Review

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Use of human pluripotent stem cell-derived cells for neurodegenerative disease modeling and drug screening platform

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Most neurodegenerative diseases are characterized by a complex and mostly still unresolved pathology. This fact, together with the lack of reliable disease models, has precluded the development of effective therapies counteracting the disease progression. The advent of human pluripotent stem cells has revolutionized the field allowing the generation of disease-relevant neural cell types that can be used for disease modeling, drug screening and, possibly, cell transplantation purposes. In this Review, we discuss the applications of human pluripotent stem cells, the development of efficient protocols for the derivation of the different neural cells and their applicability for robust *in vitro* disease modeling and drug screening platforms for most common neurodegenerative conditions.

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Neurodegenerative diseases are characterized by the chronic and progressive deterioration of neuronal functioning, resulting in cognitive impairment, memory deficits, deficiency in motor function, loss of sensitivity, affectation of autonomous brain system, changes in perception and/or mood, etc., with combination of these features present in individual patients. Several neurodegenerative diseases share common pathological pathways including abnormal accumulation of toxic-aggregated proteins, mitochondrial dysfunction, axonal transport defects and chronic inflammation, which ultimately lead to neurodegeneration [1].

Neurodegenerative conditions represent an enormous impact on global health due to their high incidence, the severity of their symptoms and, in general, the lack of effective therapies able to mitigate those symptoms and/or counteract the course of the disease [2]. In addition, the incidence of neurodegenerative diseases in developed countries is rapidly increasing, as is the case for Alzheimer's disease (AD) [3], amyotrophic lateral sclerosis (ALS) [4], multiple sclerosis (MS) [5], Parkinson's disease (PD) [6] and other forms of dementia as frontotemporal dementia, progressive supranuclear palsy or corticobasal degeneration [7]. Although the reasons for this general increase in the number of people affected by neurodegenerative diseases are not properly established, the general aging of the western-countries population and a higher exposure to certain substances as chemicals and a higher contamination, are few of the possible causes for this increase [4].

Modeling of neurodegenerative diseases has always been a difficult task, due to the complexity of the conditions, poor access to human samples and little knowledge of disease pathology. Animal models have been the most widely used, due to the intrinsic features of *in vivo* experimentation which allow, in principle, more accurate modeling. Nonetheless, disease modeling with animals requires the generation of reliable disease models, the use of a large number of animals and limits the combination of experimental conditions tested, due also to the long experimentation times required [8].

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The advent of human pluripotent stem cell (hPSC) discovery and development has represented a new scenario complementing the modeling of neurodegenerative diseases. Since their discovery, important efforts have been established in the derivation of hPSC-derived neural cells from patients and healthy donors, which allow disease modeling using human samples with inherent advantages over *in vivo* models.

In this Review, we will discuss the current state of the art in neurodegenerative disease modeling and drug testing for the most prevalent neurodegenerative conditions, and provide data predicting the development of this fast-changing and promising field of research.

Pluripotent stem cells

Since the advent of human embryonic stem cells (hESCs) derivation in 1998 [9] and the discovery of murine and human-induced pluripotent stem cells (hiPSCs) in 2006–2007 [10,11], a new field of research was opened up. Although stem cells have represented a promising strategy for their potential therapeutic uses, which have already been investigated for more than two decades, successful research performed in the past few years have revealed a prominent utility of stem cells for disease modeling and drug screening purposes [12].

Human ESCs and iPSCs are pluripotent stem cells, with hESCs deriving from the inner mass of the blastocyst. On the other hand, hiPSCs are generated by the forced expression of reprogramming factors, with the transcription factors *OCT3/4*, *SOX2*, *KLF4* and *c-MYC* constituting the initial reprogramming cocktail [11]. Both hESCs and hiPSCs have the capacity, under certain defined conditions, to differentiate toward any somatic cell type, as will be expanded later.

The employment of hPSCs for disease modeling was initially delayed due to the relatively poor yield of initial methods for their derivation, complexity and costs. Nonetheless, several improvements in methodology for their generation have been developed. Among these improvements, combination of different reprogramming factors [13] and the combined use of different compounds [14] have contributed to increase the efficiency of reprogramming in a great extent. In addition, methods to introduce reprogramming factors relied in the use of integrating viral vectors as retrovirus and lentivirus due to their efficiency, although this technology precludes a potential further use of these cells and leads to random integration events hindering the standardization of this approach among labs. Recently, other methods for transgene delivery have been developed including the delivery of reprogramming factors by means of targeting the cells with episomal vectors, non-integrating Sendai virus and the use of mRNAs. These technologies lead to the generation of iPSCs with similar efficiencies that those derived using viral vectors and allow the generation of GMP-grade iPSCs compatible with their use in the clinical setting [15].

These advances have allowed the extension in the use of hPSCs among labs for disease modeling and drug screening purposes (Figure 1). This has represented a considerable advance in the field of neurodegenerative conditions that, until the implementation of hPSC technology, had been modeled exclusively and only partially by the use of animal models without full recreation of disease phenotype [16]. hPSC technology has transformed the modeling of neurodegenerative diseases, since it has allowed the study from familial monoallelic to complex multifactorial diseases in the context of patient-derived neural cells and tissue, systems that previously were not available for experimental investigation. The ability of hPSCs to differentiate toward any somatic cell type allows the generation of neural cells implicated in the pathogenesis of neurological diseases, where insertion or correction of disease-associated genetic mutations can be performed.

Gene-editing in hPSCs

An important and sometimes poorly considered challenge of hPSC technology is the variability between lines from the same individual or from the same group of individuals in terms of their capacity to differentiate toward different lineages. These differences are due to intrinsic genetic backgrounds, differences in reprogramming methods and/or epigenetic status of the somatic cell at the time of reprogramming [17]. Therefore, the generation of isogenic pair cell lines by correction or introduction of desired disease-associated genetic modification(s) is the best approach for assessing the biological effects exerted by one or several disease-associated mutations. Making such isogenic control lines requires the use of site-specific nucleases targeting the desired location of the genome.

