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REVIEW ARTICLE

Induced pluripotent stem cells for disease modeling, cell therapy and drug discovery in genetic autonomic disorders: a review

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Abstract

The autonomic nervous system (ANS) regulates all organs in the body independent of consciousness, and is thus essential for maintaining homeostasis of the entire organism. Diseases of the ANS can arise due to environmental insults such as injury, toxins/drugs and infections or due to genetic lesions. Human studies and animal models have been instrumental to understanding connectivity and regulation of the ANS and its disorders. However, research into cellular pathologies and molecular mechanisms of ANS disorders has been hampered by the difficulties in accessing human patient-derived ANS cells in large numbers to conduct meaningful research, mainly because patient neurons cannot be easily biopsied and primary human neuronal cultures cannot be expanded.

Human-induced pluripotent stem cell (hiPSC) technology can elegantly bridge these issues, allowing unlimited access of patient-derived ANS cell types for cellular, molecular and biochemical analysis, facilitating the discovery of novel therapeutic targets, and eventually leading to drug discovery. Additionally, such cells may provide a source for cell replacement therapy to replenish lost or injured ANS tissue in patients.

Here, we first review the anatomy and embryonic development of the ANS, as this knowledge is crucial for understanding disease modeling approaches. We then review the current advances in human stem cell technology for modeling diseases of the ANS, recent strides toward cell replacement therapy and drug discovery initiatives.

Keywords ANS disease · Human pluripotent stem cells · Induced pluripotent stem cells · Embryonic stem cells · Disease modeling · Disease mechanism · Cell therapy · Drug discovery · Familial dysautonomia · Hirschsprung's disease · Neural crest · Stem cells · In vitro differentiation

Abbreviations

PNS	Peripheral nervous system
ANS	Autonomic nervous system
CNS	Central nervous system
ENS	Enteric nervous system
iPSCs	Induced pluripotent stem cells
hPSCs	Human pluripotent stem cells
hiPSCs	Human induced pluripotent stem cells
hESCs	Human embryonic stem cells
FD	Familial dysautonomia
HD	Hirschsprung's disease
NCCs	Neural crest cells
SAP	Sympathoadrenal progenitor
hOR-MSCs	Human olfactory ecto-mesenchymal stem cells
HIOs	Intestinal human organoids
GI	Gastrointestinal tract

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Introduction

The peripheral nervous system (PNS) consists of all nerves outside the brain and spinal cord (i.e. the central nervous system [CNS]), and is responsible for innervation of all organs of the body. It is divided into the somatosensory and autonomic nervous system (ANS); in simplified terms, these can be thought of as responsible for the voluntary and involuntary control of all organs. The somatosensory nervous system is responsible for transmission of signals from

the skin, skeletal muscles and sensory organs to the CNS. Both the somatosensory and autonomic neuron divisions of the PNS are derived from the embryonic neural crest, with the exception of the neurogenic placode giving rise to sensory neurons of the head and face (reviewed [1]). Both injuries to the ANS and defects during development result in diseases. Modeling disorders of the somatosensory and the ANS using human pluripotent stem cells (hPSCs) derived from patients provides a powerful platform for identifying therapeutic targets in a relevant context. Here, we review the latest advancements in the modeling and study of diseases of the ANS using hPSCs.

Anatomy of the ANS

The autonomic nervous system is divided into three parts: the enteric, the sympathetic and the parasympathetic nervous systems. The enteric nervous system (ENS) is responsible for motility of the gastrointestinal (GI) tract, secretion, absorption, blood flow and immunoregulation, among other functions [2]. It is sometimes referred to as the second brain, given the vast number of neurons it contains, the diversity in neuronal subtypes, and its complex cytoarchitecture [3]. The ENS is embedded in the intestinal lining and innervates the entire gastrointestinal tract. It has remarkable autonomy from the rest of the nervous system. To that end, the ENS has developed a large variety of neuronal cell types and employs over 30 different neurotransmitters. The sympathetic and parasympathetic nervous systems can be thought of as regulatory fight-or-flight and rest-and-digest states, respectively. However, their functions are much more complex. The anatomy of each division is arranged into preganglionic and postganglionic neurons. Preganglionic neurons have their cell bodies located within the spinal cord and project to postganglionic neurons located in ganglia along the anterior–posterior axis of the spinal cord. In the sympathetic nervous system, these ganglia are generally located close to the spinal cord and send long projections to their target organs, whereas in the parasympathetic nervous system, the ganglia are located close to or within their target organ. In the parasympathetic nervous system, both preganglionic and postganglionic neurons employ acetylcholine for neurotransmission. While preganglionic sympathetic neurons employ acetylcholine, postganglionic sympathetic neurons generally use norepinephrine for neurotransmission, with some exceptions. Notably, acetylcholine can be employed by postganglionic neurons in sweat glands and blood vessels, while renal vessels can use dopamine, which highlights the importance of the different target tissues for the function and anatomy of the ANS. Functions of the parasympathetic nervous system include slowing of the heart rate, stimulating digestion, relaxing sphincter muscles and sexual arousal. Examples of sympathetic nervous system functions are adaptation of the body to stress and threats, as well as during times of exercise.

Diseases affecting the ANS

A variety of causes can lead to ANS disease. (1) Environmental insults such as drugs and toxins, metabolic insults, traumatic injury and (auto)immune attacks lead to defective function of the ANS. These incidents are quite prevalent; for example, up to 50% of chemotherapy patients develop peripheral neuropathy [4], including autonomic symptoms. (2) Genetic mutations and rearrangements lead to ANS disorders called neurocristopathies. They arise from defective development of the neural crest [5], which is the embryonic progenitor giving rise to the PNS. These include neoplastic changes such as neuroblastoma and melanoma or genetic lesions causing Hirschsprung's disease (HD) or familial dysautonomia (FD). (3) Denervation of the ANS can be a first sign of other health problems. For example, cardiac sympathetic denervation [6] and enteric neuropathy [7] (constipation) are often a first sign and predictor of Parkinson disease. (4) Conversely, overactivity of the ANS can lead to disease as well. For example, sympathetic overactivity can lead to hypertension. It is estimated that 10–30% of patients with chronic hypertension exhibit drug resistance [8]. In such cases, treatment can involve surgical sympathetic denervation of the kidneys [9]. Thus dysfunction, hyper- or hypofunction, of the ANS can lead to disease.

Tools for studying the ANS

Traditionally available tools for studying ANS disorders have included animal models, postmortem tissue analysis and biopsies. These tools present specific advantages and disadvantages. First, animal model studies [10] have historically been instrumental in studying circuits, connectivity and regulation affected by diseases. However, identification of cellular and molecular pathologies is challenging, due to species differences and the fact that animal models often do not adequately mimic human disorders. Second, postmortem tissue analysis is a great tool for ascertaining important indicators of cellular abnormalities at the end stage of disease [11]. But while helpful in many instances, this technique is not well suited for studying the genesis and progression of diseases, where early diagnosis and intervention are important. Third, peripherally available patient biopsies such as blood or skin samples are used to study ANS diseases. In some genetic disorders, such as FD, this has enabled significant strides toward understanding the disease mechanism [12]. However, the use of these tissues for developing treatment options in ANS disorders is challenging, because the diseases do not typically manifest in blood or skin tissue. Therefore, to study the cellular, molecular and biochemical

pathophysiology of autonomic neurons, which are the ultimate target for pharmacological intervention, a large number of human patient-derived ANS neurons is required. This represents a significant roadblock for researchers, as these cells are difficult to obtain from biopsies in numbers large enough to conduct meaningful research, and they cannot be expanded in vitro. hPSCs offer an exciting, additional new tool for studying ANS disorders and for overcoming several of the above-mentioned disadvantages. These cells can be derived from patients, they can be expanded indefinitely in vitro and they can be differentiated in vitro into any cell type of interest, including ANS neurons.

