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Deriving, Regenerating, and Engineering CNS Tissues using Human Pluripotent Stem Cells

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Abstract

Progress in deriving a spectrum of central nervous system cell phenotypes from human pluripotent stem cells has spurred significant advances in *in vitro* modeling and development of regenerative therapies for neurological disorders. While the clinical impact of these advances is still being evaluated, their integration with advanced tissue engineering methodologies and therapeutic approaches that induce neural circuit plasticity, respectively, remain underexplored frontiers.

Graphical abstract

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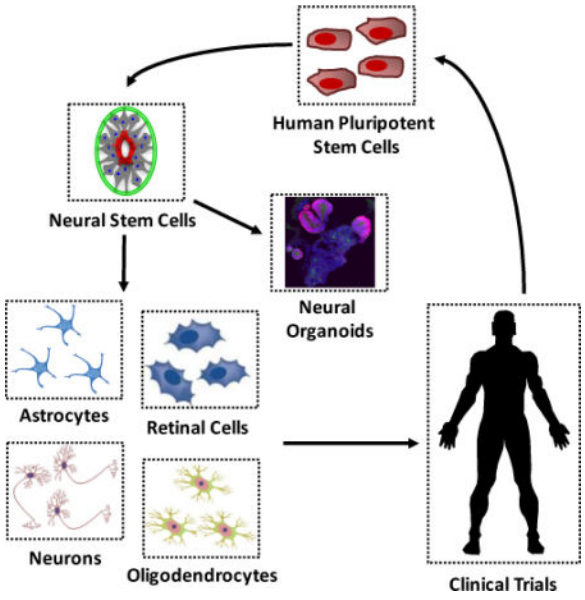

Introduction

Over the past 15 years, there has been revolutionary progress in deriving human central nervous system (CNS) cell phenotypes and tissues. Successful cultivation of human embryonic stem cells (hESCs) in 1998 [1] was quickly followed by their differentiation into neuroepithelial cells [2]. These are the germinal neural stem cells (NSCs) that constitute the embryonic neural tube from which all CNS tissues arise. This *in vitro* NSC phenotype turned out to be remarkably analogous to its *in vivo* counterpart, and through direct implementation of developmental biology principles, scientists rapidly generated numerous neuronal and glial phenotypes (Fig. 1a). Practical application of these findings was further propelled by the discovery of induced pluripotent stem cells (iPSCs) [3]. This enabled generation of patient-specific CNS cells and 2-D cultures for disease modeling as well as conception of an autologous regenerative cell therapy supply chain. More recently, NSC's have been observed to display powerful emergent properties. This is evidenced by their ability to spontaneously recapitulate extensive levels of *ex vivo* morphogenesis to generate 3-D tissues, a.k.a. organoids [4–6], with levels of cell phenotype diversity and microscale cytoarchitectures mimetic of those in the human fetal CNS.

Here, we provide a synopsis of progress in deriving human CNS cell phenotypes, translating these cells towards regenerative clinical therapies, and engineering 2-D cultures and 3-D organoids humanoid models of neurological disorders. While these are tremendous advancements, full realization of their therapeutic and modeling potential will require developing interdisciplinary approaches using technologies and methodologies from related fields, e.g. tissue engineering and neuromodulation. Such mergers could enhance engraftment of regenerative cell therapies as well as enable instructed *ex vivo* morphogenesis of anatomically and physiologically mimetic 3-D CNS tissue units that could one day serve as transplants.

Deriving CNS cell phenotypes

For decades, human neural cells and clinical transplants were solely isolated from fetal tissue sources [7]. Today, this has been almost universally supplanted by the more sustainable human pluripotent stem cell (hPSC, i.e. hESC and iPSC) source. NSC derivation protocols have advanced from re-plating hPSC-derived embryoid bodies and physically isolating polarized NSC structures, a.k.a. neural rosettes [2], to highly efficient differentiations using hPSC monolayers in the absence of [8] or while antagonizing [9] transforming growth factor-beta (TGF-β) and bone morphogenetic protein (BMP) pathway activity. Human PSC-derived NSCs are identified in culture by a polarized, columnar morphology and co-expression of Pax6/Sox2/N-cadherin [8]. Also, they default to a rostral, forebrain fate in the absence of exogenous morphogens [10].

