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Direct Neuronal Reprogramming: Achievements, Hurdles, and New Roads to Success

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The ability to directly reprogram mature cells to alternative fates challenges concepts of how cell identities are maintained, erased, and acquired. Recent advances in understanding and overcoming hurdles to direct neuronal conversion have provided new insights into mechanisms that maintain cell identity programs and have enabled high efficiency reprogramming in vivo. We discuss key cell-intrinsic molecular and metabolic constraints that influence the establishment of a new identity as well as environmental inputs from injured brains that favor or harm the conversion process. Finally, we outline the challenges ahead with a particular focus on direct neuronal reprogramming in vivo.

Introduction

How cell identity is defined is a central, yet poorly understood, question in biology. Its importance has magnified since the advent of reprogramming, which continues to suggest that almost any cell type can be turned into almost any other cell type. If this is so "easy," what keeps cells from converting into other identities? Or, if it is not so easy, which hurdles need to be overcome? Another question pertains to the robustness of the conversion process and whether we are underestimating lineage boundaries because the analysis of reprogrammed cells, i.e., induced neurons (iNs), has not yet reached full depth. These are only some of the fascinating questions associated with reprogramming that will be discussed here in the context of direct neuronal reprogramming.

The approach to turn non-neuronal cells into neurons started 15 years ago and was inspired by the potent role of the neurogenic transcription factor (TF) Pax6 (Heins et al., 2002). Since then, additional neurogenic TFs, microRNAs, and small molecules regulating key developmental pathways have been employed and largely improved the efficiency and maturity of iNs. The discovery that even mesoderm-derived fibroblasts from mice and humans can be directly converted into neurons (Vierbuchen et al., 2010) provided a huge boost to the in vitro reprogramming field, encouraging further studies using human fibroblasts for direct neuronal conversion (Karow et al., 2012; Pang et al., 2011; Vierbuchen et al., 2010). This represents a substantial improvement to the field of human disease modeling, as direct neuronal reprogramming does not reset the age of the patient-derived starter cells (Mertens et al., 2015), thus preserving the highest risk factor for most neurodegenerative diseases. Likewise, huge progress has been made since the first attempt to turn reactive glial cells into neurons after brain injury in vivo (Buffo et al., 2005; Table 1) regarding the efficiency of conversion and the maturity of the iNs (Table 1). Indeed, the field has progressed to the point that now the gold standards of reprogramming—obtaining genome-wide expression patterns and full function of the induced cell type—are becoming feasible targets.

Many cell types in several different brain regions have been targeted by various neurogenic fate determinants with the use of diverse viral vectors (Table 1), making it very difficult to identify common principles, as discussed below. Moreover, despite the huge achievements and improvements in reprogramming protocols, they are still largely based on trial and error, even though new prediction tools may help to overcome this issue (Rackham et al., 2016). Indeed, some TFs, such as the proneural factors, are common players in direct neuronal reprogramming as they appear in all protocols (see Table 1, Figure 1, and Masserdotti et al., 2016). However, molecular programs instructing defined neuronal subtypes are far from being resolved, despite the great progress in determining trajectories of neuronal subtype identity specification and differentiation during development (Bikoff et al., 2016; Hobert, 2011; Molyneaux et al., 2007; Rhee et al., 2016).

Indeed, direct neuronal reprogramming can help unravel such programs and bridge the gap in our understanding of molecular programs of neuronal subtype specification (Masserdotti et al., 2015). For example, the transcriptional changes occurring during direct neuronal reprogramming (Masserdotti et al., 2015; Smith et al., 2016; Treutlein et al., 2016; Xue et al., 2013) have highlighted the pioneer activity of key TFs and have shown that their downstream targets resemble those in development. Such studies have also revealed very early diversions in the programs for the GABAergic and glutamatergic neuronal subtypes (Masserdotti et al., 2015, and see review by Masserdotti et al., 2016). However, many important issues remain unaddressed, such as the influence of the starting population on the generation of different neurons, essential pathways that must be induced or repressed, the role of metabolic changes during the conversion process, and the influence of the environment where the conversion takes place.

Table 1. Direct Neuronal Reprogramming in the Adult CNS

Cell Source	Environment	Reprogramming Factors	Vectors Used	Type of iN	Functional Outcome	Maximum Time Investigated	Efficiency of Reprogramming	References
Astrocytes	cortex, stab wound	miR-Olig2	RV	DCX	n.d.	30 dpi	1%–2%	Buffo et al., 2005
	intact striatum	Ascl1/Brn2/Myt1L	LV	NeuN	n.d.	6 wpi	117 NeuN+/injection at 6 wpi[a]	Torper et al., 2013
	intact striatum	Sox2	LV	DCX	immature excitability	24 mpi	12,000 DCX+/injection at 5 wpi[a]	Niu et al., 2013
	intact striatum	Sox2 + BDNF/ Noggin or VPA	LV	NeuN	mature excitability, synaptic input (at 14 wpi)	14 wpi	n.d.	Niu et al., 2013
	cortex[c]	NeuroD1	RV	DCX/NeuN/Tbr1/Ctip2	mature excitability, synaptic input (at 4 wpi)	8 wpi	2.5 NeuN+/0.1 mm^2 at 2 wpi[e]	Guo et al., 2014b
	cortex, FAD mouse	NeuroD1	RV	NeuN	synaptic input	14 dpi	7–18 NeuN+/0.1 mm^2 at 2 wpi[a]	Guo et al., 2014b
	intact spinal cord	Sox2	LV	DCX/βIII-Tubulin (few MAP2/NeuN)	n.d.	8 wpi	6%–8% DCX+ at 4–5 wpi	Su et al., 2014
	hemisected spinal cord	Sox2	LV	DCX/βIII-Tubulin	n.d.	8 wpi	3%–6% DCX+ at 4–8 wpi	Su et al., 2014
	intact spinal cord	Sox2 + VPA	LV	NeuN/GABA/ GAD65/SYN1	n.d.	30 wpi	double than that achieved with Sox2 only	Su et al., 2014
	adult striatum	Sox2 + VPA/ BDNF/NOG	LV	Calretinin interneurons (through Ascl1 expression)	mature excitability, synaptic input	40 wpi	23% DCX+ at 4–5 wpi	Niu et al., 2015
	intact spinal cord	Sox2/p53KO/ p21KO	LV	NeuN	n.d.	8 wpi	10,000 DCX+/injection at 4 wpi	Wang et al., 2016
	intact spinal cord	Sox2/p53KO/ p21KO + BDNF-NOG	LV	NeuN/MAP2; VGLUT (80%); GAD6, GABA, GLYT2, 5-HT, CALB, CHAT	SYN1 presynaptic protein	8–24 wpi	30,000 NeuN+/injection at 8 wpi	Wang et al., 2016
	striatum in parkinson model (6-OHDA)	NeuroD1, Ascl1, LMX1A, miR218	RV	TH, RBFOX3, DCX, PVALB, SST, CALB2, GAD1/2, SLC6A3	mature excitability; partial behavior recovery	13 wpi	14.63 ± 8.5 TH+ cells/ section	Rivetti di Val Cervo et al., 2017
	intact striatum	NeuroD1	AAV intra-vascular	NeuN, β-III-tubulin, DCX	n.d.	10 dpi	2%–3%	Brulet et al., 2017
Ng2 glia	hemisected spinal cord	Sox2/p53KO/ p21KO	LV	NeuN	n.d.	8 wpi	6,000 NeuN+/injection at 8 wpi	Wang et al., 2016
	cortex, stab wound[d]	Sox2	RV	DCX/NeuN	immature excitability, low frequency synaptic input	12 dpi	21.8% DCX+ at 12 dpi[b]	Heinrich et al., 2014
	cortex[c]	NeuroD1	RV	DCX/NeuN	n.d.	8 dpi	42.5% NeuN+ at 8 dpi	Guo et al., 2014b

(*Continued on next page*)

Table 1. *Continued*

Cell Source	Environment	Reprogramming Factors	Vectors Used	Type of iN	Functional Outcome	Maximum Time Investigated	Efficiency of Reprogramming	References
	striatal NG2-glia	Ascl1/Lmx1a/ Nurr1	AAV	NeuN, MAP2, SYN, VGLUT + GAD65/67	mature excitability, synaptic input; integration into local circuitry	12 wpi	21% NeuN+ at 4 wpi. 47% at 12 wpi[b]	Torper et al., 2015
Unspecified proliferating glia	striatum	Neurog2 + GF	RV	DCX, NeuN, MAP2, β-III-Tubulin, HuC/D, GABA, Isl1	n.d.	28 dpi	6.6% DCX+ at 7 dpi; 3.9% NeuN+ at 14 dpi	Grande et al., 2013
	cortex, stab wound	Neurog2 + GF	RV	DCX, NeuN, GluR2/3, Bhlhb5	n.d.	28 dpi	20.6% DCX+ at 7 dpi; 0.42% NeuN+ at 14 dpi[b]	Grande et al., 2013
	cortex, stab wound	Neurog2/Bcl-2 + Vitamin E/D	RV	NeuN; Ctip2 deep-layer neurons	n.d.	8 wpi	95% NeuN+ at 10 dpi[b]	Gascón et al., 2016

FAD, Familiar Alzheimer Disease; n.d., not defined.
[a]Efficiency of reprogramming is dramatically reduced over time.
[b]Survival of reprogrammed cells is reduced over time.
[c]Injured by the injection cannula.
[d]Failed conversion without stab wound.
[e]Weeks post injection.

