Cell Tissue Res (2015) 359:5–16
DOI 10.1007/s00441-014-2048-9

REVIEW

How to make neurons—thoughts on the molecular logic of neurogenesis in the central nervous system

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Received: 16 June 2014 / Accepted: 23 October 2014 / Published online: 23 November 2014

Abstract Neuronal differentiation relies on a set of interconnected molecular events to achieve the differentiation of pan-neuronal hallmarks, together with neuronal subtype-specific features. Here, we propose a conceptual framework for these events, based on recent findings. This framework encompasses a dimension in time during development, progressing from early master regulators to later expressed effector genes and terminal selector genes. As a horizontal intersection, we propose the action of permissive fate determinants that are critical in allowing progression through the above transcriptional phases. Typically, these are widely expressed and often interact with the chromatin remodeling machinery. We conclude by discussing this model in the context of the direct fate conversion of various somatic cells into neurons.

Keywords Neurogenesis · Central nervous system · Direct conversion · Fate determinant · Transcription factor · Fate specification

Introduction

Neurogenesis is a multistep process resulting in the generation of the appropriate/adequate number and subtypes of neurons first and foremost in the developing brain when this organ is formed (Ajioka 2014; Gotz and Huttner 2005; Huttner and Kosodo 2005; Taverna et al. 2014). The brain consists of many diverse neuronal and glial subtypes that are generated either at specific time points (e.g., neurons at various layer positions in the developing cerebral cortex or neurons prior to glia) or in specific regions (e.g., the distinct dorso-ventral domains generating neuronal diversity in the spinal cord and forebrain). This prompts the key questions as to when and how the mechanisms of neurogenesis diverge to specify the various subtypes. Neurons share basic hallmarks, such as the generation of action potentials and synaptic communication, but also differ in many aspects, such as their efferent projections and synaptic contacts, their characteristic morphology, and neurotransmitters. One obvious scenario might be that pan-neuronal hallmarks are specified first by molecular factors acting at earlier stages, and that their subtype identity is determined later (Fig. 1). This mode of neurogenesis would also be compatible with the sequential generation of the different neuronal subtypes, predicting that first the stage is set for the generation of neurons, and that this mode is maintained throughout neurogenesis with additional and sequential modules specifying distinct subtypes (Fig. 1). However, another conceivable mode is that the transcriptional programs for the different neuronal subtypes might diverge at an early time point, and that the activation of the genes encoding the common neuronal genes might occur at various stages depending on the lineage. According to this model, many different ways might be available to generate neurons, and the program to generate, for example, an inhibitory γ-aminobutyric acid (GABA)-ergic neuron, might differ, right from the start, from the program for glutamatergic excitatory neurons.

These key questions about the molecular logic of making neurons are also highly relevant to forcing neurogenesis when it no longer occurs endogenously. Taking advantage of the increasing knowledge about molecular fate determinants (FDs) for neurogenesis, these have been used to direct diverse

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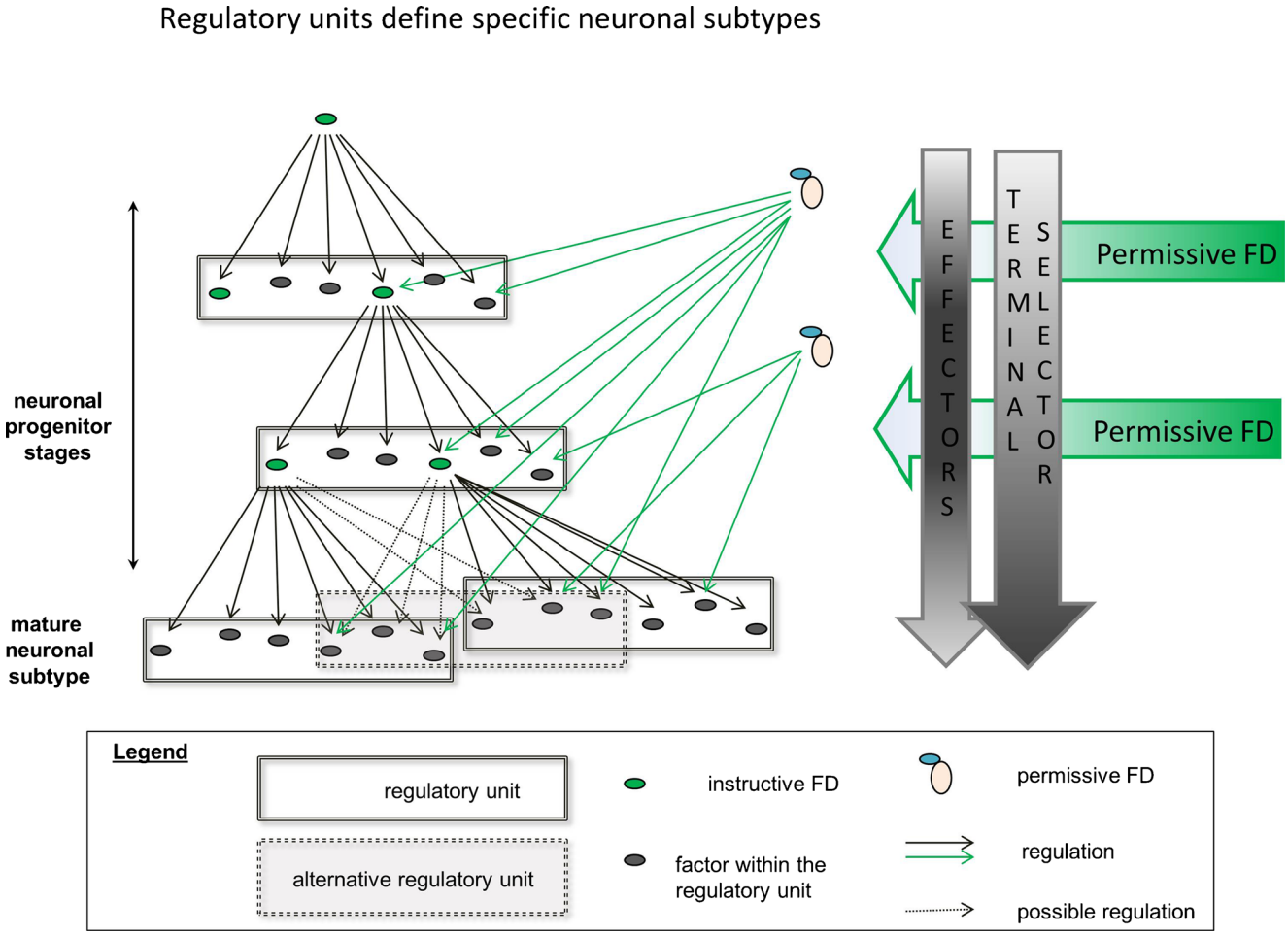

