STEM CELLS IN NEURODEGENERATION: DISEASE MODELING AND THERAPEUTICS

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PUBLISHED IN: Frontiers in Neuroscience

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88971-182-6
DOI 10.3389/978-2-88971-182-6

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STEM CELLS IN NEURODEGENERATION: DISEASE MODELING AND THERAPEUTICS

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Citation: Staats, K. A., Fink, K. D., Wakeman, D. R., eds. (2021). Stem Cells in Neurodegeneration: Disease Modeling and Therapeutics. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-182-6

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frontiers in Neuroscience

EDITORIAL
published: 08 June 2021
doi: 10.3389/fnins.2021.683122

Editorial: Stem Cells in Neurodegeneration: Disease Modeling and Therapeutics

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Keywords: stem cells, neurons, neurodegeneration, disease modeling, therapeutics

Editorial on the Research Topic

Stem Cells in Neurodegeneration: Disease Modeling and Therapeutics

With the increasing number of people with neurodegenerative disease world-wide, novel directions and paradigms are sought to understand disease-specific neuronal death and to offer novel therapeutic strategies to patients. The ability to transform a differentiated cell into a pluripotent state and to differentiate again to a defined target cell type (Takahashi and Yamanaka, 2006) has led to the hope of the development of novel therapeutics. Although this holds promise in many fields, there is an extra challenge to replace any lost post-mitotic cells, such as neurons in neurodegeneration. With the development of replacement therapy for certain neuronal subtypes, e.g. dopaminergic neurons in models of Parkinson's Disease (PD) (Wakeman et al., 2017), there are remaining challenges with the long axons grown throughout development, e.g., in motor neurons in diseases such as Amyotrophic Lateral Sclerosis (ALS). Despite that stem cell-derived neurons may not always be suitable for replacement therapies, they are extremely informative in disease modeling to understand more of human disease as well as to discover and develop novel therapeutics. This Research Topic has included original and review papers spanning both topics, therapeutics and disease modeling, and discuss a large number of neuronal cell types.

In the context of ALS and Frontal Temporal Dementia (FTD), Guo et al. summarize the disease-specific phenotypes in patient-derived IPSC-derived neurons and the variability between reports (Guo et al.), identifying the limitations of these approaches, including the variable genetic backgrounds, off-target effects of genetic corrections or targeting, the lacking cellular maturity, and the heterogeneity of differentiation techniques. This latter point is further detailed by Ghaffari et al. whom provide an impressive deep-dive into the different means of differentiation of specific neuronal and glial subtypes in detail, and compare and contrast IPSCs and direct conversion. Despite these challenges, the use of patient-derived tissue is strongly recommended in disease-modeling and preclinical ALS studies for drug discovery and development (van den Berg et al., 2019). Besides disease modeling and the replacement of neurons, stem cell derived cells can also be developed as a therapeutic strategy to support neuronal survival, instead of adopting a neuronal fate. A summary of these strategies and their (pre)clinical support in ALS is provided by the article from Forostyak and Sykova, in which they outline the terminology and cell types that have been published. In particular, they highlight the protective effects on motor neurons that mesenchymal stromal cells offer by producing neurotrophic factors upon transplantation (Forostyak and Sykova).

With the epigenetic markers of cellular maturity lost when cells are converted to iPSCs (Mertens et al., 2015; Traxler et al., 2019), it can be challenging to detect late onset disease-specific pathology in iPSC-derived neurons. Seminary et al., recapitulated an impaired heat shock response in

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Edited and reviewed by:
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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *19 March 2021*
Accepted: *12 April 2021*
Published: *08 June 2021*

Citation:
Staats KA (2021) Editorial: Stem Cells in Neurodegeneration: Disease Modeling and Therapeutics. Front. Neurosci. 15:683122. doi: 10.3389/fnins.2021.683122

iPSC-derived motor neurons harboring ALS mutations, In addition, with this model they identified an accumulation of insoluble and aggregation-prone proteins, and that the presence of these was not sufficient to induce a heat shock response or stress-granule formation (Seminary et al.). The importance of how a gene may cause disease and whether that mechanism remains present in iPSC-derived neurons is also of relevance in the context of the regulation of the gene *SNCA*, encoding for the protein alpha-synuclein, in Parkinson's Disease (Piper et al.). Piper et al. describe in-depth the different ways *SNCA* may genetically cause disease, as well as how *SCNA* may be regulated. The authors stress the importance of the understanding of temporal and cell type-specific regulation of SNCA in disease, and in disease-models (Piper et al.).

In Huntington's disease (HD), this Research Topic's contributions span the increased understanding of pathophysiology, testing of novel therapeutics, and the transplantation of cells as a potential therapeutic.

Naphade et al. used patient-derived, isogenic, and control-corrected IPSCs to generate neural stem cells to assess the role of matrix metalloproteinases (MMPs) and their inhibitors in HD (Naphade et al.). They found that MMPs' endogenous inhibitors are decreased in HD cells and are elevated by TGFb treatment (Naphade et al.), illustrating a potential new direction for HD therapeutic strategies. Rindt et al. used a similar technique to assess the potential of the pre-mRNA repair of mutant Huntingtin, and also identified a beneficial response in HD-derived IPSC neural models (Rindt et al.). Subsequnetly, Masnata and Cicchetti describe the evidence for seeding of the Huntingtin protein in *in vitro* cultures of HD, including by IPSC disease modeling, and find sufficient evidence to suggest that this occurs, prompting the conclusion that *in vivo* assessment is now needed to further assess this (Masnata and Cicchetti). Al-Gharaibeh et al. transplanted IPSC-derived neural stem cells as a potential therapeutic into the striata of aged HD model mice (YAC128) and observed a striking protective effect on pathology and behavior in these animals (Al-Gharaibeh et al.), indicating the potential for neuronal replacement therapy in HD.

To understand the potential and limitations of the use of non-primary neurons, Drouin-Ouellet et al. summarize the use of inducible neurons (iNeurons), which are derived from direct differentiation from somatic cells (Drouin-Ouellet et al.). They delineate what constitutes an iNeuron, describe the benefits of each stage of differentiation per neurological disease, discuss whether generating subtype-specific iNeurons is critical to the disease-related features of these cells, and subsequently explain the biomedical potential and limitations of the use of these cells (Drouin-Ouellet et al.). In addition, Omais et al. summarize an alternative potential cells to model neurodegenerative disease; adult neurogenesis in the olfactory bulb (Omais et al.).

To accelerate the discovery and development of novel therapeutic strategies for patients, the Montreal Neurological Institute has adopted an "Open Science" model, as described by Han et al. Open Science refers to the transparency of the research and is illustrated by Open Lab Notebooks Durcan, and the sharing of resources, including patient-derived stem cell lines (Gan-Or et al., 2020). Such initiatives will hopefully provide novel ideas and collaborations, with improved disease modeling and therapeutic development for neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

ACKNOWLEDGMENTS

Drs. Dustin Wakeman and Kyle Fink were essential for the successful completion of the Research Topic Stem Cells in Neurodegeneration: Disease Modeling and Therapeutics.

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Conflict of Interest: KS is employed by company Staats Life Sciences Consulting, LLC.

frontiers in Neuroscience

REVIEW
published: 28 September 2017
doi: 10.3389/fnins.2017.00530

Direct Neuronal Reprogramming for Disease Modeling Studies Using Patient-Derived Neurons: What Have We Learned?

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: 27 July 2017
Accepted: 12 September 2017
Published: 28 September 2017

Citation:
Drouin-Ouellet J, Pircs K, Barker RA, Jakobsson J and Parmar M (2017) Direct Neuronal Reprogramming for Disease Modeling Studies Using Patient-Derived Neurons: What Have We Learned? Front. Neurosci. 11:530. doi: 10.3389/fnins.2017.00530

Direct neuronal reprogramming, by which a neuron is formed via direct conversion from a somatic cell without going through a pluripotent intermediate stage, allows for the possibility of generating patient-derived neurons. A unique feature of these so-called induced neurons (iNs) is the potential to maintain aging and epigenetic signatures of the donor, which is critical given that many diseases of the CNS are age related. Here, we review the published literature on the work that has been undertaken using iNs to model human brain disorders. Furthermore, as disease-modeling studies using this direct neuronal reprogramming approach are becoming more widely adopted, it is important to assess the criteria that are used to characterize the iNs, especially in relation to the extent to which they are mature adult neurons. In particular: i) what constitutes an iN cell, ii) which stages of conversion offer the earliest/optimal time to assess features that are specific to neurons and/or a disorder and iii) whether generating subtype-specific iNs is critical to the disease-related features that iNs express. Finally, we discuss the range of potential biomedical applications that can be explored using patient-specific models of neurological disorders with iNs, and the challenges that will need to be overcome in order to realize these applications.

