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Review

Direct neuronal reprogramming: Fast forward from new concepts toward therapeutic approaches

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SUMMARY

Differentiated cells have long been considered fixed in their identity. However, about 20 years ago, the first direct conversion of glial cells into neurons *in vitro* opened the field of "direct neuronal reprogramming." Since then, neuronal reprogramming has achieved the generation of fully functional, mature neurons with remarkable efficiency, even in diseased brain environments. Beyond their clinical implications, these discoveries provided basic insights into crucial mechanisms underlying conversion of specific cell types into neurons and maintenance of neuronal identity. Here we discuss such principles, including the importance of the starter cell for shaping the outcome of neuronal reprogramming. We further highlight technical concerns for *in vivo* reprogramming and propose a code of conduct to avoid artifacts and pitfalls. We end by pointing out next challenges for development of less invasive cell replacement therapies for humans.

INTRODUCTION

All cells of our body have the same genes, but different cell types, such as neurons, liver cells, or pancreas cells, differ by the expression levels of distinct subsets of genes. This difference is achieved during development, at the end of which cell identity had traditionally been considered fixed and irreversible. This dogma was particularly strong for cells resident in the brain, where complex information processing was often taken as justification for tissue stability and, hence, as an argument against adult neurogenesis. By now, all of these concepts have been considerably revised. Adult neurogenesis occurs in almost all species, and cells (even in the adult brain) can be forced to convert into each other with apparent amazing ease. However, some of the miraculous fate conversions observed *in vivo* have also been challenged recently as artifacts of using viral vectors that infect many neurons. It is therefore essential to review the details of reprogramming tools, especially *in vivo*, to allow distinguishing reliable from questionable data. We provide guidance and develop a code of conduct to avoid such technical flaws in the future. Therefore, let us consider how it all started and where we are now with reliable reprogramming protocols. (Please see Box 1 for a glossary of terms referred to throughout this review.)

The discovery that a glial cell type, the radial glial cell, acts as a neural stem cell (NSC) and generates neurons in the developing murine forebrain (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001) jump-started the idea that even differentiated glial cells, such as postnatal and adult astrocytes, might be instructed to generate neurons by re-expressing neurogenic transcription factors (TFs) active in radial glial cells but downregulated during differentiation (Bertrand et al., 2002). Indeed, the re-expression of the transcription factor (TF) PAX6 was sufficient to instruct a neuronal fate in postnatal astrocytes *in vitro* (Heins et al., 2002). This finding was further supported by live imaging analysis, which revealed the morphological changes occurring during the conversion process *in vitro* (Gascón et al., 2016; Heinrich et al., 2010; Karow et al., 2012). Direct conversion was corroborated by a wealth of other reprogramming paradigms *in vitro*, such as those for generating macrophages (Xie et al., 2004), β-cells of pancreas islets (Zhou et al., 2008), and cardiac muscles (Ieda et al., 2010), highlighting the power of single or few TFs to effectively convert one differentiated cell into another. The ultimate potency of such TF-induced reprogramming has been shown by generation of induced pluripotent stem cells (iPSCs [Takahashi and Yamanaka, 2006]). This breakthrough discovery helped to overcome hesitations to attempt the conversion of cells originating from different germ layers, which usually have particularly different gene expression profiles. Germ layers are groups of cells sufficient to generate all cells of certain tissues during development (e.g., ectoderm cells differentiating into all skin and nervous system tissue; mesoderm cells differentiating into all muscle, bone, and blood cells). In 2010, mesoderm-derived mouse embryonic fibroblasts (MEFs) were reprogrammed into neurons by a cocktail of TFs (Vierbuchen et al., 2010), followed by a wave of protocols converting fibroblasts into many cell types *in vitro* (Caiazzo et al., 2015; Huang et al., 2014; Ieda et al., 2010; Kim et al., 2014; Lujan et al., 2012; Yu et al., 2013). The accessibility of fibroblasts, even from humans, opened a novel path to disease

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Box 1. Glossary

CELL TYPES

IPSCs: Induced pluripotent stem cells. Pluripotent stem cells are generated from differentiated cells—typically, fibroblasts—by the overexpression of pluripotency transcription factors, originally using Oct4, Sox2, Klf4, and c-myc.
MEF: Mouse embryonic fibroblasts.
mESC: Mouse embryonic stem cell.

DEFINITIONS

Forward programming: Differentiation of stem cells (either IPSCs or mESCs) through the forced expression of a transcription factor.
Induced neuronal cells: Reprogrammed cells that show morphological and transcriptional similarities with neurons.
Induced neurons (iNeurons; iNs): Induced neuronal cells that show electrophysiological properties similar to neurons.
Pan-neurogenic transcription factors: A subset of transcription factors induced in different cellular contexts during direct neuronal conversion. They include transcription factors such as *Hes6*, *Sox4/11*, and *NeuroD1/4*.
Pan-neuronal identity: Identity characterized by features common to most neurons irrespective of their subtype identity. Such features include the presence of 2 or more processes 3 times longer than soma and the expression of universal neuronal markers (e.g., Map2 and β3-tubulin).
Pioneer factor: A transcription factor that engages silent, unmarked chromatin to initiate transcriptional programs that lead to cell fate change.
"On-target" pioneer factor: A transcription factor that engages silent chromatin characterized by a specific epigenetic landscape to initiate transcriptional programs that lead to cell fate change.
Proneural gene/factor: bHLH transcription factor sufficient to promote neurogenesis in progenitor cells.
Starter cell: The cell type in which reprogramming is started.
Transcription factor: A protein capable of binding specific sequences on the DNA, usually promoters or enhancers, and sufficient to recruit the transcriptional machinery to induce the transcription of a gene into RNA.

EPIGENETIC MODIFICATION TERMS

Epigenetic mark: A chemical modification on a nucleotide or amino acid that results in change of function. Such modification can occur on the DNA or on the tails of histones, the proteins that constitute the nucleosome around which the DNA is wrapped.
Poised (bivalent) chromatin: The simultaneous presence of histone modifications associated with both gene activation and repression.
H3K4me1: Mono-methylation of the lysine 4 of the histone 3. Associated with a poised chromatin.
H3K27Ac: Acetylation of the lysine 27 in the histone 3. Associated with an active state.
H3K9me3: Tri-methylation of the lysine 9 of the histone 3. Associated with a repressive state and heterochromatin formation.

TECHNIQUES

ChIP-seq: Chromatin Immuno-Precipitation followed by sequencing. It aims at identifying the genomic sites (or loci) bound by a specific protein, typically a transcription factor.
Rabies virus tracing: A viral-based technique that allows the identification of the direct presynaptic partner of neurons. It requires 2 viruses: the first one infects the cells to investigate (the "starter cell") and allows the expression of an avian receptor (TVA), which confers infection capability to pseudotypes rabies virus, the glycoprotein (G), which is required for the rabies virus to retrograde spread, and a fluorescent protein. The second virus is the glycoprotein-defective (ΔG) rabies, engineered to express a second fluorescent protein. Neurons co-infected by both viruses express both fluorescent proteins—thus allowing identification of the starter cell—while the presynaptic partner is identified by the expression of the fluorescent protein encoded by the rabies virus.
RNA-seq: RNA sequencing. A technique aimed at revealing the presence and amount of specific RNAs in biological samples and thereby monitoring which genes have been transcribed. The most used methods capture mainly PolyA mRNAs and allow the detection of the transcripts' expression in a given sample.
scRNA-seq: Single-cell RNA sequencing. A technique that allows the identification and quantification of RNA molecules in single, isolated cells.

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Box 1. *Continued*

MAIN SMALL MOLECULES

CHIR99032: GSK-3 inhibitor.
DAPT: Notch pathway inhibitor.
Forskolin: Activates adenylyl cyclase and increases the intracellular levels of cAMP.
I-BET151: Inhibitors of BET family proteins.
ISX9: Activator of Ca^{2+} signaling.
Y-27632: ROCK inhibitor.
LDN193189: BMP inhibitor.
SAG: Sonic hedgehog agonist.
SB431542: TGFβ inhibitor.

modeling by using direct conversion of human fibroblasts into neurons. This approach has advantages and disadvantages compared to iPSC-derived cells for modeling diseases that will be discussed in the last section of this review. Before looking at the progress in direct neuronal reprogramming *in vitro* and *in vivo*, we turn to important conceptual questions and principles raised by the advent of cell type conversion methods that will guide us through the review.

QUESTIONS AND EMERGING CONCEPTS

Given the wealth of conversion protocols, the most obvious question is whether there are common rules and mechanisms or whether each conversion process differs. For example, why are certain TFs more effective than others? Are repressors of alternative fates rather universal or specific for each lineage? Are there common facilitators and common hurdles? On the other hand, are there mechanisms of fate stabilization and maintenance? If differentiated cells can be so readily converted into each other, which mechanisms stabilize and safeguard cell identity to avoid, for example, glial cells in the brain to spontaneously convert into neurons or even into muscle cells (Masserdotti and Götz, 2020)?

Equally relevant is the outcome of direct reprogramming: how similar is the newly established identity (e.g., the neuronal identity) to the same cell type normally generated in development? Are there traces of the original identity (e.g., astrocyte, fibroblast, or pericyte) left, and would they be deleterious for a neuron's function? Alternatively, do newly generated neurons function just as well as endogenous counterparts despite maintaining traits of their corresponding starter cells? This crucial issue has only recently been addressed using patch-seq technology (e.g., recording from a neuron converted from a glial cell and then collecting its RNA for sequencing) with surprising results. Another very relevant issue for the nervous system is the huge number of different neuronal subtypes in our brain. How can one generate the "perfect" neuronal subtype most similar to the endogenous one? Do we have as many neuronal subtypes as suggested by RNA sequencing (RNA-seq) or are there fewer subtypes that differ in gene expression depending on the brain state or functional context? Thus, direct neuronal reprogramming taps into the big question of neuronal subtype diversity and its recent challenges, as seen by the limited alignment of differences in scRNA-seq with physiology and connectivity (Scala et al., 2021).

The major advantage of direct reprogramming for repair of the central nervous system (CNS; brain and spinal cord) is the use of endogenous cells (e.g., scar-forming or neurotoxic glial cells) as a source for cell-based replacement of neurons lost upon an insult or in neurodegeneration. To do so, it is important to know which cell types are the most toxic and adverse so that their conversion into new neurons may also alleviate their detrimental role. However, these cells may not be the easiest to convert and may retain parts of the gene expression of their original identity and their active state, corrupting neuronal function. These considerations highlight the need to understand the influence of the starting cell type and actual status on the induced neuron. Initially, *in vitro* and *in vivo* reprogramming targeted proliferating glia as supposedly the easiest cell types to convert into neurons (Buffo et al., 2005; Heins et al., 2002). However, other cell types may be even easier to convert. For instance, astrocytes retain their regional identity, as discussed below, and this might make them most suited to obtain the correct neuronal subtype identity, which is also region specific. Thus, generating the most accurate neuronal subtypes may outweigh reducing scarring, which may be achieved by other means.

Given these essential questions and the progress in the expanding field of direct neuronal reprogramming, rather than mentioning all the literature in this review we first focus on the principles regulating direct conversion of different cell types into neurons *in vitro* and *in vivo* (e.g., including the role of starter cell identity and the existence of common hurdles and ways to overcome them). Then we further consider the special challenge of how to correctly instruct the plethora of different neuronal subtypes, moving from findings *in vitro* toward *in vivo* applications in the adult CNS, and finally discuss human cell reprogramming for medical needs.

DIRECT NEURONAL REPROGRAMMING *IN VITRO*: INSIGHTS INTO PRINCIPLES AND MOLECULAR MECHANISMS

Key TFs for neuronal reprogramming

As mentioned above, even a single TF is sufficient to turn a glial cell into a neuron. What makes this factor so special, and how can it achieve conversion of cell identity? Typically, the TFs used in reprogramming have been selected because of their

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known essential contribution during normal neurogenesis in development (Masserdotti et al., 2016). For instance, PAX6, the first TF reported to instruct neurons from glia *in vitro* (Heins et al., 2002), was chosen because it acts as a master regulator in organ development and forebrain neurogenesis (Bishop et al., 2000; Halder et al., 1995; Heins et al., 2002). The TF NEUROG2 was then chosen because it is downstream of PAX6 and promotes neurogenesis in development (Casarosa et al., 1999; Domínguez and Campuzano, 1993; Fode et al., 2000; Jiménez and Campos-Ortega, 1990; Ma et al., 1996). NEUROG2 is even more potent than PAX6 in inducing neurons from glia *in vitro* (Berninger et al., 2007; Heinrich et al., 2010). ASCL1 resembles NEUROG2 in its pro-neurogenic function but is expressed in neural progenitors in other brain regions and promotes neurogenesis of different neuronal subtypes (Casarosa et al., 1999; Parras et al., 2002). Indeed, the forced expression of ASCL1 or NEUROG2 converts different cell types (e.g., astrocytes, fibroblasts, and pericytes) rather efficiently into functional neurons *in vitro* (Chanda et al., 2014; Heinrich et al., 2010; Karow et al., 2012). Similarly, NEUROD1 (Guo et al., 2014b), a downstream effector of NEUROG2 (Fode et al., 2000), was selected because of its role in survival and maturation of newborn neurons (Gao et al., 2009; Hevner et al., 2006). Intriguingly, one of these TFs or their upstream regulators are included into almost all direct neuronal reprogramming protocols, highlighting their potency in cell conversion. This observation prompts the question why these TFs are so powerful and whether common features exist that make these factors so crucial in direct reprogramming.

Proneural factors as pioneer factors in direct neuronal reprogramming

Because a specific set of expressed genes defines a cell identity, changing one cell into another requires changes in gene expression. Genes specifically expressed in neurons need to be activated, and those expressed specifically in the starter cells (e.g., astrocytes or fibroblasts) have to be shut down. This task may not be as easy as it sounds because genes that are not expressed in neurons are often shut off (.e.g., silenced by chromatin marks) and wrapped around the nucleosome, whereas the DNA that is transcribed is "open" between nucleosomes. Most TFs cannot access this rather "inaccessible" DNA; however, some factors can bind DNA in closed conformation and start activating transcription. These special TFs are referred to as "pioneer" TFs because they "engage silent, unmarked chromatin to initiate transcriptional programs that lead to cell fate change" (Morris, 2016). Pioneer TFs are mobile in the nucleus and interact transiently with chromatin (Lerner et al., 2020), and recent *in vitro* studies (reviewed by Zaret, 2020) unraveled several ways in which pioneer factors engage nucleosomes and scan DNA to ultimately activate transcription at previously silenced sites.