Fig. 1 Representation of the concept of executive and permissive fate determinants (*FD*) in neuronal differentiation. A hierarchy of executive fate determinants acts at various stages in the lineage including the effector genes and terminal selector genes at later stages of differentiation (*vertical*). Permissive fate determinants intersect this vertical progression horizontally allowing the activation of several specific regulatory units and thereby progression between the different neuronal subtypes. Note that the regulatory networks defining the various neuronal subtypes (alternative regulatory units) might share one or several factors

cell types (e.g., glia, fibroblasts, hepatocytes) into functional neurons (Amamoto and Arlotta 2014). This provides a unique setting to interrogate the transcriptional logic of neurogenesis within the very same transcriptional background. For example, GABAergic and glutamatergic neurons are generated in development in highly different regions of the brain. These regions are exposed to diverse signaling mechanisms during development, with sonic hedgehog in the ventral telencephalon generating GABAergic neurons and Wnts in the dorsal forebrain generating glutamatergic neurons (Imayoshi and Kageyama 2014; Schuurmans and Guillemot 2002). Thus, the key transcriptional regulators involved in GABAergic neuron specification, such as the transcription factors (TFs) Ascl1, Dlx, and Ptf, or glutamatergic neuron specification, such as Neurog1/2 and Tlx, act in highly different transcriptional and signaling environments (Fig. 2). Indeed, distinct signals, according to the position of neurogenesis, for example, along the rostro-caudal axis in the spinal cord, directly affect repressive chromatin marks to allow the generation of distinct neuronal subtypes (Mazzoni et al. 2013a, 2013b). In reprogramming, however, the respective TFs are introduced in the same cells in the same environment (either in vitro or in vivo) allowing a comparison of their transcriptional programs within an identical environment. Thus, direct reprogramming helps the probing of the transcriptional logic of the way to make a neuron, as one can determine the similarities and/or differences of the transcriptional program elicited by, for example, Ascl1 instructing GABAergic neurons versus Neurog2 instructing glutamatergic neurons in the very same cell type (Berninger et al. 2007a; Heinrich et al. 2010).

Conversely, direct reprogramming will profit from a better understanding of the transcriptional logic eliciting neurogenesis and specifying neuronal subtypes. The latter especially is still in its infancy in the reprogramming field as little is known about the neuronal subtypes elicited from diverse cell types. The main transmitter identity (glutamate or GABA) has been determined at best, but the specific