In line with neurodevelopmental biology principles, patterning NSCs to diverse regional phenotypes along the CNS rostrocaudal (R/C) and dorsoventral (D/V) axes requires early morphogenic interventions (Fig. 2). Prior to acquisition of a $Pax6^+$ NSC state, exposure of neurally differentiating cultures to Wnts and Fibroblast growth factors (Fgfs) induces an intermediate Brachyury $(T)^+/Sox2^+/Pax6^-$ neuromesodermal progenitor (NMP) phenotype [11]. In the NMP state, the cells continually transition from a rostral to caudal CNS phenotype, and the extent of caudalization is deterministically patterned by the duration of Wnt/Fgf/Growth differentiation factor-11 (GDF-11) signaling (Fig. 2a). Activating retinoic acid (RA) signaling at any point during the caudalization time course induces full neural conversion to a $T^-/Sox2^+/Pax6^+$ NSC state and fixes its R/C positioning [11]. Concurrent with RA-induced neural induction, modulation of Wnt [10] and Tgf-β [11] (dorsal) or Sonic Hedgehog (Shh, ventral) [10] signaling can be used to pattern the NSC phenotype along the CNS D/V axis (Fig. 2b). BMP signaling is also known to play a role in dorsalizing NSCs *in vivo* [12], but has yet to be used analogously in hPSC neural differentiation protocols. Upon completion of regional phenotype patterning, NSCs can be cultured as neurospheres [2] or in monolayers [11] and immediately begin generating further differentiated region-specific neuronal progenitors. Moreover, the NSCs transition from an early epithelial to mid and late phenotype over successive passages [13].

Analogous to their *in vivo* counterparts, NSCs transition sequentially through neurogenic, astrogenic, and oligodendrogenic progenitor states. The progeny's regional phenotype is dictated by that of NSCs', and neurons can be derived after 2–4 weeks of culture [8]. As illustrated in Figure 1a, numerous CNS neuronal subtypes have been derived, including cortical glutamatergic projection neurons [14] and GABAergic interneurons [15,16], basal forebrain cholinergic [16], hippocampal dentate gyrus granular neurons [17], midbrain dopaminergic neurons [18], cerebellar granule neurons [19], and spinal cord motor [20] and sensory neurons [21]. Human PSCs can also be differentiated into retinal pigment epithelium (RPE) [22] and retinal progenitor cells (RPCs) that give rise to rod and cone photoreceptors upon transplantation [23]. Neural crest cells can be isolated shortly after neural induction from the periphery of neural rosettes [24]. Alternatively, astrocytes [25] and oligodendrocytes [26] can only be generated after 1–2 and >2 months of NSCs culture, respectively.

Regenerating CNS tissues

With access to diverse CNS cell phenotypes, clinical translation of hPSC-derived, regenerative therapies began in 2009 with Geron Corporation's FDA-approved trial of hESC-derived oligodendrocyte progenitor cells (OPCs) to treat spinal cord injury [27]. The trial was abandoned in 2011 for financial reasons, but has since been completed by Asterias Biotherapeutics (NCT01217008) along with an ongoing trial testing their own AST-OPC1 line (NCT02302157). In a January 2017 press release, Asterias announced that complete cervical spinal cord injury (AIS-A) patients administered with 10 million AST-OPC1s showed a positive safety profile and improvements in upper extremity motor function at 6- and 9-month follow-ups [28]. Since Geron's initial studies, clinical trials testing the regenerative efficacy of hESC-derived therapies for degenerative retinal disorders have predominated. Six different entities have eight different ongoing clinical trials. This includes RPE cell therapy trials by Southwest Hospital in China (NCT02748734, 2016), Astellas Institute for Regenerative Medicine (NCT01344993, NCT01345006, and NCT01469832, 2011), and Cell Cure Neuroscience LTD (NCT02286089, 2014) for various forms of macular degeneration. jCyte Inc. is conducting an RPC cell therapy trial (NCT02320812; 2014) to treat retinitis pigmentosa. Pfizer's hESC-derived RPE trial (NCT01691261, 2012) is currently on hold, and the first iPSC-derived RPE trial being conducted by the RIKEN Institute (2014) is planning to resume shortly following recent demonstration of successful allogeneic transplantation in HLA-matched primates [29]. Also, the Federal University of São Paulo (NCT02903576, 2016) is testing the regenerative efficacy of an implanted bioengineered RPE layer as compared to a standard bolus injection of a cell suspension.

Outside of the retinal space, only one additional human PSC-derived therapy for CNS regeneration is in clinical trials. International Stem Cell Corporation is currently testing whether human parthenogenetic embryonic stem cell-derived NSCs (ISC-hpNSC) can be used to treat Parkinson's patients (NCT02452723, 2015). In July 2016, they announced successful intracranial transplant of 30 million ISC-hpNSCs into their first patient [30]. Clinical trials of other hPSC-derived neuronal progenitor cell therapies are on the horizon as indicated by elegant proof-of-principle studies in animal models for Parkinson's [31], Huntington's [32], Amyotrophic Lateral Sclerosis (ALS) [33], Epilepsy [34], and learning and memory disorders [16]. A common theme throughout these studies is the critical importance of matching the cell therapy's regional phenotype with the transplantation site to effectively reconstitute degenerated neuronal circuitry.