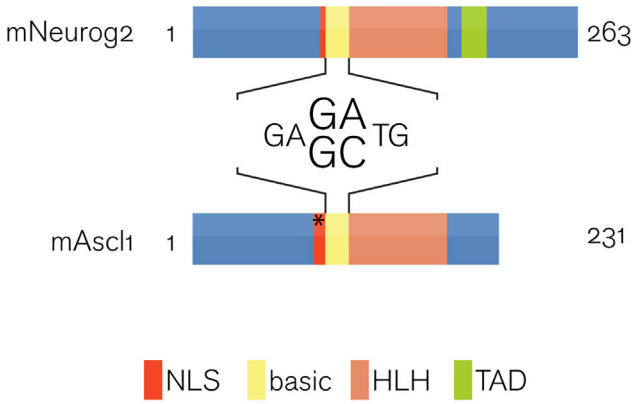
As mentioned above, reprogramming must also overcome the old program of the starting cell. Is this different for each cell type or are there common "guardians" of cell identity? If the latter is the case, we may learn about the roadblocks for direct neuronal reprogramming by analyzing obstacles identified in other reprogramming paradigms, such as the well understood process of generating induced pluripotent stem cells (iPSCs). A better understanding of the hurdles in reprogramming will not only provide new insights into the mechanisms maintaining cell identity but also open new approaches to overcome such blocks and improve reprogramming.

Here we will discuss the main principles underlying transcriptional regulation of direct neuronal reprogramming and compare the transcriptional obstacles identified in iPSCs and iNs. We will then focus on a different hurdle in reprogramming, namely metabolic conversion, which is largely responsible for increasing the efficiency in the injured brain in vivo, and conclude with a discussion of the major challenges lying ahead.

The Starter Cell: Cell-Type-Specific Prerequisites and Neuronal Subtype Generation

Given the great progress in the field of direct neuronal reprogramming, it seems appropriate to start with the achievements—first in vitro—and what we could learn from these regarding mechanisms, hurdles, and improvements for the final in vivo conversion process. A key choice in direct reprogramming is the selection of the starting cell type, which implies the cellular context (e.g., chromatin, proteome, or metabolome) where the reprogramming factors act. As these factors may ease or impede the conversion process, one consideration in choosing the starter cell is its lineage relation to neurons, assuming that developmentally closely related cells may be easiest to convert into each other, as depicted in the Waddington landscape model (Masserdotti et al., 2016). Indeed, astrocytes sharing a common origin with neurons (Kriegstein and Alvarez-Buylla, 2009) are efficiently converted into functional neurons with one TF (Berninger et al., 2007; Heinrich et al., 2010; Heins et al., 2002), while cells of non-ectodermal origin, MEFs (mouse embryonic fibroblasts), or hepatocytes require more than one factor (Marro et al., 2011; Vierbuchen et al., 2010) or additional chemical manipulation (Hu et al., 2015; Ladewig et al., 2012; Li et al., 2015; Liu et al., 2013; Zhang et al., 2015) for high-efficiency conversion. However, this is not always the case, as conversion of one neuronal subtype into another is rather difficult and has been achieved only in immature cells (Rouaux and Arlotta, 2013). Thus, the selection of the starter cells is often based on other criteria such as in vitro expandability, while its influence on the outcome of reprogramming still needs to be understood.

This influence is evident when different cells are transduced by the same TFs. Ascl1 or Neurog2, identified in *Drosophila* for their proneural activity (see review by Bertrand et al., 2002), are evolutionary conserved, particularly in the basic-helix-loop-helix (BHLH) domain (mouse: 40%–46% homology; overall homology of 20%) (Figure 1). While the HLH domain is mainly responsible for the heterodimerization with co-factors (E-proteins), the basic domain binds the genomic sequence called E-box (CANNTG) (Bertrand et al., 2002). Recent chromatin immunoprecipitation experiments have highlighted subtle but significant preferences in the E-box sequence recognized by the two proteins: Ascl1 mainly binds the CAGCTG site (Wapinski et al., 2013), while



mouse	Neurog2 (263 aa)			
Ascl1 (231 aa)	basic	HLH	C-ter	overall
	40%	40%	20%	19,3%

Neurog2	Human (272 aa)			
mouse (263 aa)	basic	HLH	TAD**	overall
	100%	100%	78,8%	83,5%

Ascl1	Human (236 aa)			
mouse (231 aa)	basic	HLH	C-ter	overall
	100%	100%	100%	91,9%

Figure 1. Proneural Transcription Factors
Protein structure of mouse Neurogenin2 (Neurog2) and Ascl1. NLS, nuclear localization signal; HLH, helix-loop-helix; TAD, TransActivator Domain (Hand et al., 2005). The table indicates the homology between the mouse protein sequences and the mouse and human orthologs. (*), predicted. (**), Hand et al. (2005).

Neurog2 mainly binds CAGATG (Smith et al., 2016), thus conferring specificity to the target selection.

During mammalian brain development, Ascl1 and Neurog2 instruct stem and progenitor cells in diverse brain regions toward different neuronal identities (e.g., GABAergic and glutamatergic neurons in the telencephalon; Casarosa et al., 1999; Fode et al., 2000). Accordingly, Ascl1 instructs a GABAergic fate and Neurog2 triggers a glutamatergic fate in astrocytes in vitro (Heinrich et al., 2010; Masserdotti et al., 2015). This has allowed investigations into whether neuron-specific programs would diverge early or late in differentiation. Ascl1 and Neurog2 instruct two almost non-overlapping programs very early during the conversion, with GABAergic or glutamatergic neuron-specific genes readily detected within a few hours after the initiation of astrocyte conversion (Masserdotti et al., 2015). However, when expressed in other cells, such as midbrain astrocytes or MEFs, Ascl1 induces a mix of glutamatergic and GABAergic neurons or mainly glutamatergic neurons, respectively (Achim et al., 2014; Chanda et al., 2014; Liu et al., 2015). This difference may be due to the requirement of Sox2 and Forkhead box G1 (FoxG1) for the induction of *Dlx2* (Colasante et al., 2015), an important downstream effector of the GABAergic lineage (Petryniak et al., 2007). In astrocytes, but not MEFs, Sox2 and FoxG1 are endogenously expressed, thus making *Ascl1* sufficient to induce the GABAergic program in astrocytes. Therefore, one may expect that midbrain astrocytes or MEFs may be heterogeneous in expression of these TFs, allowing some to convert into GABAergic neurons, while others follow a "default" glutamatergic neuronal program.

The case for Neurog2 is more extreme: in fibroblasts, whether embryonic or adult, mouse or human, this proneural factor is poorly, if at all, capable of generating neurons (Liu et al., 2013; Vierbuchen et al., 2010). In combination with other factors or small molecules, however, Neurog2 triggers the generation of somatic motor neurons (Liu et al., 2013, 2016; Son et al., 2011) as opposed to glutamatergic neurons in astrocytes.

Together, these examples highlight the importance of the crosstalk between the reprogramming factors and the cellular context in which they operate.

