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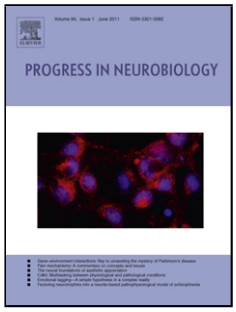

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Directing neuronal cell fate *in vitro*: achievements and challenges

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Highlights

- Recent advances in directed neuronal differentiation and reprogramming of pluripotent stem cells (PSCs) and somatic cells currently allow to obtain and study functional human neurons *in vitro* for both scientific and medical purposes.
- Chemical stimulation with morphogens, growth factors and small molecules for *in vitro* differentiation of neurons recapitulates the neurodevelopmental patterning principles that are observed in the early embryo.
- Overexpression of key-lineage specific transcription factors allow to induce dramatic changes in transcriptional networks that drive cellular conversions towards neurons.
- Chromatin modifiers, epigenetic editing and RNA interference (RNAi) allow to induce profound epigenome remodelling processes that underlie cell fate switches.
- Although various challenges remain to be overcome in order to realise their full potential, the availability of human neurons in *in vitro* has critical implications for future brain-related studies and the development of therapeutic interventions for many brain disorders.

Abstract

Human pluripotent stem cell (PSC) technology and direct somatic cell reprogramming have opened up a promising new avenue in the field of neuroscience. These recent advances allow researchers to obtain virtually any cell type found in the human brain, making it possible to produce and study functional neurons in laboratory conditions for both scientific and medical purposes. Although distinct approaches have shown to be successful in directing neuronal cell fate *in vitro*, their refinement and optimization, as well as the search for alternative approaches, remains necessary to help realize the full potential of the eventually derived neuronal

populations. Furthermore, we are currently limited in the number of neuronal subtypes whose induction is fully established, and different cultivation protocols for each subtype exist, making it challenging to increase the reproducibility and decrease the variances that are observed between different protocols. In this review, we summarize the progress that has been made in generating various neuronal subtypes from PSCs and somatic cells, with special emphasis on chemically defined systems, transcription factor-mediated reprogramming and epigenetic-based approaches. We also discuss the efforts that are being made to increase the efficiency of current protocols and address the potential for the use of these cells in disease modelling, drug discovery and regenerative medicine.

Keywords

Neuronal differentiation; pluripotent stem cells; somatic cells; directed differentiation; cellular reprogramming; transdifferentiation; brain disorders; disease modelling; epigenetics.

1. Introduction

Over the last decades, our general knowledge on human brain functions has grown exceedingly thanks to the availability of animal models and human brain tissue. Although their utility is undeniable, both are challenged with limitations that have been impeding progress in gaining complete mechanistic insights, as well as in the development of therapeutic interventions for many brain disorders (Nestler and Hyman, 2010). The numerous transgenic animal models that have been established mimic pathological mechanisms of the human brain to some extent, but they do not yet satisfactorily capture human disease phenotypes completely (Nestler and Hyman, 2010). An animal is obviously not a human being and interspecies differences might, therefore, be critical factors underlying the failure of translating a wealth of preclinical findings into clinical implementations (Akhtar, 2015; Mak *et al.*, 2014). Aside from the distinct (epi)genetic backgrounds, there are major physiological differences that could affect the development of disease phenotypes or differentially affect drug mechanisms, leading to misinterpretation of experimental findings. Human brain tissue samples, on the other hand, can generally only be obtained post-mortem, which complicates the study of disease aetiology and progression, since they simply do not allow to discriminate between cause and consequence of the disorder (Lewis, 2002). Cellular *in vitro* model systems, however, have the potential to overcome this latter challenge, owing to the possibility of manipulating the (epi)genetic architecture, as well as the environmental exposome, in culture conditions, which allows to study cause-effect relationships of pathological hallmarks in a controlled setting. Unfortunately, many early *in vitro* model systems have heavily relied on a combination of non-neuronal human cell lines, primary rodent cultures and transgenic rodent cell lines, which, similar to animal models, also all exclude the human neuronal (epi)genomic and phenotypic context (Badger *et al.*, 2014).

In view of the aforementioned limitations and the unmet necessity of finding therapeutic interventions for brain disorders, the recent availability of human pluripotent stem cell (PSC) technology and somatic cell reprogramming has offered new opportunities for human brain-related studies (Pasca *et al.*, 2014; Shi *et al.*, 2016). PSC technology is an umbrella term that encompasses both embryonic stem cells (ESCs) and induced PSCs (iPSCs) (Yap *et al.*, 2015). Aside from being derived from humans, the distinct advantages that these cells have are their unlimited proliferative capacity and the ability to differentiate towards virtually any cell type, including specific neuronal subtypes (Haston and Finkbeiner, 2016). These characteristics equip PSCs with the unique feature to provide a theoretically inexhaustible and replenishable source of cells *in vitro*. Somatic cells on the other hand, albeit restricted by their limited proliferative potential, can be obtained relatively easy from healthy individuals and patients, and can then be directly reprogrammed towards desirable neuronal subtypes in a comparative, but faster framework (Hou and Lu, 2016). Consequently, PSCs, somatic cells and their differentiated progeny can nowadays be used to model disease mechanisms in a humanized-setting, where they allow for the investigation of unique human cellular and molecular features in a cell-specific and personalised matter (Riemens *et al.*, 2017). In fact, we are currently in the middle of an exciting era where human PSC and somatic cell reprogramming studies are contributing to the understanding of underlying neurobiological processes, as well as the consequences of personal molecular variations on the development and course of brain disorders (Hou and Lu, 2016; Young-Pearse and Morrow, 2016). In addition, iPSC- and somatic cell-derived neuronal populations provide a platform for high-throughput drug screening and toxicity testing in an upcoming epoch of personalised medicine, which assists the production of therapeutic interventions and might at the same time provide cues for therapeutic resistance (Riemens *et al.*, 2017). Furthermore, such neuronal populations also

harbour therapeutic potential in the field of regenerative medicine, since they might be used for transplant therapies (Riemens *et al.*, 2017).

Given the heterogeneity of neuronal cells found in the human brain and the complex interactions between them, one of the main opportunities PSC technology, directed differentiation and somatic cell reprogramming are offering, is the study of multicellular neural cultures organized in a manner reminiscent to what is seen in distinct anatomical structures of the brain. Such a method offers a promising approach to study higher-order neuronal networks during development and allows studying single neuron connectivity, as well as various other cellular interactions found in the neuronal niche, such as those between glial cells and endothelial cells (Canfield *et al.*, 2017; Kirwan *et al.*, 2015). Another major focus in the scientific community that emerges from the unique contributions each of the neuronal subtypes have in specific brain functions and disease, has been to obtain and study pure populations of neurons free of other subtypes. Derivation of such homogenous populations is especially important for the study of disease-associated neurons, where confounding effects of other subtypes should be avoided, such as in transcriptomic- and epigenetic profiling for example. Furthermore, the acquisition of protocols that can produce highly pure populations of neurons can in turn be utilized to customize the features of a multicellular culture. Successfully directing differentiation and cellular reprogramming *in vitro* has, therefore, been an area of intense research during the past two decades (Mertens *et al.*, 2016). Many strategies have been explored utilizing mouse and human PSCs, as well as somatic cells, to generate anatomically specified neural precursor cells (NPCs) and differentiated neuronal subtypes (See Figure 1). As a definition used here, NPCs refer to the mixed population of cells consisting of all undifferentiated progeny from neural stem cells, therefore including both neural stem cells and neural progenitor cells. Altogether, these differentiation methods try to recapitulate the multistep processes of neural development that

occur in the early embryo (Mertens *et al.*, 2016). Thus, an improved understanding of developmental signalling pathways and gene regulatory networks has guided the design of neuronal differentiation and cellular reprogramming strategies (Yap *et al.*, 2015). Although significant advances have been made, *in vitro* neuronal differentiation is actually not a process that is fully disciplined yet, as very often other cell types are produced in parallel to what was first intended by the researcher. Furthermore, differentiation protocols for multiple neuronal subtypes remain either unestablished or unstable, or they do not allow to obtain the terminally differentiated neuron with its entire functional maturated characteristics (Maroof *et al.*, 2013; Zhang *et al.*, 2013).

To date, the majority of directed neuronal differentiation and direct somatic cell reprogramming protocols involve chemical stimulation through patterning cues or the use of ectopic overexpression of lineage-specific transcription factors that are known for their genuine involvement during neural development (Denham and Dottori, 2011; Erceg *et al.*, 2008; Vierbuchen *et al.*, 2010). Although it is currently accepted that these networks of patterning molecules and transcription factors orchestrate neuronal induction and differentiation, it is becoming more and more evident that cell-intrinsic mechanisms are also key players within these circuits (Gifford *et al.*, 2013; Lunyak and Rosenfeld, 2008; Qin *et al.*, 2016). Neuronal induction and differentiation generally rely on the interplay of activation and inhibition on multiple developmental signalling pathways tightly controlled by the epigenetic machinery (Imamura *et al.*, 2014; Yap *et al.*, 2015). Within these networks, the epigenetic machinery is essential for fine-tuning genetic programs that coordinate distinct developmental processes, as well as shaping the neuronal identities at a phenotypic resolution (Feng *et al.*, 2007; Fitzsimons *et al.*, 2014). With the current advances in epigenetic editing (Kungulovski and Jeltsch, 2016; Liu *et al.*, 2016; Thakore *et al.*, 2016) and RNA interference (RNAi) (Low *et al.*, 2012; Stappert *et al.*, 2013), an alternative strategy for directed neuronal

differentiation and direct somatic cell reprogramming has become available. Such approaches allow the design and construction of novel specific artificial epigenetic pathways or the redesign of existing endogenous molecular systems, in order to intentionally change epigenetic information at desired loci (Jurkowski *et al.*, 2015). Those systems can now be used as an additional tool to guide neuronal cell fate *in vitro* and also allow to address fundamental questions concerning the role of epigenetics in assigning neuronal cell fates.

In the present review, we will provide an overview on the progress made in generating various neuronal subtypes from PSCs and somatic cells *in vitro*. We will dissect the current used chemically defined systems, transcription factor-mediated reprogramming methods and epigenetic-based approaches. Furthermore, we will discuss the efforts that are being made to increase the efficiency of current protocols and highlight the potential for the use of this platform in disease modelling, drug discovery and regenerative medicine.

2. Chemically defined systems

2.1 Neural induction and differentiation

Research to develop protocols for the differentiation of PSCs into clinically relevant cell subtypes has progressed at a rapid pace. Hundreds of protocols have become available that allow to obtain early NPCs and eventually the derivation of desired neuronal populations (Chambers *et al.*, 2009; Erceg *et al.*, 2009; Erceg *et al.*, 2010; Karumbayaram *et al.*, 2009; Shi, 2013; Ying *et al.*, 2003). In the classical embryoid body (EB) method, scientists let PSC form self-assembling aggregates from dissociated suspension cultures, known as EBs (Denham and Dottori, 2011; Karanfil and Bagci-Onder, 2016). This method initially aimed to spontaneously differentiate the PSCs into mixed cellular populations encompassing the three germ-layers (Itskovitz-Eldor *et al.*, 2000). As PSCs readily differentiate into many different cell types, the efficiency of neuronal conversion is limited and culture media that enhances

neuronal production, as well as further selection procedures, are usually necessary to increase the homogeneity of a specific neuronal subpopulation (Abranches *et al.*, 2009). The second method to reconstitute neural commitment *in vitro* and to achieve efficient neural induction from PSCs has relied upon adherent monolayer culture differentiation, which eliminates the use of multicellular aggregations (Chambers *et al.*, 2009; Shi, 2013; Ying *et al.*, 2003). In this method, PSCs are dissociated into single cells and further cultured with conditioned media that also enhances neuronal conversion. Various aspects of the monolayer differentiation protocol have been extensively studied, adapted and optimized recently by different research groups, currently allowing neural induction within a few days (Günther *et al.*, 2016; Yan *et al.*, 2013). The third method to promote neural induction from PSCs can be achieved by co-cultivation of PSCs with stromal cell feeder layers (Kawasaki *et al.*, 2000; Shi, 2013). Co-culturing PSCs with other cell types is based on the idea that these surrounding cells provide cues that assign cell fates along the neural lineage. The use of stromal feeder layers is, however, only an efficient PSC differentiation strategy for certain neuronal subtypes, such as dopaminergic neurons (Lim *et al.*, 2015). Additional efforts on optimising previous methods have recently also lead to the establishment of a combinatory approach for PSC differentiation that is characterised by a chemically transitional EB-like state (Fujimori *et al.*, 2017). A low density monolayer culture on a feeder is differentiated under appropriate culture conditions and induces intermediate progenitor cells with the capability of differentiating into the three germ layers. The main advantage of this approach is that it harbours a reduced innate differentiation propensity of PSCs, even if the PSCs are known for their unfavourable differentiation (Fujimori *et al.*, 2017).

Although different strategies for neuronal conversion in PSCs have been explored, the general trend moves towards stromal-free methods combined with chemically defined culture systems. These chemically defined systems utilize culture media that are supplemented with

patterning molecules, such as morphogens and growth factors, which force enrichment of the desired neuronal cells. Additionally, an increasing number of studies have also illustrated the use of small molecules and emphasized their significant benefits for neural induction and differentiation in PSCs, as well as for somatic cell reprogramming (Zhang *et al.*, 2012). Aside from providing positive signals to induce neuronal fates, many of these factors also inhibit signalling pathways that control the differentiation into other germ layers then the ectoderm. Of note, just as *in vivo* embryonic development can be broken down into distinct stages where distinct patterning molecules are required to induce neuronal cell fates, so too can the *in vitro* specification of differentiating neuronal population from PSCs (Davis-Dusenbery *et al.*, 2014). The compositions of the chemically defined media, as well as the compound concentrations at any given time point, can, therefore, significantly redirect the anatomical and functional identity of the differentiating cells.

Neuronal conversion follows the neural induction principle that is first initiated by removing medium components that promote self-renewal, which on itself is sufficient to trigger differentiation towards all the three embryonic germ layers (Tao and Zhang, 2016). Inhibition of extraembryonic and meso-endoderm differentiation can be further enhanced by culturing the cells in serum-free medium and by the actions of early patterning molecules, through which the PSCs progressively start restricting their differentiation potential towards the neural lineage to form early NPCs (Pasca *et al.*, 2014; Tao and Zhang, 2016). Many neural induction protocols include the simultaneous inhibition of transforming growth factor beta (TGF-β)/Activin/Nodal and bone morphogenic protein (BMP) signalling pathways, *i.e.* dual SMAD inhibition, which is similar to what is observed *in vivo* (Chambers *et al.*, 2009; Patani *et al.*, 2009). Inhibition of the TGF-β and BMP pathways is thought to promote differentiation of PSCs along the neural lineage primarily through inhibition of PSC self-renewal, as well as by blocking differentiation towards alternative cellular lineages (Davis-Dusenbery *et al.*, 2014).

Consequently, signalling molecules such as Noggin (NOG), left-right determination factor (Lefty)-1, Lefty-2, Dickkopf-1 (DKK1), TGF-β inhibitor SB431542, glycogen synthase kinase 3 beta (GSK3β) inhibitor CHIR99021 and BMP inhibitor dorsomorphin homolog 1 (DMH-1) have all been used to promote neural induction from PSCs (Goulburn *et al.*, 2012; Li *et al.*, 2011). Other pathways, including epidermal growth factor (EGF), fibroblast growth factor (FGF) and wingless-type MMTV integration site family (WNT) signalling, have been described to regulate neuronal differentiation by promoting induction and survival of the NPCs (Davis-Dusenbery *et al.*, 2014; Dhara and Stice, 2008). In particular, FGF2 has been shown to enhance the neural induction phase and to increase the number of NPCs, whereas omitting it during subsequent stages promotes their differentiation into mature neurons (Wilson *et al.*, 2000).

After neural induction, second series of lineage-specific patterning molecule cocktails have been used to direct the further differentiation of the NPCs towards mature neuronal subtypes. Although the availability of protocols for subtypes within specific neurotransmitter classes is limited and different cultivation conditions for each of these classes exist, protocols for glutamatergic, dopaminergic, γ-aminobutyric acid (GABA)-ergic, serotonergic, and cholinergic motor neurons have become available over the years (See Table 1) (Eiraku *et al.*, 2008; Goulburn *et al.*, 2012; Lee *et al.*, 2007; Lu *et al.*, 2016; Yan *et al.*, 2005). Specification of the NPCs takes place both along the rostral-caudal and the dorsal-ventral axes of the brain, coordinated by the synergistic actions of temporally and spatially available patterning molecules (Imaizumi *et al.*, 2015). The presence and concentrations of these molecules define the transcriptional code and, hence, the identity of the NPCs in a particular domain along both axes. NPCs are generally specified first in the head region and extend caudally, meaning that they become committed to an anterior forebrain fate by a default programme (Tao and Zhang, 2016). Correspondingly, NPCs differentiated from PSCs, independent of the differentiation

method, carry a rostral identity that is free of caudal markers (Tao and Zhang, 2016). Indeed, this anterior phenotype is transient and NPCs will take on a definitive regional identity depending on further cues. Treatment with increasing concentrations of sonic hedgehog (SHH) has shown to promote ventralization of the NPCs, while addition of retinoic acid (RA) promotes caudal fates (Davis-Dusenbery *et al.*, 2014) and activation of WNT signalling exerts a dose-dependent effect where increasing concentrations are patterning the NPCs to forebrain, midbrain, hindbrain and anterior spinal cord identities, respectively (Davis-Dusenbery *et al.*, 2014; Kirkeby *et al.*, 2012; Tao and Zhang, 2016). Although it remains largely unknown how all the patterned NPCs acquire functional and anatomical specificity, it is this regional patterning principle recapitulating *in vivo* morphogenesis that guides PSC neuronal differentiation *in vitro* (See Figure 2) (Liu and Zhang, 2011). Moreover, a specific neurotransmitter subtype can often be generated in different parts of the human brain and at different stages, demonstrating that different spatiotemporal cues can likely converge on the same terminal selectors and thereby induce a similar terminal fate (Gabilondo *et al.*, 2016).

The anatomically directed differentiation processes seen in these neuronal differentiation protocols are characterized by the same expression and temporal regulation of lineage-specific transcription factors as observed *in vivo* (Davis-Dusenbery *et al.*, 2014). For instance, NPCs differentiated towards forebrain neurons carry an anterior identity by expressing *PAX6* and *OTX2*, but not more caudal markers like *EN1*, *GBX2* or *HOX* (Chambers *et al.*, 2009; Tao and Zhang, 2016; Zhang *et al.*, 2001). Thus, expression levels of these transcription factors represent useful markers that are widely used to assess the differentiation status of the cellular population (See Table 1). Furthermore, extensive gene expression analysis, electrophysiological characterization, biochemical assessments, and *in vivo* transplantation into rodent brains have been applied to examine the population characteristics, as well as quality and efficacy of the differentiation protocols (See Table S1). However, with many of

these protocols, *in vitro*-directed differentiation in PSCs still results in highly variable neuronal populations with fluctuating yields of neuronal cells and remarkable differences in efficiency (Zhang and Jiang, 2015). This variability emerges from clear differences between the protocols, such as the neuronal induction method used, the chemical compositions of the media, the compound concentrations and the chemical exposure times that were used, as well as more undefined differences, including the culture densities and the passage number for example (Davis-Dusenbery *et al.*, 2014).

