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AT-A-GLANCE ARTICLE

Natural and forced neurogenesis: similar and yet different?

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Introduction

The concept of direct reprogramming commonly entails forced expression of development-inspired cues that are used as a blueprint to induce conversion from one cell type into another cell type. Among other tissues, such as the heart and the pancreas, it has also been applied to the central nervous system for the generation of neurons. The employed cues are transcription factors on one side and the modulation of instructive signaling pathways recapitulating patterning events that are in place during natural neurogenesis on the other side. We will here review and discuss existing reprogramming approaches and ask the question whether and when during such forced neurogenesis a cell is taking a naturally occurring path or whether a completely new route is taken.

Intrinsic and extrinsic cues governing natural neurogenesis

In the adult mammalian brain, an amazing plethora of different kind of neurons is precisely interconnected to allow the brain to fulfill its marvelous and very diverse functions. To build a functional brain, during development the right cells have to be produced at the right time, in the right amount and at the right place. Given this diversity, it is striking to realize that this huge and precisely orchestrated neuronal heterogeneity, which can be found in the adult mammalian brain, is entirely derived from the neural tube, a homogenous sheet of epithelial cells formed during neurulation (Darnell and Gilbert 2017). To coordinate brain development, morphogen gradients along the anterior-posterior as well as the dorso-ventral axis induce patterning processes leading to the molecular regionalization of neural progenitor cells (NPCs) in the neural tube. For instance, in the telencephalon, counteracting gradients of ventral Shh and dorsal Wnt and Bmp signals pattern the forebrain, thereby inducing regional identities (Briscoe and Small 2015; Rowitch and Kriegstein 2010). This extrinsic code of signaling pathway activities is ultimately translated into a code of transcription factors (TFs) (Guillemot 2007). In the ventral forebrain, Gsx1/2 are highly expressed and induce the Dlx gene family. In turn, in combination with the proneural TF Ascl1, this results in the initiation of a GABAergic program (Wang et al. 2013). In contrast, the induction of the neuronal program in the dorsal forebrain is triggered by the expression of Pax6 and Emx1/2 and later on through upregulation of the proneural TFs Neurog1/2. This unfolds the glutamatergic program including the expression of specific target genes such as members of the NeuroD family and Tbr2 (Wilkinson et al. 2013). Interestingly, at different levels during development, cross-repressive mechanisms ensure the manifestation of one but not the other identity, such as Pax6-Gsx1/2 (Stoykova et al. 2000; Toresson et al. 2000) and Neurog2-Ascl1 (Fode et al. 2000). In the course of neurogenesis, in a next phase, cells exit the cell cycle and

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induce the expression of postmitotic neuron-specific genes, such as Tbr1 and Gad65/67 for glutamatergic and GABAergic neurons, respectively.

Adapting mother nature's program for forced neurogenesis

In past years, a range of TF combinations has been proven effective in directly reprogramming somatic cells, mostly fibroblasts, into induced neurons (iNs) in vitro. Depending on the TFs used, glutamatergic, GABAergic, dopaminergic, cholinergic neurons and motoneurons have been obtained (Masserdotti et al. 2016). Here, we will not be able to account for all the different neuronal reprogramming approaches that vary from the starting cell population (e.g., fibroblasts, astrocytes, pericytes) to the TFs used (e.g., Ascl1, Neurog2, Brn2, Myt1l, Sox2, Lmx1a) as well as the resulting neuronal subtype populations. Therefore, we will only focus on the central actors Ascl1 and Neurog2, which are part of most neuronal reprogramming approaches; their role has been extensively characterized during natural neurogenesis (Guillemot and Hassan 2017). When different combinations of TFs are tested to impose reprogramming on somatic cells, this follows the logic of mimicking processes that are in place during natural neurogenesis. However, as has been shown by Gascon et al. (2016), addressing additional cellular aspects such as the metabolic switch from an astrocyte stage to a neuronal stage can strongly improve neuronal conversion. Indeed, enhanced iN reprogramming has been achieved by co-expression of Ascl1 or Neurog2 with Bcl2 and the Vitamin D receptor, both resulting in reduced cell death and oxidative stress facilitating forced neurogenesis (Gascon et al. 2016). Similarly to the sequence of patterning events during natural neurogenesis, small-molecule-based approaches aim at modulating signaling pathways, which in turn result in neuronal conversion of a starting cell population (Zhang et al. 2015). Considering this vast diversity of reprogramming approaches and the resulting neurons, a detailed characterization of the reprogramming process as well as the reprogrammed neurons is crucial to understanding similarities and differences and to evaluate the resulting neurons. Importantly, the diversity of experimental approaches that seemingly result in the same product, namely a neuron, open up many questions (summarized in Fig. 1) such as: (1) What is the role of the starting cell population type and age and is proliferation of the starter cell required? (2) do cells undergoing reprogramming pass through a chromatin state that would resemble the chromatin state of a progenitor cell? (3) to what extent is patterning and regionalization required for specifying neuronal identity during reprogramming? (4) are natural and forced neurogenesis distinct routes that converge onto a common path to achieve neuron formation or do different routes lead to the same product? and (5), finally, how comparable are neurons and induced neurons?