Hurdles in Direct Reprogramming: From Common Mechanisms to Cell-Type Specificity

As discussed above, the induction of distinct neuronal subtypes by the same TF(s) in different starter cells is an excellent entry point to both identify the key components of the shared and subtype-specific neuronal code acting in direct reprogramming and investigate the similarities and differences in the cell identity barriers that limit reprogramming. These hurdles can be general and cell-type specific (Figure 2). The first group includes mechanisms protecting genome stability, senescence, or cell cycle re-entry (Figure 2). Some hurdles are specific for a given starting population, and they may include cell-type-specific transcriptional repressors, terminal selector genes blocking conversion into other neuronal subtypes, and heterochromatin regions preventing transcription of genes important for the new induced fate. The latter may also be more general, as the same chromatin and histone interacting proteins can be involved in permanently silencing programs of a variety of fates. Lineage specific impediments can also involve the machinery regulating 3D nuclear topology that needs to be rearranged in development as well as during reprogramming (Beagan et al., 2016). Lastly, reprogramming hurdles can also consist of specific aspects of cellular proteomes. For example, ubiquitin ligases degrade potent fate determinants (Sancho et al., 2014; Urbán et al., 2016) such that deletion of the enzyme alone may induce reprogramming (Sancho et al., 2014) or may be necessary to allow the reprogramming factor to act. The larger the differences between the proteomes of the starting and induced cell type, the more changes need to be implemented. Thus, similarities in these aspects may ease reprogramming even though cells are developmentally less related.

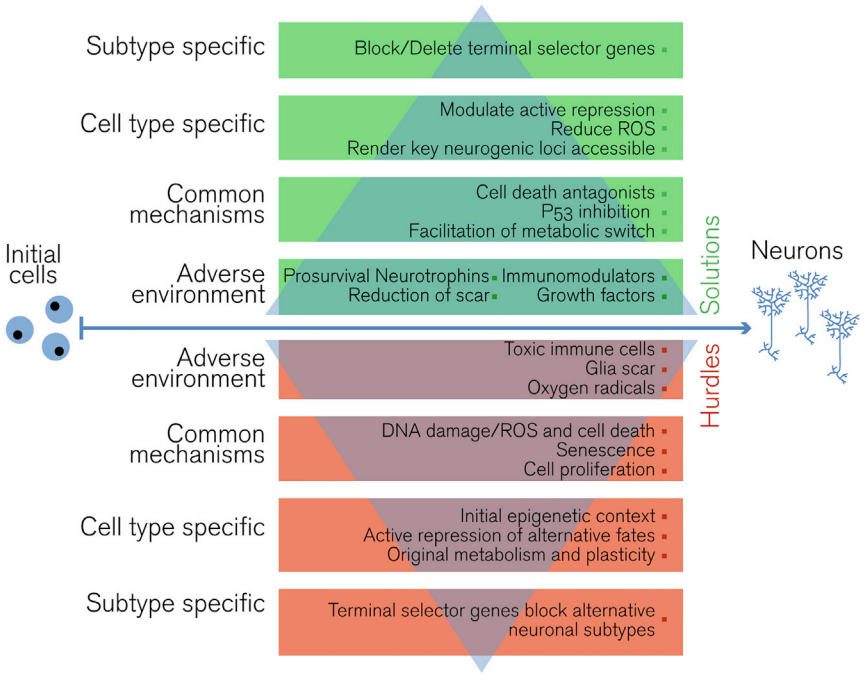


Figure 2. Reprogramming Hurdles to Direct Neuronal Reprogramming
Schematic representation of the hurdles to direct reprogramming so far identified, from most general to subtype-specific, and solutions already tested or to be explored.

Repressive Barriers Common to iPSC Generation and Direct Neuronal Conversion

Improving reprogramming efficiency is a major goal in the iPSC field, as the initial protocol was quite inefficient (Takahashi and Yamanaka, 2006). Efforts toward finding enhancers of reprogramming have led to the identification of factors whose expression boosts conversion (C/EBP, GLIS1, and Mbd3) (Di Stefano et al., 2014; Maekawa et al., 2011; Rais et al., 2013) and barriers whose removal greatly increases reprogramming efficiency. For example, ablation of the p53-p21 pathway accelerates the kinetics of iPSC generation (Kawamura et al., 2009; Utikal et al., 2009) at the expense of a higher rate of chromosomal aberrations (Marión et al., 2009). However, transient repression of p53 improves iPSC reprogramming without increased chromosomal instability (Rasmussen et al., 2014). Expression of a dominant-negative form of p53 (P53DN; Liu et al., 2014) or p53 knockdown (p53-KD; Jiang et al., 2015) also improves direct conversion of MEFs into functional dopaminergic neurons (Jiang et al., 2015; Liu et al., 2014), but their rate of chromosomal damage is not known (Figure 2).

Another interesting roadblock implicated in iPSC generation is the senescence pathway regulated by the proteins $P16^{Ink4a}$ and $P19^{Arf}$, encoded in the *INK4/ARF* locus. Remarkably, the number of iPSC colonies decreases with passaging of the starting population and inversely correlates with increased expression of $p16^{Ink4a}$ and $p19^{Arf}$ (Utikal et al., 2009). Indeed, MEFs from *Ink4a/Arf* knockout mice show much higher efficiency of iPSC colony formation (up to 40-fold; Utikal et al., 2009), similar to that observed upon p53 knockdown.

Interestingly, genes from the *INK4a/ARF* locus are not expressed in neural progenitors but are expressed in astrocytes (Bachoo et al., 2002). Accordingly, *Ink4a/Arf*-deficient postnatal mouse astrocytes and MEFs were more efficiently converted into neurons in vitro and passaging the starter cells no longer reduced reprogramming efficiency (Price et al., 2014), suggesting that the *INK4a/ARF* locus exerts general control of cell fate. Furthermore, cells from older organisms express additional reprogramming impediments, such as the TF FoxO3, which interferes with reprogramming from older, but not younger, fibroblasts (Ahlenius et al., 2016). To overcome hurdles of reprogramming aged cells is important to study iNs from patients with neurodegenerative disease that maintain their aging phenotype (Mertens et al., 2015).

Given these common hurdles in direct reprogramming, it is tempting to speculate that other hurdles identified in iPSC generation, such as specific kinases (e.g., Aurora kinase; Li and Rana, 2012), metallo-proteases (e.g., members of the ADAM family; Qin et al., 2014), or members of the ubiquitin-proteasome system (e.g., Fbxw7; Buckley et al., 2012) may also be relevant for neuronal reprogramming, thereby substantiating similarities and differences between the reprogramming paradigms.

Proliferation, Chromatin Status, and Chromatin Remodeling: Similarities and Differences in Neuronal and iPSC Reprogramming

IPSC reprogramming is eased when the starter cells are proliferating (Guo et al., 2014a; Li and Rana, 2012). Indeed, many chromatin marks need to be reestablished after cell division, providing a window of opportunity to change fate. While proliferating cells are often targeted in direct neuronal reprogramming, proliferation is neither a prerequisite nor an advantage for direct neuronal conversion (Fishman et al., 2015). Continuous live imaging revealed that most astrocytes or pericytes undergo lineage conversion without cell division (Gascón et al., 2016; Heinrich et al., 2010; Karow et al., 2012). Moreover, postmitotic cells can be converted into neurons (e.g., liver cells, postmitotic neurons, and postmitotic astrocytes; Marro et al., 2011; Rouaux and Arlotta, 2013). Attempts to force proliferation, such as via expression of Myc, even reduce neuronal conversion of fibroblasts (Fishman et al., 2015), while genetically or chemically induced cell-cycle exit improves neuronal reprogramming (Patel et al., 2012), likely due to the increased expression of Tet1 and the subsequent higher level of 5 hmC (Jiang et al., 2015). Moreover, cyclin-dependent kinases phosphorylate (for example) Ascl1 or Neurog2 at multiple serine-proline sites in proliferating cells (Ali et al., 2014; Hardwick and Philpott, 2015; Hindley et al., 2012), thus preventing the induction of target genes important for differentiation and direct neuronal reprogramming

(Ali et al., 2014; Hindley et al., 2012). Conversely, dephosphorylated forms of Ascl1 and Neurog2 can induce the expression of differentiation targets by recruiting chromatin remodelers and opening closed target sites in proliferating cells (Ali et al., 2014; Hardwick and Philpott, 2015), thus showing that post-translational modifications have a prominent impact on TF function in reprogramming.

Proliferation also impacts the chromatin status of specific genes involved in the conversion process: these may be in a repressed (R-chromatin) or open (L-chromatin) state, marked by the presence of modification at specific residues in the histone tails (e.g., methylation at H3K9, H3K27 or H3K4, or acetylation at H3K9 and H3K27), or they may be in a low-signal state, when neither active nor repressive marks accumulate (reviewed in Iwafuchi-Doi and Zaret, 2016). The comparison of Ascl1-bound sites during MEF-to-neuron reprogramming and specific histone modifications present at these sites revealed the coexistence of a trivalent chromatin state composed of two marks associated with an active state, H3K4me1 and H3K27ac, and a repressive mark, H3K9me3, in many Ascl1-bound loci (Wapinski et al., 2013). Interestingly, cells selected for the absence of such a trivalent state at predicted Ascl1 target sites could not be reprogrammed into neurons (Wapinski et al., 2013). It will now be fascinating to use epigenetic engineering techniques to introduce this trivalent state at key Ascl1 target sites to determine if this would render the previously unresponsive cells reprogrammable. Such direct proof of selective manipulation of the epigenetic landscape at reprogramming hotspots is critical now to overcome the so far rather general and non-specific chromatin changes often elicited by pharmacological treatments.

