Science Progress (2010), 93(2), 151–169
doi: 10.3184/003685010X12705764469952

Development and regeneration of projection neuron subtypes of the cerebral cortex

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ABSTRACT

The idea of repairing damaged neuronal circuitry in the mammalian central nervous system (CNS) has challenged neuroscientists for centuries. This is mainly due to the notorious inability of neurons to regenerate and the unparalleled cellular diversity of the nervous system. In the mammalian cerebral cortex, one of the most complex areas of the CNS, multipotent neural stem and progenitor cells undergo progressive specification during development to generate the staggering variety of projection neuron subtypes that are found in the adult. How is this process orchestrated in the embryo? And, can developmental signals be used to regenerate projection neuron subtypes in the adult or in the dish? Here, we first provide an overview of the diversity and fate potential of neural progenitors of the cerebral cortex during development. Further, we discuss the plasticity of neural progenitors and the roles of intrinsic and extrinsic signals over progenitor fate. Finally, we discuss the relevance of developmental signals for efforts to direct the differentiation of pluripotent stem cells into specific types of cortical projection neurons for therapeutic benefit.

Keywords: *cerebral cortex, neural progenitors, development, differentiation, neuronal identity*

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Introduction

The central nervous system (CNS) is one of the most complex tissues of the body, with unparalleled cellular diversity and very complex connectivity. Here, an extraordinary variety of glial and neuronal subtypes are generated during embryogenesis from distinct pools of tissue-specific neural stem and progenitor cells, which are progressively specified to different cellular fates. Neurons, in particular, can be classified in many distinct categories based on their molecular, electrophysiological, and functional identities, as well as their connectivity to different targets within neuronal circuits. How do multipotent neural progenitors generate this staggering diversity of neuronal populations? This is a fundamental question in the CNS. Extensive investigation of the cell intrinsic and extrinsic controls over the generation of neuronal diversity in the spinal cord[1–3] and the retina[4–6] have provided detailed models for how different subtypes of neurons are generated in these regions. This type of information is now emerging for neuronal populations in other regions of the CNS, including the cerebral cortex. The cortex is one of the most complex and cellularly heterogeneous regions of the CNS. This is where the highest level of brain functions, including cognition, sensory processing and motor coordination, reside[7,8]. In the cerebral cortex, there exist two main classes of neurons: glutamatergic projection neurons (PN), which use glutamate as neurotransmitter and are generated from neural progenitors located in the ventricular zone (VZ) and subventricular zone (SVZ) of the dorsal telencephalon (*i.e.* the most anterior region of the developing neural tube), and GABAergic interneurons, which are generated from distinct populations of neural progenitors within the ventral part of the telencephalon[9,10]. Within these two broad categories there is enormous neuronal diversity, with each population containing different classes of neurons[11]. Recent work has provided new insight into some of the molecular mechanisms that govern the generation of such neuronal diversity and has identified molecules that define individual neuronal types of the neocortex as they develop. The development of cortical interneurons has been elegantly and extensively reviewed elsewhere[12–14]. In this review, we primarily concentrate on the developmental events that lead to the fate specification of neural progenitors into projection neurons. First, we review the classification and diversity of neural progenitors in the neocortex. Next, we consider the role of extrinsic and intrinsic factors over neural progenitor fate and plasticity. Finally, we discuss

how developmental signals may be manipulated in pluripotent stem cells to direct the differentiation of cortical projection neurons for therapeutic benefit.

Neural progenitors of the cerebral cortex

Projection neurons of the cerebral cortex are generated from neural progenitor cells located in the dorsal wall of the telencephalon, within the most anterior part of the developing neural tube[8,11] (Figure 1A). Like in other regions of the developing CNS, different types of neural progenitor cells are present at different stages of development; these have distinct characteristics, different plasticity and fate potential. At the earliest stages of corticogenesis, before cortical neurons are generated, neural progenitors are defined as neuroepithelial (NE) cells due to their appearance as a sheet of polarized, cycling cells with epithelial features, including tight and adherent junctions in the apico-lateral plasma membrane. NE cells