Ultimately, this knowledge may also be instructive for understanding natural neurogenesis.

Window of opportunity

Interestingly, it has been shown that the success rate of neuronal reprogramming inversely correlates with the age of the starting cell type. Whereas cortical astrocytes obtained from postnatal mice can be instructed to change their cellular identity following overexpression of Ascl1 or Neurog2 (Heinrich et al. 2010), astrocytes isolated from adult animals are refractory to these instructive cues. Moreover, when postnatal astrocytes are aged in vitro and exposed to the neurogenic cues in a delayed manner, reprogramming fails (Masserdotti et al. 2015). This observation might be linked to the exit of the cell cycle of local parenchymal astrocytes that was described to take place within the first three postnatal weeks of mice (Ge et al. 2012). A similar observation of a restricted window of opportunity has also been shown in an in vivo reprogramming paradigm. Paola Arlotta and her team (Rouaux and Arlotta 2013) asked the question whether it is possible to convert neurons from one class into another one via overexpression of the TF Fezf2. Forced expression of Fezf2 rerouted callosal projection neurons within postnatal mouse brains into corticofugal neurons. However, this plasticity progressively declined with age and their experiments showed that the cells turned resistant to Fezf2 overexpression by postnatal day 21 (Rouaux and Arlotta 2013). Thus, a defined postmitotic window seems to exist in which the gate for direct reprogramming closes. The study of Paola Arlotta and colleagues showed that, in the case of Fezf2-mediated reprogramming, proliferation of the starter cell is not required since the neurons that were targeted by Fezf2 were postmitotic. However, in other paradigms, when mixed cell populations are targeted for reprogramming that are still in a proliferative state, it is actually not known whether the proliferative state of the starter cells is required for reprogramming. It should be mentioned here that many experimental settings, especially using retroviruses for the overexpression of the reprogramming factors, as a matter of fact do select for proliferative cells. In contrast, what has been shown in several studies is that proliferation is not required during the course of reprogramming. In fact, it has been shown that cells exit the cell cycle once the reprogramming process kicks in (Karow et al. 2012; Masserdotti et al. 2015; Treutlein et al. 2016).

Besides the proliferative capacities of developing and more adult cells, a difference in the chromatin state may be conferring different competence for reprogramming. Interestingly, a similar age-dependent progressive restriction in fate potential of neural progenitors has also been

