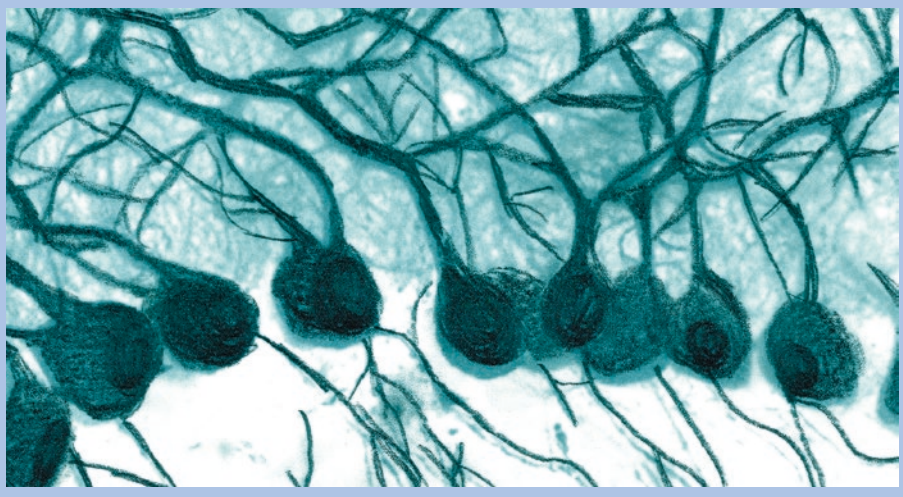
Reproducing Human Brain Development In Vitro: Generating Cerebellar Neurons for Modelling Cerebellar Ataxias

Evguenia Bekman, Teresa P. Silva, João P. Cotovio, and Rita Mendes de Almeida



G. Rodrigues, B. A. J. Roelen (eds.), *Concepts and Applications of Stem Cell Biology*, Learning Materials in Biosciences, https://doi.org/10.1007/978-3-030-43939-2_11

Contents

What You Will Learn in This Chapter

The main purpose of this chapter is to introduce the reader to the complexity of nervous system development, to better understand the huge challenge to faithfully reproduce this system in vitro for the purpose of neurodevelopmental and neurodegenerative disease modelling. The emphasis in this chapter is on the aspects of embryonic development that are needed to be mimicked in vitro to generate functionally validated neuronal cells. As an example of the latter, a case study of the generation of functionally mature cerebellar neurons from human-induced pluripotent stem cells (hiPSCs) is given.

A brief outline of crucial steps in laying out of the central nervous system (CNS) is a compilation of up-to-date knowledge based on experimental data collected mainly from animal studies, due to difficulty of studying the development of the human brain. Though the same main principles govern neural development in higher vertebrates, some important differences between murine and human cerebellar commitment have been reported. For the sake of clarity, we do not discuss these differences and focus entirely on the human cerebellar model. After reading this chapter, the reader will have acquired information on the most important features of neural development for successful in vitro modelling.

11.1 Human Brain Development

Have you ever thought that there is a galaxy of neurons inside your skull? If we compare the number of stars in the Milky Way, estimated between 100 and 400 billion ($2.5 \times 10^{11} \pm 1.5 \times 10^{11}$), with the number of neurons just in the human cerebellum (101 billion) plus some 20–25 billion neurons in the neocortex, and even without other brain areas and glial cells (which in some brain areas outnumber neurons tenfold), we will realise that we have a galaxy of neurons inside our brain.

And then we can add some extra complexity to this, with 164,000 billion synapses (~7000 synapses/neuron), ~12,000,000 km of dendrites and ~100,000 km of axons…

Thinking of these numbers the following question arises: how is this complexity generated? And maintained? And of course, how is it translated into our thoughts, memories, feelings, communication, imagination? We still do not know the answers for all these questions, but now we are able to outline, with ever-growing precision in detail, the process of the building of the brain structure. So, how does it start?

Everything starts with the egg, a wonder of nature and the origin of all the cells in the body including those that produce other eggs, upon fertilisation. The successive divisions of the fertilised egg sequentially give rise to the morula, the blastula and the primitive epiblast undergoing gastrulation to originate the three definitive germ layers, ecto-, meso- and endoderm. The formation of the ectoderm is the essential initial step in laying out the neural tissue that arises from its dorsal (axial) part. Dorsal ectoderm acquires neural identity in response to a signal from the underlying mesoderm in the process called neural induction. The inductive signal consists in the concerted action of bone morphogenetic protein (BMP) antagonists, fibroblast growth factors (FGFs) and wingless proteins (Wnts) that together efficiently inhibit ongoing transforming growth factor beta (TGFβ) signalling and induce the switch from non-neural to neural ectoderm identity. The immediate consequences induced by this switch are the onset of the expression of neural identity genes, such as *SOX1* [1], and the replacement of E-Cadherin by N-Cadherin on the cell surface. N-Cadherin local-

ises at the subapical membrane domain, where its subcellular domain contacts with the PAR3 complex to recruit β-catenin and rearranging local actin cytoskeleton [2]. As a result, elongation and apical constriction of epithelial cells occur, producing a thickened neuroepithelial sheet, called a neural plate that, due to much reduced apical surface area, starts to bend along the anteroposterior axis and forms the neural tube (Fig. 11.1). The bending and closing of the neural tube occur more easily and faster in its middle part (future spinal cord), while it is less thick than the anterior part which will give rise to brain vesicles and takes more time to close. The most posterior part of the neural tube is the last to close, and the neurogenesis here occurs in a different manner than in the rest of the neural tube and is, therefore, called secondary neurogenesis (reviewed in [3]), to distinguish from the primary neurogenesis that generates the more anterior nervous system. At the end of neurulation, three different regions are formed: neural ectoderm that originates the central nervous system (CNS); non-neural ectoderm, which will form the epidermis; and a region between neural and non-neural ectoderm, which will give rise to the neural crest [4, 5].