Neurog2 also acts as a pioneer TF binding to closed chromatin sites in human fibroblast reprogramming (Smith et al., 2016). Forskolin (FK) and dorsomorphin (DM) treatment greatly improves target gene regulation by improving Neurog2-TCF3 complex DNA binding affinity and rendering targets shared by Neurog2 and Sox4 more accessible (Smith et al., 2016). In agreement with this molecular synergism, Sox11, another HMG-box protein similar to Sox4 (Miller et al., 2013) and required for the conversion of human skin fibroblast into cholinergic neurons (Liu et al., 2013), is induced and necessary during neuronal reprogramming of astrocytes (Masserdotti et al., 2015; Mu et al., 2012; Ninkovic et al., 2013).

While the influence of chromatin status on normal neurogenesis and direct neuronal reprogramming is poorly understood, more data are available on the role of chromatin remodeling complexes (Kadoch et al., 2016). Apart from the role of miR-124 and miR-9/9* in regulating the switch between BAF53a and BAF53b during neural tube development (Yoo et al., 2009), the interaction between Pax6 and the BAF/Brg1 complex is required for adult neurogenesis. This interaction induces a neurogenic cross-regulatory transcriptional network of Brn proteins (containing a Pou domain), Sox4/11, and Nfi TFs that is also required in astrocyte-to-neuron reprogramming (Ninkovic et al., 2013). Indeed, the BAF/Brg1 complex is also important in reprogramming toward iPSCs (Kleger et al., 2012), highlighting the importance of chromatin remodeling factors for mediating accessibility and the activation of factors necessary for the fate transition.

As mentioned above, it will now be important to identify sites with relevant epigenetic marks with greater specificity using Cas9-mediated epigenetic engineering. Indeed, this technique has been recently used for activating the endogenous expression of reprogramming factors, further showing a rapid epigenetic remodeling of the respective sites in the genome, in particular the enrichment in H3K27ac and H3K4me3 at the *Brn2* and *Ascl1* loci on day 3 of reprogramming (Black et al., 2016). Together, these observations indicate that TFs can efficiently drive neuronal reprogramming when the epigenetic environment is permissive, and, if not, successful strategies are emerging to render the hostile environment more susceptible toward being reprogramed.

Cell Fate Gatekeepers and Terminal Selector Genes: From Common to Specific Guardians of Cell Identity

During cell fate commitment and differentiation, progenitor cells require the induction of cell-type-specific programs and mechanisms that prevent alternative fates. Such barriers can be established early on (e.g., during the germ layer formation) or later (e.g., during the transition between embryonic neurogenesis and gliogenesis), and they represent major hurdles for efficient and successful reprogramming protocols. An RNAi screen aimed at revealing such hurdles in *C. elegans* identified *lin-53* as a barrier for *che-1*-forced conversion of non-neuronal cells into neurons in vivo (Tursun et al., 2011) via the regulation of Polycomb Repressor Complex 2 (PRC2) (Patel et al., 2012). More recently, Chaf1a and Chaf1b have been identified as safeguards of cellular identity not only in iPSC reprogramming, but also in various direct reprogramming experiments, including neuronal conversion of MEFs (Cheloufi et al., 2015). As the mammalian ortholog of *lin-53*, Rbbp4/RBAP48, forms the CAF-1 complex together with Chaf1a and Chaf1b, it is very likely that this complex plays an important and rather general gatekeeper role in maintaining cell fate identity. Thus, it would be important to test whether other barriers identified in iPSC generation (see Ebrahimi, 2015 for a review) represent hurdles also during direct neuronal conversion or whether cell-type-specific gatekeepers exist whose removal could ease the conversion into other fates.

Another class of cell identity gatekeepers are the terminal selector genes (Hobert, 2011), whose ablation does not alter the nature of the differentiated cell (e.g., *che-1*; Etchberger et al., 2007; Uchida et al., 2003) but does alter specific function, as they can also repress alternative fates (Patel and Hobert, 2017). Thus, terminal selector genes act as guardians of specific neuronal fates and their ablation may improve neuron-to-neuron reprogramming (Figure 2).

Cell-Type-Specific Barriers: Non-neuronal and Neuron-Specific Programs Guarded by REST and Myt1

Another example of a cell-type-specific guardian relevant for reprogramming is the RE-1 transcription repressor complex (REST). REST is expressed in non-neuronal cells where it represses neuronal genes (Jørgensen et al., 2009). Therefore, REST poses an obvious hurdle in direct neuronal reprogramming when these neuronal genes should be activated. Accordingly, early ablation of REST in astrocytes dramatically improved Neurog2-induced neuronal reprogramming efficiency, with almost 90% turning into iNs (Masserdotti et al., 2015). Importantly, at later stages of astrocyte differentiation, permanent

repressive marks are established at the sites of (for example) *NeuroD1/D4* such that REST deletion no longer allows activating these essential target genes for neuronal conversion (Masserdotti et al., 2015). To remove repressive marks at these essential genes would be a new strategy to overcome this hurdle in direct neuronal reprogramming.

REST complex and the polypyrimidine-tract-binding (PTB) protein, involved in RNA processing (Boutz et al., 2007), are regulated by miR-124 and miR-9/9* (Xue et al., 2013). These miRNAs are sufficient to reprogram human fibroblasts into functional neurons (Victor et al., 2014; Yoo and Crabtree, 2009). Likewise, PTB knockdown is sufficient to convert MEFs into neurons by de-repressing neuronal genes and reducing REST activity (Xue et al., 2013). More recently, a second regulatory pathway involving the crosstalk of nPTB, Brn2, and miR-9 has been identified in human fibroblast conversion (Xue et al., 2016). Thus, cell fate re-specification critically depends on the removal of repressors of the new cell fate program.

Conversely, the newly imposed neuronal program must also negatively regulate the previous fate. Myt1l has emerged as potent repressor of non-neuronal fates during development and reprogramming (Mall et al., 2017). During MEF-to-neuron conversion, Myt1l binds preferentially to gene promoters and represses many MEF genes, including Notch-related genes. When manipulated in the developing forebrain in vivo, this either reduces the number of new neurons reaching the cortical plate (Myt1l knockdown) or, conversely, it triggers increased neuronal differentiation (Myt1l overexpression; Mall et al., 2017). Thus, a new strategy emerging to improve reprogramming when neurons are used as starter cells, e.g., in iPSC generation (Kim et al., 2011), is to knock down Myt1l as the gatekeeper of neuronal fate to ease their conversion into other fates. Conversely, when neurons are the desired induced cell fate, including Myt1l in the reprogramming cocktail should always be beneficial.

Taken together, these observations suggest an interesting antagonistic role of REST and Myt1l as fate guardians. While the former represses neuronal genes and hence guards non-neuronal fates, the latter represses non-neuronal fates, thereby helping and possibly maintaining neuronal fate. So far, it is not yet known if REST and Myt1l cross-regulate each other, which would be important for reprogramming as well as development.

Metabolic Changes and Cell Fate Decision

While the mechanisms discussed above highlight the importance of the transcriptional machinery, recent work has shown first in vitro and then in vivo that removal of a metabolic roadblock boosts the efficiency of direct neuronal reprogramming. Overcoming cell death elicited by excessive ROS allows a single neurogenic factor to reprogram reactive glial cells to more than 90% (Gascón et al., 2016), suggesting that previous inefficiency was caused by metabolic constraints rather than inefficiency of neurogenic TFs. Indeed, the importance of a functional metabolic conversion for adequate cell fate decisions has been demonstrated, not only during differentiation of embryonic and adult somatic stem cells (Folmes et al., 2012; Masserdotti et al., 2016), but also in the process of somatic cell reprogramming (Gascón et al., 2016) and iPSC generation (Teslaa and Teitell, 2015). Thus, the metabolic state of the starter cell—both in development and in reprogramming—is important for the differentiation or conversion into another cell type.