The use of site-specific nucleases to generate double-strand breaks in the genome allowed an efficient modification of the genetic sequence [18]. The complexity of the initial technologies and their scarce customization limited the wide implementation of this technology [19]. An important revolution in the field came by the employment of DNA-binding zinc finger nucleases for genomic editing, which notably increased the specificity and efficiency of the approaches, especially when cells were provided with a 'donor plasmid' (an exogenous DNA flanking the

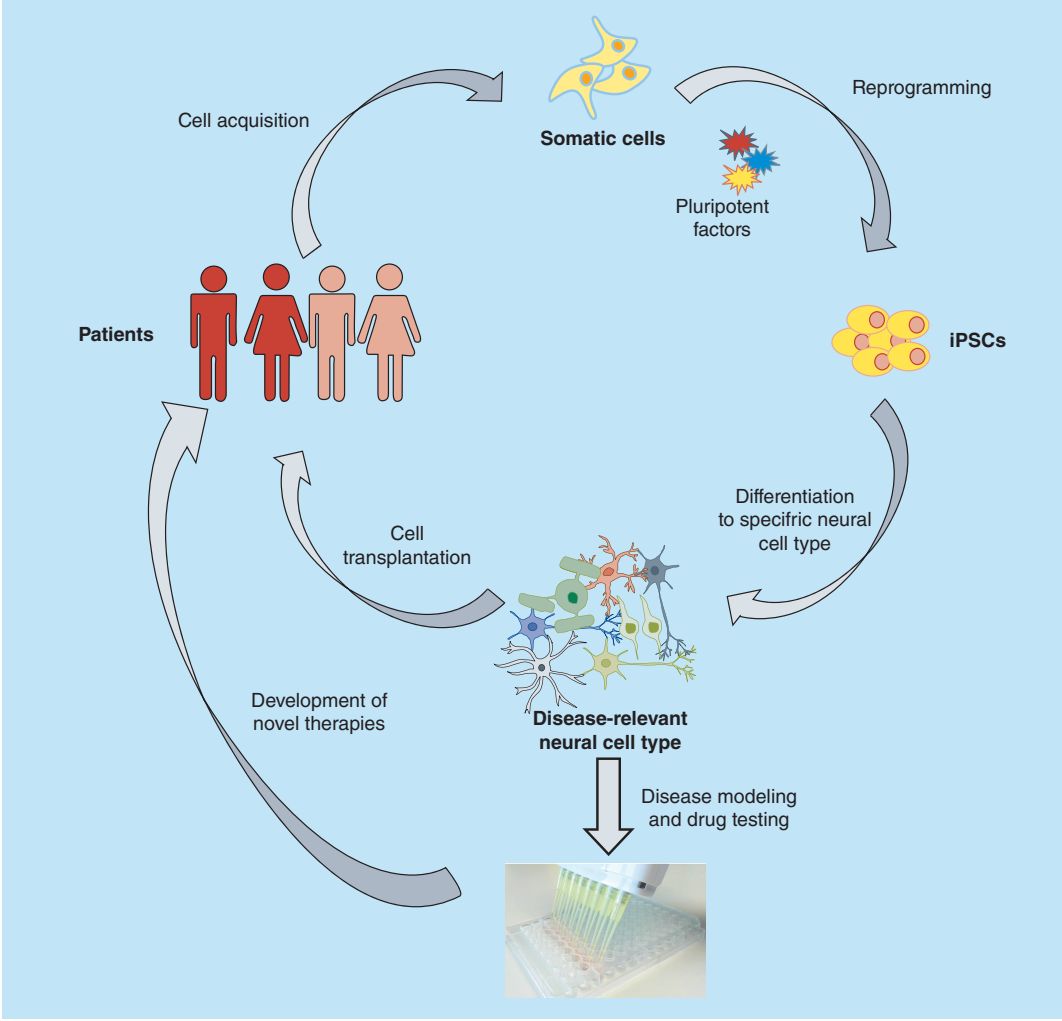

Figure 1. Schematic representation of the potential applications of induced pluripotent stem cells obtained from patients with neurodegenerative diseases. Human iPSCs can be differentiated toward disease-relevant neural cell types to be used potentially directly for cell transplantation or to be used as a platform for disease modeling and drug screening, which can lead to the development of novel and effective therapies.
iPSC: Induced pluripotent stem cell.

cutting site and with great homology with the target locus), which can be used by the cell as a template to repair the double-strand brake by homology recombination [20], a technology which can be applied to the genomic editing of hESC and hiPSCs as well [21].

In 2010, Christian *et al.* used a novel system by fusing transcription activator-like effectors (TALEs) to the catalytic domain of the Fok I endonuclease to generate TALE nucleases (TALENs) [22]. TALENs are easier to customize and less restrictive about the requirements the sequence must compile to be targeted. Moreover, this system showed a higher efficiency and accuracy over previous technologies [23].

However, the real revolution in the field came with the discovery and adaptation for genome editing of the CRISPR/CRISPR-associated (Cas) adaptive immune systems [24], with the variant CRISPR-Cas9 being the most widely used [25]. This complex is formed by the Cas9 nuclease associated with a base-paired structure formed between the activating tracrRNA and the targeting crRNA to cleave the target double-strand DNA. Site-specific cleavage occurs at places determined by both base-pairing complementarity between the CRISPR RNA (crRNA) and the target protospacer DNA and a short motif (the protospacer adjacent motif), next to the complementary region in the target DNA. As result, the Cas9 endonuclease can be programmed with single RNA molecules to

cleave specific DNA sites, leading to double-strand breaks in the genome that can be used for introducing desired modifications by co-providing a donor template DNA with desired modifications [24].

The advent of having a customizable RNA sequence (in contrast with protein sequence modifications needed for customization of zinc finger nucleases and TALENs) has resulted in a powerful gene-editing tool which, in addition, is cheaper and easier to design and implement than previous technologies. These features have made CRISPR-Cas9 system a widely used tool for gene-editing. Immediately after their discovery, they have been applied for performing gene-editing of hPSCs [26]. hPSCs are characterized by a poor efficiency of transfection, so specific gene-editing strategies should be followed to generate efficient modifications in the genome of these cells [27]. In this sense, the incorporation of selectable cassettes in safe harbor loci in the genome [28] or a gene-editing strategy including the incorporation of selectable cassettes with a subsequent removal in a seamless manner [29] allow a more efficient targeting strategy.