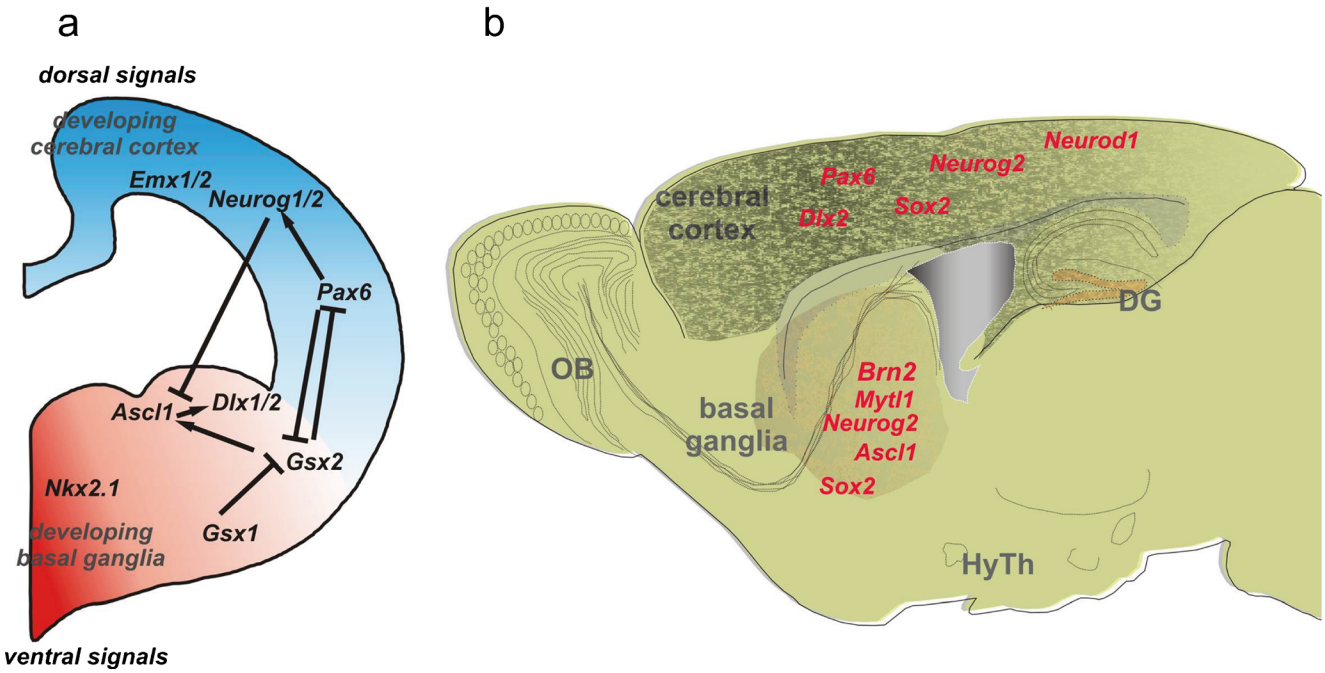

Fig. 2 Regulatory units operate in various environments during development and reprogramming in vivo. Representation of developing (**a**) and adult (**b**) mouse forebrain depicting the key factors for the establishment of neuronal subtype identity in development and factors used for reprogramming in the adult cerebral cortex and basal ganglia (*DG* dentate gyrus, *OB* olfactory bulb, *HyTh* hypothalamus)

subtypes of instructed neurons has not been addressed so far. Moreover, FDs for reprogramming have so far mostly been identified by trial and error testing candidates, but a molecular logic as to which of these might be particularly potent is largely still elusive. Pioneering factors are defined by their capacity to interact with chromatin even better than with naked DNA and, hence, to mediate the opening of the chromatin in order to set the transcriptional stage for a given lineage (Zaret and Carroll 2011). Thus, these factors are important in reprogramming; for example, Ascl1 has been suggested to act as a pioneering factor in the conversion of non-neuronal cells into neurons (Wapinski et al. 2013). However, beyond this suggestion, the molecular basis for the efficacy of most neurogenic FDs remains largely unknown. Is it those that are common neurogenic regulators shared between many different neuronal subtypes and hence eliciting pan-neurogenic features that would help to instruct neuronal identity particularly potent? Alternatively, or in addition, transcriptional regulators that are last in the lineage and determine all the mature hallmarks of a neuron might be best suited to instruct a neuron with a specific identity (Fig. 1). These TFs are referred to as terminal selector genes (Hobert 2008, 2011). Terminal selector genes regulate all the key hallmarks of a differentiated neuron, such as all aspects of specific glutamatergic neurons by homeodomain TFs (Cheng et al. 2004; Serrano-Saiz et al. 2013), of dopaminergic neurons by Ets/Dlx together with Pbx/Meis TF combinations (Agoston et al. 2014; Brill et al. 2009; Doitsidou et al. 2013), or of noradrenergic or serotonergic neurons by TFs of the Lim- or Pour homeobox family (Zhang et al. 2014). Thus, distinct combinations of TF classes not only instruct, but also maintain the defining features of a neuronal subtype, including dendritic and axonal branching and membrane properties (Deneris and Hobert 2014; Fig. 1). Likewise, the Znc finger TF Fezf2 acts as terminal selector gene for a subtype of glutamatergic projection neurons in the murine cerebral cortex, namely the cortico-spinal motor neurons, by direct regulation of many transmitter and projection hallmarks of these neurons (Lodato et al. 2014). However, so far, few of these terminal selector genes have been implicated in direct reprogramming, suggesting that they might need help from other FDs that can possibly be deduced from a better understanding of the basic principles of the molecular logic in neurogenesis. Direct reprogramming has also recently made great progress in vivo (Guo et al. 2014; Niu et al. 2013) and seems a promising avenue for the replacement of lost neurons after brain injury (Amamoto and Arlotta 2014; Dimou and Gotz 2014; Robel et al. 2011). Thus, we need to consider that transcriptional FDs elicit of

more (Guo et al. 2014) or fewer (Buffo et al. 2005; Grande et al. 2013; Heinrich et al. 2014; Niu et al. 2013) neurons in vivo in the context of the transcriptional logic underlying neurogenesis (Fig. 1).