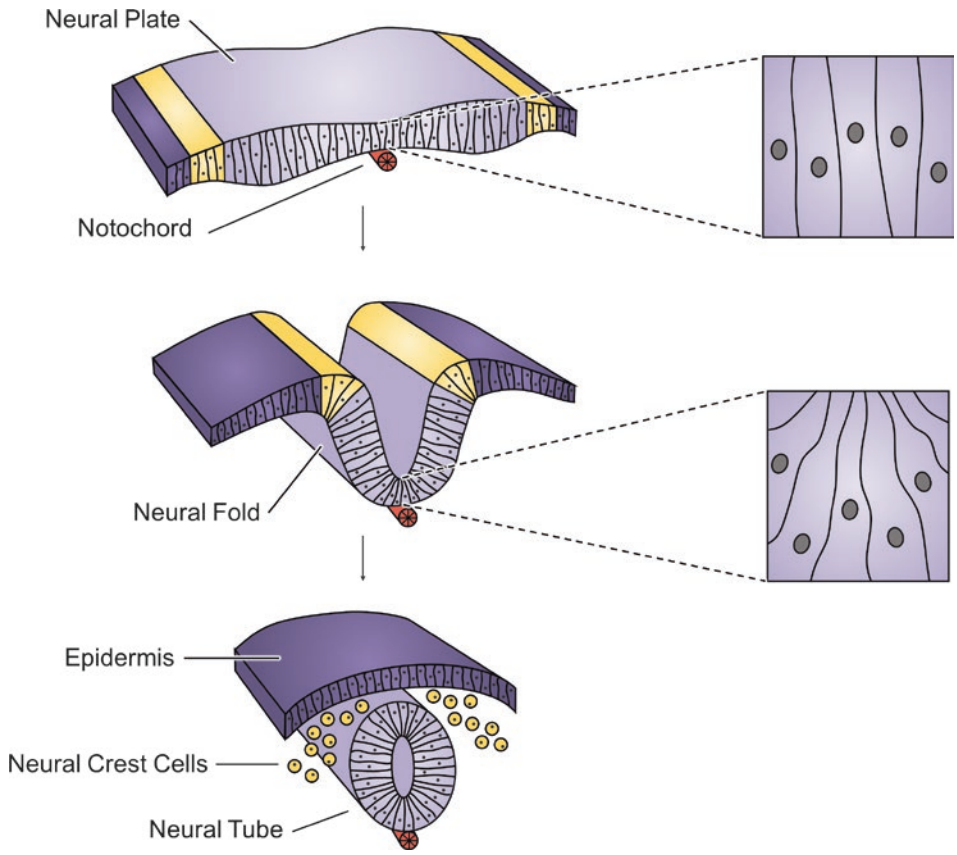


Fig. 11.1 Schematic view of vertebrate neural tube formation. Neural plate is formed by elongation of epithelial cells upon inductive signal from underlying mesoderm (notochord). Apical constriction of neuroepithelial cells facilitates the bending of neural plate and subsequent neural tube formation. As a result, three different regions are formed: neural ectoderm that originates the CNS, non-neural ectoderm (epidermis) and neural crest forming in between neural and non-neural ectoderm

11.1.1 How Billions of Neurons Are Generated

The closed neural tube is built up of a pseudostratified epithelium composed by fusiform progenitors, each of them trespassing the neuroepithelium from its apical (inner, or luminal) side to basal (outer) side (Fig. 11.2). Unlike cell nuclei of other epithelia, the nuclei of neuroepithelial cells do not lie at the same level; in fact, they are dispersed along the apico-basal axis, being in constant movement from apical side to basal and back. In this clever way, the neuroepithelium can fit a great number of progenitors in a very compact space. One of the amazing features of this peculiar organisation is that the movement of nuclei, called interkinetic nuclei migration, INM, is coupled to cell cycle, so that mitoses always occur at the apical side and S phase takes place at the basal side ([6]; reviewed in [7]). From the very beginning, and until the late embryonic stages, the neural tube is the place of intense proliferation and differentiation. For example, during the first half of pregnancy the rate of production of newborn neurons is over 200,000 neurons per minute. To keep up with such a high rate of neuronal production, neuroepithelium needs to have an efficient mechanism of the maintenance of progenitor pool. This is assured by Notch signalling, where a newborn neuron starts to express Notch ligand, Delta, that binds its receptor Notch on the surface of adjacent progenitor cell and this binding exposes the cleavage site releasing the intracellular domain of Notch (NICD) [8]. This domain goes to the nucleus where it forms a complex with CBF1 to activate target genes that will maintain the progenitor state of the cell and inhibit proneural genes necessary for the exit to differentiation (Fig. 11.3). Thus, a newborn neuron signals to neighbouring cells to prevent them from exiting for differentiation at the same time, preserving the progenitors for differentiation at later stages [9]. This mechanism of cell–cell interaction is called lateral inhibition and is widely used during development whenever a binary decision between two cell fates must be made. The importance of lateral inhibition in neural development is demonstrated by studies of Notch path-

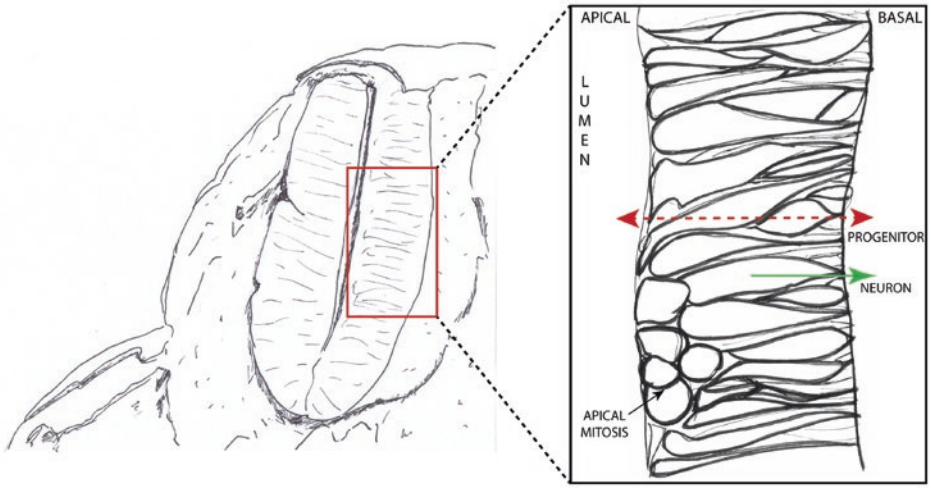


Fig. 11.2 The structure of neuroepithelium. Closed neural tube takes form of a pseudostratified epithelium with basal (outer) side and apical (inner, or luminal) side where mitoses occur. While progenitor cells stretch from apical to basal surface, neurons lose apical endfeet and accumulate at the basal side