CRISPR-Cas9-mediated gene editing is an excellent tool for modeling some monogenic diseases or to study the contribution of single or several gene variants to a certain pathology. In this sense, hiPSCs derived from patients carrying a certain mutation associated with a neurodegenerative disorder can be used not only to model the disease but through the correction of this specific mutation and generation of isogenic cell lines, the contribution of a certain genetic modification to the disease phenotype can be addressed in a human model. In the case of familial ALS cases with mutations in the *FUS* gene, Guo *et al.* using iPSC-derived motor neurons (MNs) from these patients and correcting the mutations using CRISPR-Cas9 technology demonstrated that the presence of *FUS* mutations were responsible for shortcomings in axonal transport, a defect which could be counteracted by pharmacological inhibition of the *HDAC6* gene as well, identifying a possible therapeutic target for the disease [30]. In the case of AD, studies employing the CRISPR-Cas9-mediated gene targeting in iPSCs are emerging, elucidating the pathogenic role of disease-associated mutations as those present in *PSEN2* [31], *PSEN1* [32], *APP* [33] or *APOE* [34]. Moreover, CRISPR-Cas9-mediated gene editing has promised utility in the treatment of neurodegenerative diseases, as it has been shown for PD [35]. On the other hand, CRISPR-Cas9 can be used for the introduction of pathogenic variants for disease modeling. In this sense, we have recently generated a tauopathy model by introducing three *MAPT* mutations using CRISPR-Cas9 combined with the piggybac transposase in a seamless manner in iPSCs, leading to the manifestation of neurodegenerative phenotypes in iPSC-derived cortical neurons [36]. Therefore, gene-editing strategies have evolved in parallel with the use of hPSCs for disease modeling, a fact that has just started to prove fruitful for the combination of these two tools for the discovery and validation of novel therapeutic strategies [37].

Despite the potential of the combination of gene-editing in hPSCs for disease modeling and as a platform for drug testing, there are several drawbacks that limit this technology. One of these limitations deals with the fact that most neurodegenerative diseases are caused not by monogenic mendelian mutations but are consequence of environmental factors together with multiple low-effect-size-risk alleles identified in genome-wide association studies. This makes modeling with hPSCs a difficult task since disease-associated gene variants differ among patients and are not a causative agent *per se* to develop the disease or the pathogenic phenotype. To circumvent this issue, one possible strategy would be to generate several iPSC lines from patients with different genetic backgrounds in combination with multiplexed CRISPR-Cas9-mediate gene-editing to address the implication of those variants and/or discover novel disease-driving molecular pathways [38].

Another limitation of gene-editing strategies (with special focus on CRISPR-Cas9) deals with nuclease specificity and off-target events. Several studies have determined the rate of off-target events produced by site-specific nucleases, focusing on the CRISPR-Cas9 system. Initial studies determined that the possibility of having off-target effects in a targeted cell or organism was $\geq$50% [39], limiting the use of this technology. Nonetheless, more accurate methods for measuring those off-target events [40] and the last optimizations of the CRISPR-Cas9 technology have reduced practically to undetectable the frequency of off-target mutations [41]. However, this phenomenon should always be taken into account for the experimentation performed.

Methods for the derivation of neural cells from hPSCs

One of the intrinsic features of pluripotent stem cells is that, when exposed to defined culture conditions, are able to differentiate toward (almost) any somatic cell type [42]. Based on this, soon after the derivation of hESCs, and especially after the discovery of hiPSCs, researchers have made important efforts to elucidate molecular pathways governing cell differentiation toward the different lineages, with a special focus on the derivation of neural cells, since access to primary brain tissue is scarce and, when available, expansion of the different cell types *ex vivo* is