Indeed, most of the TFs mentioned above for neuronal reprogramming, such as ASCL1, NEUROG2, and NEUROD1, have pioneering activity. For example, in MEFs, ASCL1 engages mostly nucleosome-bound DNA sites, characterized by a specific combination of histone modifications, the trivalent histone marks H3K4me1, H3K27ac, and H3K9me3 (Wapinski et al., 2013). When H3K9me3 is removed in MEFs via expression of the histone demethylase JmjD2d, ASCL1-instructed reprogramming is abolished almost completely (Wapinski et al., 2013). In line with this finding, ASCL1 fails to reprogram human dermal fibroblasts, which lack the trivalent chromatin state in ASCL1 targets (Wapinski et al., 2013). Interestingly, when expressed in mouse embryonic stem cells (mESCs), ASCL1 induces fast transcriptional changes, also favoring the addition of the epigenetic histone activator mark H3K27ac to many bound sites (Aydin et al., 2019). Remarkably, most ASCL1-bound sites in mESCs differ from ASCL1-bound sites in fibroblasts (Aydin et al., 2019), suggesting that ASCL1 binding is directed to distinct sites in the genome because of distinct epigenetic landscapes, such as chromatin marks, in different starter cells (fibroblasts versus mESCs). Thus, pioneer TFs are directed to distinct sites depending on the chromatin environment. However, in both cell types, ASCL1 causes an increase in DNA accessibility for transcription, which also allows downstream effectors (e.g., POU3F2/BRN2) to bind and activate transcription of neuron-specific genes to further sustain neuronal differentiation (Aydin et al., 2019; Wapinski et al., 2017). ASCL1 not only instructs a neuronal program, but it also seems to pattern the chromatin to facilitate binding of other TFs and, therefore, stabilizethe neuronal identity (Aydin et al., 2019).

The pioneer activity of NEUROG2 was first shown in MRC-5, a human fetal fibroblast cell line (Smith et al., 2016). However, NEUROG2-mediated chromatin accessibility is improved significantly by treatment with the small molecules forskolin, a plant-derived agonist of adenylyl cyclase (Liu et al., 2013), and dorsomorphin, an inhibitor of bone morphogenic protein (BMP) signaling (Liu et al., 2013), suggesting that other mechanisms contribute to its pioneering activity. Indeed, in cells expressing NEUROG2 and treated with such small molecules, a significant number of newly opened chromatin sites contains either cyclic AMP (cAMP) response element (CRE) half-site or high-mobility group (HMG) box motifs (Smith et al., 2016). The latter is of interest in light of the ability of the HMG box factor SOX2 to allow neuronal reprogramming of human pericytes (Karow et al., 2018; Karow et al., 2012). The combination of RNA-seq and chromatin immunoprecipitation sequencing (ChIP-seq) data revealed cooperative binding of NEUROG2 and CRE binding protein (CREB) to induce the TF *SOX4*, which, in turn, synergizes with NEUROG2 to activate expression of neurogenic genes. Indeed, *SOX4* knockdown in fibroblasts transduced with NEUROG2 and treated with small molecules nearly abolishes reprogramming (Smith et al., 2016). This observation supports the concept that *Sox4* (or *Sox11*) is required for neurogenesis (Mu et al., 2012; Ninkovic et al., 2013) and that its co-expression improves NEUROG2-mediated conversion of astrocytes into neurons (Masserdotti et al., 2015; Mu et al., 2012). Furthermore, small-molecule treatment enhances H3K27ac at sites targeted by both NEUROG2 and SOX4, contributing to increased chromatin accessibility (Smith et al., 2016). Interestingly, increased accessibility to silent DNA and addition of H3K27ac histone mark has also been found in mESCs expressing NEUROG2 (Aydin et al., 2019), indicating common mechanisms underlying NEUROG2-mediated differentiation in different cell types. These data support NEUROG2 as a pioneer factor, although its strength depends on the starter cell and may require additional

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factors (small molecules or other TFs) to induce a robust neuronal program.

NEUROD1 has been employed successfully to convert mouse glia into neurons *in vitro* and *in vivo* (Guo et al., 2014b). Its pioneer activity was first proven in forward programming experiments of mESCs (Pataskar et al., 2016). Forced expression of NEUROD1 causes the loss of the repressive histone mark H3K27me3 and the acquisition of the activating histone mark H3K27Ac, promoting transcription at many target sites, including *NeuroD1*, *Hes6*, and *Dll3* promoters, which are important neurogenic genes. In addition, NEUROD1 displaces the transcriptional repressor TBX3 from many NEUROD1-targeted promoters, allowing NEUROD1 to active its targets. Likewise, NEUROD1 displaces the methyl-CpG-binding domain protein MDB3 at specific enhancer regions, allowing their expression (Pataskar et al., 2016). In microglia, it has been shown recently that NEUROD1 binds closed chromatin regions characterized by the histone marks H3K4me3 and H3K27me3 (active and repressive marks, respectively [Matsuda et al., 2019]). Notably, these reprogramming effects have been recently called into question (Rao et al., 2021), which may be in part explained by different expression levels (Matsuda and Nakashima, 2021).

The above TFs, commonly used in direct neuronal reprogramming, show pioneer activity, which is a common mechanism in reprogramming. Without opening closed chromatin, cell fates cannot be converted. Although the power of these single TFs has come as a big surprise, they often fail to active some genes that are silenced by specific DNA and chromatin modifications (Lerner et al., 2020). Thus, it is crucial to determine not only the function but also the gene expression of the starter cell and the outcome of reprogramming to identify so-called "off-memory" genes and determine how to activate them (Hörmanseder et al., 2017). Moreover, the genes to which the pioneer TFs ultimately bind and activate are highly cell type and TF specific. It is therefore important to systematically compare their binding and their ability to regulate gene expression in different cellular contexts to disentangle general from cell-specific mechanisms and, thus, understand the principles and general rules of how these TFs function in reprogramming.

Finally, these considerations also give an inkling about how cell fate is stabilized; namely, by stringently shutting off TFs that specify alternative fates. Intriguingly, CRISPRa (clustered regularly interspaced short palindromic repeats a activator) has revealed that various genes can readily be activated by the dCas9 activator protein and a suitable guide RNA, whereas fate determinants, such as Sox1, cannot be activated as readily because they are stringently silenced, for instance by DNA methylation (Baumann et al., 2019). This implies that they are less likely to be spontaneously upregulated (e.g., in aging or by an insult), and, hence, there is no spontaneous conversion of glia into neurons. However, after brain injury, this strict silencing is partially eroded, and, for instance, the neurogenic TF PAX6 is upregulated (Götz et al., 2015). However, these levels are much lower than, e.g., in neurogenic NSCs (Götz et al., 2015) and not sufficient for endogenous conversion. If, however, additional brakes are released (e.g., deletion of Notch signaling), then conversion into immature neurons can be achieved (Magnusson et al., 2014).

The role of protein domains and post-translational mechanisms involved in direct neuronal reprogramming

In addition to regulation of gene expression at the chromatin level, the transcriptional activity of these TFs is also regulated at the protein level. All of the abovementioned TFs belong to the basic-helix-loop-helix (bHLH) TF family, characterized by 2 α helices (HLH), connected via a loop region, and a basic domain. The HLH domain is required for dimerization, either with itself, referred to as homodimerization (Li et al., 2012), or with other proteins (heterodimerization), such as the ubiquitously expressed E proteins (E2A, E2.2, and HEB, encoded by the *Tcf3*, *Tcf4*, and *Tcf12* genes [Sun and Baltimore, 1991]). Interestingly, the dimerization partner has important effects on the neurogenic activity of the bHLH TFs. For instance, E47 enhances ASCL1-mediated neurogenesis but reduces NEUROG2-induced neurogenesis in chick embryos (Le Dréau et al., 2018). Conversely, NEUROG2 homodimers are more neurogenic than ASCL1 homodimers and NEUROG2-E47 heterodimers (Le Dréau et al., 2018; Li et al., 2012). Remarkably, during embryonic neurogenesis, cell cycle-associated kinases phosphorylate NEUROG2 and ASCL1, leading to phosphorylation-dependent degradation (Ali et al., 2011, 2014; Hindley et al., 2012). However, phosphorylation facilitates NEUROG2-E47 heterodimer formation, which stabilizes NEUROG2 but makes it less neurogenic (Le Dréau et al., 2018; Li et al., 2012). When the cell cycle is prolonged, NEUROG2 is less phosphorylated, favoring its homodimerization and, indeed, preferential activation of neurogenic target genes (Ali et al., 2011, 2014; Hindley et al., 2012). Likewise, a version of the ASCL1 resistant to phosphorylation (so-called phosphomutant) shows increased neurogenic activity (Ali et al., 2014) and improves neuronal reprogramming (Rao et al., 2021). However, the mechanisms triggered by the phosphomutant factors in reprogramming have not yet been explored. It is not known whether they bind and activate expression of more neurogenic target genes and/or speed up the cell conversion process by better shutdown of the starter cell-specific gene expression.

How is the DNA binding ability of these TFs determined, and is it sufficient for their pioneering and fate conversion function? Close to the HLH domain, the basic domain of proneural factors fits in the major groove of the DNA (Bertrand et al., 2002) and is necessary for DNA binding (Bertrand et al., 2002; Dennis et al., 2019). Although the DNA binding sequence (called "E-box motif") is overall conserved (CANNTG), each TF preferentially binds a specific version of this motif, characterized by different nucleotides at its core and in the DNA sequences adjacent to the motif (Bertrand et al., 2002). Indeed, swapping bHLH domains between TFs with distinct E-box specificity results in changes in target gene activation, as shown for NEUROD2/MYOD (Fong et al., 2015) and ASCL1/NEUROG2 swaps (Aydin et al., 2019). However, when both bHLH proteins preferentially bind the same E-box (as is the case for ASCL1 and MYOD), some non-specific targets are induced (Lee et al., 2020; Treutlein et al., 2016). Indeed, to make the muscle master regulator MYOD (Tapscott et al., 1988) "neurogenic," the basic domain and the transactivator domain (TAD) at the C terminus had to be replaced with the corresponding domains of ASCL1. Chimeric proteins containing the Nterminus and HLH domain of MYOD, the basic and TAD domain of ASCL1 can reprogram MEFs into functional

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neurons (Lee et al., 2020). These observations strongly suggest that TFs require not only specific DNA-binding activity, but also interact with mostly still unknown co-factors that convey the specificity to transactivate downstream targets and, hence, to instruct a particular cell fate. Thus, protein interaction is an important determinant of TF function in neuronal reprogramming, as shown for hIPSCs (Di Stefano et al., 2016).

Unlocking neurogenesis: Repressing the repressors

Although the above factors act mainly as activators, transcriptional repressors exist, preventing the induction of neurogenic cascades. For instance, the RE1 silencing TF (REST, also known as NRSF) complex represses neuronal genes in non-neuronal cells (Schoenherr and Anderson, 1995). Accordingly, neurons cannot develop well when REST is expressed constitutively, leading to severe developmental defects *in vivo* (Paquette et al., 2000). Moreover, REST can bind and repress several microRNAs (miRNAs), including miR-124 and miR-9/9* (Ha and Kim, 2014). Indeed, both miRNAs are important for neurogenesis (Cheng et al., 2009; De Pietri Tonelli et al., 2008) because they regulate an inhibitory feedback loop on the REST repressor complex. In fact, miR-124-miR-9/9* reduce the expression of small C-terminal domain phosphatase 1 (SCP1 [Visvanathan et al., 2007]), which is recruited by REST onto neuronal genes (Yeo et al., 2005). In addition, miR-124 represses the expression of the polypyrimidine tract-binding protein 1 (*PTBP1* [Makeyev et al., 2007]), an RNA-binding protein regulating the splicing of alternative exons, including those of neuronal genes (Min et al., 1995). When *PTBP1* levels are reduced, *PTBP2* (*NPTBP*) is upregulated, which controls the splicing of mRNAs in newly born neurons (Makeyev et al., 2007; (Figure 1).

Accordingly, manipulation of several members of the miR-124-REST-PTBP feedback loop directly affects neuronal reprogramming, either in combination with other factors (e.g., REST knockdown [Drouin-Ouellet et al., 2017; Masserdotti et al., 2015; Nolbrant et al., 2020]) or alone (miR-124, miR-9/9*, or *Ptbp1* knockdown [Xue et al., 2013; Yoo et al., 2011]) *in vitro* and *in vivo* (see below). Despite being in the same feedback loop, each member triggers different mechanisms. Although REST competes with reprogramming factors for binding to downstream targets (Masserdotti et al., 2015), PTBP1 modulates the expression of components of the REST complex (*Lsd1*, *Corest*, *Hdac1*, and *Scp1* [Xue et al., 2013]). Moreover, PTBP1 regulates miRNA-mediated gene expression because the expression of many genes downregulated by PTBP1 is no longer reduced upon inactivation of AGO2 (Xue et al., 2013), a protein involved in small interfering RNA-mediated silencing (Ha and Kim, 2014). Remarkably, miR-9/9* and miR-124 have a broad effect on direct reprogramming of human adult fibroblasts. They repress *PTBP1* and downregulate *USP14*, a de-ubiquitinating enzyme (Wang and Wang, 2021) that normally degrades EZH2 (Lee et al., 2018), the enzymatic component of polycomb repressor complex 2 (PRC2 [Piunti and Shilatifard, 2021]). PRC2 cannot methylate any more REST, which is then degraded and, thus, cannot repress neuronal gene expression. This cascade strongly enhances direct conversion of human fibroblasts into neurons (Lee et al., 2018). Recent work showed that miR-124 and miR-9/9* first downregulate many genes responsible for the starter cell (e.g., fibroblast identity, day 5 of reprogramming) and only later (days 10–20) induce the expression of neuronal genes (Cates et al., 2021). Among the genes downregulated in fibroblasts are KLF4 and KLF5, whose 3′ UTR is targeted by miR-9/9* and miR-124 and whose binding sites are enriched at sites closed by miR-9/9* and miR-124 (Cates et al., 2021). Induction of the neuronal genes depends on the expression of a brain-enriched small nuclear RNA, RN7SK. Interestingly, among the neuronal genes upregulated by miR-124 is *PTBP2*, but only in the presence of ELAVL3, a neural specific RNA binding protein (Lu et al., 2021). Therefore, miR-124 triggers reprogramming by repressing components of the repressive complexes and inducing neuronal genes (Figure 1).

Another emerging common principle in reprogramming is the powerful role of repressive mechanisms to silence the starter cell fate and instruct neuronal identity in non-neural cells, resulting in efficient reprogramming from different cell types and species.