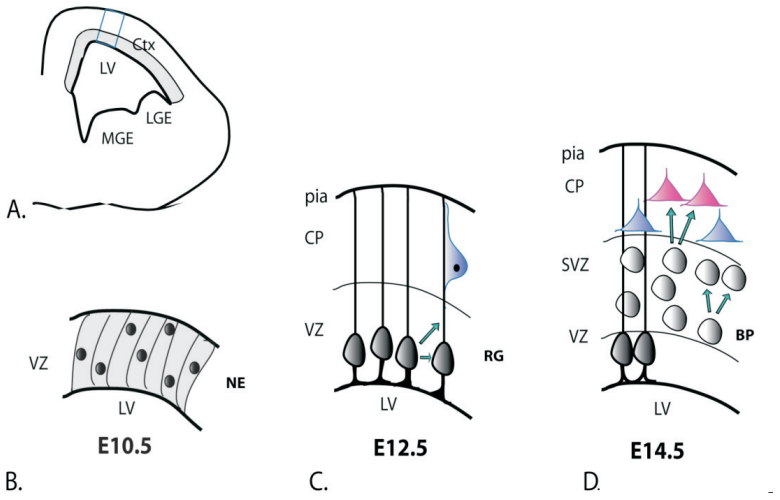

Fig. 1. *Different classes of neural progenitors generate projection neurons of the cerebral cortex.* (**A**) *Schematic representation of the developing forebrain. Cortical progenitors are located in the VZ, highlighted in grey.* (**B**) *At the earliest stages of corticogenesis, neural progenitors are defined as neuroepithelial (NE) cells.* (**C**) *Later, NE cells give rise to Radial Glia (RG) cells, which give rise to immature projection neurons (highlighted in light blue) and provide a scaffold for neuronal migration to the cortical plate (CP).* (**D**) *Basal progenitor (BP) cells appear in the subventricular zone during later stages of corticogenesis and mostly give rise to upper layer projection neurons (highlighted in pink).*

are multipotent neural progenitors that expand *via* symmetrical divisions[15–18] (Figure 1B). As corticogenesis progresses NE cells become more fate-restricted and give rise to a second type of cortical progenitor cell known as radial glia (RG) (Figure 1C). RG have a unique bipolar shape, and while their cell bodies are located in the ventricular zone (VZ), they maintain connections with both the ventricular and the pial (*i.e.* external) surface of the developing cortical wall[19,20]. RG retain some hallmarks of NE cells like the presence of adherent (but not tight) junctions and the expression of the intermediate filament protein *Nestin*[15,21,22], but they are a more fate-restricted population of neural progenitor[21,23]. When cortical neurogenesis begins, RG begin to divide asymmetrically to generate both progenitors and postmitotic neurons[15,24]. Neuronal progeny, in turn, migrate radially away from the VZ to the cortical plate (CP), following an ''inside-out'' mode of migration such that early generated neurons populate the deeper layers of the neocortex, while later-born neurons position within more superficial layers[8] (Figure 2). The laminar cytoarchitecture of the cortex is relatively similar in all mammals; however, a big expansion of the cortex occurred during recent evolution, which led to the transformation of a lissencephalic cortex (*i.e.* smooth and without folds) characteristic of rodents, into a gyrencephalic one (*i.e.* folded with gyri and sulci) characteristic of primates. The need to accommodate the disproportionate growth of the cortex appears to have been at least in part met by the appearance and enlargement of a second germinal zone, the subventricular zone (SVZ), located away from the luminal surface[25] and with it, the generation of another class of cortical neural progenitors: the basal progenitor (BP) cells (Figure 1D). BP progenitors are different from both NE and RG progenitors in that they divide in the SVZ, and they are further restricted in their fate potential[24,26–28]. It is intriguing to consider that BP progenitors may have different fate potential than RG progenitors, at least in part because they divide away from the ventricular surface, and thus do not receive VZ derived signals. Upon differentiation, progenitor cells and their postmitotic progeny behave very differently, the former remaining in the germinal zones (VZ or SVZ) to divide and generate new progeny, the latter migrating away to populate the cortical plate. A dividing progenitor cell thus bears the ''responsibility'' to choose between very different fates: undergoing a neurogenic division (*i.e.* giving rise to at least one postmitotic neuron) or continuing with proliferative divisions (*i.e.* giving rise to two progenitor cells). The mechanisms guiding these fundamental decisions are actively being investigated. Because of space limita-