Human pluripotent stem cells

Stem cells are divided into two classes, pluripotent stem cells and adult stem cells. Pluripotent stem cells are only present in the blastocyst of the early embryo and have the potential to develop into all cell types of the body via sequential development during which they lose potency and become more restricted to differentiate into specific cell types. Adult stem cells are present in the adult organism located in the niche of particular organs. They have limited potential for differentiation, such that they can only give rise to cell types related to their organ of origin.

Human embryonic stem cells

Human embryonic stem cells (hESCs) were first isolated from the blastocyst by James Thomson in 1998 [13]. They are characterized by two essential features, unlimited self-renewal and pluripotency. Their capacity for unlimited self-renewal means they can proliferate in the undifferentiated state indefinitely, thus enabling researchers to scale up their experiments indefinitely. Pluripotency, on the other hand, indicates that these cells can differentiate into all cell types of the embryo proper [13] (Fig. 1a). Thomson's discovery opened up the possibility for cell replacement therapy for degenerative disease and injury-based conditions. For example, in Parkinson disease, where midbrain dopaminergic neurons are lost due to degeneration, hESCs may be employed to produce midbrain dopaminergic neurons in vitro, which could then be transplanted in a cell therapy approach into

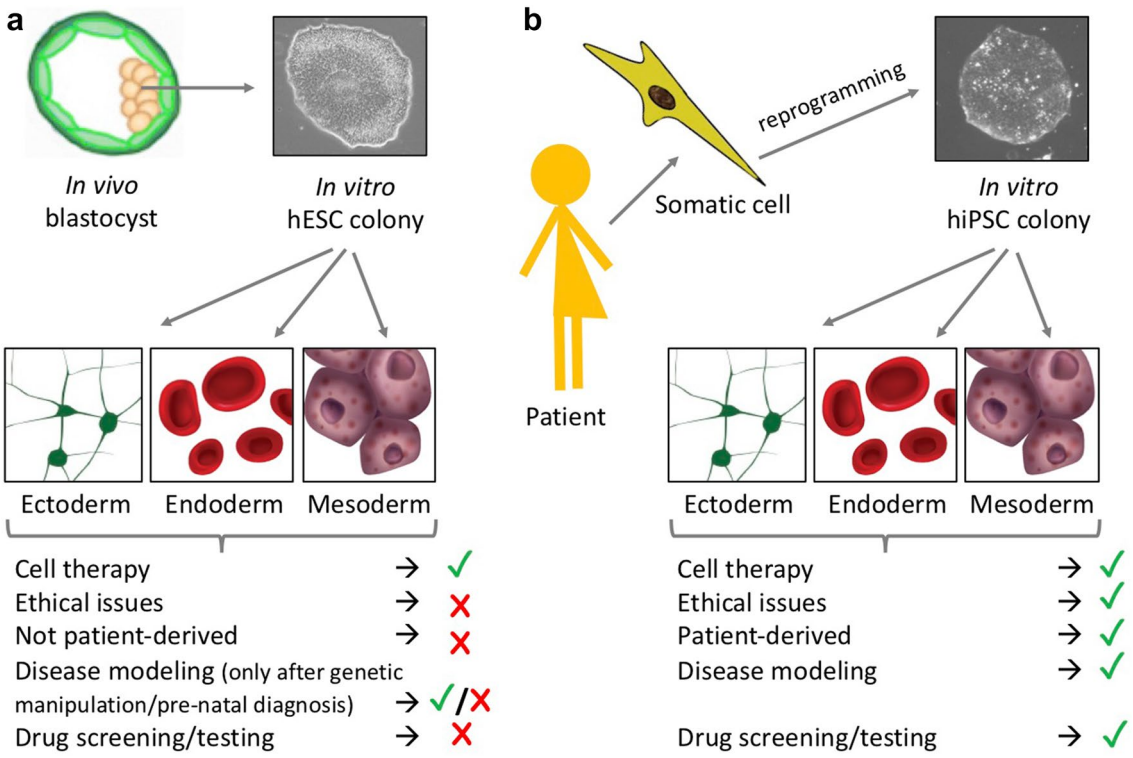

Fig. 1 Human pluripotent stem cells and their potential for disease modeling, cell replacement therapy and drug discovery. **a** Human embryonic stem cells (hESCs) are derived from the inner cell mass (yellow cells) of the blastocyst; they can be extracted and cultured in vitro indefinitely. They can be further differentiated in vitro into all cells of the ectoderm, endoderm and mesoderm lineages. **b** Human induced pluripotent stem cells (hiPSCs) are derived from a patient's biopsy of adult somatic cells, e.g. fibroblasts, via transduction with four transcription factors (SOX2, KLF-1, c-MYC, OCT4), called reprogramming. iPSCs and ESCs have the same characteristics and function similarly

the patient's brain to replace the lost cells [14]. This exciting technology, however, has some limitations. Ethical concerns around the destruction of a potentially viable human embryo for research purposes have restricted the use of these cells. Another limitation is the difficulty in obtaining hESCs from adult patients. Moreover, it is impossible to know whether the originating embryo in question would have developed the disease of interest later in life. Consequently, most early hESC-based studies presuppose a healthy genetic background. Exceptions around this problem have been made in a few studies, where the authors were able to obtain an embryo that was diagnosed via pre-implantation diagnostics with a known disease phenotype and donated for research purposes by the parents for the generation of hESCs [15]. Other exceptions include recently emerging genetically modified hESC-based disease models, where the disease-causing mutation is introduced into healthy hESCs (isogenic lines). This method allows researchers to isolate and study the effects of the particular mutation separate from the patient's genetic background [16]. However, to investigate novel disease mechanisms, it would be more clinically relevant to have the full genetic makeup of the patient in the model. Thus, the widespread use of disease modeling requires a system that allows the isolation of pluripotent stem cells directly from affected patients.