The logic of neurogenesis can be best understood by comparing the principles of neurogenesis not only in various regions, but also at different stages. Neurogenesis occurs at markedly different stages in an organism, during the development of the brain during embryogenesis, when the brain functionally matures at early postnatal stages, and in the fully mature and eventually aging brain (Garthe et al. 2009; Garthe and Kempermann 2013; Ihrie and Alvarez-Buylla 2008; Imayoshi et al. 2008; Marin-Burgin et al. 2012; Peretto and Paredes 2014; van Praag et al. 2002). Importantly, at these later stages, neurogenesis also occurs in a region-specific manner, generating various neuronal subtypes from adult neural stem cells (NSCs) located in various regions and regulating the continuation of neurogenesis in a highly region-specific manner. In humans, as in most mammals (Curtis et al. 2011, 2012; Ernst et al. 2014; Knoth et al. 2010), postnatal and adult neurogenesis is restricted to a few regions in a highly species-specific manner, such as the striatum in rabbits and human (Ernst et al. 2014; Luzzati et al. 2006), but not in mice/rats, or the dentate gyrus in most species, but not in fruit-eating bats (Amrein et al. 2007), prompting intriguing questions about the needs and regulatory mechanisms of neurogenesis, especially in these specific brain regions. This is the same in other vertebrates in which adult neurogenesis occurs in most, but not all regions (Baumgart et al. 2012; Berg et al. 2010; Kroehne et al. 2011; Kyritsis et al. 2012). Thus, neurogenesis occurs in a time- and region-specific manner in the developing and adult brain.

This brings us back to the key questions about the logic of the molecular regulators of neurogenesis. Are there common hallmarks between neurogenesis in various regions and at different stages, and if so, what is the role of such common factors? Are these particularly important for neurogenesis normally and particularly potent when forcing neurogenesis? On the other hand, are the common molecular determinants setting the stage, possibly in a more permissive manner, to allow neuronal subtype-specific specification? We shall discuss these key questions first from the phenotypes observed after the manipulation of some of the common neurogenic factors and then consider the molecular logic extracted from the manipulation of neurogenic FDs in endogenous neurogenesis for direct reprogramming.

Neuronal differentiation—FDs acting in a sequential and hierarchical manner to specify neuronal subtypes

Neurogenic FDs are proteins (often TFs) that are expressed in the neurogenic lineage and are important for neuronal differentiation from the neural stem or progenitor cells (Imayoshi and Kageyama 2014). A rather broad distinction can be made between neurogenic FDs with an extremely wide-spread expression and importance in many brain and regions of the central nervous system (CNS) and at many if not all developmental and postnatal stages, as opposed to those that are expressed and are relevant in more specific regions. For example, the homeobox TF Pax6 or the Sry-box containing TFs Sox1-3 are present in many regions and are involved in the generation of many different neuronal subtypes (see below), whereas the T-box TF Tbr2 is expressed and functionally important apparently only in glutamatergic neuron lineages, such as in the developing cerebral cortex including the hippocampus and dentate gyrus (Hevner et al. 2006; Hodge et al. 2008, 2012a, 2012b; Kowalczyk et al. 2009). Likewise, the proneural bHLH TFs Neurog2 is restricted to specific neuronal subtype lineages (e.g., the glutamatergic lineage up-stream of Tbr2 in the developing cerebral cortex), whereas the proneural factor Ascl1 is expressed and important in other lineages, such as the GABAergic neurons in the ventral telencephalon (Castro et al. 2011; Guillemot 2007; Guillemot et al. 2006; Kim et al. 2011a; Parras et al. 2002, 2004; Schuurmans and Guillemot 2002). The functional relevance of these factors in their respective lineage has been shown by both gain- and loss-of-function experiments (Fode et al. 2000; Hodge et al. 2012b; Parras et al. 2002). Indeed, the deletion of Neurog2 in the developing cerebral cortex reduces the number of glutamatergic neurons (Fode et al. 2000; Parras et al. 2002). Interestingly, this role seems to extend to adult neurogenesis, as Neurog2 and Tbr2 are still confined to the subtypes of adult progenitor cells involved in the generation of glutamatergic neurons (Brill et al. 2009; Hodge et al. 2012b) and promote the generation of glutamatergic neurons after overexpression in progenitors isolated from the adult subependymal zone (Brill et al. 2009). Moreover, the intermediate progenitors (Tbr2+ progenitors in the normal situation) fail to induce the Tbr2 downstream targets, including the next set of FDs in the lineage, such as FoxG1, Prox1, or Tbr1 (Hodge et al. 2012a) after loss of Tbr2 function. Consistent with their function, these FDs could be classified as executive FDs, as they induce the expression of genes instructing progenitor differentiation and without which the lineage progression is blocked and cells often succumb to cell death (Hodge et al. 2008, 2012a, 2012b). An important feature of the executive FDs is their sequential expression (Fig. 1; Schuurmans et al. 2004; Schuurmans and Guillemot 2002) suggesting that each of these factors controls the next set of downstream targets necessary for proper neuronal differentiation. Therefore, the molecular network of these FDs instructing various aspects of a given neuronal phenotype has a hierarchical structure (Fig. 1) for the establishment of the appropriate neuronal

subtype in a sequential manner ultimately governing neuronal subtype identity characterized by a specific set of ion channels, neurotransmitters, etc.