Keywords: induced neurons, direct neural reprogramming, disease modeling, neurological disorders, neurodegenerative diseases

INTRODUCTION

Direct reprogramming of a terminally differentiated cell into another cell type was achieved for the first time in 1987 with the conversion of fibroblasts to myoblasts (Davis et al., 1987). Following this, however, it took more than two decades to successfully directly reprogram fibroblasts to neuronal cells using the forced expression of the neuronal transcription factors *Ascl1*, *Brn2*, and *Myt1l* - a cell product termed induced neuron (iN) (Vierbuchen et al., 2010). Since then, the field of direct neuronal reprogramming has been applied to human cells and been expanding at a fast pace, and studies using patient derived iNs to model neurological disorders have started to appear.

iN cells, in contrast to induced pluripotent stem cells (iPSCs), are the result of direct reprogramming of one type of somatic cell into another without going through a pluripotent intermediate stage. Because of this feature, it was hypothesized that iNs would therefore retain some of the characteristics of the starting cell, especially related to epigenetic status and aging. Two studies have now demonstrated that this is the case—at least to some extent. Using a broad range of human fibroblasts from different age donors, Mertens et al. demonstrated that iNs exhibit an age-dependent regulation of genes associated with aging. They found that there is an age-dependant loss of nucleocytoplasmic compartmentalization in donor fibroblasts which was kept in iNs but restored in iPSCs derived from aged cells. More specifically, they further demonstrated that RanBP17, a receptor that decreases with aging, was also decreased in an age-dependent manner in iN cells—features that were both absent in iPSCs (Mertens et al., 2015a). Using different approaches to assess the age of the cell that relies on the epigenetic DNA methylation age measurements, a method that looks at a number of genomic loci becoming differentially methylated with age to predict the age of the cell (in years) (Horvath, 2013), Huh et al. have also shown that iNs retain the age of the donors at the epigenetic level. Moreover, they show that the aging signature is maintained through their microRNA expression profile and increased oxidative stress levels (Huh et al., 2016).

Neurons are especially affected by aging given that they do not regenerate in most regions of the brain, which could underlie why the majority of neurodegenerative disorders present clinically later in life. As a result, age is a prominent risk factor in many of these diseases including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD). Animal models of these disorders can mimic some aspects of these human-specific diseases but most models are toxin- or vector-based, or use mendelian forms of these diseases as their starting point, and do not recapitulate the appearance of disease phenotypes associated with human aging. As such, there is a pressing need for models that faithfully recapitulate both the sporadic and age related aspects of these common chronic neurodegenerative disorders in human cells.

As the number of disease modeling studies using iNs being published are starting to increase, here we review what has been accomplished to date, and provide an outlook of what could be achieved in the future.

Abbreviations: 5-HT, 5-hydroxytryptamine or serotonin; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AP, action potential; APP, amyloid precursor protein; ASCL1, Achaete-scute homolog 1; BD, bipolar disease; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; BRN2 or POU3F2, POU domain, class 3, transcription factor 2; C or CHIR, CHIR99021; cAMP, cyclic adenosine monophosphate; CHAT, choline acetyltransferase; ciN, chemical induced neuron; CNTF, ciliary neurotrophic factor; CNS, central nervous system; CTIP2 or BCL11B, B-Cell CLL/Lymphoma 11B; D or DM, dorsomorphin; DARPP32, dopamine- and cAMP-regulated neuronal phosphoprotein; DCX, doublecortin; DLX1/2, distal-less homeobox 1/2; F, forskolin; FACS, fluorescence-activated cell sorting; FEV, ETS Transcription Factor; FOXA2, forkhead box protein A2; FTD, frontotemporal dementia; FUS, fused in sarcoma; G, GO6983; GALC, galactosylceramidase; GDNF, glial cell-derived neurotrophic factor; GSK3β, glycogen synthase kinase 3 beta; HB9, homeobox gene 9; HD, Huntington's disease; HET, heterozygous; HOMO, homozygous; ICC, immunocytochemistry; iDAN, induced dopaminergic neuron; IGF1, insulin-like growth factor 1; iMN, induced motor neuron; iN, induced neuron; iPSc, induced pluripotent stem cell; ISL1, insulin gene enhancer; LAMP1, lysosomal-associated membrane protein 1; LDH, lactate dehydrogenase; LHX3, LIM/homeobox; LM, LM-22A4; LMX1a/b, LIM homeobox transcription factor 1, alpha or beta; LRRK2, leucine-rich repeat kinase 2; MAP2, microtubule-associated protein 2; MOI, multiplicity of infection; MYTL1, myelin transcription factor 1-like; NBIA, neurodegeneration with brain iron accumulation; NCAM, neural cell adhesion molecule; ND, not determined; NEUROD1 or 2, neurogenic differentiation 1 or 2; NF200, high molecular weight neurofilament subunit; NGN2, neurogenin-2; NLS, nuclear localization signal; NT3, neurotrophin-3; NURR1, nuclear receptor related 1; OLIG2, oligodendrocyte transcription factor 2; PD, Parkinson's disease; PGK, phosphoglycerate kinase; PINK1, PTEN-induced putative kinase 1; PKAN, pantothenate kinase-associated; PNS, peripheral nervous system; PSA-NCAM, polysialylated-neural cell adhesion molecule; PSD95, postsynaptic density protein 95; PSEN 1 or 2, presenilin 1 or 2; PTB, polypyrimidine tract-binding protein; R, repsox; RA, retinoic acid; RanBP17, RAN binding protein 17; REST, RE1-Silencing Transcription factor; RT-qPCR, real-time quantitative reverse transcription PCR; S, SP600125; SB, SB202190; SMA, spinal muscular atrophy; SNP, single-nucleotide polymorphism; SOX2, SRY (sex determining region Y)-box 2; SYN, synapsin; SYT1, synaptotagmin-1; TGFβ, transforming growth factor beta; TH, tyrosine hydroxylase; TUJ1, neuron-specific class III beta-tubulin; Ub, ubiquitin; UNO, unoprostone; V or VPA, valproic acid; VACHT, vesicular acetylcholine transporter; vGLUT, vesicular glutamate transporter; VIM, vimentin; WB, western blot; Y, Y-27632.

CAN PATIENT-DERIVED iNS PROVIDE AN AUTHENTIC CELLULAR SYSTEM TO ASSESS DISEASE-RELATED PHENOTYPES?

Patient specific neurons derived from iPSCs have shown a wide array of disease-associated phenotypes. The majority of those studies have studied mendelian forms of neurological disorders but some features could also be observed in sporadic forms of diseases such as schizophrenia, bipolar disorder and ALS (Koch et al., 2011; Burkhardt et al., 2013; Mertens et al., 2015b). To date, at least ten neurological disorders have been modeled using patient-derived iN cells (see **Table 1**) and multiple disease-associated phenotypes has been observed, although studies looking at disease features in lines from sporadic patients has yet to be reported. However, while some of these features have been uniquely seen in iN cells, other phenotypes can be detected in the starting cell before conversion or in neurons differentiated from patient-derived iPSCs. Given that each cellular system has their own merits and challenges, it will be important to decipher the benefits that iNs have for modeling neurological disorders. In addition to a much shorter and easier reprogramming route, the most important difference known to date between neurons generated from iPSCs or directly from fibroblasts is the age of the cell. It may be, though, that iPSC derived neurons will be best suited for modeling diseases associated with developmental processes whereas iNs will be most useful to study disorders associated with aging.

Disease-Associated Features Unique to Human Cells

Mouse fibroblasts, especially at the embryonic stage, are easier to reprogram than human adult fibroblasts and the resulting cells mature faster. For example, spontaneous action potentials

TABLE 1 | Neurological disease modeling in induced neurons.