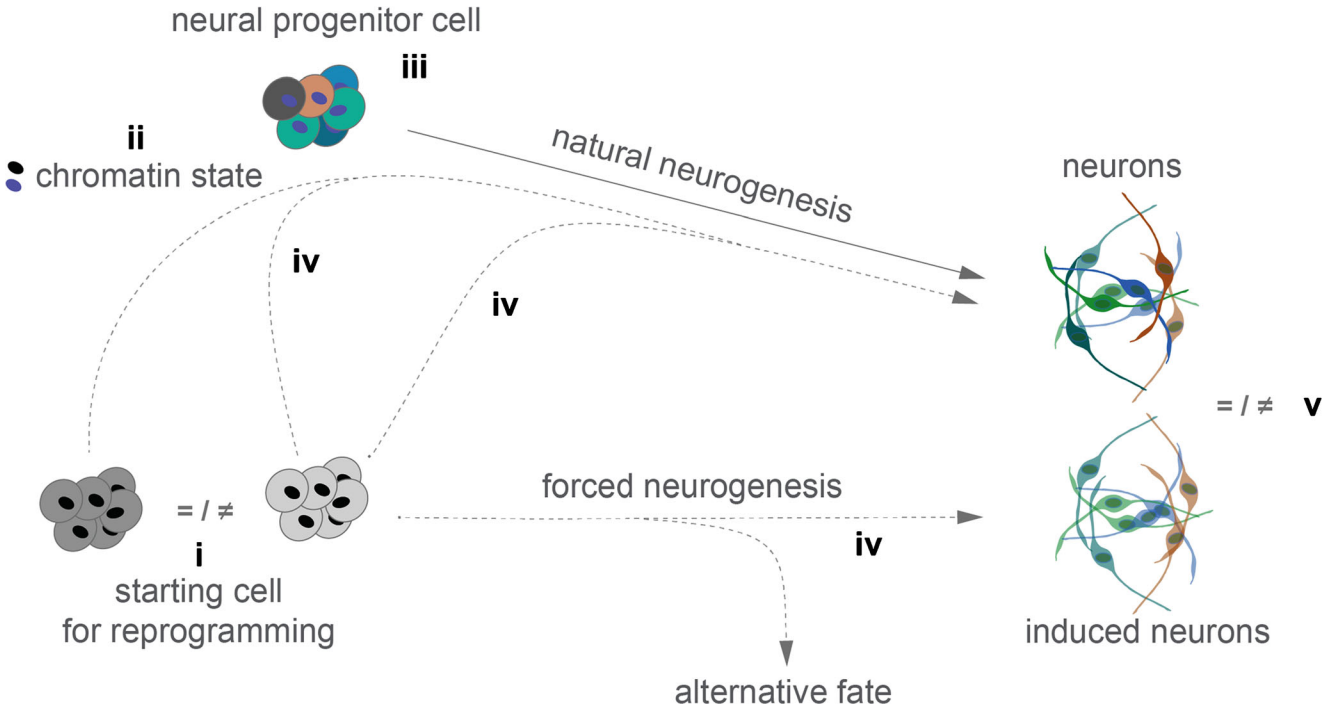

Fig. 1 Graphical abstract highlighting open questions in the field of neuronal reprogramming. Sequential differentiation cues naturally guide differently patterned NPCs (indicated by different colors) towards a specific neuron identity. In contrast, during forced neurogenesis different starter cells are converted into iNs. (i) Whether the programs elicited by the overexpressed TFs are general or dependent on the respective different starter cells remains unclear. (ii) It is also understudied whether the chromatin signature of cells undergoing reprogramming is similar to that of natural NPCs at any times during conversion. (iii) Given that appropriate patterning during natural neurogenesis is crucial for the generation of specific neuronal subtypes, an obvious question concerns whether an iN can gain a specific neuronal subtype without proper regionalization during the course of forced neurogenesis. (iv) It is unclear whether an iN is generated via a completely distinct path or whether at some point cells undergoing reprogramming use a natural route of differentiation. (v) Furthermore, to what extent an iN can acquire all features of a bona fide neuron is not understood at the moment

described during natural neurogenesis (Desai and McConnell 2000) and is attributed to chromatin changes (Kishi et al. 2012). Similarly, it is conceivable that terminally differentiated neurons targeted by Fezf2 do exhibit a vast difference in chromatin signature. In this regard, it has been shown recently that the chromatin accessibility is altered upon neuronal activity (Su et al. 2017). This suggests that a more pronounced neuronal activity in mature neurons induced chromatin changes that render the cells refractory to Fezf2 reprogramming. Similarly, divergent chromatin states between different cells such as murine embryonic fibroblasts (MEFs) and keratinocytes have been proposed to be crucial for the binding of Ascl1 to its cognate target sites (Wapinski et al. 2013). Thus, in keratinocytes in which the targets lack a trivalent state of activating and inhibiting chromatin marks (H3K4me1, H3K27acetyl and H3K9me3), Ascl1 fails at reprogramming. Interestingly, small molecules such as Forskolin and Dorsomorphin can modulate the chromatin state to infer reprogrammability on cells, which showed resistance to reprogramming without this additional treatment (Smith et al. 2016). Another way of facilitating reprogrammability is to co-express relevant factors that modulate chromatin changes, thereby changing accessibility of reprogramming-relevant genes. This might explain why adult human brain pericytes require the combinatorial expression of Ascl1 and Sox2 for reprogramming into iNs (Karow et al. 2012). In sum, it is not surprising that different starting cell types and ages require individual reprogramming factor combinations to trigger forced neurogenesis.

To what extent is patterning and regionalization required for specifying neuronal identity during reprogramming?