tions, here we will only cite some among many studies, which we have selected based on relevance to the overall goal of the present review article. Additional information on mechanisms of progenitor cell proliferation and modes of division has been previously elegantly reviewed elsewhere[15,29,30]. Differential expression of specific genes in the two daughter cells may distinguish (and possibly instruct) neurogenic from proliferative progenitor cell divisions[31]. For example, progenitor cells undergoing neurogenic divisions, but not those undergoing proliferative divisions, express the anti-proliferative gene *Tis21* during the G1-phase of the cell cycle, immediately prior to dividing[15,31]. Similarly, neurogenic, but not proliferative divisions, in the developing CNS involve polarization of the apical plasma membrane, and the subsequent asymmetric inheritance by one daughter cell of the region of the apical membrane containing the protein prominin-1 (CD133)[32]. The orientation of the mitotic spindle also has a role in affecting the balance between proliferative and neurogenic divisions, as this ultimately determines the orientation of the cleavage-plane. Specifically, the ASPM (abnormal spindle-like microcephaly-associated) protein is involved in the organisation of microtubules at the spindle poles to control the rate of symmetric divisions. Accordingly, mutations in ASPM in humans causes a condition known as primary microcephaly, characterized by a large reduction in volume of the cerebral cortex without changes in cortical architecture[33–36]. Finally, a recent elegant study has shown that asymmetric centrosome inheritance plays an important role in controlling the differential behaviour of progenitors and differentiated progeny in the cerebral cortex[37]. During asymmetric RG division, centrosome duplication generates a pair of centrosomes with differently aged mother centrioles. Intriguingly, the centrosome bearing the old mother centriole is inherited by radial glia progenitors, while the centrosome with the new mother centriole is preferentially inherited by differentiating progeny. Deregulation of this elegant process of differential centriole distribution leads to depletion of the RG population and the unbalanced preferential generation of differentiated neuronal progeny[37]. It is intriguing to note that new and old mother centrioles have a different protein composition and may thus confer the cells where they reside with a unique ability to respond to extracellular signals, possibly by regulating the ability of progenitors to generate a cilium, for example[38]. While further work is required, differential centriole inheritance might be another mechanism by which the functional and behavioural properties of neural stem[39] and progenitor cells

across the CNS are distinct from those of their differentiated progeny. Together, these data indicate that in the developing cerebral cortex, as it likely occurs in other CNS regions, several neural progenitor cell types exist that differ in their fate potential and ability to undergo symmetric versus asymmetric cell divisions. New progenitor pools have appeared in the course of evolution as a way to increase the neurogenic capacity of the tissue, which was necessary to accommodate great neuronal expansion, particularly in the cerebral cortex. Finally, the presence of different progenitor pools at different times (*i.e.* NE and RG progenitors), and the spatial separation of progenitors in different niches or compartments (*i.e.* RG and BP progenitors) observed in the cortex reflects a strategy often used in other regions of the CNS, and it may represent a way to differentially control the properties and fate potential of different progenitor pools.

Fate determination and plasticity of cortical neural progenitors

Over time, cortical neural progenitors give rise to an impressive diversity of neuronal and glial subtypes. The neurons born first are those of a structure called the preplate (PP), which later splits into the superficially located marginal zone (MZ) and the deeply located subplate (SP). Between these two structures develops the cortical plate (CP), which contains successively generated layers of projection neuron types[8] (Figure 2A). Elegant birth-dating studies using H^3–thymidine have shown that projection neurons of the different layers are generated in a specific temporal order, such that SP and deep layer VI and V neurons are born first (between E10.5 and E13.5 in the mouse) while neurons of the superficial layers IV and II/III are born later (between E14.5 and E16.5 in the mouse)[8,11]. The laminar cortex is thus generated in an ''inside-out'' manner, with late-born neurons bypassing layers of those born earlier and migrating radially and tangentially to their final location[8,40–45]. It is well known that cortical layers are heterogeneous, and contain many different subtypes of projection neurons that, in addition to having a specific laminar and area address and birth date, can be anatomically classified according to their specific axon targets, either within the cortex or to subcortical and subcerebral targets[11]. (Figure 2B). Given that different neuron types are generated at different developmental stages, this model assumes that neural progenitors (and possibly their postmitotic progeny) must make a timed series of fate decisions to ultimately differentiate into the

