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Human Pluripotent Stem Cell-Derived Cerebellar Neurons: From Development to Modeling Cerebellar Ataxias

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Abstract

The most affected cell types in cerebellar ataxias are the cerebellar neurons, which are not readily accessible for cellular and molecular investigation. Pluripotent stem cell (PSC) technology has emerged as an important tool for generating diverse types of neurons, which are used in order to better understand the human nervous system development and pathologies. In this chapter, the strategies for the differentiation of human PSCs toward cerebellar neurons are overviewed, followed by an outlook of their further optimization and diversification by implementing the knowledge from cerebellar development and new cell culture approaches. The optimization strategies are based on the recent progress made in defining the cell populations in mature and developing mouse and human cerebellum. The cellular phenotypes and organization in mouse and human cerebellum are briefly presented, followed by an overview of our current knowledge about their development, which includes patterning, proliferation, neurogenesis, gliogenesis, migration, connectivity and maturation. To date, however, relatively few studies have used induced PSCs (iPSCs) to model cerebellar ataxias and even fewer have looked directly to cerebellar neurons. The reported iPSC-derived *in vitro* models for cerebellar ataxias are reviewed, followed by an outlook of how to improve these models by generating and exploring the cerebellar neurons.

Keywords: cerebellar ataxias, iPSC-derived cellular models, cerebellar neurogenesis, Purkinje cells, cerebellar organoids

1. Introduction

Cerebellar ataxias constitute a very heterogeneous group of diseases in which the motor incoordination is caused by the dysfunction and degeneration of the cerebellar neurons. Although different causative genes or toxins have been identified and several pathological pathways have been investigated, the treatments for these conditions are still largely palliative. Therefore, it is an urgent need for disease-relevant cellular models for studying disease progression and screening for potential therapies.

The rapid development in the field of induced pluripotent stem cell (iPSC) technology offers the opportunity to combine the genetic authenticity of the

patient-derived cellular models with the disease-relevant cell types. Human iPSCs have been generated from a wide variety of easily accessible tissues, including skin and blood cells, using methods which nowadays are safer because they avoid the genomic integration of the viral vectors containing reprogramming factors. The potential of iPSCs to differentiate into any cell type of the body was previously explored by the studies with mouse and human embryonic stem cells (ESCs), which are blastocyst-derived pluripotent populations. Both iPSCs and ESCs may offer direct access to study the cells making the nervous system, but straightforward for disease models are the neurons differentiated from iPSCs, generated from patients with a variety of neurologic or neurodegenerative conditions [1, 2].

Although significant advances have been made, most of the protocols for the differentiation of human PSCs into neurons yield cellular populations which can only partially mirror the functional characteristics detected *in vivo*. In addition, most of the available neuronal characterization comes from the studies in rodents and we still know little about the phenotypes that the human neurons have in different stages of their development or degeneration. Nowadays, only few protocols generate efficiently specific neuronal classes, such as the midbrain dopaminergic neurons or the cortical neurons, while for the most neuronal types in the human brain, including the neurons of the cerebellum, the efficiency of the protocols is much lower and additional cell selection methods are required.

As it happened for the generation of other human neural or non-neural cells and especially for the generation of the cerebral cells (reviewed in [3, 4]), the improvements in the generation of cerebellar neurons will definitely come from a better knowledge of the human cerebellum and its developmental pathways.

The human adult cerebellum is the second largest brain part (after the cerebral cortex) and contains around 80 billion neurons (which represents four times more neurons than in the cerebral cortex) [5–8]. These neurons contribute to the complex cerebellar functions, including the control of movements for performing fine-tuning and coordination [9, 10], as well as of cognitive and emotional processes [11, 12]. The morphological and functional organization in the cerebellum, intensively investigated in rodents, is highly conserved across vertebrates [13]. Both human and mouse cerebella contain two lateral hemispheres connected by a region named vermis. The lateral hemispheres are subdivided into lobes and lobules and, together with vermis, covered by a uniformly pliated gray matter forming the cerebellar cortex. Cerebellar neurons have their cell bodies (somata) located in the cerebellar cortex and in the nuclei situated inside the white matter of each cerebellar hemisphere, called deep cerebellar nuclei (DCN). There are four distinctive DCN in mouse (dentate, fastigial, emboliform and globose), while the last two are fused as the interposed nucleus in human [10, 13].

The higher number of lobules in humans makes the cerebellar cortex more expanded relative to mice; in spite of the increase in size, both the volume of the cerebellum as a percentage of the total brain and the ratio of the number of neurons in the cerebellum to the cerebral cortex is remarkably constant across mammalian species, pointing to the concomitant increase of the cerebellum and the cerebral cortex in humans [6, 8, 14–17].

The morphological organization of the adult cerebellum is schematically presented in **Figure 1**. The neurons located in the cerebellar cortex form three laminar structures laying between the internal white matter and the external pia mater: the granular layer (GL, named also the inner GL), the Purkinje layer (PL) and the molecular layer (ML). The GL contains the densely packed granule cells, which are the most abundant cell type in cerebellum and in the whole brain, as well as few other cells, such as Golgi cells (with different subtypes, such as Lugaro, globular and candelabrum) and unipolar brush cells. PL is a narrow middle zone

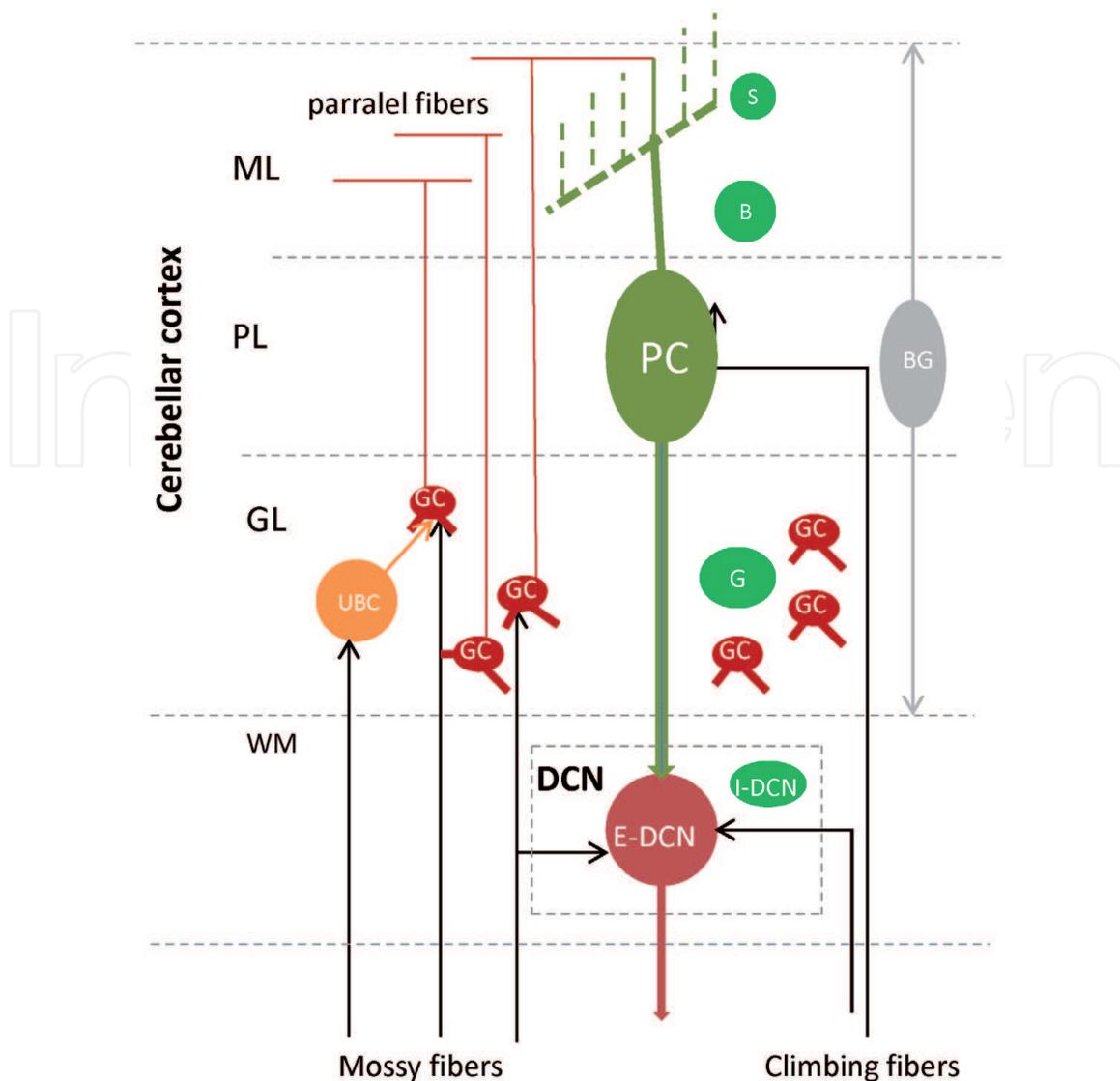


Figure 1. Cellular composition and organization in the adult cerebellum. The cerebellum contains, from exterior to interior; the cerebellar cortex with 3 layers, the molecular layer (ML), the Purkinje layer (PL) and the granular layer (GL), and the deep cerebellar nuclei (DCN) situated in the white matter (WM). Excitatory (red-orange) and inhibitory (green) neurons are located in the cortex (granule cells (GC), unipolar brush cells (UBC), Purkinje cells (PC), Golgi (G), basket (B) and stellate (S) cells) and in the DCN (E-DCN and I-DCN). GC and UBC receive external afferents via mossy fiber, while E-DCN via both mossy fibers and climbing fibers. PC receive external afferents via climbing fibers and internal afferents via parallel fibers, sending efferents to DCN. BG: Bergmann glia (gray).

that contains the large cell bodies of the Purkinje cells, together with the cell bodies of a special type of glial cells named Bergmann glia. The ML contains mainly cell projections, but also a few entire neurons such as the basket cells located near the PL and stellate cells located near the pia mater.

In addition to the shape and location of their cell bodies, the cerebellar neurons are characterized by other intrinsic properties included in their neurochemical profiles (neurotransmitters, associated neuropeptides and receptors), electrophysiological profiles and, in the recent years, in high-throughput transcriptional fingerprints. Based on the neurotransmitters used for synaptic communication, cerebellar neurons are set into two *main classes*: excitatory neurons, which release glutamate, and inhibitory neurons, which release mainly γ -aminobutyric acid (GABA). Excitatory neurons are situated in the cerebellar cortex (granule and unipolar brush cells) and in the DCN. Inhibitory neurons are localized also in the cerebellar cortex (Purkinje cells, Golgi cells, basket and stellate cells) and in the DCN (**Figure 1**).

Regarding tissue architecture and connectivity, the cerebellar neurons are arranged as repeating units in a highly regular manner, relatively identical in all areas of the cerebellar cortex. Granule cells and excitatory neurons in DCN are projection neurons, while inhibitory neurons in the cortex (Golgi cells, stellate cells and basket cells) and DCN, and the unipolar brush cells are interneurons. Granule cells receive excitatory signals from neurons of the brainstem or spinal cord, mainly with a station in the middle or inferior cerebellar peduncle, *via* the mossy fiber afferents. The information from ~25 million mossy fibers is dispersed to ~50 billion granule cells, but each dendrite apparently synapses with a single mossy fiber, in this way promoting combinatorial encoding and enhanced processing of sensory input to the cerebellum. Unipolar brush cells receive sensorimotor signals *via* mossy fibers, each cell forming a specialized giant synaptic junction with a single mossy fiber terminal. Their axons branch locally within the GL, where an intrinsic system superimposes on the canonical extrinsic mossy fiber system (reviewed in [15]).