Induced pluripotent stem cells

In 2007, Shinya Yamanaka's group reported a method for reprogramming adult fibroblasts from a subject's biopsy into induced pluripotent stem cells (iPSCs) by delivering four transcription factors, KLF-4, SOX2, c-MYC and OCT4 [17]. The discovery garnered him the Nobel Prize and spurred a huge field of research development. In this review, we will refer to pluripotent stem cells as both hESCs and hiPSCs. Over the past 10 years, scientists have used and improved this system in many ways. For example, we now know that a variety of reprogramming factor combinations result in iPSC generation, and safe delivery methods, both viral and nonviral, have been developed to prevent insertional mutagenesis from the vector [18]. iPSCs can be reprogrammed from many different cell types, including blood [19], keratinocytes [20] and lymphocytes [21], and can be derived from various species [22]. One of the most powerful features of iPSC technology is the fact that it allows the derivation of pluripotent stem cells directly from patients of interest (Fig. 1b). Therefore, it offers a technology that provides researchers with unlimited numbers of cells, cells specifically derived from patients, and the possibility to differentiate those cells into the cell type of interest. Together, this facilitates disease modeling (Fig. 2), where iPSCs are generated from patients and healthy control subjects. Both are differentiated into the cell types affected in the disease

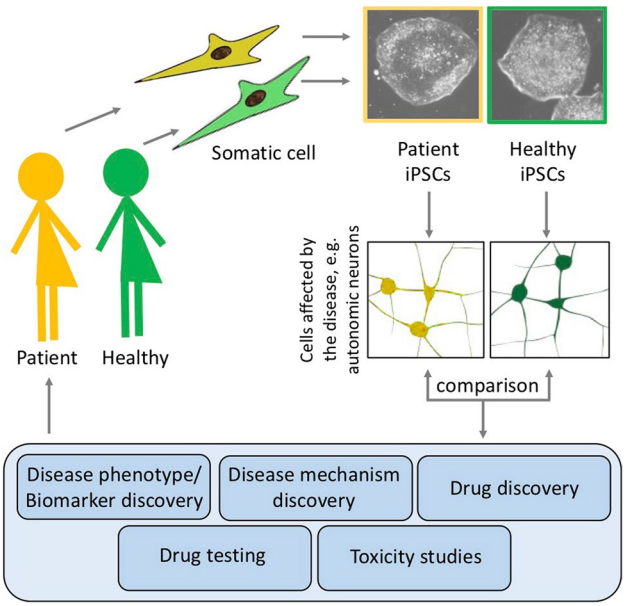

Fig. 2 Disease modeling using human pluripotent stem cells. Somatic cell biopsies (e.g. fibroblasts) from healthy subjects and patients are reprogrammed to iPSCs and differentiated into the cell types affected by the disease, e.g. autonomic neurons. Comparison of developmental capacity, functional activity, survival and other cellular, molecular and biochemical measures between healthy and diseased cell types enables the discovery of novel biomarkers and disease mechanisms. Furthermore, phenotypes that are discovered can be used for drug discovery or drug/toxicity testing, where the aim is to reverse disease phenotypes in the model

and compared in terms of developmental capacity, cellular functionality, and molecular and biochemical mechanisms. This further allows for high-throughput drug screening approaches and drug testing and toxicity evaluations, where a disease-specific phenotype is assessed in the presence of candidate drugs to evaluate whether a compound has the capacity to reverse the phenotype. This technique provides potential lead compounds for further investigation as a novel treatment option [23].

Generating specific cell types from hPSCs

To fulfill these expectations, specific hurdles have to be overcome. One of them is the knowledge bottleneck of how to differentiate the cell type of interest. This should be accomplished in an efficient and time-sensitive manner that is also feasible from a cost perspective and with respect to human-power. The subject of defining differentiation protocols has been fraught with reproducibility issues, which may partially stem from a lack of sufficiently detailed reporting, but also shows the inherent difficulty of this endeavor. Thus, most labs focus their expertise on a specific lineage or cell type, and very few labs do so for the ANS. There are currently three available approaches (and combinations

thereof) for developing an in vitro differentiation protocol to generate a specific cell type from hPSCs: monolayer differentiation, organoid differentiation and transdifferentiation (also called direct reprogramming). (i) In monolayer differentiation, hPSCs are dissociated and replated as single cells, where they are treated with specific combinations of growth factors, cytokines and/or small molecule-based activators/inhibitors targeting signaling pathways known to be important in vivo during embryonic development of the cell type of interest. To determine the identity and timing of such factors, two approaches can be taken. The first is based on screening for factors that will induce the required cell type or an upstream progenitor. For example, Chambers et al. aimed to generate peripheral sensory neurons in a time-accelerated manner. To this end, they screened a selected panel of small molecules that modulated developmental pathways, and searched for a combination of factors that would render the maximum numbers of neurons in a short period of time. They then carefully characterized the neurons to describe their exact cellular identity to be nociceptors [24]. The second, more commonly employed approach relies on our understanding of developmental processes occurring in model organisms. For example, Nostro et al. closely followed known developmental cues described in the mouse to dissect the equivalent steps in hPSCs toward the derivation of pancreatic precursors [25]. (ii) Transdifferentiation/direct reprogramming is aimed at changing the fate of one adult cell type into another without passing through the pluripotent stage. This can be done from patient cells either in vitro or in vivo (recently reviewed [26]), and promises the advantage of not erasing the epigenetic landscape acquired by environmental influences during the life of the patient/cell. However, the caveats are limited scalability and minimal control over subtype specificity. (iii) Three-dimensional, organ-mimicking organoids are being employed to study human embryonic development and disease development, and for certain disorders are particularly promising for cell replacement therapy. Organoids can be defined as stem cells (adult stem cells derived from the organ's niche or pluripotent stem cells) that spontaneously self-organize, differentiate and acquire functional aspects of the respective organ. This is achieved by instructive signaling cues given via the media, the extracellular matrix and the developing cell types themselves (recently reviewed [27]). To date, organoids have been developed mimicking tissues of organs including the prostate, cerebral cortex, intestine, liver and kidney. The disadvantages of organoid technology may be the large degree of variability in organoids as well as limited purity. Particularly with respect to the gastrointestinal (GI) tract and its enteric nervous system, however, organoid technology has become an invaluable tool for researchers and will be revisited later in this review.

Regardless of which route of in vitro differentiation is chosen for developing a cell type of interest from hPSCs, it is imperative that one have a detailed understanding of the developmental pathways leading to the establishment of the particular cell type. A deep understanding of the developmental processes leading to the establishment of the ANS is very important, not only for the generation of such cells from hPSCs, but also for the informed, critical evaluation of studies modeling ANS disorders. Thus, here we review the current understanding of the developmental process of the ANS.

Development of the autonomic nervous system

Origin: the neural crest

The three main embryonic tissue layers that develop into a full organism comprise the endoderm, mesoderm and ectoderm. These layers differentiate during early development in a process called gastrulation. The neural crest (NC) is a multipotent stem cell population induced in the ectoderm that arises at the junction of the neural tube and dorsal ectoderm during neurulation [28]. Recent advances in single-cell RNA sequencing in *Xenopus* and zebrafish have uncovered unique features of gene expression networks during development. For example, not all cell decisions are binary; multiple lineages can arise from the same pool of progenitors and exhibit hybrid developmental states, and transcription factors are reused at different developmental stages [29–31]. Although the NC arises from a defined ectodermal lineage [29], these findings underscore the complexity underlying NC differentiation. Multiple signaling pathways, including the sonic hedgehog (Shh), bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and Wnt, control NC differentiation. At this stage, NC cells (NCCs) express the markers snail1/2, foxd3, and sox10 [32–35], resulting in inhibition of cell–cell interactions [36], remodeling of the extracellular matrix [37] and changes in cytoskeleton organization [38, 39]. As a consequence, NCCs delaminate, dissociate and migrate throughout the developing embryo, guided by a combination of signaling molecules and the expression of a series of genes that allow them to interact with other cells and respond to external cues to guide them to their correct destination [40, 41] (Fig. 3a). Depending on the paths they follow, the NCCs give rise to multiple cell types, tissues and organs. Based on their contribution, the NC can be classified as cranial, vagal, trunk or sacral NC [42].