The metabolic state of a specific cell type depends on intracellular factors, like the cell-cycle phase and specialized functions, and extracellular stimuli, such as oxygen availability and the metabolic need of the tissue in a physiological or pathological condition (Ito and Suda, 2014). All mammalian cells produce energy in the form of ATP mainly via glucose degradation, the main energy source, through a balanced activation of chemical reactions generically called glycolysis and oxidative phosphorylation (OxPhos). Glycolysis is a hallmark of proliferating cells, e.g., stem and cancer cells. Despite its lower efficiency in ATP production, the faster degradation process of glucose leads to the efficient generation of precursors for amino acid, lipids, nucleotide, and co-factors like NADPH; hence it is best suited for cells with a high demand for macromolecules (Lunt and Vander Heiden, 2011). With differentiation and acquisition of specialized function, cells can catabolize substrates more efficiently through the complete oxidation of glucose inside the mitochondria, via the TriCarboxylic Acid (TCA) cycle, with the transfer of reducing equivalents to the Electron Transport Chain (ETC) (Folmes et al., 2012). Cells mainly relying on OxPhos, like neurons, cardiomyocytes, or muscle skeletal cells (Magistretti and Allaman, 2015; Shyh-Chang et al., 2013a), display a more developed mitochondrial network and express specialized antioxidant molecules to protect them against reactive oxygen species (ROS), a byproduct of the ETC.

ROS are highly reactive reduced forms of molecular oxygen, such as the superoxide radical anion (O_2^-) and hydrogen peroxide (H_2O_2), that act as signaling molecules and are physiologically mainly produced during oxidation-reduction reactions within the mitochondrial ETC. ROS can also be harmful due to lipid peroxidation, DNA damage, and cell death induction (Ito and Suda, 2014).

Metabolic Changes in Neuronal Differentiation and Direct Neuronal Reprogramming: Comparison to iPSC Reprogramming

Cell fate conversion may require either a metabolic switch from low to high glycolysis, as is seen in reprogramming of fibroblasts to iPSCs (Folmes et al., 2011; Panopoulos et al., 2012; Varum et al., 2009, 2011), or alternatively a switch from less to more OxPhos, such as occurs in the direct reprogramming of fibroblasts or astrocytes into neurons (Magistretti and Allaman, 2015) (Figure 3). Interestingly, similarities exist between neuronal reprogramming and endogenous neurogenesis, whether occurring during embryogenesis or in the adult brain. Neural stem cells (NSCs) possess a largely glycolytic metabolism despite containing functional respiratory complexes, ready to prompt ATP production through OxPhos quickly when neurogenesis is boosted (Khacho et al., 2016). Furthermore, single-cell transcriptomic analysis of NSCs isolated from the subependymal zone (SEZ) and dentate gyrus (DG) identified a set of dormant NSCs with higher expression of glycolytic genes and lipid intermediates and a concomitant shift to OxPhos during differentiation (Llorens-Bobadilla et al., 2015; Shin et al., 2015). A similar metabolic switch, from higher glycolytic metabolism toward an increased OxPhos state, occurs in neuronal differentiation of NSCs in vivo and in vitro (Candelario et al., 2013; Khacho et al., 2016), during

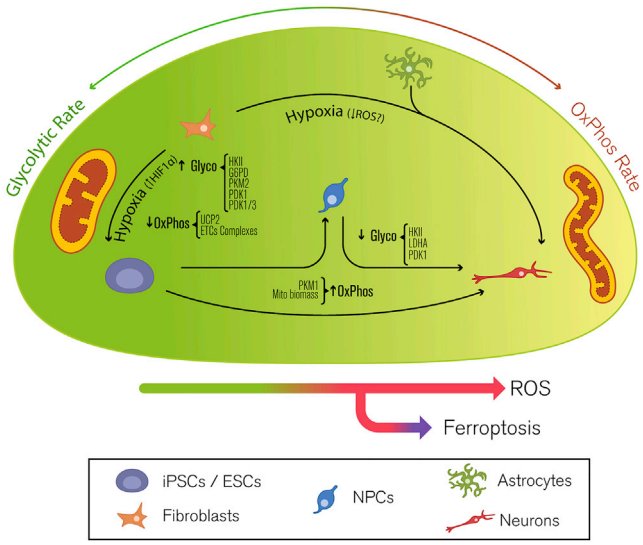


Figure 3. Metabolic Changes during Cell Reprogramming
The scheme depicts the main metabolic shifts, in terms of glycolytic versus OxPhos rate, in different paradigms of cell fate change: during normal differentiation (from NPCs to neurons), in iPSC generation, and in direct neuronal reprogramming (from astrocytes, fibroblasts, or iPSCs). Hypoxia is highlighted as a general mechanism to improve certain paradigms of cell fate conversion, while ROS increases and death by ferroptosis are general hurdles in direct neuronal reprogramming. The curly brackets specify the main metabolic changes and key players involved in these processes.

cortical neuron maturation (Agostini et al., 2016) and in iPSC differentiation (Zheng et al., 2016). These changes include the downregulation of key glycolytic enzymes (e.g., Hexokinase II [HKII], Lactate Dehydrogenase [LDHA], and Pyruvate Dehydrogenase Kinase 1 [PDK1]), alternative splicing from Pyruvate Kinase isozymes M2 (PKM2) to M1 (PKM1), and increased mitochondrial biogenesis. Moreover, enhanced OxPhos metabolism has been detected when human iPSCs differentiate into neurons (Fang et al., 2016) (see Figure 3).

The above observations would imply that forcing cells to utilize only glycolysis may interfere with neuronal differentiation. Indeed, when the inhibitor of the respiratory chain Oligomycin A was added to the culture medium, thus allowing only glycolysis, the conversion process from astrocytes to neurons was completely blocked, despite the expression of potent neurogenic reprogramming factors, such as Ascl1 or Neurog2 (Gascón et al., 2016). Interestingly, this effect was not due to selection, as there was virtually no increase in cell death, demonstrating a true block in converting to a neuronal fate when the respiratory chain is blocked. Conversely, treatment with Oligomycin A or stimulating glycolysis with D-fructose-6-phosphate (F6P) accelerated iPSC generation from MEFs, in terms of efficiency and kinetics (Panopoulos et al., 2012; Son et al., 2013), while inhibition of glycolysis with 2-deoxyglucose (2-DG) or sodium oxamate blocked iPSC conversion without affecting cell growth or viability (Folmes et al., 2011; Son et al., 2013; Zhang et al., 2011; Zhu et al., 2010).

Overall, these observations strongly suggest that changes in metabolism precede changes in cell fate in both directions, from progenitors or somatic cells into neurons (Candelario et al., 2013; Gascón et al., 2016) and from somatic cells into iPSCs (Folmes et al., 2011; Shyh-Chang et al., 2013b). Moreover, these results further highlight that the reprogramming TFs are unable to mediate fate conversion in an environment where the metabolic switch is inhibited. These data not only explain why in some conditions (low oxygen or certain metabolic states) reprogramming is not possible, but also open the door for powerful improvement of reprogramming by manipulation of the key metabolic enzymes via cell-intrinsic genetic and/or cell-extrinsic factors.

Intrinsic Regulators: Metabolic Genes as Direct Targets of Neuronal Transcription Factors

The metabolic conversion from more glia-like NSCs to differentiating neurons occurs during normal development, so transcriptional regulators of neuronal differentiation should also target genes regulating metabolic conversion.

Indeed, the neuronal TF NeuroD6 (Schwab et al., 1998) induces neuronal differentiation toward a glutamatergic fate while promoting survival through the expression of anti-apoptotic mitochondrial regulators and mitochondrial biogenesis (Baxter et al., 2012). Interestingly, NeuroD6 regulates factors of the antioxidant response, thus generating a reservoir of antioxidant molecules ready to inactivate ROS in situations of stress. This is coordinated with the expression of key mitochondrial biogenesis regulators, such as PINK1 (phosphatase and tensin homolog-induced kinase 1), PGC-1α (peroxisome-proliferator-activated receptor gamma coactivator-1α), TFAM (Mitochondrial Transcription Factor A), and SIRT1, and this explains how NeuroD6 stimulates the mitochondrial biomass and ATP level during the early stage of neuronal differentiation (Baxter et al., 2012; Uittenbogaard et al., 2010).

Thus, increasing the mitochondrial biomass and mitochondrial fission may help neuronal reprogramming. Indeed, overexpression of the mitochondrial fission factor DRP1 increases hippocampal neurogenesis in vivo (Steib et al., 2014), while its conditional deletion is detrimental for adult neurogenesis and different neuronal subtypes, due to impaired mitophagy, mitochondrial biomass distribution, and synapse formation (Berthet et al., 2014; Khacho et al., 2016; Steib et al., 2014).