The axons of granule cells project to the ML, where they form the parallel fibers, which intercept the dendrites of Purkinje cells at right angles. There are ~200 granule cells per Purkinje cell in mice, while in humans there are 3000 granule cells *per* Purkinje cell [8]. Purkinje cell bodies form a monolayer in the middle of PL, each neuron sending a monoplane-oriented expansive dendritic tree with thousands of little spines into the ML, while its axon projects towards and connects with one neuron in the DCN. In addition to the inputs from granule cells, each Purkinje cell receives excitatory signals from climbing fibers arising from the inferior olive neurons in the medulla (which receives sensory information from the cortex). Purkinje cells convey the results of the analysis of afferent information to the excitatory neurons in DCN, which form the main cerebellar output. Each excitatory neuron in DCN receives inputs from several Purkinje cells, but also inputs from the spinocerebellar tract *via* the mossy fibers and from the inferior olive *via* the climbing fibers. Excitatory neurons in DCN send projections back to the brainstem and to motor cortex *via* the thalamus [18, 19].

Remarkably, Purkinje cells can exhibit two distinct types of action potential, with simple and complex spikes. The simple spikes represent an autonomous pacemaker activity, with very little variability between spiking intervals, firing in absence of synaptic inputs. The simple spikes can be modulated by inputs from mossy fiber *via* the parallel fibers. Inhibitory interneurons in the ML, i.e. the stellate and basket cells, also influence circuit topography by making synapses with the dendritic tree and modulating the activity level of Purkinje cells. Additionally, Purkinje cells can evoke complex climbing fiber inputs. Integration of the inputs from climbing fibers and parallel fibers in Purkinje cells generates a unique form of heterosynaptic plasticity, that has been shown to underlie the motor learning [18, 20, 21]. In line with the recent multimodal characterization of the cerebral cortical neurons [22], a deeper investigation of the electrical profiles in human cerebellum is expected from the new Patch-seq techniques [23, 24].

A more extensive neuronal characterization was recently performed by high throughput sequencing, including single-cell sequencing for mouse and human cerebellar tissue [25, 26]. In spite of their quite regular morphology, the cerebellar neurons in each subclass appear as a heterogeneous population, different subsets being defined by several molecular cues, including co-neurotransmitters (e.g. glycine) and neuromodulators (e.g. calbindin, parvalbumin). Markers of some subclasses are related to the position in the cerebellar areas (reviewed in [27]). In addition, a comparative high throughput analysis of mouse versus human cerebellar cells using single cell-RNA sequencing showed that several genes are expressed in human but not in mouse Purkinje cells and confirmed at protein level the expression of novel and specific human Purkinje cell markers, in line with the data from the cerebral cortex [28, 29].

Recent progress in genetic technologies has significantly clarified how the cerebellar cells and their circuits are formed in model organisms, especially in mouse [30–33]. Remarkable advances were made not only in defining of the molecular phenotypes and the differentiation pathways for most of the neural progenitors, but also in understanding of how these synchronize for forming neuronal circuits. Purkinje cells have major roles also during development [34]. They orchestrate the long lasting neurogenesis of the granule cells, the most abundant local excitatory neurons, and the maturation of the local inhibitory neurons, which reciprocally respond by helping in their own maturation.

The human-specific morphological and functional attributes were intensively studied over the last two decades, including for the development of the cerebellum. Mouse mutants for different genes related to developmental diseases affecting the cerebellum in humans demonstrated a considerable evolutionary conservation of the molecular programs across species, but also revealed some human-specific differences. Recent investigations of the developing human cerebellum have emphasized some differences in the organization of the cerebellar progenitor pools. Other human specific differences have been outlined by the single-cell sequencing of different brain cells, including cells in the cerebellum. These high throughput results point out that we still have much to learn about the human cerebellar development, composition and functions.

To what extent can or could the cellular diversity in the adult human cerebellum, and, in the same time, the spatial precision in its organization *in vivo* be reproduced by the PSC-related differentiation protocols? Which would be a proper human model for cerebellum development and cerebellar diseases?

The reported strategies for the differentiation of human PSCs toward cerebellar neurons, especially toward Purkinje cells, are reviewed in this chapter, followed by an outlook of their further optimization and diversification by implementing the knowledge from cerebellar development and new cell culture approaches. This outlook includes an overview of the recent progress made in defining the cell populations in developing mouse and human cerebellum, followed by our current knowledge about their development, which includes pattering, proliferation, neurogenesis, gliogenesis, migration, connectivity and maturation. This knowledge is also the basis for the establishment and optimization of the PSC-derived models for cerebellar ataxias. An overview of the reported *in vitro* patient-derived iPSC approaches for modeling cerebellar ataxias is presented, followed by an outlook of some challenges that remains to be overcome.

2. Differentiation of pluripotent stem cells toward cerebellar neurons

Over the past 20 years, human PSCs, including the ESCs and the iPSCs [35–38], have revolutionized the research on human development and diseases, particularly for the nervous system. Considerable progress has been made in converting human PSC into different types of neural progenitors, from which some continued to differentiate toward different classes of neurons, *in vitro* or after xenotransplantation.

Most of the reported human PSC-based protocols are an adaptation of the protocols that were previously developed for mouse ESCs, which reflect, to a various extent, different stages of neural differentiation in mouse embryo. On this line, the differentiation of the human PSCs is expected to reflect different stages of neural differentiation in human embryonic and fetal stages. Remarkably, recent data have demonstrated that several protocols starting from human PSCs produced authentic neurons and structured brain-like tissues, including the cerebral cortex,

the most complex structure in the human brain. However, many questions remain about the extent to which the relative simplistic *in vitro* settings could reproduce the high complexity of the adult brain structures, both in cell diversity and connectivity (reviewed in [3, 39]).

For the neurons making the human cerebellum, the progress of *in vitro* differentiation protocols was a lot slower comparing to other neuronal populations, such as the spinal cord motoneurons, midbrain dopaminergic neurons, and glutamatergic and GABAergic cortical neurons, between many others. The main reason is the complexity of the cerebellar development, which was only partially and only recently deciphered (overviewed in the next section), while the developmental mechanisms for the spinal cord, midbrain and cerebral cortex were much faster and deeper investigated [40–43].

Increasing understanding of cerebellar development has allowed the elaboration of several protocols in the last years, which made the production of some classes of cerebellar neurons possible, with increasing efficiencies. These protocols were implemented in 2D and 3D cell cultures, or in their combination. As for other brain regions, the differentiation protocols include “directed” steps, meaning controlled differentiation by using extrinsic manipulation approaches, but also steps in which the differentiation advances spontaneously. Most of the protocols use morphogens/growth factors or small molecules with similar functions, which are sequentially administered to mimic the environment *in vivo*.

Two early studies implemented the mouse ESCs differentiation into cerebellar neurons, using different approaches [44, 45], which were followed by several protocols aimed to increase their efficiency. Su et al. [45] used non-adherent ESC cell clusters in serum-free medium supplemented with fibroblast growth factor 2 (FGF2) and insulin. The cellular spheroids, named serum-free embryoid bodies (SFEB, even though they contained mainly undifferentiated cells in this stage), gradually differentiated into more complex 3D cell aggregates containing a mixture of progenitor cells and neurons, which included some granule cell progenitors and few neurons expressing early Purkinje cell markers. Following the same conditions, Muguruma et al. [46] showed that the FGF2-treated neural progenitors presented a broad fate, but some cells organized in tissue-like structures resembling the cerebellum origin in the embryo. These 3D cell aggregates further formed brain organoids, which contained some areas organized as a primitive cerebellar tissue. When cyclopamine, a sonic hedgehog (SHH) antagonist, was added to block the spontaneous ventralization, the proportion of cerebellar cells was increased, including 35–42% Purkinje cell progenitors by day 11 of ESC differentiation. Additionally, this study introduced the selection of the cerebellar progenitor cells, addressing to a cell-surface marker expressed in this population (Kirrel2/Neph3). The selected cells survived and integrated into the mouse cerebellum following *in utero* transplantation at embryonic day (e) 15.5, but their surviving and differentiation into Purkinje cells *in vitro* was possible only in co-culture with dissociated mouse postnatal cerebellar cells [47]. Following the same protocol, Tao et al. [48] showed that the cerebellar organotypic slices prepared from mice at postnatal day (p) 6–8 supply an appropriate trophic environment for the differentiation and maturation of ESC-derived Purkinje cells. Remarkably, after 28 days in co-culture, they showed the same characteristics as the neonatal Purkinje cells.

Salero and Hatten [44] succeeded in generating mouse ESC-derived granule cells at a relatively high efficiency by implementing a protocol in 2D culture based on step-related treatments with different morphogens. FGF8, WNT1 and retinoic acid (RA) were used in the first step, while bone morphogenic proteins (BMPs) were used in the next step to obtain the granule cell progenitors, which were next proliferated with SHH and Jagged1 and showed markers expressed in GL *in vivo*.

Again, for differentiation and maturation, granule cell progenitors were co-cultured with either postnatal mouse cerebellar neurons or glial-conditioned medium and the resulted neurons resembled the neonatal counterparts.

The pioneering studies of mouse ESC cerebellar differentiation were next translated to human PSCs and subsequently refined (**Table 1**). The protocol of Muguruma et al. [46] in 3D culture was applied to human ESC and iPSCs [49, 50, 52]. Human progenitor cells self-organized in polarized neuroepithelium containing around 10% KIRREL2+ cells after 20 days. Muguruma et al. [50] also refined this protocol and followed a long-term ESC differentiation in 3D culture, an approach which resembled the first generation of human brain organoids. They found that the dorsal hindbrain patterning is more efficient for human cells without cyclopamine. Sequential addition of FGF19 and stromal cell-derived factor 1 (SDF1) generated approximately 28% KIRREL2+ cells (representing the progenitors of the cerebellar inhibitory neurons) and 18% ATOH1+ cells (representing the progenitors of the cerebellar excitatory neurons) by day 35. As for the mouse protocol,

General procedure	Hindbrain patterning	Cerebellar progenitors		Cell selection	Neuronal maturation	References
		VZ	RL			
3D cell cultures <ul style="list-style-type: none"> spontaneous IsO induction cerebellar organoids 	FGF2, insulin, cyclopamin	KIRREL2+		—	on organotypic cerebellar slices (rat, human)	Wang [49]
	<ul style="list-style-type: none"> FGF2, insulin FGF19 SDF1 	KIRREL2+	ATOH1+	KIRREL2+ VZ progenitors by FACS	co-culture with postnatal mouse granule cell progenitors <150 days	Muguruma [50], Ishida [51]
	<ul style="list-style-type: none"> FGF2, insulin, TGFβ antagonist 	KIRREL2+	ATOH1+	—	co-culture with e18.5 mouse cerebellar progenitors <70 days	Watson [52]
2D cell cultures <ul style="list-style-type: none"> controlled neural induction (Noggin, SB431542) directed dorsal hindbrain patterning selection of neurons 	FGF8b and RA	KIRREL2+	ATOH1+	ATOH1-GFP+ by FACS	cell transplantation in mouse brain	Erceg [53]
	<ul style="list-style-type: none"> D0–4: WNT agonist (CHIR-99021) D4–12: FGF8b 	KIRREL2+	—	THY+ immature neurons by MACS	co-culture with mouse granule cells	Sundberg [54]
	<ul style="list-style-type: none"> D0–4: WNT agonist (CHIR-99021) 1.5 μM D4–12: FGF8b (100 ng/ml) D12–24: BDNF 	EN1/2 GBX2 (D6)	—	(D22) Negative selection for GD3 by immunopanning Positive selection for NCAM1+ immature neurons by MACS	co-culture with mouse cerebellar glia < 65 days and next with granule cells < 89 days	Buchholz [28]

Table 1.
 Reported protocols for the differentiation of human PSCs toward cerebellar neurons.