The cranial NC gives rise to the facial skeleton and glia. The vagal NC gives rise to the enteric nervous system. A subpopulation called cardiac NC contributes to the development of the valves and septa of the heart and other cardiac

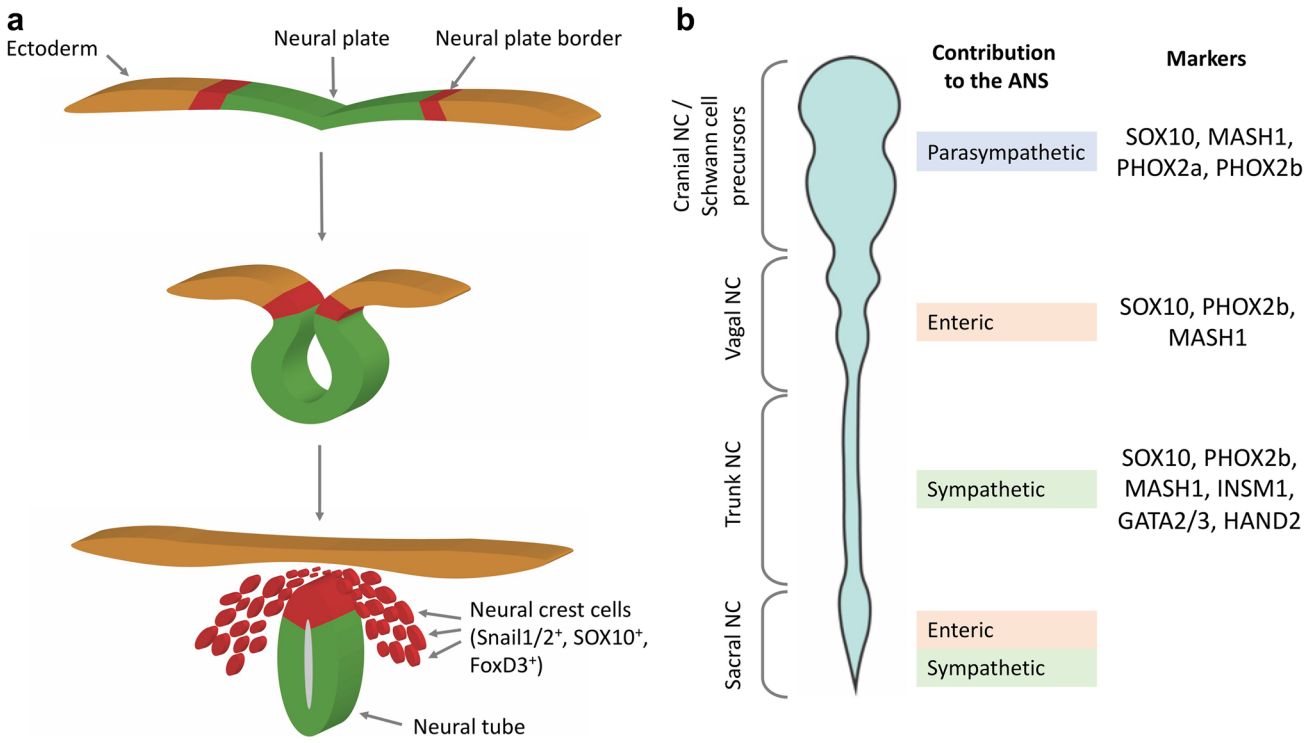

Fig. 3 Development of the ANS. **a** During neurulation, the neural plate folds to form the neural tube. As the neural plate closes, the neural crest cells (NCCs) begin differentiating, which triggers NCCs to delaminate, dissociate and begin migrating to their target tissues. **b** NCCs migrate through the anterior–posterior axis of the developing embryo. Depending on their migration pattern, NCCs differentiate into different derivatives (Schwann cell precursors, cranial, vagal, trunk and sacral NC). Each derivative contributes to different components of the ANS as well as other cells not depicted here. A summary of the markers expressed by progenitor cells of each ANS component is shown

tissues. The trunk NC gives rise to the dorsal root and sympathetic ganglia of the peripheral nervous system as well as chromaffin cells of the adrenal gland and melanocytes. Finally, the sacral NC is involved in the enteric nervous system development in conjunction with the vagal NC [43, 44]. These subpopulations of NCCs give rise to the regionalization of the anterior–posterior axis (Fig. 3b).

Development of the enteric nervous system

Developmentally, the majority of the ENS arises from vagal NC that derive from the dorsal neural tube and migrate ventrally to colonize the foregut at around embryonic week 4 in humans. Subsequently, the NCCs proliferate extensively and migrate caudally to colonize the entire GI tract by the seventh week of human gestation [45]. Vagal NCCs express the transcription factor *SOX10*, which is required to maintain an undifferentiated and proliferative state [46]. Once the vagal NCCs enter the gut mesenchyme, they express the transcription factor *PHOX2b* [47], which is required for the formation of the enteric ganglia [46]. *SOX10*- and *PHOX2b*-expressing NCCs migrate from the neural tube toward the foregut, where they are committed to an enteric lineage by the secretion of retinoic acid from the mesoderm. However, more recent studies using single-cell RNA sequencing suggest that the ENS derives from progenitor cells with gliogenic and neurogenic trajectories [48]. Retinoic acid, in conjunction with *SOX10* and *PHOX2b* [33, 47], activates the expression of the receptor tyrosine kinase *RET*, which is critical during enteric nervous system development [49]. Glial-derived neurotrophic factor (GDNF) then activates *RET*, which activates a signaling cascade promoting migration, proliferation and survival of enteric neuron precursors [46]. The transcription factor *MASH1* (also called *ASCL1*) is expressed upon arrival in the foregut and is critical for neurogenesis [45] (Fig. 3b). Endothelin receptor B (*EDNRB*) signaling opposes RET signaling by promoting vagal NCC proliferation and prevents their differentiation into neurons [50, 51]. Thus, a balance between *RET* and *EDNRB* signaling is necessary for proper development of the ENS. Indeed, mutations in *EDNRB* and its ligand, endothelin-3, have been identified in Hirschsprung's disease [52]. Additionally, netrin-1 and Shh (which regulate BMP4 expression), secreted by the intestinal epithelium, regulate migration and guide vagal NCCs to the developing distal intestine [53, 54]. Finally, BMP4 is important not only for cell migration, but

also for neuronal differentiation, and influences neuronal subtype diversity by promoting the development of $TrkC^+$ neurons [55].

Development of the sympathetic nervous system

The sympathetic nervous system is formed from trunk NCCs; however, certain ganglia are formed from vagal and sacral NC as well [56]. Trunk NC give rise to the sympathoadrenal progenitor (SAP), the precursor of sympathetic neurons, adrenal gland medulla (chromaffin cells) and small intensely fluorescent cells (SIF) [57]. Migration of the trunk NC is guided by secretion of factors by the target tissue [58]. The first trunk NCCs migrate toward the ventral middle section of the developing embryo, guided by ephrin-B ligands [59], followed by a dorsolateral migration [60]. BMP expressed from the dorsal aorta seems to be the major driver of sympathetic determination [61], along with neuregulin [62], in concert with intracellular signals such as cyclic adenosine monophosphate (cAMP) [63]. For decades, it was thought that sacral NC mainly generated parasympathetic neurons; however, recently it was shown that all sacral outflow is sympathetic [64]. The first transcription factor expressed in sympathetic neuron precursors is *MASH1* [65]. *MASH1* then promotes expression of *PHOX2b*, which is required to maintain *MASH1* and is critical for the development of sympathetic neurons [47]. *PHOX2b* activation promotes expression of *INSM1*, which in turn down-regulates earlier markers such as *MASH1* [66]. Finally, expression of the *GATA* family of transcription factors occurs late during sympathetic development. *GATA2* and *GATA3* are expressed in the sympathetic ganglia and are responsible for activating the noradrenaline-synthesizing enzymes [44] (Fig. 3b). The transcription factor *HAND2* plays important roles in the expression and maintenance of dopamine β-hydroxylase and tyrosine hydroxylase (required to process norepinephrine and dopamine, respectively) [67], however its exact position in the hierarchy of the regulatory network remains unclear. The final phenotype of sympathetic neurons, including the neurotransmitters, neuropeptides, and receptors, is influenced by the target tissue [60].