Disease	Mutations	References	Target cell type	Reprogramming strategy	Days post transduction	% Conversion efficiency	% Purity	iN characterization	Electrophysiology	Disease phenotype
PD	*PINK1* Q456X	Fiesel et al., 2015	iN	LV.shPTB, bFGF, BDNF, GDNF, NT3, CNTF	12–14	ND	ND	ICC: TUJ1 WB: MAP2, TUJ1	ND	No pS65-Ub accumulation upon mitochondrial damage
	PINK1 p.G411S HET, p.Q456X HET or HOMO	Puschmann et al., 2017	iN	LV.shPTB bFGF, BDNF, GDNF, NT3, CNTF	12–14	ND	ND	ICC: TUJ1 WB: TUJ1	ND	Reduced pS65-Ub levels in p.G411S over time elevated parkin levels
FTD and parkinsonism	*MAPT* (K298E)	Iovino et al., 2014	iN	LV.ASCL1-BRN2-MYT1L	30–53	ND	ND	ICC: TUJ1	ND	3R and 4R tau isoform expression
PKAN	*PANK2*	Santambrogio et al., 2015	iDAN	LV.ASCL1-NURR1-LMX1a	20	5	ND	ICC: TUJ1, NCAM, MAP2 TH (50% of TUJ1+)	ND	Altered oxidative status mitochondrial dysfunction
AD	*APP* (V717I) or *PSEN* (I167 or A434T or S169del)	Hu et al., 2015	ciN or iN	small molecules VCRFSGYD bFGF, cAMP, BDNF, GDNF, NT3 or LV.ASCL1-NGN2 and cAMP, SB, noggin, LDN, CHIR, BDNF, GDNF, NT3	14–28	10–13	ND	ICC: TUJ1, DCX, MAP2, TAU, NEUN, SYN vGLUT RT-qPCR; Single-cell sequencing; microarray; FACS	YES	Abnormal Aβ production increased pTau and Tau levels in APP
HD	*HTT* 68Q or 86Q	Liu et al., 2014	iN	LV.shPTB bFGF, BDNF, GDNF, NT3, CNTF	19–30	ND	8–14	ICC: TUJ1, NEUN (10%), GABA, DARPP32 (60-80%)	ND	Neuritic breakdown, Abnormal neuritic branching, increased cell death aggregation of mutant huntingtin
BD	ND	Bavamian et al., 2015	iN	LV.miR9/9*-124 + NEUROD2-ASCL1-MYT1L VPA	38–40	ND	ND	ICC: MAP2, TUJ1	ND	Increased miR-34a levels
Schizophrenia	16p11.2 duplication 22q11.21 deletion	Passeri et al., 2016	iN	LV.ASCL1-BRN2-MYT1L, SB, Noggin, CHIR, cAMP, VPA, BDNF, GDNF, NT3	21	ND	ND	ICC: MAP2	ND	Toxoplasma gondii infection and characterization

(Continued)

TABLE 1 | Continued

Disease	Mutations	References	Target cell type	Reprogramming strategy	Days post transduction	% Conversion efficiency	% Purity	iN characterization	Electrophysiology	Disease phenotype
	22q11.2 deletion; 16p11.2 duplication and/or 22q13.3 duplication	Passeri et al., 2015	iN	LV.ASCL1-BRN2-MYT1L, SB, noggin, CHIR, cAMP, VPA, UNO, GDNF, BDNF, NT3	21	ND	20–40	ICC: MAP2	ND	Neurons with a similar morphological complexity
	SNPs rs1198588, rs1625579, rs2660304, rs2802535	Siegert et al., 2015	iN	LV.ASCL1-BRN2-MYT1L, bFGF	28	ND	ND	FACS sort (0.01-0.001% iN cells/all FACS events)	ND	Minor allele SNPs cause miR-137 gain of function miR-137 genetic risk
SMA	*SMN1*	Zhang et al., 2017	iMN	LV.ASCL1-NEUROD1-BRN2-MYTL1-NGN2-ISL1-HB9-LHX3 bFGF, BDNF, GDNF, IGF1, cAMP	23–62	6	2–5	ICC: TUJ1, CHAT	ND	Reduced neurite outgrowth, disintegrated neurons, neurodegeneration (day 60), increased caspase-3 levels, high LDH activity
ALS	C9orf72 repeat expansion	Su et al., 2014	iN	LV.shPTB, bFGF, BDNF, GDNF, NT3, CNTF	15–19	ND	ND	ICC: MAP2, TUJ1, SYN, PSD95, SMI32, Drebrin	ND	Cytoplasmic poly(GP) inclusions
	FUS p.G504Wfs*12 p.R495* or p.Q519e	Lim et al., 2016b	iN	LV.shPTB, bFGF, BDNF, GDNF, CNTF, NT3	10–21	80–90	ND	ICC: TUJ1, MAP2, NEUN, SYN	ND	Reduced endogenous FUS levels in nucleus, increased cytoplasmic FUS levelsneuropathology of FUS mutations with a disrupted NLS region
	FUS R522R or H517Q or R521G	Liu et al., 2016	iMN	LV.NGN2-SOX11-ISL1-LHX3 FSK, DM, bFGF, BDNF, GDNF, NT3	14–49	80–93	95–97	ICC: TUJ1, MAP2, NF200, SYT1, HB9, CHAT, VACHT RT-qPCR	YES	Mislocalization of FUS, shrunken somas, deficits in AP firing and reduced membrane capacitance, impaired control of muscle contraction
Krabbe-disease	*GALC* (p.K563*;L634S) or (p.N228_S232delinsT; G286D)	Lim et al., 2016a	iN	LV.shPTB bFGF, BDNF, GDNF, CNTF, NT3	8–10	ND	ND	ICC: MAP2, TUJ1, SYN, vGLUT, Phalloidin, TAU WB: TUJ1	ND	Diminished GALC activity, increased psychosine levels, neurite fragmentation, abnormal neuritic branching, higher LAMP1 level, enlarged and fragmented LAMP1+ vesicles, mitochondrial morphology altered

can be detected in iNs originating from MEFs as early as 8 days into conversion (Vierbuchen et al., 2010), whereas the earliest time point when spontaneous action potentials could be detected to date in human iNs is 46 days (Xu et al., 2015), suggesting that human cells take longer to become fully mature. Therefore, mouse embryonic fibroblasts have been used to study the disease mechanisms in iNs in monogenic disorders (Chanda et al., 2013). While this approach may be a starting point through which to study the impact of a specific mutation on disease pathogenesis, it has been reported that some disease-related phenotypes only have a pathology in human iNs. For example, iNs derived from ALS patients carrying a mutation in the fused in sarcoma (FUS) protein recapitulated the localization of the mutated protein in the cytoplasm instead of the nucleus following stress induction, a feature that rat primary neurons carrying the same mutation failed to express (Lim et al., 2016b). In another study investigating iron metabolism in neurodegeneration with brain iron accumulation (NBIA), mitochondrial iron and energetic dysfunction were observed in both pantothenate kinase-associated (PKAN) patient derived fibroblasts and iNs (Santambrogio et al., 2015), whereas these features were not seen in fly or mouse models of the disease (Rana et al., 2010; Brunetti et al., 2012). While there are only a limited number of such reports published to date, they do highlight some species-specific differences and favor the use of human-based cellular system(s) in which the disease-associated phenotypes will be assessed, especially given these diseases are all uniquely human.