2.2 Glutamatergic neurons

Despite the differences in developmental principles that underlie the specification of their subpopulations, excitatory glutamatergic neurons can be found throughout the whole central nervous system, such as in the cerebral cortex (Costa and Muller, 2014), as well as in subcortical regions like the thalamus (Song *et al.*, 2015), and even in the spinal cord among others (Thomas Cheng, 2010). To date, strategies that have been established to successfully generate glutamatergic neurons from PSCs (See Table 1) are mainly based on the derivation of cortical glutamatergic neurons of which the vast majority originates from dorsal telencephalic regions (Li *et al.*, 2009). As mentioned earlier, forebrain identity is a default programme for neuronal differentiation of PSCs, and existing protocols yield neurons with a glutamatergic identity without the need of an extra second series of patterning molecules. However, attributed to endogenous SHH signalling, mouse ESCs have been shown to differentiate into anterior NPCs with a ventral phenotype under serum- and morphogen-free culture conditions, resulting in a neuronal population of which only a minority was considered to be glutamatergic (Gaspard *et al.*, 2008). Consequently, inhibition of intrinsic SHH signalling with the small molecule cyclopamine has been shown to prevent ventralization of the mouse NPCs and significantly increases the derivation of dorsal glutamatergic neurons (Gaspard *et al.*, 2008). In a separate study on the other hand, it has also been demonstrated

that mouse ESCs cultured in the presence of retinoic acid (RA) induced highly homogenous neuronal populations, which, similar to previous report, where consistent with an identity of cortical pyramidal neurons (Bibel *et al.*, 2004). Pyramidal neurons constitute more than 80% of the cerebral cortex neurons and are further diversified in distinct cortical layers that establish specific patterns of axonal output and dendritic input, providing the essential substrate of cortical circuitry (Espuny-Camacho *et al.*, 2013). In a study by Eiraku *et al.* (2008) for example, it was also demonstrated that human ESC-derived cortical neurons stained positive for transcription factors corresponding to the development of the cortical layers in a temporal manner, including RELN, TBR1, CTIP2 and CUX1. However, opposite to the ventral phenotype observed in mouse ESCs, human PSCs have shown to differentiate by a default programme into synchronised populations of NPCs that predominantly express anterior dorsal markers (Gaspard *et al.*, 2008; Li *et al.*, 2009). This dorsal phenotype has been attributed to expression of endogenous WNT ligands, and, as a consequence, inhibition of WNT signalling or activation of SHH signalling has shown to almost completely convert primitive dorsal telencephalic NPCs to ventral progenitors at the expense of glutamatergic neuron identity (Li *et al.*, 2009).

Various studies have also addressed the potential of using the derived glutamatergic cultures for fundamental research. Kim *et al.* (2011c) suggested an approach for efficient differentiation of human glutamatergic neurons based on a spin EB protocol in ESCs and iPSCs. Interestingly, when co-cultured with human embryonic kidney cells (HEK293T) expressing *NLGN3* and *NLGN4*, but not those containing autism-associated mutations, the iPSC-derived neurons were able to form functional synapses, demonstrating that these neuronal populations are a potential model for the study of synaptic differentiation and function under normal and disorder-associated conditions (Kim *et al.*, 2011c). Also in their differentiation approach it was demonstrated that the human spin EB-derived NPCs acquired

an anterior dorsal forebrain character by a default pathway. While addition of the SHH agonist purmorphamine (PUR) during the EB stages ventralized the NPCs, inhibition of SHH, however, did not enhance expression of dorsal markers as seen in mouse ESCs (Kim *et al.*, 2011c). Consistent with this finding, a more recent study also showed that cyclopamine treatment was not required for induction of the dorsal telencephalic fate in a human PSC monolayer system (Espuny-Camacho *et al.*, 2013). By determining the time of onset for the expression of layer-specific markers during the course of differentiation, it was demonstrated that most of the pyramidal neurons generated here displayed an identity corresponding to deep cortical layers, while upper layer neurons were underrepresented (Espuny-Camacho *et al.*, 2013). This contrasts with a more recent report describing a robust culture system in human ESCs for the generation of both electrophysiological active deep- and upper-layer pyramidal neurons in equivalent proportions that were cultured in the presence of retinoids (Shi *et al.*, 2012a; Shi *et al.*, 2012b). In the context of dual SMAD inhibition, they found that vitamin A is crucial for the efficient induction of cortical NPC differentiation and subsequent cortical neurogenesis. These findings agree with previous report (Bibel *et al.*, 2004), which demonstrated that derivatives of retinoids have important roles in the acquisition of NPCs and telencephalic glutamatergic neurons from mouse ESCs. By using this approach, the efficiency of cortical neural induction from PSCs even approaches 100% (Shi *et al.*, 2012b). Although various organisations are still missing, such as interactions with glial cells that are essential partners in synaptic functioning, these systems provide the first steps towards functional studies of human cerebral cortex development and the generation of patient-specific cortical networks *in vitro*. These future applications will be particularly interesting for modelling disorders that are known for their cortical synaptic dysfunctions, including epilepsy, schizophrenia and dementia, and allow high-throughput testing for therapeutic interventions (Shi *et al.*, 2012a; Shi *et al.*, 2012b).

2.3 GABAergic neurons

Various subtypes of inhibitory GABAergic neurons exist in the brain and spinal cord, which can be categorised based on their developmental origins, localization, synaptic connections, co-expression of molecular and neurotransmitter markers and electrophysiological properties (Liu *et al.*, 2013). During development, GABA interneurons are synaptically integrated into neuronal networks in the forebrain that originate mostly from the medial ganglionic eminence and, to a lesser extent, from the ventral lateral ganglionic eminence and the anterior dorsal ganglionic eminence (Fishell and Rudy, 2011; Liu *et al.*, 2013; Nobrega-Pereira *et al.*, 2008). These GABAergic NPCs migrate by following radial or tangential pathways, they differentiate into post-mitotic neurons and make connections with local glutamatergic neurons (Kriegstein and Noctor, 2004; Liu *et al.*, 2013; Marin and Rubenstein, 2003). The vast majority of forebrain GABAergic interneuron progenitors express *Nkx2-1* (Sussel *et al.*, 1999) in addition to the telencephalic transcription factor *Bf1*, also known as *Foxg1* (Goulburn *et al.*, 2012), and they can be distinguished from other types of GABAergic neurons, including the striatal GABAergic projection neurons, which originate from the lateral ganglionic eminence (Campbell, 2003; Liu *et al.*, 2013). Forebrain GABA interneurons can be divided into many subgroups on the basis of molecular markers and their expression of neuropeptides or calcium-binding proteins, including somatostatin (SST), parvalbumin (PV), calretinin, calbindin and neuropeptide Y, although the medial ganglionic eminence progenitors mostly give rise to SST and PV interneurons (Liu *et al.*, 2013).

The majority of studies on the differentiation of GABAergic neurons *in vitro* have focused on guiding PSCs toward ventral telencephalic NPCs, primarily defined by the co-expression of *Bf1* and *Nkx2-1* (See Table 1) (Goulburn *et al.*, 2012; Li *et al.*, 2009; Watanabe *et al.*, 2005). As stated in the previous section of glutamatergic neurons, ventral telencephalic pecursors have been generated from mice ESCs without the need of additional patterning factors,

leading to a neuronal population that is enriched in cortical GABAergic interneurons (Gaspard *et al.*, 2008).Ventralization of human dorsal telencephalic NPCs on the other hand, has been achieved by addition of concentrated SHH or inhibition of WNT by DKK1 together with low concentrations of SHH, leading to the generation of enriched populations of human cortical GABAergic interneurons (Li *et al.*, 2009). Interestingly, various groups have tried to purify the generated GABAergic NPCs using cellular selection systems. For instance, Maroof *et al.* (2010) described a protocol for the generation of cortical GABAergic interneurons from mouse ESCs based on EB formation and SHH signalling. In this study, a *Lhx6-GFP* bacterial artificial chromosome reporter construct was used, which allowed for the isolation and enrichment of the newly generated NPCs. Using a similar approach with a previously established *NKX2-1::GFP* human ESC reporter line, two other groups (Maroof *et al.*, 2013; Nicholas *et al.*, 2013) have developed a protocol based on the combination of small molecules with the timed activation of SHH signalling. In both studies they showed that the human PSCs develop into GABAergic interneurons with mature physiological properties, both *in vitro,* as well as after transplantation into rodent brains. Liu *et al.* (2013) on the other hand, have described a protocol without transgenic modification or cell sorting that involves treatment with SHH or its antagonist PUR for directed GABAergic differentiation of human PSCs with high efficiency. After 2 weeks of differentiation, more than 90% of the neurons were estimated to be GABAergic interneurons, which was confirmed by immunostaining and electrophysiological analysis (Liu *et al.*, 2013).

Aside from the cortical GABAergic interneurons, several studies have also managed to acquire striatal GABAergic projection neurons, also known as medium spiny neurons (Carri *et al.*, 2013). In one of the procedures, neural induction via BMP/TGF-β inhibition was coupled with exposure to SHH and DKK1 to drive ventral telencephalic specification in human ESC and iPSCs, followed by the terminal differentiation towards authentic medium

spiny neurons (Carri *et al.*, 2013). Authenticity of the resulting neuronal population was monitored by the appearance of BF1/GSX2-positive progenitor cells typical for the lateral ganglionic eminence, followed by appearance of CTIP2-, FOXP1- and FOXP2-positive cells. These precursor cells then matured into MAP2/GABA-positive neurons with 20% of them co-expressing DARPP-32 and CTIP2, and also carried electrophysiological properties expected for fully functional medium spiny neurons (Carri *et al.*, 2013). Most recently, a reliable and simplified two- and three-step protocol to derive striatal GABAergic neurons from immortalized NPCs has also been established, using valproic acid (VPA) or SHH and DKK1, respectively (Lin *et al.*, 2015). The differentiated cells expressed appropriate GABAergic markers and responded to ionotropic glutamate receptor stimulation. In accordance, the cells also expressed various glutamate receptor subunits and released GABA upon stimulation (Lin *et al.*, 2015). In relation to disease modelling, the derivation of these GABAergic neurons represents a possible critical resource for the study of Huntington's disease and Rett syndrome for example (Lin *et al.*, 2015; Tang *et al.*, 2016).

2.4 Dopaminergic neurons

Dopaminergic neurons are localized in the diencephalon, mesencephalon and the olfactory bulb (Chinta and Andersen, 2005), although the most prominent group resides in the mesencephalon, containing approximately 90% of the total number of brain dopaminergic neurons (Chinta and Andersen, 2005). Differentiation protocols for dopaminergic neurons, in particular for the ones originating from the midbrain, have received a lot of attention due to their applicability into regenerative medicine for Parkinson's disease, with numerous differentiation protocols published over the last years (See Table 1) (Ono *et al.*, 2007; Pasca *et al.*, 2014; Placzek and Briscoe, 2005). Studies in mice have shown that dopaminergic midbrain NPCs are specified from the floor plate in the mesencephalon, which is located at the ventral midline of the neural tube (Ono *et al.*, 2007; Placzek and Briscoe, 2005). These

NPCs are transcriptionally characterised by the expression of *Lmx1a*, *Foxa2*, *En1* and *Otx2*, and are controlled by two regulatory feedback loops also involving WNT and SHH signalling (Chung *et al.*, 2009; Xi *et al.*, 2012). In more detail, WNT1 induces expression of *Otx2*, which represses *Gbx2* to coordinate the mid-hindbrain organizer and represses *Nkx2-2*, which defines the midbrain dopaminergic NPC domain from the lateral located progenitors of serotonergic neurons (Prakash *et al.*, 2006). Thereby, it induces the expression of *Lmx1a* which either induces the pro-neural gene *Ngn2* through *Msx1* or inhibits the NPCs from acquiring alternative cell fates by repressing *Nkx6-1* (Andersson *et al.*, 2006; Chung *et al.*, 2009).

It is this developmental principle in mice described above that currently also forms the guideline for differentiating midbrain dopaminergic neurons from human PSC (Xi *et al.*, 2012). However, initial reports for the differentiation of dopaminergic neurons have also heavily relied on the use of PA6 and MS-5 feeder cells (Barberi *et al.*, 2003; Kawasaki *et al.*, 2000; Lim *et al.*, 2015). Furthermore, studies have shown the successful differentiation of TH (tyrosine hydroxylase)-expressing neurons from mouse ESCs and iPSCs based on chemically defined systems that relied on the generation of EBs (Lee *et al.*, 2000;) and the activation of key signalling pathways by SHH and FGF8, a morphogen important for the formation of the isthmus (Barberi *et al.*, 2003; Lee *et al.*, 2000; Pasca *et al.*, 2014; Ye *et al.*, 1998). Gradually, studies have tailored and applied these initial studies in primate (Xi *et al.*, 2012) and human iPSCs (Cai *et al.*, 2010;; Swistowski *et al.*, 2010; Yan *et al.*, 2005), and demonstrated efficient induction of neurons with a dopaminergic phenotype. Importantly, in most of the previous mentioned reports it was actually not conclusively determined whether the obtained TH-positive neurons were really representing midbrain dopaminergic neurons, at least they did not always carry abundant midbrain markers, suggesting that the combination of FGF8 and

SHH had the potency to induce the dopaminergic identity but was possibly not sufficient to restrict the neurons to the midbrain fate (Xi *et al.*, 2012).

A better understanding of essential signalling pathways and transcriptional networks important for dopaminergic neuron midbrain differentiation, as well as their more precise temporal implementation, has improved the protocols over time. Incorporation of WNT/β-catenin signalling due to the availability of the GSK3β inhibitor CHIR99021 eventually led to an improved midbrain specification in a reliable and efficient manner (Arenas *et al.*, 2015; Denham *et al.*, 2012; Doi *et al.*, 2014; Kirkeby *et al.*, 2012; Kriks *et al.*, 2011; Xi *et al.*, 2012). This was first demonstrated by Kriks *et al.* (2011), who produced cultures containing around 75% of floor plate-derived dopaminergic neurons, assessed by immunostaining of markers such as FOXA2 and TH. Further expression analysis in this study also demonstrated abundant co-expression of lineage-specific genes necessary for appropriate dopaminergic neuron specification (Kriks *et al.*, 2011). Furthermore, the cells were able to be efficiently engraft in rodent brains and survived *in vivo* without overgrowing, a phenomenon that was previously only observed with very poor performance using human PSC-derived dopaminergic neurons (Kriks *et al.*, 2011). Their findings were later also confirmed in different human ESC lines, iPSCs, and rhesus monkey iPSCs, showing that a narrow range of CHIR99021 at a particular developmental stage restricts the cells to form midbrain floor plate progenitors which, in the presence of FGF8, acquire a dopaminergic neuron identity (Xi *et al.*, 2012). Based on the latter approaches, differentiated dopaminergic populations from Parkinson's disease patient-derived iPSCs have even demonstrated to mimic several pathological mechanisms of the neurological disorder *in vitro* (Fernandez-Santiago *et al.*, 2015; Woodard *et al.*, 2014). Interestingly, disease phenotypes were only observed in the PSC-derived dopaminergic neurons and not in patients' fibroblasts, which emphasizes the

significance of directed dopaminergic differentiation protocols for disease modelling *in vitro* (Woodard *et al.*, 2014).

2.5 Serotonergic neurons

Serotonergic neurons are found in the raphe nuclei that arise from progenitors in the rhombencephalon during development and can be divided into two main clusters: A rostral division located just caudal to the isthmus and a more caudal division situated in the myelencephalon (Deneris and Gaspar, 2018). While the rostral division has widespread innervating projections throughout the brain, the caudal division mainly projects down to the spinal cord (Deneris and Gaspar, 2018). These primary anterior and posterior clusters are further segmented along the rostro-caudal axis according to 9 rhombomeric divisions, where specific transcriptional codes confer positional identities. For instance, the progenitors located at rhombomeric segments 2–3 are distinguished from segment 1 by expression of *Hoxa2* but not *En1*, while progenitors in segment 4 express *Phox2b*, leading into an intervening gap between cluster 3 and 5, where serotonergic identity is repressed and visceromotoneurons are formed instead (Alenina *et al.*, 2006; Lu *et al.*, 2016). Specification of the distinct segments is thought to be induced by different combinations of morphogen gradients of which the most important ones include SHH, FGF8 and FGF4 (Vadodaria *et al.*, 2016). In addition, WNT- and TGF-beta signalling have also been shown to be important for determining boundaries and specifying a hindbrain fate (Dias *et al.*, 2014; Kirkeby *et al.*, 2012; Rhinn and Brand, 2001). Developing serotonergic NPCs gradually start expressing *Nkx2-2, Ascl1* and *Foxa2*, which constitutes a primary gene regulatory network for serotonergic specification (Deneris and Gaspar, 2018). Although it is evident that their postmitotic neurons in different regions are transcriptional and functional heterogeneous, this primary genetic cascade activates a secondary network that consists of a set of core transcription factors, including GATA2,

GATA3, INSM1, LMX1B and PET1 in mice or FEV in humans, which is key in their terminal specification (Deneris and Gaspar, 2018).

Thus far, there have been only few attempts to differentiate serotonergic neurons in chemically defined systems from PSCs (See Table 1). Mice studies have given primary knowledge on the combinations of developmental signals that allow the generation of serotonergic neurons *in vitro* (Vadodaria et al., 2016). One of the first approaches to induce serotonergic neurons from ESCs was based on the formation of EBs in combination with the activation of SHH and FGF8 signalling (Lee *et al.*, 2000). The protocol was primarily intended to enrich for dopaminergic neurons, resulting in considerably low yields of serotonergic neurons (±11%) compared to the total neuronal population (±72%). Mouse ESCs have then also been co-cultured with stromal cells in the presence of SHH and FGF4, yielding a substantially higher proportion (±57%) of serotonergic neurons (Barberi *et al.*, 2003). More recently, a simplified method to generate serotonergic neurons from mouse PSCs in monolayer cultures has also been developed (Shimada *et al.*, 2012). The cells were cultured on a layer of matrigel in the presence of NOG, a BMP inhibitor, and reached 80% pure serotonergic cultures after cell sorting (Shimada *et al.*, 2012). Although their approach increased the homogeneity of serotonergic neurons and appears to be an appealing simpler alternative to culturing mouse ESCs on feeder cells, effectively only ±6% of the initial neuronal cells were considered serotonergic, which remains a relatively low proportion of cells that can be generated from mouse PSCs *in vitro*.

Studies have consequently demonstrated that the key developmental signalling molecules involved in serotonergic differentiation could also be applied for directing their differentiation utilizing human PSCs. One of the first protocols established in order to obtain serotonergic neurons from human ESCs was based on the induction and enrichment of NPCs that differentiated toward serotonergic neurons under empirically determined culture conditions

(Kumar *et al.*, 2009). In this study, neural differentiation was estimated to be around 20% with up to 70% of all neurons staining positive for serotonin (5-HT), generating 14% of serotonergic neurons *in vitro*. Interestingly, acidic FGF, which is localized in raphe neurons in rats (Stock *et al.*, 1992), and 5-HT, which is known to contribute to the development of serotonergic neurons *in vivo* (Migliarini et al., 2013), were added to the growth factor cocktail for differentiation and maturation of the NPCs. Using a differentiation protocol based on FGF8 and SHH, another study (Vadodaria *et al.*, 2015) found that approximately 8% of the differentiating human ESCs committed to a serotonergic fate, which was determined by a co-staining of 5-HT and tryptophan hydroxylase (TPH).

Thus, although these first protocols in mice and human PSCs were very promising, they only met limited success considering their low yields, which limits the ability to use these cells for further applications. However, most recently, Lu *et al.* (2016) were the first to develop a robust chemically defined system to induce human iPSC to enriched populations of serotonergic neurons with very high efficiency. Induction of ventral hindbrain NPCs was achieved by maintaining PSCs in medium containing SB431542, DMH-1 and CHIR99021. Following neural induction, the NPCs were exposed to FGF4 together with SHH to promote the acquisition of a serotonergic cell fate, eventually reaching more than 60% serotonergic neurons (Lu *et al.*, 2016). The key aspect here, is the activation of the WNT pathway by CHIR99021 that, within a narrow window of concentrations, gives rise to ventral hindbrain NPCs that further differentiate into serotonergic with a typical identity for rhombomeric divisions 2-3. Interestingly, treatment with the FDA-approved antidepressants tramadol and escitalopram oxalate in this latter study resulted in release or uptake of 5-HT in a dose- and time-dependent manner, which emphasized their utility for the evaluation of drug candidates in depression (Lu *et al.*, 2016). Dysregulation of the serotonergic system is typical in

depression and a common target for antidepressants (Licinio and Wong, 2016; Sierksma *et al.*, 2010).

