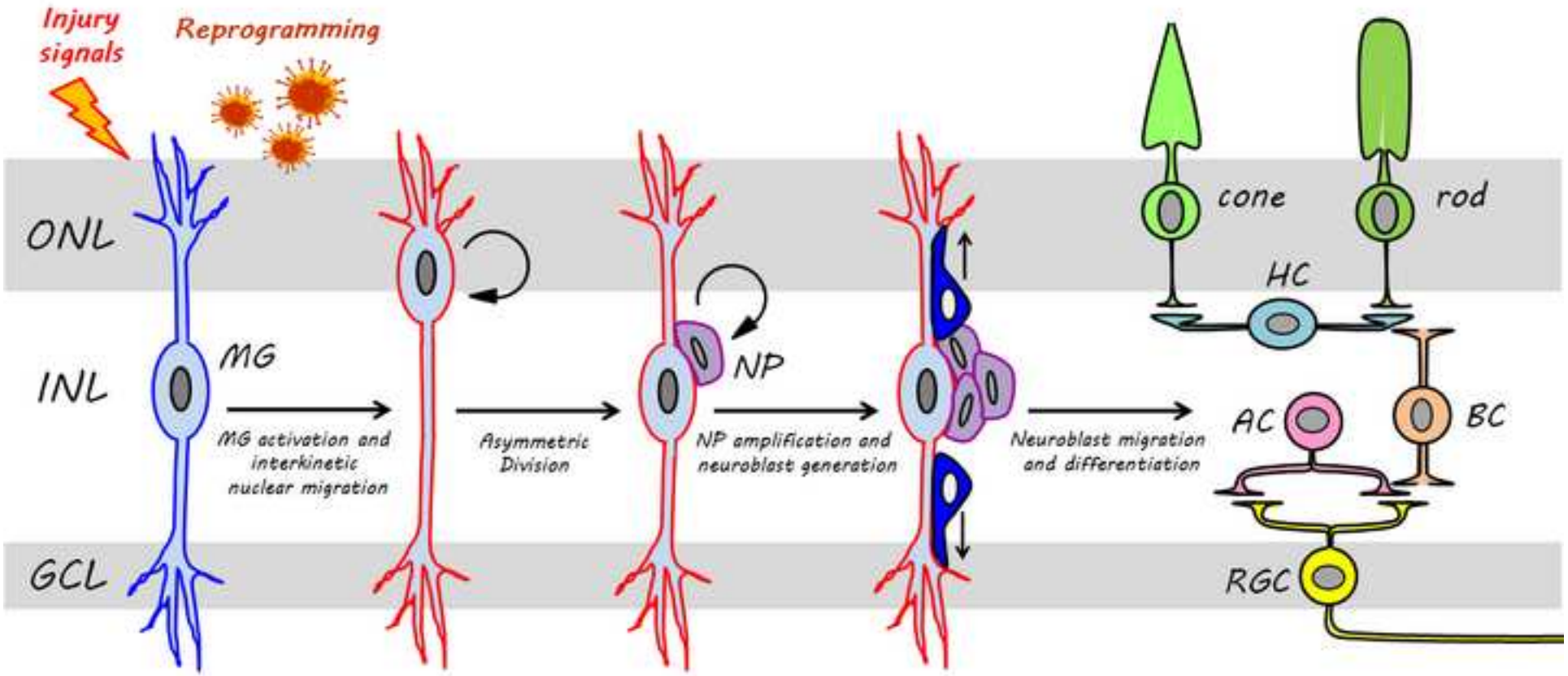

Figure 3

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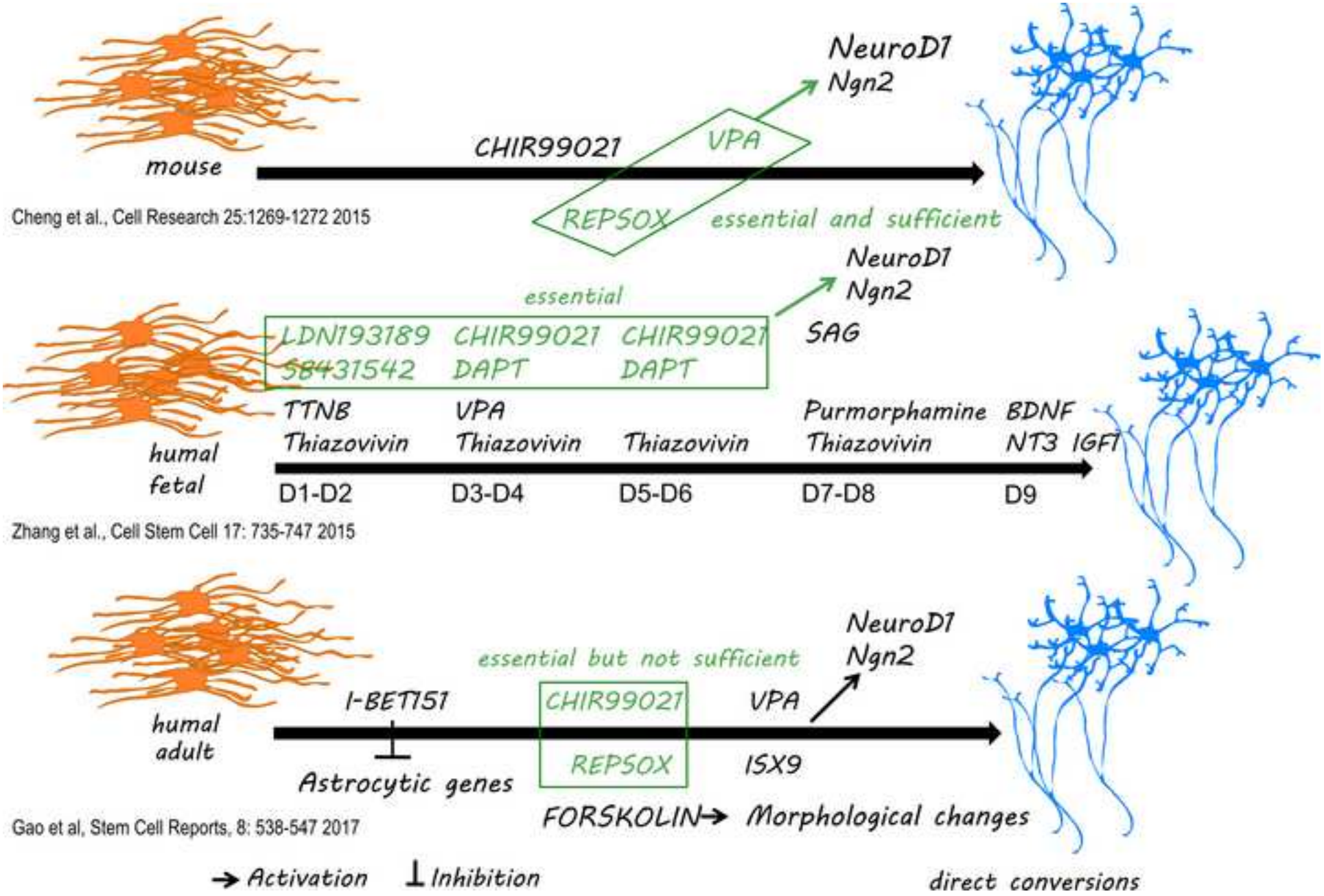

*Graphical Abstract (for review)

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