The nuclear-cytosolic TF FoxG1 is another TF that directly regulates metabolism by localizing within the mitochondria. Import of FoxG1 into isolated mitochondria appears to be membrane potential dependent, and its overexpression increases mitochondrial membrane potential ($\Delta\Psi m$) and promotes mitochondrial fission and mitosis. Thus, FoxG1 acts as a major modulator of cellular and mitochondrial functions such as proliferation, differentiation, mitochondrial membrane potential, and oxygen consumption rate (Pancrazi et al., 2015) and it potently promotes neuronal reprogramming (Colasante et al., 2015).

NRF2 factor (NF-E2-related factor 2) is another TF associated with metabolic regulation and controls the antioxidant defense and NSC commitment (Bell et al., 2015; Khacho et al., 2016). Normally repressed in mature neurons, it is activated concurrently with changes in mitochondrial dynamics and a subsequent ROS increase that allows adult NSC differentiation and expression of proneuronal genes. Interestingly, NRF2 is also strongly induced by treatments aimed at increasing direct neuronal reprogramming that dampen ROS production (Gascón et al., 2016). Another interesting gene with important implications in the reprogramming field is Sox2, as it improves mitochondrial

function and reduces cell death by activating the transcription of *Bcl-XL*, at least in cancer cells (Chou et al., 2013). This is relevant given the beneficial role of Bcl-2 and related proteins in the metabolic switch during neuronal reprogramming (Gascón et al., 2016).

Thus, factors specifically potent in direct neuronal reprogramming may be the ones that also regulate metabolic aspects of fate specification, an area still rather neglected when target genes of major neurogenic factors are analyzed.

Extrinsic Regulators: Effect of Oxygen Tension and ROS Production in Neuronal Reprogramming

Experimental evidence indicates that direct neuronal reprogramming benefits from low oxygen (Davila et al., 2013). Likewise, low oxygen pressure enhances efficiency of iPSC reprogramming as well (Yoshida et al., 2009). In the latest paradigm, Hif1α transcription and downstream activation of PDK1 (Prigione et al., 2010; Zhu et al., 2010) play essential roles in promoting the glycolytic switch required for the acquisition of pluripotency (Kim et al., 2006; Papandreou et al., 2006). In the case of neuronal direct reprogramming, the activation of glycolytic pathways mediated by HIF in hypoxic conditions would play against neuronal conversion rather than promoting it. However, a hypoxic environment is not necessarily linked to a complete shut-down of OxPhos metabolism, which at 5% O_2 can still occur, but rather leads to a drastic reduction in ROS production, that can be even higher in basal O_2 conditions (21%). This effect may explain the beneficial role of low oxygen pressure in direct neuronal reprogramming, as increased ROS levels are detrimental for the conversion of many somatic cells into neurons (Gascón et al., 2016) (Figure 3). Indeed, excessive ROS levels may result from attempts in the starting cell to change metabotype and activate OxPhos at a rate that exceeds the upregulation of the protective machinery (e.g., antioxidant molecules) needed to cope with the new metabolic profile.

Studies on embryonic and adult neurogenesis may provide interesting hints on the mechanisms underlying increased ROS production. For instance, in vitro commitment of neuronal progenitors toward differentiation is associated and necessarily linked to an increase in ROS (Agostini et al., 2016), which acts as a signaling molecule rather than as a sign of oxidative damage, as also observed during development (Khacho et al., 2016). Influencing ROS levels or pushing pro- or anti-oxidative conditions can also affect the differentiation potential of cells toward different fates. Embryoid bodies derived from human iPSCs change fate according to the oxygen tension levels, with values closer to physiological condition (2%) inducing neural, vascular, and skeletal differentiation. Furthermore, lowering O_2 concentration of hESC-derived NSCs induces a shift in differentiation toward a gliogenic fate. This is driven by HIF2α stabilization, mainly acting on MYC targets such as LIN28/let-7 (Xie et al., 2014), a central mediator in metabolic reprogramming in other contexts also (Shyh-Chang et al., 2013c) that is involved in activating the glycolytic program (DeBerardinis et al., 2008), a hallmark of astrocytes in vivo (Magistretti and Allaman, 2015).

NSCs, normally prone to neurogenesis, shift to a gliogenic fate under mild non-toxic pro-oxidative conditions, directly affecting neuronal gene regulation by activating Sirt1 and inducing *Hes1* that, in turn, represses *Ascl1* targets and hence favors the glial lineage (Prozorovski et al., 2008). The Sirtuin family is sensitive to the oxidative/reduced state in the cytoplasm and acts as master regulator of stem cell differentiation, from satellite cells to NSCs (Rafalski and Brunet, 2011; Ryall et al., 2015). On the other hand, anti-oxidative conditions increase neurogenesis at the expense of gliogenesis (Prozorovski et al., 2008). This might be also due to a different susceptibility of astrocytes and neurons to oxidative conditions, with the latter being more prone to death if ROS increase in a non-controlled manner (Bell et al., 2015). The role of ROS in regulating NSC fate is thus difficult to determine, taking into account the different sources of ROS (mitochondria, cytosol, peroxisomes, and endoplasmic reticulum) and how they can affect gene expression and neuronal differentiation. Indeed, dosage, time of application, and cell-intrinsic properties are fundamental in this regard (Prozorovski et al., 2015; Rafalski and Brunet, 2011).

The influence of ROS production on the neurogenic potential of different cell types has a great impact in terms of clinical translation of direct neuronal reprogramming in vivo. Indeed, different pathological environments, such as acute traumatic injuries, stroke, or neurodegeneration such as that in Alzheimer disease (AD) or Parkinson's disease (PD), each exhibit a different degree and temporal dynamic of inflammatory cell infiltration and consequent ROS induction that all affect the neuronal conversion process and the proper in vivo integration.

In Vivo Neuronal Reprogramming: The Challenges and Opportunities

Reprogramming in vivo not only has to take place in a challenging (e.g., injured, inflamed, and highly oxidative) environment, but also in a complex mixture of many different cell types (Figure 4). Moreover, each of these cell types reacts specifically to a given set of injury conditions, thus being very different from the in vitro situation, where typically a single cell type is reprogrammed, e.g., cultured astrocytes, pericytes, fibroblasts, or hepatocytes. Furthermore, while in vitro reprogramming always occurs in a controlled environment, the surrounding cellular and molecular milieu in vivo varies with the kind of injury or neurodegenerative disease. Accordingly, we shall discuss three major aspects of in vivo reprogramming: (1) the choice of the starter cell in different areas of the CNS; (2) the influences of the neighboring cells, surrounding milieu in a damaged environment, and, therefore, the best time window for direct neuronal reprogramming after injury (Figure 4); and (3) the correct phenotype, subtype identity, and integration of iNs in vivo.

Before getting into these more specific considerations, we should consider the need or advantages of in vivo neuronal reprogramming over other approaches for neuronal repair. There are three approaches toward neuronal replacement therapies: transplantation, recruitment of neurons from neural stem cell niches, and conversion of local glial cells into neurons. Currently, the approach that is best studied and closest to clinical application is transplants of fetal progenitor cells of the neuronal subtype affected in disease (Barker et al., 2013; Goldman, 2016; Kefalopoulou et al., 2014). Recent advances in deriving these neurons also from human ESCs/iPSCs could result in excellent sources from which to replace (for example) the missing dopaminergic input to the striatum in PD. Excitingly, direct reprogramming of striatal astrocytes has recently resulted in their conversion into dopaminergic neurons reaching some

