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Review

In vivo modeling of neuronal function, axonal impairment and connectivity in neurodegenerative and neuropsychiatric disorders using induced pluripotent stem cells

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ABSTRACT

Modeling neurological diseases using human embryonic or patient-derived induced pluripotent stem cells (iPSCs) improves the understanding of molecular and cellular changes underlying these diseases and can lead to new, potentially personalized therapies. Changes in expression of axon guidance cues and altered cytoskeletal maintenance have been implicated in neurodegenerative and neuropsychiatric disorders. To date, most of the iPSC patient-derived cellular dysfunction and phenotypic studies have been performed in vitro. To study the intrinsic axonal impairments and neuronal connectivity deficits in human disease iPSC-derived neurons we propose to graft these cells into the physiological three-dimensional multi-structural environment of the central nervous system of rodent models to obtain relevant in vivo data. Such human iPSC in vivo chimeric models can allow for neuronal maturation, capture neuropathological phenotypes of axonal and connectivity impairments, and serve as target engagement and drug validation studies using human cells, thus highly relevant for advancement of the drug development process in the late pre-clinical stages.

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1. Introduction

The increased life expectancy and the size of today's world population contribute to the increased prevalence of neurodegenerative and neuropsychiatric diseases, with a large impact on societal health costs. In

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general, the practiced therapies for most of these diseases are referred to as symptomatic treatments and the causes of these diseases are still mostly unknown. One of the main challenges in the field of neuroscience research, in particular in the field of neuropsychiatric disease research, is the lack of adequate in vitro and in vivo models. Recent developments in disease modeling using patient-derived induced pluripotent stem cells (iPSCs) contribute to better understanding of the molecular and cellular changes causal to these diseases and new, potentially personalized therapies.

The adult central nervous system is thought to maintain neuronal circuitry patterns in part through continuous expression of axon guidance cues (Mironova and Giger, 2013). There is a potential dysregulation of axon guidance signaling and cytoskeletal structure in the adult central nervous in Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), schizophrenia and autism, which could have an impact on the etiology of these diseases by inducing alternative axonal plasticity and impairment in neuronal connectivity (further discussed below and reviewed by Van Battum et al., 2015). Transformed synaptic maintenance may trigger early degenerative changes in the years preceding the clinical diagnosis. Altered guidance during brain development may introduce a primary delay in growth or changes in neuronal circuits that can contribute to the development of neurological disease. It is possible that even minor fluctuations in neuronal circuitry can eventually lead to failure in accurate