2.6 Cholinergic motor neurons

Cholinergic motor neurons can be broadly divided into two main groups according to the location of their cell body: (I) Upper motor neurons located in the motor regions of the cerebral cortex, and (II) lower motor neurons, which are located in the brainstem and spinal cord (Davis-Dusenbery *et al.*, 2014). Upper motor neurons have ascending pathways to lower motor neurons, which project to the musculature, where they control muscle contractions via neuromuscular junctions (Davis-Dusenbery *et al.*, 2014). Spinal motor NPCs originate from a highly restricted foci in the ventral neural tube in response to RA, FGFs and SHH (Patani, 2016). These cells express the basic helix-loop-helix transcription factor *Olig2*, which, together with *Ngn2*, direct the expression of motor neuron fate consolidating genes such as *Hb9* and *Isl1*. For a more detailed description of the underlying developmental cascade that results in the acquisition of these cells, we would like the reader to refer to other excellent reviews (Davis-Dusenbery *et al.*, 2014; Patani, 2016). Motor neurons can be further developmentally allocated to discrete motor columns, which extend along the rostral-caudal neural tube and contain motor neuron pools that are responsible for innervating a single skeletal muscle, each of which is also arranged by an anatomical logic corresponding to their targets (Patani, 2016). Retinoid signalling plays key roles in the diversification of motor neuron subtypes from the common NPC pool and additionally contributes to spinal cord columnar organisations, which are then again characterized by unique transcriptional codes that define the regional identity of the neuronal subtypes (Patani, 2016).

By recapitulating the developmentally rationalized programme of morphogenic cues, considerable advances using chemically defined systems have primarily been made in differentiating PSCs into lower spinal motor neurons (See Table 1). The desire to regenerate

in vitro motor circuitry in the contexts of motor neuron disease and spinal cord injury has been motivating the attempts to produce motor neurons for translational research (Davis-Dusenbery *et al.*, 2014). Initial studies have outlined methods to derive functional cholinergic motor neurons from mice (Barberi *et al.*, 2003) and human ESCs (Lee *et al.*, 2007; Li *et al.*, 2005; Singh Roy *et al.*, 2005), while more recent studies have applied similar methods to human iPSC lines (Dimos *et al.*, 2008; Karumbayaram *et al.*, 2009; Qu *et al.*, 2014; Shimojo *et al.*, 2015). The numerous protocols that have been developed utilize various directed differentiation methods, including co-culture with stromal feeders (Barberi *et al.*, 2003; Lee *et al.*, 2007), adherent monolayer cultures (Qu *et al.*, 2014), or the use of EB induction followed by neuralization, cholinergic neuron differentiation and neuronal maturation (Karumbayaram *et al.*, 2009; Shimojo *et al.*, 2015). In most cases, studies have reported the use of RA treatment with addition of recombinant SHH or small molecule agonists of the SHH signalling pathway to induce differentiation of PSCs into cholinergic motor neurons. Patterning NPCs by RA and SHH confers caudal and ventral anatomical identities, respectively, and gives rise to *OLIG2* expressing neurons, which in turn begin to express *CHAT*, *HB9* and *ISL1* (Shimojo *et al.*, 2015). Cholinergic motor neurons obtained through these methods have been shown to possess numerous characteristics of their *in vivo* equivalents, including electrophysiological properties, the possibility to engraft into the developing spinal cord and the presence of correctly labelled neuromuscular junction complexes, demonstrating the potential ability to form functional muscular junctions *in vitro* (Davis-Dusenbery *et al.*, 2014). However, in contrast to the generic developmental principles that allow cholinergic motor neuron specification, the process determining how individual motor neuron subtypes can be generated is relatively less well understood (Davis-Dusenbery *et al.*, 2014). Nevertheless, in the context of mouse ESC differentiation for example, protocols based on a treatment with RA typically result in spinal motor neurons with a rostral cervical

character, as judged by expression of *Hoxc5* and *Hoxc6*, but not *Hoxc8* (Wichterle *et al.*, 2002). In the context of human motor neuron differentiation on the other hand, a recent report demonstrated a shift in the proportion of motor neurons expressing the median motor column marker *LHX3* or the lateral motor column marker *FOXP1*, when SHH signalling was activated via a combination of smoothened agonist (SAG) and PUR instead of recombinant SHH (Amoroso *et al.*, 2013). This sensitivity of the differentiating NPCs emphasizes the need for thorough evaluation of the differentiation protocols and also presents the opportunity for the optimization of motor neuron subtype specification (Davis-Dusenbery *et al.*, 2014).

3. Transcription factor-mediated reprogramming

3.1 Cellular reprogramming and transdifferentiation

For long it was thought that cellular differentiation and lineage commitment were irreversible processes established during embryonic development (Vierbuchen and Wernig, 2011). However, the cloning of animals by nuclear transfer demonstrated that maturated molecular mechanisms are reversible and that a nucleus from the most differentiated cell bears the potential to generate an organism (Vierbuchen and Wernig, 2011). These cell fusion experiments have proved that transcriptional reprogramming can occur by exposing a distinct nucleus to cytoplasmatic components of cells from distinct lineages, although the exact mechanisms underlying these processes remain challenging to address. Substantial interest in transcriptional reprogramming has been rejuvenated upon the discoveries by Dr. Takahashi and Prof. Dr. Yamanaka, who provided the foundation that somatic cells can be reprogrammed to iPSCs. The cells were initially generated by reprogramming fibroblasts via recombinant overexpression of four transcription factors, including *Oct3/4*, *Sox2*, *Klf4*, and *Myc* (OSKM, Yamanaka factors) (Takahashi and Yamanaka, 2006). The use of only four transcription factors was sufficient to induce dramatic cell fate changes and to reprogram fully differentiated cells into a more embryonic cell state. The derivation of iPSCs has been

substantially adapted and improved by using other sets of transcription factors, including *LIN28* and *NANOG* (Yu *et al.*, 2007), by introducing non-integrative transgene expression and by using different types of somatic cells (Schlaeger *et al.*, 2015; Takahashi and Yamanaka, 2016). Earlier work on the other hand, has demonstrated that increased activity of a single transcription factor, namely *Myod1*, is sufficient to directly convert fibroblasts into myocytes by a process known as transdifferentiation (Weintraub *et al.*, 1989). This has supported the notion that cell fate conversions can be direct without the need of precedent de-differentiation. Consequently, these studies have raised the question whether transcription factor-mediated reprogramming could also directly induce neuronal fates in somatic cells or even PSCs (See Figure 3).

Notably, and especially important in the context of disease modelling, these direct conversion modalities may prove to be invaluable in the study of late-onset neurodegenerative disorders because the age of somatic cells is maintained in the converted neurons, thus allowing to model the aging process *in vitro* (Mertens *et al.*, 2015). In addition, while chemically defined differentiation protocols in PSCs are known for their long multistep protocols, PSCs exposed to specific sets of transcription factors have shown to differentiate much faster without additional culturing steps, therefore providing an appealing, simpler and possibly more effective differentiation strategy for PSCs *in vitro* (Busskamp *et al.*, 2014). One of the major disadvantages of direct somatic cell reprogramming compared to directed differentiation of PSCs however, is that the former skips the pluripotent state and does not allow expansion of the cells before further applications, thereby limiting the accessibility of cells readily available *in vitro*. Nevertheless, somatic cell reprogramming into an intermediate state that allows substantial proliferation, such as NPCs, has already been considered (Hou and Lu, 2016; Raciti *et al.*, 2013). For instance, mouse and human fibroblasts have been partially reprogrammed by introducing the four Yamanaka factors, which gave rise to NPCs that were

capable of differentiating into both neuronal and glial cells in the presence of leukemia inducible factor (LIF) and FGF2 (Matsui *et al.*, 2012). The direct differentiation of partially reprogrammed cells may be useful for rapidly preparing high numbers of NPCs that could be expanded before terminal differentiation into target neurons.

3.2 Induced glutamatergic neurons

Starting from a pool of nineteen candidates, Vierbuchen *et al.* (2010) were the first to identify a combination of only three transcription factors, including *Brn2*, *Ascl1*, and *Myt1l* (BAM), which could rapidly and efficiently convert mouse embryonic fibroblasts (MEF) into induced neuronal (iN) cells (See Table 2). These iN cells expressed multiple neuron-specific markers, generated action potentials and were able to form functional synapses (Vierbuchen *et al.*, 2010). Electrophysiological recordings demonstrated that mainly excitatory postsynaptic potentials could be recorded, providing functional proof that a large majority of the iN cells exhibited a glutamatergic phenotype. Some cells also expressed GABAergic markers at earlier time points, including *Gaba* and *Gad67*, suggesting that both neuronal subtypes could be obtained but culture conditions probably favoured the glutamatergic phenotype (Vierbuchen *et al.*, 2010; Vierbuchen and Wernig, 2011). These findings in murine somatic cells led to follow-up experiments using human fibroblasts, which eventually led to the successful generation of human iNs with the addition of *Neurod1* to the BAM pool (Pang *et al.*, 2011). Just like the generation of iPSCs, during that time it was thought that a combination of factors was necessary to fully reprogram iN cells from fibroblasts and the use of a single transcription factor was considered insufficient (Chanda *et al.*, 2014). However, later it was shown that *Ascl1* alone is satisfactory to generate populations of pre-dominantly glutamatergic iN cells from mouse and human fibroblasts, as well as ESCs (Chanda *et al.*, 2014; Mall *et al.*, 2017). A clear hierarchical role of the reprogramming factors has, therefore, been suggested, demonstrating that ASCL1 acts as a key factor to activate the neuronal

program, whereas access of BRN2 to the chromatin is apparently more cell-context-dependent and facilitates reprogramming later on (Chanda *et al.*, 2014). Moreover, although *Ascl1* alone is sufficient to generate iNs, endogenous *Mytl* is subsequently induced during reprogramming, and exogenous *Myt1l* has, therefore, demonstrated to greatly improve the efficiency of reprogramming and the functional maturity of the resulting iN cells (Mall *et al.*, 2017). In fact, it has been shown that MYT1L exerts its pro-neuronal function by direct repression of many different somatic lineage programs except the neuronal program. This repressive function of MYT1L is mediated via recruitment of a complex containing SIN3B by binding to a previously uncharacterized N-terminal domain (Mall *et al.*, 2017). In addition, knockdown of *Myt1l* in primary postmitotic neurons removed the repression of non-neuronal programs and impaired neuronal gene expression and function, indicating that many somatic lineage programs are actively and persistently inhibited by MYT1L to maintain neuronal identity (Mall *et al.*, 2017). Aside from fibroblast, for murine astrocytes (Heinrich *et al.*, 2010), mouse ESCs (Chanda *et al.*, 2014; Thoma *et al.*, 2012) and human PSCs (Chanda *et al.*, 2014; Zhang *et al.*, 2013), it has also been shown that single neurogenic factors, such as *Neurod1* and *Ngn2*, alone are sufficient to rapidly induce the neuronal fate. Although *Ascl1*-induced iN cells displayed slower maturation kinetics at early developmental stages, their functional properties and neuronal gene-expression profile at later time points are surprisingly similar to that of *Ngn2* or BAM iN cells (Chanda *et al.*, 2014).

After the establishment of the BAM pool and the other neurogenic transcription factors, researchers have been attempting to derive other neuronal subtypes by transcription factor-mediated reprogramming and, thanks to that, additional factors have been identified with the ability to induce dopaminergic neurons, GABAergic neurons, serotonergic neurons, cholinergic neurons and adrenergic neurons (See Table 2) (Colasante *et al.*, 2015; Mong *et al.*, 2014; Pfisterer *et al.*, 2011; Son *et al.*, 2011; Xu *et al.*, 2015). Identical to the chemically

defined systems, all of these obtained cultures have been subjected to various bioassays and assessments in order to examine their population characteristics (See Table S2). Furthermore, studies have demonstrated that supplementation of chemically defined systems with transcription factor-mediated reprogramming can significantly increase the efficiency of obtaining differentiated neuronal cells, as well as *vice versa* (Hester *et al.*, 2011; Kim *et al.*, 2011b; Mong *et al.*, 2014). Notably, blockade of TGFb/SMAD signalling using Noggin and molecules such as SB431542 and LDN193189, as well as pharmalogical promotion of calcium signalling with cAMP and Forskolin have not only shown to increase iN yield, but have also been used to successfully generate transgene-free iNs (Gascón *et al.*, 2017; Vadodaria *et al.*, 2016; Xie *et al.*, 2017). The fast progress in the field of chemically-mediated reprogramming and transdifferentiation provides us new ways to manipulate neuronal fates both *in vitro* and *in vivo*. These methods on their own and/or in combination with other approaches may accelerate the eventual applications of patient-specific human neurons generated *in vitro*, by facilitating the potency and timelines of the protocols, and by aiding in the specification of regional subtypes within neurotransmitter classes.

3.3 Induced dopaminergic neurons

By combining the BAM factors with *Lmx1a* and *Foxa2*, which are typically expressed in midbrain dopaminergic NPCs, human fibroblasts have been converted into induced dopaminergic (iDA) neurons (Pfisterer *et al.*, 2011). This provided proof-of-principle that other subtypes of iN cells can be produced by transcription factor-mediated fate instructions. Since then, iDA neurons have been obtained by ectopic overexpression of various combinations of transgenes encoding midbrain-specific transcription factors (See Table 2). For instance, a minimal set of three transcription factors, *i.e. Ascl1*, *Nurr1* and *Lmx1a*, was sufficient to generate functional iDA neurons from murine and human fibroblasts (Caiazzo *et al.*, 2011). The three factors were able to elicit dopaminergic neuronal conversion, resulting in

iDA neurons that were highly enriched in genes of the dopaminergic phenotype, released dopamine, exhibited proper electrophysiological profiles, and, in case of the murine iDA neurons, were able to integrate into neonatal mouse brains. Interestingly, the endogenous *Th* and *Vmat2* promoter regions were highly demethylated in the iDA neurons, whereas they were fully methylated in the fibroblasts, indicating their epigenetic reactivation during dopaminergic conversion (Caiazzo *et al.*, 2011). In addition to fibroblasts, astrocytes have also been efficiently converted into iDA by using a single polycistronic vector containing *ASCL1*, *LMX1B* and *NURR1* (Addis *et al.*, 2011). The fact that different somatic cells can be reprogrammed towards iDA using similar sets of transcription factors highlights their importance in reprogramming processes and reassigning cell fate. Reprogramming mouse fibroblast based only on *Ascl1* and *Pitx3* in another study resulted in immature iDA neurons after 4 weeks of culture (Kim *et al.*, 2011b). However, inclusion of additional factors, such as *En1*, *Foxa2*, *Lmx1a*, and *Nurr1*, could fully reprogram fibroblasts into iDA neurons that were more similar at the molecular level to *bona fide* dopaminergic neurons (Kim *et al.*, 2011b). Furthermore, the murine iDA neurons were able to alleviate symptoms in a mouse model of Parkinson's disease, demonstrating their therapeutic potential for transplant therapies (Kim *et al.*, 2011b). Although transdifferentiation of fibroblasts in the latter study could be achieved by ectopic overexpression of only 2 factors, the results suggested that additional factors are required to ensure proper maturation of the iDA neurons (Kim *et al.*, 2011b). Furthermore, by adding SHH and FGF8 to the culture media, iDA neuron reprogramming could be enhanced up to 2 fold when using only 2 factors, and up to 3 fold when using the combination of 6 factors (Kim *et al.*, 2011b). These findings demonstrate that patterning molecules can act as critical enhancing components in promoting the generation of iDA neurons from fibroblasts. Notably, Theka *et al.* (2013) have established a fast protocol to obtain dopaminergic neurons by overexpressing *Ascl1*, *Nurr1*, and *Lmx1a* in human iPSCs. They were able to generate

mature and functional dopaminergic neurons in as few as 21 days, avoiding all the intermediate steps of induction and selection of EBs and NPCs. Strikingly, the resulting neuronal conversion process was very efficient, since approximately 93% of all the co-infected iPSCs were forced to differentiate into postmitotic iDA neurons (Theka *et al.*, 2013). The iPSC-derived neurons expressed all the critical molecular markers of midbrain dopaminergic neurons at the molecular level and exhibited sophisticated functional features, including spontaneous electrical activity and dopamine release (Theka *et al.*, 2013).

3.4 Induced GABAergic neurons

In the first study that achieved to obtain iNs (Vierbuchen *et al.*, 2010), some cells expressed markers of GABAergic differentiation (Vierbuchen and Wernig, 2011). However, whether enriched populations of induced GABAergic interneurons (iGNs) can be obtained has not been addressed until recently (See Table 2). Wasko (2013) was the first to demonstrate that mouse fibroblasts could be directly reprogrammed to iGN-like cells using different pools of transcription factors, including *Dlx1, Dlx2, Lbx1, Lhx1, Lhx2, Pax2, Pitx2 and Pft1a* in combination with members of the BAM factors. The author states that different groups containing these transcription factors have demonstrated some capacity for the derivation of iGNs, although the most efficient factor combination remains to be determined. Additional experiments will be required to fully characterize the efficiency of the different transcription factor pools, as well as to assess the functional properties and maturity of the eventual derived iGNs. Aside from fibroblasts, iGNs neurons have also been derived by overexpressing the ventral telencephalic fate determinant *Dlx2* in murine astrocyte cultures (Heinrich *et al.*, 2010). Interestingly, they found that the overall efficiency of *Dlx2*-mediated neuronal reprogramming towards iGNs is much lower compared to *Ngn2*-mediated reprogramming towards induced glutamatergic neurons, suggesting that cortical astrocytes possess a higher competence to respond to the dorsal telencephalic fate determinant (Heinrich *et al.*, 2010). In

addition, five different factors have been identified, including *Ascl1*, *Bf1*, *Dlx6*, *Lhx6* and *Sox2*, which were able to convert mouse and human fibroblast, as well as human iPSCs, into iGNs that possessed characteristics of telencephalic GABAergic interneurons (Colasante *et al.*, 2015). Molecular profiling showed pronounced activation of forebrain-specific (epi)genetic markers, required for GABAergic fate specification (Colasante *et al.*, 2015). Furthermore, the iGNs displayed progressively maturing firing patterns comparable to cortical GABAergic interneurons, formed functional synapses, and released GABA upon stimulation (Colasante *et al.*, 2015). The iGNs also survived and matured upon engraftment into the mouse hippocampus and optogenetic stimulation demonstrated functional integration of the grafted iGNs into the host circuitry, triggering inhibition of host granule neuron activity (Colasante *et al.*, 2015). This latter study also elegantly demonstrated how human PSCs can be harnessed to generate GABAergic neurons.

3.5 Induced serotonergic neurons

There have only been two studies published that were able to directly obtain induced serotonergic (i5HT) neurons by transcription factor-mediated reprogramming of somatic cells (See Table 2). In the first study (Xu *et al.*, 2015), human fibroblasts could be directly converted to i5HT neurons by the ectopic expression of *ASCL1*, *FEV*, *FOXA2*, and *LMX1B*. The transdifferentiation was enhanced by p53 knockdown and appropriate culture conditions, including hypoxia (Xu *et al.*, 2015). Addition of the small-molecule compounds dorsomorphin (DOR), SB431542, ROCK inhibitor Y-27632 and the CDK4/6 inhibitor PD0332991 improved both the conversion efficiency and morphology of the obtained i5HT neurons, whereas addition of brain derived neurotrophic factor (BDNF) and glial derived neurotrophic factor (GDNF) slightly improved the morphology but not the conversion efficiency (Xu *et al.*, 2015). The i5HT neurons expressed markers for mature serotonergic neurons, had calcium-dependent 5-HT release and selective 5-HT uptake, and exhibited

spontaneous action potentials, as well as spontaneous excitatory postsynaptic currents (Xu *et al.*, 2015). Moreover, application of 5-HT significantly increased the firing rate of spontaneous action potentials (Xu *et al.*, 2015). In the second study, it was demonstrated that overexpression of the transcription factors *FEV*, *GATA2*, *LMX1B* and *NKX2-2* in combination with *ASCL1* and *NGN2* directly and efficiently generated i5HT neurons from human fibroblasts (Vadodaria *et al.*, 2015). The i5HT neurons showed increased expression of specific serotonergic genes known to be expressed in the raphe nuclei, displayed spontaneous action potentials, released 5-HT *in vitro* and functionally responded to selective serotonin reuptake inhibitors (SSRIs) (Vadodaria *et al.*, 2015).