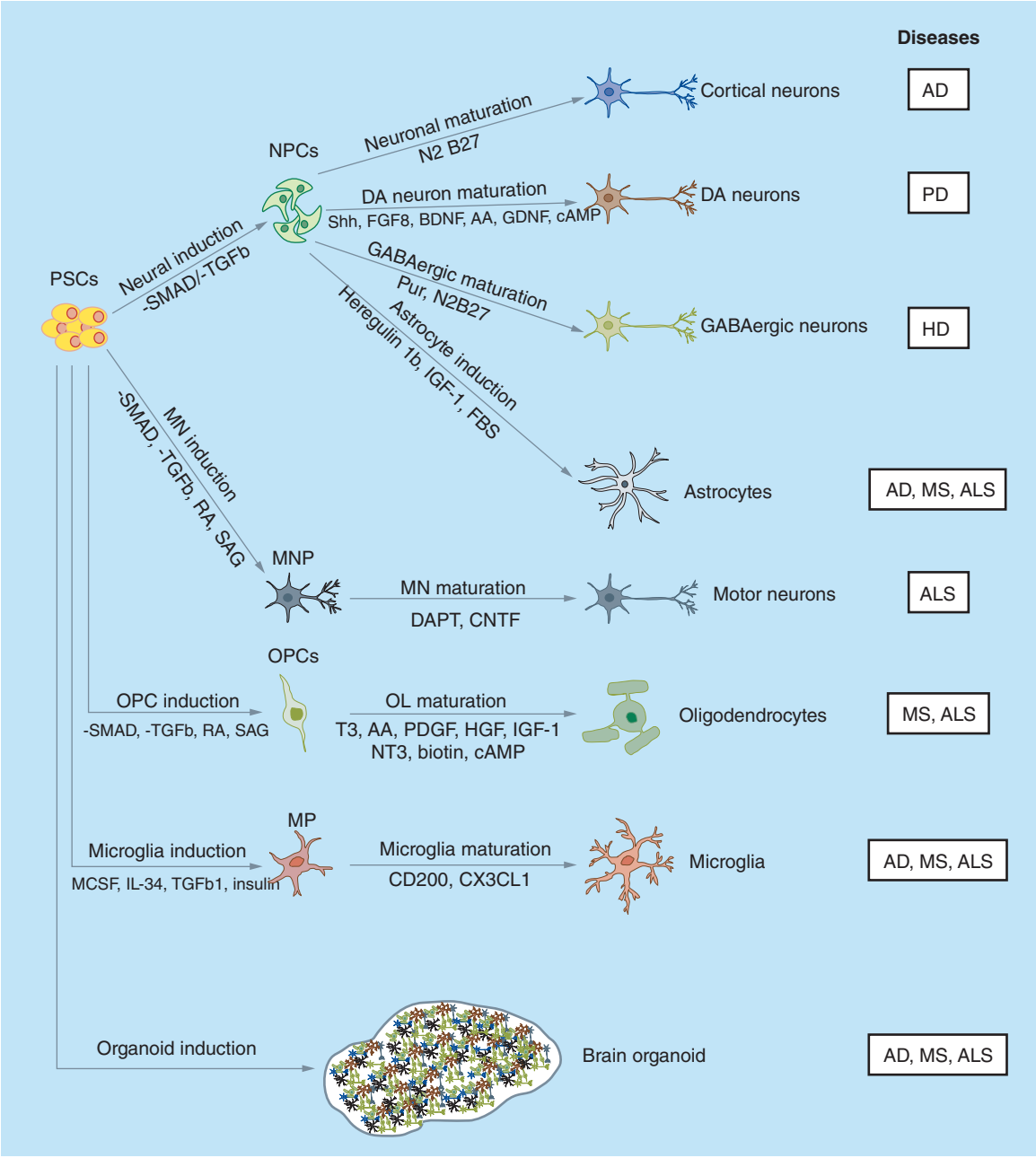

Figure 2. Layout of culture conditions for the derivation of human pluripotent stem cells into the different neural cell types and relevance of each cell type to neurodegenerative diseases.
AD: Alzheimer's disease; ALS: Amyotrophic lateral sclerosis; HD: Huntington's disease; MS: Multiple sclerosis; PD: Parkinson's disease.

very limited. Next, we are going to discuss the progress made for the optimization of the differentiation methods developed to generate the different neural cell types from hPSCs (see Figure 2).

Induction of a neural fate & neuronal differentiation

Soon after hESCs derivation, different attempts were made to fate pluripotent stem cells toward a neural phenotype. It was observed that when hPSCs are cultured in standard conditions without pluripotency-sustaining factors, neural fate is spontaneous induced, with the TGFβ-related signaling negatively inducing this differentiation [43]. Later on, using specific TGFβ antagonists to abolish the SMAD signaling, Chambers *et al.* improved and defined

the differentiation method obtaining a robust and efficient neural conversion from both hESCs and hiPSCs [44]. Nonetheless, these derivation methods did not recreate human neurogenesis in a temporal manner, with important limitations for disease modeling. In 2012, Shi *et al.* circumvented these issues since they developed an improved method for neuronal specification based on known rodent cortical development that included, after neural progenitor specification based on TGFβ-signaling inhibitors, an extended period of cortical neurogenesis and terminal neuronal maturation, which recreate human corticogenesis with the generation of deep and upper layer cortical neurons in a specified temporal manner [45]. This methodology enables studies to unravel the development of human cerebral cortex and the generation of individual-specific neurons that could be used for disease modeling and therapeutic approaches. Further improvements in the methodology to obtain electrophysiological active neurons have been described [46], also using defined and standard culture conditions without the need of co-culturing the generated neurons with exogenous cells [47].

In addition, protocols for generating specific neuronal subtypes have been established. In this sense, specific protocols have been developed to generate dopaminergic (DA) neurons from hPSCs. Nguyen *et al.* achieved efficient generation of DA neurons by a protocol performing neural induction by dual-SMAD inhibition, followed by culturing the cells in specific DA neurons cues including Sonic hedgehog and FGF8b signaling, supporting the cells with additional growth factors as BDNF, ascorbic acid, GDNF and cAMP; obtaining DA neurons able to reproduce key PD features *in vitro* [48]. Further optimizations of the protocol have been performed, leading to a more efficient generation of DA neurons able to engraft murine and non-human primates [49–51].

MNs have been another neuronal population widely studied due to their implication in several neurodegenerative conditions as ALS, where selective MN death occurs [52]. Since the discovery of hiPSCs, several protocols have been developed for the specific differentiation of these cells into functional MNs [53–56]. Maury *et al.* tested several small molecules targeting developmental cues for the derivation of different neuronal subtypes [57]. They obtained an improved method for MN generation consisting of neural specification induced by SMAD inhibition and Wnt pathway activation, followed by caudalization and ventralization induced by retinoic acid and smoothened agonist, respectively; culturing the cells in the presence of the gamma secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) and ciliary neurotrophic factor [30]. Although cell-replacement therapies are far from being achieved due to the complex and still unsolved pathology of ALS, hPSC-derived MNs can be used to resolve the complex pathology and serve as a platform to test altered pathways [37].

Apart from DA or MNs, other neuronal subtypes have been generated by finely exposing pluripotent cells to the signals governing telencephalic development, in a time and concentration-dependent manner [58].

Generation of astrocytes from hPSCs

Astrocytes are the most common glial cells present in the CNS, with essential functions in neuronal metabolic support, synapse formation and regulation of blood flow, among others [59]. Astrocytes are a heterogeneous cell type emerging from neural precursors in common with neurons that results in mixed generation of neurons and astrocytes in several protocols of hPSC differentiation [47]. Nonetheless, specific methods have been developed for efficient generation of functional astrocytes from hPSCs in defined culture conditions that resemble primary astrocytes and maintain their functionality after *in vivo* transplantation [60]. Further optimization in the protocols has been performed, obtaining more efficient methods to generate astrocytes able to unleash immune responses and able to support phagocytosis mediated by microglial cells [61]. hPSC-derived astrocytes can be generated from individuals suffering from different neurodegenerative diseases and can reproduce several phenotypes encountered *in vivo*, a feature that can be employed for effective disease modeling and the discovery of new therapeutic targets [62–64].