Importantly, these regulatory units might not be mutually exclusive. Indeed, phenotype switches between neuronal subtypes imply some common regulatory networks diverging only at the end of neuronal subtype specification. This is supported by direct conversion, including the electrophysiological properties and efferent projections (De la Rossa et al. 2013), of layer II/III callosal projection neurons into layer V corticospinal neurons after the overexpression of the terminal selector TF Fezf2 (Lodato et al. 2014; Molyneaux et al. 2005; Rouaux and Arlotta 2010, 2013). Importantly, this inter-conversion between particular neuronal subtypes (neuron-to-neuron reprogramming) is possible only in an extremely narrow developmental window, suggesting that the overexpressed FD requires a specific state of neurogenic regulatory networks, redundantly active in callosal and corticospinal projection neurons. Thus, common neurogenic regulatory networks allow cells to differentiate into a common class of neurons, e.g., into a cerebral cortex glutamatergic projection neuron, with separate regulators then specifying the layer-specific subtype of the glutamatergic pyramidal neurons (Greig et al. 2013). These experiments support the concept of some redundant regulatory units being common to several different lineages that are ultimately fixed into one specific neuronal subtype by the terminal selector gene which acts last, i.e., hierarchically low in the lineage (Fig. 1). This concept further implies that every cell type is defined by a specific set of regulatory units, whereas a single regulatory unit can be shared by one or several cell types (Serrano-Saiz et al. 2013; Zhang et al. 2014).

In this context, the regulation of survival is important. An increasing number of examples highlight a role of the TFs that act at earlier stages in the lineage but are then maintained in expression throughout adulthood in regulating the survival of the differentiated neurons. This is, for example, the case for the TF Pax6 in the dopaminergic neurons of the olfactory bulb and for the TF engrailed in midbrain dopaminergic neurons, which are, in both cases, critical for the survival of these fully mature neurons (Alberi et al. 2004; Brill et al. 2008; Hack et al. 2005; Ninkovic et al. 2010; Sonnier et al. 2007). Importantly, the gene *pitx* specifies and maintains the identity of serotonergic neurons in planarians (März et al. 2013), suggesting that this principle is conserved throughout the animal kingdom. An intriguing possibility is that, during neuronal network formation, survival is often regulated by extrinsic factors tuning the number of neurons to the need of the emerging network (Southwell et al. 2012), whereas neuronal survival in the adult brain is regulated by intrinsic factors.

Permissive FDs horizontally intersect the network of executive FDs to allow progression between different neuronal subtypes

As described above, the generation of specific different neuronal subtypes is organized in time with the sequential generation of increasingly specific regulatory networks. However, how can this transcriptional hierarchy then explain the sequential generation of different neurons? For example, in the developing cerebral cortex, the generation of deep layer neurons precedes the generation of the upper layer neurons (Greig et al. 2013; Molyneaux et al. 2007). This requires additional FDs that retain the potential for the differentiation of the various neuronal subtypes (permissive FDs). Transplantation experiments have, for example, shown that the neuronal progenitors isolated from the early stages of cerebral cortex development and transplanted into later stage cerebral cortex adopt the fate of the new environment and generate the late, upper layer neurons (Desai and McConnell 2000). This shows that they have the potential to generate upper layer neurons while they are normally still engaged in the generation of the lower layer neurons. Thus, some factors are permissive for upper layer neuron fate, even during the time when lower layer neurons are generated (Molyneaux et al. 2007). Indeed, such factors might be neurogenic FDs, such as Pax6, that are expressed at early stages of cerebral cortex development. Pax6 is important for neurogenesis in various regions of the CNS (Ericson et al. 1997; Heins et al. 2002; Stoykova et al. 2000) and at various stages of development reaching even into adulthood (Hack et al. 2005). Interestingly, Pax6 forms a functional regulatory complex with the BAF (BRG1- or HRBM-associated factors) chromatin remodeling factors at both developing and adult stages (Ninkovic et al. 2013; Tuoc et al. 2013a, 2013b). The complex of Pax6 with BAF has been shown to be important for the specification of the intermediate progenitors at the onset or mid-neurogenesis to generate the appropriate pool of the upper layer neurons in the developing cortex (Berger et al. 2007; Pinto et al. 2009; Tarabykin et al. 2001; Tuoc et al. 2009, 2013a, 2013b). Importantly, the Pax6/BAF complex modulates the capacity of the Pax6/REST co-repressor complex to bind Pax6 targets (Tuoc et al. 2013a) suggesting a transient transcriptional regulation inhibiting alternative fates. Thus, one way of viewing Pax6 function in this context is as a permissive FD suppressing alternative fates when they are not appropriate. Therefore, the molecular network of the FDs involved in the neuronal specification is not only a linear hierarchical structure with sequential modules being activated, but also a complex interface of permissive FDs intersecting the temporal progression of the executive FDs as illustrated in Fig. 1.