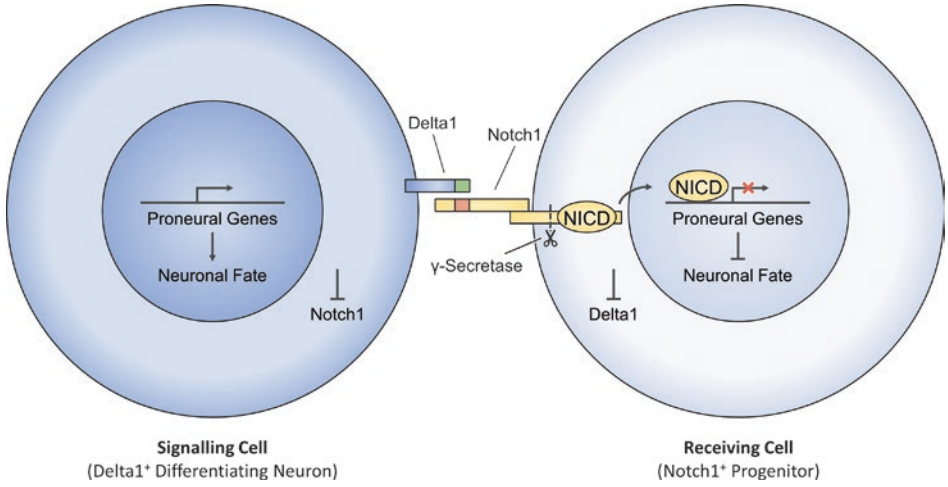


Fig. 11.3 Notch signalling pathway. Newborn neuron expresses Notch ligand, Delta1, which binds its receptor Notch on the surface of adjacent progenitor cell. Cleavage releases the intracellular domain of Notch, NICD, which goes to the nucleus and inhibits proneural genes necessary for the exit to differentiation. As a result of this inhibition, cells adjacent to the newborn neuron remain as progenitors until the next cell cycle, in which, due to interkinetic nuclear migration and to the movement of neurons towards mantle layer, a new combination of neighbouring cells is generated and another cell is singled out for differentiation and lateral inhibition exerted on surrounding progenitors

way mutants, where excessive signalling results in overproliferation of neural progenitors and dramatic decrease in neuronal production, while the lack of signalling causes massive premature neuronal differentiation and reduction of neural tube thickness due to exhaustion of progenitor pool [8, 9].

With the progress of neurogenesis in the neural tube its thickness increases, so the later stage progenitors, called radial glia, are obliged to stretch out and form a very long basal endfeet that plays an important role in guiding neuronal migration [10]. Radial glia is thought to divide asymmetrically, each division giving rise to another radial glial cell and a transit-amplifying cell that will divide several times to produce neurons [10]. With time, the neural tube subdivides into the apical ventricular zone, where progenitors persist, and basal mantle layer, where differentiating neurons accumulate.

The maintenance of the progenitor pool throughout embryonic development is one of the key mechanisms underlying the diversity of neural cell types in the CNS. Once the constant supply of progenitors is assured, the diversity is generated by conjugation of spatial and temporal cues combined with gradual changes in progenitor competence, as will be discussed below.

11.1.2 How Is Neuronal Diversity Obtained?

The great diversity of neuronal subtypes is obtained by a combination of intrinsic and extrinsic cues that together will determine which type of neuron will be born at a specific time and place, meaning that both positional and temporal cues are in charge of this process.

The most important and probably the earliest positional cue is the location of the progenitor along the rostro-caudal (R-C) axis. The regional patterning of neural progenitor cells starts with the most rostral identity, the "primitive identity" [11–14]. While the forebrain territory is specified in the absence of all major signalling molecules, more caudal fates require the action of some morphogens (see below), including retinoic acid (RA), WNT and FGF [15–17]. For the midbrain/hindbrain identity, FGF signalling is essential, while RA confers spinal cord identity [18]. As a result of the concerted action of different morphogens, four major regions are created along the R-C axis of the neural tube: forebrain (prosencephalon), midbrain (mesencephalon), hindbrain (rhombencephalon) and spinal cord (Fig. 11.4). Within these four regions, the same morphogen gradients induce overlapping expression of Hox homeodomain proteins that generate a segmented pattern of positional identities determining neuronal fates [19]. *Positional cues along the R-C axis define the functional specificity of neural cells with respect to different body segments.*

In addition to the R-C axis, dorsal–ventral (D-V) direction is determined by different concentrations of morphogens provided by different organising centres. Morphogens are diffusible molecules that are able to establish a graded concentration distribution to elicit distinct cellular responses in a dose-dependent manner.

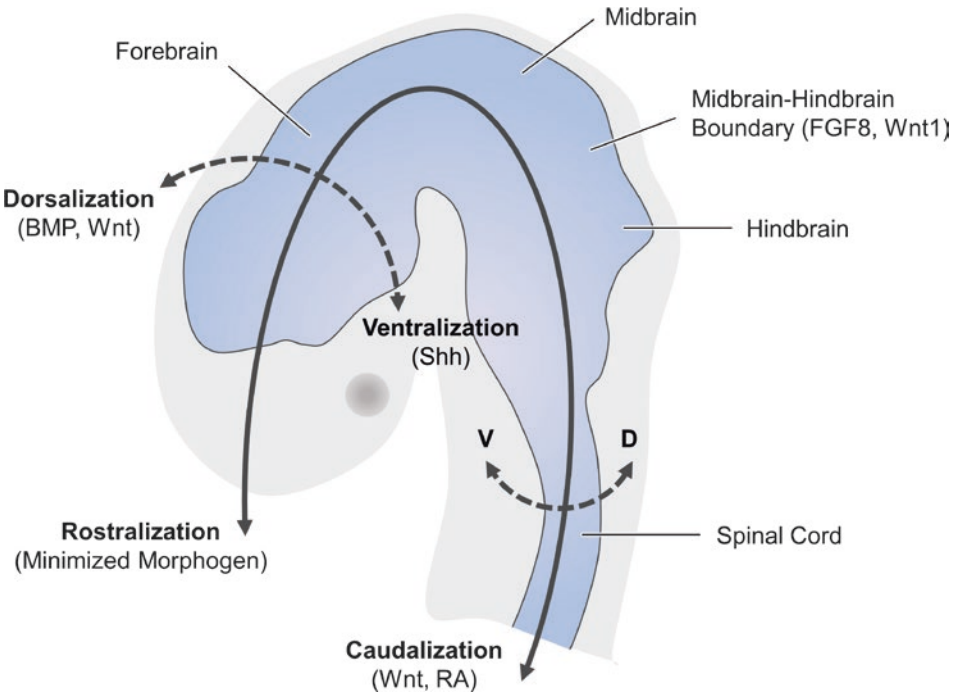


Fig. 11.4 Regional patterning of neural tube. Rostral–caudal (R-C) and dorsal–ventral (D-V) axis are determined by the action of various morphogens from different organising centres. Forebrain identity is established in the absence of major signalling molecules, while midbrain requires FGF activity, where FGF8 in particular is essential for the positioning of the midbrain–hindbrain boundary. Spinal cord identity is conferred by concerted action of Wnts and RA. In the dorsoventral plane, BMPs produced by overlying epidermis and Wnts coming from the roofplate oppose ventrally produced Shh establishing a dorsoventral gradient according to which different types of neurons will be generated