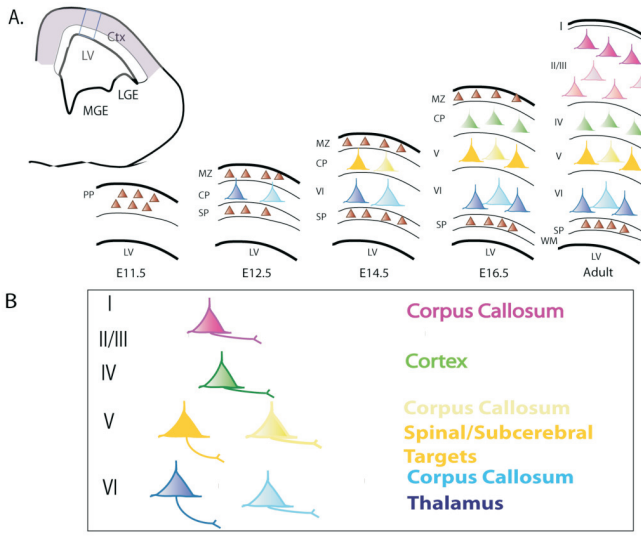

Fig. 2. *The cortex contains many subtypes of cortical projection neurons.* (**A**) *Projection neuron types are born at different stages of development. Layers contain more than one type of projection neuron (indicated by the different colours).* (**B**) *Different projection neurons connect to different targets within the cortex (*via *the corpus callosum and to local ipsilateral targets), and to subcortical (exemplified by the thalamus) and subcerebral (exemplified by the spinal cord) targets.*

right type of neuron at the right time. The mechanisms that instruct and regulate this process are not well defined and several questions remain unanswered. Do neural progenitors undergo genetic and possibly epigenetic changes that actively restrict their fate choices over time? How do cell-intrinsic and cell-extrinsic factors interplay to control the acquisition of neuron type-specific fate? In the retina and during motor neuron development in *Drosophila* a precise temporal sequence of competence states restricts and defines the spectrum of differentiated neuronal progeny that progenitors generate[4,46]. In the cortex, a similar process of fate-restriction affects neural progenitors such that early-stage progenitors that normally generate neurons of the deep layers are more plastic, and still retain the potential to generate all projection neuron types of the cortex[47]. In contrast, later in corticogenesis, progenitors are more restricted in the type of neuronal progeny that they can generate and are not able to generate earlier (deep layer) neuronal fates even if grafted in the early germinal zone[48]. It is likely, however, that while becoming fate-restricted over time, cortical progenitors do not undergo chromatin changes that permanently

prevent them from acquiring new, alternative, fates. This is supported by the observation that overexpression of *Fezf2* (a transcription factor required for the generation of subcerebral projection neurons) in late-stage cortical progenitors can fate-switch them to generate deep layer cortical neurons[49]. Similarly, when cortical progenitors are exposed to BDNF during generation of upper layer neurons and before the S-phase of the cell cycle, they switch to generate neurons of an early deep layer identity[50]. This work supports a model in which neural progenitors respond to timed developmental stimuli and precisely change their fate potential over time to ultimately produce different types of progeny at different times. However, in doing so they still retain enough plasticity to change fate if otherwise instructed. This may be a more general concept applicable to other types of neural progenitors in the nervous system. For example, neural progenitors of the basal ganglia in the ventral forebrain, which normally generate striatal medium spiny neurons and different classes of interneurons, can acquire different fates when exposed to local extracellular cues of a different developing brain region[51–53]. Specifically, if transplanted in the germinal zone of the developing cortex, they generate neurons of cortical identity that extend axonal projections to the spinal cord and through the corpus callosum[52,53]. Similarly, if transplanted in the hindbrain germinal zone, at least within a certain developmental window, they are capable of responding to the new local cues and generate neurons that are appropriate for their new location[51]. Together, these studies also indicate that both environmental and cell-intrinsic cues play active roles in instructing neural progenitor fate. Additional demonstration that progenitors have ''built-in'' mechanisms to cell-autonomously control the generation of different cortical projection neuron types came from elegant experiments conducted by the Temple's group. In this work, cortical progenitors that are isolated from the cortex and cultured *in vitro* give rise to projection neuron populations in a temporal order that respects the timing of sequential generation of the same neurons observed *in vivo*[54,55]. Of note, even in this culture system the possibility does exist that neuronal fate is in some part affected by the postmitotic projection neuron progeny produced, *via* a ''feedback'' loop mechanism. This is in line with a recent report in the developing cortex that *Sip1*, a transcription factor expressed in postmitotic cortical neurons, controls progenitor layer-specific fate in a cell-extrinsic manner, likely *via* the production of neurotrophin-3 (Ntf3)[56].