3.6 Induced cholinergic motor neurons

Transcription factor mediated reprogramming has also been used to derive cholinergic motor neurons (See Table 2). Son *et al.* (2011) reported that the forced expression of the BAM factors, in combination with *Hb9*, *Isl1*, *Lhx3* and *Ngn2* was sufficient to convert mouse fibroblasts into induced motor neurons (iMNs). The iMNs were identified based on an *HB9::GFP* reporter and exhibited a morphology, gene expression signature, electrophysiological profile, synaptic functionality, *in vivo* engraftment capacity and sensitivity to degenerative stimuli, similar to ESC-derived cholinergic motor neurons (Son *et al.*, 2011). By adding *NEUROD1* to the 7 transcription factors they demonstrated that also human fibroblasts could be converted to iMNs (Son *et al.*, 2011). Other studies have also coupled chemically defined systems with transcription factor-mediated reprogramming in PSCs in order to improve the efficiency and timing to obtain cholinergic motor neurons. For instance, adenoviral delivery of *Isl1, Lhx3* and *Ngn2* combined with exposure to RA and SHH signalling allowed rapid and efficient (>55%) acquisition of electrophysiological active human iMNs within 11 days (Hester *et al.*, 2011). In a separate study, the same set of transcription factors were used to sufficiently differentiate mouse ESCs to a cholinergic motor

neuron identity (Mazzoni *et al.*, 2013). Interestingly, replacement of *Lhx3* by *Phox2a* led to specification of cranial, rather than spinal motor neurons, emphasizing the possibility to direct subtype specification. *Phox2a* and *Phox2b* have also been used to generate both visceral motor neurons and noradrenergic neurons from mouse ESCs in the presence of appropriate patterning molecules (Mong *et al.*, 2014). Culturing *Phox2b* expressing cells under the signalling influence of FGF8 and BMPs promoted the generation of enriched noradrenergic cultures, while culturing *Phox2a* or *Phox2b* expressing cells with FGF8 and SHH generated cholinergic motor neurons instead (Mong *et al.*, 2014). The authors demonstrated that the obtained neurons were suitable for drug testing *in vitro* and, therefore, harbour the potential for the discovery of therapeutic interventions. To conclude, these studies add up to the growing body of protocols, allowing to produce clinically relevant neuronal cells and demonstrate that combinations of distinct methods can work synergistically in obtaining the desired neuronal subtypes.

4. Epigenetic-based approaches

4.1 Epigenetics in directed differentiation and cellular reprogramming

Even though every cell in the human brain shares an identical genotype, it consists of many neuronal subtypes with distinct, yet stable, profiles of gene expression patterns. As addressed above, this diverse repertoire of neurons is produced by extrinsic patterning cues and lineage-specific transcription factors that define and reinforce these neuronal subtype-specific expression patterns. The neuronal phenotypes are further stabilized by the epigenetic machinery that maintains their genetic profile over a lifetime (Barrero *et al.*, 2010; Vierbuchen *et al.*, 2010). Epigenetics can be defined as stable and heritable modifications on the chromatin that occur without changes in the underlying DNA sequence (Delgado-Morales and Esteller, 2017). It is generally accepted that the epigenetic machinery includes multiple levels of transcriptional control by (re)organizing the chromatin structure and architecture

(Goldberg *et al.*, 2007; Lardenoije *et al.*, 2015). Histone modifications and DNA methylation are the most predominant examples of chromatin modifications that have been studied over the last years. Both of these modifications play an important role in neuronal cell fate determination and differentiation (Imamura *et al.*, 2014). In continuously self-renewing ESCs, genes that regulate pluripotency are activated, whereas genes that regulate neuronal differentiation are repressed in a stable and heritable manner over many cell divisions (Avgustinova and Benitah, 2016; Coskun *et al.*, 2012; Lilja *et al.*, 2013; Olynik and Rastegar, 2012). Moreover, this activation and repression needs to be inverted upon initiation of neural induction and differentiation. In addition to these chromatin modifications, noncoding RNAs are other important modulators that regulate gene expression patterns at the post-transcriptional level (Lardenoije *et al.*, 2015). A growing body of evidence is revealing that these also represent strong mediators of neuronal cell fate determination (Liu *et al.*, 2012; Lukovic *et al.*, 2014). Examples of noncoding RNAs include small interfering RNAs (siRNAs), small modulatory RNAs, ribosomal RNAs, transfer RNAs, natural antisense transcripts, enhancer RNAs and microRNAs (miRNAs), but many other subclasses have been described (Esteller, 2011; Guil and Esteller, 2012; Lardenoije *et al.*, 2015).

Based on the key role of the epigenetic machinery in assigning neuronal fate and identity, it has currently become evident that the underlying mechanisms of both directed differentiation and direct cellular reprogramming encompass epigenetic phenomena (Qin *et al.*, 2016; Smith *et al.*, 2016). A significant degree of transcriptional regulation takes place, where epigenetic mechanisms communicate with each other in collaboration with the extrinsic patterning cues and transcription factors to guide neuronal cell fate conversions. The profound epigenome remodelling processes in PSCs and somatic cells eventually lead to the acquisition and stabilization of neuronal subtype-specific gene expression profiles, which reinforce their cellular phenotypes. Although the epigenetic machinery has been extensively studied in ESC

differentiation along the neural lineage, please refer to Hirabayashi and Gotoh (2010), little is known about the extent in which these epigenetic mechanisms are identical in iPSC differentiation. The field is still in its infancy and we are just starting to understand similarities and differences in epigenetic and transcriptional states between iPSCs and ESCs (Huang and Wu, 2013; van den Hurk *et al.*, 2016; Watanabe *et al.*, 2013). In addition, the underlying epigenetic mechanisms of direct reprogramming in somatic cells are also still incompletely understood and the interactions between transcription factors and the chromatin architecture are currently under investigation (Firas *et al.*, 2015; Qin *et al.*, 2016). Nevertheless, considering the crucial role of chromatin modifications and transcriptional regulations by non-coding RNAs in modulating neuronal cell fate and identity, (re)programming and interfering with the epigenetic machinery offers an alternative approach for directing neuronal fates *in vitro* (See Figure 4, Table 3 and Table S3).

4.2 Chemical compounds targeting chromatin-remodelling proteins

First proof-of-concept on epigenetic-based approaches for neuronal differentiation *in vitro* came from experiments where beneficial effects on direct cellular reprogramming by chemical compounds that target chromatin-remodelling proteins were found (See Table 3). Genome-wide profiling of PSCs and their differentiated progeny has suggested a global, progressive transition from euchromatin to heterochromatin at various loci during differentiation (Chen and Dent, 2014). The epigenetic state of a differentiated cell is, therefore, considered to be more condensed and has significantly less dynamic exchange with transcriptional regulators (Chen and Dent, 2014). However, by targeting remodelling proteins, heterochromatin enriched regions can be altered and recruitment of transcriptional activators can be orchestrated to DNA sites that were previously inaccessible, allowing them to induce dramatic cell fate changes. As a consequence, epigenetic research in PSC differentiation and somatic cell reprogramming has supplied a plethora of potential drug targets to improve

neuronal cell fate conversions. For instance, NPCs were induced from mouse fibroblasts by ectopic overexpression of *Pax6* and *Bf1* (Raciti *et al.*, 2013). Treatment with a combination of small molecules that inhibit histone deacetylases (HDACs), H3K27 methyltransferases, and H3K4me2 demethylases accelerated the direct conversion of fibroblasts into NPCs up to ten times (Raciti *et al.*, 2013). Remarkably, simultaneous inhibition of BMP- and TGF-β-signalling almost doubled the frequency of NPCs, again demonstrating that combinations of different approaches can significantly enhance the derivation of the desired cell types *in vitro*. HDAC inhibitors and histone demethylase inhibitors coupled with other appropriate chemical patterning cues have also shown to turn mouse fibroblasts and astrocytes, as well as human urinary cells, into NPCs or neurons under physiological hypoxia conditions and without the need of additional transgene overexpression (Cheng *et al.*, 2015a; Cheng *et al.*, 2015b). Analysis of global gene expression patterns revealed a high degree of similarity between the induced NPCs and control NPCs (Cheng *et al.*, 2015b), whereas the neurons induced from cultured astrocytes were electrophysiological active and expressed various subtype specific markers for dopaminergic neurons, GABAergic neurons, glutamatergic neurons and cholinergic motor neurons (Cheng *et al.*, 2015a). The delivery of small molecules or systematic administration of drug cocktails enabling astrocytic-to-neuronal conversions bear the potential for direct induction of desirable cells from resident astrocytes *in situ*, while bypassing possible adverse effects of genome integrating constructs. Taken together, drugs targeting the chromatin can improve reprogramming efficiency and might function as useful adjuvants in currently used reprogramming protocols, thereby providing a possible alternative strategy to produce patient-specific neuronal cells.

4.3 Epigenetic editing

Epigenetic editing systems (Kungulovski and Jeltsch, 2016; Thakore *et al.*, 2016) offer an alternative tool to supplement, or in some cases even replace components of, current widely

used directed differentiation and cellular reprogramming protocols (Jurkowski *et al.*, 2015). Making locus-specific alterations to the epigenetic code allows to (re)shape the mechanistic relationships among chromatin state, gene regulation, and cellular phenotype by the natural dynamics of gene expression (Thakore *et al.*, 2016). For this reason, these epigenetic editing systems allow probing of signatures responsible for cellular identity and provide intelligent control to direct neuronal cell fates in PSCs and somatic cells (Black *et al.*, 2016; Chavez *et al.*, 2015; Kearns *et al.*, 2014; Victor *et al.*, 2014). DNA-targeting platforms based on the initially established zinc finger proteins (ZFPs), transcription activator-like effector nucleases (TALENs) and the clustered, regularly interspaced, short palindromic repeats (CRISPR)-Cas9 systems, have allowed the recruitment of transcriptional modulators and epigenome-modifying factors to any genomic locus (Kungulovski and Jeltsch, 2016; Laufer and Singh, 2015; Thakore *et al.*, 2016). Virtually any DNA sequence can be targeted with these customizable synthetic epigenetic tools (Thakore *et al.*, 2016). The direct fusion of transcriptional effector domains to designed DNA-targeting domains can induce transcriptional activation or repression of endogenous key-lineage-determinant genes (Thakore *et al.*, 2016). Transcriptional effector domains include epigenetic effectors that directly catalyse covalent modifications to DNA or histones, or that recruit other histone modifying enzymes, as well as interfere with chromatin-binding proteins. For instance, epigenetic effectors that directly catalyse covalent modifications to DNA, such as DNA methyltransferases (DNMTs) or ten-eleven translocation (TET) enzymes, can methylate or demethylate CpGs at non-neuronal and neuronal target promoters, leading to transcriptional repression or induction, respectively (Jurkowski *et al.*, 2015; Liu *et al.*, 2016). Other effector domains such as VP64 can recruit histone remodelling factors, leading to increased chromatin accessibility and to the deposition of activating histone modifications at desired neuronal loci (Chakraborty *et al.*, 2014; Wei *et al.*, 2016). Alternatively, localization of DNA-targeting

domains without an epigenetic effector to promoter regions or regions downstream of the transcription start sites can silence non-neuronal gene expression by steric hindrance of lineage-specific transcription factor binding and RNA polymerase elongation (Chavez *et al.*, 2015; Jurkowski *et al.*, 2015). Thus, custom epigenetic and transcriptional regulation by epigenetic editing-based approaches offer refined control over cell fate decisions, providing an invaluable tool for applications such as directed differentiation and cellular reprogramming along the neural lineage (See Table 3).

Several pioneer studies utilizing these epigenetic editing systems have shown successful and precise deposition or removal of different chromatin modifications to induce directed differentiation and cellular reprogramming for multiple cell types, including iPSCs, myocytes and neurons. Gao *et al.* (2013) used TALE-based transactivators targeting distal enhancers of *Oct4* in concert with *Sox2*, *Klf4* and *Myc* transgene overexpression to generate mouse iPSCs. More recently, a protocol for the direct conversion of mouse fibroblasts to skeletal myocytes using a dCas9-based transactivator targeting the endogenous *Myod1* gene has been developed (Chakraborty *et al.*, 2014). Other groups have also applied dCas9-based transcriptional regulation to direct the differentiation of human PSCs (Balboa *et al.*, 2015; Chavez *et al.*, 2015). In an elegant study, human iPSCs were derived from human skin fibroblast by replacing *OCT4* overexpression with dCas9-mediated activation of the endogenous promoter (Balboa *et al.*, 2015). The authors demonstrated that directed endodermal differentiation of the iPSCs could be achieved by targeting proximal promoters of endodermal and pancreatic key-regulatory transcription factors, including *FOXA2*, *SOX17*, *GATA4*, *PDX1*, and *NKX6-1* (Balboa *et al.*, 2015). Directed neuronal reprogramming through multiplex endogenous gene activation using an engineered VP64-dCas9-VP64-based transcriptional activator has also been achieved (Black *et al.*, 2016). Induced activation of the BAM factors successfully converted mouse fibroblasts to iN cells and their expression sustained in high levels during

later stages of reprogramming despite the transient delivery of the guide RNAs (gRNAs) (Black *et al.*, 2016). In a separate study, it was demonstrated that rapid and robust neuronal differentiation of human iPSCs could be achieved by targeting *NGN2* and *NEUROD1* with a VP64-p65-Rta-dCas9-mediated transcriptional activator (Chavez *et al.*, 2015). Although many of the aforementioned reports include targeted activation of a single gene in combination of concurrent overexpression of multiple transcription factors, these latter examples also demonstrate that multiplex activation with a collection of gRNAs against a set of genes can be used to direct somatic cell reprogramming.

Previous studies have demonstrated that targeted epigenetic editing of the regulatory elements controlling expression of lineage-specific transcription factors is sufficient for direct conversion between cell types, emphasizing the feasibility and potential advantages of using these synthetic epigenetic systems to direct neuronal cell fate of PSCs and somatic cells *in vitro*. Moreover, epigenetic editing in isolation has incredible promise as a platform for disease modelling both in *vitro*, as well as *in vivo*, whereas using it for transdifferentiation within the native physiological niche of the human brain might provide an alternative strategy to achieve cell fate conversions for applications in regenerative medicine (Cano-Rodriguez and Rots, 2016; Fu *et al.*, 2014a; Liu *et al.*, 2016). Several labs have recently pioneered *in vivo* reprogramming in the brain and spinal cord by converting endogenous glial cells (Gascón *et al.*, 2016; Guo *et al.*, 2014; Torper *et al.*, 2013) and NPCs (Niu *et al.*, 2013; Ohori *et al.*, 2006) into functional neurons. For a more in-depth review on more surpassed studies, please refer to Li and Chen (2016) and Srivastava and DeWitt (2016). Epigenetic editing systems applied in a safe and efficient manner that target similar lineage-specific transcription factors, might allow to induce any desirable neuronal subtype *in vivo* and could eliminate undesired issues in cell transplantations that may arise due to precedent *in vitro* cultures. To conclude, ground-breaking advances in this field are beginning to yield novel opportunities in the

context of inducing neuronal phenotypes and bear excessive potential for many different applications in fundamental research and biomedicine.

4.4 RNA interference using miRNAs

On the one hand, the acquisition of distinct histone and DNA modifications at neuronal genes and non-neuronal genes plays a role in determining neuronal identity. On the other hand, neuronal identity is also determined by synergistic actions of extrinsic cues and the combined expression of transcription factors that are modulated by transcriptional regulators, including non-coding RNAs. Among these, miRNAs have been most extensively studied in relation to cellular identity and although most of the other subclasses are also anticipated to play important roles in regulating neuronal cell fate determination, the exact contribution of many remains elusive. Currently, it is accepted that miRNAs promote the transition from ESC self-renewal to differentiation by either directly suppressing the self-renewal state or by stabilizing the differentiated state (Ong *et al.*, 2015). In addition, multiple miRNAs target components or modulators of neural developmental signalling pathways, such as BMP and TGF-β signalling, and have been identified to either positively or negatively affect entry along the neural lineage (Stappert *et al.*, 2015). In mature neurons, it has been proposed that the cellular miRNA milieu might even be unique in each subtype and required to facilitate developmental transitions during differentiation (Lopez-Ramirez and Nicoli, 2014). Some miRNAs even exhibit region-specific expression patterns in the brain, suggesting that neuronal subtypes residing in these regions may express different miRNA profiles (Stappert *et al.*, 2015). This is exemplified by a study of He *et al.* (2012), in which they demonstrated substantial differences between the miRNA repertoire expressed in glutamatergic neurons and GABAergic interneurons that co-expressed either PV or SST. Specifically, miR-133b and miR-187 were found to be expressed higher in GABAergic interneurons as compared to glutamatergic

neurons, where miR-133b was more abundant in PV expressing and miR-187 in SST expressing GABAergic interneurons (He *et al.*, 2012).

Based on the emerging role of miRNAs during neural induction, neuronal differentiation and neuronal subtype specification, techniques such as RNAi using miRNAs holds great promise as an alternative tool to direct neuronal cell fate *in vitro* (See Table 3) (Low *et al.*, 2012; Stappert *et al.*, 2013). RNAi is a post-transcriptional gene silencing technique that has therapeutic opportunities for the treatment of various human disorders and has extensively been employed in translational studies to address fundamental biological questions (Low *et al.*, 2012). The potential of RNAi lies in its capacity to virtually target any RNA molecule of interest, which allows fine-tuning of expression of key-determinant factors in neuronal fate determination. Approaches to downregulate endogenous miRNA expression to influence gene expression opposite of RNAi are also available and could for example be achieved by using anti-sense oligonucleotides (ASOs) (See Table 3) (Davis *et al.*, 2006). The temporal control of miRNA regulation might facilitate the induction of neuronal subtype-specific transcriptional networks and aids in recapitulating the natural dynamics of transcriptional regulation during neuronal cell fate commitment. Each miRNA has multiple molecular targets that might play essential roles in the derivation of specific neuronal subtypes and modulating a single miRNA could, therefore, guide entire neural developmental processes. Moreover, successful delivery of miRNAs could be achieved in many different ways depending on the needs of the experiments, as each method has different transfection efficiencies and transgene expression duration. Representative methods that allow expression of a miRNA construct include non-viral delivery systems such as lipid-based transfection, electroporation or the use of microvesicles, or viral delivery systems such as lentiviruses and adeno-associated viruses (Yang, 2015).

The potential of miRNAs to complement current practiced directed neuronal differentiation protocols from PSCs was first demonstrated by Kim *et al.* (2007). Interestingly, in this study they reported an unexpected negative impact of miR-133b on the generation of dopaminergic neurons from mouse ESCs. MiR-133b was found to be enriched in the human midbrain, while overexpression in this study impaired the generation of TH-positive cells (Kim *et al.*, 2007). Inhibition of miR-133b on the other hand, resulted in an increased dopaminergic differentiation. The authors speculated that miR-133b regulates the maturation and function of midbrain dopaminergic neurons within a negative feedback circuit that includes the dopaminergic transcription factor *Pitx3*. A similar negative impact on the differentiation of dopaminergic neurons from mouse ESCs has been reported in a separate study (Yang *et al.*, 2012). Inhibition of miR-132 promoted the differentiation of dopaminergic neurons, while ectopic expression of miR-132 decreased the derivation of TH-positive cells without affecting the total number of neuronal cells. Through a bioinformatics assay they identified *Nurr1* as a potential molecular target of miR-132, which also represents a key transcription factor of dopaminergic neuron specification. Stappert *et al.* (2013) showed that miR-125b and miR-181a specifically promote the generation of neurons of dopaminergic fate from NPCs derived from human ESCs, whereas miR-181a* inhibits the development of this neurotransmitter subtype. By using a set of miRNA-mimics and –inhibitors, they also demonstrated that inhibition of miRNA-124 enhances the development of dopaminergic neurons (Stappert *et al.* 2013). Although other studies on additional neuronal subtypes have not been published, these studies demonstrated that time-controlled modulation of specific miRNA activities can contribute to the derivation of defined neuronal cells *in vitro*.