Engineering CNS tissues

While still awaiting full clinical implementation, hPSC-derived CNS cells have drastically enhanced our ability to create *in vitro* models of human neural development, physiology, toxicity, and disease. Novel insights into signaling pathways and cell phenotypes involved in development of various regional CNS tissues have been elucidated through analysis of *in vitro* hPSC differentiation processes [11,13]. Cells, tissues, and microphysiological systems derived from hPSCs have been shown to recapitulate facets of *in vivo* CNS physiology, and thus, have been useful in screening for neurotoxicity [35] and investigating molecular underpinnings of traumatic injury [36] and viral infections [37]. Also, since first revealed as

feasible in 2009 [38], numerous studies have observed facets of neurodegenerative [39–42], neurodevelopmental [43], and psychiatric disorders [44] in 2-D cultures of CNS cells derived from patient-specific iPSCs (Fig. 1b). Such disease-in-a-dish models have tremendous potential for drug screening applications; however, their utility as clinically predictive screening platforms remains to be demonstrated.

The mere fact that CNS diseases believed to have a mid-to-late adult onset pathology, e.g. Alzheimer's [39], Parkinson's [40], and Huntington's Disease [41], Amyotrophic Lateral Sclerosis (ALS) [42,45], and Macular Degeneration [46], can be modeled by iPSC-derived cultures that do not mature past a fetal phenotype is a ground breaking discovery (Fig. 1b). This enables the possibility of clinically implementing patient-specific disease models for personalized medicine strategies. However, full *in vivo* disease pathology, e.g. targeted neuronal subtype death, is not routinely and robustly observed, possibly due to the limited biomimicry achievable in standard 2-D culture. A cell death phenotype is generally only observable upon application of an exogenous physiological stressor, and a subset of pathological facets are typically used as disease indicators instead [40,41,45]. Improving standard 2-D disease-in-a-dish models potentially requires overcoming their lack of biomimetic tissue cytoarchitecture and cell phenotype diversity as well as progressing past a fetal maturation state.

To create more biomimetic CNS models, tissue engineering techniques are being integrated with stem cell culture. By shifting from 2- to 3-D Matrigel hydrogel culture, neural tissues derived from fetal NSCs overexpressing pathological Alzheimer's protein precursors were able to deposit amyloid-β plaques *in vitro* for the first time [47]. Using 3-D aggregate culture, neurally differentiating hESCs were discovered to possess innate abilities to spontaneously morph, i.e. differentiate and self-organize, and recapitulate remarkable levels of cortic- and retinogenesis *in vitro* [4,5] (Fig. 1c). The extensive morphogenesis capabilities of hPSC aggregates was further revealed by embedment within 3-D Matrigel hydrogels and long-term culture in stirred-tank bioreactors to enhance interstitial transport of oxygen and nutrients. This allowed the organoids to grow to millimeters in diameter, and within a single organoid, generate diverse cerebral tissues that enable novel disease in a dish models, e.g. microcephaly [6]. Extended culture of cerebral organoids can generate even further biomimetic CNS tissue microenvironments containing laminated, interconnected, and electrophysiologycially active neuronal tissue cytoarchitectures with interspersed astrocytes [48]. Moreover, neurodevelopmental biology principles discussed previously can be applied to morphing, NSC-stage aggregates to derive organoids from other CNS regions, e.g. midbrain-like [49] and hypothalamic [50] organoids.

Conclusion

Advancements in our ability to efficiently derive CNS cells and tissues foreshadow a new era in brain, eye, and spinal cord regenerative medicine. While such hPSC-derived cell therapies are still in the early stages of clinical trials, pre-clinical consideration should be given to their integration with neuromodulation [51] and acute intermittent hypoxia (AIH) approaches [52]. Patients treated with both of these approaches have demonstrated remarkable recovery of function via activation of endogenous plasticity mechanisms that

induce adaptive neuronal circuitry changes. Thus, a combinatorial cell therapy utilizing all of these methods would likely create an optimally supportive trophic and plastic microenvironment for facilitating transplant engraftment.