Gradients of these signalling molecules direct tissue patterning during embryogenesis [20, 21]. For instance, while sonic hedgehog (SHH) is produced at the ventral side of the neural tube [22, 23], at the opposite side BMPs and WNTs constitute dorsal signals [24–26] (◘ Fig. 11.4). In this way, the neural tube is organised into different zones along the D-V axis: roof plate (dorsal-most), alar plate (dorsal), basal plate (ventral) and floor plate (ventral-most), where at each given point of the D-V axis a combination of opposing ventralising and dorsalising signals specifies a unique type of progenitor [27]. Subsequently, different types of neural progenitors are formed with the capacity to originate specific types of neurons and glial cells. For example, in the spinal cord, several classes of interneurons are produced by progenitors in dorsal and intermediate domains while motor neurons arise from the ventral motor neuron (MN) domain. The interneurons of each longitudinal spinal cord segment integrate circuits that will orchestrate the coordinated action of body muscles by regulating motor neuron activity. Examples of these are discrete circuits commanding trot and gallop, i.e. simultaneous or alternate leg movements, composed by different interneuron types produced in different D-V domains of the same R-C segment of the spinal cord. Thus, *the diversity of neuronal types produced at the level of each segment of the neural tube is essential for the formation of local neuronal circuits and provides functional specificity in each segment.*

11.1.2.1 Timing and Competence

Individual neural progenitors possess spatial identity determined by their position within the neural tissue, defining the type of neuronal cell they can originate. In addition to this, they are able to give rise to distinct cell types over time, further increasing neural diversity in the CNS. This temporal switch of the progenitor identity is determined by the expression of specific subsets of transcription factors and results from two different processes: specification of temporal identity by changing intrinsic or extrinsic cues and progenitor competence, i.e. the ability of progenitor to respond to these cues [28]. With time, neural progenitors undergo competence restriction, gradually losing the ability to specify earlier-born cell fates and acquiring the competence to make later-born cell types. *This means that every neural cell type has a restricted time window during which it can be specified.*

Multiple studies both in Drosophila and mammals have shown that early progenitors are able to give rise to later neuronal fates when transplanted to later embryonic stages, but the opposite is not always true. This happens because the switch of progenitor competence is reinforced through gene silencing, either by repositioning of a given genomic locus into a gene-silencing hub such as the nuclear lamina or by recruitment of Polycomb repressive complexes (PRCs) which promote heritable gene silencing (reviewed in [28]).

Temporal patterning is best understood in Drosophila melanogaster neuroblasts, where sequential expression of transcription factors Hunchback (Hb), Kruppel (Kp), POU domain protein (Pdm) and Castor (Cas) determines the transition from early to late neuronal fates [29]. After the first two neuroblast divisions, Hb expression is downregulated and by the fifth division the Hb locus is relocated to the nuclear lamina and permanently silenced [30]. This relocation coincides with the time window of expression of Distal antenna (Dan), a member of the Centromere protein B (CENP-B)/transposase family of proteins. Although the exact role of Dan in this

relocation is still unknown, this is one of the few examples where the mechanism of the temporal switch of neuroblast competence has been elucidated.

In mammals, orthologues of *Drosophila* transcription factors (TFs) have also been shown to define the temporal identity of progenitors in several contexts. In the developing retina, a timely succession of seven cell types has been described [31]. The Hb orthologue Ikaros is expressed in retinal progenitors where it specifies early-born neuronal fates [32]. Interestingly, while *Ikaros* mRNA is expressed throughout entire retinal development, the protein is detected only in early progenitors, suggesting that temporal restriction of progenitor competence occurs via post-translational regulation [32]. Misexpression of Ikaros in the older retina is able to restore some but not all early neuronal types blocking the differentiation of the latest cell type, Muller glia [32]. Temporal regulation of different retinal cell type production acts in parallel with stochastic mechanisms generating progenitor heterogeneity to which Notch-mediated lateral inhibition is thought to contribute [33]. By the concerted action of these mechanisms, several cell types are produced in retina simultaneously, allowing the proper laying out of complex neuronal circuitry.

The structural and functional complexity of the mammalian cortex is also generated by several mechanisms. Cortical excitatory neurons are generated from radial glial progenitors in the ventricular zone (VZ) of the dorsal telencephalon, often with an intermediate amplification step via the proliferation of basal progenitors in the subventricular zone (SVZ) [10]. Inhibitory neurons, in contrast, originate from the ventral telencephalon and populate cortical layers by concerted radial migration that is tightly coupled with their birthdate, with early-born neurons populating deep layers and later-born ones settling in the outer layers. Pioneering heterochronic transplantation studies of Susan McConnell demonstrated that young cortical progenitors generate late-born neuronal types when transplanted into the old cortical environment [34, 35] suggesting that early neural progenitors can respond to late extrinsic cues by generating temporally matched neuronal types. Older cortical progenitors, in contrast, were not able to produce younger, deep-layer neuronal types even when they had undergone cell divisions in a younger cortical environment [36]. However, when progenitors of layer VI (late-born) neurons were transplanted into layer IV (earlier-born), they were able to give rise to layer V (but not layer IV) neurons despite that at the time of transplantation the production of layer V neurons already ceased. This demonstrates an important property of neural progenitors: the interval of their competence to specify temporal identity spreads beyond the time of a given cell fate transition [28].

11.1.2.2 Neuron-to-Glia Switch

The most common switch in progenitor competence is the transition from neuronal to glial production that occurs in the different brain and spinal cord regions [37]. Neuronal identity of the cell is assured by the expression of proneural basic helix–loop–helix (bHLH) transcription factors known to promote neurogenesis and inhibit gliogenesis [38]. Glial identity is promoted by the gliogenic factor SOX9, and cytokines such as leukemia inhibitory factor (LIF), and Notch and BMP signalling [39]. Chromatin regulators also play a role in this switch, by demethylating the *GFAP* (glial fibrillary acidic protein) promoter and silencing proneural genes at the end of neurogenesis [40].