Disease-Associated Features Present in iNs and Absent in Parental Fibroblasts

Disease-associated features are not always unique to the neurons and as a result, several of these phenotypes can be observed in both iNs and fibroblasts (Santambrogio et al., 2015; Lim et al., 2016a). However, as iNs adopt a neuronal-like morphology and at least some functional properties of neurons, they provide an opportunity to study diseases in the cell type primarily clinically affected. For example, iNs derived from adult-onset Krabbe disease had the same lysosomal storage defects as the starting fibroblasts, but unique to the iNs was the abnormal neuronal branching, which may be more relevant to the clinical expression of this disorder (Lim et al., 2016a). In fact, a few studies have now reported that disease-associated features could only be seen in iNs. For example, Lim et al. (2016b) reported that mutant FUS-associated pathology was observed in iNs derived from familial ALS patients, but not transfected cells or patient-derived fibroblasts (Lim et al., 2016b). In line with this, an independent study in induced motor neurons (iMNs) also reported such disease-associated phenotype (Liu et al., 2016). In another report, *Toxoplasma gondii* infection of iN cells derived from patients with childhood onset schizophrenia resulted in cyst formation due to *T. gondii* differentiation in the iN soma, whereas the infected parental fibroblasts were completely lysed by parasite infection (Passeri et al., 2016). Given that the conversion of the fibroblasts to iN cells allowed the formation of cysts, this argues in favor of the specific need of the relevant neuronal cell type to assess disease pathogenesis. Other examples illustrating this point include a study showing that changes at the level of pathological protein expression have also been observed, with elevated levels of Aβ42 as well as phosphorylated Tau in iNs derived from patients with familial AD as compared to fibroblasts (Hu et al., 2015). Finally, the investigation of phenotypes associated with the repeat expension $r(GGGGCC)_{exp}$ in *C9orf72* that leads to frontotemporal dementia (FTD) and ALS resulted in the detection of cytoplasmic poly(GP) as well as poly(PR) inclusions in iNs but not in fibroblasts (Su et al., 2014).

One important caveat with most of these studies, however, is that the iNs that have been used for disease modeling were mostly at early stages of conversion, and thus rather immature neurons in terms of function, marker expression and morphology. Additionally, most studies have been performed on a pan-neuronal or unspecified neuronal subtype rather than on a specific subtype of neuron. When using iPSCs for disease modeling, subtype specific disease-related features have been reported to be important. For instance, abnormal neuronal arborization was observed in dopaminergic neurons bearing a *Leucine-rich repeat kinase 2 (LRRK2)* mutation but not in sensory neurons differentiated from the same cell source (Schwab and Ebert, 2015). It is thus likely that iNs of different subtypes may express distinct disease-related phenotypes, which will be important to study given that most of these diseases have pathology that is region specific in the CNS/PNS. This ability to generate subtype specific neurons has now been achieved for many types of neurons, including dopaminergic (Caiazzo et al., 2011; Pfisterer et al., 2011a), striatal medium spiny (Victor et al., 2014), cholinergic (Liu et al., 2013; Zhang et al., 2017), nociceptive (Wainger et al., 2015), spinal motor (Son et al., 2011; Liu et al., 2016), GABAergic interneurons and serotoninergic neurons (Xu et al., 2015; Vadodaria et al., 2016) (see **Figure 1**). Furthermore, the generation of such subtype specific iNs provides the advantage to expand the array of functional assays that can be performed. For example, the formation of functional neuromuscular junctions by iMN could be evaluated in co-cultures with primary mouse skeletal myotubes, as well as through more conventional approaches such as electrophysiologically, a functional aspect which has been shown to be impaired in iMNs derived from ALS patients (Liu et al., 2016).

Ideally, investigation of disease-associated phenotypes should be done using neuron specific subtypes and comparing subtypes of cells that are, or are not, affected in the disease process. For this purpose, the generation of additional subtypes of neurons as well as the optimization of current reprogramming protocols to produce iNs that mimic more closely the cellular phenotypes of the diverse human neuronal subtypes is needed. This, however, will remain challenging as long as the mechanisms behind fate specification during direct reprogramming are not better understood. Furthermore the production of a high yield of subtypes specific iNs is technically very challenging given that this often requires the delivery of a greater number of reprogramming factors and as a result, only a small subset of cells expresses the full set.

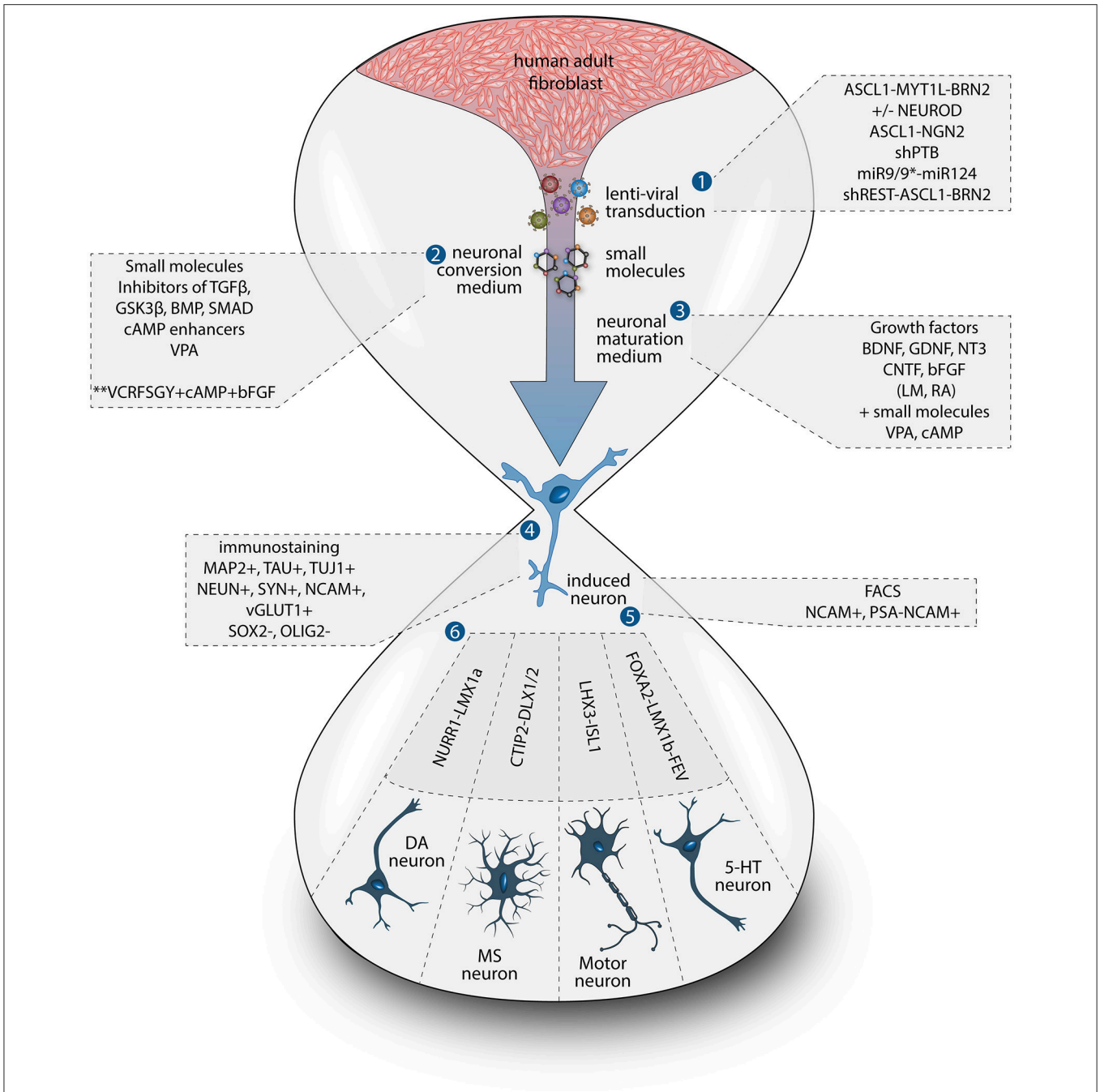

FIGURE 1 | Methods for direct neuronal conversion. During direct neuronal conversion, adult human fibroblasts undergo progressive conversion into iNs. This process is initiated by a lentiviral transduction (1) to deliver the reprogramming factors and/or the addition of chemical compounds (2, 3). Neuronal identity can be confirmed by the expression of pan-neuronal markers (4). iN cultures can further be purified using antibiotic selection and/or cell sorting (5). Subtype specific neurons can also be obtained by the addition of fate determinant reprogramming factors (6).

HOW TO DEFINE AN iN?

The term iN has been used to describe neurons generated from multiple cell sources and through multiple methods. A first important distinction should be made between neurons differentiated from iPSCs using extrinsic factors, neurons obtained from pluripotent stem cells through the forced expression of programming factors or via the direct reprogramming of somatic cells to neuronal progenitors and further differentiated into mature neurons. Here, we define iNs as the product of directly reprogrammed neurons starting from somatic cells, such as a fibroblast, and avoiding a pluripotent or

progenitor stage intermediate and we focus on iNs produced from adult human fibroblasts due to their utility in disease modeling.