Development of the parasympathetic nervous system

Traditionally, it was thought that neurons of the parasympathetic nervous system arose from cranial and sacral NC [68]. In that line of thought, cranial NC gives rise to postganglionic neurons and glial cells of the cranial, cardiac and gut-innervating parasympathetic ganglia. The parasympathetic ganglia in the head and face arise from cranial NCCs that migrate ventrolaterally from the midbrain and hindbrain toward the optic vesicle [69], whereas the cardiac parasympathetic ganglia arise from cranial NCCs that migrate dorsolateral to the somites and travel in the mesenchyme associated with the aorta [60, 70]. Parasympathetic ganglia that innervate the lungs, pancreas and gall bladder come from a subpopulation of cranial NC that migrate from the hindbrain along the gut [60]. Sacral NC give rise to postganglionic neurons in the pelvic ganglia [60]. However, doubt was recently cast on this developmental origin of the parasympathetic nervous system, at least in the mouse, partially due to the discordant timing of parasympathetic neuron emergence (mouse E12.5) and NC migration to their ganglia (mouse E9-10). Using selective nerve ablation, two elegant studies spearheaded in 2014 [71, 72], postulate that parasympathetic neurons arise from Schwann cell precursors that travel along preganglionic nerves to their respective ganglia destination, rather than from vagal and sacral NCCs. The developmental origin of these Schwann cell precursors, however, remains unclear. Furthermore, as stated above, sacral NC are shown not to produce parasympathetic neurons, instead giving rise to sympathetic neurons [64]. Similar to sympathetic ganglia, BMP signaling induces the expression of *MASH1*, *PHOX2b* and *PHOX2a* in parasympathetic ganglia [73]. Neurturin, GDNF and RET signaling are required for proper migration and differentiation of parasympathetic neurons [74]. As a consequence, parasympathetic ganglia express dopamine β-hydroxylase and tyrosine hydroxylase [60]. However, *HAND2* or *GATA2* are not expressed in parasympathetic ganglia [73] (Fig. 3b).

Development and specification of the ANS is a complex and tightly regulated process. Mutations leading to mis-regulation of these steps results in various neuropathies. Thus, understanding the mechanism driving ANS neurogenesis is necessary to identify therapeutic targets and develop therapies.

Modeling autonomic nervous system diseases with hPSCs

Differentiation of ANS cell types from hPSCs

The exciting discovery of iPSCs has quickly spurred many research groups to work toward modeling diseases unique to their expertise. However, it has become apparent that one of the main bottlenecks impeding success is the differentiation of specific cell types that can then be investigated to reproduce disease-specific phenotypes in vitro. Particularly for developmental disorders, such cell types should ideally be available at various developmental stages, thereby allowing the study of disease progression. For degenerative disorders, on the other hand, mature stages are preferred, since these are ideal for drug discovery, and they represent the target in patients. All cell types in the ANS are derived from the

NC; thus the first cornerstone for modeling disease of the ANS was laid in 2007 when Lee et al. reported the differentiation of NCCs from hESCs [75]. This was followed by several works that refined the technique in terms of efficiency and purity [76–79]. These hPSC-derived NCCs have migration capacity, express appropriate marker genes and have the ability to differentiate into NC derivatives. However, it remained unclear from which NC subtype they originated, i.e. whether they had cranial, vagal, trunk or sacral NC identity. One way to distinguish NC subtypes is based on expression of the *HOX* gene code, which indicates location along the anterior–posterior (head–tail) body axis. For example, cranial NC do not express any *HOX* genes, while vagal NC express *HOX 3–5*, trunk NC express *HOX 3–11* and sacral NC express *HOX 3–13* [80]. Thus, subsequent studies focused on the generation of such specific NC subtypes and their further differentiation into cell types arising thereof. For example, Mica et al. derived trunk NC-like cells and further defined conditions for generating melanocytes from them [81], whereas Fattahi et al. produced vagal NC giving rise to enteric neurons [82], which they further employed to investigate treatment options for Hirschsprung's disease (HD, more on this study below). Reports have only recently emerged regarding the generation of sympathetic neurons using various differentiation strategies and degrees of characterization [83–86]. Only one of those studies has been used to model an ANS disorder, familial dysautonomia [83]. Interestingly, differentiation of parasympathetic neurons from hPSCs has not yet been attempted, and thus no disease involving these cells has been investigated to date. Here, we discuss in detail the studies that generated ANS cell types from hPSCs and employed those to study specific ANS disorders. We divide the studies into three categories based on their achievements in discovery of novel aspects of disease mechanisms, cell therapy approaches or drug discovery (Table 1).

Disease mechanistic discoveries

Familial dysautonomia (FD) is one of the most extensively studied ANS disorders. FD is caused by poor development and survival and progressive degeneration of sensory and autonomic neurons [87], resulting in symptoms such as defective lacrimation, excessive sweating, hypertension, defective pain sensation and difficulties regulating the physiological stress reaction. In 2001 it was discovered that 99.5% of all FD patients harbor a mutation in the *IKBKAP* gene that leads to a splicing defect [88, 89]. The result is dramatically lowered wild-type IKAP protein. For reasons yet to be elucidated, this selectively affects neural tissues [12]. IKAP protein (also called ELP1) is the scaffolding protein of the transcriptional elongator complex [90] that is primarily involved in transcriptional acetylation and elongation as well as tRNA modification [91]. Interestingly, IKAP has been implicated in many cellular processes, and it remains to be determined how those processes interact to cause FD symptoms and whether there are additional factors leading to FD pathology. Despite great strides toward the mechanistic understanding of FD, several issues have posed difficulties. It is difficult to study both the developmental defects and the pathology of sensory and autonomic neurons derived from patients. Thus, FD is the ideal disease to investigate using hPSCs, which was first reported by Lee et al. in 2009. They reprogrammed fibroblasts from three FD patients into iPSCs and showed tissue-specific splicing defects in *IKBKAP*, defective migration and neural differentiation potential of FD-NCCs. The model was also used to validate kinetin as a potential therapeutic compound, showing that it increased the splicing ratio toward wild type in NCCs and significantly increased the NCCs' capacity to differentiate toward peripheral neurons. However, kinetin treatment had these effects only when it was used during the entire differentiation period, and not when it was used for 1 or 5 days after the NC emerged, suggesting that kinetin treatment would be most effective in utero, and its effects may be limited after birth [92]. Lefler et al. and Valensi-Kurtz et al. subsequently used a hESC line derived from a pre-implantation embryo that was determined to be mutant for *IKBKAP*. They analyzed NCCs and neurons derived from that FD-hESC line and confirmed NC migration defects. They further showed that in FD, IKAP protein in neurons is not correctly co-localized with vesicular proteins. Transcriptomic analysis of human fetal tissue in conjunction with the FD-hESC-derived neurons confirmed that synaptic and vesicular transport genes were affected by reduced IKAP levels, suggesting that IKAP may function as a vesicular-like protein involved in neuronal transport. Furthermore, the authors showed that kinetin increased IKAP as well as its associated protein levels [15, 93].