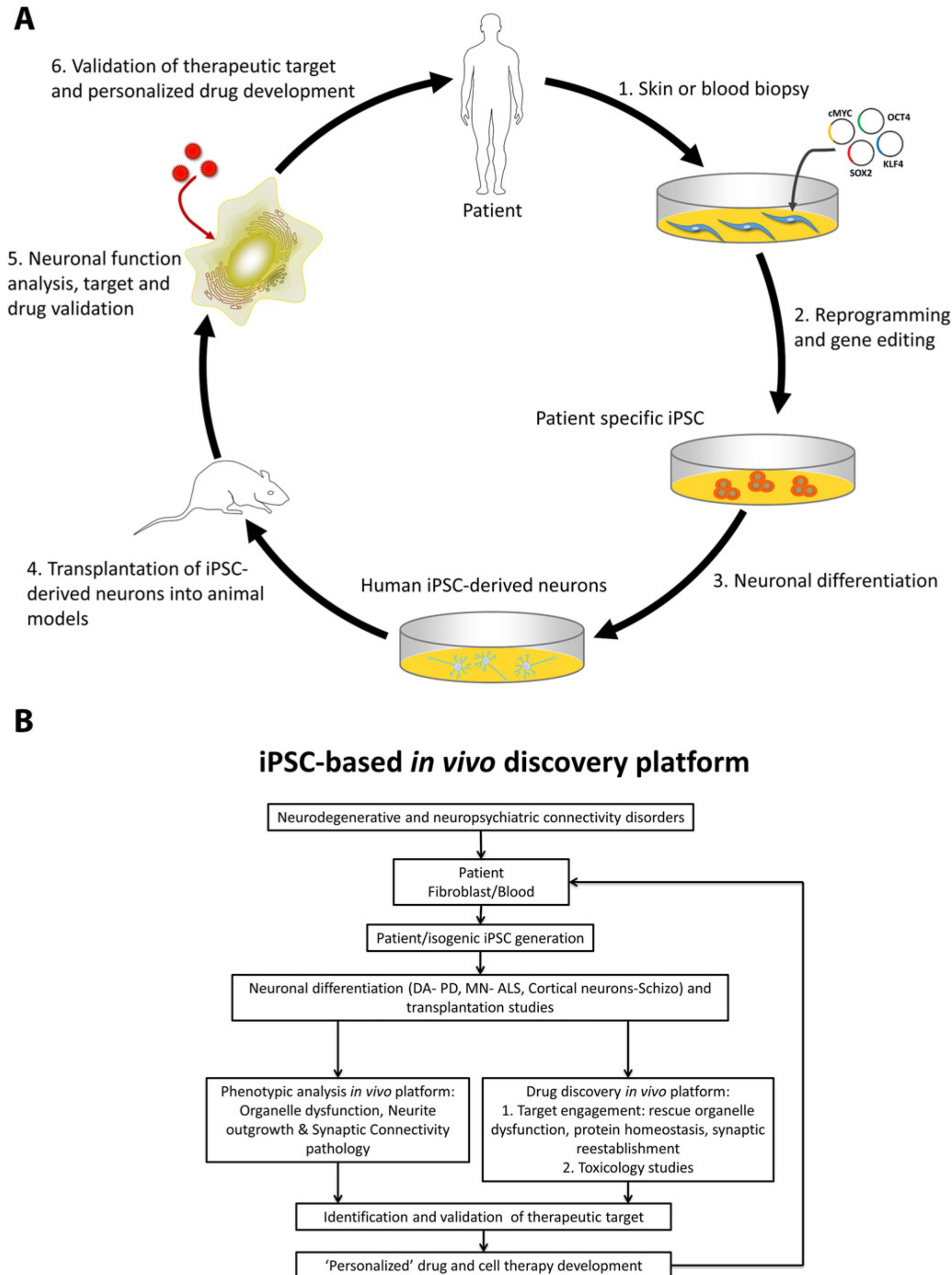


Fig. 1. Generation of iPSC based in vivo discovery platform. A. Flow and **B.** chart diagram showing the use of human iPSCs and iPS-derived young neurons from patients or patients at risk as tools to obtain a relevant in vivo phenotypic bioassay discovery platform used for analyses of neuronal functions altered in neurodegenerative and neuropsychiatric diseases. These models can further allow for drug target validation in pre-clinical research and personalized drug development. Abbreviations: iPSC- induced pluripotent stem cells, PD — Parkinson's disease, DA — dopaminergic neuron, MN — motor neuron, ALS — amyotrophic lateral sclerosis, Schizo — schizophrenia.

connectivity, trophic support or producing insufficient control of neurotransmitter release. How can we improve our understanding of the neuronal circuitry changes resulting in the development of axonal neuropathology and how can we identify molecules responsible for the development of axonal pathology? In this review we discuss that modeling neurite outgrowth and synaptic connectivity of healthy and patient iPSC-derived neurons in the physiological in vivo 3D environment can bring us closer to understanding the causes of this neuropathology (Fig. 1).

2. Axonal dysfunction in neurodegenerative diseases

2.1. Axon guidance and connectivity deficits in Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder involving multiple neuronal systems, including loss of dopaminergic neurons in the substantia nigra (SN) directly accounting for the motor symptoms. Approximately 5% of PD cases are familial, caused by mutations in genes such as SNCA, Parkin, DJ-1, PINK1, LRRK2 and PARK9, while the rest of the patients suffer from sporadic forms of the disease (Bonifati, 2007; Hardy, 2010; Trinh and Farrer, 2013). The principal risk factor for PD is increase in age, with incidence rising significantly after 50 (Driver et al., 2009; Rocha et al., 2015). The etiology of PD appears to be multifactorial, involving both biological and environmental components, yet the specific cellular and molecular changes occurring in the early stages of the disease still need to be elucidated.

In recent years more evidence has been composed for axonal pathology underlying changes in axon transport and connectivity in PD (reviewed in Tomiyama, 2011; and Van Battum et al., 2015). Synaptic and axonal degeneration appears to take place first, before the loss of neuronal soma in the SN in the human brain (Chu et al., 2012). This can also be seen in a number of PD rodent models such as models based on overexpression of the mutated A53T human α-synuclein (SNCA) protein (Chung et al., 2009), or overexpression of two LRRK2 mutations (G2029S and R1441G) (Li et al., 2009; MacLeod et al., 2006), as well as MPTP toxicity models (Burke and O'Malley, 2013; Korecka et al., 2013). Gene expression studies (Bossers et al., 2009), genome wide association studies (GWAS) of single-nucleotide polymorphism (SNP) variations (Kim et al., 2011; Lin et al., 2009; Maraganore et al., 2005; Srinivasan et al., 2009) and pathway analysis studies (Edwards et al., 2011; Lesnick et al., 2007; Sutherland et al., 2009) identified axon guidance signaling pathways to be associated with PD development. Through comparison of GWAS and pathway analyses, five axon guidance genes were identified to be significantly associated with the development of PD pathology: DCC, EPHB1, NTNG1, SEMA5A and SLIT3 (Lin et al., 2009). In addition to GWAS and gene expression studies, functional studies have been published showing a role of single axon guidance molecules playing a role in the development of axonal PD pathology such as LINGO-1 (Inoue et al., 2007).

2.2. Is axon guidance signaling part of ALS pathology?

ALS is a rapidly progressing disease characterized by motor neuron degeneration in the central nervous system (CNS). The denervated muscles weaken and atrophy, inducing paralysis and respiratory failure at the end stage. The average age of onset of the disease is 55. Most ALS cases are categorized as sporadic, with 5–10% caused by inherited genetic mutations in SOD1 (Rosen et al., 1993), alsin, senataxin, angiogenin, profiling VAMP-associate protein B, dynactin, TDP-43, FUS and more being discovered in the last years (Bettencourt and Houlden, 2015; Guerreiro et al., 2015; Robberecht and Philips, 2013). Recently, the intronic hexanucleotide repeat sequence in C9ORF72 has been identified as a large contributor to familial ALS (Renton et al., 2011). Similar to PD, ALS etiology of the sporadic forms of the disease are still unknown, with a variety of environmental and genetic factors identified that contribute to the pathophysiology of the disease.