Adult neurogenesis

Indeed, the concept of Pax6 as a permissive FD allowing neurogenesis and the acquisition of various neuronal subtype identities to occur is further substantiated in adult neurogenesis. A major difference between NSCs during development and in adulthood is that the former express high levels of neurogenic FDs, such as Pax6, Dlx factors, and Arx (Bibel et al. 2004, 2007; Heins et al. 2002; Pinto et al. 2008; both permissive or executive), whereas the later adult NSCs express such low levels of neurogenic FDs that the respective proteins are largely undetectable in NSCs but only appear later in the lineage (Beckervordersandforth et al. 2010; Brill et al. 2009; de Chevigny et al. 2012; Hack et al. 2005; Ninkovic and Gotz 2013; Ninkovic et al. 2013). When Pax6 is deleted in adult neurogenesis, this results in the conversion of neuronal progenitors into glial cells (Ninkovic et al. 2013), whereas this is not the case upon Pax6 deletion during development when impairments to differentiate into neurons result in an increase in stem and progenitor numbers or neuronal cell death (Bibel et al. 2004; Gotz et al. 1998; Mi et al. 2013; Nikoletopoulou et al. 2007). These data further support the concept that the default lineage in the adult brain is (oligodendro)gliogenesis, whereas neurogenesis is the default stage during most embryonic development (Ninkovic and Gotz 2013). Moreover, even when Pax6 is deleted at late stages in the adult neuroblast lineages, these progenitors still convert into glial cells (Ninkovic et al. 2013), demonstrating that Pax6 is required throughout the lineage to allow progression along the neuronal lineage. It does so by activating a cross-regulatory effector network of neurogenic factors directly promoting neuronal differentiation, namely Pou3f2/4, Sox4/11, and Nfia/b (Ninkovic et al. 2013). Thus, Pax6 establishes the permissive chromatin state to allow the executive FDs (effector and terminal selector genes) to specify neuronal fate in a molecular logic similar to that of development (Fig. 1; Hodge et al. 2012a).

Importantly, however, key differences are apparent in the molecular logic of setting up the permissive chromatin state in adult versus developmental neurogenesis, and this is related to the glial nature of the adult NSCs. In the developing brain, the permissive FDs help to sustain alternative fates (upper vs. deeper cortical neurons, for example), whereas in the adult brain, the gene expression of neurogenic FDs that have been epigenetically silenced during gliogenesis at early postnatal stages has to be activated (Hirabayashi et al. 2009). Interestingly, the silencing appears to differ in fully differentiated glial cells such as astrocytes from the brain parenchyma and adult neural stem cells that resemble astrocytes in many ultra-structural, cell biological aspects and in their genome-wide expression profile (Beckervordersandforth et al. 2010). Despite their many similarities, fully differentiated astrocytes residing in the brain parenchyma do not exhibit neurogenic priming (low level of expression of neurogenic FDs), whereas NSCs do (Ninkovic and Gotz 2013). Intriguingly, however, parenchymal astrocytes can re-activate, at least in part, low level expression of permissive neurogenic FDs after brain injury (e.g., Pax6 in the cerebral cortex [Sirko et al. 2009] or Ascl1 in the striatum [Magnusson et al. 2014]) or after the deletion of inhibitory factors, such as the Notch signaling mediator Rbpjk (Magnusson et al. 2014). Taken together, these data suggest that an important role of the permissive FDs in adult neurogenesis is to open the chromatin by interacting with chromatin remodeling factors, such as the BAF complex (Feng et al. 2013; Ninkovic et al. 2013), to allow additional factors access to these sites and the up-regulation of executive neurogenic FDs. This is supported by the phenotype that is observed after the inducible genetic deletion of the ATPase subunit Brg1 of the BAF complex interacting with Pax6 and that also results in the conversion of adult neurogenesis to gliogenesis, because of the failure to increase the cross-regulatory neurogenic executive FDs, just as happens after the deletion of Pax6 (Ninkovic et al. 2013). The data therefore support the concept that Pax6 interacting with the BAF complex in adult neurogenesis acts as a permissive factor setting up the appropriate state of the chromatin necessary for progression along the neuronal lineage, thereby acting on a broad range of gene sets in the lineage (Ninkovic et al. 2013). As many neurogenic FDs are epigenetically silenced toward the end of neurogenesis, complexes containing the permissive FDs and the chromatin remodeling factor engage in the activation of these loci (Feng et al. 2013; Lim et al. 2009; Ninkovic et al. 2013; Wang et al. 2011) and the establishment of the neuronal lineage-specific regulatory networks. Moreover, the deletion of Brg1 also results in the reduced expression of members of the polycomb complex (Ninkovic et al. 2013) responsible for the repression of the alternative glial lineage (Hack et al. 2005; Hirabayashi et al. 2009) and could be, in part, responsible for the fate change observed after the genetic deletion of Pax6 (Ninkovic et al. 2013). Thus, permissive FDs in adult neurogenesis not only allow neurogenesis to proceed by opening the chromatin at neurogenic target gene sites, but also activate repressors of alternative fates, namely glia.