KIRREL2⁺ cells were subsequently selected by fluorescence activated cell sorting (FACS) and differentiated into Purkinje cells in co-culture with murine granule cell progenitors. The *in vitro* differentiation of the KIRREL2⁺ cells for 10 days generated ~45% Purkinje cell progenitors.

Other approaches aimed to increase the proportion of human ESC-derived cerebellar cells by applying the hindbrain patterning conditions tested for mouse ESCs [44]. Erceg et al. [53, 55] treated human ESCs aggregates with FGF8b and RA, followed by a manual selection of the neuroepithelial cells organized in polarized structures. This procedure yielded, after further differentiation, a heterogeneous population expressing markers of granule cells, Purkinje cells and glial cells. In a more directed differentiation approach, Sundberg et al. [54] used the WNT agonist CHIR99021, FGF8b and FGF2 for patterning the neuroepithelial cells resulted from the parallel neural induction of human ESCs with dual-SMAD inhibition [56]. The patterned progenitors gradually express the hindbrain, cerebellar and Purkinje cell progenitor markers, such as EN1/2, GBX2, PTF1a, KIRREL2 and SKOR2. Between days 24 and 48 of differentiation, markers of GABAergic phenotype and markers of immature Purkinje cells, such as PCP2, were detected. In order to enrich for the Purkinje cell population, instead of the previously used cell sorting for KIRREL2, Sundberg et al. [54] implemented the THY1⁺ cell selection, a method previously used to purify mouse Purkinje cells from primary cerebellar cultures [57]. The sorted THY1⁺ cells further matured into Purkinje cells expressing the early Purkinje cells marker PCP2 (or L7). The same team further optimized the directed differentiation protocol [28], by quantifying the effect of patterning molecules on directing the cerebellar cell phenotypes. They found that the combination of the GSK3 inhibitor CHIR99021 (1.5 μ M) for 4 days with FGF8b (100 ng/ml) between days 5 and 12 of differentiation generated the highest proportion of Purkinje cell progenitors. From days 12 to 24, neural cells expressing the cerebellar marker KIRREL2 gave rise to increasing numbers of adjacently located cells expressing Purkinje cell markers. As early as day 35 of differentiation, subpopulations of iPSC-derived cells expressed markers of the primary cerebellar progenitor cells. The postmitotic Purkinje cell marker PCP2 was observed starting from day 18 onward. Flow cytometry analysis showed that ~23% of cells expressed PCP2 at day 24 of differentiation. A changing element of this protocol was the selection of the immature human PSC-derived Purkinje cells in two steps, a negative selection by GD3 immunopanning and a positive selection by magnetic cell sorting (MACS) with NCAM antibodies [28].

As for the mouse cerebellar neurons, the conditions used for the *in vitro* maturation of the Purkinje cells and granule cells generated from human PSC were undefined, based on co-culture with different cerebellar tissue-derived populations (**Table 1**). The maturation into functional Purkinje neurons has so far been achieved in undefined conditions by co-culturing with either cerebellar granule cell precursors isolated from murine embryos [50–52], or with fetal or postnatal cerebellar organotypic slices [48, 49]. A protocol adapted from Muguruma et al. [50] eliminated the KIRREL2⁺ cell sorting and employed the differentiation of human cells in co-culture with e18.5 mouse cerebellar progenitors [52]. Again, markers of the cerebellar proliferative zones were detected at early times of differentiation and around 10% Calbindin⁺ Purkinje cells were detected from day 50 onward. Following long-term co-culture (up to 150 days), these neurons expressed the Purkinje cell markers L7, Calbindin, Aldolase C and LHX5 [50]. In the study of Sundberg et al. [54], the selected Purkinje cells were co-cultured with mouse cerebellar glia and then with mouse granule cells. With this methodology, human PSC-derived Purkinje cells formed synapses with mouse granule cells and had more differentiated morphologies. However, significant electrophysiological activity,

comparable with that of Purkinje cells *in vivo* of the iPSC-derived neurons, was observed only following co-culture with human fetal cerebellar slices [49].

3. Strategies for the optimization of the human PSC-derived cerebellar cultures

Even though the reported protocols have advanced in the generation of cerebellar neuron from human PSCs, they still need a lot of optimization in order to generate homogeneous population of cerebellar neurons in 2D cultures or cerebellar tissue-like aggregated in 3D cultures. Looking at the previous optimized protocols for generating other neuronal populations, such as the midbrain neurons, the cortical neurons or the cortical organoids, it is relevant to follow again the steps which were gradually applied in order to achieve the efficiency and complexity they offer today (reviewed in [3, 4]). Following this aim, here the development principles of the cerebellar neurons are overviewed, from progenitor specification to neuronal assembles, followed by an outlook of how these principles could be applied for the optimization of the protocols generating cerebellar neurons from human PSCs.

During early embryo development, the human neural tube is formed by the folding of a sheet of neuroepithelium and is progressively closed and regionalized under the control of temporally and spatially coordinated gradients of morphogens secreted by organizer centers. At the end of the neurula stage, corresponding to embryonic day (E) 28, the neural tube is entirely closed and contains, from anterior to posterior, the three primary brain vesicles (forebrain, midbrain and hindbrain) and the spinal cord. Soon after the definition of the midbrain-hindbrain boundary (MHB), cerebellum starts to form at the most anterior and dorsal hindbrain territory. In humans, the cerebellar development is highly protracted, extending from E30 to the end of the second postnatal year. In mice, cerebellum almost completes over a period of around one month, starting from embryonic day (e) 9 and including the first three postnatal weeks (reviewed in [15, 58–60] (**Figure 2**)). However, as for the whole brain, the mechanisms of cell differentiation and histogenesis in cerebellum are mainly conserved in mammals. While the development of the mouse cerebellum was intensively studied [15, 30, 32–34, 58, 61–65], the embryonic and fetal stages in human cerebellar development were only recently described in details [13, 16, 59, 60]. Notably, as for the other parts of the human brain, the embryonic and fetal stages of development are not available for cellular and functional studies, and their histological and clinical images represent only snapshots in time for one individual. Conversely, developmental time-course experiments in mice can be conducted on multiple mice of identical genotypes. These studies revealed that the ontogenesis of all neurons and glial cells in the nervous system, including the ones in the cerebellum, follows the same steps of (1) patterning and specification of the progenitor cells, (2) neurogenesis/gliogenesis and (3) migration, histogenesis, formation of the neuronal circuits and neuronal maturation (reviewed in [15, 27, 58, 61, 66, 67]). However, in contrast to other CNS areas, including the cerebral cortex, in which gliogenesis follows neurogenesis [68, 69], glia generation in cerebellum parallels or precedes the long-lasting generation of the granule cells and inhibitory neurons [15, 30, 32, 65, 68]. Even though the main developmental programs are conserved from mice to humans, some important specie-specific differences responsible for the expansion of the human cerebellum have been recently identified [59, 60]. In the following brief presentation, the main morphological, cellular and molecular events in mouse are complemented with the available information in human.

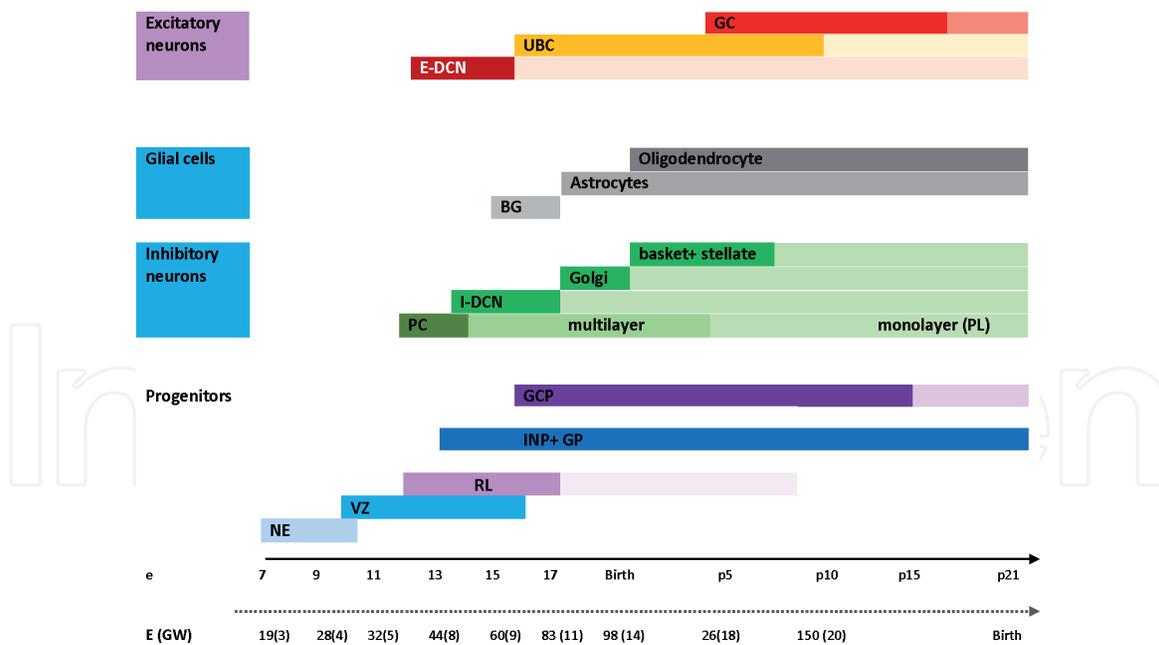


Figure 2.

Timing and the aligned stages in mouse and human cerebellar development. Embryonic days in mouse (e) and human (E). GW-gestational weeks. NE- neuroepithelium (light blue). The cerebellar ventricular zone (VZ) (blue) is the origin of the inhibitory neurons and glial cells. Inhibitory neurons (green) are the Purkinje cells (PC), Golgi, basket and stellate cells, and the inhibitory neurons in the deep cerebellar nuclei (I-DCN). The rhombic lip (RL) (Lila) is the origin of the excitatory neurons in the cortex (Granule cells (GC) and unipolar brush cells (UBC) and in the DCN (E-DCN). Long-lasting progenitor stages for the GC progenitors (GCP-violet), and inhibitory interneuron and glial progenitors (INP and GP, blue). Long-lasting maturation of inhibitory neurons (light green) and of excitatory neurons (light pink-orange), and gliogenesis (gray) stages.