Can neuronal regeneration learn from embryonic development?

During embryonic development a highly orchestrated process of cell-fate specification and neuronal differentiation progressively shapes the diversity of neuronal types that populate the mature CNS. Over time, the ''portrait'' of each neuronal population is achieved through the combinatorial effects of cell intrinsic programs of fate specification (often acting through ''master'' transcription factors), and cell extrinsic signals that pattern the niche where each neuronal population develops[11,57–61]. Most of these signals are present in the CNS during development and are deregulated or turned-off in the adult. As we begin to understand in greater detail the molecular pathways that direct neural progenitors in the embryo to generate individual types of neurons, it is reasonable to imagine that some of the critical molecular ''effectors'' of this complex developmental process may be induced again in the mature CNS or in the culture dish to achieve the directed differentiation of the same neuronal classes.

There are several important experimental precedents supporting the general idea that signals shaping neuronal diversity in the embryo may be used to regenerate neuronal populations in a tissue culture system. Morphogens that instruct neural progenitors patterning in the developing spinal cord, for example, have been successfully used in the dish to generate spinal motor neurons from pluripotent embryonic stem (ES) and induced pluripotent (iPS) cells[62–64]. Similarly, cell-intrinsic signals driving neuron type-specific development, when known, have also been manipulated with success *in vitro* to direct the differentiation of clinically relevant neuronal types. These include the master transcription factor *Lmx1a*, which is critical for the developmental generation of dopaminergic (DA) neurons in the midbrain, and has been successfully used (with best results when paired with *Msx1*) to instruct ES cells to generate midbrain DA neurons in the dish[65]. These studies can be taken as the demonstration of two very important points: (1) embryonic signals can be used to pattern and direct the fate of pluripotent stem cell populations towards generating specific types of neurons; and (2) despite the complexity of the overall sequence and combination of signals that are used during development to produce a given neuronal population, it may not be necessary to precisely and fully recapitulate every one of them to regenerate neuron types *in*

vitro. Rather, a small number of ''master'' signals may be sufficient to direct the generation of distinct types of neurons.