Generation of oligodendrocytes from hPSCs

Since the successful isolation and culture of hESCs, different groups developed specific protocols for the generation of oligodendrocyte precursor cells (OPCs) and oligodendrocytes (OLs), which are the cells responsible for myelin synthesis in the CNS. OLs and OPCs are a heterogeneous population of cells existing at different maturation states that, apart from being the cells responsible for myelination, have important trophic and metabolic functions [65]. Nistor *et al.* in 2005 was the first to describe the generation of OLs from hPSCs through neurosphere formation after a 10–15 week protocol, using only one hESC line [66]. Further improvements in the methodology were made, but all these protocols remained inefficient (very long differentiation times and low yield) and their robustness was in debate due to reduce number of cell lines tested [67,68]. All these protocols attempted to recapitulate the different processes and signals that occur during development. However, the different protocols differed in the combinations

of growth factors, cytokines and other molecules needed, which highlight that OL specification *in vivo* is achieved in different developmental regions that respond to specific local concentration of different factors [69]. Since the discovery of iPSCs, several groups developed specific protocols to obtain OPC/OLs from hiPSCs. Initial studies adapted previous protocols developed for hESCs to hiPSCs [70,71]. In 2013, Wang *et al.* developed an optimized protocol for OPC/OL generation from hiPSCs, accounting for a total differentiation time of 150 days, time after which they obtained 70–80% of OPCs, with a small fraction of those differentiating toward mature MBP^+ OLs able to engraft and myelinate the myelin-deficient shiverer mice [72]. Douvaras *et al.* in 2014 published an improved version of the protocol, consisting in a first stage generating $OLIG2^+$ OPCs in suspension, which were dissociated and plated to mature till day 75–90, when MBP^+ mature OLs started to appear [73]. Generated OPCs/OLs using this protocol were able to myelinate neurons *in vivo* and could be generated from MS patient lines. In line with this, Piao *et al.* showed the clinical applicability of hPSC derived-OPCs that were able to remyelinate and rescue an irradiated rat model of brain tumor radiation [74].

Although the last protocols reduced the required time needed for OPC/OL generation and increased the efficiency, the generation of OLs from hPSCs remained inefficient and required long differentiation times in culture, limiting the use of these cells. To circumvent these issues, we and others have developed specific protocols that notably increase the efficiency of OL generation and reduced the differentiation time [75,76]. We demonstrated that forced expression of *SOX10* alone leads to efficient OL generation within 20 days from hPSC stage, with generated OLs able to myelinate neurons both in *in vivo* and *in vitro* contexts. Moreover, hPSC-derived neuron-OL cocultures can be used for addressing the effect on myelin production of myelinating drugs in a high-throughput manner, serving this system as a platform where to test and/or validate the effects of candidate drugs [76].

Generation of microglia from hPSCs

The developmental origin of microglia has recently been elucidated, with few studies demonstrating that microglia arise from early yolk sac-derived hematopoietic progenitors, which migrate to the brain early in the development [77]. This fact has delayed the development of specific protocols to generate microglial cells from hPSCs. Several protocols were developed generating $CD14^+$ monocytes/macrophages from hPSCs [78,79]. These cells express phagocytic markers, are morphologically similar to blood-derived monocytes and macrophages, produce cytokines in a similar fashion as macrophages depending on the activation status of the cell and display high phagocytic capacity [80]. Other protocols tried to generate specifically microglial cells, but these protocols failed to resemble *bona fide* microglia since they did not recapitulate precisely microglial ontogeny [81,82].

In the past 2 years, a number of different protocols for generating microglial-like cells from hPSCs have been developed [83–86]. Although the methodology differed among the different approaches developed, these protocols have in common a first phase where early mesodermal progenitors are induced from hPSCs, followed by a maturation state where microglial-specific signaling pathways as IL34 and TGFb are induced [83–86]. This results in the generation of microglial-like cells resembling primary microglia at the transcriptome level, able to response to immune inducers and able to phagocytose amyloid-β, serving these cells as a platform for disease modeling and possibly drug testing [86].

Brain organoids

Much of our current understanding of human brain development and functioning relied on experimental studies performed mostly on mouse models, which do not fully mimic the features present in the human brain. Three-dimentional CNS organoids possess the unique advantage of recapitulating multiregional and region-specific cytoarchitecture seen in the early human fetal brain development, becoming a strong complement for studying brain development and pathology, to develop new therapies for treating neurodevelopmental diseases [87]. In the past few years, different groups have reported the generation of human brain organoids from PSCs. Lancaster *et al.* were the first to demonstrate that brain organoids from hPSCs can be generated that recapitulate the human brain development generating different discrete but connected brain areas defined by the expression of specific cell markers and transcription factors, which can be used as well for modeling microcephaly [88]. After this discovery, other groups have used this approach to generate brain organoids that have resulted useful for modeling different developmental diseases as autism spectrum disorders [89], schizophrenia [90], microcephaly induced by Zika virus infection [91] and others [87]. In addition, 3D culture systems have been proved useful for modeling neurodegenerative diseases as AD [92,93] and dys-/demyelinating diseases [94].

Use of hPSC-derived cells for drug screening & validation platform

For decades, drug discovery and validation relied on experimental testing of a certain number of compounds in animals to observe the effects produced on phenotypes, processes that were costly and time-consuming, allowing the test of a limited number of compounds. Advances made in chemical synthesis and automation platforms altered the paradigm of drug discovery, allowing the development of high-throughput screenings (HTS). HTS allow testing of hundreds or even thousands of compounds in a short period of time and with relative low costs. Classical HTS have been based on simple platforms where the effect of candidate compounds were tested on purified proteins, RNA, DNA or a simple mixture of a few molecules [95]. More complex platforms relied on the use of simple organisms (as yeasts) or cell lines that allow to study the effect of candidate drugs on alive organisms [96]. Although efficient, these assays usually present a major problem that is the poor validation rates of initially identified hits in *in vivo* models reproducing the disease, due mostly to the scarce biological relevance of the screening platforms related to the disease to treat and the use of non-human derivatives, which do not hold specific human biology. This problem may be circumvented by the use of more reliable models as screening platforms. In this sense, hPSCs may represent an excellent system where to perform drug validation and even being used for drug discovery. Although inefficient protocols for the derivation of specific cell types have precluded the use of this technology for drug testing [65], we believe recent literature indicates that it is the right moment to perform this technology translation, and several small biotech companies as well as big pharma industries are developing stem cell-derived platforms for drug testing.