Recent developments in direct somatic cell reprogramming also highlighted the potential of miRNAs as mediators for transdifferentiation along the neural lineage. The convergence of transcriptional control by miRNAs that leads into direct cellular transitions is exemplified by

miR-9/9* and miR-124, which both belong to a set of brain-enriched miRNAs that are activated upon initiation of neurogenesis (Abernathy *et al.*, 2017). It has been shown that overexpression of miR-124 along with *BRN2* and *MYT1L* is able to reprogram human fibroblasts into functional neurons in the absence of other cell types (Ambasudhan *et al.*, 2011). These iNs exhibited typical neuronal morphology, appropriate electrophysiological properties and were able to form functional synapses between each other (Ambasudhan *et al.*, 2011). In a separate study, it was reported that the expression of miR-9/9* and miR-124 in human fibroblasts induced their direct conversion into neurons, a process which was enhanced by the addition of several transcription factors, including *ASCL1, MYT1L* and *NEUROD2* (Yoo *et al.*, 2011). Importantly, they found that the expression of these transcription factors alone without the miRNAs was inefficient to induce a neuronal phenotype, suggesting that this miRNA-induced neuronal state is indulgent to subtype-specific transcription factors that can initiate and advance differentiation towards mature neuronal identities (Abernathy *et al.*, 2017). Co-expression of miR-9/9* and miR-124 with transcription factors enriched in the developing striatum, including *CTIP2*, *DLX1*, *DLX2*, and *MYT1L*, guided the conversion of human fibroblasts into enriched populations of GABAergic neurons analogous to striatal medium spiny neurons (Victor *et al.*, 2014).

Synergism between miR-9/9*, miR-124 and two other neuronal subtype-specific transcription factors, *i.e. ISL1* and *LHX3*, has also been shown to be able to induce a highly homogeneous population of spinal cord motor neurons in adult human fibroblasts (Abernathy *et al.*, 2017). Longitudinal analyses of the transcriptome, genome-wide DNA-methylation, and chromatin accessibilities in the latter study revealed that miR-9/9* and miR-124 trigger reconfiguration of the epigenome, including activation of a pan-neuronal program and the reconfiguration of chromatin accessibilities (Abernathy *et al.*, 2017). Neurons solely induced by miR-9/9*- and miR-124 demonstrated to be functionally excitable and uncommitted toward specific

subtypes, but possess open chromatin structures at neuronal subtype-specific loci that can be activated upon further instructions (Abernathy *et al.*, 2017). The authors, therefore, also suggested that expression of bot miRNAs in somatic cells initiates gradual but active changes in the activities of multiple chromatin modifiers while simultaneously repressing anti-neuronal genes and activating neuronal genes, resulting in the binary cell fate switch. The fact that pre-existing neuronal loci within the heterochromatic regions opened-up in response to miR-9/9* and miR-124, suggests that miRNA-mediated reprogramming could indeed stem from their ability to induce remodelling of the epigenome. Taken together, these findings demonstrate that miR-9/9* and miR-124 control the neurogenic potential of somatic cells and provide a platform for the foundation of subtype-specific neuronal conversions of human cells. For a further in depth description of all other brain-enriched miRNAs, their target genes and exact functions, please refer to excellent reviews by Åkerblom and Jakobsson (2014), Coolen *et al.* (2013), Meza-Sosa *et al.* (2014) and Stappert *et al.* (2015).

To conclude, miRNA-mediated neuronal differentiation and transdifferentiation have enlarged our current toolkit for directing neuronal cell fate *in vitro* and have the potential to widen our understanding on the transcriptional regulations in cell fate decisions. Further insights could in the end be exploited to develop new protocols in order to obtain enriched populations of the complete repertoire of neurons found in the human brain.

5. Discussion and future directions

The establishment of efficient stepwise protocols to obtain functional neurons *in vitro* is highly essential for the study of human brain functions, as well as disease modelling, drugs discovery and regenerative medicine. In this review, we have highlighted the advances that have been made over the last two decades in obtaining neuronal cells from PSCs and somatic cells. Insights from basic research and developmental biology have guided the design of current strategies and numerous protocols for glutamatergic, dopaminergic, GABAergic,

serotonergic, and cholinergic/motor neurons have become available. The use of chemically defined systems and ectopic overexpression of key lineage-specific transcription factors have been first-choice to direct neuronal fates *in vitro*. In the chemically defined systems, PSCs have been exposed to a variety of cocktails containing patterning cues and small molecules that induce differentiation towards early NPCs and eventually specific mature neuronal subtypes. Classical experiments such as nuclear transfer or cell fusion on the other hand, have demonstrated that differentiated cells are not irreversibly committed to their fate. Very recent work has built on these conclusions and discovered that ectopic overexpression of defined transcription factors can directly generate iNs from distinct somatic cell types, as well as from PSCs. Other groups have combined both approaches and have shown successful derivation of neuronal populations and increased protocol efficiencies in both PSCs and somatic cells.

Scientists have also uncovered the existence of intrinsic mechanisms that influence the responsiveness to patterning cues and transcription factors. As we have outlined above, recent work has demonstrated a significant contribution of the epigenetic machinery to assigning neuronal fate and identity. From cancer studies, it is also becoming apparent that shifts in epigenetic signatures underlie phenotypic changes, and can induce stem cell-like properties in cancer cells due to transcriptional reprogramming (Mateo *et al.*, 2017). Several pioneering studies have now used this knowledge in combination with the current growing availability of epigenetic editing systems and RNAi, enabling the modification of epigenetic marks at key-determinant loci that allow to direct neuronal fates *in vitro*. Additionally, lists of potential drugs and drug targets such as histone-modifying enzymes have been suggested to improve neuronal conversions. Such approaches on their own or in combination with others could lead to an accelerated application of the obtained neuronal populations. It is therefore, anticipated that epigenetic editing systems and RNAi will be increasingly involved in PSC-differentiation and cellular reprogramming in the near future. These epigenomic editing tools even have the

potential to become a golden standard for probing interactions among specific chromatin modifications, transcriptional programmes and cellular phenotypes. However, care should be taken in terms of advantages and disadvantages that come together with each of the aforementioned methods. For instance, off-target effects with the use of epigenetic-based strategies are common and should be reduced at all times to prevent experimental bias and undesirable outcomes, especially when one would consider the use of these cells in regenerative medicine. Such systems in combination with genome-integrating techniques can affect the genome in a way that it might lead to adverse changes in the biology of the cell, including changes in its differentiation potential. Genome-integrating constructs are randomly incorporated into the host genome and the copy number of the exogenous DNA per cell may vary to great extent, since there are often no specific genetic elements or no apparent logic for their integration (Medvedev *et al.*, 2010). Furthermore, integration can occur into various chromatin-regulatory elements and interfere with gene-coding sequences, possibly affecting endogenous transcriptional regulation and three-dimensional chromatin structures. Finally, there is a possibility that transgenes maintain their activity or reactivate in the progeny of the initial targeted cell type. Nevertheless, studies have already shown to circumvent the issue with genomic-integrating techniques by the use of removable constructs, non-integrating systems such as adeno-associated vectors, transfections of mRNAs, transduction of reprogramming proteins and the use of small molecule compounds. Furthermore, promoters controlled by chemical compounds that allow to regulate transgene transcription, such as doxycycline (DOX) for example (See Table 2 and Table 3), might provide an alternative to prevent continuously expressed transgenes and, thus, allows better temporal control of transgene activation.

Despite the achievements in directing neuronal cell fate *in vitro*, several other challenges also need to be addressed before their full potential in fundamental research and biomedicine can

be utilized. Different cultivation protocols for each neuronal subtype currently exist and the majority of these protocols result in heterogeneous neuronal populations with remarkable differences in efficiency. Furthermore, we are currently also unable to enrich for the full repertoire of neurons found in the human brain, especially when considering specific anatomical subtypes within neurotransmitter classes. For these reasons, the refinement and the search for alternative approaches that will allow us to obtain all known neuronal subtypes found in the human brain remains an ongoing demand. Although multicellular cultures will be pivotal for brain-related studies, optimizing the used parameters and devising strategies to enrich for specific neuronal subtypes will also be essential in order to obtain pure populations of neurons, which will allow mechanistic studies and clinical applications in which confounding effects from other cell types can be kept to a minimum. In this respect, one could think of combining different protocols or adding additional factors to existing protocols that may act synergistically in mimicking the complete molecular processes that (re)assign cell fates along the neural lineage. As has been shown in many available approaches, every single driving factor fulfils a crucial role as a part of a bigger network and absence of a single component can completely redirect the terminal differentiation, as well as induce incomplete differentiation or reprogramming processes, leading to the production of immature cells that may not fully recapitulate *bona fide* neurons. By combining directed differentiation with transcriptional reprogramming and/or epigenetic-based approaches, one might be able to control these cellular conversions in a robust way, which could, in turn, also lead to a greater diversity of neuronal subtypes to be specified *in vitro*. Another option to enhance culture purity could be achieved by finding more effective ways to isolate the desired cell types in different developmental stages. The use of specific cellular markers or expressing constructs combined with cell-sorting techniques, such as fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) might provide suitable solutions (See Table S1-3).

The choice of the initial tissue and cell type for generating the neuronal populations also needs considerable attention and could significantly affect the efficiency of a neuronal differentiation protocol. For instance, it is currently recognized that there are epigenetic differences between different PSC lines that can induce lineage differentiation bias (Nishizawa *et al.*, 2016). This is exemplified in the study by Kim *et al.* (2011c), where they observed that the majority of neurons generated from one iPSC line were glutamatergic, whereas populations generated from another iPSC line mainly consisted of GABAergic neurons, when exposed to the same chemical culture conditions. Scientists have committed to the challenge to find markers that will allow them to predict this bias. The presence of such a marker could reveal which cell line has the highest neuronal differentiation capacity, leading to an increased neuronal conversion efficiency later on. It is noted that the origin-dependent epigenetic and transcriptional patterns of the pluripotent state can render iPSC lines with different neuronal differentiation potential. Cell lines that harbour epigenetic signatures, which were maintained and are characteristic of the somatic tissue of origin, have been shown to favour differentiation along lineages related to the donor cell, while restricting alternative cell fates (Kim *et al.*, 2010). Furthermore, it has been show that expression levels of other transcriptional regulators in iPSCs, such as miR-371-3, can predict neuronal differentiation propensities (Kim *et al.*, 2011a). Aside from presenting the concept of epigenetic memory that may influence efforts in directed differentiation in iPSCs, this also emphasizes the role of epigenetic mechanisms in neuronal differentiation and addresses the complexity of the neuronal fate determination that needs the warrant for more comprehensive comparisons between different PSC lines.

Another debate in relation to starting cell choice, but based on direct reprogramming, arises from the initial assumption that developmentally related cells convey a higher conversion efficiency, as compared to cells that originate from distinct germ layers (Masserdotti *et al.*,

2016). Indeed, previous studies have shown that cells derived from the same lineage, such as astrocytes and neurons, can be converted with minimal sets of transcription factors, whereas cells of non-ectodermal origin require more than one factor or additional chemical stimulation (Gascón *et al.*, 2017). Even though this is not always the case, as conversion of one neuronal subtype into another is rather difficult and has been achieved only in immature cells (Gascón *et al.*, 2017; Rouaux and Arlotta, 2013), lineage boundaries established during cellular differentiation and specification might be overcome, depending on the potency of the factors that are employed (Masserdotti *et al.*, 2016). However, the questions to what extent direct reprogramming recapitulates the natural dynamics of neuronal differentiation and whether the developmental origin and, more specifically, the epigenetic memory of the starting cell type is negligible when using the proper reprogramming factors remain unanswered. The underlying principle of direct reprogramming is based on the expression of key lineage-specific transcription factors that are essential during development, but their action during direct reprogramming can be rather different, since these factors are operating in a completely different context (Masserdotti *et al.*, 2016). Further studies on how factors function in various reprogramming environments may bring new insight that can lead into the establishment of more robust neuronal differentiation protocols in various somatic cell subtypes, as well as in PSCs. Additionally, detailed characterization of the cellular and molecular characteristics involved in guiding PSC-differentiation and somatic cell reprogramming along the neural lineage is expected to contribute, not only to enhance our understanding on the developmental aspects, but also to develop more efficient protocols and rational interventions (Kee *et al.*, 2017; Kirkeby *et al.*, 2017). Continuous characterization of patterning cues, small molecules and other driving factors, as well as a comprehensive understanding of the underlying molecular pathways that they target will be necessary to achieve a higher efficiency, decrease culture heterogeneity and increase neuronal subtype availability. Single cell analysis and

direct comparison of differentiated cells versus undifferentiated counterparts will be crucial in order to find signalling mechanisms, as well as to develop a comprehensive understanding of the epigenetic state and transcriptional programmes involved in neuronal fate specification. Such knowledge can in the end be exploited to manipulate single molecules or even complete molecular networks in the developmental processes and, hence, could aid the production of specific neuronal cells of interest. With the current advances of next-generation sequencing technology we are now also able to define genome-wide expression patterns and epigenetic modifications in each cell type. Such approaches have already demonstrated that different cellular subtypes display unique epigenetic signatures that persist as ESCs differentiate into the neuronal lineage. However, how these unique signatures are acquired in specific gene promoters or to what extent they are involved in shaping neuronal fates remains to be elucidated.

A final point of consideration with regard to the use of PSC- and somatic cell-derived neuronal populations in fundamental research and biomedicine, is that the conventional cell culture systems do not fully resemble the *in vivo* cellular microenvironment, where three-dimensional cell-to-cell interactions form the foundation of the human brain. The simplicity of an *in vitro* culture system is an advantage, as well as a significant disadvantage, when cellular homogeneity becomes a reliability. Studying pure populations of neuronal subtypes that are in principle part of a more complex integrated cellular network, might lead to under- or overrepresentation of experimental findings depending on the research question. Organoids or three-dimensional culture systems in combination with bioprinting might offer a way to circumvent this issue (Fatehullah *et al.*, 2016; Lancaster and Knoblich, 2014; Murphy and Atala, 2014). Several studies have already succeeded in establishing organoids that imitate many features of human cortical development in a precise and complex manner (Dezonne *et al.*, 2017; Lancaster *et al.*, 2013; Li *et al.*, 2017). Such culture systems derived from human

iPSCs hold great potential for the investigation of developmental and evolutionary features of the human brain and provides a useful platform for drug screening and disease modelling (Dezonne *et al.*, 2017; Nascimento and Martins-de-Souza, 2015). Additionally, the technology of decellularization and recellularization on obtained tissue matrixes to create entire organs *in vitro* is currently also under development (Fu *et al.*, 2014b). Unfortunately, despite these advances, the challenge of how to generate organs that cherish the highly integrated cellular complexities like in the human brain persists, and will require collaborative multidisciplinary expertise to overcome. Nevertheless, neuronal *in vitro* differentiation techniques in combination with these advanced three-dimensional culture systems represent powerful tools for future brain-related studies. The potential to manipulate (epi)genetic and environmental factors in culture conditions, with the possibility to characterize cellular functions, electrophysiological properties and cellular connectivity of various neuronal subtypes in isolation, as well as organized in a multi-layered dimension, will be of great utility to enhance our current understanding on brain disorders and will undoubtedly contribute to the development of therapeutic interventions.

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Conflict of interest statement

The authors declare no conflict of interest.

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List of abbreviations

5-HT, serotonin (5-hydroxytryptamine);

A83-01, TGF-β kinase/activin receptor-like kinase inhibitor;

ACTB, actin beta/beta-actin;

ASCL1, achaete-scute homolog 1 (MASH1);

ASOs, anti-sense oligonucleotides;

Bcl-xL, b-cell lymphoma-extra large;

BDNF, brain derived neurotrophic factor;

BF1, brain factor 1/forkhead box protein G1 (FOXG1);

BIO, GSK3β inhibitor 6-bromoindirubin-3'-oxime;

BIX-01294, histone-lysine methyltransferase inhibitor;

BMP, bone morphogenic protein;

BMP5, bone morphogenic protein 5;

BMP7, bone morphogenic protein 7;

BMPRIA-Fc, bone morphogenetic protein receptor 1a-fragment crystallizable;

BMPs, bone morphogenic proteins;

BRN2, brain-specific homeobox/POU domain protein 2 (POU3F2);

CALB1, calbindin 1;

Cas9, CRISPR associated protein 9;

CD4, cluster of differentiation 4;

CDK4/6, cyclin-dependent kinase 4/6;

CHAT, choline o-acetyltransferase;

CHIR99021, GSK3β inhibitor;

CpGs, cytosine-phosphate-guanine sites;

CRISPR, clustered regularly interspaced short palindromic repeats;

CTIP2, b-cell CLL/lymphoma 11b (BCL11B)/ COUP-TF-interacting protein 2 (COUP-TFII);

CUX1, cut like homeobox 1;

DARPP-32, dopamine and cAMP-regulated neuronal phosphoprotein 32;

dCas9, catalytically inactive CRISPR associated protein 9;

DCX, doublecortin;

DI, diencephalon;

DKK1, dickkopf-1;

DLX1, distal-less homeobox 1;

DLX2, distal-less homeobox 2;

Dlx5, distal-less homeobox 5;

DMH-1, dorsomorphin homolog 1;

DNA, deoxyribonucleic acid;

DNMTs, DNA methyltransferases;

DOR, Dorsomorphin;

DOX, doxycycline;

dsRed, discosoma sp. red fluorescent protein;

EB, embryoid body;

EBs, embryoid bodies;

EGF, epidermal growth factor;

EGFP, enhanced green fluorescent protein;

En1, homeobox protein engrailed 1;

ePet, enhancer of the mouse Pet-1 (human FEV) gene;

ESCs, embryonic stem cells;

FACS, fluorescence-activated cell sorting;

FEV, ETS transcription factor (PET1);

FGF, fibroblast growth factor;

FGF10, fibroblast growth factor 10;

FGF2, fibroblast growth factor 2/basic fibroblast growth factor (bFGF);

FGF4, fibroblast growth factor 4;

FGF8, fibroblast growth factor 8;

FGF8b, fibroblast growth factor 8 isoform b;

FOXA2, forkhead box A2;

FOXP1, forkhead box P1;

FOXP2, forkhead box P2;

GABA, γ-aminobutyric acid;

GAD65/67, glutamic acid decarboxylase isoform 65/67 (GAD2/1);

GAD67, glutamic acid decarboxylase isoform 67 (GAD1);

GATA2, GATA binding protein 2;

GATA3, GATA binding protein 3;

GATA4, GATA binding protein 4;

GBX2, gastrulation brain homeobox 2;

GCaMP3, a GFP-based calcium sensor for imaging calcium dynamics;

GDNF, glial derived neurotrophic factor;

GFAP, glial fibrillary acidic protein;

GFP, green fluorescent protein;

GIRK2, G protein-activated inward rectifier potassium channel 2 (KCNJ6);

gRNAs, guide RNAs;

GSK3β, glycogen synthase kinase 3 beta;

H3K27, histone H3 lysine 27;

H3K4me2, histone H3 dimethyl K4;

HB9, homeobox HB9/motor neuron and pancrease homeobox 1 (MNX1);

HDAC, histone deacetylase;

HDACs, histone deacetylases;