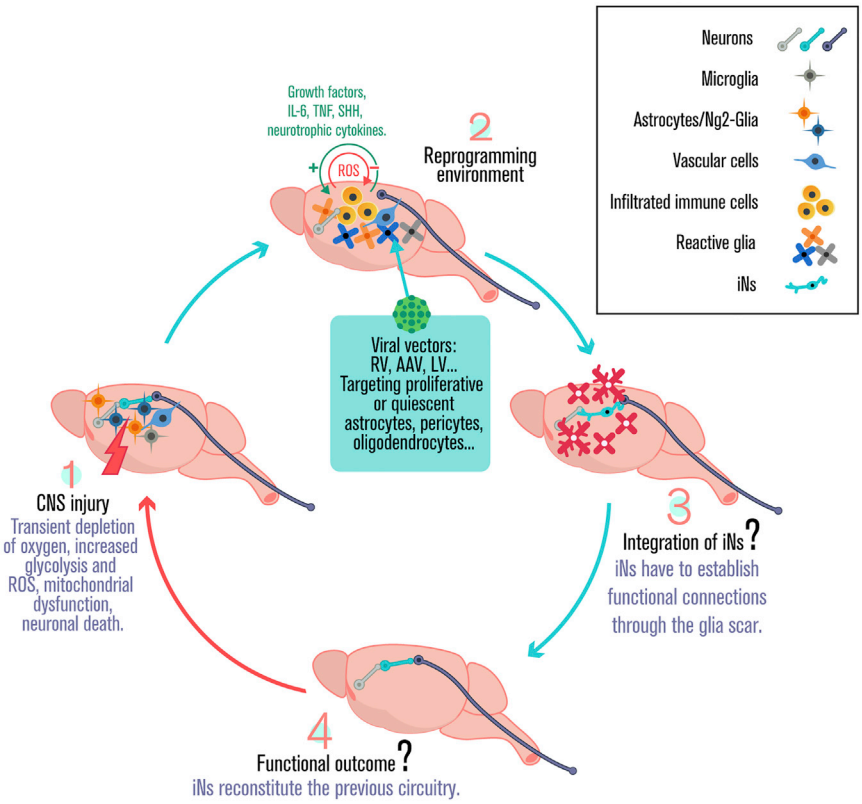


Figure 4. Effect of the In Vivo Environment in Neuronal Reprogramming

The scheme illustrates the different phases that reprogramming has to face in a damaged environment in vivo. (1) Tissue damage leads to oxygen and nutrient deprivation, increased glycolytic rate, and ROS levels. This environment is harmful for neuronal survival and reprogramming. (2) Several cell types surrounding the lesion area can be targeted and reprogrammed by viral vectors. Glia hypertrophy, proliferation, infiltration of immune cells, release of chemokines, and increased ROS can be detrimental (–) or beneficial (+) for reprogramming. (3) After reprogramming is accomplished, iNs must establish new connections and integrate into the damaged neuronal circuitry. (4) INs can improve the deficits in brain function.

degree of behavioral recovery (Rivetti di Val Cervo et al., 2017). While this is still only a starting point, these achievements further challenge the key question about the advantages of transplantation over reprogramming, given that the recruitment of neurons from NSC niches could so far not produce neurons with long-term survival. Endogenous cell conversion avoids the need for immunosuppression, required in allogeneic transplantation, and the GMP (Good Manufacturing Practice) and complex production schemes for cultured cells used for transplantation. Beyond that, reprogramming of endogenous glia into neurons offers the possibility of targeting larger cell numbers. Indeed, transplantation can hardly replace the widespread loss of cortical projection neurons in AD. Innovative approaches for targeting many glial cells with the aim to induce widespread neuronal reprogramming are just emerging, such as the use of small molecules potent in neuronal reprogramming in vitro (Hu et al., 2015; Li et al., 2015; Zhang et al., 2015) also being used for reprogramming in vivo, or the systemic injection of AAVs (Adeno-associated viral vectors) (Brulet et al., 2017; Foust et al., 2009) capable of targeting specific cell types. However, these strategies must still be scrutinized for effects on other organs. While these approaches are still in their infancy, they represent major novel strategies for fully exploiting the power of neuronal reprogramming in neuronal replacement therapies that should ultimately be achieved in a non-invasive manner. Thus, direct neuronal reprogramming could have several advantages over allogenic transplantations—if the iNs can differentiate as well and integrate as adequately as the primary fetal progenitors and their equivalents from iPSC cultures.

Macroglia and Reactive Gliosis as an Entry Point for Direct Neuronal Reprogramming

To date, in vivo neuronal reprogramming has largely focused on two sets of macroglial cells, the astrocytes and the oligodendrocyte progenitors/NG2 glia (Table 1). Given recent reviews on their role after injury (Dimou and Götz, 2014; Filous and Silver, 2016) and their suitability to being reprogramed (Torper and Götz, 2017), we shall focus on reprogramming including recent data. After injury, astrocytes resume and NG2 glia accelerate their proliferation, opening a window to their being targeted and reprogrammed by retroviral vectors, which transduce only proliferating cells. Indeed, most direct neuronal reprogramming after brain injury has used MLV (Moloney-Leukemia-Virus)-based retroviral vectors and has an induction rate of over 90% neuronal conversion of the targeted glial cells (as compared with a usual 20%) (Table 1). Neuronal identity is largely defined by immunostaining for neuron-specific proteins, such as βIII-Tubulin, MAP2, or NeuN expression (Buffo et al., 2005; Grande et al., 2013; Guo et al., 2014b; Heinrich et al., 2014) (Table 1), but also by electrophysiology (Guo et al., 2014b; Heinrich et al., 2014) and in very few studies a clear recognizable neuronal morphology of pyramidal neurons (Gascón et al., 2016). Thus, a major aim is to determine the full projection patterns of the iNs, their axonal output, their dendritic arborization, and their brain-wide synaptic input as achieved for transplanted neurons (Adler et al., 2017; Falkner et al., 2016; Tornero et al., 2017). Targeting proliferating glial cells comes with the advantage that they will not be depleted, which is particularly evident for the NG2 glia that have a strong homeostatic drive (Dimou and Götz, 2014). Moreover, depending on the timing of reprogramming, the conversion of some of the neurotoxic reactive astrocytes and/or chondroitin-sulfate proteoglycan-rich NG2 glia may be beneficial (Dimou and Götz, 2014; Liddelow et al., 2017; Robel et al., 2011) (Figure 4).

Importantly, regional diversity of glial cells must be considered in reprogramming, in regard to the ease of conversion and the generation of the correct neuronal subtype. For example, astrocytes in the murine striatum are more amenable for neuronal

reprogramming than astrocytes in the cerebral cortex (Table 1). Likewise, NG2 glia or astrocytes in the intact cerebral cortex cannot be reprogrammed by Sox2 (Heinrich et al., 2014), while they can in the striatum (Niu et al., 2015). In further support of this region-specific plasticity, deletion of the central Notch signaling mediator Rbpj allows astrocytes to activate a neurogenic program only in the striatum and in a very restricted area of the medial cortex (Magnusson et al., 2014). This also implies that astrocytes in these regions can convert into neurons virtually in absence of proliferation, as they hardly proliferate in the intact brain. AAVs or pseudotyped lentivirus could be used to target non-proliferative astrocytes or slow-proliferating NG2 glia specifically in the intact brain that are also amenable for reprogramming (Buffo et al., 2008; Torper et al., 2013, 2015). Thus, both in vitro and in vivo direct neuronal reprogramming does not require proliferation, but regional differences strongly influence the success of the process.

Brain injury provides an opportunity for reprogramming, as it makes glial cells more plastic. Indeed, reactive astrocytes show a low level of activation of NSC gene expression (Götz et al., 2015; Sirko et al., 2015), and, when cultured in vitro, they give rise to self-renewing multipotent neurospheres (Buffo et al., 2008; Sirko et al., 2009, 2013, 2015). Thus, selective targeting of these rather plastic astrocytes may greatly ease direct neuronal reprogramming. However, much needs to be learned about astroglial heterogeneity after brain injury, and new markers will need to be identified to target selectively the proliferative plastic subtypes.

The Brain Injury Environment: Opportunities and Challenges for Direct Neuronal Reprogramming

Traumatic brain or spinal cord injury and stroke induce a temporal restriction of nutrients and oxygen in a specific region of the CNS (Giri et al., 2000; Woodruff et al., 2011) (Figure 4). The reduction of oxygen tension inactivates oxidative metabolism (Singh et al., 2006) and favors glycolytic pathways (Hertz, 2008; Hovda et al., 1991). As discussed above, a transient boost of glycolysis and low oxygen pressure may facilitate a partial dedifferentiation processes, while sustained glycolytic metabolism will obstruct neuronal reprogramming.

Pro-inflammatory factors also seem to be beneficial, as NF-kB activation through TNF derived from immune cells promotes conversion of astrocytes into neuronal progenitors (Gabel et al., 2016). Likewise, the growth factors EGF and FGF2, which are typically released during inflammation (Cotman et al., 2007), exert a significant beneficial effect in reprogramming proliferating glial cells into neurons in vivo (Grande et al., 2013). Interestingly, injury and released interleukins also facilitate reprogramming in other organs (Chiche et al., 2017; Fu and Srivastava, 2015), suggesting that the inflammatory environment after injury increases cell plasticity and contributes to the breakdown of cellular barriers in direct reprogramming in vivo (Figure 4).