Suppressing non-neuronal fates

As mentioned above, an important step during reprogramming is to repress the starter cell identity. Could repression be easier in some cells than in others? Are some cells more stable and others more "vulnerable" or "plastic" in their identity? This prompts the question of whether there are mechanisms repressing the identity of the starter cells and mechanisms capable to inhibit all alternative fates or specific repressors of distinct lineages in different donor cell types (e.g., a repressor for fibroblast fate, another repressor for glial fate, etc.). A definitive answer to these questions is still missing, but accumulating evidence suggests the existence of pan-repressors of alternative fates. For example, the first combination of factors used to reprogram fibroblasts into neurons comprised ASCL1, BRN2, and the transcriptional repressor MYT1L (Vierbuchen et al., 2010; Yee and Yu, 1998). MYT1L improves neuronal maturation by repressing non-neuronal genes (e.g., genes expressed specifically in fibroblasts), Notch-related genes (e.g., *Hes1*), effectors of proliferation (e.g., Jak/Stat and Shh), and regulators of several non-neuronal processes (Mall et al., 2017; Vierbuchen et al., 2010). Members of the myelin TF family are induced during *in vitro* differentiation of NPCs and embryonic neurogenesis in the subventricular zone (SVZ), largely overlapping with the neuronal marker β3-tubulin (Vasconcelos et al., 2016). Accordingly, *Myt1l* knockdown during embryonic neurogenesis leads to defects in neuronal differentiation (Mall et al., 2017). The MYT1L DNA binding motif is depleted substantially from neuronal gene promoters but present in the promoters of many genes related to non-neuronal fates. Therefore, MYT1L acts as a repressor for "all lineages other than neurons" (Mall et al., 2017), supporting the concept that "all but" repressors may exist for each lineage, such as repressors of all non-fibroblast genes or all non-B cell genes. Alternatively, MYT1L may be a special case, and neurons may require specific safeguards for their identity. Stay tuned—the jury is still out on this one.

Hurdles in direct reprogramming: Proliferation and metabolic shift

Live imaging analysis of *in vitro* astrocyte-to-neuron reprogramming led to the identification of several hurdles during this

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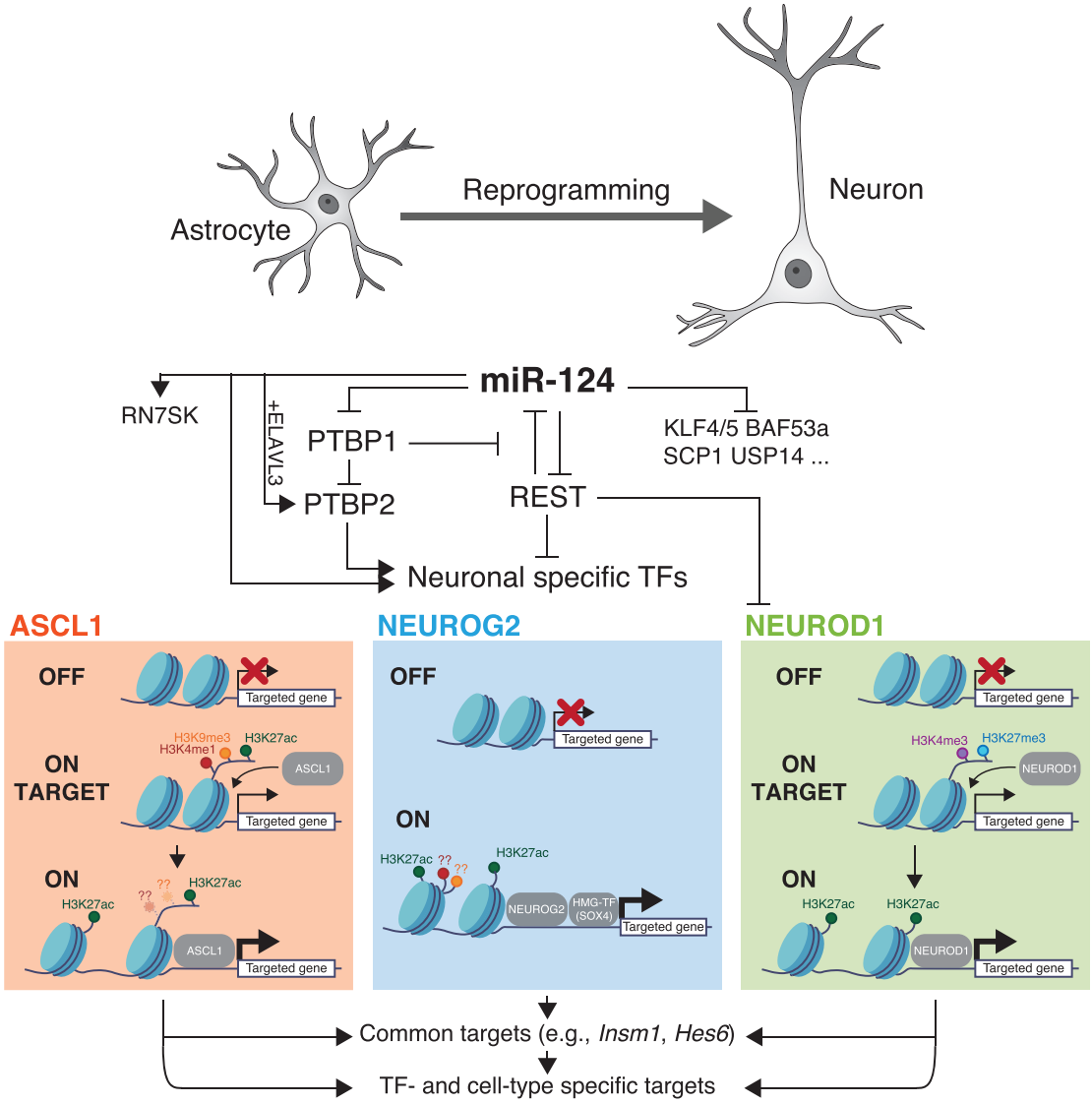

Figure 1. Molecular mechanisms underlying direct neuronal reprogramming

Shown is a schematic of the neurogenic factors and fate repressors that play a crucial role in direct *in vitro* neuronal reprogramming, defining a hierarchical trajectory of feedback loops and converging on regulation of pioneer factors (e.g., ASCL1, NEUROG2, and NEUROD1).

A unique trivalent chromatin signature (H3K4me1, H3K9me3, and H3K27ac) in the starter cell predicts the permissiveness for ASCL1 to bind promoter regions and initiate chromatin remodeling/opening. Binding of ASCL1 enhances H3K27 acetylation.

A similar increase in H3K27 acetylation is observed after NEUROG2 and NEUROD1 overexpression. In the latter case, NEUROD1 causes also loss of the repressive histone marker H3K27me3 at target sites.

process (Gascón et al., 2016; Heinrich et al., 2010). First, it showed that cells undergoing conversion into neurons do not proliferate or quickly stop proliferating, probably because of early induction of anti-proliferation genes (e.g., *Cdkn1c*) by the neurogenic reprogramming TF (e.g., ASCL1 [Kempf et al., 2021; Masserdotti et al., 2015]). Indeed, proliferation seems to impair conversion into neurons, which are postmitotic cells (Jiang et al., 2015; Liu et al., 2013); accordingly, inhibiting proliferation (e.g., via Jak2) improves fibroblast-to-neuron reprogramming (Herdy et al., 2019). Conversely, proliferation promotes reprogramming into a highly proliferative cell type, namely, iPSCs (Guo et al., 2014a; Li and Rana, 2012), possibly because of partial erasure of epigenetic marks during proliferation. Thus, the effect of proliferative status of the starter cells on reprogramming seems to depend on the nature of the induced fate: if this is a postmitotic cell, then proliferation may be a hurdle rather than a benefit.

A second bottleneck in astrocyte-to-neuron reprogramming discovered by live imaging is cell death (Gascón et al., 2016). This phenomenon is to a large extent due to the increased production of reactive oxygen species (ROS) during the conversion process (Gascón et al., 2016), which elevates lipid peroxidation and causes mostly the converting neurons to die by ferroptosis (Gascón et al., 2016), a ROS-dependent form of regulated cell

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death (Jiang et al., 2021). Expression of anti-apoptotic genes (e.g., *Bcl2* or *BclXl*), which also prevent ferroptosis (Gascón et al., 2016), or pharmacological treatments aimed at reducing cell death potently improve the conversion rate *in vitro* and *in vivo* (Gascón et al., 2016; Victor et al., 2014).

Mitochondria are key players in cell metabolism and differ profoundly between glia and neurons (Fecher et al., 2019; Russo et al., 2021), especially in the ability to control ROS levels and, hence, ferroptosis (Gao et al., 2019). This observation prompted the analysis of the mitochondrial proteome of astrocytes and neurons, unveiling a 20% difference and, therefore, the need of a major change of the mitochondrial proteome during neuronal reprogramming (Russo et al., 2021). However, the downregulation of astrocyte-enriched mitochondrial proteins and the upregulation of neuron-enriched mitochondrial proteins occur late in astrocyte-to-neuron conversion, days after the major wave of death (Gascón et al., 2016; Russo et al., 2021). Remarkably, the early induction of neuron-enriched mitochondrial protein-coding genes by CRISPRa improves the speed and efficiency of direct reprogramming (Russo et al., 2021). In line with this observation, blocking the oxidative phosphorylation inhibitor HIF1α boosts fibroblast-to-neuron conversion efficiency (Herdy et al., 2019). These data highlight the key role of mitochondrial proteome conversion in direct neuronal reprogramming, a concept applicable to reprogramming into various cell fates that differ in their metabolism. Importantly, this concept may also apply to other organelles that need to change according to the new identity. Thus, the emerging principle is that universal organelles also need to adapt their protein composition according to the new fate, and the closer the proliferation state of the starting cell is to the reprogramming outcome the better.

Small molecules as facilitators or inducers of direct reprogramming

The high variability of reprogramming efficiency and the possibility of partial or incomplete reprogramming are major obstacles for its use in cell-based therapies (see subsection How to utilize direct neuronal reprogramming with human cells: From disease modeling to neuron replacement therapies). BMP signaling is an obstacle common to many reprogramming paradigms; its inhibition (e.g., via noggin or dorsomorphin, as mentioned earlier) improves the neuronal conversion of human fibroblasts, pericytes, and mouse astrocytes (Ambasudhan et al., 2011; Karow et al., 2018; Kempf et al., 2021; Ladewig et al., 2012; Liu et al., 2013). BMP inhibitors are often combined with other small molecules, such as the abovementioned forskolin, which activates cAMP synthesis, or CHIR99021, a GSK-3β inhibitor that improves neuronal differentiation (Chambers et al., 2009), and highlight a general role of these signaling pathways in favoring non-neuronal fates. Thus, there are common signaling pathways that act as hurdles to conversion into neuronal fates. These are likely different for fate conversion into other cell types (e.g., muscles etc.) and, again, are reminiscent of development when BMP signaling often inhibits neurogenesis (Gross et al., 1996).

The use of small molecules to improve the conversion and survival rate raised the question of whether a purely chemically based protocol could be established to generate neurons. Sequential screening of small molecules led to identification of a cocktail of four compounds (forskolin, ISX9, CHIR99021, and I-BET151) sufficient to reprogram MEFs into functional neurons with very high efficiency (Li et al., 2015). Among them, ISX9 is essential to induce the expression of *NeuroD1* and *Neurog2* but not *Ascl1*, which might explain the high proportion of induced glutamatergic neurons. The activation of the neurogenic program might occur most likely via Ca^{2+}-mediated derepression of MEF2, a consequence of increased nuclear export of the histone deacetylase HDAC5, as shown previously in hippocampal NSCs from adult rats (Schneider et al., 2008). Complementary to the induction of the neuronal program, I-BET151 leads to the suppression of the fibroblast program, probably via inhibition of BRD4, a member of the BET family important for the maintenance of cell-specific gene expression (Wu et al., 2015). More recently, a similar cocktail of small molecules (forskolin, ISX9, CHIR99021, I-BET151, and Y-27632) has been used to reprogram astrocytes into neurons *in vitro* and *in vivo* (cortex and striatum), resulting in neurons that express region-specific genes *in vivo* (Ma et al., 2021).

A combination of small molecules could also reprogram human astrocytes of fetal origin (from the cortex or midbrain but not spinal cord) into neurons (Zhang et al., 2020). Sequential treatment with various compounds (DAPT, SB431542, LDN193189, and SAG) triggers activation of *NEUROG1/2*, *ASCL1*, and *NEUROD1* at early stages (days 4–6 after the initial treatment). Such induction is probably the result of epigenetic reorganization at neurogenic loci (e.g., an increase in H2K27me3 at the *NEUROG2* promoter) and at glial promoters (e.g., increased methylation of the *GFAP* promoter). Interestingly, this cocktail shares few compounds with the abovementioned combinations, and it is not sufficient to reprogram mouse astrocytes, suggesting the need to manipulate different signaling pathways in different species to obtain a similar effect.

Overall, use of small molecules is a promising approach to greatly improve, or even induce on its own, direct conversion of cells into neurons. However, more evidence is needed to support the observation that the sole use of small molecules is sufficient to induce neurons. It will be important to fully understand the molecular mechanisms modulated by each molecule and whether a synergism exists among the molecules. Along these lines, it remains uncertain whether a full neuronal identity is established just by small molecules and whether remnants of the original identity may be maintained, so-called on-memory expression (Hörmanseder et al., 2017), as reported in neuronal reprograming of hepatocytes (Marro et al., 2011) and spinal cord-derived astrocytes (Kempf et al., 2021). Finally, so far, only glutamatergic and GABAergic neurons have been reported to be induced by small molecules, highlighting the need to search further to induce other neuronal subtype identities. Indeed, generation of specific neuronal subtypes remains the main challenge in the neuronal reprogramming field.

Neuronal subtype specification in direct reprogramming

The brain consists of many distinct neuronal subtypes whose properties (specific input and output connections, firing properties, etc.) are crucial for information processing. Thus, reprogramming into neurons faces the fascinating challenge to

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generate the desired and correct neuronal subtype fitting in the pre-existing circuit (Figure 2). Therefore, a major aim is to identify the factors sufficient for generating specific neuronal subtypes, a topic of great interest, especially for cell-based replacement therapies and disease modeling (see subsection How to utilize direct neuronal reprogramming with human cells: From disease modeling to neuron replacement therapies).

Single factors

As mentioned above, NEUROG2 and ASCL1 play essential roles in neurogenesis, specifying different neurons in distinct brain regions. In the telencephalon, the most rostral part of the forebrain, NEUROG2 specifies glutamatergic projection neurons, whereas ASCL1 generates GABAergic inhibitory neurons (Casarosa et al., 1999; Fode et al., 2000). Accordingly, NEUROG2 reprograms astrocytes from the postnatal cerebral cortex (the most prominent region in the dorsal telencephalon) into glutamatergic neurons, whereas ASCL1 induces GABAergic neurons (Heinrich et al., 2010; Hu et al., 2019; Masserdotti et al., 2015). Interestingly, NEUROG2 and ASCL1 induce very different programs in cortical astrocytes, with only a small subset of genes commonly regulated by both TFs (Masserdotti et al., 2015). Even in undifferentiated cells (mESCs), ASCL1 and NEUROG2 induce partially different neuronal cascades for specific neuronal subtype differentiation (Aydin et al., 2019). However, this is not always the case. For instance, the identity of neurons induced by the forced expression of ASCL1 or NEUROG2 is very similar in spinal-cord-derived astrocytes, as discussed below (Kempf et al., 2021). In contrast to its main role as a GABAergic fate determinant, ASCL1 induces mainly glutamatergic neurons from MEFs (Chanda et al., 2014), as it does in human fibroblasts together with NEUROG2 (Herdy et al., 2019; Ladewig et al., 2012; Mertens et al., 2021). These data suggest that the cellular context (e.g., the epigenetic, transcriptional, and proteomic status of the starter cells) influences the ability of TFs to induce specific neuronal programs (Influence of the starter cell on direct reprogramming).