Axonal degeneration is an early feature of ALS. In one of the larger GWAS studies done on ALS patient cohorts, 3 SNP variants in the SARM1 gene were identified, with its function tied to Wallerian degeneration (Fogh et al., 2014). Interestingly, genomic pathway analysis of GWAS datasets implicated axon guidance pathway genes to be highly predictive of ALS susceptibility, partially overlapping with the SNP variant analysis in PD (Lesnick et al., 2008). There is growing evidence supporting alterations of neuronal connectivity and distal axonopathy to play major roles in ALS progression (reviewed in Moloney et al., 2014; and Schmidt et al., 2009). mRNA levels of EphrinA4 (Van Hoecke et al., 2012) and Nogo-A (Dupuis et al., 2002; Jokic et al., 2005), both of which are repulsive axon guidance molecules, are increased in ALS patients in the motor neurons and skeletal muscles respectively. Nogo-A expression levels were proposed to be used as a prognostic marker for the disease (Pradat et al., 2007). Modeling genetic forms of ALS in animals identified a significant role of semaphorin 3A and neuropilin signaling in the progression of the ALS phenotype. Sema3A shows increased expression in the neuromuscular junction of pre-symptomatic ALS SOD1 mice (De Winter et al., 2006) and mice treated with antibodies blocking sema3A–neuropilin signaling display improved motor neuron function (Venkova et al., 2014). Finally, Rho GTPases signaling, important in multiple neuronal functions such as dendritic arborization, spine morphology, growth cone development and axon guidance, has also been linked to ALS etiology (reviewed in Stankiewicz and Linseman, 2014).

2.3. Cytoskeletal and connectivity changes are shared in schizophrenia and autism

Schizophrenia, a multi-factorial neuropsychiatric brain disorder includes patient groups experiencing both positive (such as hallucinations) and negative (such as disruption of normal emotions) symptoms. The cause of the disease onset is unknown, with evidence indicating heredity and environmental components to play a role. Recent discoveries strongly suggest involvement of neurodevelopmental etiologies in schizophrenia (reviewed in Insel, 2010; Owen et al., 2011).

GWAS and gene expression analysis studies bring evidence implicating molecular changes in the regulation of connectivity and axon outgrowth signaling pathways. An axon guidance cue netrin isoform NTNG1, has recently been validated both in the Chinese (Zhu et al., 2011) as well as North American population (Wilcox and Quadri, 2014) as an associated risk factor for schizophrenia. NDST3, a critical enzyme for heparan sulfate metabolism, has also been implicated (Lencz et al., 2013). Heparan sulfate binding is critical for neurite outgrowth, axon formation and synaptic plasticity. Genetic mutations modifying neurite outgrowth through cytoskeletal protein activity and myelination are linked to schizophrenic syndromes. Such genes include DISC-1 regulating the rate of neurite outgrowth (Ozeki et al., 2003) and its functional binding with APP regulating neuronal migration during development (Young-Pearse et al., 2010), Nogo receptor (NRG) mediating myelin inhibition of axonal sprouting (Budel et al., 2008; Sinibaldi et al., 2004) and its binding partner LINGO1 (Fernandez-Enright et al., 2014), which is also implicated in PD (Inoue et al., 2007).

Many shared risk variants have been identified in schizophrenia, bipolar disorder and autism, suggesting molecular commonalities between these disorders. In fact, altered regulation of molecular signaling in axonal outgrowth has been associated with autism and intellectual disability (Hussman et al., 2011). Loss of function mutation in KIAA2022 in a group of autistic patients has been linked to impaired axonal and dendritic outgrowth in vitro (Van Maldergem et al., 2013), whereas mutations associated with X-linked mental retardation in polyglutamine-binding protein 1 regulate dendritic outgrowth (Wang et al., 2013). Interestingly, in the mouse model of fragile X syndrome, the most common form of hereditary mental retardation, there is evidence of altered protein expression

regulating neurite outgrowth and immature presynaptic maturation (Klemmer et al., 2011).