Conversely, the effect of executive neurogenic FDs that are activated by Pax6 (Ninkovic et al. 2013) in adult neurogenesis is different. For example, the deletion of the effector genes Sox4 and Sox11 (Mu et al. 2012) or Tbr2 (Hodge et al. 2012b) does not result in fate conversion to glial cells, but rather failure to differentiate fully into mature neurons. Thus, these FDs act as executers of the neurogenic program (as effector or terminal selector genes), whereas permissive FDs allow the program to occur and suppress alternative programs (directly or indirectly).

Notably, the definition of the FD as an executive or permissive FD is context-dependent, and the same FD, such as

Pax6, can act at various stages as a permissive or executive FD. Pax6 is also key for the specification of periglomerular dopaminergic neurons (Hack et al. 2005; Kohwi et al. 2005) through the interaction with Dlx2 and Meis2 later in the lineage when neurons differentiate into neuronal subtypes in the olfactory bulb (Agoston et al. 2014; Brill et al. 2008). In this case, interference with Pax6, Dlx2, or Meis2 or their target gene activation does not convert neuroblasts to glia but decreases the differentiation and survival of dopaminergic neurons in the olfactory bulb (Brill et al. 2008; Ninkovic et al. 2010). Interestingly, for the function in neuronal survival, Pax6 does not require interaction with the BAF chromatin remodeling complex (Ninkovic et al. 2013). A similar dual or multiple role also seemingly emerges for Ascl1, which can act not only very early in the lineage as a pioneer factor, but also later specifying neuronal subtypes in a rather more instructive manner (Schuurmans et al. 2004; Schuurmans and Guillemot 2002; Wapinski et al. 2013).

Direct fate conversion—same or different transcriptional logic?

The above-described process of neurogenesis and neuronal differentiation is a sequence of progressive restrictions in the potency of the NSC and progenitors to establish a specific set of regulatory units, associated with progressive epigenetic changes. The epigenetic mechanisms, therefore, fix the established regulatory units not only in the final state, but also at the progenitor stages (Ninkovic et al. 2013) defining the point of "no return" in the lineage. According to the epigenetic landscape model, the restrictive barriers are established during lineage progression in order to restrict more differentiated cells into their specific fate. Epigenetic mechanisms do indeed regulate the temporal progression of neuronal subtypes (Tan et al. 2012) consistent with their cooperation with the permissive FDs (Fig. 1). Importantly, recent advances in the stem cell field have demonstrated that cells can also go "up-stream" within their epigenetic landscape, from differentiated to more undifferentiated stem cell fates, a process referred to as reprogramming (Takahashi and Yamanaka 2006). Moreover, differentiated cells can also trans-differentiate, i.e., changing their fate by a direct route from one epigenetic valley to the other without returning to the start of the valley in a bi-potent progenitor state (Heins et al. 2002; Marro et al. 2011; Vierbuchen et al. 2010). This process of tunneling through the epigenetic barrier is referred to as direct lineage reprogramming (direct conversion).

Direct fate conversion into functional neurons was originally achieved first from postnatal glia (Heins et al. 2002) and myoblasts (Watanabe et al. 2004) by the overexpression of a single factor, namely Pax6 or REST-VP16, respectively. However, the permissive FD Pax6 was less efficient at stimulating maturation into neuronal subtypes (Heins et al. 2002; Ninkovic et al. 2013), whereas this can be achieved by the executive FDs Neurog2 or Ascl1 and Dlx2 (Berninger et al. 2007b; Heinrich et al. 2010). Thus, a single FD is sufficient to drive postnatal astrocytes into fully functional neuronal subtypes, attaining electrophysiological and immunohistochemical characteristics of glutamateric neurons with fully functional glutamateric synapse formation upon transduction with Neurog2, whereas transduction with Ascl1 and/or Dlx2 leads to a full GABAergic neuronal subtype identity (Berninger et al. 2007a, 2007b; Heinrich et al. 2010). Thus, the final neuronal subtype can be predicted based on the function of the FD in the process of neuronal differentiation (Amamoto and Arlotta 2014; Heinrich et al. 2012). Interestingly, the cell of origin also appears to determine the neuronal subtype generated, as Ascl1 converts fibroblasts into somatic motor neurons (Liu et al. 2013), whereas its combination with additional factors, Brn2 and Myt1L, turns these instead into glutamatergic neurons of a subtype with an identity that is as yet unknown (Vierbuchen et al. 2010). Indeed, the cell of origin and signals from the environment, e.g., various injury conditions, influence neuronal subtype identity during the conversion of non-neuronal cells resident in the brain in vivo into neurons (Guo et al. 2014; Vierbuchen et al. 2010). Although the permissive FD Pax6 achieves a rather limited number of neurons from adult glial cells in vivo (Buffo et al. 2005; Kronenberg et al. 2010), the instructive factors Neurog2 and Ascl1 are more efficient (Grande et al. 2013) and are most efficient in combination with other factors, such as Brn2 and Myt1L (Torper et al. 2013) or Sox2 (Heinrich et al. 2014; Karow et al. 2012; Niu et al. 2013). Interestingly, a late executive TF, namely NeuroD1, seems to be most efficient in converting glial cells into neurons in the adult mouse cerebral cortex, at least in the healthy brain and under non-invasive injury conditions (Guo et al. 2014; Fig. 2b).