As highlighted above, an intimate and tightly timely controlled interplay between patterning events and differentiation waves is crucial for natural neurogenesis. Accordingly, cortical GABAergic interneurons such as those expressing Parvalbumin or Somatostatin originate from Nkx2.1-positive progenitors in the ventral medial ganglionic eminence (MGE). During the course of natural neurogenesis, these progenitors progressively differentiate and tangentially migrate towards the cortex where they functionally integrate (Marin 2013). Interestingly, it has been shown that murine embryonic

fibroblast-derived in vitro-reprogrammed GABAergic iNs, although being endowed with a forebrain regional identity expressing Foxg1 and Sox2, rarely express Nkx2.1 (Colasante et al. 2015), suggesting that these cells may not pass through a MGE-progenitor-like state. However, only tight monitoring of the global transcriptomal changes occurring during conversion will allow addressing this open question. An interesting approach to phenocopy natural neurogenesis is to sequentially express the applied reprogramming factors. In this case, the starter cells would be first exposed to temporally restricted cues that are normally active in NPCs and, later on, once the conversion has started, more differentiating cues should be imposed on the cells. Such approaches would also have the advantage of counteracting mechanisms that are active in a progenitor-like state, which block terminal neuronal differentiation. Indeed, this latter aspect was partly addressed in the afore-mentioned study in which five reprogramming factors (Sox2, Foxg1, Ascl1, Lhx6, Dlx5) were used to induce conversion of fibroblasts into GABAergic iNs. Here, the authors could show that turning off the expressing of the progenitor markers, Sox2 and Foxg1, although not resulting in a higher efficiency of conversion, increased the maturation into more mature GABAergic iNs (Colasante et al. 2015). Ideally, such approaches could also be combined with small-molecule strategies that mimic patterning events, as specified earlier. Similarly to the open question of how fibroblasts following overexpression of Sox2, Foxg1, Ascl1, Lhx6 and Dlx5 can adopt a GABAergic identity without passing through an MGE progenitor-like state, it is still an enigma why Ascl1 alone, albeit during natural neurogenesis being instrumental for the generation of inhibitory neurons, generates predominantly iNs exhibiting an excitatory glutamatergic fate from murine embryonic fibroblasts (Treutlein et al. 2016; Vierbuchen et al. 2010; Wapinski et al. 2013). Yet, this may be another example of how the starter cell is influencing the outcome of the reprogramming process, since, in astrocytes, forced expression of Ascl1, also by itself, results in an inhibitory GABAergic phenotype (Heinrich et al. 2010; Masserdotti et al. 2015). Similarly, Ascl1 is able to induce an inhibitory GABAergic neuron identity in human brain pericytes; however, it needs additional expression of Sox2 (Karow et al. 2012).

Are natural and forced neurogenesis distinct routes that converge onto a common path to achieve neuron formation or do different routes lead to the same product?

During natural neurogenesis, progenitors undergo patterning events, which lead to the formation of a range of different progenitors that ultimately give rise to distinct neuronal subtypes. In order to address whether a starter cell targeted for neuronal reprogramming adopts transcriptome signatures reminiscent of naturally occurring progenitors, only a detailed molecular characterization of the trajectory of cells undergoing reprogramming can ultimately be telling. However, considering that the starter cells not only have to acquire a new identity but also have to abandon their original identity to fully adopt a new cell fate, this is a challenging task. Taking advantage of the single-cell RNA-sequencing (scRNA-seq) technology, it has actually been shown that, during Ascl1-mediated MEF-to-iN reprogramming, cells never pass through a canonical NPC stage marked by the expression of Sox2 or Pax6 but rather adopt a cellular intermediate progenitor stage that is different from starter and target cells (Treutlein et al. 2016). These data suggest that the cells would rather take a different path than progenitors would undertake naturally (see Fig. 2a, b). Other evidence came from a study in which the early reprogramming phases of Ascl1- and Neurog2-mediated reprogramming of postnatal murine astrocytes were directly compared and evaluated on a transcriptome level (Masserdotti et al. 2015). Interestingly, Ascl1 and Neuorog2 induced distinct neurogenic cascades early on in the same starter cells. It could have also been conceivable that these neurogenic factors would induce a pan-neuronal program, which would split up only later during reprogramming to give rise to either GABAergic or glutamatergic cells. However, this was not the case since only a very few targets overlapped when comparing the transcriptional programs induced in cells undergoing reprogramming. Moreover, the authors could show that, although seemingly natural neurogenic cascades were induced, only a minor fraction of natural target genes of those TFs were activated, further suggesting a rather artificial progenitor program (Fig. 2c). Ultimately, when comparing all the different reprogramming approaches, it is striking that different neuronal fates can be observed when overexpressing the same factors in different starter cells but even more striking that one and the same factor overexpressed in the same starter cell can induce alternative fates that are not related (Fig. 2d). Again, using scRNA-seq, it has been uncovered that a fraction of the fibroblast starter cell population, following overexpression of Ascl1 only, converted into a neuronal population, whereas another fraction surprisingly turned into myogenic cells (Treutlein et al. 2016). This finding raises the obvious question of whether heterogeneity of the starter cell would be responsible for these alternative fates and infer different competence for reprogramming. However, except for being mostly heterogenous in regard to cell cycle status, the starter cell population appeared relatively homogenous. Most importantly, at early stages following forced Ascl1 expression, the cells responded uniformly to the stimulus, suggesting that the competing trajectories are established at later stages during reprogramming (Treutlein et al. 2016). Interestingly, co-expression of Ascl1 with Brn2 and Myt1l (referred to as the BAM factor combination) in MEF cells resulted in virtually