These type of adult iNs have been generated using four main methods: (i) by the forced expression of transcription factors (Caiazzo et al., 2011; Pfisterer et al., 2011b; Iovino et al., 2014; Mertens et al., 2015a; Passeri et al., 2015; Siegert et al., 2015; Liu et al., 2016), (ii) by knocking down of the RNA-binding proteins PTB/nPTB (Xue et al., 2016) or p16-p19 (Sun et al., 2014), (iii) by the forced expression of neuronal specific microRNAs (Victor et al., 2014; Richner et al., 2015; Huh et al., 2016), (iv) by chemically manipulating pathways involved in neuronal fate and functions (Hu et al., 2015) or by a different combination of these strategies (Ambasudhan et al., 2011; Liu et al., 2013; Hsu et al., 2014; Wang et al., 2014; Xu et al., 2015; Drouin-Ouellet et al., 2017) (**Figure 1**). Each of these methods has been proven effective in generating functional neurons in which it is possible to evoke action potentials as well as observe spontaneous synaptic activity within a timeframe ranging from 4 to 12 weeks when co-cultured with astrocytes or primary cortical neurons or after transplantation (Hu et al., 2015; Huh et al., 2016; Liu et al., 2016; Xue et al., 2016; Drouin-Ouellet et al., 2017), and even spontaneous action potentials in some cases (Mertens et al., 2015a). The resulting iNs have also been shown to express mature neuronal markers including MAP2, TAU, and NEUN with complex neuronal morphology.

To date, the predominant method by which to isolate/identify the iN population to assess disease relevant phenotypes in patient derived iNs, has involved either an antibiotic selection to remove cells that are not expressing the reprogramming construct(s) (Liu et al., 2014; Su et al., 2014; Bavamian et al., 2015; Lim et al., 2016a) or based on the expression of neuronal markers such as TUJ1 (βIII-Tubulin) (Iovino et al., 2014; Liu et al., 2014; Fiesel et al., 2015; Lim et al., 2016a,b; Puschmann et al., 2017). However, direct neuronal reprogramming studies that have used antibiotic selection to purify the neuronal culture have consistently reported that a significant percentage of cells do not convert even though the reprogramming constructs are expressed (Victor et al., 2014; Mertens et al., 2015a; Huh et al., 2016; Liu et al., 2016; Xue et al., 2016), which presents the need to identify the iN population even after antibiotic selection. Furthermore, using a method that combines the forced expression of Ascl1 and Brn2 with the knockdown of the neuronal repressor complex REST, we show that although an important proportion of cells expressing stronger levels of TUJ1 can be observed very early on, only a few MAP2+ or TAU+ cells are detectable around day 18 and this number increases at day 25 (**Figure 2**). Notably, a striking and progressive change in cell morphology toward a more mature neuronal appearance is observed over time—e.g., a decrease of the size of the nucleus and cell body, thinning and elongation of the processes and increase in the number of branches. As expected, some markers of fibroblasts and non-mature neurons such as Vimentin can be co-expressed with mature neuronal markers in iNs even at later time points (**Figure 3A**), whereas the marker TE7, which is fibroblast-specific, is not co-expressed in TUJ1+ cells as early as day 10 (**Figure 3B**). Indeed Xue et al. (2016) have reported that TUJ1 is expressed as early as 3 h following shPTB and plateaus at 1 day post transduction - at a time when a fibroblast marker such as fibronectin is still strongly expressed and when the transduced cells do not exhibit a full neuronal morphology (Xue et al., 2016). These authors have also shown that knocking down PTB is not sufficient to induce the expression of mature neuronal markers such as MAP2 and NEUN in adult human fibroblasts but that the full maturation of iNs requires sequential nPTB knockdown. As a result, the neuronal identity of iNs used in disease modeling studies that have both knocked down PTB and used TUJ1 as a neuronal marker or only antibiotic selection to obtain the iN population, has not been confirmed.

These challenges with iN reprogramming can lead to potential bias when assessing neuron-specific phenotypes associated with different disorders as cells not fully converted or not fully mature may not be an ideal system for modeling of diseases in which the primary cell population affected are neurons. As such, we suggest that disease modeling studies should use:

1. One of the methods that have been proven to generate mature neurons in adult human fibroblasts (Hu et al., 2015; Mertens et al., 2015a; Richner et al., 2015; Xue et al., 2016; Drouin-Ouellet et al., 2017), or new methods where the neuronal identity and function is well documented.
2. The expression of at least one of the following markers (MAP2, TAU, and/or NEUN) to identify the neurons and
3. Morphological criteria for neuronal identification and
4. Conversion protocols with maturation times *in vitro* of at least 4 weeks.

FUTURE OUTLOOK

Although the field of modeling neurological disorders with iN cells is still in its early stages, the results reported thus far support the need for further development of the iN technology, as it has already been shown to be useful in studying some neuronal specific age related human diseases. Up until very recently, the methods used to obtain iNs have been rather inefficient when applied to adult human fibroblasts, which has greatly hampered their utility for disease modeling. To circumvent this issue, we have developed a simple single-step and single-vector based approach that can generate very high yields of iNs from patients with neurodegenerative disorders independent of the passage number of the fibroblasts (Drouin-Ouellet et al., 2017). This new approach results in cells that fulfill the above criteria for iN cell suitable for disease modeling as outlined above (expression of mature neuronal markers, neuronal morphology, more than 4 weeks maturation *in vitro*) and overtime the cells develop functional properties of neurons including post-synaptic currents and the ability to fire action potentials. The simplicity and high efficiency of the method should facilitate the application of direct neuronal reprogramming for disease modeling studies.

The use of iNs is advantageous in terms of allowing studies of large cohort of patients and controls including patients with sporadic diseases within shorter time, with relatively little work and cost compared to iPSC-based modeling. Another advantage

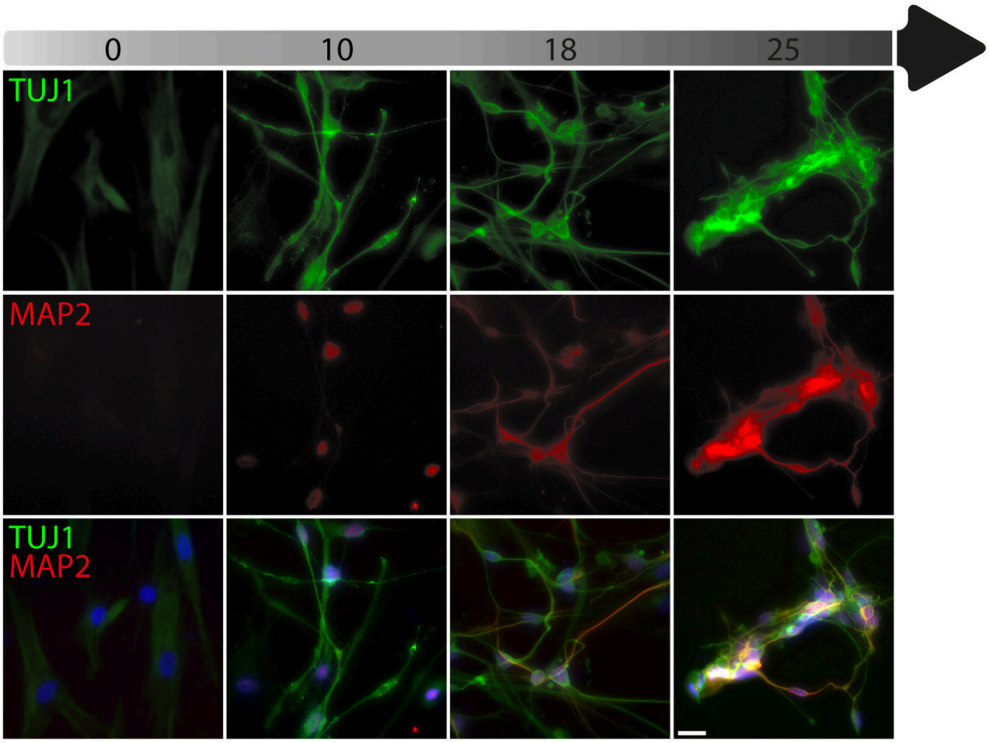

FIGURE 2 | Timeline of neuronal marker expression during reprogramming. Representative images of TUJ1 and MAP2 double immunostaining counterstained with DAPI (in blue) showing low levels of TUJ1 in dermal fibroblasts (in green), followed by intensification of expression at day 10, which is sustained until day 25 post-transduction with the U6.shREST.PGK.BRN2.PGK.ASCL1.WPRE construct. MAP2 expression (in red) is detectable in the nucleus at day 10 and is incrementally expressed in the processes from day 18 to 25. Scale bar = 25 μm.

of iN cells is that they maintain, at least partially, the aging signature of the cell, and are therefore more likely to provide insights into the molecular mechanisms underlying the age-dependent pathogenesis of some neurological disorders, as well as the pathological basis of their clinical heterogeneity.