Taken together, the described work allows further insights into the transcriptional logic and the regulatory unit model described above. First, the more different from the neuronal fate or the more differentiated (adult versus postnatal versus embryonic) the cell of origin is, the more FDs seem to be needed to achieve the proper set of regulatory units defining the specific cell type. Second, the combination of three FDs (Brn2, Ascl1, Myt1L) sufficient to convert embryonic fibroblasts into functional neurons comprises FDs acting at various stages in the hierarchical model combined with a factor having pioneer activity in opening closed chromatin sites (Ascl1; see Wapinski et al. 2013) and with FDs acting later in the lineage as effector genes (Brn2; Ninkovic et al. 2013) in

an instructive manner. Indeed, Brn2/Pou3f2 is part of the neurogenic effector network in adult neurogenesis and is important for the stabilization of neuronal differentiation (Ninkovic et al. 2013). Least is known about Myt1l, which is expressed late in differentiating neurons and might be helpful in instructing late neuronal hallmarks.

Interestingly, Brn2 and Myt1l are rather widespread in neurogenesis and might thus qualify as common neurogenic factors involved in neurogenesis in many regions at various stages. This prompts the concept that executive FDs that are involved in many neuronal subtype lineages are particularly potent in direct reprogramming, a testable prediction. Moreover, the observation that these FDs are active at various hierarchical nodes suggests a possibly important principle, namely, that efficient direct conversion can be achieved best if a set of regulatory units that are compatible with each other is activated. Therefore, direct conversion is a two-step process consisting of the activation of the broader spectrum of regulatory units and the selection of the compatible active regulatory units that are then fixed by the epigenetic mechanisms necessary for reprogramming. This might indeed explain the higher potency of the hierarchically low executive FD to induce the fate conversion compared with the either permissive or hierarchically high executive FD (Heinrich et al. 2010; Mu et al. 2012; Ninkovic et al. 2013). However, these factors might not be able to open closed chromatin sites, an event that can be achieved only by pioneer and permissive TFs. These not only activate a higher number of the regulatory units, but also increase the probability of activation of one or several incompatible regulatory units that cannot be epigenetically fixed. These considerations predict the best efficiency by combining the right combination FDs acting in a pioneer/permissive fashion with those acting at relatively late stages in an instructive manner. This combination is also present in the TF cocktail containing Ascl1, Nurr1, and Lmx1a used to convert fibroblasts to dopaminergic neurons (Caiazzo et al. 2011; Pfisterer et al. 2011). However, only the larger cocktail containing Ascl1, Pitx3, Lmx1a, Nurr1, Foxa2, and En1 directly converts fibroblasts into functional dopaminergic neurons able to alleviate some symptoms upon transplantation into animal models of Parkinson's disease (Kim et al. 2011b). The comparison of the last conversion cocktails with the BAM cocktail stresses the necessity of activating at least some aspects of progenitor regulatory units by Ascl1 in combination with the late regulatory units. This principle of the necessity for the establishment of compatible regulatory units is further substantiated by experiments with the microRNAs miR-9/9* and miR-124 increasing the efficiency of the direct conversion by NeuroD2, Ascl1, and Mytl1 (Yoo et al. 2011). As these microRNAs regulate a number of factors involved in neuronal differentiation, their anticipated function during reprogramming should be in the selection of the compatible regulatory units activated by the FD.

Closing remarks

The above considerations not only highlight our increased understanding of the molecular regulation of neurogenesis, but also shed light on areas of a lack of understanding. In particular, the molecular basis of direct reprogramming is still poorly understood. Here, we propose a model encompassing TFs acting in a hierarchical and instructive manner progressing via effector genes and terminal selector genes (that we refer to as executive factors) to the final differentiated neuronal subtype fate. These are intersected by permissive factors that allow different instructive modules to act and hence to allow the generation of neuronal subtypes in regional or temporal sequences during development (Figs. 1, 2). Although the only study monitoring transcriptional events during direct neuronal reprogramming so far (Wapinski et al. 2013) has suggested a hierarchical mode of reprogramming with pioneer TFs at the tip of the hierarchy, much remains to be done. First, the early events in direct neuronal reprogramming have never been addressed, as the above study by Wapinski et al. (2013) started 48 h after transduction, and the events underlying the generation of the different neuronal subtypes in reprogramming have not yet been investigated. An understanding of the extent to which neuronal conversion indeed occurs in a hierarchical manner is also important during reprogramming and development and by direct progression towards distinct neuronal subtypes. The isolation of as many intermediate stages as possible during the process of conversion and the identification of the regulatory units present at the consecutive stages are also important, as is their correlation to the regulatory networks active during the differentiation of particular neuronal subtypes from the neural stem cells. Such experiments will not only resolve the basic principles of the direct conversion, but also ultimately identify the selection of the appropriate combination of regulatory networks that need to be simultaneously active in order to establish the most efficient and timely conversion between the various cell types. Thus, an understanding of the transcriptional logic in endogenous and forced neurogenesis will not only allow insights into key mechanisms of fate determination, but will also pave the way to more effective cell replacement strategies.

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