As mentioned above, NEUROD1 is another TF sufficient to generate functional neurons *in vitro* and *in vivo* from different starter cells (Chen et al., 2020; Guo et al., 2014a; Matsuda et al., 2019). In most reprogramming contexts, it induces mainly a glutamatergic identity but also GABAergic neurons, in line with its role in neurogenesis (Boutin et al., 2010; Roybon et al., 2009). Importantly, many different subtypes of glutamatergic and GABAergic neurons exist, and their specification requires additional factors in reprogramming. Moreover, some other factors, like miR-124-miR-9/9*, as discussed above, are not sufficient to specify a certain neuronal subtype (e.g., GABAergic or glutamatergic), but they rather induce a pan-neuronal identity from fibroblasts without a subtype specification (Cates et al., 2021). Together with additional factors, they can then achieve reprogramming into neurons of a precise subtype identity (Cates et al., 2021; Victor et al., 2014). Therefore, the use of several factors is essential to adequately specify neuronal subtypes adequately.

Cocktails

Several cocktails of factors have been reported to generate different neuronal subtypes, including dopaminergic, motor, medium spiny, and peripheral nervous system sensory neurons from fibroblasts and non-fibroblasts (reviewed in Colasante et al., 2019; Kim et al., 2021; Masserdotti et al., 2016). Rather than listing all the successful combinations, here we focus on the principles that guide the selection of candidates, such as their temporal expression and specific activity during embryonic neurogenesis. For instance, *Lmx1a*, *Nurr1*, *FoxA2*, and *Pitx3* and miR-218 are expressed sequentially in mesencephalic progenitors and regulate the commitment, differentiation, maturation, and survival of dopaminergic neurons (Arenas et al., 2015). Combinations of these factors induce dopaminergic neurons from mouse and human non-neuronal cells (Addis et al., 2011; Caiazzo et al., 2011; Pfisterer et al., 2011; Rivetti di Val Cervo et al., 2017). Likewise, the co-expression of *Lhx3*, *Isl1*, and *Hb9*, which are essential for spinal cord motor neuron development (Cho et al., 2014; Lee et al., 2009; Thaler et al., 2002), induces motor neurons when expressed together with ASCL1, NEUROG2, or miR-124-miR-9/9* in fibroblasts (Cates et al., 2021; Liu et al., 2016; Son et al., 2011).

An intriguing hallmark to consider for reprogramming is the timing of fate determinant activity. During embryonic neurogenesis, TFs essential for specification of certain cell types are often expressed earlier than factors involved in neuronal maturation. Interestingly, TFs can also elicit such cascades in direct reprogramming. For instance, NEUROG2 first activates TBR2 (Eomes), a glutamatergic progenitor TF, followed by the neuronal differentiation TF TBR1 (Berninger et al., 2007; Heinrich et al., 2010). However, in direct reprogramming, several TFs are often expressed at the same time (e.g., driven by a constitutive promoter or doxycycline-inducible promoter). This may not be advantageous because sequentially expressed factors often cross-repress each other to specify the neuronal subtype and promote neuronal maturation. On the other hand, expressing TFs together could also lead to cooperative effects and speed up the conversion process. Although this possibility has not been investigated systematically, some aspects could be highlighted. For instance, ISL1/LHX3 induce late target gene expression in fibroblast-to-neuron reprogramming and do not contribute to the initial neuronal program or erase the fibroblast identity (Cates et al., 2021). Thus, neuronal subtype specification factors may first require accessibility to their target sites during reprogramming because they are often not endowed with pioneer activity, and then act to specify neuronal subtype identity. Further evidence comes from direct reprogramming of fibroblasts into -GABAergic neurons. Transient expression of FOXG1 and SOX2, usually expressed in progenitors, instructs a forebrain identity and improves the morphology of ASCLI1-DLX5-LHX6-induced neurons (Colasante et al., 2015). Overall, these data suggest that a tightly controlled transcriptional hierarchy exists and that this cannot be easily overcome by forcing simultaneous expression of multiple TFs. Therefore, timely regulated expression of TFs mimicking the developmental sequence would be beneficial in direct reprogramming.

Predictive network-based tools to identify combination of genes for direct reprogramming

Despite the increasing number of reported cocktails of factors, only a fraction of neurons present in the nervous system can so far be generated via direct conversion. For most neurons,

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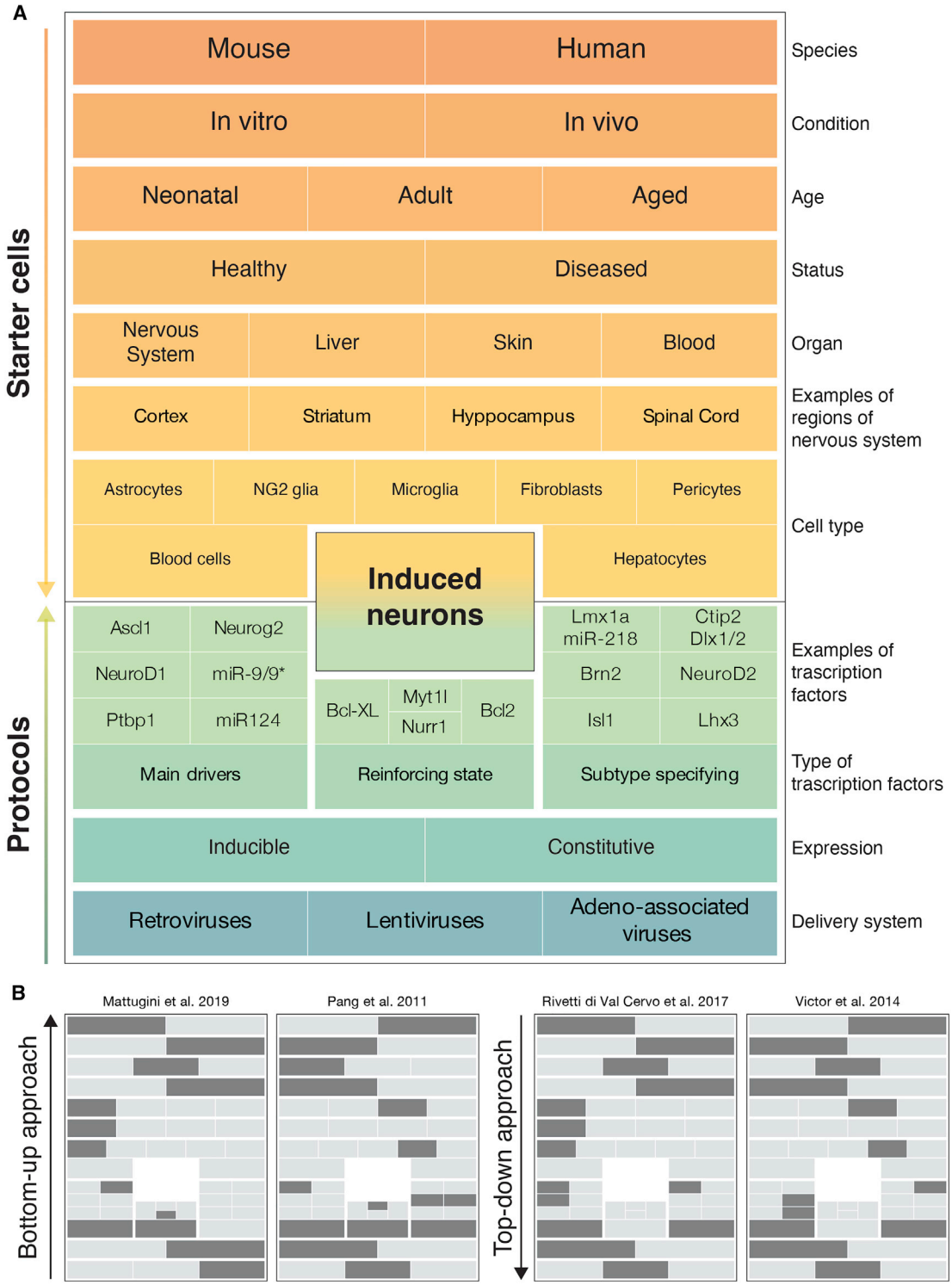

Figure 2. Key point for designing a direct neuronal reprogramming protocol

(A) The table summarizes the main aspects that need to be considered for a reprogramming protocol. The approach could be bottom-up (e.g., to investigate the mechanisms of direct reprogramming in a given starter cell) or top-down (e.g., to generate a specific neuronal subtype irrespective of the starter population).

(B) Examples of paradigms used in experiments mentioned in the review, *in vitro* and *in vivo*.

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no combination has yet been identified, likely because of insufficient information about their developmental trajectories and the theoretically infinite possibilities to combine TFs. Indeed, more rational approaches have been pursued, and algorithms have been developed to identify key players for cell identity and predict combinations of factors that could convert one cell type into another one (e.g., CellNet, Mogrify, and Epimogrify, signaling perturbation; Cahan et al., 2014; Kamaraj et al., 2020; Rackham et al., 2016; Zaffaroni et al., 2019). The advent of scRNA-seq and the generation of transcriptional profiles of thousands of adult neurons (La Manno et al., 2016; Macosko et al., 2015; Tasic et al., 2016, 2018; Zeisel et al., 2018) and their developmental trajectories (Di Bella et al., 2021; La Manno et al., 2021) are invaluable sources of data to decipher the transcriptional master regulators of specific neuronal identities and generate new algorithms capable of integrating this information to generate more precise combinations of TFs that could then be tested experimentally.

Influence of the starter cell on direct reprogramming

As mentioned previously, distinct brain regions contain different neuronal subtypes. In development, these subtypes derive from neural stem and progenitor cells, the radial glial cells, which correspondingly differ in different brain regions (Taverna et al., 2014). Astrocytes are the only glial population that seemingly inherits this patterning information. Contrary to other glial cells (oligodendrocytes and microglia), astrocytes, especially some subtypes, possess strong regional identity (Batiuk et al., 2020; Ben Haim and Rowitch, 2017; Boisvert et al., 2018; Denis-Donini and Estenoz, 1988; Emsley and Macklis, 2006; Itoh et al., 2018; Ohlig et al., 2021). Remarkably, individual reprogramming TFs convert astrocytes from different brain regions into different types of neurons *in vitro* and *in vivo* (Hu et al., 2015; Mattugini et al., 2019). Astrocytes cultured from distinct brain regions have long been known to differ in supporting distinct neuronal morphology (Denis-Donini and Estenoz, 1988). Significant transcriptional differences can be detected in astrocytes acutely isolated from the postnatal cerebral cortex versus the spinal cord, and these differences also persist *in vitro* (Kempf et al., 2021) and *in vivo* (Batiuk et al., 2020; Ben Haim and Rowitch, 2017; Boisvert et al., 2018; Denis-Donini and Estenoz, 1988; Emsley and Macklis, 2006; Itoh et al., 2018; Ohlig et al., 2021). About 2%–4% of all genes expressed by astrocytes differ between regions (for adult astrocytes see Ben Haim and Rowitch, 2017; Morel et al., 2017; Ohlig et al., 2021)) and include TFs important for patterning the brain in development, such as *Emx1*, *Emx2*, *Otx2* (enriched in cortex astrocytes), and *Hox* genes (expressed specifically in spinal cord astrocytes). Their expression persists in induced neurons, as identified by scRNA-seq of neurons reprogrammed from spinal cord-derived astrocytes by ASCL1 or NEUROG2 *in vitro* (Kempf et al., 2021). Accordingly, the forced expression of NEUROG2 or ASCL1 in spinal cord-derived astrocytes instructs transcriptional programs shared with spinal cord neurons; namely, V2 interneurons (Kempf et al., 2021). Indeed, both TFs are involved in the development of this interneuron subtype in the spinal cord (Misra et al., 2014; Parras et al., 2002) but also in other neuronal subtypes in the spinal cord (Lu et al., 2015), highlighting the need to better understand the factors present in each starter cell that could contribute to instruct the cells toward specific identities. However, it is important to note that neither NEUROG2 nor ASCL1 is sufficient to elicit the full V2 identity from spinal cord-derived astrocytes, as will be reviewed below in the chapter assessing the quality of the neurons. Generally it is very clear that the outcome of reprogramming differs when starting from astrocytes of different brain regions, as shown not only for the spinal cord, but also the cerebellum (Chouchane et al., 2017; Hu et al., 2019) and cortical gray matter (Heinrich et al., 2010) *in vitro*. Thus, a general emerging concept is that regionalization of astrocytes is a potent contributor to the regional identity of the induced neuron.

Despite the abovementioned differences, factors commonly induced in various cellular context have also been identified. For example, *NeuroD4* is a necessary downstream effector of NEUROG2 and ASCL1 in cortical gray matter-derived (Masserdotti et al., 2015) and midbrain-derived (Rao et al., 2021) astrocytes and can hence be considered a key ''pan-neurogenic'' factor independent of the neuronal subtype identity. Consistent with this observation, its expression could generate functional neurons (Masserdotti et al., 2015) and partially replace ASCL1 in midbrain-derived astrocyte direct neuronal conversion (Rao et al., 2021). Indeed, the comparison with published datasets on ASCL1-mediated reprogramming of other cell types (MEFs [Wapinski et al., 2013]; cortical astrocytes [Masserdotti et al., 2015]) highlights the existence of a subset of genes induced by ASCL1 in different cellular contexts besides the induced pool of cell context-dependent genes (Rao et al., 2021). Thus, region-specific and pan-cellular targets and functions of reprogramming TFs need to be better understood to improve the choice of adequate factors specifying correct neuronal subtype identity. Ultimately, however, key aspects of neuronal subtype identity can only be assessed *in vivo*, such as correct targeting of their axonal connections. This brings us to the achievements in reprogramming *in vivo*.

IN VIVO ACHIEVEMENTS: GLIA-TO-NEURON REPROGRAMMING

Testing *in vivo* direct reprogramming protocols allows exploration of the influence of the diseased environment on this process as well as connectivity and integration into preexisting circuits, as already done for transplanted neurons (Falkner et al., 2016). Stroke, traumatic brain injury, and neurodegenerative disease conditions generate altered and distinct environments with different facets of reactive gliosis and inflammation that cannot be reproduced *in vitro*. Thus, analysis of *in vivo* reprogramming is necessary to achieve the ultimate goal of efficiently replacing lost neurons from endogenous non-neuronal cells and restoring brain function as well as understanding the mechanisms underlying this process *in vivo*.