HEK293T, human embryonic kidney 293 cell line expressing a SV40 large T antigen;

Hh-Ag1.3, small molecule agonist of SHH signalling;

HOX, homeobox;

Hoxa2, homeobox a2;

Hoxa5, homeobox a5;

Hoxc4, homeobox c4;

Hox8-11, homeobox 8-11;

hp53shRNA, human p53 small hairpin RNA;

i5HT neurons, induced serotonergic neurons;

iDA neurons, induced dopaminergic neurons;

iGNs, induced GABAergic interneurons;

iMNs, induced motor neurons;

iN cells, induced neuronal cells;

INSM1, INSM transcriptional repressor 1;

iPSCs, induced pluripotent stem cells;

IRES, internal ribosome entry site;

ISL1, ISL LIM homeobox 1;

ISL1/2, ISL LIM homeobox 1/2;

KLF4, kruppel-like factor 4;

Lbx1, ladybird homeobox 1;

LDN193189, selective BMP signalling inhibitor;

Lefty-1, left-right determination factor 1;

Lefty-2, left-right determination factor 2;

Lhx1, LIM homeobox 1;

Lhx2, LIM homeobox 2;

LHX3, LIM homeobox 3;

LHX6, LIM homeobox 6;

Li2CO3, lithium carbonate;

LiCl, lithiumchloride;

LIF, leukemia inhibitory factor;

LIN28, lin-28 homolog a;

LMX1A, LIM homeobox transcription factor 1 alpha;

LMX1B, LIM Homeobox transcription factor 1 beta;

MACS, magnetic-activated cell sorting;

MAP2, microtubule-associated protein 2;

MAP2ab, microtubule-associated protein 2ab;

Msx1, msh homeobox 1;

MEF, mouse embryonic fibroblast;

MES, mesencephalon;

MET, metencephalon;

miRNA, microRNA;

miRNAs, microRNAs;

mRNA, messenger RNA;

MS-5, stromal cell line derived from irradiated murine bone marrow cultures;

MYC, myc/cMyc gene or protein;

MYE, myelencephalon;

MYOD1, myogenic differentiation 1;

MYT1L, myelin transcription factor 1 like;

NaB, sodium butyrate;

NANOG, homeobox protein NANOG;

NES, nestin;

NEUN, neuronal nuclei antigen;

NEUROD1, neurogenic differentiation 1;

NEUROD2, neuronal differentiation 2;

NGN2, neurogenin 2;

NKX2-1, NK2 homeobox 1;

NKX2-2, NK2 homeobox 2;

NKX6-1, NK6 homeobox 1;

NLGN3, neuroligin 3;

NLGN4, neuroligin 4;

NOG, Noggin;

NPCs, neural precursor cells;

NURR1, nuclear receptor related 1 protein;

OCT3/4, octamer-binding transcription factor 3/4;

OLIG2, oligodendrocyte transcription factor 2;

OSKM, Oct4, Sox2, Klf4 and Myc (Yamanaka factors);

OTX2, orthodenticle homeobox 2;

p53, tumour protein p53;

p65, nuclear factor nuclear factor kappa beta (NF-κB) p65 subunit;

PA6, stromal cell line derived from newborn calvaria tissue of the C57BL/6 mice;

Pax2, paired box 2;

PAX6, paired box 6;

PD0332991, cyclin-dependent kinase 4/6 inhibitor;

PDX1, pancreatic and duodenal homeobox 1;

PET1, ETS transcription factor (FEV);

Pft1a, pancreas specific transcription factor 1a;

PHOX2A, paired-like homeobox 2a;

PHOX2B, paired-like homeobox 2b;

Pitx2, paired-like homeodomain 2;

PITX3, paired-like homeodomain 3;

PSC, pluripotent stem cell;

PSCs, pluripotent stem cells;

PUR, purmorphamine;

PV, parvalbumin;

RA, retinoic acid;

RELN, reelin;

REST, RE1-silencing transcription factor;

RFP, red fluorescent protein;

RNA, ribonucleic acid;

RNAi, RNA interference;

RNAs, ribonucleic acids;

ROCK, rho-associated coiled-coil containing protein kinase;

Rta, R transactivator;

RUES1-EGFP, human ESC line expressing EGFP;

SAG, smoothened agonist;

SB431542, transforming growth factor beta inhibitor;

SHH, sonic hedgehog;

SHH-C24II, recombinant human SHH;

SHH-C25II, recombinant mouse SHH;

siRNAs, small interfering RNAs;

SMAD, transcription factor and member of the BMP and TGF-β signalling pathways;

SOX1, SRY box 1;

SOX17, SRY box 17;

SOX2, SRY box 2;

SOX3, SRY box 3;

SSRIs, selective serotonin reuptake inhibitors;

SST, somatostatin;

SYN1, synapsin 1;

t2PCPA, trans-2-phenyl-cyclopropylamine hydrochloride;

TALENs, transcription activator-like effector nucleases;

TALEs, transcription activator-like effectors;

TAU, microtubule-associated protein tau (MAPT);

TBR1, T-box brain 1;

TdT, tandem dimer tomato red fluorescent protein;

TEL, telencephalon;

TET, ten-eleven translocation;

TGF-β, transforming growth factor beta;

TH, tyrosine hydroxylase;

TPH, tryptophan hydroxylase;

TPH2, tryptophan hydroxylase 2;

Trp53, tumor protein p53;

TSA, trichostatin A;

TUJ1, neuron-specific class III beta-tubulin (TUBB3);

VACHT, vesicular acetylcholine transporter;

VGAT, vesicular GABA transporter;

VGLUT1, vesicular glutamate transporter 1;

VGLUT2, vesicular glutamate transporter 2;

Vmat2, vesicular monoamine transporter 2 (SLC18A2);

VP64, tetrameric VP16 transcription activator domain;

VP64-dCas9-VP64, dCas9 with N-terminal and C-terminal VP64 transactivation domains;

VP64-p65-Rta-dCas9, dCas9-based transcriptional activator containing VP64, p65 and Rta;

VPA, valproic acid;

WNT, wingless-type MMTV integration site family;

XAV939, WNT/β-catenin inhibitor;

Y-27632, ROCK inhibitor;

ZFPs, zinc finger proteins.

Figure 1. Overview of methods and applications for neurons obtained by directed differentiation and (direct) cellular reprogramming in embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and somatic cells. (A) The figure shows the complete directed differentiation and (direct) cellular reprogramming toolkit currently available, including the use of patterning factors, chemical compounds, small molecules, transcription factors, epigenetic editing and RNA interference (RNAi) with the use of microRNAs (miRNAs) as example. The neurodevelopmental transitions starting from PSCs towards neural precursor cells (NPCs) and eventually mature neurons are depicted. Directed differentiation protocols can generally be divided into three methods: monolayer methods, co-culture methods and embryoid body (EB) methods. Reprogramming of somatic cells towards iPSCs can be achieved by increased expression of transcription factors, such as *Oct3/4*, *Sox2*, *Klf4* and *Myc* (OSKM, Yamanaka factors). Direct reprogramming of somatic cells towards specific neuronal subtypes can be achieved by co-expression of the pan-neuronal factors *Brn2*, *Ascl1*, *Myt1* (BAM) and *Neurod1* among others, and neuronal subtype-specific transcription factors. (B) The direct applications of the obtained neurons in fundamental research and biomedicine, including the use for disease modelling, drug discovery and regenerative medicine, are shown.

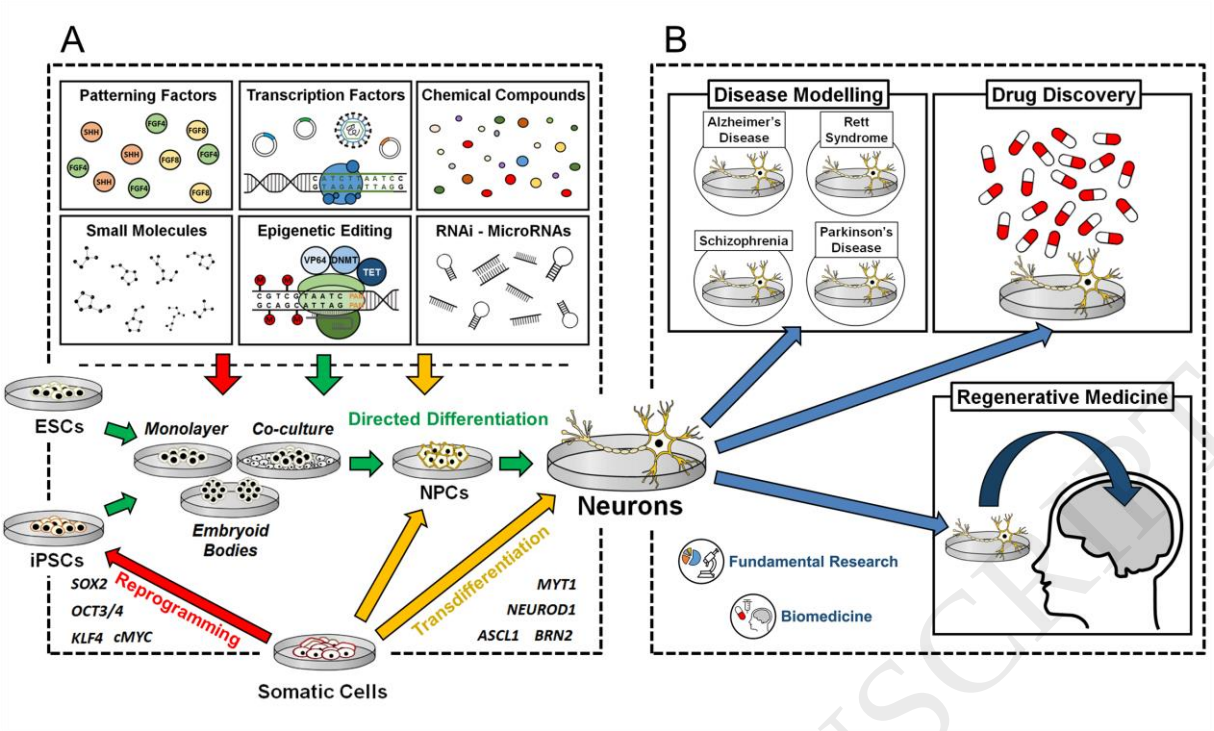
Figure 2. Schematic overview of the working mechanisms of chemically defined neuronal differentiation systems and the underlying neurodevelopmental principles that they recapitulate *in vitro*. (A) The figure shows how various morphogen signalling gradients, including bone morphogenic protein (BMP), wingless-type MMTV integration site family (WNT), fibroblast growth factor (FGF), sonic hedgehog (SHH) and retinoic acid (RA), as well as inhibitors/antagonists (α) of these pathways, pattern the various brain regions during early embryonic development both along the rostral-caudal and dorsal-ventral axes. The depicted brain regions include the telencephalon (TEL), diencephalon (DI), mesencephalon (MES), metencephalon (MET), myelencephalon (MYE) and spinal cord. (B) By using the

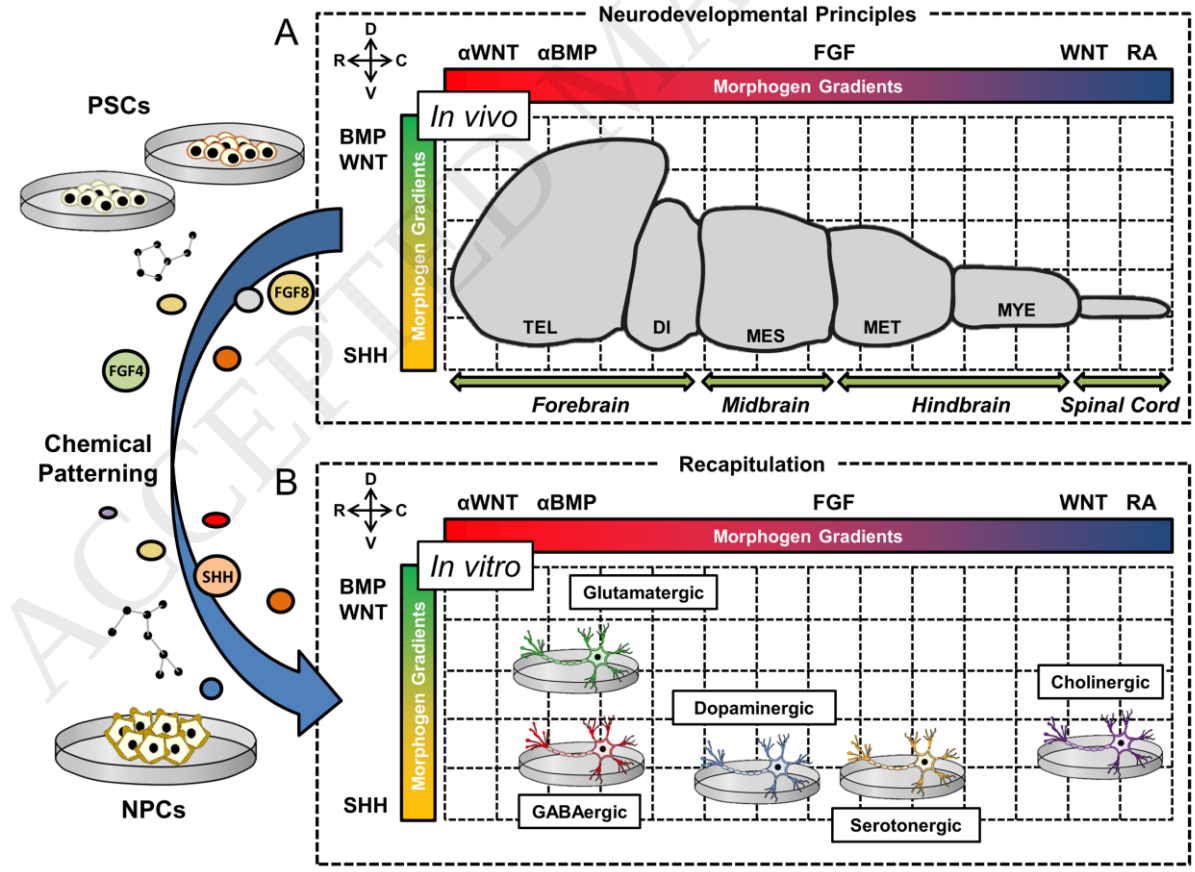
same chemical patterning principles as seen *in vivo*, pluripotent stem cells (PSCs) and neural precursor cells (NPCs) can be patterned towards neuronal subtypes *in vitro*, corresponding to the brain regions where they typically originate from.

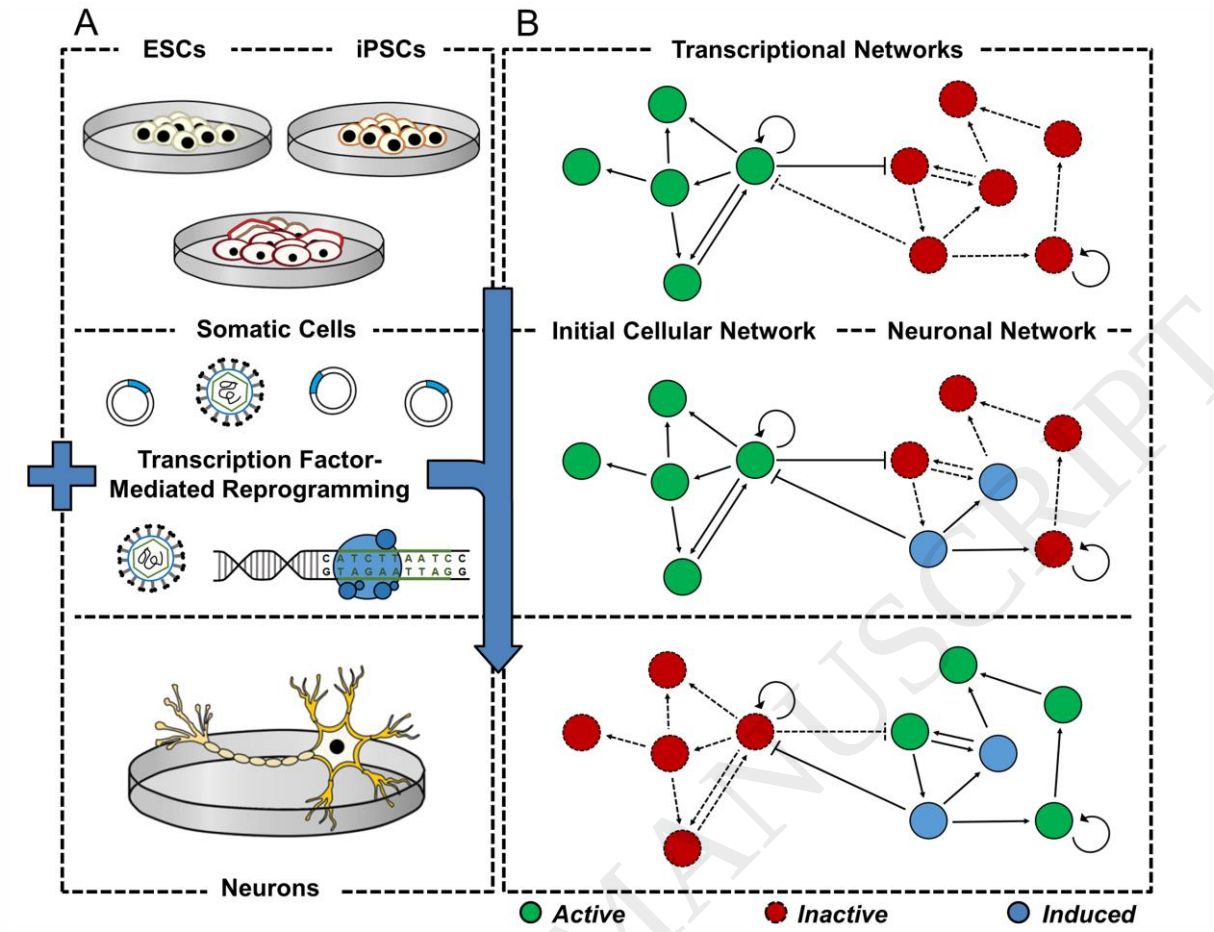
Figure 3. Schematic overview of the working mechanisms of transcription factor-mediated reprogramming along the neural lineage *in vitro*. (A) The figure shows how embryonic stem cells (ESCs), induced pluripotent stem cells (PSCs) and somatic cells can be reprogrammed towards neurons by transcription factor-mediated fate instructions. (B) The initial active (in green) transcriptional network that defines and reinforces cellular identity can be perturbed by induction of exogenous key-lineage determinant factors (in blue), leading to the activation (in green) of the neuronal transcription factor network and deactivation (in red) of the initial cellular network, which underlies the cellular fate switch.

Figure 4. Schematic overview of the working mechanisms of epigenetic-based approaches for neuronal differentiation *in vitro*. (A) The figure shows how somatic cells, pluripotent stem cells (PSCs) and neural precursor cells (NPCs) can be differentiated or reprogrammed into neurons. (B) In each of these cells, histone and DNA modifications orchestrate transcriptional activity of pluripotency genes, self-renewal genes, neuronal genes and somatic genes, which define their cellular identity. While somatic genes are only active in somatic cells (in green), genes that regulate pluripotency and self-renewal are activated in PSCs (in green), whereas genes that regulate neuronal differentiation are repressed and poised (in red with green modifications) for activation upon further developmental cues. This activation and repression is inverted upon initiation of neural induction and differentiation, leading to the activation of neuronal genes (in green) and repression of PSC genes (in red). By using epigenetic editing and molecules that allow modifying the chromatin structure, activity of genes that redefine and reinforce cellular identity can be altered, offering control over neuronal fate determination *in vitro* by the natural dynamics of endogenous gene regulation. (C) Aside from chromatin

modifications, it has been proposed that the cellular microRNA (miRNA) milieu is unique in each cellular subtype and required to facilitate developmental transitions during neuronal differentiation. For PSC regulation, miRNAs such as miR-371 and miR-302 are important, while miR-9/9* and miR-124 belong to brain-enriched miRNAs that are activated during neuronal specification. By using RNA interference (RNAi), miRNA profiles that redefine and reinforce cellular identity can be manipulated, leading to changes in transcriptional regulation that could aid neuronal differentiation and specification *in vitro*.