In 2016, Zeltner et al. followed with a study showing that disease severity differences seen in FD patients could be accurately reproduced in the iPSC model [83], proving the exceptional sensitivity of the hPSC technology and providing a tool that led to further elucidation of the FD disease mechanism. FD presents an interesting phenomenon, where 99.5% of all patients harbor the identical homozygous point mutation in *IKBKAP*, yet some patients present with much more severe symptoms than others. This work aimed at reproducing this phenomenon in vitro. iPSCs were reprogrammed from FD patients with severe or mild symptoms, based on three criteria: (i) subjective assessment of the patient's disease severity by their physician, (ii) the degree of pain insensitivity and (iii) the frequency of dysautonomic crisis. Surprisingly, it was found that development of NCCs, sensory and autonomic neurons from mild FD patients was intact, whereas development in severe patients was defective at each cell type and developmental stage. Further, it was

Table 1 Selected studies that employed hPSCs to model ANS disease

Year	Title	Author	Journal	Disease	Summary	Disease modeled	Disease mechanistic discovery	Drug discovery	Cell therapy
2009	Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs	Lee and Studer	Nature	Familial dysautonomia (FD)	They generate iPSCs from FD patients, show *IKBKAP* splicing, migration and differentiation potential defects in iPSC-derived neural crest cells (NCCs). Validate kinetin in FD-NCCs	Yes	Validation of FD-NC defects (migration, differentiation)	Validation of kinetin in FD-NC cells	No
2010	Enriched population of PNS neurons derived from human embryonic stem cells as a platform for studying peripheral neuropathies	Valensi-Kurtz and Weil	Plos One	Familial dysautonomia	They generate sensory neurons from healthy and FD-hESCs and characterize IKAP localization	Yes	No	No	No
2012	Large-scale screening using familial dysautonomia induced pluripotent stem cells identifies compounds that rescue *IKBKAP* expression	Lee and Studer	Nature Biotechnology	Familial dysautonomia	They generate FD-NC cells, miniaturize culture conditions, screen 6912 chemical compounds for *IKBKAP* splicing. Validate hits for splicing, migration and differentiation potential in NC. Top hit SKF86466 was found to induce *IKBKAP* transcription via cAMP and pKA-dependent CREB phosphorylation	No	No, but mode of action of hit compound is assessed	Yes	No
2015	Familial dysautonomia (FD) human embryonic stem cell derived PNS neurons reveal that synaptic vesicular and neuronal transport genes are directly or indirectly affected by *IKBKAP* downregulation	Lefler and Weil	PLOS One	Familial dysautonomia	hESC line is made from preimplantation embryo and differentiated into NC and PNS neurons. They confirm FD-NC migration defect, *IKBKAP* missplicing in neurons. Discover reduced intracellular localization of IKAP and vesicular proteins. Vesicular and neuronal transport genes are affected by reduced IKAP	Yes	Yes	No	No

Table 1 (continued)

Year	Title	Author	Journal	Disease	Summary	Disease modeled	Disease mechanistic discovery	Drug discovery	Cell therapy
2015	Modeling pain in vitro using nociceptor neurons reprogrammed from fibroblasts	Wainger and Woolf	Nature Neuroscience	Pain and familial dysautonomia	They use 5 transcription factors to generate nociceptive sensory neurons directly from fibroblasts via direct reprogramming. Show chemotherapy- and inflammation-induced hypersensitivity of nociceptors (pain). Show that FD direct reprogrammed nociceptors are made inefficiently, have shorter and fewer neurites	Yes	Yes	No	No
2016	Deriving human ENS lineages for cell therapy and drug discovery in Hirschsprung disease	Fattahi and Studer	Nature	Hirschsprung's disease	They generate vagal/enteric-NC and enteric neurons. Inject NC into adult mouse colon, show migration, differentiation and survival of enteric neurons. NC injection into HD mouse colon rescues disease-associated defects. A high-throughput chemical screen identifies pepstatin A to rescue migration defects in *EDNRB*-/-NC cells both in vitro and in vivo after 72 h exposure and rescues survival and gut transit time	Yes	No	Yes	Yes
2016	Functional coupling with cardiac muscle promotes maturation of hPSC-derived sympathetic neurons	Oh and Lee	Cell Stem Cell	Sympathetic-cardiac connection	They generate hESC-based reporter lines for *SOX10*, *ASCL1* and *PHOX2B* to track and purify cells differentiating into sympathetic neurons and characterize them extensively. Co-culture of sympathetic neurons with murine cardiomyocytes shows the neuron's ability to stimulate myocyte contraction and maturation of the neurons	No, but connection between cardiomyocytes and sympathetic neurons is modeled	No	No	No

Table 1 (continued)

Year	Title	Author	Journal	Disease	Summary	Disease modeled	Disease mechanistic discovery	Drug discovery	Cell therapy
2016	Capturing the biology of disease severity in a PSC-based model of familial dysautonomia	Zeltner and Studer	Nature Medicine	Familial dysautonomia	iPSCs from FD patients with severe or mild disease are generated and phenotypes in NC, sensory and sympathetic neuron development, functionality and degeneration are assessed. Mild FD patients have degenerative, but not developmental defects. Severe patients have both developmental and degenerative defects. *IKBKAP* mutation is genetically reversed and whole exome sequencing reveals a modifier mutation in *LAMB4* in severe FD patients	Yes	Yes	No, but platform established to assess drugs for rescue of degenerative phenotypes	No
2017	Correction of Hirschsprung-associated mutations in human induced pluripotent stem cells via clustered regularly interspaced short palindromic repeats/Cas9, restores neural crest cell function	Lai and Ngan	Gastroenterology	Hirschsprung's disease	They generate iPSCs from short-segment HD patients with unknown mutations. By combining genetic and transcriptomic approaches, they identify a novel mutation in the vinculin gene that leads to differentiation and migration defects in enteric NC	Yes	Yes	No	No
2017	Engineered human pluripotent-stem-cell-derived intestinal tissues with a functional enteric nervous system	Workman and Wells	Nature Medicine	Hirschsprung's disease	They generate HIOs from hPSCs that contain enteric NCCs. They show that when transplanted in vivo, the HIOs form 3D neural plexus that mediates contractile activity. They model HD caused by a *PHOX2B* mutation with HIOs and NCCs	Yes	No	No	No

Table 1 (continued)

Year	Title	Author	Journal	Disease	Summary	Disease modeled	Disease mechanistic discovery	Drug discovery	Cell therapy
2017	Novel mutations in *dystonin* provide clues to the pathomechanisms of HSAN-VI	Manganelli and Santoro	Neurology	HSAN-VI	They describe a family of 3 affected siblings who have sensory (pain) and autonomic disturbances and lack of those nerve fibers. Identify novel mutations in the dystonin gene. iPSCs differentiated to neurons show low levels of dystonin protein and short/no neurites	Yes	From clinical and sequencing but not iPSC data	No	No