3.1 Patterning and specification of the cerebellar progenitor cells

Several studies in mouse showed that all cerebellar neurons and glial cells originate from the hindbrain region corresponding to the dorsal (or alar) part (or plate) of the first rhombomere (r1) [30, 70]. The anterior limit of the cerebellum is defined by the MHB, named also isthmus, where an organizer center, named the isthmus organizer (IsO), forms early in development and has a major role in the anterior/posterior (A/P) patterning of the midbrain and hindbrain. IsO formation is preceded by a series of patterning events that start in the forming neural plate, where two transcription factors, *Otx2* (Orthodenticle Homeobox 2) and *Gbx2* (Gastrulation Brain Homeobox 2) define the primitive anterior and posterior domains, respectively [71]. They are further co-expressed in early IsO and then differentially express in the midbrain and hindbrain domains [72]. WNT signaling has a main role in the A/P patterning of the neural tube but also in IsO induction, showed by the loss of IsO in WNT1 homozygous mutants ([73]; reviewed in [74]). Shortly after the primary brain vesicles formation, Fibroblast Growth Factor 8 (FGF8) secreted by IsO patterns the adjacent territories [71, 75–80]. Additional A/P patterning by extra-neurally secreted retinoic acid (RA) defines the metencephalic and myelencephalic secondary hindbrain vesicles. The metencephalon expresses the homeobox gene *Hoxa1*, and formed the first hindbrain rhombomere (r1), where the FGF8 blocks the expression of other *Hox* genes. Next, the selective expression of negative regulators of the activated Ras–ERK pathway in r1 stops the local action of FGF8 [81]. In parallel with the A/P patterning, whole neural tube is patterned also dorsoventrally (D/V). The main ventralizing factor is Sonic Hedgehog (SHH), which by e9 is produced in the floor plate of the metencephalon [15, 74, 82, 83] and secreted into the neural tube's lumen, which at this level becomes the 4th ventricle. Consequently, the alar plate of the r1 territory

is patterned into the cerebellar domain (anlagen) (**Figure 3**), while anteriorly situated territory becomes the tectum domain, posteriorly, the r2 domain, and ventrally, the pons domain.

Between e9 and e12.5 and, the cerebellar neuroepithelium undergoes morphological changes: the midline remains as a single cell layer and forms the roof plate, while each lateral part forms two primary proliferative zones, known as the origins of the neural populations in the mouse cerebellum: the cerebellar ventricular zone (VZ) and rhombic lip (RL) (**Figures 2 and 3**) [30]. By e10, the roof plate becomes the second cerebellar organizer center and secretes factors belonging (TGF)- β family, such as the bone morphogenetic proteins (BMPs), the most important dorsalizing factors in the cerebellum, and gradually transforms into the choroid plexus epithelium (ChPe). By e12.5, ChPe additionally produces SHH. Genetic fate mapping proved that the morphogens secreted by IsO, roof plate and floor plate define the cerebellar domains which, in addition to the hindbrain restricted

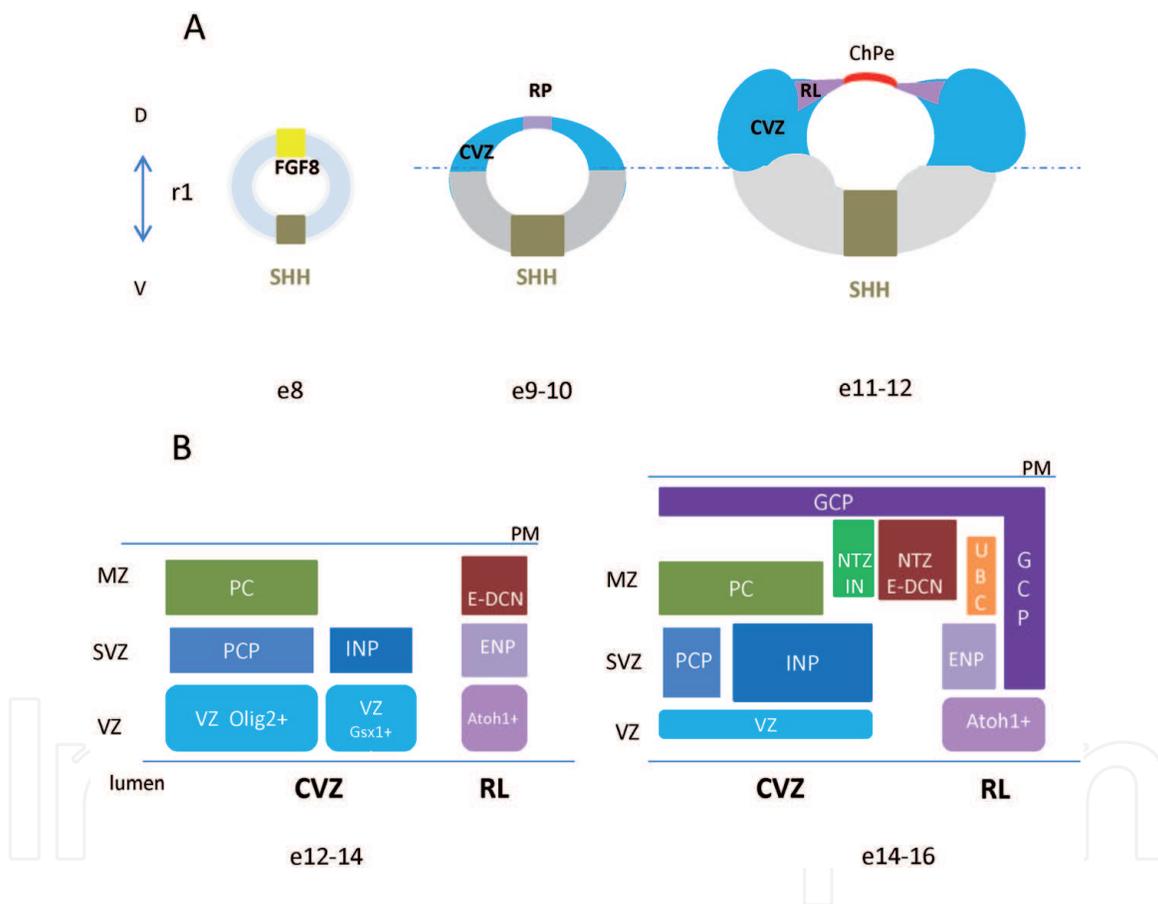


Figure 3.

Stages and distribution of cell populations in mouse early cerebellar development. Formation and differentiation of the cerebellar populations from embryonic day (e) 8 to e16, when all the neuronal populations or their long-lasting progenitors are formed. (A) Between e8 and 12, in the dorsal part of the first rhombomere (r1) of the hindbrain neural tube, the cerebellar ventricular zone (CVZ) (light blue) forms at e9–10, due to the dorsal FGF8 signal and ventral SHH signal, while the rhombic lip (RL) forms at after e10, being visible at the border between the CVZ and the roof plate (RP) (light Lila), due to the BMP signaling from the RP, which forms the choroid plexus epithelium (ChPe) (red). (B) Between e12 and 16, different progenitors arrive in the subventricular zone (SVZ) and mantle zone (MZ) of the neural tube. At e12–14, the Ptf1+ ventricular zone (VZ) of the CVZ primary domain contains the Olig2+ and the Gbx1+ subdomains, which generate the Purkinje cell progenitors (PCP) and the interneuron progenitors (INP, blue) domains, respectively, while the first postmitotic Purkinje cells (PC) already exit the SVZ. The VZ in RL contains Atoh1+ progenitors, which gradually form progenitors of the excitatory neurons in SVZ. They generate first the excitatory neurons for the deep cerebellar nuclei (E-DCN) and at later time points (e14–16), they start to generate the unipolar brush cells (UPC). The RL generates also the progenitors of the granule cells progenitors (GCP-violet), which migrate in waves in the MZ close to the pia mater (PM). In the CVZ, cells representing a subpopulation of the INP domain migrates in the MZ and join the E-DCN in a nuclear transitory zone (NTZ), where they start to differentiate into the inhibitory neurons of the DCN (I-DCN).

expression of *Gbx2*, show the differential expression of two basic-helix-loop-helix (bHLH) transcription factors: Pancreatic transcription factor 1 (*Ptf1*) specifies the VZ domain and Atonal homolog 1 (*Atoh1*, also called *Math1*), specifies the RL progenitor domain [15, 58, 61, 84, 85].

Each cerebellar progenitor zone forms subdomains with their own spatial and temporal identities, which produce specific neuronal subtypes. VZ-derived progenitors give rise to all GABAergic neurons and glial cells of the cerebellum. VZ-derived neurogenesis starts at e10.5 and continues until e17 in mouse. Before neurogenesis starts (~e9), the VZ progenitor domain corresponds to the neuroepithelial cells localized in the VZ of the r1 neural tube (**Figure 3**). Most of the earliest *Ptf1a*⁺ progenitors upregulate *Kirrel2/Neph3* and oligodendrocyte-specific bHLH gene *Olig2* expression [82, 86], while a small proportion in the early rostral VZ express homeodomain-containing transcription factor gene *Gsx1*. As the neural tube grows, the neuroepithelial cells gradually transform into radial glial progenitors and a subventricular zone appears evident in the VZ domain (SVZvz in **Figure 3**). *Ptf1a*⁺ and *Olig2*⁺ radial glial cells start to express *Lhx1*, *Lhx5* and *Skor2*, and become Purkinje cell progenitors, located in the SVZvz, which gradually express Neurogenin 1 and 2, start neurogenesis and migrate from the SVZvz. *Ptf1a*⁺ and *Gbx1*⁺ radial glial cells gradually commit to inhibitory interneuron and glial progenitors. The interneuron progenitors express *Lhx1*, *Lhx5* and *Pax2*. By e14.5, they become predominant in the SVZvz [87] and soon after start to migrate out of the SVZvz and form transient amplifying progenitor pools. Once all the neurons and transit amplifying progenitors exit the SVZvz, the remaining radial glial cells differentiate into Bergmann glia. The VZ-derived transit amplifying progenitors generate inhibitory interneurons, astrocytes and oligodendrocytes [58].

The neuroepithelium of the RL gives rise to all glutamatergic neurons in the cerebellum (**Figures 2 and 3**), but also to extracerebellar neurons such as the pontine neurons [66, 70]. RL *Atoh1*⁺ neuroepithelial cells situated between the roof plate and the VZ domain start their proliferation after the adjacent VZ progenitors (~e10). Also the RL neuroepithelial cells gradually acquire a radial glial phenotype and are patterned in subdomains, which express the paired box gene *Pax6* in combination with the zinc finger genes *Zic* and the homeobox gene *Meis*. First, *Pax6* and *Meis2* expressing progenitors commit to neurogenesis, when they gradually express *Tbr2* and *Tbr1* and generate the glutamatergic neurons in DCN. Later, the remaining RL progenitors co-expressing *Pax6*, *Meis1*, *Zic1/2* and *Barhl1* commit to granule cell progenitors, in parallel with the unipolar brush cell progenitors, which upregulate the *Tbr2* expression, downregulate *Pax6* expression and become unipolar brush cells [15, 88].

The cerebellar proliferative zones in human embryos have been only recently investigated. The human cerebellar VZ (gradually forming the SVZ_{vz}) undergoes massive expansion which covers the second month (E30–56), afterwards extinguishing its proliferative potential and remaining as a single cell layer. Conversely, the RL germinal zone remains small during the peak expansion of the VZ progenitors, but starts a significant expansion at around gestational week (GW) 11, when it forms the SVZ_{RL}, which persists long after birth [59, 60].