Certainly, the use of hPSCs for drug testing has been earlier implemented in the case of hPSC-derived hepatocytes [97] and cardiomyocytes [98], possibly because the phenotypes to be observed after drug treatment are robust and easy to evaluate. For neurodegenerative diseases, this implementation has been slower due mostly to the inherent complex disease pathology, the inefficient protocols for the generation of neural cells and the lack of clear phenotypes to be evaluated. Next, we are going to discuss recent advances in disease modeling and drug screening for the main neurodegenerative diseases focusing in the employment of hiPSCs.

Alzheimer's disease

AD is the most prevalent neurodegenerative disease and form of dementia, characterized by the accumulation of amyloid-beta (Ab) plaques and TAU-laden neurofibrillary tangles, with huge impact on society [99]. The pathology of AD is very complex and still not fully understood, with a recently described key role for microglial dysfunction in the disease [100–102]. Due to this fact and because it is a noncell autonomous disease, the disease modeling with hPSC-derived cells in AD has been hindered.

Most of the studies performed have focus on reproducing the Ab pathology present in AD (aberrant extracellular accumulation of the Ab42 peptide). Several groups have demonstrated that neurons generated from iPSCs of AD patients show an increased secretion of Ab42 [103] and this platform can be exploited for testing of drugs diminishing this phenomenon. Yahata *et al.* in 2011 generated hiPSC-derived cortical neurons, able to produce Ab40 and 42 peptides. They inhibited the function of beta and gamma secretases (enzymes implicated in the synthesis of Ab) by employing beta- and gamma-secretase drug inhibitors as well as a nonsteroidal anti-inflammatory drug, finding that those drugs reduced Ab40–42 content [104]. Using a similar platform, Xu *et al.* tested over 100 drugs, finding that a Cdk2 inhibitor was able to reduce Ab toxicity [105]. Nonetheless, drugs targeting Ab production pathways have resulted inefficient in clinical trials [106], evidencing a more complex disease pathology. In this sense, Mertens *et al.* found that iPSC-derived neurons do not respond to clinical doses of nonsteroidal anti-inflammatory drug-based gamma-secretase modulation [107]. Kondo *et al.* established an iPSC-derived neuronal drug screening platform where they tested over 1000 compounds for their ability of reducing Ab load within the culture. They obtained 27 hits that resulted in six leading compounds and, to maximize the anti-Ab effect, a combination of three compounds (bromocriptine, cromolyn and topiramate) was selected as anti-Ab cocktail, which showed efficacy in reducing Ab levels in iPSC-derived neurons, suggesting that this approach could be translated to clinical trials [108].

Israel *et al.* demonstrated that neurons derived from iPSCs of both sporadic and familial forms of AD behave differently, with not only anomalous Ab expression but also aberrant expression of the aGSK-3b and hyperphosphorylated TAU forms, linking both Ab and TAU pathological hallmarks in iPSC models. In addition, treatment with b-secretase inhibitors led to a reduction in phosphorylated TAU and aGSK-3b levels [109]. TAU pathology is present in AD and other dementias, and iPSC-derived neurons with mutations in the *MAPT* gene showed altered TAU isoform expression, hyperphosphorylated TAU aggregates as well as different phenotypes associated with neurodegeneration [110]. Recently, we generated an iPSC-derived mutant TAU model that shows several phenotypes

associated with neurodegeneration, among them the accumulation of pathogenic TAU. The characteristics of this iPSC-derived model allow the use of this platform for drug screening purposes [36].

As mentioned before, AD is a noncell autonomous disease, with involvement of several neural cell types. In fact, iPSC-derived astrocytes show pathological phenotypes in those derived from AD patients in comparison with those derived from healthy subjects [62,63], although still this platform has not been translated into the drug discovery field. As mentioned earlier, only recently specific protocols for the generation of iPSC-derived microglial cells have been developed [83–86], which have impeded modeling the role that microglia has in AD pathology. iPSC-derived microglia express main immunological risk variants associated to sporadic AD and are activated after Ab treatment [86]. Moreover, iPSC-derived microglia are starting to be used as a model where to test the effect of genetic variants in the microglial functionality [111,112], which can lead to the discovery of novel disease-mediated mechanisms. Finally, due to the involvement of several neural cell types in AD pathology, few studies have reported the generation of 3D organoid cultures that do not only reproduce AD pathology, but are also sensitive to drugs reverting disease phenotype [92,113]. Nonetheless, these approaches are far for being translated to HTS for drug discovery.

Parkinson's disease

PD symptoms are caused by a specific loss of DA neurons in the ventral midbrain [114]. Due to this localized neuron loss, important efforts have been made to generate and evaluate the correct neural population to be transplanted into the putamen of assessed models. The use of neural cells from human origin for therapeutic purposes in terms of promoting recovery after injury or for cell replacement purposes has been long tested in PD, due to the specific neuronal population and region to be targeted. Already in the early 1980s, researchers demonstrated that transplantation of fetal DA neurons successfully engrafted and restored functionality in a PD rat model [115]. This and other studies paved the way for the translation of this approach to patients 10-years later, when fetal DA neurons were successfully transplanted in a severe PD case, which engrafted and improved motor functions [116].

Since then, numerous studies have been performed employing neural cells from different origins [117]. Among them, the transplantation of DA neurons derived from iPSCs is taking shape to become a therapeutic possibility for PD patients. In this sense, recent protocols for obtaining DA neurons from hPSCs have notably improved the efficiency of obtaining these cells [118]. Preclinical studies have demonstrated that generated DA neurons, after purification, are successfully engrafted in a PD rat model [49]. The clinical suitability of hPSC-derived DA neurons has been further demonstrated, since these cells were transplanted in nonhuman primates treated with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), engrafting and improving motor function, surviving for at least 2 years without evidences of tumor formation [51]. This therapeutic approach is currently being translated to PD patients in a Phase I clinical trial.