Another potential advantage of iNs is that they do not rely on clonal selection and while this could result in a higher heterogeneity of the final neuronal population, the end product as a whole is more likely to be biologically relevant than a few selected iPSC clones. Furthermore, as methods for producing glial cells by direct reprogramming are emerging (Caiazzo et al., 2015; Tian et al., 2016), we can expect a greater sophistication of the induced cellular systems and with this, a more comprehensive assessment of specific non-cell autonomous interactions involving multiple neural cell types during disease processes on a patient-specific basis.

Once models for a diverse range of neurological disorders using iNs have been well established, we anticipate that there will be an expansion of the field toward early and differential diagnostics, drug target validation as well as drug screening assays. However, for this to become a reality, a number of challenges need to be overcome. For instance, careful characterization of the cell product should be carried out in terms of neuronal phenotypes as well as subtype authenticity to mimic as close as possible the types of neurons that are found and affected in the human brain. In that respect, more molecular studies at the single cell level are warranted to better understand the relationship between the reprogramming and the endogenous factors, as well as the target level of expression needed to perfect the end cell product. In support of this, novel reprogramming strategies which ensure that the full set of factors are expressed in each starting cell, and which provide a better control of their expression level will improve the yield of the target iN subpopulations. Finally, further insights into the mechanisms of direct reprogramming will undoubtedly help shed light on how best to bring iN technology to the point where it becomes a routine tool, as well as possible therapeutic approach in its own right.

METHODS

An adult dermal fibroblast line derived from a skin biopsy from a neurologically healthy 71-year-old male was obtained from the Parkinson's Disease Research clinic at the John van Geest Centre for Brain Repair (Cambridge, UK) and used under local ethical approval (REC 09/H0311/88). Written informed consent was obtained from the participant, and the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. For

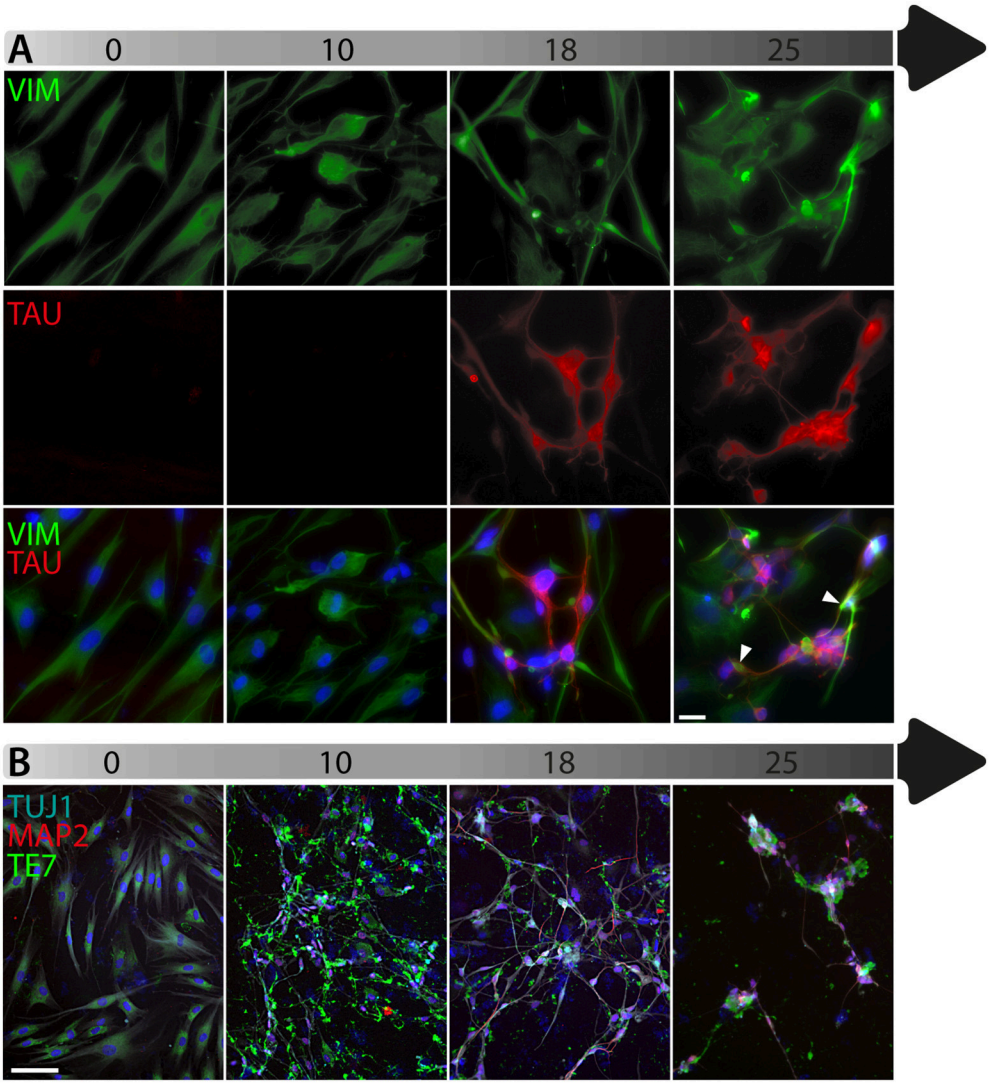

FIGURE 3 | Timeline of fibroblast marker expression during reprogramming. **(A)** Double immunofluorescent staining of the fibroblast and neuronal progenitor marker Vimentin (VIM; in green) and the neuronal marker TAU (in red) counterstained with DAPI (in blue) showing Vimentin expression in dermal fibroblasts as well as in a subpopulation of cells that are not expressing TAU at day 18 and 25 post-transduction. The majority of TAU expressing cells do not express VIM except for a few cells, which are double TAU/VIM+ (white arrowheads). **(B)** TUJ1 (cyan), MAP2 (red) and TE7 (green) triple immunostaining counterstained with DAPI (in blue) showing expression of the fibroblast marker TE7 in fibroblasts before transduction, whereas TUJ1+ and MAP2+ cells are negative for TE7, which is only detectable extracellularly at later time points during conversion. Scale bar in A = 25 μm, B =100 μm.

details on the skin biopsy sampling and the fibroblast cultures, refer to Drouin-Ouellet et al. (2017).

Neuronal reprogramming was done as described before (Drouin-Ouellet et al., 2017) using a single third-generation lentiviral vector expressing a combination of Ascl1 and Brn2 with short hairpin RNA (shRNA) targeting REST. It was generated with a non-regulated ubiquitous phosphoglycerate kinase (PGK) promoter produced as previously described (Zufferey et al., 1997) and titrated by quantitative PCR (qPCR) analysis (Georgievska et al., 2004). Transduction was performed at a multiplicity of infection (MOI) of 20. The virus titer was 1.93E + 09.