GABAergic phenotypes. Cerebellar inhibitory neurons, including Purkinje cells and interneurons (Golgi, stellate, basket and inhibitory neurons of the DCN) originate from different subdomains in cerebellar VZ (*Ptf1a*⁺), in different waves (**Figures 2 and 3**). Purkinje cell progenitors (expressing *Skor2*, *Lhx1/5* and *Corl2*) gradually express Neurog1/2 and start neurogenesis, which in mouse is completed at e12.5. Once in the postmitotic stage, Purkinje cells start a short distance radial migration alongside the radial glial processes toward the mantle zone where they stack in a transient multilayered structure named the Purkinje cell plate and

gradually express markers such as Purkinje cell protein 2 (Pcp2, named also L7), Pcp4 and Calbindin 1 (Calb1) [15, 64]. In postnatal stages, due to extensive cerebellar expansion, multilayered Purkinje cells gradually form a monolayer while each neuron starts the development of its characteristic extensive and flattened dendritic arbor and the expression of mature markers synaptic markers [30, 58, 89].

In humans, all Purkinje cells are generated before the 8th GW, which places them among the earliest-born central neurons. They start to migrate at E44 outwards from the VZ along radial glial projections to the pial surface. A broad Purkinje cell multilayer extending in the mantle zone is evident between the GW 10 and 13 GW, while a monolayer distribution is achieved by GW 20–24 (**Figure 2**). Human Purkinje cells start to develop their characteristic extensive and flattened dendritic arbors and long axons in the early fetal stages, their final maturation being achieved postnatally, in a 6-fold longer period than in mice [59, 60, 90, 91].

Contrary to the Purkinje cells, which are postmitotic already into the cerebellar SVZvz, the Gbx1+ progenitors expressing the paired homeobox gene *Pax2* migrate in several waves from the SVZvz to the mantle zone, where they start to express the neurogenic genes *Neurog1* or *Ascl1* and differentiate into Pax2+ interneurons. In the first wave (from ~e10.5), the interneuron progenitors migrate to the rostral end of the cerebellar anlage in a Nuclear Transitory Zone (NTZ), which is transient zone for the DCN assembly [15]. After the progenitors settle near the already established excitatory neurons, they produce the inhibitory interneurons of the DCN. In later stages of development, NTZ is gradually organized into distinct DCN. In the second wave (from ~e13.5), the interneuron progenitors migrate to the Purkinje cell multilayer, continue their migration in the developing white matter and postnatally reach the developing granular layer where they generate postmitotic Golgi cells. At later stages, interneuron progenitors migrate radially in the white matter, continue to proliferate in a transit amplifying center and eventually generate the stellate and basket cells in the ML [78, 92]. In parallel with the late interneurons progenitors, the progenitors of astrocytes and oligodendrocytes continue to proliferate in the developing white matter (**Figure 2**).

Glutamatergic cerebellar neurons (excitatory neurons in DCN, granule cells and unipolar brush cells) originate from different subdomains of the RL, in different waves (**Figures 2 and 3**). The first cells leaving from the RL are the newborn excitatory neurons in DCN. Next, the granule cell progenitors migrate in waves out of the RL, where they continue the proliferation. In the first wave (e10.5–12.5), discrete subpopulations of rostrally situated Atoh1+ cells gradually upregulate *Pax6*, *Meis2*, *Lhx9* *Tbr2* and *Tbr1* and become newborn glutamatergic neurons, which migrate rostrally and tangentially to the NTZ [15, 88, 93]. The allocation of a temporal framework of different DCN components is accompanied by a characterized sequence of transcriptional maturation that results in the first born neurons for the lateral nucleus (projecting to midbrain and thalamus), followed by neurons for the medial (fastigial) group.

The second wave covers middle to late embryonic stages, when Pax6+ granule cell progenitors leave the RL, migrate out toward the pial surface and undergo a prolonged expansion in a secondary germinal zone, or a second transit amplifying center, named the external granular layer (EGL) [64]. Granule cell progenitors retain the expression of *Atoh1* and migrate into the mantle zone where they express *Tbr2* and continue to proliferate to form the EGL [88]. During the early postnatal period, multiple mitogenic pathways expand the EGL. Peak EGL proliferation occurs around p7 and is complete by p15 (**Figure 2**). The main mitogen is the SHH, secreted by the underlying Purkinje cells [94], but also Jag1, a ligand the Notch2, acts locally in the EGL [95]. Exponential granule cell proliferation in the EGL drives cerebellar growth and foliation [96]. BMP4 and WNT3 secreted by the ChPe promote cell-cycle exit and

neurogenesis [15]. The postmitotic granule cells downregulate *Atoh1* and upregulate *NeuroD1* [15]. Newborn granule cells migrate tangentially within the EGL and then exit the EGL migrating radially inwardly along Bergmann glial fibers, trailing a long T-shaped axon behind, interact with the flat, elaborate dendrites of Purkinje cells and form the parallel fibers in ML. Migrating granule cells settle below the developing PL to form the internal granule layer (IGL, corresponding to the adult GL), achieving the final laminar arrangement of the mature cerebellum, from where they extend dendrites to form synapses with mossy fiber afferent axons [15, 58].

Unipolar brush cell differentiation parallels the granule cell progenitor waves (**Figure 2**). Unipolar brush cells are born starting with e13.4, while continuing to p0–1. Progenitors of the unipolar brush cells express *Wnt1* early in development (e10.5–13.5), but this expression is downregulated before they migrate from the RL. The newly generated neurons remain in the RL for an additional 1–2 days, after which they exit RL and migrate dorsally through the white matter to their final destination. Most unipolar brush cells reach the IGL by p10, several days before granule cell neurogenesis is complete. Their final maturation occurs between p2 and p28, which seems to coincide with the establishment of the first synaptic contacts with external mossy fibers [15, 27, 88].

3.2 Coordinated formation of the cerebellar circuits

The successful construction of the neuronal circuitry relies on the coordinated generation of functionally opposed neurons. Accordingly, the differentiation programs of cerebellar excitatory and inhibitory neurons are interdependent and defined as the coordinated integration of the VZ and RL-derived lineages in local circuits, in both the cortex and DCN. For the DCN, the cell fate of the excitatory neurons appears determined at the RL, in a temporal pattern, while the interneuron progenitors migrate, differentiate and integrate in the NTZ after receiving local signals from the excitatory neurons.

Purkinje cells have a remarkable capacity to regulate developmental events by sending *SHH* signals bi-directionally. Starting at e16.5 and continuing throughout adulthood, *SHH* expression in cerebellum is restricted to Purkinje cells and Bergmann glia [97]. Dendritic-derived *SHH* drives the granule progenitor cell proliferation, while axon-derived *SHH* disseminates to the neonatal white matter and contributes to the expansion of the VZ-derived progenitors for the late-born interneurons and glial cells during the postnatal period [98]. Additionally, Purkinje cells are critical for the terminal differentiation and morphogenesis of the interneurons in the ML, the basket and stellate cells. On the other side, signaling from differentiating granule cells influences the planarity and the elaborate branching pattern of the Purkinje cell dendritic tree, which occurs from p5 to p15 [99, 100]. Additionally, the dendritic differentiation of the interneurons in ML is sensitive to the granule cell-derived inputs, including BDNF signaling [15].

In the third trimester and postnatally, human cerebellum undergoes its major growth, primarily due to the prolonged expansion of the granule cell progenitors. By 10–11 GW, streams of cells which form the external GL (EGL) were observed along the pial surface connecting to the RL. Due to extensive EGL proliferation, human cerebellum increases 5 fold in size between GW 24–40 [90]. Differentiation and maturation of the human cerebellar neurons progress mainly as in the mouse, but there are some species-specific features. Foliation correlates with EGL proliferation and increases dramatically between GW 20–32, as the cerebellum rapidly increases in size and volume. The formation of the Purkinje cell monolayer coincides with the peak of EGL proliferation [89, 90]. The human cerebellar cortex still has a prominent EGL at birth. EGL gradually decreases in thickness as a result of

migration of granule cells into the internal GL. By the end of the second postnatal year, EGL is depleted while the thickness of the molecular layer and the length of the PL increase, concomitant with the increasing cerebellar volume [89, 90]. To date, there are few studies about the development of the human interneurons, both inhibitory and excitatory, which represent a minority comparing to the granule cells, but with a major role in the maturation of Purkinje cells and circuit formation [15, 34, 58, 91, 101].

In addition, the single-cell sequencing techniques have been applied for analyzing different stages of mouse cerebellar development [62, 102]. Carter et al. [62] performed single-cell RNA-sequencing and unbiased classification of around 40 thousand murine cerebellar cells from eight embryonic samples (at e10-e17) and 4 postnatal samples (at p0, p4, p7 and p10). Such approach allows for a more comprehensive detailing of the transcriptional and cellular heterogeneity among lineages of interest and can provide a valuable resource for answering further questions related to cerebellar development and diseases. In a similar study, Peng et al. [102] analyzed around 20 thousand cells from mouse postnatal cerebella and looked in addition to the dynamics of interneuron differentiation but also mitochondrial markers and ataxia risk genes. In a complementary approach, gene expression in the postnatal stages of mouse cerebellar development were analyzed by Buchholtz et al. [28] in Purkinje cell populations selected from mice expressing a *Egfp-Pcp2* reporter gene. Again, the dynamics of different pathways of mitochondrial and autophagy genes correlated with the developmental stages of Purkinje cells, which suggest their implication in several neurodevelopmental diseases.

3.3 From development of the cerebellum to the optimization of the human PSC differentiation protocols

There are several steps to be considered for the cerebellar protocols, which practically cover all the developmental stages: from neural induction and dorsal hindbrain patterning to the patterning and proliferation of the VZ-like and RL-like progenitors, to the neurogenesis of the selected progenitors, and lastly to the maturation of the neurons and the formation of the neuronal circuits. Are the previously used neural induction and early patterning conditions (in both 2D and 3D approaches) optimal for the generation of progenitors similar to the ones in the dorsal r1 in the neurula stage, which represent the origin of the neurons making the cerebellum? Are the previously used conditions optimal for the uniform generation of early VZ and RL progenitors? Which factors and what timing would be necessary for a uniform patterning towards VZ or RL subpopulations? Which conditions would be efficient to produce a uniform neurogenesis from different progenitors? What would the defined conditions for the neuronal maturation be? How can the neuronal maturation be faster? How can other neuronal subtypes, such as the interneurons in the cerebellar cortex and in the DCN, be generated uniformly and efficiently?

Some recent strategies were successful for the optimization of the protocols for the cerebral neurons and cerebral organoids. It remains to be checked whether these strategies can be extrapolated for the cerebellar cultures. Again, the solutions may come from the development principles. The main trajectories that could be followed from the human iPSC to the neuronal cell types contained in the cerebellum are outlooked in **Figure 4** and detailed in the following paragraphs.