Several groups have generated DA from iPSCs of PD patients and healthy controls and compared altered pathways between them with the intention of use these cells for disease modeling (reviewed in [118]). When compared with healthy controls or PD patients from different genetic backgrounds, different altered pathways were identified, with some of them susceptible of therapeutic modulation.

Hartfield *et al.* described the generation of mature and physiologically active DA neurons, which not only formed functional synapses but were susceptible to the DA-specific toxin 1-methyl-4-phenylpyridinium (MPP^+) that reduced mitochondrial membrane potential and altered mitochondrial morphology, suggesting the usefulness of this system for the testing of drugs [119]. Moreover, iPSC-derived DA neurons from PD patients with familial forms of the disease are characterized for presenting several pathogenic phenotypes, suggesting that a combination therapy may be necessary for PD, employing patient-derived cells for its elucidation [120]. Therefore, for the development of effective therapies for PD, iPSC-derived platforms seem suitable to test candidate drugs, which will most likely result in a combination of cell and drug therapy for the treatment of PD symptoms and disease course.

Huntington's disease

Huntington's disease (HD) is an inherited neurodegenerative disorder caused by an expanded stretch of CAG trinucleotide repeats in the huntingtin gene that results in the loss of GABAergic neurons in the striatum [121].

iPSC lines from HD patients have been generated that after differentiation toward NSCs or neurons exhibited decreased cell adhesion and ATP production, increased apoptosis and cell death after prolonged culture or BDNF withdrawal, and increased vulnerability to stressors. Moreover, the severity of these disease-associated phenotypes are directly influenced by the extension of CAG repeats [122]. These phenotypes can be reversed after specific

CRISPR-Cas9-mediated gene correction [123], suggesting that those isogenic cell lines are particularly suitable for screening of drugs or identifying mechanisms that target phenotypes caused by the disease mutation [124].

Among the different phenotypes to be tested, aggregation of the mutated huntingtin protein has been the main readout employed for the search of effective drugs [125]. Another important phenotypes tested have been cell death and neuronal toxicity, as in HD, neuronal cell loss is observed in the cortex and striatum of patients [124]. Using this approach, several effective compounds have been identified, which need to be tested in more relevant HD models before translation to clinical trials for addressing their effectiveness to counteract HD [126]. In this sense, Nekrasov *et al.* reported in HD-derived neurons mutant huntingtin protein aggregation, increased number of lysosomes and autophagosomes and enhanced neuronal death during cell aging, with part of phenotypic reversal when cells were treated with a quinazoline derivative, EVP4593 [127]. In conclusion, iPSC-derived cells represent a more reliable platform where to perform screenings for finding drugs able to counteract pathogenic mechanisms associated to HD.

Amyotrophic lateral sclerosis

ALS is a fatal disease characterized by the degeneration of both upper and lower MNs that lead to death after 3–5 years of diagnosis [128]. Due to its devastating consequences and the lack of effective therapies counteracting effectively the disease progression, considerable actions are being taken for the development of effective therapies using different approaches. As mentioned before, several protocols have been developed for the specific differentiation of hPSCs into functional MNs [53,54,56,57,129,130].

Several groups have used iPSC-derived MNs as a drug screening platform using cells derived from familial forms of the disease with mutations in *TDP-43* [131,132], in the *C9ORF72* gene [133] and in the *SOD1* gene [37], which are the most common genes associated with familial ALS [134]. Among the different phenotypes encountered using iPSC-derived cells as a platform, several studies point out toward a disrupted membrane excitability in MNs derived from different ALS patients, suggesting that the development of effective drugs counteracting these phenotypes may have an important clinical relevance.

Burkhardt *et al.* generated iPSC-derived MNs from controls and sporadic ALS cases, finding that MNs derived from patients showed TDP-43 aggregates that recapitulate the pathology in *post mortem* tissue from one of the donor patients. Afterward, they established a high-content chemical screen measuring TDP-43 aggregation in MN-like cells, identifying an US FDA-approved small molecule (digoxin) showing efficacy [132]. Working with familial ALS cases with mutations in the *SOD1* gene, Kiskinis *et al.* developed a high-throughput functional characterization of iPSC-derived MNs using optogenetic techniques and the employment of isogenic genetically corrected iPSC lines, highlighting the suitability of this system as a simple readout to evaluate the effect of candidate drugs on disease phenotypes [135].

In addition, it is worthy considering that ALS is not a cell-autonomous disease, as other neural cell types as astrocytes [136] or OLs [137] contribute to disease pathology, so they should be considered as well for the development of reliable drug testing platforms.

MS & other myelin-deficient diseases

MS is an immune-mediated disease that leads to neuroinflammation, demyelination and neurodegeneration, leading to important and diverse functional deficits depending on the brain area affected [138]. In MS and other myelin-deficient diseases as leukodystrophies (group of congenital diseases where the structure and/or function of myelin is defective due mostly to mutations in myelin-related genes), one of the therapeutic strategies has been to develop specific drugs able to increase the myelin production by OLs within the CNS. Since this is a relatively easy phenomenon to be monitored, several approaches have been made to develop a reliable platform for drug screening purposes [139]. An important number of studies have developed myelinating platforms employing rodent primary OLs in combination with artificial homogeneous scaffolds [140] or presence of neurons [141]. Deshmukh *et al.* developed a HTS platform using primary rat optic nerve-derived OPCs, where they tested the effect of over 100,000 molecules on maturing these OPCs toward MBP^+ OLs. As a result of this HTS, they found several molecules that significantly improved the maturation of OPCs, but most of them were not adequate for their potential clinical uses as they had off-target activities, toxicity, poor brain exposure and/or demonstrated lack of *in vivo* efficacy. Therefore, the researchers focused on benztropine, a compound with a low effective concentration which is FDA-approved, orally available and crosses the blood–brain barrier, finding that benztropine improved remyelination in the experimental autoimmune encephalomyelitis model of MS [142].