The above considerations raise the important point about the extent to which this increase in plasticity is also present in conditions of chronic damage and, if there is none, when this window of opportunity is closed. Activation of immune cells, neuronal loss, and monocyte infiltration can last for months or years after injury (Holmin and Mathiesen, 1999; Loane et al., 2014). TNF-α, interleukins, and pro-inflammatory pathways are activated in the injured parenchyma after acute damage (Holmin and Mathiesen, 1999) or in chronic diseases such as AD, PD, and others (Berjaoui et al., 2015; Hemmer et al., 2004; Morales et al., 2010; Stolp and Dziegielewska, 2009). However, there are differences in the inflammatory responses activated during acute and chronic damage, which, together with other factors (e.g., the age of the subject when the damage occurs, the type of injury, or the time window when the reprogramming protocols are applied), may have consequences on the outcome of direct reprogramming (Cherry et al., 2014; Kumar et al., 2013). For example, a rather neurotoxic environment characterized by increased ROS production has been observed at longer times after traumatic acute injury or in chronic injury conditions (Hu et al., 2012; Kumar et al., 2013; Wang et al., 2013). This may act against neuronal reprogramming and survival of young iNs (Figure 4). Importantly, besides microglia and immune cells, astrocytes also exhibit different behavior in acute and chronic inflammation, the latter more associated with neurodegeneration than the former (Zamanian et al., 2012). However, so far, few studies have rigorously compared neuronal reprogramming in different injured environments (Guo et al., 2014b; Heinrich et al., 2014; Su et al., 2014) (Table 1). Direct neuronal reprogramming is rather effective in a mouse model of amyloidosis (Guo et al., 2014b), proving its feasibility in this special disease environment. However, the precise effects of specific immune-modulators (Grimmig et al., 2016) have not been yet examined in direct reprogramming either in vitro nor in vivo.

While the injury condition may activate beneficial growth and immune-modulatory factors, it also poses a metabolic challenge. The temporal reduction of oxygen in stroke results in the inactivation of antioxidant defenses (Ying et al., 1999) and gives rise to mitochondrial depolarization due to energy deprivation (Abramov et al., 2007). Subsequently, the restoration of oxygen levels and mitochondrial respiratory chain function raise ROS production (Chen et al., 2008) and induce a very oxidative environment with deleterious consequences for neurons. This is a serious hurdle for direct neuronal reprogramming, as it interferes with the acquisition of a neuronal oxidative metabolism and leads to cell death by ferroptosis (Gascón et al., 2016). ROS also interferes with other pathways and molecules that are required for the differentiation of neurons, such as unsaturated fatty acids (Kang and Gleason, 2013), that are particularly susceptible to peroxidation by O_2^- (Butterfield et al., 2002). Overall, the effect of ROS in the injured brain may explain why many reprogramming factors that efficiently drive neuronal conversion in vitro (Heinrich et al., 2010; Vierbuchen et al., 2010) exhibit a poor effect, if any, when they are applied to models of reprogramming after acute brain injury in vivo (Gascón et al., 2016; Grande et al., 2013; Torper et al., 2013). Indeed, the low efficiency of conversion mediated by Neurog2 in a murine model of acute brain injury could be increased up to ~90% by adding survival and antioxidant molecules to the reprogramming cocktail in vivo (Gascón et al., 2016). While this is a breakthrough in efficiency at this time point after a larger injury, future strategies are needed to replace the application of Bcl-2, which could possibly occur via newly developed ferroptosis inhibitors.

All the above considerations highlight the importance of the timing of reprogramming after injury. For example, excessive ROS levels may be avoided with a longer wait after the insult. As summarized in Table 1, most in vivo reprogramming has been done either immediately (Grande et al., 2013; Guo et al.,

2014b) or 3 days after the insult (Buffo et al., 2005, 2008; Gascón et al., 2016; Heinrich et al., 2014). As the injury was minimal in the approach of Guo et al., 2014b, we would conclude that, in a larger TBI (traumatic brain injury) or stroke injury, high conversion efficiency of reprogramming requires survival and antioxidant treatments (Buffo et al., 2008; Gascón et al., 2016; Grande et al., 2013; Heinrich et al., 2014). In contrast to the fast and strong expression from these retroviral vectors, AAV-mediated expression of the reprogramming factors has a rather slow onset and kinetics, reaching levels sufficient for reprogramming typically 7–10 days after transduction (Torper et al., 2015). This implies that conversion takes place when the major injury reaction, including monocyte invasion and microglia and macroglia proliferation, has largely vanished. Thus, the reduced inflammatory environment at later stages (Figure 2) may contribute to the efficient reprogramming rate by these viral vectors (Rivetti di Val Cervo et al., 2017; Torper et al., 2015).

Another important aspect is the long-term survival of reprogrammed neurons in order to assess the conversion stability and the integration in the pre-existing circuitry. Indeed, long-term survival of iNs for more than 4 weeks in an injured environment has been only observed when Neurog2 was combined with the expression of the pro-survival protein Bcl-2 and treatment with antioxidants (Gascón et al., 2016) (see Table 1). Likewise, iNs survived up to 8 weeks in a hemisected spinal cord injured environment, helped by inhibition of the p53 pro-apoptotic pathway and treatment with BDNF and Noggin, factors known to ameliorate ROS effects and promote neuronal survival and maturation (Hachem et al., 2015; Wang et al., 2016). Thus, the use of antioxidants and pro-survival factors potently boosts in vivo reprogramming and survival of the resulting neurons (Figures 2 and 4), at least when conversion occurs in the ROS-rich environment shortly after injury (Table 1).

Induction of Neuronal Subtypes In Vivo

While neuronal reprogramming has by now reached high efficiency of conversion and shown the mature nature and long-term survival of the iNs (at least in some cases), the identification of the exact neuronal subtype induced is still in its infancy. As shown in Table 1, iNs are mostly characterized by a few pan-neuronal markers. In some studies neurotransmitter phenotypes, such as GABA and glutamate, have been examined (Niu et al., 2013, 2015; Wang et al., 2016), showing a range of neuronal identities to be induced. Only few studies have so far reported the generation of a specific neuronal subtype, e.g., the induction of Ctip2+ deep layer pyramidal neurons after stab wound injury in the cerebral cortex (Gascón et al., 2016), or the induction of dopaminergic neurons in the striatum capable of improving some aspects of the motor deficits elicited by ablation of midbrain dopaminergic neurons (Rivetti di Val Cervo et al., 2017). While it is not yet known whether this is due to the correct integration of the iNs (for tracing of the input connectome of iNs, see Torper et al., 2015), this study shows that iNs can be produced in sufficient number and quality to affect behavior, a crucial advance for the entire field of neuronal reprogramming.

Outlook

While remarkable achievements have been made in direct neuronal reprogramming in vitro and in vivo, reaching high efficiency and decent maturity of the iNs, still very little is known about the mechanisms underpinning fate conversion in vitro and in vivo or the integration and neuronal subtype identity of the iNs. It is thus important to apply the gold standard of the reprogramming field also to the neuronal conversion process—namely comparing the full genome-wide expression pattern of iNs with those of the endogenous neurons. Likewise, the brain-wide input connectome needs to be determined for the iNs, as missing or aberrant connections may lead to aberrant function. The input connectome of striatal iNs has been determined in a pioneering study, revealing so far exclusively local input (Torper et al., 2015). This highlights the fact that the analysis of integration and function of the iNs in vivo may indeed reveal further obstacles that cannot be identified in vitro. Therefore, a better mechanistic understanding of the in vivo reprogramming is required to identify and overcome potential deficiencies of the iNs in vivo. This implies also a more systematic evaluation of the best time of conversion (earlier or later after the injury) and the adequacy of the connectivity formed in these different paradigms may help to identify the best window of opportunity. Moreover, a thorough molecular understanding of the reprogramming process will help to utilize new tools, such as precise epigenetic engineering, to target hurdles of the conversion process in the chromatin. In this regard, however, many of the more general hurdles unraveled in vitro have not yet been tackled in vivo, such as blocking p53, deleting CAF1, etc.

Likewise, an important advance in our understanding of reprogramming is single-cell analysis, as it reveals and allows tackling subpopulations of cells resistant to reprogramming (Treutlein et al., 2016). More of such studies are needed both in vitro and ultimately also in vivo. Likewise, live imaging following single cells through the conversion process has been an eye opener in vitro and has led to the discovery of death by ferroptosis and its solution, reaching 90% conversion efficiency in vivo (Gascón et al., 2016). As the in vivo conversion process takes place within many different cell types, live imaging of the in vivo conversion process may again provide unprecedented insights in beneficial and adverse interactions with other cell types—e.g., attack by macrophages or microglia. Last but not least, it will be exciting to follow the road of non-invasive approaches, moving direct neuronal reprogramming toward more feasible therapeutic approaches. Given the sensational achievements within the last decade of neuronal reprogramming, thrilling times lay ahead for bringing these approaches to neural circuit analysis, epigenetic engineering, and pharmacology closer toward achieving neuronal repair from endogenous cells in the brain.

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