3. iPSC technology and generation of patient-derived neuronal models to study neurological diseases

Through technological advancement of somatic cell reprogramming using four defined transcription regulators (Oct4, Sox2, Klf4 and c-Myc) Prof. Yamanaka and his team (Takahashi et al., 2007; Takahashi and Yamanaka, 2006) facilitated the generation of iPSC from somatic animal and human cells. The stem cell field rapidly progressed towards the development of sophisticated protocols for diverse cellular differentiations. By comparing cellular phenotypes from patients and controls, valuable insights are gained into the biological mechanisms of human disorders including neurodegenerative and neuropsychiatric disorders. Fibroblast and blood samples have been collected from neurological and psychiatric patients, reprogrammed into iPSCs and differentiated into multiple neuronal subtypes, even from centenarians (Yagi et al., 2012). These patient-derived somatic and iPSCs are available to many laboratories interested in studying cellular processes involved in neurodegenerative and neuropsychiatric diseases. Human iPSCs relevant for PD, ALS and neuropsychiatric disease research were differentiated by various laboratories including ours to generate neuronal (Brennand et al., 2011), dopaminergic (Cooper et al., 2010; Hartfield et al., 2014; Kriks et al., 2011), motor (Burkhardt et al., 2013; Dimos et al., 2008; Toma et al., 2015), excitatory cortical (Zhang et al., 2013) and GABAergic (Liu et al., 2013) neuronal populations.

3.1. Using human iPSC-derived neurons to model specific cellular dysfunctions

The use of iPSC-derived neurons has been extensively reviewed as in vitro models for neurological disorders (Badger et al., 2014; Beevers et al., 2013; Cooper et al., 2012a; Imaizumi and Okano, 2014), their potential to become a new drug discovery and screening platform (Egawa et al., 2012) and adapted as personalized medicine (Ebben et al., 2011). Phenotypic characterization of iPSC-derived neurons can advance knowledge on cellular pathology and further evaluate the dysfunctional or adaptive cellular functions involved in neurodegeneration.

In the field of Parkinson's disease, iPSC technology proved to be fruitful for deepening the understanding of dopaminergic neuron vulnerability to genetic and environmental stressors. Mitochondrial dysfunction and increased oxidative stress levels (Chung et al., 2013a; Cooper et al., 2012c; Flierl et al., 2014; Ryan et al., 2013; Sanders et al., 2014), ER stress (Chung et al., 2013a), lysosomal dysfunction (Sanchez-Danes et al., 2012; Schondorf et al., 2014), and neurite outgrowth (Reinhardt et al., 2013; Sanchez-Danes et al., 2012), have all been found to be altered in iPSC-derived neurons carrying genetic mutations that contribute to PD development; such as LRRK2, GBA and SNCA and SNCA overexpression.

IPSC-derived motor neurons from ALS patients carrying TARDBP, C9ORF72 and SOD1 mutations have also been extensively characterized on their neuronal function. The iPSC-derived ALS motor neurons' main intrinsic phenotypes are their hyperexcitability (Devlin et al., 2015; Wainger et al., 2014) but also increased oxidative stress, reduced mitochondrial function, altered cellular transport, and activation of the ER stress and unfolded protein response pathways (Kiskinis et al., 2014). iPSC-derived neurons from schizophrenia patients present aberrant migration indicating altered cytoskeletal remodeling and increased oxidative stress (Brennand et al., 2015), deficits in adherens junctions and apical polarity (Yoon et al., 2014). iPSC-derived forebrain neurons from individuals carrying a frame shift mutation in the disrupted in schizophrenia gene (DISC1) show a synaptic pathology of vesicle release deficit and transcriptional dysregulation of synaptic signaling (Wen et al., 2014).

3.2. Connectivity and neuronal maturation — limitations of in vitro studies and advantages of in vivo modeling using iPSC-derived cells

To date most of the cellular dysfunction and phenotypic studies have been performed in vitro by comparing healthy subject or isogenic control cells to patient iPSC-derived neurons by focusing on organelle and neuronal function. Although informative, it may be argued that these culture conditions do not represent the more relevant physiological 3D multi-structural environment in the central nervous system, and