Improving neural induction and hindbrain patterning. The first step for all the protocols regarding the neural differentiation of human PSCs implies the removing of the pluripotent cell proliferation factors, such as FGF2 and TGF β . The additional use of several inhibitors such as BMP/Activin/TGF β pathway inhibitors, alone (such as Noggin) or in combination (dual-SMAD inhibition by small molecules such as

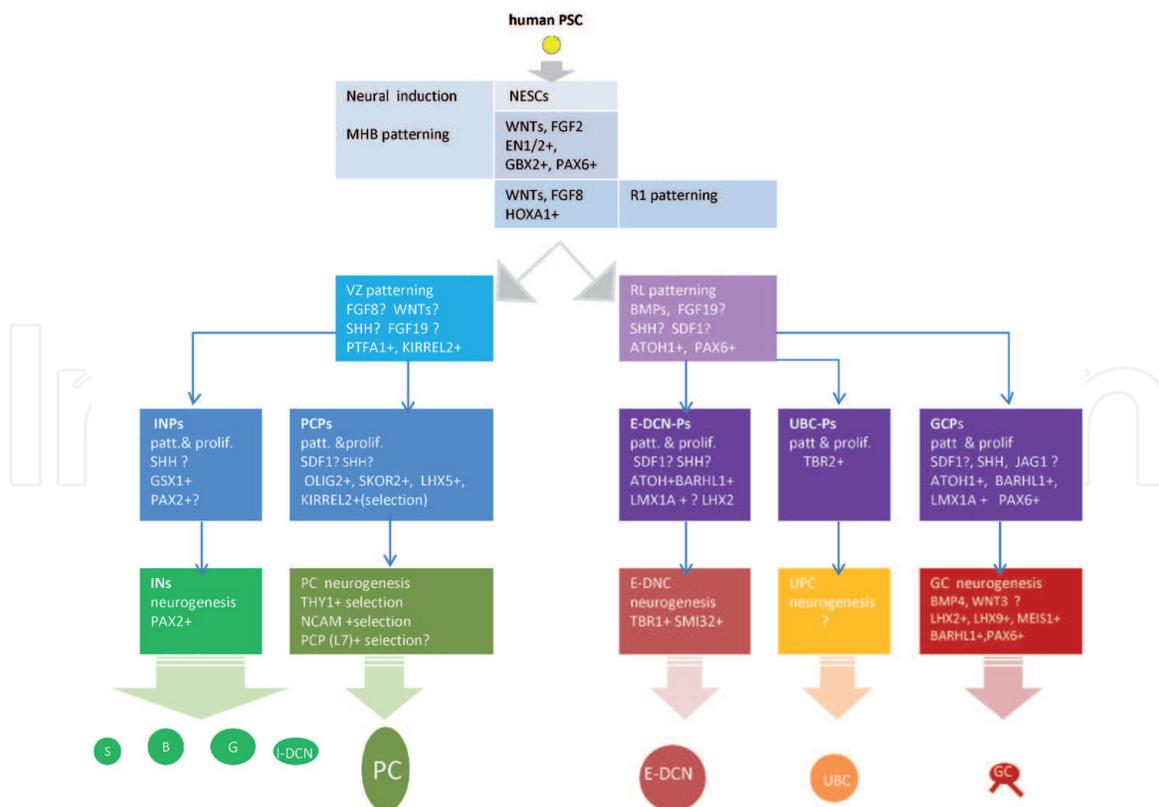


Figure 4.

In vitro trajectories from human induced pluripotent stem cells to cerebellar neurons by combining the differentiation protocols and the developmental principles. The differentiation conditions for some stages (meaning the combination of extrinsic factors, their concentration and time of action in the protocol) are previously established. However, for several steps, it remains to be established which treatments are necessary for patterning and proliferation of progenitor subpopulation in VZ and RL and in the secondary proliferation domains. Some factors which are known to act in the mouse cerebellar development could work also for the patterning and proliferation of human progenitor cells, but many question marks remain. These questions address both the treatments and the specific markers for subpopulation of progenitor cells and neurons.

dorsomorphin or LDN and SB431542) [56], significantly increased the yield of neural induction in human PSCs cultured in serum-free medium, both in 2D and 3D systems [40]. Shortly after neural induction, human PAX6⁺ neuroepithelial cells acquire a primitive anterior identity, expressing *OTX2*, but no more caudal markers, like *EN1*, *GBX2*, or *HOX* genes [56, 103]. However, this anterior phenotype is transient and, depending on the presence of added or endogenously secreted morphogens such as WNTs, FGFs, and RA, neuroepithelial cells take on a definitive regional identity [41, 104–106].

Some previous protocols used FGF2 for amplifying the neuroepithelial population and showed that, although an anterior phenotype is kept for a few passages in the presence of FGF2, longer exposure gradually patterns human progenitors toward midbrain and hindbrain fates [105, 107, 108]. FGF2 was used by Muguruma et al. [50] for inducing a brought midbrain-hindbrain patterning, including the IsO-like cells, in 3D spontaneously differentiating human PSCs in serum-free medium, for a time approximating the MHB formation in human embryos. However, the reproducibility of this protocol is limited and the efficiency of the neural induction and patterning was not investigated, many cells in the 3D clusters could present a more anterior phenotype (and maybe non-neural phenotypes). Watson et al. [52] proposed the parallel neural induction and hindbrain patterning by using FGF2 in combination with the SMAD inhibitor SB431542 for around 20 days. Even though it showed an increased expression in hindbrain and cerebellar markers, yet the efficiency and the selectivity of this approach was not reported.

The implementation of WNT signaling was shown to increase the midbrain and hindbrain patterning and reduce the spontaneous forebrain patterning in human PSC-derived neural cultures [28, 41, 54, 109, 110]. In Kirkeby et al. [41] and Kirkeby et al. [110], neural induction with dual-SMAD inhibition and patterning were applied in parallel for 9 days. The GSK3 inhibitor CHIR99021 was used at 1–2 μM concentration for patterning the anterior r1 fate. Following this protocol with some modifications, Sundberg et al. [54] applied the neural induction and hindbrain patterning by WNT in the same time, for 12 days, with noggin and 1.7 μM CHIR99021, while in a following study coming from the same group [28], neural induction and patterning with CHIR99021 1.5 μM was applied for only 4 days. In both studies, FGF8b (100 ng/ml) was added from day 4 to day 12 of differentiation, while FGF2 applied at day 10–12 in Sundberg et al. [54] was excluded in the next protocol [28]. However, the resulted cell populations in both studies were not directly phenotyped, but after 16 or 32 days of differentiation, when they contained KIRREL2+ or THY1+ cells, respectively, which were selected by FACS. Further optimization for neural induction and hindbrain patterning requires a deeper investigation, including negative markers for forebrain, midbrain, hindbrain (excepting the r1), and ventral markers (especially for the r1). The dorsal r1 cells should concomitantly and uniformly express GBX2 and EN1/2. Obviously, reporter lines for different genes expressed solely in r1, such as HOXA1, would be very useful tools.

In addition, a study using human hindbrain tissue from embryos at GW 5–7 showed that the hindbrain neuroepithelial cells were stably expandable in FGF2 and EGF conditions, but the short treatment with FGF8 and WNT (for 1 passage) hugely increased the expression of GBX2, EN1 and EN2 [111]. A deeper investigation of the human embryonic dorsal hindbrain tissue could provide hints for the optimization of the human PSC differentiation protocol toward cerebellar cells. The human embryonic hindbrain neuroepithelial cells can be further patterned *in vitro* by BMPs (BMP6, BMP7 and GDF7) and WNT3A to RL progenitors (ATOH1), which generated granule cells after transplantation into the rat cerebellum [111]. Some additional hints are revealed by the patterning of the human embryonic hindbrain tissue. ATOH1 was not expressed if FGF8 was added together with BMPs or if FGF2 and EGF were maintained, FGF signaling appearing to counteract the BMP stimulation [111]. The same factors were applied for the RL patterning from human PSCs (reviewed in [40]). It appears clear that ATOH is not expressed by default, but only after BMP signaling, in spontaneous or directed differentiation approaches. Again, developing human PSC reporter lines for *ATOH* and a deeper phenotypic investigation, including negative markers such the ones express in vicinity of the RL, (e.g. in pons, tectum and neural crest), would be of great help. The same approach is necessary for the optimization of cerebellar VZ progenitors, which are favored by FGFs and SHH treatments. It remains to be established which treatments with extrinsic factors (combination, concentration and time) are necessary for patterning and proliferation of progenitor subpopulation in VZ and RL, as well as out of them, in the secondary proliferation domain, as long-term proliferative populations (such as granule progenitor cells, interneuron progenitor cells and glial progenitor cells). Some factors known to act in the mouse cerebellar development could work also for human progenitor cells, but many question marks remain. These questions address both the treatments and the specific markers for subpopulation of progenitors and neurons (**Figure 4**).

Increasing maturation of the cerebellar neurons in defined conditions. One of the most consistent observations about human PSC-derived neurons is that they mature relatively slow and often incomplete (reviewed in [3]). An obvious reason is the time in culture: human PSC-derived Purkinje cells are usually kept in culture around

4 months, while they need over 2 years for maturation *in vivo*. An important challenge is the long-term culture and maturation of human PSC-derived cerebellar neurons without the presence of mouse cell/tissue co-cultures. Mature phenotypes of PSC-derived Purkinje cells and granule cells have so far only been demonstrated in co-culture or, more convincingly, by transplantation of differentiated cells into mouse cerebellum. While some of the *in vitro* and transplantation procedures demonstrated the potential of the PSC-derived neurons to mature into functional cerebellar neurons, they also highlighted the need to better understand the factors that promote their maturation. Significant variability in the efficiency to obtain functional Purkinje cells using different feeder cell sources was reported. For instance, feeder-free and co-culturing with rat granular progenitors failed to sustain Purkinje cell maturation and survival, while co-culture with rat cerebellar slices sustained Purkinje cells that nevertheless were devoided of any action potential or spontaneous post-synaptic currents. In contrast, co-culture with human fetal cerebellar slices resulted in electrophysiological active Purkinje cells [49], suggesting that human specific factors, as well as interactions with glial cells [112] are needed for proper maturation. The use of the co-culture system has limitations *per se*, feeder cells introducing inherent variability to the procedure [49]. A growing number of methods for reverse-engineering specific cellular micro-environments and the cells and molecules which constitute these [113] will definitely extend into the cerebellar field. It is likely that the combination of these technologies will help in elucidating key conditions for long-term survival and maturation of PSC-derived cerebellar neurons.

Another approach can come for the optimization of long-term cultures of cerebellar organoid, in line with the extensively investigated field of cerebral organoids [39]. As shown in different previous reports, functional synaptic connections are necessary for maturation and activity of the human PSC-derived neurons, which include glia and target neurons, all of these could be provided in the same cerebellar organoid.

Again, one limitation for most of the human PSC-derived neurons, as for the human neurons in general, is the lack of transcriptomic signatures, to rigorously identify specific types of neurons and to compare their development across species. A recent Metagene projection analysis of global gene expression patterns revealed that differentiating human PSC-derived Purkinje cells share classical and developmental gene expression signatures with developing mouse Purkinje cells. Remarkably, it revealed that the human PSC-derived Purkinje cells matured in co-culture for around two months are closest to late juvenile (p21) mouse Purkinje cells, suggesting that they are relatively mature. Gene expression profiling also identified human-specific genes in human PSC-derived Purkinje cells. Protein expression for one of these human-specific genes CD40LG, a tumor necrosis factor superfamily member, was confirmed in native human cerebellar tissue, arguing for the bona-fide nature of the human PSC-derived cerebellar neurons [28]. Obviously, the routine applications of the single-cell transcriptomics into the optimization steps of the human PSC-derived cerebellar differentiation protocols will hugely contribute to the progress in the field.

4. iPSC-derived models for cerebellar ataxias

The iPSC technology together with the cerebellar differentiation protocols offer the opportunity to indirectly generate and to directly study the most affected cells in patients with cerebellar ataxias, the cerebellar neurons. As schematically presented in **Figure 5**, somatic cells such as skin fibroblasts or white blood cells obtained from patients are reprogrammed into iPSCs, which can be theoretically differentiated

into any type of neurons. Ideally, the neuronal differentiation should address the most affected subpopulation in each disease, by following the existing protocols or optimized protocols in the desired direction (using development principles and combining efficient selection methods). Remarkably, for the inherited ataxias, the patient iPSC-derived neurons express the disease mutation in the authentic genetic background and cellular environment, which is not the case in the animal models.