Human PSC-derived organoids have become the premier 3-D platform for investigating human CNS disorders *in vitro* due to constituent microscale tissue structures displaying unprecedented biomimicry. However, their derivation relies primarily on spontaneous, uncontrolled morphogenesis. This can limit reproducibility in cellular/tissue composition as well as the ability to acquire an anatomically mimetic cytoarchitecture throughout the entire organoid, i.e. at the macroscale [6]. Transitioning to an instructed, controlled morphogenesis will be necessary to reproducibly derive organoids with a biomimetic macroscale anatomy containing multiple CNS tissues that interconnect to make physiologically relevant neurological circuits. In progressing toward this goal, synthetic matrices capable of supporting organoid morphogenesis are being developed to replace widely used but ill-defined and heterogeneous Matrigel hydrogels [53]. Tissue engineered platforms that enable spatiotemporal control of hPSC-derived CNS tissue morphology during morphogenesis are being developed for both 2-D [54] and 3-D culture [55]. In theory, platforms that instruct R/C and D/V morphogenetic patterning of developing organoids by exogenous application of morphogen gradients will also be needed. However, recent work describing spontaneous D/V patterning in mouse ESC-derived cysts may indicate that this is not necessary [56]. Regardless, intimate integration of tissue engineering methodologies with organoid derivation protocols will be needed to advance towards instructed organoid morphogenesis and create next generation CNS *in vitro* models and potentially even organ transplants.

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• of special interest

•• of outstanding interest

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Highlights

- Developmental biology principles can be applied to generate diverse CNS cell types.
- Clinical trials for diverse CNS cell therapies are either ongoing or imminent.
- 2- and 3-D CNS tissue models expand the scope of clinical relevance.

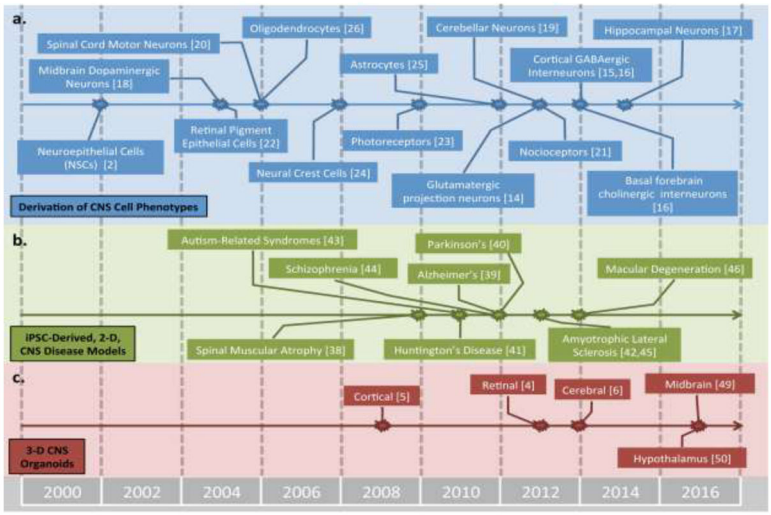

Figure 1.

Chronology of progress in deriving CNS cells and tissues. Timeline list seminal studies describing derivation of human CNS (a) regional cell phenotypes, (b) 2-D disease-in-a-dish models, and (c) 3-D organoids from hPSCs. Includes references cited in the review; it is not a comprehensive list.

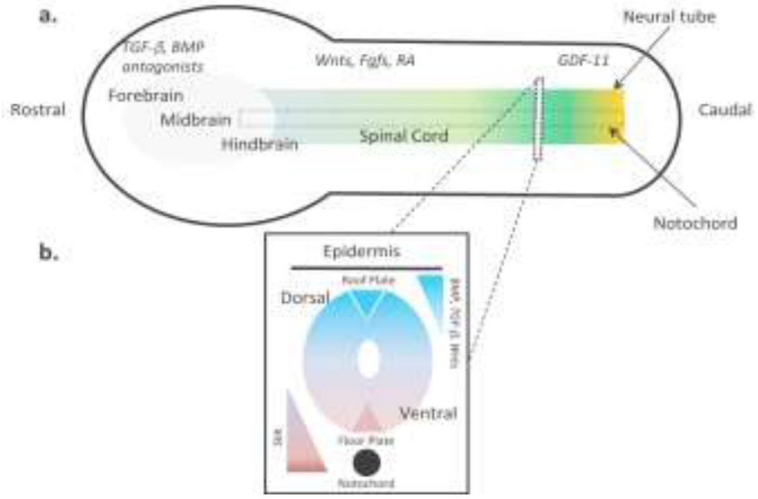

Figure 2.

Schematic of signaling pathways modulated by researchers to mimic CNS development *ex vivo*. a) Morphogens gradients for patterning regional phenotypes along the rostrocaudal (R/C) axis. Forebrain tissues are patterned in the absence of TGF-β/BMP signaling, and Wnts/Fgfs/RA signaling is modulated to pattern more caudal tissues. GDF-11 is added to access the most caudal spinal cord phenotypes. b) Morphogens gradients for patterning regional phenotypes along the dorsoventral (D/V) axis. The ventral neural tube is patterned by graded Shh first from the notochord and then the floor plate. The dorsal neural tube is patterned by graded BMP, TGFβ, and Wnt signaling first from the epidermis and then the roof plate.