Generation of cortical neuron types from pluripotent stem cells

In the cerebral cortex, the signals that drive the early steps of specification and overall development of individual types of neurons are largely not defined for most populations. However, among the projection neurons, corticospinal motor neurons (CSMN), and, to a smaller extent, callosal projection neurons (CPN) are notable first exceptions[11,66–70]. CSMN are a clinically relevant population of neurons that die in Amyotrophic Lateral Sclerosis (ALS), and are permanently injured in spinal cord injury (SCI). CSMN are located in layer Vb of motor cortex and they extend axonal projections through the internal capsule and the pons to the spinal cord, where they synapse with lower motor neurons (*via* interneurons in rodents)[70,71]. Together with highly related neuronal types that make axonal projections from layer Vb to targets below the brain, CSMN are also classified as subcerebral projection neurons[11]. Recently, the development of experimental approaches to label and isolate CSMN and other projection neuron subtypes from the cortex has led to the identification of the first series of genes that, with different degrees of restricted expression, distinguish CSMN at the molecular level[66]. Genes that define the CSMN population include transcription factors (*e.g.*, *Ctip2*, *Bcl6*, *Sox5*, *Fezf2*); cell surface proteins (*e.g.*, *Encephalopsin*, *Itm2a*, *Daf1*); calcium signalling proteins (*e.g.*, *Pcp4*, *S100a10*); cell adhesion proteins (*e.g.*, *Cdh22*, *Cdh13*, *Cntn6*), and axon guidance molecules (*e.g.*, *Neto1*, *Netrin-G1*)[66]. Most importantly, beyond their roles as CSMN molecular markers, some of these genes have been shown to control central steps of development of CSMN, including the timing of birth, fate specification and axonal connectivity[66]. Among key transcription factors, the zinc finger protein *Fezf2* has been shown to be essential for CSMN early specification, *in vivo*[67,72,73]. In the absence of *Fezf2* in null-mutant mice, CSMN and all subcerebral projection neurons are not specified and are absent from the cortex. In line with this finding, the corticospinal tract (and all cortical connections to the brainstem) do not form[67]. This effect is neuron type-restricted since upper layer IV and II/III neurons appear to develop normally, and layer VI neurons, despite some morphologic and molecular abnormalities, are specified and locate correctly in layer VI of the *Fezf2*$^{-/-}$ cortex[67].

Further adding to the ''master'' role of *Fezf2* in CSMN development, elevated levels of *Fezf2* expression in progenitor cells of upper layer neurons of the cortex (mostly CPN), is in part sufficient to instruct a switch of fate and the generation of deep layer neurons that make connections to subcortical and subcerebral targets[67,49]. Likely acting after *Fezf2*, another CSMN specific transcription factor, *Ctip2*, is important for the establishment of appropriate axonal connectivity by CSMN to the spinal cord[49,66]. Similarly, *Sox5* and *Bhlhb5* have been demonstrated to control the timing of generation and the arealization of the CSMN population, respectively[74,75]. As the molecular determinants of CSMN birth and differentiation are uncovered, they support the possibility that a small number of the same transcription factors that instruct and regulate CSMN development in the embryo may be used to direct the birth of new CSMN in the postnatal brain or in the dish. This latter possibility was made more concrete by the recent demonstration that ES cells exposed to embryonic patterning signals give rise to dorsalized (likely cortical) neural progenitors, which, like their endogenous counterparts in the cortex, can sequentially produce all major projection neuron types *in vitro*[54,55]. The order of generation of ES-derived projection neuron populations respects that observed in the embryo, with deep layer neurons born before upper layer ones. Importantly, when grafted into the neonatal cerebral cortex, some of the ES-derived neurons develop specific projections to intracortical, subcortical and subcerebral targets[76–78]. This exciting work provides the demonstration that, albeit within heterogeneous cultures, discrete numbers of cortical projection neurons can be generated from pluripotent stem cells in the presence of developmentally relevant extracellular signals. Several challenging tasks now lay ahead. Can the acquisition of subtype-identity be controlled to instruct the selective generation of homogeneous classes of projection neurons? This would be very important for downstream applications that rely on large numbers of pure neuronal populations, like high-throughput chemical screenings and transplantation experiments, among others. One limitation of grafting heterogeneous populations is the potential heterogeneity of targets innervated by the donor neurons. Consequently, it is not currently known, for example, whether ES-derived cortical projection neurons establish and maintain projections to more than one target, although this could be established by cumbersome experiments of retrograde labelling of the grafted neurons from multiple targets within the same animal. Most importantly, there is no easy approach to guarantee selective innervations of only targets of