Immunocytochemistry was performed at day 0, 10, 18 and 25 as previously described (Drouin-Ouellet et al., 2017). The following primary antibodies were used in the blocking solution overnight at 4°C: chicken anti-MAP2 (1:15,000; Abcam, ab5392); mouse anti-TUJ1 (1:1,000; Promega, G7121); rabbit anti-TUJ1 (1:1,000; BioLegend, 801201); chicken anti-VIM (1:5,000; Millipore, AB5733); mouse anti-TAU clone HT7 (1:500, Thermo Scientific, MN1000); mouse anti-TE7 (1:100, Millipore, CBL271). On the second day, after washing twice with PBS, Cyanine-conjugated secondary antibodies (1:200; Jackson ImmunoResearch Laboratories) were added and counterstained with DAPI (1:1,000; Sigma-Aldrich). Images were captured from

a PBS-filled well at 20X using an inverted microscope (Leica, DFC360 FX-DMI 6000B).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This research has received funding from the New York Stem Cell Foundation, the European Research Council under the European Union's Seventh Framework Programme: FP/2007-2013 Neuro Stem Cell Repair (no. 602278), ERC Grant Agreement no. 30971, the Swedish Research Council treatment of the future grant agreement K2012-99X-22324-01-5, the Swedish Research Council 70862601/Bagadilico, Swedish Parkinson Foundation (Parkinsonfonden), the Strategic Research Area at Lund University Multipark and StemTherapy. JJ is supported by the Swedish Foundation for Strategic Research (#FFL12-0074). JD is supported by a Canadian Institutes of Health Research (CIHR) fellowship (#358492), and RB is supported by an NIHR Biomedical Research Centre grant to the University of Cambridge/Addenbrooke's Hospital. MP is a New York Stem Cell Foundation—Robertson Investigator.

ACKNOWLEDGMENTS

We thank Marie Persson Vejgården and Ulla Jarl for technical assistance.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AC and handling Editor declared their shared affiliation.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 10 October 2017
doi: 10.3389/fnins.2017.00544

Optimization of *trans*-Splicing for Huntington's Disease RNA Therapy

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Huntington's disease (HD) is a devastating neurodegenerative disorder caused by a polyglutamine (polyQ) expansion in exon 1 of the *Huntingtin* (*HTT*) gene. We have previously demonstrated that spliceosome-mediated *trans*-splicing is a viable molecular strategy to specifically reduce and repair mutant HTT (mtHTT). Here, the targeted tethering efficacy of the pre-mRNA *trans*-splicing modules (PTM) in HTT was optimized. Various PTMs that targeted the 3′ end of HTT intron 1 or the intron 1 branch point were shown *trans*-splice into an HTT mini-gene, as well as the endogenous HTT pre-mRNA. PTMs that specifically target the endogenous intron 1 branch point increased the *trans*-splicing efficacy from 1–5 to 10–15%. Furthermore, lentiviral expression of PTMs in a human HD patient iPSC-derived neural culture significantly reversed two previously established polyQ-length dependent phenotypes. These results suggest that pre-mRNA repair of mtHTT could hold therapeutic benefit and it demonstrates an alternative platform to correct the mRNA product produced by the mt*HTT* allele in the context of HD.

Keywords: Huntington's disease, HD, *Huntingtin, HTT, trans*-splicing, therapy

OPEN ACCESS

Edited by:
Kyle David Fink,
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Reviewed by:
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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *02 August 2017*
Accepted: *19 September 2017*
Published: *10 October 2017*

Citation:
Rindt H, Tom CM, Lorson CL and Mattis VB (2017) Optimization of trans-Splicing for Huntington's Disease RNA Therapy. Front. Neurosci. 11:544. doi: 10.3389/fnins.2017.00544

INTRODUCTION

Huntington's Disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expanded polyglutamine repeat (polyQ) in one allele of the *Huntingtin* (*HTT*) gene (The Huntington's Disease Collaborative Research Group, 1993). More than 35 polyQs causes disease, and the number of repeats is inversely correlated to the onset age and severity (Andrew et al., 1993; Duyao et al., 1993; The Huntington's Disease Collaborative Research Group, 1993). The disease affects ∼6 per 100,000 in Caucasian populations (Pringsheim et al., 2012), with devastating consequences including progressive motor dysfunction, chorea, cognitive impairment, psychiatric abnormalities (Vonsattel et al., 2008), sleep disturbances (Arnulf et al., 2008), eventually leading to death (DiFiglia et al., 1995). These symptoms are due to the dysfunction and eventual death of specific projection neurons within the brain (Ross and Tabrizi, 2011). In particular, medium spiny neurons of the striatum, followed by the pyramidal neurons of the cortex, are most affected by disease. The loss of these neurons can occur years before overt clinical symptoms (Rosas et al., 2008; Aylward et al., 2011).

The *HTT* gene encodes a protein that is evolutionarily conserved, with homologs found as distant as the multicellular amoeba *Dictyostelium discoideum* (Eichinger et al., 2005). This degree of conservation implicates *HTT* as a necessary gene for the survival of multicellular organisms. Consistent with this notion, a *Htt*-null in murine models results in early embryonic lethality (e7.5) (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). *HTT* is ubiquitously expressed and has roles in a multitude of cellular functions including protein transport (DiFiglia et al., 1995; Gutekunst et al., 1995; Block-Galarza et al., 1997), protein-protein interactions

(Takano and Gusella, 2002), transcriptional regulation (Kegel et al., 2002; Sugars and Rubinsztein, 2003), inhibition of apoptosis (Rigamonti et al., 2000), and embryonic development (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). While the expanded allele is associated with disease development, from an evolutionary perspective, the increasing CAG trinucleotide repeat length in *HTT* homologs correlates with an increase in brain evolution (Zuccato and Cattaneo, 2016). Potentially, the expanded repeat assists in the development of more complex nervous systems and that further pathogenic expansion of HD is an unintended side effect of this evolutionary process (Zuccato and Cattaneo, 2016). Like many other neurodegenerative diseases, it is currently unknown why this ubiquitously expressed protein specifically causes neuronal degeneration. There are a multitude of theories of the contribution of mutant HTT (mtHTT) protein to cell death including: specific mtHTT cleavages (Lunkes et al., 2002; Graham et al., 2006; Ratovitski et al., 2009) and modifications (Steffan et al., 2004; Gu et al., 2009; O'Rourke et al., 2013; Vicente Miranda et al., 2016), buildup of toxic aggregates (DiFiglia et al., 1997; Scherzinger et al., 1997) or soluble mtHTT oligomers (Takahashi et al., 2008; Leitman et al., 2013; Ramdzan et al., 2017), and haploinsufficiency of wild-type HTT (Humbert, 2010).

RNA splicing is the editing of nascent pre-mRNA, thereby producing mRNA by removing the intronic regions. Part of this highly dynamic and regulated process involves recognition of intronic regions by the spliceosomal complex, including: a "GU" donor site at the 5′ end of the intron, an "A" branchpoint near the 3′ end of the intron, and a poly-pyrimidine tract with an "AG" splice acceptor site at the 3′ end of the intron (**Supplementary Figure 1**). *cis*-Splicing occurs when introns are spliced out of a pre-mRNA and the flanking exons of the gene are ligated together. However, splicing can also occur between exons of two different RNA molecules, termed *trans*-splicing. *trans*-Splicing generates a chimeric mRNA and has been demonstrated to occur naturally in trypanosomes (Murphy et al., 1986; Sutton and Boothroyd, 1986), rat hepatocytes (Caudevilla et al., 1998), and even human cells (Flouriot et al., 2002; Wu et al., 2014). A technology predicated upon these naturally occurring *trans*-splicing events was developed in 1999, where an endogenous transcript was modified via spliceosome-mediated pre-mRNA *trans*-splicing to include an exogenous exon (Puttaraju et al., 1999). Previously, we have presented proof-of-principle experiments demonstrating that exon one replacement of *HTT* by *trans*-splicing can be achieved in cultured cells, both into a *HTT* mini-gene and the endogenous *HTT* pre-mRNA, suggesting a novel mechanism for mtHTT repair (Rindt et al., 2012).

These exogenous exons were termed pre-mRNA *trans*-splicing molecules (PTMs) and have three main modalities: a region that specifically binds to the target endogenous RNA, an artificial intron, and the exogenous "replacement" exon. Each sequence can be modified accordingly to improve efficacy and specificity to the *trans*-splicing event into the endogenous RNA (Garcia-Blanco, 2003). To further refine and increase the efficacy of HTT *trans*-splicing, a novel series of PTMs were designed to specifically bind to either the 5′ or 3′ ends of HTT intron 1. The coding sequence of each PTM-replaced exon 1 with a non-disease exon 1 sequence including 21 CAG trinucleotide repeats. PTMs that targeted the 3′ end of HTT intron 1 or the intron 1 branch point resulted in enhanced levels of *trans*-spliced RNA compared to the original HD *trans*-splicing RNA, demonstrating a correction of the HTT allele at the molecular level. To determine whether delivery of the optimized PTMs increased functional HTT protein, PTMs were expressed in a neural culture derived from HD patient induced pluripotent stem cells (iPSCs). In each instance PTM expression correlated with improvements in two previously established HTT CAG-expanded dependent phenotypes: susceptibility to BDNF withdrawal and ATP deficiency. Together, these results demonstrate that *trans*-splicing can be optimized to increase functional HTT protein, suggesting that *trans*-splicing could potentially serve as a therapy for HD.