11.1.3 Cerebellar Development

Cerebellar specification starts early in human embryonic development, at 6 weeks, while its final cytoarchitecture is only achieved postnatally [41, 42]. During early development, when the neural tube is being regionalised, the hindbrain or rhombencephalon is presented as a segmental structure, containing 11 different rhombomeres [43, 44]. The cerebellum primordium, called "cerebellar anlage", originates from one of the hindbrain segments, the rhombomere 1 – r1 [45], which comprises the most anterior zone of the hindbrain caudally to the mid-hindbrain boundary (MHB), the isthmic organizer (IsO) [46]. This boundary appears to be maintained by the differential expression of transcription factors OTX2 and GBX2, which are important for the development of forebrain/midbrain and anterior hindbrain respectively [47–50]. The organising activity of the IsO is essentially mediated by the secretion of FGF8, which is strongly expressed in the MHB and its confined localisation is induced by the interaction of different transcription factors [50–54]. The organising action of IsO plays an important role in the formation of the cerebellum, because it regulates expression of different transcription factors involved in r1 patterning, including EN2, PAX2 and WNT1 [55]. The limits of the cerebellar territory are determined by the rostral expression of OTX2 and caudal expression of HOX genes, particularly HOXA2, in the hindbrain region, also in response to FGF8 signalling from MHB [55, 56].

After cerebellar territory formation, the cerebellar anlage is divided into two germinal centres that originate all GABAergic and glutamatergic cerebellar neurons, the ventricular zone (VZ) and the rhombic lip (RL) [57, 58]. The VZ is characterised by the expression of pancreas-specific transcription factor 1a (PTF1a) and gives rise to all inhibitory GABAergic neurons (Purkinje cells, Golgi, Lugaro, Stellate, Basket, Candelabrum, mid-sized GABAergic inhibitory projection neurons and small GABAergic interneurons) present in the adult cerebellum [59]. In a similar way to described above for the cerebral cortex, cerebellar excitatory neurons have a separate origin. Thus, in the cerebellum, the RL is a source of all excitatory glutamatergic neurons (Granule cells, unipolar brush cells and large glutamatergic projection neurons) and is essentially generated by atonal homolog 1 (ATOH1, also known as MATH1)-expressing progenitors [60, 61]. The cerebellar regionalisation is achieved by radial and tangential migration of post-mitotic neurons from the different germinal zones that will contribute to the final shape and size of the cerebellum [43, 46]. The appearance of a temporary layer containing ATOH1^{+} proliferative progenitors derived from RL, at the surface of the developing cerebellum [62, 63], the external germinal layer (EGL), is a key feature in cerebellar development [57]. Already at a postnatal stage, the EGL-derived granule cells differentiate and migrate radially from the molecular layer across the Purkinje cell layer to their final destination, the granular cell layer. When granule cell migration is completed, the final stage of cerebellum foliation is achieved [57, 58].

The adult cerebellum is anatomically arranged into the cerebellar cortex that surrounds the white matter and the cerebellar nuclei. The cerebellar cortex is composed of different cell layers containing several types of neurons with an organised arrangement. This includes the Purkinje cell layer, containing a monolayer of the Purkinje cell bodies, Bergmann glial cells and a lower number of Candelabrum cells; between the innermost dense layer of Granule cells and Interneurons (Golgi cells, Unipolar

Brush cells, and Lugaro cells), constituting the Granular layer; and the outermost layer with the inhibitory Interneurons (Stellate cells and Basket cells), which is the molecular layer. Cerebellar nuclei are constituted by three major different neuronal types: large glutamatergic projection neurons, mid-sized GABAergic inhibitory projection neurons and small GABAergic interneurons [57, 58, 64]. The involvement of this brain structure in motor functions is well established, comprising the maintenance of balance and posture and the coordination of voluntary movements [65–67]. More recently, the cerebellum has also been associated with non-motor functions, including auditory processing tasks [68], reward expectation [69] and other forms of emotional processing [70].

The dysfunction of the cerebellum is translated into ataxia, a symptom detected in different neurodegenerative disorders consisting of motor dysfunction, balance problems, as well as limb movement and gait abnormalities. Thus, there is a phenotypically and genotypically heterogeneous group of disorders called cerebellar ataxias characterised by neurodegeneration of the cerebellum [71]. Up to date, there is no effective cure available for ataxias, and the majority of recently performed trials have failed, mostly because the assessed drugs did not target a specific deleterious pathway [72]. The identification of the molecular and cellular mechanisms involved in disease pathogenesis is necessary for the development of therapies aimed to target relevant pathogenic pathways.

11.2 Case Study: Generating Cerebellar Neurons for Modelling Cerebellar Ataxias

Different model systems used until now have been important for providing information about the function of the cerebellum, the pathogenesis of cerebellar disorders and have also given some clues about therapies for cerebellar ataxias. For neurodegenerative disorders in general, most of the current knowledge about disease-related neuronal phenotypes is based on post mortem studies hampering the understanding of disease progression and development [73]. Besides that, the current pre-clinical models used to test the potential positive effects of some drugs and to study the molecular and cellular pathways of cerebellar ataxias include animal models and immortalised human cell lines [74, 75]. Although these models help in understanding the various mechanisms of cerebellar neurodegeneration, differences in anatomy, metabolism and behaviour between animals and humans make it difficult to fully recapitulate the human disease [76]. Furthermore, many candidate drugs that presented significant effects in these models have failed to show relevant positive effects in clinical trials [74]. On the other hand, human pluripotent stem cells (PSCs) provide a human cell source that has demonstrated great potential for disease modelling, drug screening and toxicology, since they have unlimited in vitro expansion potential and differentiation capacity [77, 78]. The knowledge about the signalling pathways involved in the maintenance of pluripotency as well as the generation of different germ layer derivatives has allowed the manipulation and control of PSC commitment to different lineages and further differentiation into specific cell types, including brain cells. In recent years, advances in our understanding of cerebellar development and differentiation have fostered the generation of techniques for obtaining different types of cerebellar neurons from PSCs [79–81].