The neuropathological events in hereditary cerebellar ataxias affect both cerebellar and extracerebellar territories. Nevertheless, degeneration and ultimate loss of cerebellar neurons is a neuropathological hallmark in cerebellar ataxias. The affected cerebellar neurons and the responsible genes for several cerebellar ataxias are presented in **Table 2**. Spinocerebellar ataxias (SCAs) are a family of over 40 currently described late-onset dominant diseases, manifesting clinically at middle age and gradually progressing with neurodegeneration in cerebellum and other CNS areas, [136–139] while in other genetic ataxias, such as the autosomal recessive Friedreich ataxia (FRDA) and ataxia-telangiectasia (AT), the disease manifests a lot earlier and, in addition to the nervous system, extraneural territories are affected [137, 138]. FRDA is considered a multi-systemic condition, including central and peripheral neuropathies, diabetes and cardiomyopathy [140, 141].

In cerebellum, SCA1, SCA3 and FRDA involve mainly the DCN, especially the dentate nucleus, but also extracerebellar territories such as the Clarke's column, which present with severe neuronal loss (reviewed in [142]). SCA2 predominantly affects the pontine nuclei, while the Purkinje cells and DCN seem to be secondarily affected. SCA31 is relatively restricted to the Purkinje cells. Although Purkinje cells are predominantly involved in SCA6, degeneration is evident also in the dentate nucleus and granule cells. Therefore, patients with SCA6 show more severe ataxia than those with SCA31. Several SCA subtypes have CAG repeat expansions in the coding region of different genes (<http://www.scabase.eu/>; [143–146]), resulting in PolyQ elongations in the respective proteins, the elongation size being correlated with the intensity of clinical manifestations. In other SCAs (SCA12, SCA31 and SCA36) or non-SCA monogenic ataxias, such as FRDA, the repeat expansion is intronic, but also in these diseases the cerebellar dysfunction is correlated with the elongation size [147].

Modeling these human genetic disorders in mice has reproduced to a certain extent the neuropathological aspects and has provided some insights into disease mechanisms. Many disease mechanisms that have been explored in mouse models are expected to be recapitulated in patient iPSC-derived neurons. However, some ataxias could not be modeled in mice using the same mutation as in the patients, suggesting that the human-specific environment is essential for the disease to

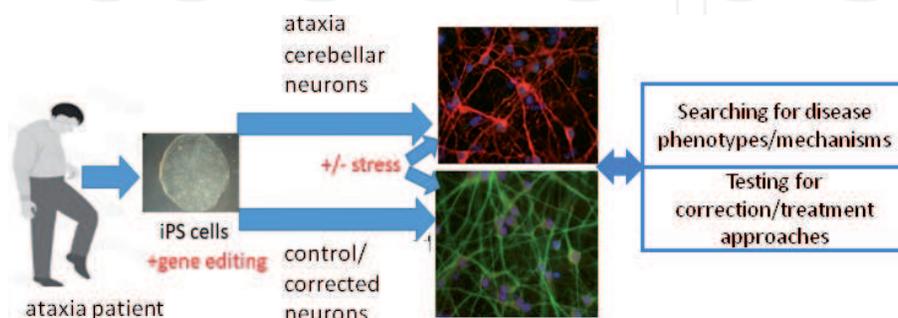


Figure 5.

From ataxia patients to neuronal disease models. Somatic cells from patients with cerebellar ataxias are reprogrammed into induced pluripotent stem (iPS) cells, which can be genetically modified in order to correct the mutation. Patient and control/corrected iPS cells can be differentiated into neurons that are relevant for the cerebellar diseases, such as Purkinje cells. Additional stress or forced aging can be equally applied to the patient and control/corrected neurons or their progenitors, in order to amplify the phenotypic differences resulted from the ataxia's specific mutation.

Ataxia Type	Affected cerebellar neurons	Gene, mutation&location	Affected protein	Human iPSC-derived neurons	Human iPSC-derived models References
SCA1	PCs ⁺⁺ , DCN ⁺⁺	<i>ATXN1</i> , (CAG) ₃₉₋₈₃ exon 8	Ataxin-1	—	[114, 115]
SCA2	PCs ⁺⁺⁺ , DCN ⁺⁺⁺	<i>ATXN2</i> , (CAG) ₃₅₋₇₉ exon1	Ataxin-2	CNS	[116, 117]
SCA3	PCs ⁺ , DCN ⁺⁺⁺	<i>ATXN3</i> , (CAG) ₄₅₋₈₇ exon8	Ataxin-3	CNS	[117-122]
SCA6	PCs ⁺⁺⁺ , GCs ⁺ , DCN ⁺⁺	<i>CACNA1</i> , (CAG) ₂₀₋₃₃ exon 47	α1A & α1ACT	cerebellar	[51, 107]
SCA7	PCs ⁺⁺⁺ , DCN ⁺⁺⁺	<i>ATXN7</i> , (CAG) ₃₈₋₁₅₀ exon3	Ataxin-7	CNS	[123, 124]
SCA12	PCs ⁺⁺⁺ , DCN ⁺⁺⁺	<i>PPP2R2B</i> , (CAG) ₅₅₋₇₈ intron	PP2R2B	—	[125]
SCA17	PCs ⁺⁺⁺ , DCN ⁺⁺⁺	<i>TBP</i> , (CAG) ₄₇₋₆₃ exon3	TBP	—	—
SCA31	PCs ⁺⁺⁺	<i>BEAN1</i> , (TGGAA) _n , (TAGAA) _n , (TAAAA) _n , or (TAAAATAGAA) _n intron	—	—	—
SCA36	PCs ⁺⁺⁺ , DCN ⁺⁺⁺	<i>NOP56</i> (GGCCTG) ₁₅₀₀₋₂₀₀₀ intron	—	CNS	[126]
SCA42	PCs ⁺⁺⁺ , DCN ⁺⁺⁺	<i>CACNA1G</i> , exon 5144G > A; R1715H missense	Cav3.1	cerebellar	[127]
FRDA	PCs ⁺ , DCN ⁺⁺⁺	<i>FXN</i> , (GAA) _n , intron 1	Frataxin	PNS, CNS	[128-134]
A-T	PCs ⁺⁺⁺ , GCs ⁺⁺⁺	<i>ATM</i> , exon, truncating, splicing or missense mutation	ATM	cerebellar	[135]

PCs-Purkinje cells, GCs-granule cells, DCN-deep cerebellar nuclei, +++high ++ medium, +low.

Table 2.
Affected cerebellar neurons and iPSC-derived models for different ataxias.

develop. Additional mechanistic understanding of the network of events produced by the mutation is crucial for the development of effective therapies, as none of the cerebellar ataxias is yet curable, treatable or preventable [143, 145, 147-149].

For modeling cerebellar ataxias, the iPSC-based models present three main advantages. First, most of cerebellar ataxias are monogenic diseases. Second, neurons bearing the mutation, which are not directly available from patients, can be generated *in vitro* from the patient iPSCs. Third, the human neurons generated *in vitro* seem to acquire a molecular profile close to the postnatal age in mouse, as in the previously mentioned Metagene analysis of key gene pathways, which showed that the human Purkinje cells generated *in vitro* have the closest molecular expression with the Purkinje cells in p21 mouse cerebellum [28]. As for many mouse models for cerebellar ataxias a disease phenotype was found close to this age, the *in vitro* generated human neurons are expected to behave similarly and to reveal the disease phenotype in early stage of maturation.

However, as presented in **Table 2**, relatively few studies have succeeded in generating iPSC-based models for cerebellar ataxias. An additional important question for the iPSC-based models is to what extent the mutated gene is expressed in the neurons generated *in vitro*. The most vulnerable and affected cells are neuronal subpopulations, most of them being located in cerebellum. From the reported iPSC-derived models, only a very few implemented the cerebellar differentiation protocols, including the pathways for generating the specific cerebellar cells affected in disease.

A handful of studies published to date addressed iPSC models of PolyQ SCAs (such as SCA1, 2, 3, 6, 7 and 12), non-PolyQ SCAs (such as SCA36 and 42), and other ataxias (such as FRDA and A-T). Most of the iPSC-based models used a generic differentiation towards the neural lineage, as opposed to the generation of specific neuronal subtypes, and very few characterized the neuronal phenotypes. The only reported iPSC-derived models addressing the cerebellar neurons were for SCA6 [51], SCA42 [127] and A-T [135].

For SCA1 and SCA12, only the generation of patient-derived iPSCs were until now reported [114, 115, 119, 125]. Several other SCA models have already addressed the neural phenotypes. SCA2 was modeled by Xia et al. [116] and by Chuang et al. [117] using patient iPSC-derived neural progenitors and central neurons. No cerebellar protocol has yet addressed SCA2, in which both Purkinje cells PCs and DCN neurons are affected. Whereas patient and control fibroblasts showed comparable levels of expression of the disease-causing protein Ataxin-2, its expression was decreased in patient iPSC-derived neural stem cells, which survived shorter in cell culture. Chuang et al. [117] reported that SCA2 neurons exhibited a glutamate-dependent disease phenotype, which are suppressed by anti-glutamate drugs and a calcium stabilizer treatment.

One of the first studies using the generation of neurons from patient iPSCs addressed to SCA3, also called Machado-Joseph disease (MJD) [118]. In this model, neuronal excitation by glutamate promoted an increase in intracellular calcium concentration and proteolysis of Ataxin-3, triggering its aggregation—a hallmark of the disease in patients. This intraneuronal aggregation, (which was also found to depend on sodium and potassium channel function, as well as on ionotropic and voltage-gated calcium channel function), was abolished by calpain inhibition, pointing to a key role of this protease in Ataxin-3 cleavage. Furthermore, intracellular aggregations were not observed in patient iPSCs, fibroblasts or iPSC-derived glial cells, providing a clue for the neuron-specific phenotype observed in SCA3 patients. Hansen et al. [120] differentiated the SCA3 patient-derived iPSCs further into hindbrain neurons that expressed *GBX2* and *HOXA2*. They reported that glutamate loading or calcium increase by ionomycin did not induce Ataxin-3 accumulation in these hindbrain neurons. It remains to be investigated whether this discrepancy comes from a difference in cell types or in the applied protocols. In another study [121], SCA3 iPSCs differentiated into NeuN-positive (postmitotic) neurons showed accumulation of Ataxin-3 in the absence of stress. The activation of autophagy by rapamycin was effective for degradation of Ataxin-3, suggesting that autophagy could be a key for development of therapeutic treatments. Chuang et al. [117] reported that SCA3 iPSC-derived neurons again showed glutamate-dependent phenotypes, which were suppressed by anti-glutamate drugs. Ouyang et al. [122] applied gene editing techniques for the deletion of the expanded CAG in the *ATXN3* gene in SCA3 patient-derived iPSCs, which were further characterized. Such corrected iPSCs will be useful for SCA3 isogenic models. However, no further studies have addressed SCA3 iPSC-derived cerebellar neurons and a directed protocol for the DCN neurons, the most affected in SCA3, is not yet available.