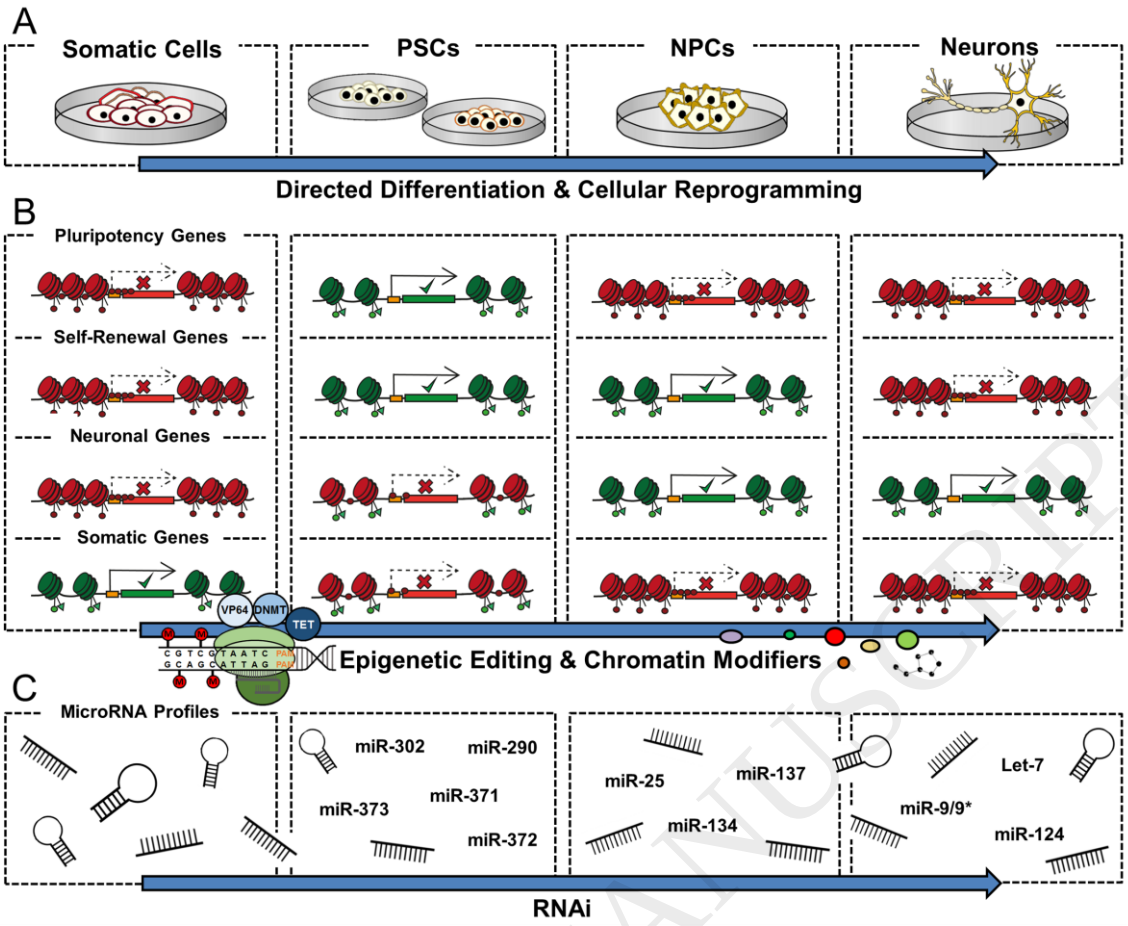


Table 1. Chemically defined differentiation systems for neural induction and differentiation *in vitro* per neuronal phenotype.

Chemically defined systems						
Phenotypes	**Species**	**Starting cell types**	**Culture methods**	**Chemical driving factors**	**Phenotypic markers (% cells)**	**References**
Glutamatergic neurons	Mouse	ESCs	EB	RA	±85% TUJ1+ 93 ± 4.7% VGLUT1+/TUJ1+	Bibel *et al.* (2008)
		ESCs (*TAU::GFP*)	Monolayer	Cyclopamine	±70% TUJ1+ ±70% VGLUT1+/TUJ1+ ±13% VGLUT2+/TUJ1+ <50% TBR1+/TUJ1+ <35% CTIP2+/TUJ1+	Gaspard *et al.* 2008
	Mouse and human	ESCs (*Sox1::GFP* and *Bf1::Venus*)	EB	DKK1, Lefty-1 and SB431542	*Mouse* 83% VGLUT1+/TUJ1+	Eiraku *et al.* (2008)
	Human	ESCs	EB	None	±79% TBR1+/TUJ1+ ±57% CTIP2+	Li *et al.* (2009)
		ESCs (*ACTB::GFP*) and iPSCs	Monolayer	NOG	*ESCs* <65% TUJ1+ ±60% VGLUT1+/TUJ1+ <75% TBR1+/TUJ1+ <72% CTIP2+/TUJ1+ <18% CTIP2+/TBR1+/TUJ1+	Espuny-Camacho *et al.* (2013)
		ESCs and iPSCs	(Spin) EB	DOR, DKK1, EGF, FGF2 and NOG	*iPSCs (8011)* 94.7 ± 2.5% VGLUT1+/MAP2+ 9.9 ± 4.7% GABA+/MAP2+ *iPSCs (BJiPS#1)*	Kim *et al.* (2011c)

					32.8 ± 8.1% VGLUT1+/MAP2+ 71.1 ± 5.5% GABA+/MAP2	
			Monolayer (Dual SMAD inhibition)	DOR, FGF2, NOG, SB431542 and Vitamin A	*ESCs* ±27% TBR1+ ±28% CTIP2+ ±34% BRN2+ *iPSCs* 22-29% TBR1+ 25-30% CTIP2+ 28-36% BRN2+	Shi *et al.* (2012a, 2012b)
GABAergic neurons	Mouse	ESCs	Co-culture (MS-5)	FGF2, FGF8 and SHH	±68% GABA+/TUJ1+	Barberi *et al.* (2003)
		ESCs (*Lhx6::GFP*)	EB	FGF2, NOG and SHH-C25II	91.6 ± 4.4% BF1+/Lxh6-GFP+ 70.5 ± 7.7% DLX2+/Lxh6-GFP+ 97.7 ± 3.1% LHX6+/Lxh6-GFP+ 94.6 ± 3.7% GABA+/Lxh6-GFP+	Maroof *et al.* (2010)
		ESCs (*Sox1::GFP*)	EB	DKK1, Lefty-2 and SHH-C25II	60−70% NKX2-1+/BF1+ 20-30% GAD65/67+/TUJ1+ 20-30% GABA+/TUJ1+	Watanabe *et al.* (2005)
	Human	ESCs	EB	DKK1 and SHH	±84% NKX2-1+	Li *et al.* (2009)
		ESCs and iPSCs	EB	SHH-C24II, SHH-	*ESC and iPSCs*	Liu *et al.* (2013)

				C25II and PUR	±90% TUJ1+ ±90% NKX2-1+ >90% BF1+ >90% GABA+ ±25% CALB1+ ±15% SST+ ±13% PV+	
			Monolayer	DKK1, DOR, NOG, SHH-C25II and SB431542	*ESC and iPSCs* ±58% BF1+ ±51% MAP2+ ±80% TUJ1+ ±78% GABA+/MAP2+ ±60.3% CTIP2+/MAP2+ ±86% GABA+/CTIP2+/MAP2+ ±53% CALB1+/MAP2+ ±70.6% CTIP2+/CALB1+/MAP2+	Carri *et al.* (2013)
		ESCs (*NKX2-1::GFP*) and iPSCs	EB	BMPRIA-Fc, DKK1, PUR and SB431542	*ESCs* 74.9% ± 2.1% NKX2-1-GFP+ 81.5 ± 3.6% BF1+/NKX2-1-GFP+ 75.8 ± 2.3% GABA+/NKX2-1-GFP+ 31.1 ± 5.4% CALB1+	Nicholas *et al.* (2013)
		ESCs (*NKX2-1::GFP*) and iPSCs	Monolayer (Dual SMAD inhibition)	DKK1, FGF2, LDN193189, NOG, PUR, SB431542, SHH-C25II and XAV939	*ESCs* ±90% BF1+ ±80% NKX2-1+ ±90% BF1+/NKX2-1-GFP+ <88% GABA+ <16% CALB1+	Maroof *et al.* (2013)

		Neural precursor cell line (ReNcell VM)	Monolayer	EGF, FGF2 and VPA[1]	68 ± 4% MAP2+ 90% GABA+/MAP2+ 54% CALB1+/MAP2+	Lin *et al.* (2015)
				DKK1, EGF, FGF2 and SHH	63 ± 4% MAP2+ 96% GABA+/MAP2+ 84% CALB1+/MAP2+	Lin *et al.* (2015)
Dopaminergic neurons	Mouse	ESCs	Co-culture (MS-5)	FGF2, FGF8 and SHH	50 ± 10% TH+/TUJ1+	Barberi *et al.* (2003)
			Co-culture (PA6)	None	52 ± 9% TUJ1+ 30 ± 4% TH+/TUJ1+	Kawasaki *et al.* (2000)
			EB	FGF2, FGF8 and SHH	71.9 ± 6.9% TUJ1+ 33.9 ± 5.5% TH+/TUJ1+	Lee *et al.* (2000)
	Human	ESCs	Co-culture (MEF-NOG, MS-5-NOG and MS-5-SHH)	FGF2	52.5 ± 2.56% TUJ1+ 38.2 ± 2.15% TH+ 75.0 ± 3.02% TUJ1+/TH+	Lim *et al.* (2015)
			Dual SMAD inhibition with EB	CHIR99021, NOG, SB431542 and SHH-C24II	*After in vivo transplantations* 54.2 ± 2.5% TH+ 81% LMX1A+/FOXA2+	Kirkeby *et al.* (2012)
			EB	FGF2, FGF8 and SHH	50-60% TH+/TUJ1+	Yan *et al.* (2005)
		iPSCs	EB	FGF2, FGF8 and SHH	30 ± 5% TH+ ±100% GIRK2+/TH+	Swistowski *et al.* (2010)
				FGF2	6.5 ± 1.4% TH+	Cai *et al.* (2010)

[1] Epigenetic factor

			Monolayer (Dual SMAD-inhibition)	A83-01, CHIR99021, FGF8, LDN193189 and PUR	42 ± 4.4% TH+ 19.9% ± 6.9% NURR1+ 70%–75% FOXA2+	Doi *et al.* (2014)
		ESCs and iPSCs	Monolayer (Dual SMAD-inhibition) and Co-culture (MS-5)	CHIR99021, FGF8, LDN193189, NOG, PUR, SB431542 and SHH-C25II	*ESCs* ±75% TH+ ±50% NURR1+ ±80% FOXA2+ ±60% LMX1A+	Kriks *et al.* (2011)
	Human and primate	ESCs and iPSCs	Monolayer (Dual SMAD inhibition) with subsequent cellular aggregations	CHIR99021, FGF8b, LDN193189, SB431542 and SHH-C25II	*Human ESCs and iPSCs* 43.6 ± 6.2% TH+ 95.3 ± 2.4% NURR1+/TH+ 96.7 ± 1.8% FOXA2+/TH+ 96.5 ± 2.3% LMX1A+/TH+ 56.3 ± 6.7% GIRK2+/TH+	Xi *et al.* (2012)
Serotonergic neurons	Mouse	ESCs	Co-culture (MS-5)	FGF2, FGF4 and SHH	±57% 5-HT+/TUJ1+	Barberi *et al.* (2003)
			EB	FGF2, FGF8 and SHH	11 ± 0.5% 5-HT+/TUJ1+	Lee *et al.* (2000)
		ESCs (*ePet::EGFP*) and iPSCs	Monolayer	NOG	*ESCs* ±6% 5-HT+/TUJ1+	Shimada *et al.* (2012)
	Human	ESCs	EB	5-HT, FGF2, FGF10, Forskolin and RA	±20% TUJ1+ <69 ± 4% 5-HT+/TUJ1+ 40% TPH+/MAP2+ 40 ± 4% MAP2+/5-HT+	Kumar *et al.* (2009)

		ESCs (*TPH2::GFP, TPH2::TdT and SYN1*::dsRed)	Dual SMAD inhibition with EB	FGF8, LDN193189, NOG, SB431542 and SHH	±8% TPH+/MAP2ab+ ±27% 5-HT+/TPH+ ±64% TPH+/TPH-GFP+ ±60% TPH-GFP+/TPH+ ±5% 5-HT+/MAP2ab+	Vadodaria *et al.* (2015)
		ESCs and iPSCs	Monolayer	CHIR99021, DMH-1, FGF4, SB431542 and SHH-C25II	*ESCs and iPSCs* ±52% TPH2+ >60% 5-HT+/TUJ1+	Lu *et al.* (2016)
Cholinergic motor neurons	Mouse	ESCs	Co-culture (MS-5)	FGF2, RA and SHH	±60% HB9+/TUJ1+	Barberi *et al.* (2003)
		ESCs (*Hb9::GFP*)	EB	Hh-Ag1.3, RA and SHH	25% ± 5% HB9+ 25% ± 5% HB9-GFP+ >70% ISL1+/ HB9+	Wichterle *et al.* (2002)
	Human	ESCs	EB	FGF2, RA and SHH	>50% ISL1+/TUJ1+/MAP2+ ±50% HB9+/ISL1/2+ ±21% HB9+	Li *et al.* (2005)
		ESCs (*Hb9::EGFP*)	EB	RA and SHH	35.3 ± 24.9 TUJ1+ 56.1 ± 9.9% ISL1+/CHAT+/TUJ1+ 37.4 ± 3.3% HB9+/TUJ1+ 96.3 ± 12.5% HB9+/Hb9-GFP+ 88.7 ± 7.4% TUJ1+/Hb9-GFP+	Singh Roy *et al.* (2005)
		ESCs and iPSCs	EB	RA and SHH agonist	*iPSCs* 20% HB9+ >90% ISL1/2+/HB9+ >50% CHAT+/ISL1/2+/HB9+	Dimos *et al.* (2008)
			Monolayer	DOR, RA and SHH	*ESCs*	Qu *et al.* (2014)

					69.5 ± 11.2% HB9+/CHAT+/TUJ1+ 78.3 ± 3.5% ISL1+	
					iPSCs	
					51.6 ± 5.7% HB9+/TUJ1+ 65.4 ± 5.1% ISL1+	
		ESCs and iPSCs (*Hb9::(E)GFP*)	EB	PUR and RA	*ESCs*	Karumbayaram *et al.* (2009)
					59.1 ± 7.07% OLIG2+/SOX3+ 28.2 ± 5.7% ISL1+/TUJ1+	
					iPSCs	
					57.6% ± 9.88% OLIG2+/SOX3+ 33.6% ± 12% ISL1+/TUJ1+	
			Dual SMAD inhibition with EB	LDN193189, PUR, RA, SAG, SB435142 and SHH-C25II	*ESCs*	Amoroso *et al.* (2013)
					83 ± 1% TUJ1+ <29 ± 4% HB9-GFP+ 98 ± 0% HB9-GFP+/TUJ1+ 30 ± 6% ISL1+/HB9-GFP+ 16 ± 5% HB9+/HB9-GFP+ 37 ± 2% ISL1+/HB9+/HB9-GFP+	
		ESCs and iPSCs (*HB9::Venus*)	Dual SMAD inhibition with EB	BIO, CHIR99021, DOR, LDN193189, PUR, RA and SB431542	*ESCs*	Shimojo *et al.* (2015)
					50-58% HB9+ 45-50% ISL1+ 43-68% CHAT+ 84.1 ± 2.4% HB9+/HB9-Venus+ 39.5 ± 13.2% ISL1+/HB9-Venus+ 83.4 ± 1.7% CHAT+/HB9-Venus+	
					iPSCs	

					42-48% HB9+ 37-48% ISL1+	
	Human and primate	ESCs (*RUES1-EGFP*)	Co-culture (MS-5)	NOG, RA and SHH	*Human* 20% HB9+ ±26% CHAT+ *Primate* 43% HB9+ 65% HB9+/TUJ1+	Lee *et al*. (2007)

Neuronal phenotypes, species, starting cell types, culture methods, chemical driving factors and representative phenotypic markers that have been used to assess the differentiation efficiency and culture homogeneity are broadly summarized. + indicates the percentage of cells in the population that stained positive for a certain marker. *Abbreviations*: 5-HT, serotonin (5-hydroxytryptamine); A83-01, TGF-β kinase/activin receptor-like kinase inhibitor; ACTB, actin beta/beta-actin; BF1, brain factor 1/forkhead box protein G1 (FOXG1); BIO, GSK3β inhibitor 6-bromoindirubin-3'-oxime; BMPRIA-Fc, bone morphogenetic protein receptor 1a-fragment crystallizable; BRN2, brain-specific homeobox/POU domain protein 2 (POU3F2);; CALB1, calbindin 1; CHAT, choline o-acetyltransferase; CHIR99021, GSK3β inhibitor; CTIP2, b-cell CLL/lymphoma 11b (BCL11B)/ COUP-TF-interacting protein 2 (COUP-TFII); DKK1, dickkopf-1; DLX2, distal-less homeobox 2; DMH-1, dorsomorphin homolog 1; DOR, Dorsomorphin; dsRed, discosoma sp. red fluorescent protein; EB, embryoid body; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; ePet, enhancer of the mouse Pet-1 (human FEV) gene; ESCs, embryonic stem cells; FGF10, fibroblast growth factor 10; FGF2, fibroblast growth factor 2/basic fibroblast growth factor (bFGF); FGF4, fibroblast growth factor 4; FGF8, fibroblast growth factor 8; FGF8b, fibroblast growth factor 8 isoform b; FOXA2, forkhead box protein A2; GABA, γ-aminobutyric acid; GAD65/67, glutamic acid

decarboxylase isoform 65/67 (GAD2/1); GFP, green fluorescent protein; GIRK2, G protein-activated inward rectifier potassium channel 2 (KCNJ6); HB9, homeobox HB9/motor neuron and pancrease homeobox 1 (MNX1); Hh-Ag1.3, small molecule agonist of SHH signalling; iPSCs, induced pluripotent stem cells; ISL1, ISL LIM homeobox 1; ISL1/2, ISL LIM homeobox 1/2; LDN193189, selective BMP signalling inhibitor; Lefty-1, left-right determination factor 1; Lefty-2, left-right determination factor 2; LHX6, LIM homeobox 6; LMX1A, LIM homeobox transcription factor 1 alpha; MAP2, microtubule-associated protein 2; MAP2ab, microtubule-associated protein 2ab; MEF, mouse embryonic fibroblast; MS-5, stromal cell line derived from irradiated murine bone marrow cultures; NKX2-1, NK2 homeobox 1; NOG, Noggin; NURR1, nuclear receptor related 1 protein; OLIG2, oligodendrocyte transcription factor 2; PA6, stromal cell line derived from newborn calvaria tissue of the C57BL/6 mice; PUR, purmorphamine; PV, parvalbumin; RA, retinoic acid; RUES1-EGFP, human ESC line expressing EGFP; SAG, smoothened agonist; SB431542, transforming growth factor beta inhibitor; SHH, sonic hedgehog; SHH-C24II, recombinant human SHH; SHH-C25II, recombinant mouse SHH; SMAD, transcription factor and member of the BMP and TGF-β signalling pathways; Sox1, SRY box 1; SOX3, SRY box 3; SST, somatostatin; SYN1, synapsin 1; TAU, microtubule-associated protein tau (MAPT); TBR1, T-box brain 1; TdT, tandem dimer tomato red fluorescent protein; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; TPH2, tryptophan hydroxylase 2; TUJ1, neuron-specific class III beta-tubulin (TUBB3); VGLUT1, vesicular glutamate transporter 1; VGLUT2, vesicular glutamate transporter 2; VPA, valproic acid; XAV939, WNT/β-catenin inhibitor.