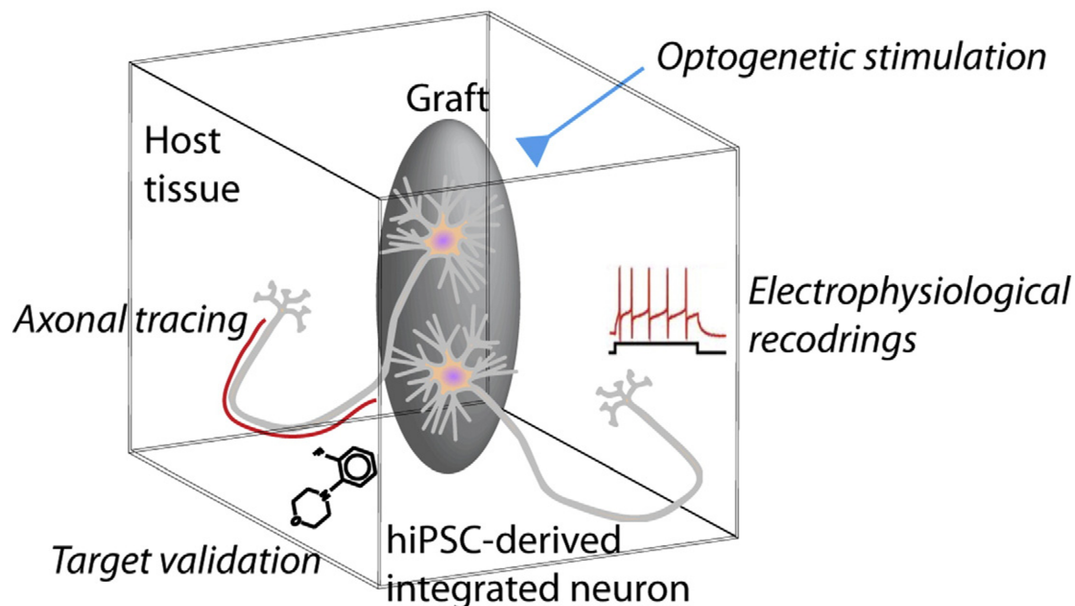


Fig. 2. Using patient hiPSC-derived neuronal graft in a rodent brain as an in vivo bioassay platform for phenotypic connectivity dysfunction in neurological disorders. Human iPSC-derived grafts integrate into the host brain and projects axonal connections into target zones. With the use of axonal tracing, electrophysiological recordings and optogenetic stimulation, the survival and the integrity of the iPSC-derived graft can be analyzed, further elucidating the neuropathological connectivity phenotypes intrinsic to neurological diseases such as PD, ALS, schizophrenia and autism. Furthermore, this graft-based bioassay can be used as chimeric model for drug target validation, where traditional in vivo testing can be combined with target engagement in human neurons.

that these cells do not have the opportunity for neuronal maturation and outgrowth as observed in vivo. These can be important factors when studying neurological and psychiatric disorders, where structural connectivity may be changed as seen in PD, ALS and schizophrenia.

Recently new in vitro culturing techniques emerged aimed at the development of iPS-derived organoid neuronal culture systems in an attempt to simulate the growth of three-dimensional structures resembling that of a developing brain. Organoid cultures previously shown to be quite successful to simulate the growth of intestines (Sato et al., 2009; Spence et al., 2011), stomach (McCracken et al., 2014), kidney (Takasato et al., 2014) or liver buds (Takebe et al., 2013). In 2013, a first report was published of a 3D cerebral organoid culture derived from human pluripotent stem cells (Lancaster and Knoblich, 2014; Lancaster et al., 2013). As a tool for disease modeling, others have used these cultures to study gene expression dysregulation and developmental regulation of neuronal subpopulations in idiopathic autism spectrum disorder (Mariani et al., 2015). A major advantage of these cultures is the growth and development of a mutli-cellular environment within these organoid structures, containing both neuronal and glial progenitors, young neurons and radial glia cells, indeed simulating the complex aspect of multicellular support system present in the developing brain. Some of the internal structures of the cerebral organoids resemble and express developmental markers characteristic to specific brain regions such as hippocampus, prefrontal lobe, ventral cortex or choroid plexus (Lancaster et al., 2013). It is not established yet if axonal connectivity and guidance signaling are present in these organoids and how closely this mimics the development of brain connectivity. Additionally, these cultures most closely resemble the early stages of brain development, being most suited to study the neurodevelopmental disorders, rather than functions of a mature neuron.

To study neuronal maturation, outgrowth and axonal impairments, and neuronal connectivity deficits in human disease such as PD, ALS and schizophrenia, we propose to take advantage of studying the intrinsic characteristics of iPSC-derived neurons by grafting them into a rodent animal model and develop in vivo phenotypic discovery methodology. Such an in vivo bioassay platform can more accurately capture axonal and connectivity neuropathological phenotypes in neurodegenerative and neuropsychiatric disorders (Figs. 1 and 2).