SCA6 is a very interesting case, first, by being one of the three diseases in which patient iPSC-derived cerebellar neurons were generated to date, and second, because of the bicistronic nature of the affected gene, *CACNA1A*. It encodes the $\alpha 1A$ subunit of P/Q-type voltage-dependent calcium channel Cav2.1, and the $\alpha 1ACT$, with an identical sequence with the PolyQ bearing C-terminal segment of the longest isoform of $\alpha 1A$ [150]. In addition, the gene is expressed mainly in neurons, contrary to the other ataxia-related genes, that are ubiquitously expressed. Utilizing the differentiation method for the cerebellar neurons [50], Ishida et al. [51] differentiated Purkinje cells from iPSCs derived from hetero- and homozygous SCA6 patients [51]. They found that SCA6-derived Purkinje cells exhibit decreased expression of $\alpha 1ACT$ and its target molecules, TAF1 and BTG1. They further constructed a disease model in which SCA6 patient-derived Purkinje cells specifically degenerate by depletion of the thyroid hormone triiodothyronine (T3), which is necessary in late stages of maturation. Bavassano et al. [107] differentiated SCA6 patient-derived iPSCs into neurons expressing Cav2.1 and $\alpha 1ACT$, using the same differentiation and stress model as for the SCA3 [118]. The glutamate loading decreased the viability of SCA6 neurons, pointing toward a common pathway of stress response in PolyQ SCAs. In addition, SCA6 neurons showed differences in the expression of several genes previously reported to depend on the transcriptional regulation by the $\alpha 1ACT$, and showed no differences in the electric response of the Cav2.1 channel. Recent high-throughput investigations in the mouse and human cerebellum revealed complex functions of $\alpha 1ACT$ [26] and further studies are expected to clarify the role of the mutated $\alpha 1ACT$ in cerebellar neurons, especially in Purkinje cells.

For SCA7, in which cerebellar and retinal cells are degenerated [151], Luo et al. [123] reported the generation of iPSCs and neurons from a SCA7 patient, but did not characterize the neuronal phenotype and the disease phenotype. Ward et al. [124] generated SCA7 patient-derived iPSCs and their isogenic lines transduced with either normal or expanded ATXN7. They reported that SCA7 iPSC-derived neural progenitors exhibit altered metabolism and mitochondrial dysfunction.

SCA36 and SCA42 are non PolyQ autosomal dominant diseases, affecting the cerebellar neurons and other neurons. Matsuzono et al. [126] generated motor neurons from the patient-derived iPSCs and recapitulated an increase in RNA foci-positive cells that can be markedly suppressed by treatment of antisense oligonucleotide. SCA42 is caused by a mutation in *CACNA1G*, which encodes T-type voltage-dependent calcium channel Cav3.1 [127]. In addition to identifying the affected gene, [127] reported a model disease for which patient-derived iPSCs were differentiated into Purkinje cells. The SCA42-derived Purkinje cells would provide a useful tool for further phenotype analysis of the mutated CAV3.1, for which the investigation was till now limited to the HEK293 cell line.

For the FRDA, a pioneering work revealed that abnormal expansion of GAA repeats led to upregulation of the DNA mismatch repair protein MSH2 in FRDA patient-derived iPSCs [130]. They reported that the functional inhibition of *MSH2* by shRNA suppresses the repeat expansion. They further reported an inhibitor of histone deacetylase HDACi 109 increased the expression of *FXN* gene and Frataxin protein, pointing to the involvement of histone H3 lysine 9 in *FXN* expression. Polak et al. [131] also focused on epigenetic modifications in FRDA-derived iPSCs and performed drug evaluations. They found that an inhibitor of lysine-specific demethylation enzyme 1 (called Parnate or Tranylcypromine), and the HDAC inhibitor sodium butyrate have transient effects on decreasing the repeats and increasing *FXN* gene expression. Bird et al. [132] also reported a decrease in Frataxin expression in neurons differentiated from FRDA iPSCs, but could not

detect abnormality in mitochondrial functions. Hick et al. [133] reported decreased expression of *FXN* and Frataxin, a decrease in mitochondrial membrane potential and degeneration of mitochondria in FRDA iPSC-derived neurons. Eigentler et al. [128] showed a cell-specific decrease of frataxin in disease-vulnerable FRDA iPSC-derived peripheral neurons. Lai et al. [129] and Mazzara et al. [134] generated FRDA isogenic lines. Mazzara et al. [134] demonstrated that the entire intron 1 removal, and not solely the elongation, was necessary for the recovery of the *FXN* expression level in peripheral sensory neurons. Although several studies have provided insights into the pathogenesis of FRDA in cardiomyocytes and peripheral neurons, additional work is required to elucidate the role of Frataxin in other affected cell types, such as the neurons of the DCN.

For the A-T is caused by several mutations in the *ATM* gene [152], Nayler et al. [135] differentiated A-T patient-derived iPSCs into cerebellar neurons and performed RNA sequencing analysis with them. Remarkably, they found that the generated neurons acquired properties of the cerebellum at GW 22 and exhibited disrupted gene regulatory networks related to synaptic vesicle dynamics and oxidative stress.

5. Strategies for optimizing the neuronal models of cerebellar ataxias

Of particular interest in future research in the cerebellar ataxias is the comparison between affected and unaffected neuronal types, in order to identify particular characteristics that render specific neuronal populations vulnerable to a genetic insult which is ubiquitously presented. One of the most crucial needs is to establish a reliable and consistent disease phenotype in a relevant cell population, and those cell types to be generated in relatively large quantities *in vitro* [153].

Differentiation into specific and mature neurons that are the disease targets, such as Purkinje cells for several SCAs, or solely DCN neurons for some ataxias, or both of them for the most of SCAs (**Table 2**), will enable the construction of more reliable disease models [154]. However, the suitability of iPSC-derived neurons for modeling late-onset conditions remains controversial, particularly given the immature, fetal-like phenotypes of the neurons generated from these cells.

Remarkably, in contrast to the immature morphology observed for human PSC-derived Purkinje cells, a recent bioinformatics analysis of their gene expression and developing showed that they most closely resembled late juvenile p21 mouse expression mouse Purkinje cells, when most of the cerebellar disease phenotypes in several animal models start to manifest. This finding suggests that the Purkinje cells are among the most mature human PSC-derived central neurons analyzed to date. This approach also underscores the utility of transcriptomic analysis for analyzing the maturation of human PSC-derived neurons and validates the use of hPSC-neurons for modeling cerebellar ataxias.

Still, it is possible that the disease phenotypes of adult-onset conditions, as the most of genetic SCAs are, may never be fully recapitulated under 2D cell culture conditions, even with directed protocols and optimized maturation. Generation of 3D cerebellar-like tissues as the cerebellar organoids may allow to increasing the neuronal maturation *in vitro*. The next generation or organoids or “assembloids”, which will allow the proper combination of different cell types, including vascularization, can offer a good perspective but also limitations by increased heterogeneity. The multiomics approaches at single-cell level can definitely contribute to understand and quantify this heterogeneity and in the same time decipher the cell-type related disease phenotype.

Another way to model the late-onset diseases is the addition of neural stressors, such as reactive oxygen species, pro-inflammatory factors, and toxins or forced aging, as schematically presented in **Figure 5**. These approaches were already used for modeling several SCAs or other neurologic diseases [153, 155–157]. However, in an ideal situation, these stressors should only exacerbate the disease phenotype, which can be evident in a good model solely by the expression of the mutation in the disease-relevant cells. Another approach is to genetically manipulate the system for forcing the aging, such as by overexpression of progerin in neural progenitors. By this approach, the disease phenotype is expected to manifest *in vitro* in earlier stages of neuronal maturation [155, 156] (reviewed in [158]).

On the other side, recent evidence from cell and animal models indicates that abnormalities in early Purkinje cell development may contribute to the pathogenesis of the ataxias. Purkinje cell developmental abnormalities are clearly evident in a wide range of ataxic mouse mutants, including models of the degenerative SCAs [26]. The observed Purkinje cell developmental defects commonly include impaired dendritic arborization, resulting in synaptic deficits affecting CF and PF connections and ultimately altering Purkinje cell physiology. Similar impairments in Purkinje cell dendritogenesis and synapse formation have been described in mouse models of SCA5, and in cell and mouse models of SCA14, SCA1, SCA3 and SCA5. Given the increasing evidence for Purkinje cell developmental abnormalities in cerebellar ataxias, it seems likely that iPSC-derived models, which are capable of recapitulating early developmental events *in vitro*, will be invaluable in unraveling the pathogenic complexities of these conditions. It will be important to better understand the underlying—likely common—molecular mechanisms, by which mutations in distinct genes cause abnormal Purkinje cell development and function [159]. These could offer attractive future therapeutic targets to alleviate motor dysfunction in cerebellar ataxia.

Another limitation in the field of modeling cerebellar ataxias is that most of the studies implemented the production of iPSCs from a few patients. On one hand, addressing to larger patient cohorts may allow to identifying more accurate phenotypes. On the other hand, for investigating the pathological function of a mutation, the ideal situation is to compare the cells bearing the mutation with control cells with an identical genetic background. The rapid development of CRISPR/Cas9-mediated genome editing is likely to result in significant advances in the field, allowing the correction of disease-causing mutations into iPSCs, which can then be used to create paired isogenic lines to produce better disease models in which far less patient-derived cell lines will be necessary [160]. This was already performed even for the ‘difficult to correct’ elongations, like in SCA3, SCA7, it is expected in the near future to constitute ‘the norm’ for all iPSC-derived disease models.

The establishment of efficient, reproducible cellular models of cerebellar dysfunction and degeneration will be important not only in elucidating the molecular basis of these diseases, but also in the development of effective therapies. Establishment of special cell cultures, such as Purkinje cells from patients with cerebellar ataxia, provides opportunities to screen for drugs that may correct the observed disease phenotypes. These cell cultures can be combined with stressors capable of eliciting phenotypes in late-onset conditions and genotypic modifiers of disease progression and drug response. In addition, these cerebellar cell cultures may be used for toxicity screens, to assess the effects of novel compounds on relevant cell types, or for differentiation screens, to identify compounds capable of enhancing self-renewal, maturation or survival of specific cerebellar cells (**Figure 5**).

6. Final remarks

Recent technologies for producing iPSCs from patients combined with the differentiation of PSCs into neural cells and the self-organizing 3D neural tissues have provided a new way to experimentally investigate the developmental and disease mechanisms of the human brain. While several challenges have hindered the generation of cerebellar neurons *in vitro*, starting from human PSCs, some important steps have been made. These protocols, combined with the patient-derived iPSCs, have been further applied for the investigation of several cerebellar diseases. In addition to the “classical” protocols aimed to generate specific types of neurons in two-dimensional (2D) cell cultures, recent progress has been made in culturing cells in three-dimensional (3D) structures, which may better reproduce the tissue organization and complexity *in vivo*, such as the PSC-derived brain organoids. Despite promising results, a number of issues remain to be addressed before the iPSC-based models to be widely adopted. Generation of the disease-relevant cerebellar cells and tissue *in vitro* remains a challenge, requiring a precise understanding of the complex molecular events during the development of each neuronal subtype, and an accurate set of markers by which to identify and characterize the generated cells. The 3D brain models in general and the 3D cerebellar models in particular still wait for improvements, including a better cellular characterization and an increased reliability, in order to contribute to better disease models.

However, human PSC-based models offer distinct advantages for the study of cerebellar ataxias. Cerebellar neuronal models are likely to provide valuable insights into the selective vulnerability of distinct neuronal subtypes, particularly the Purkinje cells. More directed and/or complex approaches will allow for the generation of accurate, disease-relevant models for the study of the molecular mechanisms underlying cerebellar ataxias, and the development of the long-awaited therapies.

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