Efficiency of induced neurons in the CNS

Targeting proliferating cells: Use of retroviruses

The first attempts to convert glia into neurons in a murine brain employed retroviral vectors (RVs) for gene delivery because of their capacity to integrate their genome only in diving cells.

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Nearly two decades have passed since the first pioneering evidence of *in vivo* conversion of proliferating glial cells into doublecortin (DCX)-positive neuroblasts upon a stab wound (SW) injury (Buffo et al., 2005). This was achieved by RV-mediated forced expression of the abovementioned neurogenic TF PAX6 combined with suppression of the gliogenic TF OLIG2, targeting proliferating reactive glia in the adult mouse neocortex (Table 1). These lesions and injections of viral vectors were at coordinates distant from the endogenous NSC niches to ensure absence of migration of NSCs or their neuroblast derivates to the injury site (Buffo et al., 2008; Sirko et al., 2013). Indeed, inducing a larger lesion or moving it more rostrally faces the issue of NSC-derived contamination (Benner et al., 2013; Faiz et al., 2015).

It is interesting to note that there are TFs, such as SOX2, sufficient to convert some reactive glia to neurons *in vivo* but not in postnatal astroglia *in vitro*. RV-mediated expression of the stem cell TF SOX2 could convert about a third of transduced cortical NG2 glia after a SW injury into DCX^+ neuroblasts in the cortex (Heinrich et al., 2014) or spinal cord (Su et al., 2014; Tai et al., 2021; Table 1). A small fraction of induced cortical neuroblasts matured into $NeuN^+$ functional neurons, showed action potentials, and made synaptic connections with endogenous neurons, as shown by detection of synaptic potentials. This result may be due to some neurogenic factors that are upregulated after injury, as discussed above, and SOX2 can then help to convert these cells into neurons, as demonstrated *in vitro* for the cooperative role of SOX2 and ASCL1 in pericyte conversion (Karow et al., 2012, 2018). Moreover, *in vitro* data suggested that the identity of the targeted cell could also play an important role during the process of *in vivo* reprogramming. This was corroborated *in vivo* by RV-mediated expression of NEUROD1 in proliferating NG2 glia, oligodendrocyte progenitor cells, or astrocytes using *NG2* or *GFAP* promoter elements, respectively (see Table 1 and discussion of the specificity of promoter element-driven viral vectors below). The former induced glutamatergic and GABAergic neurons, whereas expression of the same factor in proliferating astrocytes after cortex injury gave rise only to glutamatergic neurons (Guo et al., 2014b). NEUROD1-induced neurons showed spontaneous and evoked synaptic responses, suggesting that they were integrated into the pre-existing circuitry. Other TFs mentioned above as being efficient *in vitro*, such as NEUROG2 and ASCL1, were also able to convert proliferating cells in the injured cortex and striatum into $NeuN^+$ neurons (Gascón et al., 2016; Grande et al., 2013), albeit rather inefficiently. Thus, *in vivo* direct neuronal conversion of adult glia seemed, in most cases, with the exception of SOX2, to be less efficient than *in vitro* reprogramming of postnatal glia. This observation is consistent with *in vitro* data showing that only a few days of further maturation of astrocytes significantly reduces the efficiency to convert them into neurons with ASCL1 or NEUROG2 (Masserdotti et al., 2015; Price et al., 2014).

After the initial results with single-factor reprogramming, various approaches were pursued to improve efficiency using combinatorial treatments. For example, combining RV-mediated expression of NEUROG2 with local exposure to growth factors (fibroblast growth factor 2 [FGF2] and epidermal growth factor [EGF]) improved the number and efficiency of induced neurons (Grande et al., 2013). Co-expressing NEUROG2 with the pro-survival factor BCL2 and applying antioxidative treatments substantially increased the conversion efficiency, reaching more than 90% $NeuN^+$ neurons among the transduced proliferating reactive glia (Gascón et al., 2016; Figure 3). Intriguingly, co-expression of BCL2 and antioxidant treatment sped up conversion from immature DCX^+ to mature $NeuN^+$ neurons with pyramidal morphology that acquired neuronal subtype identity *in vivo*, consistent with *in vitro* treatments of antioxidants (Gascón et al., 2016), and promoted expression of neuron-specific mitochondrial antioxidant proteins (Russo et al., 2021). Given the success of these treatments, other viral vector approaches were pursued to explore conversion of non-proliferating glial cells, aiming for safer gene delivery methods.

Targeting non-proliferating cells: use of lentiviruses

Because in the adult mouse brain only a minority of cells divide, even after injury, several studies have exploited the capacity of lentiviruses (LVs) to transduce postmitotic cells to increase the number of targeted cells and, therefore, the number of induced neurons. Moreover, pseudotyped LVs allow targeting of specific cell types, such as astrocytes (Buffo et al., 2008; Watson et al., 2002). LVs carrying specific coat proteins on their surface dock to specific cell surface receptors. For example, LVs carrying Mokola or LCMV coat proteins specifically infect different glial cells in the adult murine cerebral cortex (Buffo et al., 2008). If such specificity is not required, then typically vesicular-stomatitis virus G protein (VSV-G) is used because it conveys high stability of the viral vectors and, hence, high titers (Kato and Kobayashi, 2020). Because VSV-G protein causes viral envelope-to-cell membrane fusion, it targets all cells. For RVs, this feature is less of a problem because they need nuclear membrane breakdown during cell division to integrate their DNA, as mentioned above. For LVs, however, this ability of membrane fusion causes a problem for neuronal reprogramming because they can transport their retrotranscribed genome through the nuclear pores and, hence, mediate expression in postmitotic cells, including neurons. In this case, specific expression may be achieved using promoter elements, such as the *GFAP* promoter to direct, e.g., SOX2 to reprogram some resident astrocytes into DCX^+ neuroblasts in the mouse striatum (Niu et al., 2013) and in the injured spinal cord (Su et al., 2014; Table 1). Because such promoter elements may not be entirely cell-type-specific and even be regulated via the neurogenic factors in *cis* (Wang et al., 2021), it is important to include additional controls, as listed in Table 1 and discussed below.

Using LVs to express SOX2 via the *hGFAP* promoter, the authors found an intermediate $ASCL1^+$ progenitor state and proliferation of the induced neuroblasts to be arguing against mistargeting of postmitotic neurons (Niu et al., 2015). Notably, these neuroblasts eventually matured into synapse-forming neurons *in vivo*, even though SOX2 was expressed in a non-inducible manner and, hence, never turned off. SOX2-induced neurons in the spinal cord were mostly $VGLUT2^+$ excitatory interneurons (Wang et al., 2016), whereas GABAergic interneurons were obtained mainly in the striatum (Niu et al., 2015). Thus, also *in vivo*, astrocyte regionalization seems to play a key role in the neuronal subtypes achieved by reprogramming (see also below). Furthermore, the p53-p21-mediated signaling pathway was identified

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Table 1. List of *in vivo* studies with the respective control experiments to support glia-to-neuron direct reprogramming

Publication	Viral vector	Promoter-driving factors	Cre from	Factors	Intermediate state	Fate mapping	Labeling endogenous neurons	Labeling proliferating cells (BrdU/EdU)	Behavioral recovery
Buffo et al., 2005	RV	CAG	not needed	Pax6/Olig2-dn	+	N/D	N/D	N/D	N/D
Grande et al., 2013	RV	LTR (pMXIG)	not needed	Neurog2	+	N/D	N/D	+	N/D
Niu et al., 2013	**LV**	**hGFAP**	**none**	**Sox2**	**+**	**+**	**+**	**+**	**N/D**
Torper et al., 2013	*LV*	*PGK-flex*	*mGFAP-Cre mouse line (Jackson 24098)*	*Ascl1+Brn2a+ Myt1l*	+	*(+)*	*N/D*	*N/D*	*N/D*
	RV	hGFAP or hNG2	not needed	NeuroD1	+	N/D	N/D	N/D	N/D
Heinrich et al., 2014	RV	CAG	not needed	Sox2	+	+	N/D	+	N/D
Su et al., 2014	**LV**	**hGFAP**	**none**	**Sox2**	**+**	**(+)**	**N/D**	**+**	**N/D**
Liu et al., 2015	*AAV*	*hGFAP or hGFAP-flex*	*Aldh1l1-Cre mouse line*	*Ascl1*	+	*N/D*	*N/D*	–	*N/D*
Niu et al., 2015	**LV**	**hGFAP**	**none**	**Sox2**	**+**	**+**	**N/D**	**+**	**N/D**
Torper et al., 2015	*AAV*	*CBA or SYN*	*mNG2-Cre and GFAP-Cre mouse lines*	*Ascl1+Lmx1a+ Nurr1*	*N/D*	*N/D*	*N/D*	*N/D*	*N/D*
Gao et al., 2016	RV	unknown	not needed	Oct4+Klf4+ Sox2+c-Myc	+	N/D	N/D	N/D	N/D
Gascón et al., 2016	RV	CAG	not needed	Neurog2+Bcl2	N/D	N/D	N/D	N/D	N/D
Wang et al., 2016	**LV**	**hGFAP**	**none**	**Sox2**	**+**	**N/D**	**N/D**	**+**	**N/D**
Pereira et al., 2017	*AAV*	*CBA-flex or SYN-flex*	*NG2-Cre mouse line*	*Ascl1+Lmx1a+ Nurr1*	*N/D*	*N/D*	*N/D*	*N/D*	+
Rivetti di Val Cervo et al., 2017	*LV*	*Tet-O*	*GFAP-tTA mouse line (Jackson 5964)*	*NeuroD1+Ascl1+ Lmx1a+miR218*	+	*N/D*	*N/D*	–	+
Yoo et al., 2017	**LV**	**GFAP**	**none**	**Ascl1+Pitx3+ Lmx1a+Nurr1**	**N/D**	**(+)**	**N/D**	**N/D**	**N/D**
Matsuda et al., 2019	**LV**	**hCD68**	**none**	**NeuroD1**	**+**	**N/D**	**N/D**	**N/D**	**N/D**
Mattugini et al., 2019	*AAV*	*CAG-flex*	*mGFAP-Cre mouse line (Jackson 24098)*	*Neurog2+Nurr1*	*N/D*	*N/D*	+	+	+
Yamashita et al., 2019	RV	LTR (pMIG)	not needed	Ascl1+Sox2+ NeuroD1	+	N/D	N/D	N/D	+
Chen et al., 2020	**AAV**	**hGFAP or CAG-flex**	**hGFAP AAV**	**NeuroD1**	**N/D**	**N/D**	**N/D**	**N/D**	**N/D**
Ge et al., 2020	**AAV**	**hGFAP**	**none**	**NeuroD1**	**N/D**	**N/D**	**N/D**	**+**	**N/D**
Liu et al., 2020	**AAV**	**hGFAP**	**none**	**NeuroD1**	**+**	**N/D**	**N/D**	**N/D**	**+**
Puls et al., 2020	**AAV**	**CAG-flex**	**hGFAP AAV**	**NeuroD1**	**N/D**	**N/D**	**N/D**	**N/D**	**N/D**
Qian et al., 2020	*AAV*	*CMV-flox*	*mGFAP-Cre mouse line (Jackson 24098)*	*shPTB1*	*N/D*	+	*N/D*	*N/D*	+
Wu et al., 2020	*AAV*	*CAG-flex and hGFAP*	*mGFAP-Cre mouse line (Jackson 24098)*	*NeuroD1+ Dlx2*	*N/D*	*N/D*	*N/D*	*N/D*	+
Zhang et al., 2020	**AAV**	**CAG-flex**	**hGFAP AAV**	**NeuroD1**	**N/D**	**N/D**	**N/D**	**N/D**	**N/D**
Zhou et al., 2020	**AAV**	**GFAP**	**none**	**gPTB1**	**N/D**	**N/D**	**N/D**	**N/D**	**+**
Herrero-Navarro et al., 2021	RV	CAG	not needed	Neurog2+ Bcl2	N/D	N/D	N/D	N/D	N/D

(*Continued on next page*)

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Table 1. *Continued*

Publication	Viral vector	Promoter-driving factors	Cre from	Factors	Intermediate state	Fate mapping	Labeling endogenous neurons	Labeling proliferating cells (BrdU/EdU)	Behavioral recovery
Lentini et al., 2021	RV	CAG	not needed	Ascl1+Dlx2	+	N/D	N/D	+	+
Liu et al., 2021	*AAV*	*hGFAP or hGFAP-flex*	*Aldh1l1-Cre mouse line*	*Neurog2/Sox2*	+	*N/D*	*N/D*	-	*N/D*
Tai et al., 2021	**LV**	**hNG2 or hNG2-flex**	**Pdgfra-CreER mouse line (Jackson18280)**	**Sox2**	**+**	**+**	**N/D**	**+**	**N/D**
Wang et al., 2021	**AAV**	**hGFAP**	**none**	**shPTB1/ NeuroD1**	**–**	**–**	**–**	**–**	**N/D**

Studies shown in bold are those that used a promoter-driven construct. Studies shown in italics are those that used a mouse line to drive expression of the reprogramming factors. +, experiment performed with positive results; –, experiment performed with negative results; N/D, no data (experiment not done). We defined as "intermediate state" if the authors analyzed the expression of markers normally expressed in immature neurons, such as DCX, bIII-tubulin, and HuC/HuD.

as the upstream repressor of SOX2-induced neuroblasts because its downregulation increased the number of SOX2-induced neuroblasts and, subsequently, of mature neurons (Tai et al., 2021; Wang et al., 2016). Another powerful TF for *in vivo* reprogramming already used with RVs is NEUROD1. Using a mouse line to express the tetracycline-regulated activator (tTA) under the *hGFAP* promoter and LVs expressing NEUROD1, ASCL1, LMX1A, and mi-R218 under the tetracycline-dependent operon (Tet-O) resulted in appearance of dopaminergic neurons in the 6-OHDA-induced Parkinson's disease (PD) mouse model (Rivetti di Val Cervo et al., 2017; Table 1). However, promoter-driven constructs may have problems with their specificity, as shown for adeno-associated virus (AAV) vectors, which we will discuss now.

Targeting non-proliferating cells: Use of adeno-associated viruses

AAVs are an even more promising alternative to target non-dividing cells for *in vivo* reprogramming because they very rarely integrate into the genome and, if so, only into specific genomic loci (Hamilton et al., 2004), minimizing the risk of mutations (De Ravin et al., 2014; Wu et al., 2003). Moreover, AAV injections into the adult murine cerebral cortex lead to low levels of inflammation and moderate reactive gliosis compared with those caused by injections of LVs and RVs, where $CD45^+$ leukocytes are abundant and reactive gliosis ($IBA1^+$ and $GFAP^+$ cells) was very strong at the injection site (Mattugini et al., 2019). Clearly, use of vectors eliciting such strong inflammation is not possible for humans and may lead to many of the converted neurons succumbing to cell death (Gascón et al., 2016). Therefore, for clinical use, AAVs will probably be the best option.