Mei *et al.* developed a HTS-compatible drug screening system based on the capacity of primary murine OLs to surround and ensheath synthetic micropillars. Based on the expression of PDGFRa or MBP proteins, the authors tested the effect of 1000 bioactive compounds in the promotion of OPC (PDGFRa) or mature OLs (MBP), identifying a cluster of antimuscarinic compounds that enhance OL differentiation and remyelination, being the most efficacious compound clemastine [143], a widely available first-generation antihistamine, used for the treatment of seasonal allergies. Promotion of OL maturation induced by clemastine treatment showed beneficial in the cuprizone mouse model of demyelination [144] and for the treatment of hypoxic brain injury [145]. These evidences have led to the development of a crossover clinical trial where the researchers have evaluated the effect of clemastine on chronic MS patients, which showed efficacy in reducing delay in visual-evoked potentials [146].

Recently, *in vitro* myelination systems formed by OLs derived from murine ESCs have been developed [147], which are compatible with HTS platforms as well. Najm *et al.* employed mESC-derived OLs to constitute a drug screening platform. They tested over 700 FDA-approved drugs in their primary screen, which led to the identification of seven drugs that function at nanomolar doses selectively to enhance the generation of mature OLs (MBP^+) from progenitor cells *in vitro*. Among them, two drugs, miconazole and clobetasol, enhanced myelination in *ex vivo* and *in vivo* models. Moreover, both drugs, when administered at the peak of the pathology in experimental autoimmune encephalomyelitis models, lead to reversal of disease severity, suggesting that they could be used as therapeutics for the treatment of MS or other de-/dysmyelinating diseases [148]. In a continuation work, this group studied the mechanism of action of miconazole and other previously identified drugs promoting myelin production, finding that all those drugs blocked an enzyme called CYP51, encouraging stem cells to form new OLs. CYP51 is involved in cholesterol synthesis, and the boost in OL production appeared to be due to the accumulation of the cholesterol precursors 8,9-unsaturated sterols that, *per se*, leads to an increased OL maturation. Using OLs derived from primary tissue and hPSC-derived organoids, researchers newly screened over 3000 drugs for their ability to mature OLs, finding that the top ten all caused a buildup of 8,9-unsaturated sterols, having found a druggable therapeutic target for the treatment of MS [149].

These studies indicate that the development of myelinating platforms for drug screenings can lead to novel discoveries susceptible to be translated to the clinical practice, which would benefit from the existence of more human-relevant drug testing platforms. In this sense, we have recently developed a methodology for an efficient generation of a myelinating platform formed by all human PSC-derived neurons and OLs, which is compatible with a HTS setting where the effect of candidate myelinating compounds can be evaluated [76]. Recently, a 3D myelinating system has been generated, where validation of candidate drugs could be performed in a human model close to *in vivo* situation [94].

Since it is believed that restoring myelin integrity could, at least partially, reverse the clinical symptoms, cell transplantation strategies have been as well tested for MS and other myelin-deficient diseases. In initial studies, researchers used human fetal tissue from where they isolated functional OLs. In 1987, Gumpel *et al.* generated cell homogenates from human fetal brain and transplanted these cells into the myelin-deficient shiverer mice, resulting in an effective myelination [150]. Recently, several studies have described specific OL/OPC isolation from human tissue, both from fetal [151] and adult origin [152,153]. However, the limited access to human tissue, the limited expansion capacity of these cells *ex vivo*, together with cell heterogeneity, make these cells not desirable for clinical uses. Human tissue-derived neural stem cells (NSCs) have also been used to study remyelination and recovery in several mouse models. Brustle *et al.* demonstrated that NSCs derived from human tissue are able to engraft, migrate, differentiate to OLs and promote myelination after transplantation in rats [154]. Since then, different studies have reported that human tissue-derived NSCs are able to differentiate, myelinate and improve functioning in different animal models of dys-/demyelination [155–157]. In spite of the positive results obtained when somatic neural cells are transplanted in animal models of different neurodegenerative conditions, the limited access to the tissue, the poor yield in terms of cell number and the great heterogeneity among samples remains a major hurdle for the use of these cells in the clinical setting, so alternative sources for the procurement of OLs are highly desirable.

Conclusion

The discovery and development of hPSC technology, especially when combined with gene-editing strategies, represents a novel and more reliable platform helpful for neurodegenerative disease modeling, a system that can be employed as well for performing screenings to identify new therapies able to counteract disease course of these devastating pathologies.

Future perspective

Modeling of neurodegenerative diseases is a difficult task due to their complex and poorly elucidated pathology and the scarce access to human primary samples where to test hypothesis and candidate drugs. For these reasons, the development of hPSCs has revolutionized the field, though its technological progress has been slowed down due to the lack of efficient and robust protocols for the generation of specific disease-relevant neural cell types. Nonetheless, recent advances in the development of better protocols and the current existence of several iPSC banks from patients of selected neurodegenerative diseases with different genetic backgrounds should allow an extensive implementation of hPSC technology for disease modeling and drug testing efforts, which, most probably, will derive in better success rates of novel and effective therapies.

Executive summary

- Neurodegenerative diseases are complex pathological conditions and the elucidation of the underlying mechanisms has been precluded by the scarce access to human tissue and the lack of reliable models.
- Human-induced pluripotent stem cells (hiPSCs) represent an impactful technology for the generation of patient-specific relevant cell types to the disease.
- The development of effective gene-editing strategies as CRISPR-Cas9 together with iPSCs technology is allowing the elucidation of pathogenic pathways governing neurodegeneration.
- Since the discovery of iPSCs, specific and efficient protocols are being developed for the generation of the different disease-relevant neural cell types.
- The lack of efficiency and robustness for the derivation of iPSC-derived neural cell types has delayed the implementation of this technology as drug screening/validation platforms.
- In the past few years, iPSC-derived neural cells have started to be used as a platform where to test the effect of candidate drugs to counteract pathogenic mechanisms associated with neurodegenerative diseases.
- A review of the leading studies using iPSC-derived neural cells for disease modeling and drug screening platforms is provided for the main neurodegenerative conditions: Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and multiple sclerosis and myelin-deficient diseases.

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