Studies listed were selected based on a PubMed search with the following search terms: pluripotent stem cells OR embryonic stem cells OR induced pluripotent stem cells OR iPSC AND enteric neurons OR sympathetic neurons OR parasympathetic neurons OR genetic autonomic disorder OR HSAN. Studies were included when they contained PSC-derived autonomic neurons and when they addressed modeling of an ANS disease, disease mechanism discovery, drug screening or cell therapy approaches. Studies that did not address such criteria were excluded, for example, studies employing non-pluripotent stem cells, non-human stem cells, when ANS cells were made but no disease was modeled, or infectious disease was modeled in organoids that did not contain ANS cells. Only a few exceptions were made to those strict criteria: Wainger et al. generated sensory, not autonomic, neurons; however, they modeled FD. Oh et al. did not model a disease, but they reported an important co-culture system that may lead to enhanced ANS disease modeling. Authors are cited as first and last author

possible to show that both mild and severe FD-derived sensory neurons degenerated (died) in vitro over time, together suggesting that severe FD patients suffer both developmental and degenerative defects, while mild patients suffer only degenerative defects. This study also established a drug validation platform that enabled the validation of the positive effects of kinetin and SKF86466 (described below) on the survival of sensory neurons derived from mild FD patients, which could prove important for testing of other compounds in the future [83].

In the second part of the story, Zeltner et al. used the FD model to understand the mechanistic basis of severe FD. Interestingly, by genetically correcting the *IKBKAP* mutation, they was found that *IKBKAP* splicing could not explain severity differences, and they concluded that there must be a secondary player important in FD. Whole-exome sequencing of the three severe and three mild FD patients finally revealed that all three severe patients harbored a modifier mutation in the *LAMB4* gene. Lamininβ4 is an extracellular matrix protein, highly expressed in sensory and autonomic ganglia, and laminins are crucial for important processes affecting FD development, including cell migration, adhesion, differentiation and neuronal development. Together, these results suggest that severe FD is influenced by the *LAMB4* modifier mutation, which is absent in mild FD patients. It will be important to assess the frequency of the *LAMB4* mutation in the general FD population and further elucidate its mechanism of action. These examples highlight the powerful tool that hPSC technology offers for increasing our understanding of FD.

A few other studies have worked toward establishing cellular models to study ANS or related disorders. Oh et al. did not explicitly model a disorder, but through their extensive characterization of sympathetic neurons generated from hPSCs and co-culture of such neurons with mouse cardiac tissue, they established a platform to study cardiac regulation by the sympathetic nervous system [84]. This work further highlighted the importance of target tissue innervation by sympathetic neurons, in that they showed the vastly improved neuron maturation and functional response to stimulants and inhibitor that cultures without target tissues have rarely achieved. Wainger et al. used direct reprogramming to generate pain-sensing nociceptors directly from fibroblasts and employed them to show inflammation- and chemotherapy-induced hypersensitivity, laying the groundwork for studying molecular mechanisms involved in these processes. They were also able to transdifferentiate FD fibroblasts and show *IKBKAP* splicing phenotypes [94]. Several studies have employed human olfactory ecto-mesenchymal stem cells (hOR-MSCs), an adult stem cell population that can be extracted via biopsy from the patient's nasal mucosa and has the potential to differentiate into olfactory neurons, glia and oligodendrocytes [95, 96]. Transcriptional analysis of

FD hOR-MSCs revealed dysregulated genes in FD [96–98]. They found that overactivity of the 26S proteasome lowered IKAP levels in FD patients, leading to the suggested use of proteasome inhibitors for FD [99]. Manganelli et al. [100] generated iPSCs from patients with hereditary sensory and autonomic neuropathy type VI (HSAN-VI) and showed that the mutation in the dystonin gene that they identified leads to lowered protein and shortened neurites. Lai et al. used iPSCs derived from patients with short-segment HD, the most common form of HD, which is usually not associated with known mutations. By combining genetic and transcriptomic data, they identified a novel mutation in the vinculin gene that leads to differentiation and migration defects in patient-derived enteric NC [101].

These studies show the high value of hPSC technology for investigation and elucidation of disease mechanisms affecting ANS disorders. Such knowledge will spur the rational design of novel drug and treatment options. Furthermore, it simultaneously provides in vitro screening platforms on disease-relevant cell types that can be employed for drug testing and toxicity studies as well as drug discovery.

Drug discovery

Lee et al. conducted one of the first high-throughput drug screening approaches in an hPSC-based disease model [102], using their previously established FD model based on NC-specific phenotypes [92]. The rationale for using NCCs is supported by tissue-specific (neural) missplicing of *IKBKAP* in FD [89]. After adapting NCC culture to a 384-well high-throughput screening platform, they treated FD-iPSC-derived NCCs with 6912 chemical compounds from several libraries. The screen readout was based on increased *IKBKAP* splicing in a qRT-PCR assay. Eight hit compounds were further pursued, of which several reversed the FD-specific *IKBKAP* splicing defect after short-term (48 h) treatment. However, to rescue the neural differentiation defect, the compounds had to be present throughout the NC differentiation process. None of the compounds were able to rescue the migration defect, which remains unexplained. The top hit SKF86466, an α2-adrenergic receptor (α_2AR) antagonist, and its analogs yohimbine and imiloxan increased *IKBKAP* expression, whereas its agonist xylazine decreased it. SKF86466 was confirmed to induce *IKBKAP* expression via α_2AR signaling and cAMP-mediated activation of pKA and pCREB. Notably, the effect of SKF86466 is different from that of kinetin, as it does not change splicing efficiency, but rather increases overall *IKBKAP* expression. A note of caution, however, about SKF86466 is warranted. Its analog yohimbine stimulates norepinephrine release. FD patients in crisis have elevated norepinephrine and are treated with clonidine, which has the opposite effect, i.e. it lowers norepinephrine release. Thus, yohimbine/SKF86466 have the potential to be detrimental in this patient population. Nevertheless, this work established the proof of concept that an hPSC-based disease model can be instrumental in drug discovery, and subsequently opened the door for such approaches to be employed for various others diseases, including FD. Another drawback of a drug discovered in NCCs is that to be relevant for improving the patient's condition, it likely must be given in utero in order to positively influence embryonic development. Thus, such a drug may not be practical in preventing or halting ongoing degenerative changes, nor will it reverse current phenotypes. Therefore, it is important to develop drug screening platforms of cell types that are present and affected in patients after birth. Zeltner et al. provided such a platform in sensory neurons from FD patients, where the authors were able to validate the positive impact of current drug candidates kinetin and SKF86466 on degeneration of sensory neurons [83].

Fattahi et al. discovered a drug for the potential treatment of HD using an hPSC model [82], which is described in more detail below. Intestinal human organoids (iHOs, more on this below) are being explored for modeling human pathogen infections such as rotavirus [103], *Clostridium difficile* [104] and *Salmonella enterica* [105], with the goal of establishing a platform for the study of infection mechanisms and screening/testing drugs for prevention or treatment. Together, these studies show that hPSC disease models can be instrumental for screening and validating new and existing drugs for ANS disorders.