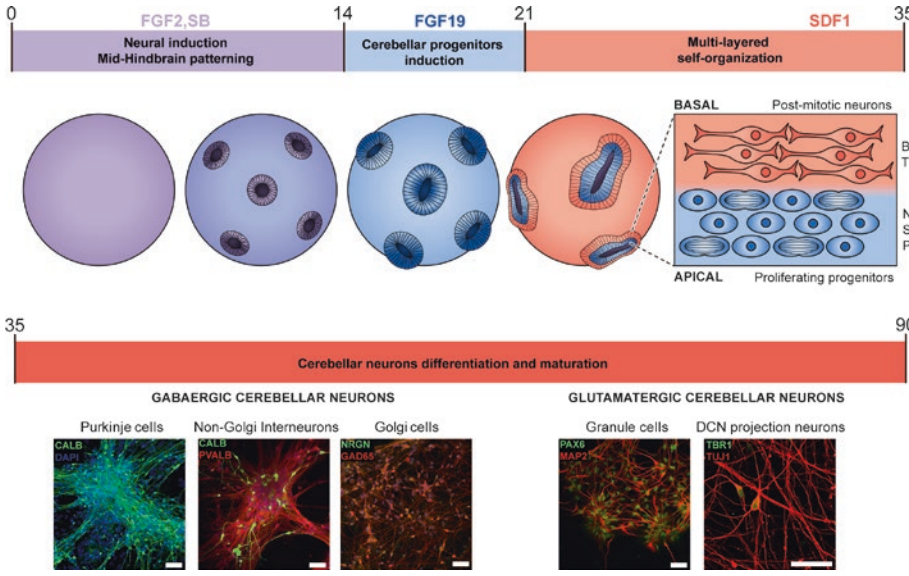


Fig. 11.5 Generation of cerebellar neurons from human pluripotent stem cells. Upper panel: schematic representation of cerebellar differentiation from human pluripotent stem cells. Neural tube-like structures form within floating aggregates, with apical domains delineated by NCAD, Sox2 and PAX6 staining and basal layer of post-mitotic neurons (Tuj1$^+$/BARHL1$^+$). Lower panel: immunostaining analysis for the indicated markers, supporting the presence of different mature cerebellar neurons. Scale bars 50 μm

For cerebellar commitment, an efficient neural induction is required as the first step of differentiation. To achieve this, the Nodal/Activin signalling inhibition by a chemical antagonist SB431542 (SB) of TGFβ signalling is used to prevent the meso-endodermal differentiation and drive neural commitment of PSC [82, 83]. After this step, the PSC-derived neural progenitors are ready to acquire their regional identity, and different regions of the human neural tube can be mimicked in vitro by adding specific morphogens to the culture medium. For cerebellar patterning, the sequential addition of defined morphogens, including FGF2, FGF19 and SDF1, can reproduce the sequential progression of human cerebellar development (Fig. 11.5). FGF2 has an inductive role in cerebellar commitment, acting as a moderate caudalising factor and leading to an efficient generation of mid-hindbrain progenitors. After the establishment of cerebellar territory, FGF19 signalling promotes the spontaneous generation of rostral hindbrain-like structures with apico-basal polarity, which are reorganised into different layers after the SDF1 addition as seen at the developmental stage when cerebellar neurogenesis occurs. By initiating differentiation using PSC-derived aggregates, after 14 days of neural induction, aggregates are mostly composed of neural progenitors expressing typical neural markers NESTIN and PAX6, that organise into small neural rosettes structures, similar to the embryonic neural tube (Fig. 11.5). After 21 days in culture and upon the action of FGF19 signalling, these neural rosettes reorganise into larger neuroepithelium with apico-basal polarity, strongly expressing the apical marker N-Cadherin (NCAD) on the apical side of the neural rosette. Going onwards until day 35 of differentiation, neural rosettes reorganise into polarised neuroepithelial structures with different layers, with prolif-

erating cerebellar progenitors expressing PAX6 and SOX2 on the apical (luminal) side, and more mature post-mitotic neurons on the basal side, expressing TUJ1 and BARHL1 (◘ Fig. 11.5). By promoting further maturation of the PSC-derived cerebellar progenitors, different types of functional cerebellar neurons can be obtained, including Purkinje cells (Calbindin (CALB)$^+$), Non-Golgi interneurons (Parvalbumin (PVALB$^+$; CALB$^-$), Golgi cells (Neurogranin (NRGN)$^+$), Granule cells (PAX6$^+$ and MAP 2$^+$) and large glutamatergic projection neurons (TBR1$^+$; ◘ Fig. 11.5). This procedure represents a differentiation strategy to generate different types of cerebellar cells in a well-organised structure that can form functional cerebellar neurons. This strategy gives the opportunity to study cerebellar development together with the possibility to efficiently generate cerebellar neurons from patient-derived iPSCs for the purpose of drug screening and for the study of specific pathways involved in cerebellar dysfunctions.

Take Home Message

- Billions of neurons composing our CNS are generated in an orderly fashion in accordance with spatial and temporal cues.
- To sustain the continuous generation of neural cells during CNS development, the progenitor pool must be maintained by asymmetric progenitor divisions and by lateral inhibition via Notch signalling.
- Neural development can be efficiently reproduced in vitro using pluripotent cell differentiation in a controlled environment.
- Many neurodevelopmental and neurodegenerative disorders have cerebellar ataxia as one of the major symptoms, and there is still no effective cure for ataxia.
- Functionally mature cerebellar neurons can be efficiently produced in vitro using human iPSCs as a source, being an excellent model for studying diseases affecting the cerebellar function.

References

1. Pevny LH, Sockanathan S, Placzek M, Lovell-Badge R. A role for SOX1 in neural determination. Development. 1998;125(10):1967–78.
2. Afonso C, Henrique D. PAR3 acts as a molecular organizer to define the apical domain of chick neuroepithelial cells. J Cell Sci. 2006;119(20):4293–304. https://doi.org/10.1242/jcs.03170.
3. Henrique D, Abranches E, Verrier L, Storey KG. Neuromesodermal progenitors and the making of the spinal cord. Development. 2015;142(17):2864–75. https://doi.org/10.1242/dev.119768.
4. Gammill LS, Bronner-Fraser M. Neural crest specification: migrating into genomics. Nat Rev Neurosci. 2003;4(10):795–805.
5. Sadler TW. Embryology of neural tube development. Am J Med Genet C Semin Med Genet. 2005;135C(1):2–8.
6. Sauer FC. Mitosis in the neural tube. J Comp Neurol. 1935;62(2):377–405.
7. Spear PC, Erickson CA. Interkinetic nuclear migration: a mysterious process in search of a function. Develop Growth Differ. 2012;54(3):306–16.
8. Louvi A, Artavanis-Tsakonas S. Notch signalling in vertebrate neural development. Nat Rev Neurosci. 2006;7(2):93–102.
9. Henrique D, Hirsinger E, Adam J, Le Roux I, Pourquié O, Ish-Horowicz D, et al. Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. Curr Biol. 1997;7(9):661–70.