However, despite the possibility of generating variants of AAV capsids to increase target cell specificity (Davidsson et al., 2019), so far no selective AAVs targeting glial cells are known for *in vivo* use (Challis et al., 2019; Nonnenmacher et al., 2020). Until now, mostly AAV2/5 serotypes have been used for intracranial injection for direct neuronal reprogramming, as pioneered by Torper et al. (2015). However, AAV2/5 are neurotropic and, hence, infect mostly neurons (Petrosyan et al., 2014). Therefore, promoters such as *hGFAP* or *mGFAP(and their shorter versions GfapABC(1)D and gfa2;* (Lee et al., 2008)) are used to restrict reprogramming TF expression to astrocytes (Table 1; Chen et al., 2020; Mattugini et al., 2019; Qian et al., 2020; Zhang et al., 2020; Zhou et al., 2020(Liu et al., 2020)). Alternatively, mouse lines expressing Cre recombinase under astrocyte-specific promoters (e.g., the *mGFAP*-Cre line) have been used together with vectors with the TFs of interest in inverted orientation ("FLEx"), requiring Cre-mediated recombination for expression (Mattugini et al., 2019; Pereira et al., 2017; Qian et al., 2020; Torper et al., 2015). However, caution is needed because promoter/enhancer element expression is often not selective in a given cell type. For example, scRNA-seq showed some level of expression of the neuronal gene *Map2* in astrocytes and the astrocyte gene *Gfap* in neurons (Kempf et al., 2021). Accordingly, the marker GFP, delivered as FLEx in AAV2/5, was also expressed in some endogenous neurons upon GFAP-mediated expression of Cre in a transgenic mouse line (Mattugini et al., 2019; Qian et al., 2020). In addition, the observed leaky expression of GFP in neurons also depends on the titer of the injected AAVs (e.g., the amount of viral particles injected per animal; Xiang et al., 2021), which has been reported to even impair the physiology of targeted cells above certain amounts (Suriano et al., 2021). However, these technical concerns may be outweighed by the much better safety of the AAVs for clinical use and the potential to improve reprogramming by lower immunogenicity.

Recent studies reported many more neurons generated when the reprogramming factors were delivered by AAVs (e.g., NEUROD1 under the *hGfap* promoter in AAVs [Chen et al., 2020] or controlled by a GFAP-Cre-expressing AAV [Zhang et al., 2020]) compared with LV-mediated delivery (Guo et al., 2014b; Matsuda et al., 2019). In addition, cortical ischemic stroke (Chen et al., 2020) and cortical SW injury (Zhang et al., 2020) were nearly completely repaired upon AAV-mediated delivery of NEUROD1. Remarkably, the induced neurons exhibited cortical-layer-specific identities, formed functional neural circuits, and rescued motor and memory deficits (Chen et al., 2020). Neuronal conversion of astrocytes upon NEUROD1 also led to regeneration of beneficial astrocytes, restoration of the blood-brain barrier, and reduction of neuroinflammation (Zhang et al., 2020). In

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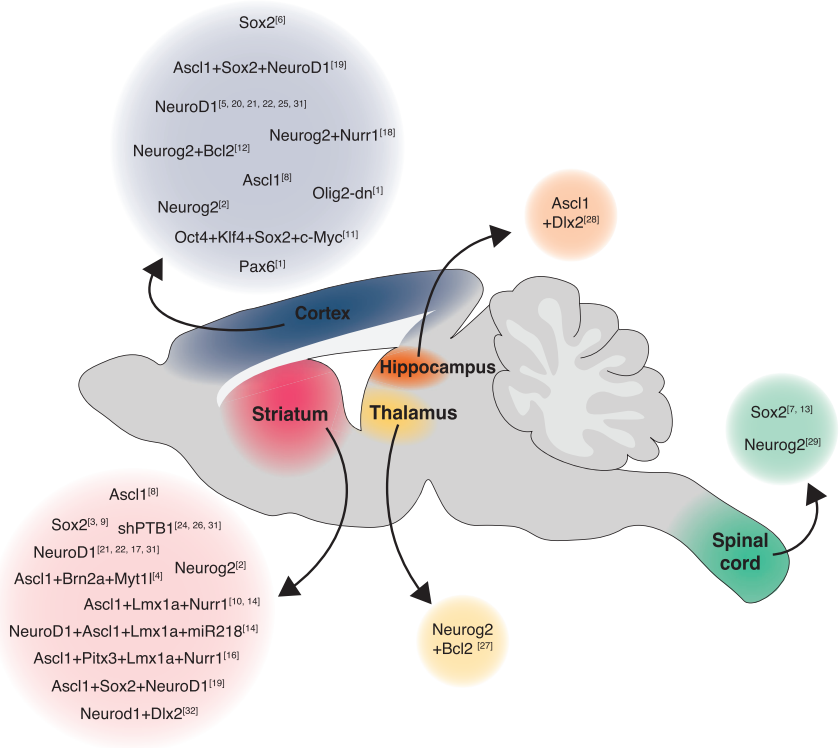

Figure 3. Combinations of neurogenic factors used for *in vivo* reprogramming

The illustration provides a comprehensive list of all TFs (and their combinations) used so far for *in vivo* reprogramming in different regions of the brain. 1, Buffo et al., 2008; 2, Grande et al., 2013; 3, Niu et al., 2013; 4, Torper et al., 2013; 5, Guo et al., 2014b; 6, Heinrich et al., 2014; 7, Su et al., 2014; 8, Liu et al., 2013; 9, Niu et al., 2015; 10, Torper et al., 2015; 11, Gao et al., 2016; 12, Gascón et al., 2016; 13, Wang et al., 2016; 14, Pereira et al., 2017; 15, Rivetti di Val Cervo et al., 2017; 16, Yoo et al., 2017; 17, Matsuda et al., 2019; 18, Mattugini et al., 2019; 19, Yamashita et al., 2019; 20, Chen et al., 2020; 21, Ge et al., 2020; 22, Liu et al., 2020; 23, Puls et al., 2020; 24, Qian et al., 2020; 25, Zhang et al., 2020; 26, Zhou et al., 2020; 27, Herrero-Navarro et al., 2021; 28, Lentini et al., 2021; 29, Liu et al., 2021; 30, Tai et al., 2021; 31, Wang et al., 2021; 32, Wu et al., 2020.

therapeutic applications, AAV-based expression of NEUROD1 under the *GFAP* promoter was very efficient in repairing damaged brain regions after ischemic stroke in adult non-human primates; although mostly astrocytes were targeted by the control virus, 90% of NEUROD1-infected cells were neurons (Ge et al., 2020). Given that AAV2/5 in these studies could also infect endogenous neurons, it may be that low levels of NEUROD1 in neurons, even when controlled by a *GFAP* promoter, could improve neuronal survival and be beneficial for the outcome in various aspects.

Indeed, it has been shown that expression of NEUROD1 as well as PAX6, NEUROG2, ASCL1, and MYC (but not SOX2) could transactivate different *GFAP* promoter elements in *cis* (e.g., in a cell-autonomous manner) in infected cells (Wang et al., 2021). The possible mechanisms for in *cis* regulation have not yet been investigated. In this regard, it is intriguing that the coding sequence of many reprogramming factors (e.g., *NeuroD1* or *Neurog2*) contains regulatory elements, which is not so frequent in other genes. These regulatory sites could be recognized and bound by TFs expressed in neurons, which, in turn, could induce expression of the reporter over time. This possibility would explain why single point mutations affecting *NeuroD1* did not abrogate *cis* regulation—the regulatory elements were not affected by a single mutation—and how confounding results when evaluating the direct conversion were obtained.

In this study, the authors tested AAV-mediated overexpression (e.g., NEUROD1) or knockdown (e.g., PTBP1) reported previously to induce astrocyte-to-neuron conversion *in vivo* (Chen et al., 2020; Qian et al., 2020; Zhang et al., 2020; Table 1). Using four criteria for conversion of astrocytes to neurons, they found that expression of the marker gene used to trace infected astrocytes occurs in endogenous neurons. First, because reactive astrocytes proliferate, the authors checked whether reprogrammed neurons had incorporated the DNA base analog bromodeoxyuridine (BrdU) and, hence, were derived from cells proliferating prior reprogramming but found no evidence of this. Second, they could not detect the transient marker for immature neurons, DCX. Third, astrocyte lineage tracing experiments were performed to label virtually all astrocytes (crossing Aldh1l1-CreERT2 with R26R-YFP mice). The reporter gene expressed from the ROSA locus in the Aldh1l1-CreERT2 mouse line via tamoxifen induction showed no overlap with the supposedly induced neurons, suggesting that induced neurons were wrongly labeled endogenous neurons. Fourth, a retrograde labeling approach was used to trace endogenous neurons prior reprogramming. Many neurons, obtained by AAV-mediated overexpression of NEUROD1 or knockdown of PTBP1, were positive for the retrograde reporter, suggesting that the alleged reprogrammed neurons were endogenous and already existed before the reprogramming process (Wang et al., 2021). It is intriguing that expression of the virally delivered reporter gene in pre-existing neurons in the presence of the reprogramming factor takes some time and is not detected a few days after transduction, as it would when transduced alone. One mechanism to explain this phenomenon may be linked to protein-protein interactions of the bHLH neurogenic reprogramming factor (see e.g., Sun et al., 2001). Low expression levels of the TF could progressively deplete proteins from the GFAP promoter and cause its activation or derepression in endogenous neurons. Normally, recruitment of CBP/p300-SMAD1 by NGN1 leads to repression of the GFAP promoter (Sun et al., 2001), but this may then result in activation of the cryptic regulatory sequences in the coding sequences of the neurogenic factors discussed above.

Thus, this important work sheds light on possible artifacts using promoter-driven cell-type-specific expression and calls for controls to verify the truthfulness of cell fate conversion. Some previous studies had already implemented some of these

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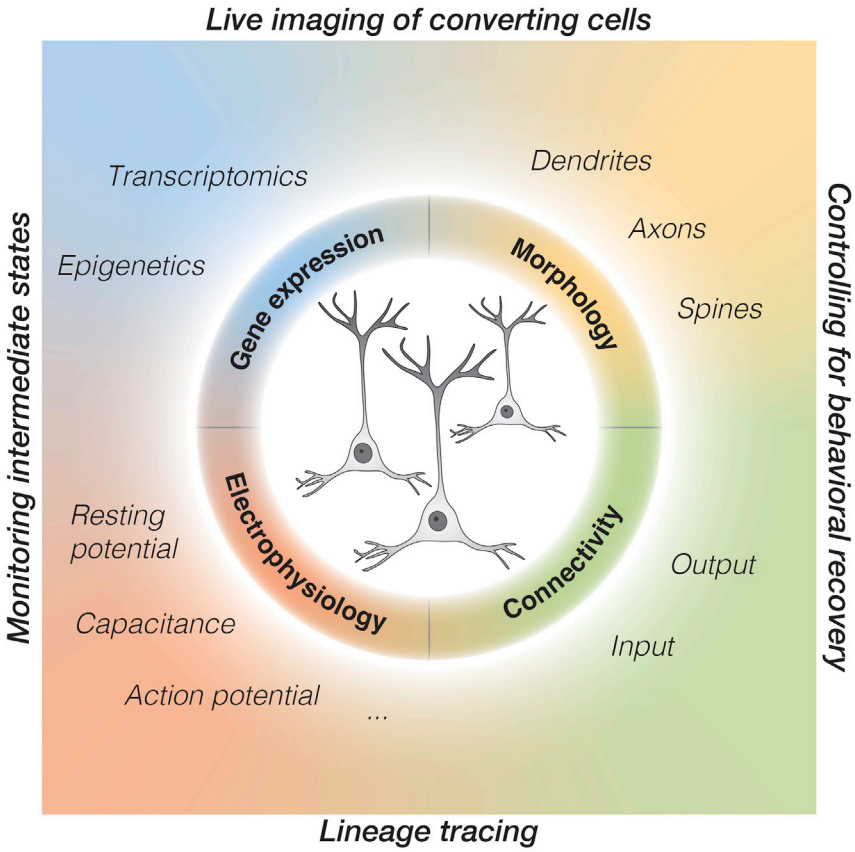

Figure 4. Defining the perfect induced neurons

Four main parameters should be used to access the identity of induced neurons: gene expression, morphology, electrophysiology, and connectivity. Ideally, the "perfect" induced neuron should be transcriptionally and morphologically indistinguishable from the endogenous counterpart. In addition, it should receive the correct inputs, process them, and generate adequate action potentials to properly integrate into the pre-existing circuitry. Around the square are highlighted the main proposed controls (the code of conduct) to avoid artifacts and pitfalls during *in vivo* reprogramming.

controls, as summarized in Table 1. In the next section, we first discuss possible controls for *in vivo* reprogramming protocols to set a code of conduct and then mention the vectors and controls when discussing further *in vivo* reprogramming experiments.

Call for obligatory controls and standards when using viral vectors targeting neurons

To ensure *in vivo* conversion of resident glia into neurons, it is essential to distinguish induced neurons from pre-existing neurons and ascertain that the source of induced neurons are indeed the targeted cells (e.g., resident glial cells). We will discuss the controls necessary to keep the desired scientific standard (Figure 4).

Monitoring the transition of glia to neurons: Live in vivo *imaging or labeling proliferating cells and immature transitory stages of conversion*

The best proof of conversion is to follow the process by continuous live imaging. This experiment has so far only been done *in vitro* (Gascón et al., 2016; Heinrich et al., 2010; Karow et al., 2012; Russo et al., 2021) and is urgently needed *in vivo*; it will require following individual glial cells for several weeks to monitor and ensure their conversion. This detailed analysis will also provide fascinating insights into the similarities and differences in differentiation and neurite development of induced neurons compared with the reported maturation of transplanted neurons (Falkner et al., 2016). For example, the apical dendrite of a transplanted pyramidal neuron grew surprisingly fast and in a directed manner, whereas basal dendrites appeared later and were subjected to massive pruning (Falkner et al., 2016). Would this phenomenon also be the same for induced neurons or would they develop following a different sequence of events elicited by forced expression of specific TFs? Is cell death a major hurdle as it is *in vitro*, and what is the influence of inflammation on death; e.g., would blocking inflammation be sufficient to also improve survival of new neurons *in vivo*? Thus, *in vivo* live imaging of targeted glial cells will not only confirm the conversion process by observing loss of the glial cytoarchitecture and gradually adopting a neuronal morphology but also provide unprecedented insights into the mechanisms underlying this process.