Cell therapy

Hirschsprung's disease (HD) is the most common genetic enteric neuropathy and arises from defective migration of enteric neural crest cells, leading to the functional obstruction of the distal part of the colon [2]. It is treated by surgical resection of the distal aganglionic colon, which reduces mortality but does not resolve all symptoms [106]. Currently, there are two approaches being developed for treatment of HD and other intestinal disorders involving hPSCs. The first is transplantation of enteric neural crest cells for restoration of the missing enteric ganglia in the distal colon. Fattahi et al. showed the derivation and extensive characterization of vagal NC from hPSCs, by induction with retinoic acid. Vagal NCCs were found to express appropriate *HOX* genes as well as specific genetic markers, and migrated correctly in chick transplants. These cells were able to be further differentiated via enteric NC into a variety of enteric neurons following a 4-day expansion period as neural spheres. Enteric neurons expressed appropriate genetic markers at high efficiency and showed expression of a variety of neurotransmitters and other characteristics for the enteric nervous system. Additionally, these neurons mediated contraction of smooth muscle (also generated from hPSCs) by means

of co-culture and optogenetic stimulation, demonstrating functional connectivity between enteric neurons and smooth muscle. The authors then transplanted enteric NC into the proximal colon of adult mice and showed migration along the colon, differentiation into enteric neurons, integration into the myenteric and mucosal layers, and proper survival of the neurons. They further investigated this transplantation paradigm in an EDNRB$^{s-l/s-l}$ HD mouse model, where they were able to show that after injection of enteric NC into the colon, all mice survived. Enteric NC migration and differentiation into enteric neurons was found to occur, as well as integration into the mucosa, and finally, the gastrointestinal transit time in grafted mice improved compared to control injected animals. In an effort to develop patient-matched cell therapy for HD, the authors further conducted a drug screen designed to reverse the NC migration defect. They first generated an *EDNRB* knockout hESC line that was defective in migration. They then conducted a high-throughput screen based on restoration of in vitro migration (scratch assay). Pepstatin A, an acid protease inhibitor that acts through *BACE2*, was found to rescue migration in vitro. Furthermore, when *EDNRB−/−* enteric NC were treated for 72 h with pepstatin A before transplantation into the adult colon, migration in vivo was rescued as well [82]. This extensive study provides a novel combination of pharmacological and cell therapy treatment options for HD patients. Additionally, it offers a platform for testing of drugs affecting the GI tract.

The second approach involving the use of hPSCs in treating intestinal disorders entails the generation of intestinal organoids (HIOs) for transplantation into HD patients. HIOs, cellular aggregates generated from hPSCs, self-organize and expand into a 3-D intestinal tissue [107, 108]. When transplanted to the mouse kidney capsule, they develop an array of cell types and structures normally found in the GI tract, including crypts and villi, intestinal stem cells and smooth muscle [109]. Bioengineered and transplanted HIOs have similarly been shown to develop into tissue nearly identical to adult intestine [110]. However, none of the HIO enteric neurons developed, and thus contractile activity was not achieved. Workman et al. addressed this limitation by combining NCCs generated from hPSCs in vitro with HIOs. They first generated vagal NCCs by the addition of retinoic acid, similar to previous reports [82]. Via centrifugation, the vagal NCCs were incorporated into the HIOs and further developed for 1 month in vitro, leading to the emergence of neurons and glia integrated into the myenteric and submucosal layers of the HIOs. However, only after transplantation under the mouse kidney capsule did the HIOs + enteric NC mature and develop ganglia, neuroglial networks within the smooth muscle layer and inhibitory neurons, which restored contractile activity. Conversely, the presence of the ENS promoted development and maturation of the intestinal cell types. Workman et al. then used *PHOX2B* (a mutation found in HD patients) mutant hPSCs to show that enteric NC do not migrate or differentiate properly in HIOs [111]. Schlieve et al. followed this work with hPSC-derived bioengineered HIOs co-implanted early with enteric NCCs in vivo, and showed additional maturation of both tissues [112].

Together, these papers advance the field into an era where cell replacement therapy can become a tangible possibility, particularly for patients with HD and other intestinal disorders. In addition, both systems provide exciting platforms for drug screening, toxicity testing and the testing of novel treatment approaches, while expanding our current understanding of the mechanistic details of the modeled disorders.

Conclusions

Despite the fact that hPSC technology is still in its infancy, the work published to date highlights its great potential and promise to move the field of ANS disorders and treatment approaches forward. The exciting studies reviewed here also demonstrate that this technology is a valuable asset for advancing multiple biological disciplines, including disease mechanism studies, drug discovery and testing, and finally cell therapy.

Since the discovery of PSCs, great strides have been made toward improving the technology (reviewed in [113]). However, there are several challenges and roadblocks that still must be overcome before reaching the goal of novel drugs or therapies for patients with ANS disorders. The establishment of in vitro differentiation protocols to make a particular cell type remains challenging, highlighted by the fact that only a few labs in the world have this expertise, particularly in the PNS, and thus new protocols only emerge slowly. For example, parasympathetic neurons have not yet been generated from hPSCs, and thus no disease affecting those cells has been modeled to date. The major challenge here is in generating well-characterized cell types whose exact identity and developmental stage is known and that are functional and thus responsive to physiological cues. The maturity of the generated cells is a major challenge as well. Most differentiation protocols render immature, embryonic cell types, which makes it difficult to study diseases occurring in adult or even elderly patients. Miller et al. have addressed this issue by artificially aging their cultures using progerin, and showed enhanced modeling of Parkinson disease [114]. Several studies are currently investigating how similar success could be achieved in a way that more closely resembles healthy aging (reviewed in [115]). To date, most differentiation protocols result in isolated, purified cell types. This has advantages for their use in mechanism discovery, but it often hinders maturation and, with that, reaching functionality. One approach for overcoming this is the use of co-cultures with physiologically relevant

cell types. Oh et al. achieved this by co-culturing sympathetic neurons with cardiomyocytes, leading to enhanced maturity of the neurons and improved functionality [84]. Another approach involves organoid cultures, reviewed above, which more closely resemble the physiological environment. Another challenge is developing a disease model that not only reproduces disease phenotypes, but is also ideal for fostering treatment of the specific disease. For example, the organoid system seems ideal for modeling intestinal and enteric nervous system disorders, both for modeling the full spectrum of the intricate interplay of multiple cell types for working toward cell replacement therapy for patients with intestinal disorders such as HD. Developing novel drug compounds that target a specific molecular phenotype, such as *IKBKAP* splicing in FD, on the other hand, may be more easily accomplished in purified, two-dimensionally grown cell types. Lastly, a significant challenge is the often unreported variability and reproducibility issues between labs, within labs and among differentiations, which leads to variation in experimental observations. Many factors have been proposed as the root of such issues, including poorly defined media conditions and inter-clonal genetic or epigenetic variations (reviewed in [113]). These issues are being addressed and slowly resolved, with the hope that variability issues can be eliminated altogether in the near future. Looking to the future of ANS disease modeling, the field will hopefully soon be able to report success stories, where novel drugs/treatments for patients have been discovered with the involvement of hPSCs, have moved through clinical trials and have become available for patients.

hPSC technology, particularly its disease modeling division, provides a new tool for investigating ANS disease using human cells derived from patients. This tool is scalable for screening approaches, and specific cell types can more easily be investigated. The power of hPSC technology may be maximized by combining this technology with other scientific tools, such as biopsies, cell lines, postmortem tissue analysis and animal models. Together, these tools will provide the greatest opportunity for scientists to move our knowledge forward and to develop treatments and cures to improve the lives of patients with ANS disorders.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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