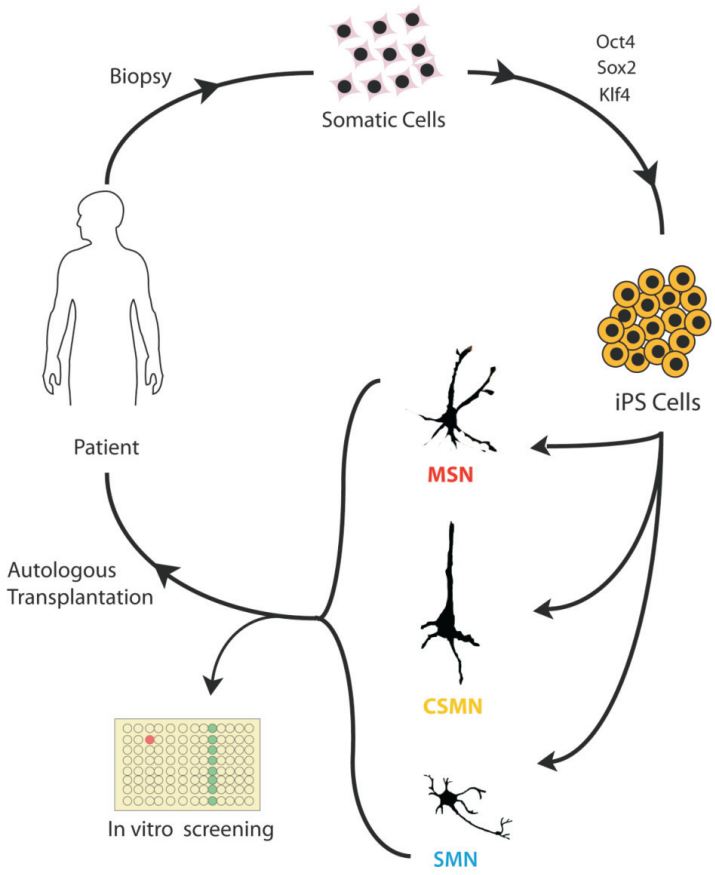

Fig. 3. *iPS cells can be derived from differentiated cells of a patient and directed to differentiate into specific types of clinically relevant neurons (exemplified by MSN, CSMN, and SMN) for chemical screenings and potential autologous transplantation. CSMN, corticospinal motor neurons; SMN, spinal motor neurons; MSN, medium spiny neurons.*

interest (the spinal cord, for example). However, it is reasonable to imagine that it may be possible to refine the current *in vitro* differentiation protocols to include modulation of genes that cell autonomously bias the fate of one neuron type versus another. For example, genes like *Fezf2*, *Ctip2*, *Sox5* and *Bhlhb5* (all proven to have roles in CSMN development)[11,74,75] could be used to favour ES directed differentiation into CSMN and other subcerebral projection neurons. Equally important, inhibition of genes that direct the differentiation of other projection neuron types, for example *Satb2*, proven to be critical for CPN development[79,80],

may be used to prevent ES directed differentiation into neurons of an unwanted identity.

There are explicit clinical implications for this type of work. Not the least, the fact that induced pluripotent stem (iPS) cells, which share many properties with ES cells, can today be obtained from differentiated cells types of the adult, and thus can have a patient-specific origin. Using iPS cells derived from patients as the starting population of pluripotent stem cells enables the directed differentiation of neurons that may not be rejected upon autologous transplantation. In addition, iPS cells derived from a patient with specific, late-onset neurodegenerative diseases could be used to generate the exact types of neurons that die in these pathologies. This offers the unique opportunity to investigate whether neurons are defective from early stages of differentiation, far before clinical symptoms are typically detected. Along these lines of thought, iPS cells generated from patients with familial ALS have been successfully differentiated into spinal motor neurons and studied *in vitro* to gain insight into the pathogenesis of the disease[63]. Similarly, spinal motor neurons have been generated from patients with spinal muscular atrophy (SMA)[81], Parkinson disease (PD)[82], Huntington disease (HD), Down syndrome, juvenile-onset type 1 diabetes mellitus, and a growing number of diseases across different organ systems[83], This enormous progress in the field of nuclear reprogramming, combined with the growing understanding of the signals that drive differentiation of specific subtypes of clinically relevant neurons, seem to indicate that, even in an organ-system as complex as the CNS, regenerative medicine is gaining great momentum.

Acknowledgements

We would like to thank members of the Arlotta laboratory for their suggestions and critical reading. This work was partially supported by the NIH (NS062849), the Harvard Stem Cell Institute and the Spastic Paraplegia Foundation to P.A.

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