4. In vivo modeling of axonal neuropathy using iPSC-derived neurons

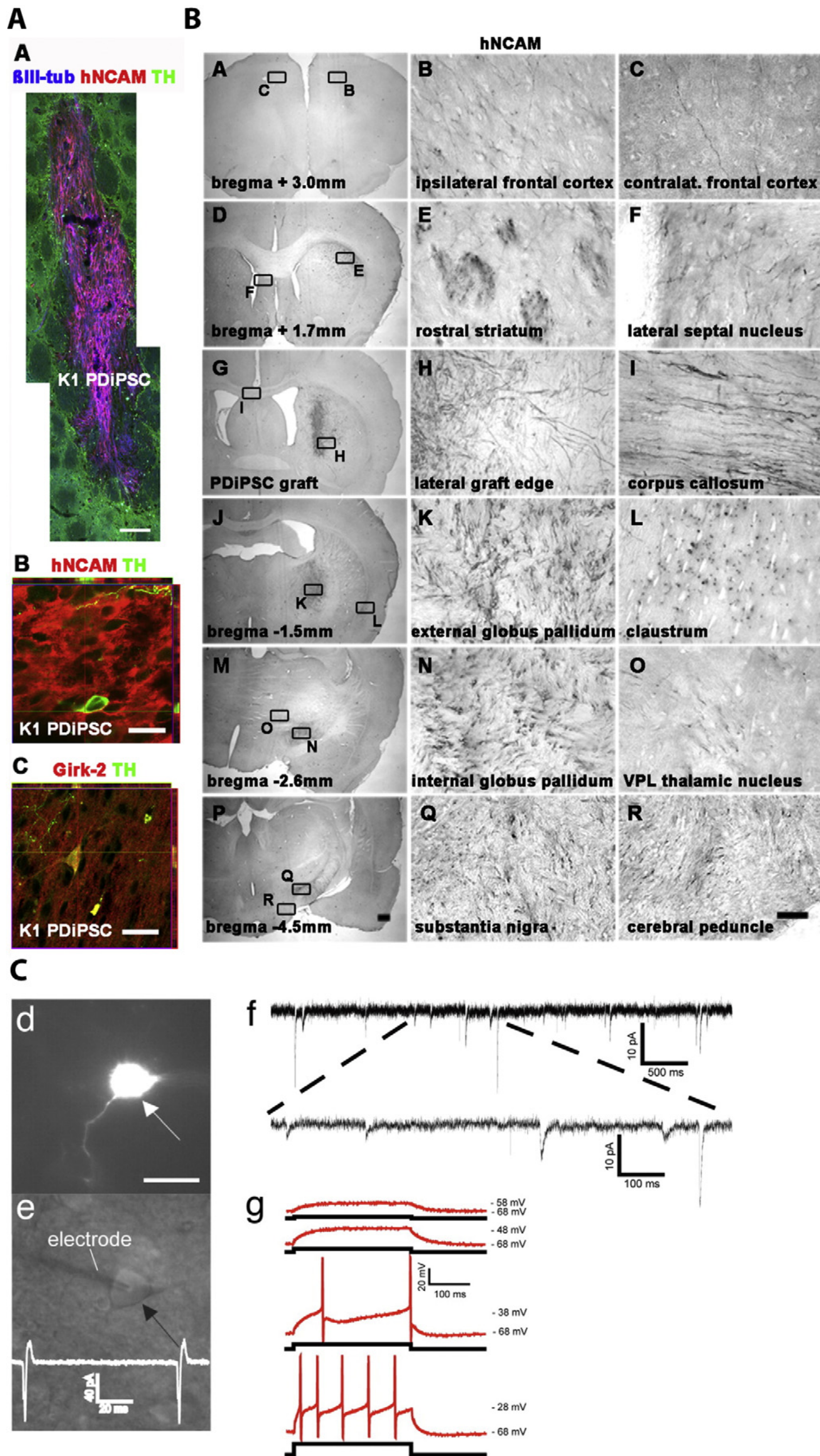
4.1. Stem cell- and iPSC-derived neuronal grafting into the CNS

The uninjured adult central nervous system allows for neuronal reinnervation and stabilization of connectivity most probably through maintained expression of axon guidance cues (Deacon et al., 1999; Isacson and Deacon, 1996). This important feature could allow for the examination of neuronal integration and axonal biology of healthy and diseased human iPSC-derived neurons. To date, the most well characterized CNS grafts include dopaminergic neurons, as transplantation of these neurons into a denervated striatum has a high potential for therapeutic application (see reviews Cooper et al., 2012b; Steinbeck and Studer, 2015; Tsui and Isacson, 2011). Transplanted dopaminergic neurons derived from human and primate iPSCs have the capacity to innervate and form synapses in the rat and primate striata (Deleidi et al., 2011; Hallett et al., 2015; Hargus et al., 2010; Hallett et al., 2013; Sanchez-Pernaute et al., 2005; Wernig et al., 2008). The 3-dimensional in vivo environment created by interactions of multiple cell types is permissive for the functional maturation of neurons, and allows for long term survival months to years post-transplantation (Hallett et al., 2015; Hargus et al., 2010; Hallett et al., 2013; Sanchez-Pernaute et al., 2005). In the adult brain transplanted neurons do not migrate out of the transplant zone but grow axons and release dopamine into their original target zone — the dorsolateral striatum (Hargus et al., 2010, Fig. 3). Similarly, other non-dopaminergic neurons also grow and extend their axons out of the graft into their distant target zones (Hargus et al., 2010, Fig. 3). It is well established, also with the use of optogenetic technology (Steinbeck et al., 2015), that dopamine neuron grafts functionally integrate into the denervated rodent striatum and improve motor behavior (Bjorklund et al., 2002). Human stem cell-derived and mouse embryonic ventral midbrain dopaminergic neurons are responsive to guidance cues such as netrin and slits, essential for path finding towards their appropriate targets (Lin and Isacson, 2006; Lin et al., 2005). In fact, human and porcine neurons, whether fetal or the equivalent embryonic stem cell-derived dopamine neurons transplanted into the adult rat SN, survive and extend their axons along the medial forebrain bundle and reinnervate their striatal targets (Grealish et al., 2014; Isacson and Deacon, 1996; Isacson et al., 1995). Further, human embryonic stem cell (ESC)-derived cortical neurons transplanted into a mouse neonatal brain integrate, mature and survive up to 6 months in vivo (Espuny-Camacho et al., 2013). Finally, iPSC-derived neuron precursor cells transplanted into mouse fetal brain migrate and differentiate into glia and neurons, including glutamatergic, GABAergic, and catecholaminergic neuronal subtypes, upon which they integrate into the host brain, establishing specific and appropriate axonal projections and synaptic integration (Wernig et al., 2008, Fig. 3). All of these studies indicate that human ES or iPSC-derived neurons are functionally sensitive to axon guidance signaling present in the host brain and have the capability to functionally integrate into the host neuronal network (Fig. 3).