However, not every reprogramming experiment can include the technically challenging chronic *in vivo* imaging. As a first approach to label specifically proliferating reactive glia, DNA base analogs (e.g., BrdU or ethynyldeoxyuridine [EdU]) can be given during the period of reactive glia proliferation after an insult; e.g., during the first week after the insult. For example, after SW injury, about 20% of all astrocytes proliferate, and a similar fraction has been observed among induced neurons achieved by NEUROG2 and NURR1 expression using FLEx AAV2/5 injection into a mGFAP-Cre mouse line (Mattugini et al., 2019). However, astrocyte-to-neuron conversion could also potentially occur without undergoing an intermediated proliferative stage, as is the case *in vitro* (Gascón et al.., 2016; Heinrich et al.., 2010) and as demonstrated *in vivo* for retinal Müller glia (Hoang et al., 2020; Jorstad et al., 2017) and most astrocytes in the cortex (Mattugini et al., 2019). In this case, markers for immature neurons can be used, such as DCX, βIII-tubulin, HuC/HuD, and others (Heinrich et al., 2014; Lentini et al., 2021). Because these are also often induced in endogenous neurons in stages of plasticity or injury, labeling proliferating cells is a more stringent criterion in this case. Notwithstanding, DNA base analogs also have limitations because they are also integrated into damaged DNA (Taupin, 2007). Therefore, these controls are not sufficient on their own and require additional evidence for conversion of cell fates.

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Labeling pre-existing endogenous neurons

As mentioned above, the key is to discriminate induced neurons from endogenous neurons. This has been introduced by Mattugini et al. (2019) by labeling pre-existing neurons with continuous EdU administration during embryonic neurogenesis (Mattugini et al., 2019). Giving EdU for 8 consecutive days from embryonic day 7.5 (E7.5) to E15.5 resulted in EdU labeling of about 80% of all pyramidal neurons in the cortex. Among NEUROG2/NURR1-induced neurons, however, only 20% were labeled with embryonic EdU administration, suggesting that most of them were not endogenous neurons (Mattugini et al., 2019). The proportion of EdU^+ neurons corresponded to the proportion of endogenous neurons labeled under the control condition, where only AAV FLEx-GFP was injected, consistent with a certain degree of leakiness of *GFAP*-Cre-driven recombination, as discussed above. However, given the long-term experimental procedure of this protocol (waiting 3 months after EdU application for mice to become adults and perform injury and reprogramming), other techniques for labeling endogenous neurons are desirable. For example, retrograde labeling by AAVs (Oh et al., 2014; Wang et al., 2021) can be used to back-trace pre-existing neurons. However, this approach typically traces only a few neurons, as also reported by Wang et al. (2021). Alternatives would be inducible mouse lines and labeling neurons prior to applying an injury or viral vector, such as CAMKCreERT2 (Madisen et al., 2010) for cortical pyramidal neurons. Finally, neuron-specific AAVs may be used to label pre-existing neurons prior to an injury condition and reprogramming attempt.

Genetic fate mapping or lineage tracing of the starter cell type

Another important control is to permanently label the starter cells (e.g., via fate mapping) so that they would still express the marker after their conversion into neurons. For example, astrocytes could be labeled by crossing mouse lines expressing the Cre recombinase protein specifically only in astrocytes (e.g., Cst3-CreERT2, ALDH1L1-CreERT2, GFAP-CreERT2) with Cre-dependent reporter mouse lines (e.g., R26R-YFP mice, R26R-tdTOMATO [Niu et al., 2013, 2015; Qian et al., 2020]). Using these mouse lines to label astrocytes, neurons induced by GFAP-driven SOX2 or PTBP1 knockdown have been reported to be derived from astrocytes (Table 1; Heinrich et al., 2014; Niu et al., 2013, 2015; Qian et al., 2020; Tai et al., 2021). In this regard, surprisingly, Wang et al. (2021) obtained the opposite result (no double labeling of AAV-targeted neurons with astrocyte fate mapping) using a different transgenic mouse line following PTBP1 knockdown. GFAP-CreERT2;R26R-tdTOMATO was used by Qian et al. (2020), and the ALDH1L1-CreERT2;R26R-YFP and mGFAP-Cre;R26R-YFP mouse lines were used by Wang et al. (2021). These contrasting data show how important it is to carefully characterize the recombination achieved by a specific amount of tamoxifen in a specific mouse background to evaluate its recombination specificity under different conditions (e.g., in healthy condition, upon injury, over time, etc.) to assure reliability during the reprogramming process because some of these and other transgenic lines can also label neurons to some extent (the leaky gene expression also described above). However, it is important to highlight that this approach also depends on the promoter elements driving expression of the reporter after the STOP cassette was excised by Cre-recombinase in the reporter mouse line. These elements are typically ubiquitous promoters, such as those present in the R26R locus or CAG (chicken-β-actin [Nakamura et al., 2006]). Also, reporter mouse lines show different degrees of specificity and, most importantly, they have not yet been examined for silencing in cells or neurons expressing high levels of neurogenic TFs. The ideal case would therefore be to turn on proteins with an extra-long half-life (such as histones) that would still be present for months even when their expression was turned off.

Single-cell transcriptomic analysis of converting glial cells

So far, very little is known about the molecular mechanisms and transcriptional changes during *in vivo* reprogramming. Single-cell transcriptomics analysis at different stages of conversion would provide a better understanding of how the conversion is achieved and provide a more unbiased view on intermediate stages rather than using a single marker (e.g., DCX). Moreover, expression analysis is essential to ultimately reveal how induced and endogenous neurons are molecularly similar (see below). Importantly, these methods could also be combined with genetic fate mapping or scar tracing technology to ensure the cellular origin of the induced neurons (Alemany et al., 2018; Heinrich et al., 2014; Niu et al., 2013; Zhou et al., 2020). However, scRNA-seq would not be able to fully discriminate immature gene expression as a transitory state derived from glia or possibly induced in endogenous neurons even though state-of-the-art analysis and tracing methods may overcome this issue.

Controlling for cell fusion or the effects of behavioral recovery

Finally, the gold standards achieved in transplantation should be also adopted for the *in vivo* reprogramming field; namely, controlling for cell fusion and confirming the effects of behavioral recovery by silencing or ablating newly generated neurons (Andreoli et al., 2020). Controlling for cell fusion is easy for exogenous cells. For example, male donor cells can be transplanted into female hosts, alternatively cells containing floxed-stop reporter alleles can be transplanted into mice with ubiquitous Cre expression (Falkner et al., 2016; Grade and Götz, 2017). In some *in vivo* reprogramming experiments, astrocytes were transplanted and underwent conversion *in vivo* (Chouchane et al., 2017; Lentini et al., 2021). However, cell fusion controls have not been used in this case. Controlling for cell fusion is very difficult in direct reprogramming of endogenous cells because it would occur between cells that all have the same genotype. Checking for double chromosomes and DNA content would be one possibility, and it is worth noting that scRNA-seq data analysis usually removes cells with high numbers of genes expressed; e.g., double DNA content. Inducible genetic fate mapping (e.g., in neurons and astrocytes) performed prior to reprogramming experiments could be another possible approach. However, the latter would require dual recombination systems (e.g., Cre recombinase for astrocytes and Flipase for neurons), highlighting the difficulty of controlling for fusion of cells with the same genetic background.

Silencing the transplanted neurons to assess whether behavioral recovery is abolished has become a powerful method to

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ensure that behavioral recovery is indeed due to transplanted neurons (Andreoli et al., 2020). In the reprogramming field, this has been done by Qian et al. (2020) using a chemogenetic approach; the lesion-induced motor phenotype disappeared 2 months after reprogramming but reappeared when the induced dopaminergic neurons were specifically silenced. However, it is worth noting that this functional analysis is useful only after proving that the reprogramming transgene is not expressed in pre-existing neurons because silencing endogenous neurons that were spared or rescued from death by the neurogenic TF would also abrogate the behavior improvement.

Given the complexity of some experimental protocols and the inconclusive nature of some controls, we suggest to always use several of them. We propose to monitor proliferation, intermediate phenotypes, and labeling endogenous neurons as the most crucial and feasible controls that should become standard in any direct reprogramming study. For the reader to judge reliability of the *in vivo* reprogramming protocols, we refer to Table 1 and focus our discussion on studies that avoid use of promoter-driven viral vector constructs and/or performed at least one of the abovementioned controls.

Neuronal subtype specificity

To improve reprogramming efficiency and achieve neuronal subtype specificity, combinatorial approaches have also been pursued using AAVs *in vivo*; for instance, to induce dopaminergic neurons (Pereira et al., 2017; Torper et al., 2013, 2015). Therefore, it came as a surprise that sole knockdown of PTBP1 was sufficient to induce various specific types of neurons depending on the regions to which they were applied, including dopaminergic neurons in the ventral midbrain (Table 1). Given the above concerns with possible in *cis* regulation of glial promoters in neurons (Wang et al., 2021) and work deleting PTBP1 in brain astrocytes and not observing any astrocyte-to-neuron conversion (Thanh Hoang et al., 2021), we focus here on neuronal subtypes obtained by use of AAV constructs driven by Cre-expressing mouse lines (Table 1). For instance, in the *NG2*-Cre mouse line, FLEx AAV-mediated expression of the TFs ASCL1, BRN2, and MYT1L or ASCL1, LMX1A, and NURR1 triggered the appearance of neurons that could functionally integrate into the local circuits (Pereira et al., 2017; Torper et al., 2015). However, none of the above controls were performed (Table 1). The latter combination of TFs resulted in a greater proportion of labeled neurons than reported previously and failed to show dopaminergic neurons in the striatum (Torper et al., 2015; Figure 3). Wu et al. (2020) also used GFAP-Cre transgenic mouse lines to activate FLEx AAV-mediated expression of NEUROD1 along with DLX2, reporting induction of GABAergic DAPR32^{+} medium spiny neurons, which promote improvement in motor neuron functions in two models of Huntington's disease. However, none of the abovementioned controls were used.

Following cortical SW injury, AAV-mediated combinatorial expression of FLEx NEUROG2 and NURR1 in the mGFAP-Cre mouse line resulted in highly efficient reprogramming of targeted astrocytes into functional pyramidal neurons (Mattugini et al., 2019). As mentioned above, this study confirmed that some induced neurons were derived from proliferating cells (that is, cells other than postmitotic neurons), and most of the induced neurons were not endogenous pre-labeled neurons (Table 1). Remarkably, these induced neurons developed, over time, the lamina-specific hallmarks of cortical pyramidal neurons, including an appropriate layer-specific molecular identity and long-distance axonal projections. However, despite the controls mentioned above, further experiments, such as live *in vivo* imaging and scRNA-seq, would be helpful to ensure reliability of this approach. This approach will also help to elucidate to which extent these neurons truly resemble their endogenous counterparts and how they function within the neural network.

Integration of newly formed neurons into pre-existing circuits and behavioral recovery

The possibility of successful repair relies on functional integration of induced neurons into the pre-existing network and, ultimately, on restoration of neural circuit function to ameliorate the deficits caused by neuronal loss. Currently, transplantation studies are the gold standard because they have shown that embryonic neurons transplanted into postnatal and adult brains display a remarkable capacity to integrate by extending axonal projections to their respective target regions and receive adequate inputs (Andreoli et al., 2020; Czupryn et al., 2011; Fricker-Gates et al., 2002; Gaillard and Jaber, 2011; Gaillard et al., 2007; Grealish et al., 2015; Michelsen et al., 2015; Shin et al., 2000). Importantly, a comprehensive analysis of the input connections within the whole brain revealed no aberrant input to neurons transplanted into the injured adult visual cortex but unveiled adequate and even correct topographic specificity of the afferent innervation (Falkner et al., 2016). The precise connectivity is reflected by functional accuracy; the transplanted visual cortex neurons acquired direction- and orientation-selective receptive field properties, indicating fully functional integration and restoration of the appropriate circuitry (Falkner et al., 2016).

The first evidence that induced neurons can acquire a mature electrophysiological state and receive synaptic connections was provided by two independent studies, where RV- and LV-mediated expression of NEUROD1 and SOX2 induced neurons that evoked spontaneous action potentials (Guo et al., 2014b; Heinrich et al., 2014). Following studies using different pro-neurogenic cocktails further demonstrated synaptic action potentials in the induced neurons (Gao et al., 2016; Liu et al., 2015; Niu et al., 2015; Pereira et al., 2017), although some studies may face the issue of *cis* activation of the viral promoter elements (Table 1). Particularly impressive is the finding that induced neurons from adult endogenous non-neuronal cells (mostly astrocytes) can also re-grow axons without being affected by the inhibitory cues of the adult brain (Chen et al., 2020; Guo et al., 2014b; Mattugini et al., 2019; Qian et al., 2020). Although some of these studies may be affected by labeling endogenous neurons, Mattugini et al. (2019) had controlled for this issue (Table 1) and found long-distance axonal projections even reaching the spinal cord.

In contrast, a comprehensive analysis of the input connectivity labeled by monosynaptic retrograde rabies virus tracing (Callaway and Luo, 2015) has so far been mapped only in the hippocampus and the striatum (Lentini et al., 2021; Torper et al., 2015). Although only local input neurons to the neurons induced in the

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striatum were found (Torper et al., 2015), reprogramming proliferating glia by RV-mediated expression of ASCL1 and DLX2 in the epileptic hippocampus showed largely adequate input connectivity but less than endogenous neurons (Lentini et al., 2021). Most importantly, and further corroborating functional integration, the induced interneurons were sufficient to reduce seizures in this model of medial temporal lobe epilepsy (Lentini et al., 2021). Mapping the input connectivity of *in-vivo*-induced neurons is important to reveal adequate rather than disturbed circuit function. This is of great importance in some brain regions, such as the cerebral cortex, which tends to suffer from overexcitation and epilepsy, especially after injury. In other disease conditions, such as in PD, nonspecific release of dopamine in the striatum from transplanted cells can already have some beneficial effects (Kikuchi et al., 2017; Kim et al., 2020). Dopaminergic neurons allegedly converted from striatal astrocytes (Table 1) improved dopamine levels and reduced motor deficits in mouse models of dopaminergic neurons loss in the *substantia nigra* (Rivetti di Val Cervo et al., 2017; Torper et al., 2015; Zhou et al., 2020).

Nowadays there are methods to assist with evaluation of brain-wide connectivity, such as scRNA-seq. This technique could be used, for example, to determine the exact molecular identity of induced neurons to further understand why, in some cases, correct connectivity was not established properly and may help to identify other deficits of the induced neurons compared with endogenous neurons. Furthermore, myelination of newly formed axons, which is essential for proper circuit function (Bei et al., 2016; Wang et al., 2020), has not yet been examined for induced neurons. Thus, many aspects to achieve adequate circuit integration of induced neurons remain to be tackled.

scRNA-seq to monitor neuronal identity and on- or off-target memory from the starter fate

As mentioned above, so far mostly electrophysiology has been used as a gold standard for proving a fully converted neuronal identity, and it is certainly a crucial functional readout. However, it was not clear until recently how good electrophysiology is as a predictor of complete fate conversion. Recently, electrophysiological recordings were combined with single-cell transcriptomics of the same cell (so-called "patch sequencing" experiments) reprogrammed *in vitro* (Kempf et al., 2021). This analysis revealed little correlation between the transcriptional profile and the electrophysiological properties of neurons reprogrammed from astrocytes (Kempf et al., 2021). Interestingly, mature repetitively firing neurons sometimes had remnant astrocyte gene expression (on-memory gene expression), whereas transcriptionally fully converted neurons were occasionally immature at electrophysiological levels. These findings may be surprising at first glance but may be less so in light of the reported regulation of ion channels and transmitter receptors mostly at the protein level by recycling at the membrane (Dörrbaum et al., 2018), which may explain the minor contribution to the overall transcriptome. Thus, incomplete or partial conversion requires transcriptional readouts beyond electrophysiological quality controls and should ideally also become a standard for *in vivo* reprogramming experiments.