10. Kriegstein A, Alvarez-Buylla A. The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci. 2009;32(1):149–84. https://doi.org/10.1146/annurev.neuro.051508.135600.
11. Levine AJ, Brivanlou AH. Proposal of a model of mammalian neural induction. Dev Biol. 2007;308:247–56.
12. Stern CD. Initial patterning of the central nervous system: how many organizers? Nat Rev Neurosci. 2001;2(2):92–8.
13. Suzuki IK, Vanderhaeghen P. Is this a brain which I see before me? Modeling human neural development with pluripotent stem cells. Development. 2015;142(18):3138–50. https://doi.org/10.1242/dev.120568.
14. Wilson SW, Houart C. Early steps in the development of the forebrain. Dev Cell. 2004;6:167–81.
15. Imaizumi K, Sone T, Ibata K, Fujimori K, Yuzaki M, Akamatsu W, et al. Controlling the regional identity of hPSC-derived neurons to uncover neuronal subtype specificity of neurological disease phenotypes. Stem Cell Reports. 2015;5(6):1010–22.
16. Irioka T, Watanabe K, Mizusawa H, Mizuseki K, Sasai Y. Distinct effects of caudalizing factors on regional specification of embryonic stem cell-derived neural precursors. Dev Brain Res. 2005;154(1):63–70.
17. Kirkeby A, Grealish S, Wolf DA, Nelander J, Wood J, Lundblad M, et al. Generation of regionally specified neural progenitors and functional neurons from human embryonic stem cells under defined conditions. Cell Rep. 2012;1(6):703–14.
18. Petros TJ, Tyson JA, Anderson SA. Pluripotent stem cells for the study of CNS development. Front Mol Neurosci. 2011;4:30.
19. Lumsden A, Krumlauf R. Patterning the vertebrate neuraxis. Science. 1996;274(5290):1109–15.
20. Crick F. Diffusion in embryogenesis. Nature. 1970;225(5231):420–2.
21. Wolpert L. Positional information and the spatial pattern of cellular differentiation. J Theor Biol. 1969;25(1):1–47.
22. Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, et al. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature. 1996;383(6599):407–13.
23. Martínez S, Puelles E, Puelles L, Echevarria D. Molecular regionalization of the developing neural tube. In: The mouse nervous system. London/Waltham: Elsevier/Academic Press; 2012. p. 2–18.
24. Basler K, Edlund T, Jessell TM, Yamada T. Control of cell pattern in the neural tube: regulation of cell differentiation by dorsalin-1, a novel TGFβ family member. Cell. 1993;73(4):687–702.
25. Dickinson ME, Selleck MA, McMahon AP, Bronner-Fraser M. Dorsalization of the neural tube by the non-neural ectoderm. Development. 1995;121(7):2099–106.
26. Ulloa F, Martí E. Wnt won the war: antagonistic role of Wnt over Shh controls dorso-ventral patterning of the vertebrate neural tube. Dev Dyn. 2010;239:69–76.
27. Jessell TM. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet. 2000;1(1):20–9.
28. Kohwi M, Doe CQ. Temporal fate specification and neural progenitor competence during development. Nat Rev Neurosci. 2013;14(12):823–38.
29. Isshiki T, Pearson B, Holbrook S, Doe CQ. Drosophila neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. Cell. 2001;106(4):511–21.
30. Kohwi M, Lupton JR, Lai SL, Miller MR, Doe CQ. Developmentally regulated subnuclear genome reorganization restricts neural progenitor competence in Drosophila. Cell. 2013;152(1–2):97–108.
31. Turner DL, Snyder EY, Cepko CL. Lineage-independent determination of cell type in the embryonic mouse retina. Neuron. 1990;4(6):833–45.
32. Elliott J, Jolicoeur C, Ramamurthy V, Cayouette M. Ikaros confers early temporal competence to mouse retinal progenitor cells. Neuron. 2008;60(1):26–39.
33. Rocha SF, Lopes SS, Gossler A, Henrique D. Dll1 and Dll4 function sequentially in the retina and pV2 domain of the spinal cord to regulate neurogenesis and create cell diversity. Dev Biol. 2009;328(1):54–65.
34. McConnell SK, Kaznowski CE. Cell cycle dependence of laminar determination in developing neocortex. Science. 1991;254(5029):282–5.
35. Desai AR, McConnell SK. Progressive restriction in fate potential by neural progenitors during cerebral cortical development. Development. 2000;127(13):2863–72.
36. Frantz GD, McConnell SK. Restriction of late cerebral cortical progenitors to an upper-layer fate. Neuron. 1996;17(1):55–61.