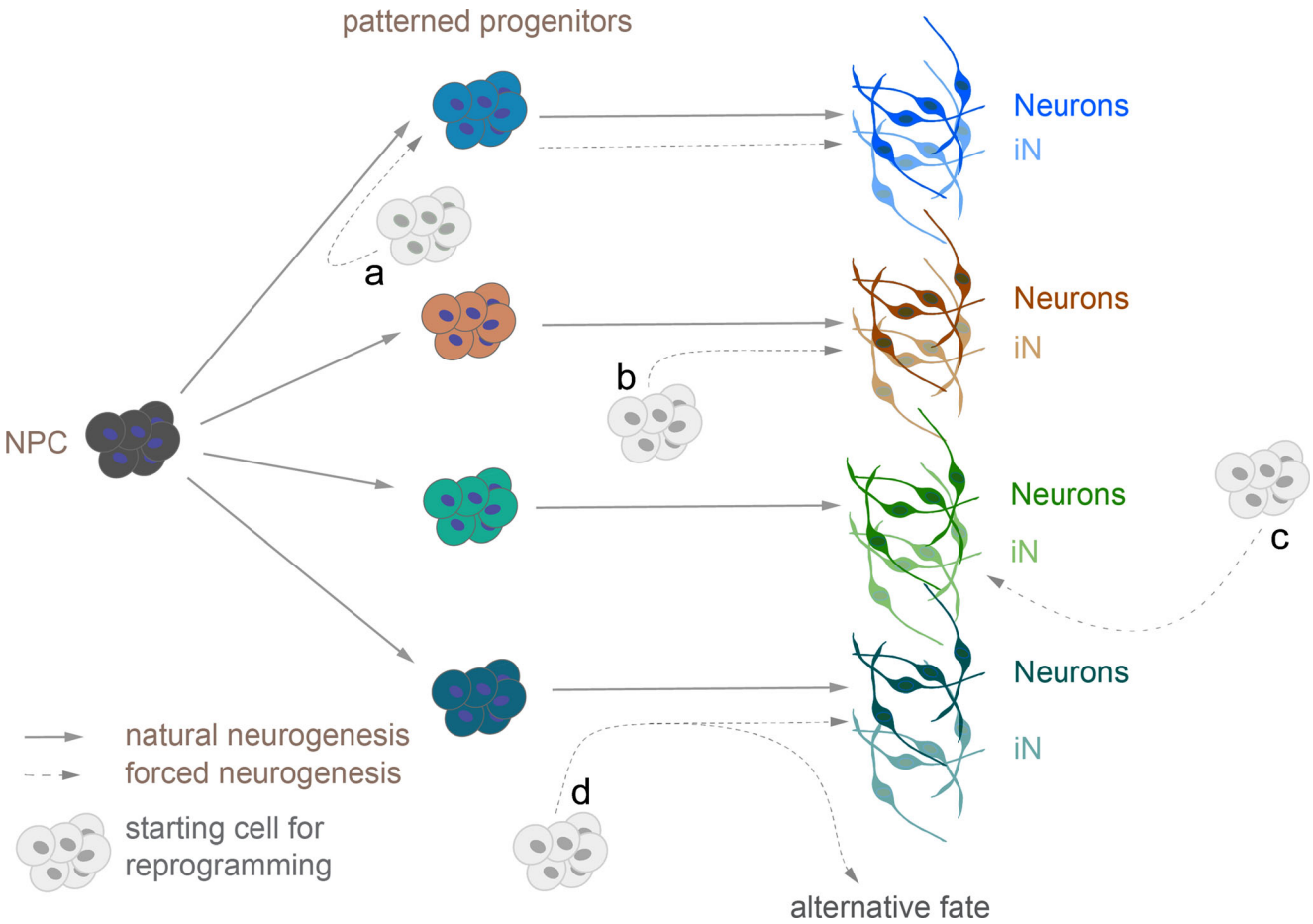

Fig. 2 Potential reprogramming routes. During natural neurogenesis, NPCs are differently patterned and give rise to different neuronal subtypes, e.g., dorsal forebrain progenitors generate glutamatergic excitatory neurons and ventral forebrain progenitors generate GABAergic inhibitory neurons. The different trajectories are indicated by different colors. During forced neurogenesis, different potential scenarios are conceivable. *a* Starter cells can be redirected to become a specific patterned progenitor similar to natural progenitors and then further differentiate towards a specific neuronal subtype. *b* The starter cells bypass a canonical progenitor stage and fuse into the natural route at later stages. *c* Neuronal subtypes are generated by imposing an artificial differentiation path distinct from naturally occurring trajectories. *d* Bypassing a properly patterned progenitor stage is a likely explanation for the emergence of an alternative fate from the same starter cell, even when the same reprogramming factor is applied

only neuronal fates. As has been discovered very recently, in this particular paradigm, Myt1l acts as transcriptional repressor blocking conversion into other than the neuronal lineage (Mall et al. 2017).

How comparable are neurons and induced neurons?

Considering the therapeutic utilization of iNs as the ultimate goal of direct reprogramming strategies raises the question of how comparable these neurons are. To evaluate this, we have to take into account different aspects such as transcriptomal identity, the proteome, electrophysiological properties, the capability of activity-dependent gene expression and finally their functional integration. A major challenge, especially working with human cells, is posed by the lack of positive controls since human neurons are hard to derive and culture in vitro. Furthermore, it is questionable whether the above-mentioned aspects and in particular the ones regarding the functional properties, can be modeled at all in a 2D system in vitro. As an approximation, human iNs can be transplanted into the (developing) rodent brain to study their function within a 3D in vivo context. Indeed, in a meticulous study of Vanderhaeghen and colleagues (Espuny-Camacho et al. 2013), it has been shown that in vitro-derived human cortical progenitor-like cells programmed from human-induced pluripotent stem cells can be transplanted in the neonatal mouse brain, where they differentiate into neurons and form functional synapses with the host circuitry. They even observed proper myelination of the human neurons by the host, supporting the notion of functional integration. Of note, the authors could also show that the duration of neuron maturation (up to 6 months) was encoded intrinsically to these human projection neurons and not changed to one of the murine counterparts (Espuny-