The role of intrinsic and extrinsic factors in reprogramming *in vivo*

The process of *in vivo* reprogramming and the obtained induced neuronal subtypes could be influenced by cell-intrinsic (e.g., the cell identity of the starting cell) and extrinsic factors (e.g., the extracellular environment where reprogramming occurs). As mentioned previously, glial heterogeneity has been shown at morphological, functional, and, more recently, single-cell level in various areas of the CNS (Bayraktar et al., 2020; Lanjakornsiripan et al., 2018; Häring et al., 2018; Zeisel et al., 2018). Beyond that, the surrounding environment provided by neighboring cells, such as the extracellular matrix, cytokines, neurotransmitters, and morphogens (which differ among brain regions) under healthy or disease conditions could also influence the *in vivo* reprogramming process. *In vivo*, it is difficult to separate intrinsic and extrinsic factors because the reprogramming outcome differs by transducing astrocytes in different brain regions. For example, in the striatum, LV-driven expression of SOX2 from a *GFAP* promoter in astrocytes induced GABAergic interneurons (Niu et al., 2015), whereas in the spinal cord, the same factor reprogrammed astrocytes into $VGLUT2^{+}$ excitatory neurons (Wang et al., 2016). However, it is not clear whether these results depend on intrinsic differences among astrocytes or on the environment. Intriguingly, transducing astrocytes in different compartments within a given region also results in distinct neuronal subtypes, such as astrocytes in different cortical layers converting into different subtypes of neurons with the correct layer-specific properties (Mattugini et al., 2019), but failed to reprogram cortical white matter astrocytes (Liu et al., 2020; Mattugini et al., 2019). However, despite the molecular diversity of astrocytes within cortical layers (Bayraktar et al., 2020; Lanjakornsiripan et al., 2018), the environment along the cortical column also has profound differences in cell type compositions, particularly between white matter and gray matter. It is therefore not clear to which extent the molecular diversity of the targeted astrocytes and the different local environment shape the reprogramming outcome *in vivo*.

This issue was addressed more directly in a recent study of thalamic inter- and intra-regional diversity of astrocytes, which confers an exceptional degree of spatial specification during astrocyte-to-neuron reprogramming (Herrero-Navarro et al., 2021). Upon RV-mediated NEUROG2 overexpression, proliferating glia from post-natal day 3 cortices and thalami were selectively reprogrammed into cortical and thalamic neurons *in vitro* and *in vivo*, respectively. This was attributed to the fact that astrocytes and neurons, given their common developmental origin, share expression of region-specific patterning TFs (Herrero-Navarro et al., 2021), such as *Gbx2*, a TF expressed by thalamic astrocytes and neurons (Mallika et al., 2015). Accordingly, upon forced expression of GBX2 and NEUROG2 in cultures of cortical astrocytes, cortex-specific neuronal markers, such as TBR1 and CTIP2, were not induced, in favor of the thalamic neuronal markers POU2F2 and SLC17A6 (Herrero-Navarro et al., 2021). These data strongly indicate that developmentally defined cues can be manipulated to achieve regional subtype identity of neurons. It would be interesting to induce this identity switch *in vivo*, which would suggest that establishment of cellular identities happens independent of the environment, or, in case of a

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different outcome, would demonstrate that the environment contributes to shape the region-specific identity.

It is also important to note that the injured environment influences glial cells. Reactive glia differs under specific injury conditions (e.g., traumatic or ischemic) or during neurodegenerative conditions, changing their morphology, gene expression, and proliferation properties (Götz et al., 2015; Sofroniew, 2009). SW injury prior to virus injection is a prerequisite to achieve reprogramming of cortical NG2 glia by SOX2 delivered by RVs (Heinrich et al., 2014). In line with these findings, a lower number of induced neurons was found after overexpression of NEUROG2 and NURR1 in the intact brain compared with injection of these TFs close to a SW injury site (Mattugini et al., 2019). Similarly, ischemic lesions also slightly enhanced NEUROG2-induced neuronal production, survival, and maturation (Grande et al., 2013). Last, the number of neurons was increased upon viral promoter-driven NEUROD1 overexpression in the cortex of an Alzheimer's disease model compared with a healthy brain, which correlated well with the number of disease-induced reactive glial cells in an age-dependent manner (Guo et al., 2014b; Table 1).

These results support the idea that the cellular and molecular identity of glial cells together with the surrounding environment, in particular pathophysiological conditions, potently influence the outcome of neuronal reprogramming and will require much more work to be understood and targeted adequately in humans.

How to utilize direct neuronal reprogramming with human cells: From disease modeling to neuron replacement therapies

Besides the exciting conceptual insights into fate conversion processes and their feasibility even in an injured brain *in vivo*, the overarching aim is to use this approach to improve human health by disease modeling to find treatment strategies or, ultimately, replacing lost neurons in humans. The first direct conversion of human fibroblasts into functional neurons (Pang et al., 2011) was a breakthrough because it now allows envisioning direct reprogramming-based strategies for humans (for non-human primates, see Ge et al., 2020). This and following studies (e.g., Caiazzo et al., 2011; Drouin-Ouellet et al., 2017; Yoo et al., 2011) highlighted important differences between direct conversion of mouse and human cells into neurons. For instance, the conversion of human cells takes more time and requires optimized conditions to increase reprogramming efficiency and generate functional neurons; e.g., treatment with small molecules (Ladewig et al., 2012), systematic medium change over 4 or more weeks (Drouin-Ouellet et al., 2017), use of additional TFs (e.g., NeuroD2), and co-culture with mouse astrocytes (Pang et al., 2011). These improved protocols for human cell reprogramming now pave the way to two major areas of application: disease modeling *in vitro* and *in vivo* (e.g., xenotransplantation) and neuronal replacement of degenerated neurons ideally by non-invasive viral vector application.

The need for adequate models of human diseases stems from the observation that animal models often do not sufficiently recapitulate the complexity of human conditions, which limits such models for developing drug-based therapies that pass clinical trials (e.g., BACE inhibitors for Alzheimer's disease [AD]; Egan et al., 2018). There are several reasons for the poor phenocopy of diseases in animal models: different genetic background, higher complexity of the human brain, crosstalk between the brain and the body, distinctive susceptibility to environmental agents, diverse lifespans, and different effects of the ageing process, just to mention a few. Although many limitations cannot be overcome in a dish (e.g., brain complexity), *in-vitro*-cultured human cells retain cell autonomous properties that contribute to the etiology of many diseases, such as the genetic background and the age of the individual. Moreover, *in vitro* models with increasing complexity and cell-cell interactions are being developed by co-culturing neurons and various glial cells (Guttikonda et al., 2021).

Direct reprogramming of human differentiated somatic non-neural cells (e.g., fibroblasts, pericytes, and astrocytes) into neurons has several advantages (as well as disadvantages) over human iPSC-based neuronal differentiation for disease modeling. First, it does not require generation of hiPSCs, which can cause chromosomal aberrations (Araki et al., 2020) and resets the entire epigenetic and aging signature (Yang et al., 2015). Direct neuronal reprogramming of human cells maintains many hallmarks of aging, such as epigenetic marks, nuclear lamina, and mitochondrial dysfunction (Horvath, 2013; Huh et al., 2016; Kim et al., 2018; Mertens et al., 2015). This feature makes induced human neurons more suited to model age-related diseases, such as AD, PD, amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD) because murine models of AD and PD only partially recapitulate the phenotypes observed in affected individuals (Drummond and Wisniewski, 2017). Second, the availability of fibroblasts or blood cells from individuals carrying specific mutations allows the generation, in a relatively short time, of neurons and studying their phenotypes. The disadvantage of direct reprogramming over hiPSC-mediated generation of neurons is the limited number of cells achieved by direct somatic reprogramming because neurons are post-mitotic and do not proliferate anymore. However, recent approaches to turn fibroblasts into proliferative NSC allows cell expansion (Sheng et al., 2018; Thier et al., 2012), later followed by differentiation of these NSCs into neurons. Importantly, direct reprogramming of cells from older individuals also comes at a price; the age of donor fibroblasts is a hurdle for efficient conversion (Drouin-Ouellet et al., 2017), and it has been associated with increased REST activity, an inhibitor of neuronal reprogramming, as described above. Because *in vivo* data suggest that injury helps reprogramming, it would be interesting to test whether inducing a reactive state in aged human cells would improve their reprogramming or, rather, whether this is yet another difference between murine and human cells.

Over the years, several neurological diseases have been modeled via direct *in vitro* conversion of human cells, and, although not completely recapitulating the disease, some disease-associated phenotypes could be observed. For instance, fibroblasts carrying a mutation in the APOEε3/4 gene were converted into neurons via forced expression of ASCL1, BRN2, MYT1L, and NEUROD1 to model AD. Induced neurons with the ApoE mutation showed increased accumulation of β-amyloid 42, tau hyperphosphorylation, and more pronounced cell death upon peroxide treatment (Kim et al., 2017). Likewise, neurons

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induced from fibroblasts carrying mutations in the *PINK1* gene, which accounts for 5%–10% of all PD cases (de Lau and Breteler, 2006), showed overall no stress signs (pS65-Ub accumulation) following valinomycin treatment (Fiesel et al., 2015), in sharp contrast to control neurons. The absence of a stress-related signature prevents mutant cells from sensing mitochondrial stress, which contributes to exacerbated neuronal loss. In directly reprogrammed AD models of human neurons, *APP* mutant fibroblast-converted neurons revealed an accumulation of β-amyloid 42 (Aβ42) and increased phosphorylated tau, but not in all mutant cells tested (Hu et al., 2015). More recently, detailed analysis of induced neurons from fibroblasts obtained from individuals with AD revealed similarities between AD-related changes and age-dependent cellular programs, including a stress-related de-differentiation signature and epigenetic changes underlying a hypomaturation state (Mertens et al., 2021).

Although the abovementioned studies highlight the contribution of direct neuronal reprogramming to disease modeling, some caveats also need to be highlighted. First, induced neurons were analyzed within a month of their conversion, and, in many cases, the electrophysiology was not assessed, thus not fully covering the influence of the mutations in neuronal activity. Second, some mutations affect specific neuronal subtypes (e.g., in PD, mainly dopaminergic neurons in the *substantia nigra*), and these subtypes were not yet examined. Third, induced neurons have been analyzed only *in vitro*, and additional defects might depend on the surrounding *in vivo* environment. This could be overcome by transplanting the reprogrammed cells at an early stage into mice or non-human primates or performing the entire conversion after transplantation. For instance, when human fibroblasts converted into medium spiny neurons (MSNs) were transplanted into the mouse striatum, DARPP32-, FOXP1-positive MSN could be detected for up to 6 months *in vivo* and had postsynaptic potentials similar to the endogenous MSNs, suggesting their integration into the preexisting circuitry (Victor et al., 2014). Given the functional similarity of reprogrammed MSNs and the endogenous counterparts, the conversion protocol was applied to fibroblasts carrying different expansions of CAG codons in the Huntingtin (*HTT*) gene (Victor et al., 2018). *HTT*-iNeurons showed mHTT aggregates, mitochondrial disfunctions, and spontaneous degeneration not present in hiPSC-derived neurons carrying the same mutation. Interestingly, induced MSNs from pre-symptomatic younger individuals showed mHTT aggregates at levels similar to MSNs derived from symptomatic individuals, but they were less vulnerable to mHTT-induced toxicity, revealing age-dependent phenotypes detectable via direct neuronal reprogramming (Victor et al., 2018).

Thus, the technology is now ready for using direct somatic cell-to-neuron conversion *in vitro* by utilizing some of the advanced co-culture systems creating increasingly complex, multicellular environments or *in vivo* after transplanting the converting neurons. The price to pay for *in vivo* modeling of human neurons in xenotransplantation approaches is the suppression of the immune system, which is a natural part of the disease process. However, transplantation of non-converted human cells followed by the induction of neurogenic factors *in vivo* will allow monitoring of the conversion of human non-neuronal cells into neurons *in vivo* and then following their connectivity, as pioneered by the transplantation of iPSC-derived neurons (Hallett et al., 2015) and employing the transplantation of murine astrocytes in *in vivo* reprogramming (Chouchane et al., 2017; Lentini et al., 2021).

Overall, direct neuronal reprogramming could provide important insights into disease modeling, but there is also a lot of room for improvement. For example, more specific combinations of reprogramming TFs need to be identified to induce specific neuronal subtypes. Moreover, comprehensive molecular analysis and transplantation experiments will be required to evaluate non-cell autonomous effects of human mutations. Given that astrocytes residing in the right compartment of the mouse brain can be converted to specific neuronal subtypes, an important task will be to understand human astrocyte regionalization and ideally mimic it *in vitro* or by TF combinations to enable conversion into neuronal subtypes appropriate and adequate for specific human brain regions.

Furthermore, studies should investigate how the disease affects brain circuitry, how human induced neurons can integrate properly and re-establish correct circuitry, and how many neurons are necessary to obtain therapeutic effects. Last but not least, appropriate vectors for use in humans need to be developed. They should be cell type specific, tunable, non-integrating, and ideally delivered by systemic rather than intracranial injection. Answering these questions also requires non-human primates as *in vivo* models. Use of direct neuronal reprogramming in human cells holds great potential for disease modeling of neurological diseases, drug screening, or even gene replacement as therapeutic intervention, but a deeper understanding and better models are needed.

Concluding remarks

Progress in direct neuronal reprogramming has been so impressive over the last 20 years that efficiency of reprogramming even *in vivo* seems no longer an issue. Clear steps lie ahead, such as achieving appropriate neuronal subtypes that connect in an adequate manner to re-establish the circuitry that has been damaged. The future for this task looks bright; CRISPRa technology now allows regulating many genes simultaneously or even sequentially, enabling manipulation of entire gene expression networks and modules. Advanced sequencing technology allows detection of mismatches between induced and pre-existing neurons to correct mistakes in induced neurons. Last but not least, the approach of direct reprogramming may allow replacement of lost neurons by non-invasive technology if blood-brain barrier-permissive AAV capsids can be developed to generate cell type specificity (Nonnenmacher et al., 2020). Interdisciplinary approaches of virologists, circuit neuroscientists, and developmental experts are now needed to further move this promising approach toward a use in medicine.

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AUTHOR CONTRIBUTIONS

R.B., G.M., and M.G. conceived the work and wrote the manuscript.

DECLARATION OF INTERESTS

M.G. is a member of the advisory board of *Neuron*.

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