Table 2. Transcription factor-mediated neuronal reprogramming protocols *in vitro* per neuronal phenotype.

Transcription factor-mediated reprogramming							
Phenotypes	**Species**	**Starting cell types**	**Culture methods**	**Transcription factors**	**Chemical driving factors**	**Phenotypic markers (% cells)**	**References**
Glutamatergic neurons	Mouse	Fibroblasts (*Tau::EGFP)*	Monolayer	*Ascl1, Brn2* and *Myt1l*	DOX	>20% TUJ1+ 53% TBR1+/TUJ1+	Vierbuchen *et al.* (2010)
		Astrocytes	Monolayer	*Ngn2*	None	70.2 ± 6.3% TUJ1+ 85.4 ± 5.0% VGLUT1+ ±48.2% TBR1+	Heinrich *et al.* (2010)
		ESCs	Monolayer	*Ngn2*	None	<40% TUJ1+	Thoma *et al.* (2012)
	Mouse and human	Fibroblasts (*Tau::EGFP*) and ESCs	Monolayer	*Ascl1*	DOX	*Mouse embryonic fibroblasts* ±45% VGLUT1+/TAU-EGFP+	Chanda *et al.* (2014)
	Human	Fibroblasts (*Tau::EGFP*), ESCs and iPSCs	Monolayer	*Ascl1, Brn2, Myt1l* and *Neurod1*	DOX	*Fetal fibroblasts* ±60% TUJ1+ >50% VGLUT1+/TUJ1+ >50% VGLUT2+/TUJ1+ 17 ± 8% TBR1+/TUJ1+ *Postnatal fibroblasts* 81 ± 17% TBR1+/TUJ1+	Pang *et al.* (2011)
		ESCs and iPSCs	Monolayer	*Ngn2 and Neurod1*	DOX	*Ngn2- ESCs* ±80% MAP2+ *Ngn2 - iPSCs* ±90% MAP2+	Zhang *et al.* (2013)

Dopaminergic neurons	Mouse	Astrocytes (*MAP2::CD4* and *MAP2::GCaMP3*) and fibroblasts	Monolayer	*ASCL1*, *LMX1B* and *NURR1*	DOX	*Astrocytes* 35.1 ± 1.5% TUJ1+ 50.9 ± 3.3% TH+/TUJ1+ 18.2 ± 1.5% TH+ *Fibroblasts* 14.9 ± 2.3% TUJ1+ 9.1 ± 0.9% TH+	Addis *et al.* (2011)
		Fibroblasts (*Pitx3::EGFP*)	Monolayer	*Ascl1*, *En1*, *Foxa2*, *Lmx1a*, *Nurr1* and *Pitx3*	DOX, FGF8 and SHH	9.1% Pitx3-EGFP+	Kim *et al.* (2011b)
	Mouse and human	Mouse fibroblasts (*TH::GFP*) and human fibroblasts	Monolayer	*Ascl1*, *Lmx1a* and *Nurr1*	DOX	*Mouse* ±22% TUJ1+ ±17% TH+ *Human* 10 ± 4% TUJ1+ 6 ± 2% TH+	Caiazzo *et al.* (2011)
	Human	Fibroblasts	Monolayer	*Ascl1*, *Brn2*, *Myt1l*, *Foxa2* and *Lmx1a*	DOX	±15% TUJ1+/MAP2+ ±10% TH+/TUJ1+/MAP2+	Pfisterer *et al.* (2011)
		iPSCs	Monolayer	*Ascl1*, *Lmx1a* and *Nurr1*	DOX	*Fetal fibroblast-derived iPSCs* 51 ± 4% TUJ1+ 65 ± 5% TH+/TUJ1+ ±30% CALB1+/TH+ ±40% GIRK2+/TH+ *Parkinson's disease patient-derived iPSCs* 48 ± 4% TUJ1+	Theka *et al.* (2013)

						26 ± 3% TH+/TUJ1+	
GABAergic neurons	Mouse	Fibroblasts	Monolayer	*Ascl1, Brn2, Dlx1, Dlx2, Lbx1, Lhx1, Lhx2, Myt1l, Pax2, Pitx2* and *Pft1a*	None	<35 ± 4% GABA+/TUJ1+	Wasko (2013)
		Astrocytes	Monolayer and neurosphere	*Dlx2*	None	*Monolayer* 35.9 ± 13.0% TUJ1+ 33.7 ± 3.6% VGAT+	Heinrich *et al.* (2010)
	Mouse and human	Mouse fibroblasts (*GAD67::GFP*), human fibroblasts and human iPSCs	Monolayer	*Ascl1, Dlx5, Bf1, Lhx6* and *Sox2*	DOX	*Mouse fibroblasts* ±94% GAD65/67+/GAD67-GFP+ ±97% GABA+/GAD67-GFP+ ±93% PV+/GAD67-GFP+ ±3% SST+/GAD67-GFP+ *Human fibroblasts* ±70% GABA+/TUJ1+ ±90% PV+/TUJ1+ *Human iPSCs* ±50% GABA+/MAP2+ ±90% PV+/MAP2+ ±2% SST+/GABA+	Colasante *et al.* (2015)
Serotonergic neurons	Human	Fibroblasts	Monolayer	*ASCL1, FOXA2, FEV, LMX1B* and *hp53shRNA*	DOR, DOX, PD0332991 and SB431542	<49% TUJ1+ <23% 5-HT+	Xu *et al.* (2015)
		Fibroblasts (*TPH2::GFP* and *SYN1::dsRed*)	Monolayer	*ASCL1, FEV, GATA2, LMX1B, NGN2* and *NKX2-2*	A83-01, CHIR99021, DOX, Forskolin, LDN193189, NOG and	58.4 ± 4.2% TUJ1+ ±60% MAP2ab+ 61 ± 15% TPH+/MAP2ab+ 38 ± 2% 5-HT+	Vadodaria *et al.* (2015)

					SB431542		
Cholinergic motor neurons	Mouse	ESCs	EB	*Isl1, Lhx3, Ngn2* and *Phox2a*	DOX	*Ngn2, Isl1 and Lhx3* 99.82 ± 0.17% HB9+/ISL1+ 0.24 ± 0.28% PHOX2B+/ISL1+ *Ngn2, Isl1, Phox2a* 0.11 ± 0.11% HB9+/ISL1+ 99.03 ± 0.08% PHOX2B+/ISL1+	Mazzoni *et al.* (2013)
	Mouse and human	ESCs	Monolayer (mouse) and EB (human)	*Phox2a* and *Phox2b*	FGF2, FGF8b and Hh-Ag1.3	*Mouse* 61% PHOX2B+/ISL1+/TUJ1+	Mong *et al.* (2014)
		Mouse fibroblasts (*Hb9::GFP*) and human fibroblasts	Monolayer	*Ascl1, Brn2, Myt1l, Lhx3, Isl1, Hb9, Ngn2* and *NEUROD1*	None	*Mouse* 5-10% Hb9-GFP+ 97.6% VACHT+/Hb9-GFP+	Son *et al.* (2011)
	Human	ESCs (*Hb9::GFP*) and iPSCs	EB	*Isl1, Lhx3* and *Ngn2*	Forskolin, RA and SHH	*ESCs* 55% Hb9-GFP+ ±50-62% HB9+/CHAT+ *iPSCs* 49-72% HB9+/CHAT+	Hester *et al.* (2011)
Noradrenergic neurons	Mouse and human	ESCs	Monolayer (mouse) and EB (human)	*Phox2b*	BMP5, BMP7, Cyclopamine, FGF2 and FGF8b	*Mouse* 17.9% TH+/PHOX2A+/TUJ1+	Mong *et al.* (2014)

Neuronal phenotypes, species, starting cell types, culture methods, transcription factors, chemical driving factors and representative phenotypic markers that have been used to assess the differentiation efficiency and culture homogeneity are broadly summarized. + indicates the percentage of cells in the population that stained positive for a certain marker.

Abbreviations: 5-HT, serotonin (5-hydroxytryptamine); A83-01, TGF-β kinase/activin receptor-like kinase inhibitor; ASCL1, achaete-scute homolog 1 (MASH1/HASH1); Bf1,

brain factor 1/forkhead box protein G1 (FOXG1); BMP5, bone morphogenic protein 5; BMP7, bone morphogenic protein 7; Brn2, brain-specific homeobox/POU domain protein 2 (POU3F2); CALB1, calbindin 1; CD4, cluster of differentiation 4; CHAT, choline o-acetyltransferase; CHIR99021, GSK3β inhibitor; Dlx1, distal-less homeobox 1; Dlx2, distal-less homeobox 2; Dlx5, distal-less homeobox 5; DOX, doxycycline; dsRed, discosoma sp. red fluorescent protein; EGFP, enhanced green fluorescent protein; En1, homeobox protein engrailed 1; ESCs, embryonic stem cells; FEV, ETS transcription factor (PET1); FGF2, fibroblast growth factor 2/basic fibroblast growth factor (bFGF); FGF8, fibroblast growth factor 8; FGF8b, fibroblast growth factor 8 isoform b; FOXA2, forkhead box protein A2; GABA, γ-aminobutyric acid; GAD65/67, glutamic acid decarboxylase isoform 65/67 (GAD2/1); GAD67, glutamic acid decarboxylase isoform 67 (GAD1); GATA2, GATA binding protein 2; GCaMP3, a GFP-based calcium sensor for imaging calcium dynamics; GFP, green fluorescent protein; GIRK2, G protein-activated inward rectifier potassium channel 2 (KCNJ6); HB9, homeobox HB9/motor neuron and pancrease homeobox 1 (MNX1); Hh-Ag1.3, small molecule agonist of SHH signalling; hp53shRNA, human p53 small hairpin RNA; iPSCs, induced pluripotent stem cells; ISL1, ISL LIM homeobox 1; Lbx1, ladybird homeobox 1; LDN193189, selective BMP signalling inhibitor; Lhx1, LIM homeobox 1; Lhx2, LIM homeobox 2; Lhx3, LIM homeobox 3; Lhx6, LIM homeobox 6; Lmx1a, LIM homeobox transcription factor 1 alpha; LMX1B, LIM Homeobox transcription factor 1 beta; MAP2, microtubule-associated protein 2; MAP2ab, microtubule-associated protein 2ab; Myt1l, myelin transcription factor 1 like; NEUROD1, neurogenic differentiation 1; NGN2, neurogenin 2; NKX2-2, NK2 homeobox 2; NOG, Noggin; NURR1, nuclear receptor related 1 protein; Pax2, paired box 2; PD0332991, cyclin-dependent kinase 4/6 inhibitor; Pft1a, pancreas specific transcription factor 1a; PHOX2A, paired-like homeobox 2a; PHOX2B, paired-like homeobox 2b; Pitx2, paired-like homeodomain 2; Pitx3, paired-like

homeodomain 3; PV, parvalbumin; RA, retinoic acid; SB431542, transforming growth factor beta inhibitor; SHH, sonic hedgehog; Sox2, SRY box 2; SST, somatostatin; SYN1, synapsin 1; Tau, microtubule-associated protein tau (MAPT); TBR1, T-box brain 1; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; TPH2, tryptophan hydroxylase 2; TUJ1, neuron-specific class III beta-tubulin (TUBB3); VACHT, vesicular acetylcholine transporter; VGAT, vesicular GABA transporter; VGLUT1, vesicular glutamate transporter 1; VGLUT2, vesicular glutamate transporter 2.

Table 3. Epigenetic-based neuronal conversion approaches *in vitro* per neuronal phenotype.

Chemical compounds targeting chromatin remodelling proteins								
Phenotypes	**Species**	**Starting cell types**	**Culture methods**	**Epigenetic factors**	**Transcription factors**	**Chemical driving factors**	**Representative phenotypic markers (% cells)**	**References**
NPCs	Mouse	Fibroblasts (*Trp53*+/+, *Trp53*−/−, *Sox1*+/+, *Sox1EGFP*/+, *Tau*+/+ and *TauEGFP*/+)	Monolayer	BIX-01294, t2PCPA and VPA	*Pax6* and *Bf1*	DOX, LDN193189 and SB431542	30% Sox1-EGFP+	Raciti *et al.* (2013)
	Mouse and human	Mouse fibroblasts and human urinary cells	Monolayer and neurospheres	NaB, TSA and VPA	None	CHIR99021, LIF, Li2CO3, LiCl, Repsox, SB431542 and Tranilast	*Mouse* <96% NES+ <96% SOX2+ <96% PAX6+ <93% NES+/SOX2+ <93% NES+/PAX6+	Cheng *et al.* (2015b)
Dopaminergic-, GABAergic-, glutamatergic- and cholinergic motor neurons	Mouse	Astrocytes (*GFAP::GFP* and *Neurod1::GFP*)	Monolayer	VPA	None	CHIR99021, FGF2, FGF8, Repsox, SHH and Tranilast	<13% DCX+ <15% NEUN+ ±5% GAD67+ ±4% CHAT+ ±3% TH+ ±6% VGLUT1+	Cheng *et al.* (2015a)
Epigenetic editing								
Phenotypes	**Species**	**Starting cell types**	**Culture methods**	**Epigenetic factors**	**Transcription factors**	**Chemical driving factors**	**Representative phenotypic markers (% cells)**	**References**
Neurons	Mouse	Fibroblasts (*SYN1::RFP*)	Monolayer	VP64-dCas9-VP64 gRNAs: *Brn2,*	None	CHIR99021, LDN193189 and SB431542	±4% TUJ1+ ±75% MAP2+/TUJ1+	Black *et al.* (2016)

				Ascl1, and *Myt1l*				
	Human	iPSCs	Monolayer	VP64-p65-Rta-dCas9 gRNAs: *NGN2* and *NEUROD1*	None	DOX	7% TUJ1+	Chavez *et al.* (2015)
RNAi								
Phenotypes	**Species**	**Starting cell types**	**Culture methods**	**Epigenetic factors**	**Transcription factors**	**Chemical driving factors**	**Representative phenotypic markers (% cells)**	**References**
Neurons	Mouse	NPCs	Monolayer and neurospheres	siRNA-REST	None	FGF2 and RA	<80% TUJ1+ <80% MAP2+	Low *et al.* (2012)
Dopaminergic neurons	Mouse	ESCs (*TH::GFP*)	EB and co-culture (PA6)	miR-132-ASOs	None	None	>25% TH+/MAP2+ 70% TH+/TH-GFP+	Yang *et al.* (2012)
		ESCs	EB	miR-133b-ASOs	None	FGF2	20% TH+/PITX3+	Kim *et al.* (2007)
	Human	NPCs	Monolayer	miR-124-inhibitor, miR-125b, miR-125b-mimic, miR-181a-mimic, miR-181a*-inhibitor and miR-181a/a*	None	FGF8b and SAG	<30% TUJ1+ <15% TH+	Stappert *et al.* (2013)
GABAergic neurons	Human	Fibroblasts (*SYN1::EGFP*)	Monolayer	miR-9/9*, miR-124 (*Bcl-xL)* and VPA	*CTIP2*, *DLX1*, *DLX2*, and *MYT1L*	DOX and RA	*Postnatal fibroblasts* 87% TUJ1+ 90% MAP2+ 72.3% GABA+/MAP2+ 80% BF1+/MAP2+ 60% DLX2+/MAP2+ *Adult fibroblasts*	Victor *et al.* (2014)

							82% MAP2+ 86% GABA+/MAP2+	
Glutamatergic- and GABAergic neurons	Human	Fibroblasts	Monolayer	miR-9/9*, miR-124 and VPA	*ASCL1*, *MYT1L* and *NEUROD2*	DOX and FGF2	80% MAP2+ ±24% TBR1+/MAP2+/TUJ1+ ±13% CTIP2+/MAP2+/TUJ1+ ±38% VGLUT1+/MAP2+/TUJ1+ ±30% GAD67+/MAP2+/TUJ1+ ±38% DLX1+/MAP2+/TUJ1+	Yoo *et al.* (2011)
				miR-124 (IRES-RFP)	*BRN2* and *MYT1L*	Cumate, FGF2, NOG and DOX	*Postnatal fibroblasts* 55% MAP2+/RFP+ 46% NEUN+/RFP+ 8% GABA+/RFP+ 12% VGAT+/RFP+ *Adult fibroblasts* 28% RFP+/VGLUT1+	Ambasudhan *et al.* (2011)
Cholinergic motor neurons	Human	Fibroblasts	Monolayer	miR-9/9*, miR-124 (*Bcl-xL)* and VPA	*ISL1* and *LHX3*	DOX and RA	±80% TUJ1+ ±80% MAP2+ ±80% CHAT+/TUJ1+	Abernathy *et al.* (2017)

Neuronal phenotypes, species, starting cell types, culture methods, epigenetic factors, transcription factors, chemical driving factors and representative phenotypic markers that have been used to assess the differentiation efficiency and culture homogeneity are broadly summarized. + indicates the percentage of cells in the population that stained positive for a certain marker. *Abbreviations*: ASCL1, achaete-scute homolog 1 (MASH1/HASH1); ASOs, anti-sense oligonucleotides; Bcl-xL, b-cell lymphoma-extra large; BF1, brain factor 1/forkhead box protein G1 (FOXG1); BIX-01294, histone-lysine methyltransferase inhibitor; BRN2, brain-specific homeobox/POU domain protein 2 (POU3F2); CHAT, choline o-

acetyltransferase; CHIR99021, GSK3β inhibitor; CTIP2, b-cell CLL/lymphoma 11b (BCL11B)/ COUP-TF-interacting protein 2 (COUP-TFII); DCX, doublecortin; DLX1, distal-less homeobox 1; DLX2, distal-less homeobox 2; DOX, doxycycline; EB, embryoid body; EGFP, enhanced green fluorescent protein; ESCs, embryonic stem cells; FGF2, fibroblast growth factor 2/basic fibroblast growth factor (bFGF); FGF8, fibroblast growth factor 8; FGF8b, fibroblast growth factor 8 isoform b; GABA, γ-aminobutyric acid; GAD67, glutamic acid decarboxylase isoform 67 (GAD1); GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; gRNAs, guide RNAs; iPSCs, induced pluripotent stem cells; IRES, internal ribosome entry site; LDN193189, selective BMP signalling inhibitor; Li2CO3, lithium carbonate; LiCl, lithiumchloride; LIF, leukemia inhibitory factor; MAP2, microtubule-associated protein 2; MYT1L, myelin transcription factor 1 like; NaB, sodium butyrate; NES, nestin; NEUN, neuronal nuclei antigen; NEUROD1, neurogenic differentiation 1; NEUROD2, neuronal differentiation 2; NGN2, neurogenin 2; NPCs, neural precursor cells; PA6, stromal cell line derived from newborn calvaria tissue of the C57BL/6 mice; PAX6, paired box 6; PITX3, paired-like homeodomain 3; RA, retinoic acid; REST, RE1-silencing transcription factor; RFP, red fluorescent protein; SAG, smoothened agonist; SB431542, transforming growth factor beta inhibitor; SHH, sonic hedgehog; siRNAs, small interfering RNAs; Sox1, SRY box 1; SOX2, SRY box 2; SYN1, synapsin 1; t2PCPA, trans-2-phenyl-cyclopropylamine hydrochloride; Tau, microtubule-associated protein tau (MAPT); TBR1, T-box brain 1; TH, tyrosine hydroxylase; Trp53, tumor protein p53; TSA, trichostatin A; TUJ1, neuron-specific class III beta-tubulin (TUBB3); VGAT, vesicular GABA transporter; VGLUT1, vesicular glutamate transporter 1; VP64-dCas9-VP64, dCas9 with N-terminal and C-terminal VP64 transactivation domains; VP64-p65-Rta-dCas9, dCas9-based transcriptional activator containing VP64, p65 and Rta; VPA, valproic acid.