37. Okano H, Temple S. Cell types to order: temporal specification of CNS stem cells. Curr Opin Neurobiol. 2009;19(2):112–9.
38. Ross S, Greenberg M, Stiles C. Basic helix-loop-helix factors in cortical development. Neuron. 2003;39(1):13–25.
39. Rowitch DH, Kriegstein AR. Developmental genetics of vertebrate glial-cell specification. Nature. 2010;468:214–22.
40. Hirabayashi Y, Suzki N, Tsuboi M, Endo TA, Toyoda T, Shinga J, et al. Polycomb limits the neurogenic competence of neural precursor cells to promote astrogenic fate transition. Neuron. 2009;63(5):600–13.
41. Cho KH, Rodríguez-Vázquez JF, Kim JH, Abe H, Murakami G, Cho BH. Early fetal development of the human cerebellum. Surg Radiol Anat. 2011;33(6):523–30.
42. Millen KJ, Wurst W, Herrup K, Joyner AL. Abnormal embryonic cerebellar development and patterning of postnatal foliation in two mouse Engrailed-2 mutants. Development. 1994;120(3):695–706.
43. Andreu A, Crespo-Enriquez I, Echevarria D. Molecular events directing the patterning and specification of the cerebellum. Eur J Anat. 2014;18(4):245–52.
44. Morales D, Hatten ME. Molecular markers of neuronal progenitors in the embryonic cerebellar anlage. J Neurosci. 2006;26(47):12226–36.
45. Zervas M, Millet S, Ahn S, Joyner AL. Cell behaviors and genetic lineages of the mesencephalon and rhombomere 1. Neuron. 2004;43(3):345–57.
46. Butts T, Green MJ, Wingate RJT. Development of the cerebellum: simple steps to make a "little brain". Development. 2014;141(21):4031–41. https://doi.org/10.1242/dev.106559.
47. Broccoli V, Boncinelli E, Wurst W. The caudal limit of Otx2 expression positions the isthmic organizer. Nature. 1999;401(6749):164–8.
48. Wassarman KM, Lewandoski M, Campbell K, Joyner AL, Rubenstein JL, Martinez S, et al. Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function. Development. 1997;124(15):2923–34.
49. Millet S, Campbell K, Epstein DJ, Losos K, Harris E, Joyner AL. A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer. Nature. 1999;401(6749):161–4.
50. Wurst W, Bally-Cuif L, Bally-Cuif L. Neural plate patterning: upstream and downstream of the isthmic organizer. Nat Rev Neurosci. 2001;2(2):99–108.
51. Heikinheimo M, Lawshé A, Shackleford GM, Wilson DB, MacArthur CA. Fgf-8 expression in the post-gastrulation mouse suggests roles in the development of the face, limbs and central nervous system. Mech Dev. 1994;48(2):129–38.
52. Crossley PH, Martin GR. The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development. 1995;121(2):439–51.
53. Joyner AL, Liu A, Millet S. Otx2, Gbx2 and Fgf8 interact to position and maintain a mid-hindbrain organizer. Curr Opin Cell Biol. 2000;12:736–41.
54. Chizhikov VV, Millen KJ. Neurogenesis in the cerebellum. In: Comprehensive developmental neuroscience: patterning and cell type specification in the developing CNS and PNS. Amsterdam: Academic Press; 2013. p. 417–34.
55. Irving C, Mason I. Signalling by FGF8 from the isthmus patterns anterior hindbrain and establishes the anterior limit of Hox gene expression. Development. 2000;127(1):177–86.
56. Gavalas A, Davenne M, Lumsden A, Chambon P, Rijli FM. Role of Hoxa-2 in axon pathfinding and rostral hindbrain patterning. Development. 1997;124(19):3693–702.
57. White JJ, Sillitoe RV. Development of the cerebellum: from gene expression patterns to circuit maps. Wiley Interdiscip Rev Dev Biol. 2013;2(1):149–64.
58. Marzban H, Del Bigio MR, Alizadeh J, Ghavami S, Zachariah RM, Rastegar M. Cellular commitment in the developing cerebellum. Front Cell Neurosci. 2015;8(January):1–26.
59. Hoshino M, Nakamura S, Mori K, Kawauchi T, Terao M, Nishimura YV, et al. Ptf1a, a bHLH transcriptional gene, defines GABAergic neuronal fates in cerebellum. Neuron. 2005;47(2):201–13.
60. Wang VY, Rose MF, Zoghbi HY. Math1 expression redefines the rhombic lip derivatives and reveals novel lineages within the brainstem and cerebellum. Neuron. 2005;48(1):31–43.
61. MacHold R, Fishell G. Math1 is expressed in temporally discrete pools of cerebellar rhombic-lip neural progenitors. Neuron. 2005;48(1):17–24.

62. Ben-Arie N, McCall AE, Berkman S, Eichele G, Bellen HJ, Zoghbi HY. Evolutionary conservation of sequence and expression of the bHLH protein Atonal suggests a conserved role in neurogenesis. Hum Mol Genet. 1996;5(9):1207–16.
63. Ben-Arie N, Bellen HJ, Armstrong DL, McCall AE, Gordadze PR, Guo Q, et al. Math1 is essential for genesis of cerebellar granule neurons. Nature. 1997;390(6656):169–72.
64. Hoshino M. Neuronal subtype specification in the cerebellum and dorsal hindbrain. Dev Growth Diff. 2012;54:317–26.
65. Ito M. The modifiable neuronal network of the cerebellum. Jpn J Physiol. 1984;34:781–92.
66. Glickstein M. Motor skills but not cognitive tasks. Trends Neurosci. 1993;16(11):450–1.
67. Schmahmann JD. Rediscovery of an early concept. Int Rev Neurobiol. 1997;41:3–27.
68. McLachlan NM, Wilson SJ. The contribution of brainstem and cerebellar pathways to auditory recognition. Front Psychol. 2017;8:265. https://doi.org/10.3389/fpsyg.2017.00265.
69. Wagner MJ, Kim TH, Savall J, Schnitzer MJ, Luo L. Cerebellar granule cells encode the expectation of reward. Nature. 2017;544(7648):96–100.
70. Adamaszek M, D'Agata F, Ferrucci R, Habas C, Keulen S, Kirkby KC, et al. Consensus paper: cerebellum and emotion. Cerebellum. 2017;16:552–76.
71. Klockgether T. Update on degenerative ataxias. Curr Opin Neurol. 2011;24(4):339–45.
72. Marmolino D, Manto M. Past, present and future therapeutics for cerebellar ataxias. Curr Neuropharmacol. 2010;8(1):41–61.
73. Durnaoglu S, Genc S, Genc K. Patient-specific pluripotent stem cells in neurological diseases. Stem Cells Int. 2011;2011:1–17.
74. Manto M, Marmolino D. Animal models of human cerebellar ataxias: a cornerstone for the therapies of the twenty-first century. Cerebellum. 2009;8:137–54.
75. Ziv Y, Danieli T, Amiel A, Ravia Y, Shiloh Y, Etkin S, et al. Cellular and molecular characteristics of an immortalized ataxia-telangiectasia (group AB) cell line. Cancer Res. 1989;49(9):2495–501.
76. Cendelin J, Schmahmann J, Sherman J, Manto M, Manto M, Marmolino D, et al. From mice to men: lessons from mutant ataxic mice. Cerebellum Ataxias. 2014;1(1):4.
77. Kropp C, Massai D, Zweigerdt R. Progress and challenges in large-scale expansion of human pluripotent stem cells. Process Biochem. 2017;59:244–54.
78. Yamamoto T, Takenaka C, Yoda Y, Oshima Y, Kagawa K, Miyajima H, et al. Differentiation potential of Pluripotent Stem Cells correlates to the level of CHD7. Sci Rep. 2018;8(1):241.
79. Muguruma K, Nishiyama A, Kawakami H, Hashimoto K, Sasai Y. Self-organization of polarized cerebellar tissue in 3D culture of human pluripotent stem cells. Cell Rep. 2015;10(4):537–50.
80. Salero E, Hatten ME. Differentiation of ES cells into cerebellar neurons. Proc Natl Acad Sci U S A. 2007;104(8):2997–3002.
81. Wang S, Wang B, Pan N, Fu L, Wang C, Song G, et al. Differentiation of human induced pluripotent stem cells to mature functional Purkinje neurons. Sci Rep. 2015;5:9232.
82. Laping NJ, Grygielko E, Mathur A, Butter S, Bomberger J, Tweed C, et al. Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542. Mol Pharmacol. 2002;62(1):58–64.
83. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol. 2009;27(3):275–80.