IPSC-derived cells have been grafted in other in vivo models of neurological disorders such as spinal cord injury or ALS models, exploring the possibility for therapeutic applications. A number of transplantation studies of human iPSC-derived neuronal precursor cells have been done in spinal cord injury rodent models, with approximately half of them being successful in improving motor behavior by axonal regrowth through the injury and reestablishment of connectivity (reviewed in Lee-Kubli and Lu, 2015). By investigating the axonal growth properties and morphology of both the disease genotype and healthy iPSC-derived neurons that integrate into a rodent central nervous system, it may be possible to generate a growth and connectivity paradigm for the neuron of interest. IPSC-derived glia can also be transplanted and used for in vivo modeling of neurological disorders. Glia-rich human iPSC-derived progenitors were transplanted into the spinal cord of an ALS mouse model prolonging the survival rate of mice, most likely through an increase in neurotropic factors and activation of Akt signaling (Kondo et al., 2014). The recently reported development of human glia chimeric mice opens up the possibilities of studying patient specific glial function and interactions in the CNS, modeling the pathology of glia in neurological diseases (Goldman et al., 2015; Windrem et al., 2014).

4.2. Axonal outgrowth and connectivity analysis in the living brain

As discussed above, compelling evidence suggests that axon outgrowth and guidance are aberrant in neurological disorders. IPSC-based modeling of neurological diseases such as Parkinson's disease, schizophrenia, autism and spinal motor dystrophy also display axonal neuropathy. IPSC-derived neurons from PD patients with LRRK2 mutations present axonal dysfunction such as decreased neurite outgrowth and neurite number (MacLeod et al., 2006; Reinhardt et al., 2013). LRRK2 has also been shown to play a critical role in the WNT signaling pathway (Berwick and Harvey, 2012). These data strongly suggest that LRRK2 may play a regulatory role in axonal outgrowth and the neuronal circuitry in PD patients carrying these mutations can be significantly altered. In iPSC neurons derived from patients with schizophrenia, neurite number and connectivity were diminished, with altered gene expression profiles seen in the cAMP and WNT signaling pathways (Brennand et al., 2011). iPSC-derived forebrain neurons from fragile X syndrome autistic patients carrying an FMR1 mutation showed a defective neurite outgrowth initiation and extension (Doers et al., 2014). Cortical neurons generated from iPSCs of Rett syndrome patients carrying a


A
B
A
ßIII-tub hNCAM TH
K1 PDiPSC
B hNCAM TH
K1 PDiPSC
C Girk-2 TH
K1 PDiPSC
hNCAM
bregma + 3.0mm
ipsilateral frontal cortex
contralat. frontal cortex
bregma + 1.7mm
rostral striatum
lateral septal nucleus
PDiPSC graft
lateral graft edge
corpus callosum
bregma -1.5mm
external globus pallidum
claustrum
bregma -2.6mm
internal globus pallidum
VPL thalamic nucleus
bregma -4.5mm
substantia nigra
cerebral peduncle
C
d
e
electrode
40 pA
20 ms
f
10 pA
500 ms
10 pA
100 ms
g
- 58 mV
- 68 mV
- 48 mV
- 68 mV
20 mV
100 ms
- 38 mV
- 68 mV
- 28 mV
- 68 mV

MECP2 mutation displayed impaired neuronal maturation including reduced dendritic complexity, and impaired synaptic function (Djuric et al., 2015), whereas iPSC-derived neuronal cultures from patients with type 1 spinal muscular atrophy show severely delayed neurite outgrowth (Chang et al., 2011). Although informative, in vitro analyses can only give a representative image of basic neurite outgrowth parameters. On the other hand, by taking advantage of the in vivo environment of the adult brain, persistently expressing axon guidance cues, grafting neurological patient iPSC-derived neurons into the rodent brain can give us a true image of the intrinsic connectivity capabilities of these cells. With the natural maturation of the fetal and iPSC-derived neurons in the host environment, axonal and dendritic outgrowth would be able to follow the evolutionarily conserved circuitry pathways and functionally integrate into the host brain (Hargus et al., 2010; Isacson and Deacon, 1996). Grafting of iPSC-derived neurons from patients with neurological disease in this bioassay paradigm can be highly informative as to the intrinsic outgrowth and connectivity dysfunction, modeling a more precise image of the neuronal circuitry in the disease-affected brain (Fig. 2).

As the graft is chimeric for species, tracing axons in the rodent tissue can be done using human species-specific antibodies against membrane bound proteins such as human NCAM or NCAM-L1 (Figs. 2 and 3). Alternatively, prior to transplantation, human iPSC-derived neurons can be transduced with a reporter gene, such as eGFP and later monitored for axonal outgrowth. Such traditional immunohistochemical analysis provides a detailed representation of human or porcine axonal projections within the rat tissue (Hargus et al., 2010; Isacson and Deacon, 1996), but has its limitations, as cutting of the tissue does not allow for continuity in axonal tracing. Recently, new whole brain immunohistochemistry and imaging techniques were developed such as CLARITY (Chung et al., 2013b), CUBIC (Susaki et al., 2014) or iDISCO (Renier et al., 2014), allowing for single axon tracing within uncut brain tissue. Although very promising, these techniques still need to verify that despite the de-lipidization of the brain tissue and the size changes of the cleared brain during this process, the traced axonal outgrowth pattern is indeed conserved within the tissue.