Camacho et al. 2013). Whereas, in the latter study, following transplantation of progenitor-like cells, neuronal maturation occurred within the neonatal mouse brain, Colasante et al., grafted in vitro directly reprogrammed murine GABAergic neurons at later stages of reprogramming into the adult mouse hippocampus. Here, the acquisition of electrophysiological firing capabilities was also shown (Colasante et al. 2015). However, cells that had turned on Parvalbumin expression ex vivo lost their Parvalbumin expression following transplantation, which so far is due to unknown mechanisms but, as suggested by the authors, is potentially due to the cultivation period in vitro (Colasante et al. 2015). A possibility to avoid an ex vivo culturing period is to directly target brain-resident cells in vivo for reprogramming into iNs, as has been shown following stereotactic administration of retroviruses encoding reprogramming factors in the adult mouse brain. Following overexpression of Sox2 and, more efficiently, Sox2 plus Ascl1, NG2 glia could be converted into electrophysiologically active iNs in vivo as shown by patch-clamp recordings (Heinrich et al. 2014). Furthermore, using an AAV-based delivery and reporter system it has been shown that NG2 glia can be targeted and reprogrammed into iNs via overexpression of Ascl1, Lmx1a and Nurr1. Here, the authors revealed functional integration of the newly formed neurons by rabies virus-based tracing techniques (Torper et al. 2015). One aspect, which has not been addressed to date, concerns myelination of either transplanted in vitro-derived reprogrammed iNs or directly in vivo-reprogrammed iNs. However, such myelination would be imperative for proper functional integration and a prerequisite for contributing to functional recovery, for instance in mouse models of neurodegeneration.

In summary, despite the fact that natural and forced neurogenesis result in similar functional end products, namely neurons, the routes taken by the starter cells are manifold. Whether or not this is relevant for ultimately applying the iN technology will be unraveled in the future. In other words, *omnes viae Neuronum ducunt*!

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