Studying the integration and connectivity of the grafted patient-derived iPSC neurons can further deepen the understanding of the synaptic dysfunction in neurological disorders such as schizophrenia. Matured and integrated iPSC-derived neurons can be analyzed for their synaptic integration with the use of electrophysiological recordings in the transplanted brain slices (Wernig et al., 2008), or optogenetic functional manipulations (Steinbeck et al., 2015). Using these readouts we may be able to identify synaptic dysfunction in iPSC-derived neurons isolated from patients carrying genetic mutations altering synaptic output, and potentially influence this phenotype by chemical or genetic manipulation (Fig. 2).

4.3. In vivo iPSC-based bioassays as a drug validation platform

Human iPSC-derived neuronal grafts cannot only be used as phenotypic bioassays of neurological disorders, but also as in vivo chimeric species models for drug validation and target engagement studies (Fig. 2). Drug and dosage testing in animals transplanted with iPSC-derived neurons from patients with neurological disorders such as PD, ALS or schizophrenia and many others, can validate the potential of the drug to ameliorate the desired phenotype of these *human* cells such as increase in protein degradation or stabilization of synaptic excitability. At the same time target engagement can be assessed in a model system containing the blood–brain barrier. Additionally, toxicology analyses can be performed, indicating whether the new drug has a potential to induce stress responses in human neurons. The use of these models will advance drug development in the pre-clinical testing stage, possibly with a higher efficacy for success in the clinical phase, as the drug will be already tested in vivo in a human-related biological system. Such advantages can be of a great impact in the drug development process, as often the progression from a successful in vitro and in vivo pre clinical testing does not translate to a positive outcome in clinical application (Geerts, 2009; Henderson et al., 2013; Hyman, 2013; Miller, 2010).

5. Conclusion

In summary, we propose that the use of human embryonic, healthy and patient iPSC-derived neurons by grafting in a physiological chimeric in vivo 3D environment can improve our understanding of the early neuronal changes in synaptic connectivity in the neuronal circuitry that contribute to the development of neurodegenerative and neuropsychiatric diseases.

Until now, studies primarily used iPSC technology to model disease related cellular and molecular pathology in a dish. Such in vitro models typically test very immature cells. We propose that the chimeric models using human cells and neurons can provide more mature systems to test both mechanistic questions and late-stage validation of drugs that are aimed at modifying targets of human neurons and glia. In particular, we reason that the physiological environment of the central nervous system including the blood–brain barrier can be utilized by grafting the patient iPSC-derived neurons (or disease gene-edited human stem cells) into rodent animal models. Such in brain phenotypic bioassays can more accurately capture the neuropathological phenotypes of intrinsic axonal and connectivity impairment in neurodegenerative and neuropsychiatric disorders and allow assessment of new intervention strategies in a more relevant physiological environment.

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Fig. 3. High survival and integration of transplanted human dopaminergic neurons in the rat striatum. A. Survival assessment one month post-transplantation of human PD midbrain neurons derived from K1 iPSC. Images adapted from (Hargus et al., 2010) (A) tiled image of a PD DA neuron graft histologically stained for hNCAM (red), TH (green) and B-III-tubulin (blue). (B) Z-stack image within the graft showing colocalization of TH (green) and hNCAM (red) (C) Z-stack image within the graft showing colocalization of TH (green) and the DA neuron marker Girk2 (red) 12 weeks post-transplantation. Scale bars 100 μm in (A), 20 μm in (B) and (C). **B**. Already 4 weeks post-transplantation PD DA neurons derived from S1 iPSC extend their axons to their targets in the rodent brain, following the intrinsic axon guidance patterning of the brain. Adapted from Hargus et al. (2010) (A–R). Photomicrographs from different brain areas stained against hNCAM 4 weeks post-intrastriatal DA neuron transplantation. Graft-derived axons project to gray matter zone targets through white matter tracks (E, I and R). The boxed areas in the left panels are shown at high magnification in the two images in the middle and to the right. Scale bars: left 500 μm, right 25 μm. **C.** Electrophysiological analysis of neuronal synaptic integration in the P20 mouse brain after in utero transplantation on iPSC-derived neuronal precursor cells. Adapted from Wernig et al. (2008). (D) Image of graft-derived GFP positive neuron from iPSC neuronal precursor cells in the dorsal midbrain. Scale bar 20 μm. (E) Identification of GFP-positive neuron and illustration of electrode recording. The trace indicates spontaneous generation of action potential in the cell. (F) Voltage clamp recording at −70 mV. Spontaneous slow and fast currents indicate receiving of synaptic contacts by the transplanted neuron. (G) Current-clamp recordings during current injection. Superimposed membrane potential changes (upper traces, red) are illustrated demonstrating the capability of the grafted neurons to fire action potentials in response to a series of current injection (lower traces, black) from a holding potential of −68 mV (data from 6 GFP positive neurons).

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