MATERIALS AND METHODS

Cloning of Constructs

HTT Mini-Gene Construct

The HTT mini-gene has been previously described in Rindt et al. (2012). It contained exon 1 with 42 CAG repeats and exons 2–3, separated by intervening sequences. The sequences were based on Genbank accession number NT_006051. The two introns were shortened to 860 and 109 bp, respectively. The mini-gene was subcloned into pCI-neo (Promega, E1841) and expression was driven by the cytomegalovirus (CMV) promoter/enhancer.

PTM Constructs

The PTM constructs consisted of three portions: (1) the replacement exon 1 of HTT with 21 CAG repeats, (2) the splicing domain with an U1 snRNP binding site at the 3′ end of exon 1 and a triplet repeat of an intronic splice enhancer (ISE), and (3) the tether which binds to intron 1 by antisense base pairing. The constructs were generated by custom gene synthesis (Geneart). The PTM was inserted behind the CMV promoter into pMU1 (Coady et al., 2008). pMU1 also contained an eGFP expression module expressed from a separate promoter. For viral delivery, the PTMs were inserted into the lentiviral vector pSIN18 (Gropp et al., 2003) at the EcoRV site, where they were expressed off of a CMV promoter. This vector also contains an EF1a promoter-driven GFP upstream of the PTM. All tether sequences are included in Supplementary Table 1.

Transient Expression of PTMs

HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, 11965-092) containing high glucose and supplemented with 10% fetal bovine serum (Hyclone) and 100 U penicillin/100 μg streptomycin (Invitrogen) per mL. Cells were transiently transfected when they had reached ∼90% confluency using PEI (Polysciences, 23966-1) or lipofectamine 2000 (Invitrogen, 12566014), according to the manufacturer's recommendations. Mini-gene and PTM plasmids were co-transfected at a 1:1 ratio. Empty vector controls were extensively performed, but never demonstrated a positive response (data not shown). Alternatively, the PTM plasmid was transfected by itself

in experiments designed for *trans*-splicing of endogenous HTT pre-mRNA. Cells were harvested 24–48 h post-transfection.

RNA Isolation and RT-PCR

RNA was isolated using Tri-Reagent (Sigma Aldrich, T9424) following the manufacturer's instructions. RNA was resuspended in 10 mM Tris–HCl pH 8.2, 1 mM EDTA, and concentrations were measured using a Nanodrop (Thermo Fisher). cDNA was synthesized using 1 μg of RNA and random primers following the SuperScript III protocol (Invitrogen, 18080-044). PCR was performed using two different procedures. For amplifications outside the HTT exon 1 CAG repeat and the adjacent GC-rich region, Pfu enzyme (prepared in-house) with Thermopol buffer (New England Biolabs, B9004S) was used. For amplification across the exon 1 CAG repeat, Taq PCRx with 2 × enhancer solution (Invitrogen, 11495-017) was used, per manufacturer instructions. Primer sequences are all included in Supplementary Table 1.

Generation, Characterization, and Propagation of iPSC Lines

Human fibroblast lines were obtained from one HD patient with an expanded CAG *HTT* allele of 180 CAG repeats (CS97iHD180n) and from two non-HD "controls" with 33 (CS83iCTR33n) or 28 (CS14iCTR28n) repeat CAG alleles. Reprogramming was conducted by non-integrating methods, as previously described (Mattis et al., 2015). Neural progenitor aggregates were generated by manually lifting iPSC colonies from the feeder layers directly into 70:30 DMEM (Gibco, 11965-092):F12 (Gibco, 11765-054) plus 2% B27 without vitamin A (Gibco, 12587-010) supplemented 100 ng/mL basic fibroblast growth factor (bFGF, Peprotech, 100-18B), 100 ng/mL epidermal growth factor (EGF, Millipore, GF316), and 5 μg/mL heparin (Sigma Aldrich, H3393-50KU) in polyhema-coated flasks to prevent attachment, as previously described (Ebert et al., 2013). iPSC-derived neural progenitors were expanded as spherical aggregates and passaged weekly with a chopping technique (Svendsen et al., 1998).

Lentiviral Generation and Infection

Virus was produced by triple transfection of HEK293 FT cells with pSIN18-PTM, the helper plasmid psPAX2 (originally developed by D. Trono and obtained from Addgene #12260) and the envelope plasmid pVSV-G for pseudotyping. After 48 h, cell culture supernatant was collected and filtered through a 0.45-μm PES membrane, followed by centrifugation at 53,000 g for 90 min to pellet viral particles. Pellets were resuspended in phosphate-buffered saline (PBS) and stored at 4°C until use.

Neural progenitor spheres were infected with lentivirus encoding the PTMs by first allowing the spheres to settle in a flask, then carefully removing the majority of the media from the flask. Cells were then moved into a conical with TrypLE (Gibco, 12604-013) in order to dissociate the aggregates. After a 5 min incubation at 37°C, the spheres were washed and triturated into a single cell suspension. 3×10^6 cells were infected in a total of 1 mL of conditioned media with 100 ng p24/ mL of virus. An additional 1 mL of fresh media was added the next day. The cells were given a complete refeed 3 days later.

Lentiviral generation and infection were carried out in accordance of the respective safety practices, equipment and facility requirements outlined by the Institutional Biosafety Committees at the authors' respective institutes.

iPSC Striatal-like Differentiations

iPSC colonies grown on Matrigel in TeSR media (feeder-free) were scraped into EGF/FGF2 (100 ng/mL each) containing media (70:30 DMEM:F12 plus 2% B27 without vitamin A) and grown as floating neural progenitor spheres for at least nine passages. Cells were then plated on PLO/laminin coated coverslips and differentiated in DMEM:F12 with 1% N2 (Gibco, 17502-048) (neural induction media; NIM) for 5 days. BDNF (20 ng/mL; Peprotech 450-02) was then added for 2 days. For the next 21 days cells were differentiated in 20 ng/mL BDNF, rhShh (200 ng/mL; R&D 1845-SH), and Dkk1 (100 ng/mL; R&D 1096-DK-010) to promote a rostral forebrain fate. Afterwards, cells were then matured in 20 ng/mL BDNF, dibutyryl cyclic AMP (dbcAMP, 0.5 mM; Sigma D0260) and valproic acid (VPA, 0.5 mM; Sigma P4546) for the rest of the differentiation (until day 42). Medium was half-changed twice per week, or as needed.

Immunocytochemistry

Neural progenitor spheres were plated on poly-lysine and laminin-coated coverslips from 24 h to 43 days (striatal differentiations) before being fixed in 3.2% paraformaldehyde (EMS). Cells were then permeabilized using 0.2% Triton X-100 in PBS for 10 min at room temperature before incubation with mouse monoclonal anti-HTT (Millipore MAB5374; 1:1,000), rabbit polyclonal anti-GFP (LifeTechnologies A11122; 1:1,000), and chicken anti-βIIITubulin/TUJ1 (Aves labs, TUJ; 1:200) overnight at 4°C and then washed in PBS. The slides were further incubated with Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (Invitrogen, A21206; 1:500), Alexa Fluor 647-conjugated donkey anti-mouse secondary antibody (Invitrogen, A31571; 1:500), and Alexa Fluor 594-conjugated goat anti-chicken secondary antibody (Invitrogen, A11042; 1:500) for 60 min at room temperature, followed by PBS washes. After a 5 min incubation with 4′,6-diamidino-2-phenylindole (DAPI), slides were mounted with fluoromount (Sigma-Aldrich, F4680) and observed under fluorescence microscope (Leica).

Western Blot

Neural progenitor sphere pellets were lysed by incubation in 20–40 μl SDP buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Igepal, 0.1% SDS, 40 mM B-glycerophosphate (Sigma Aldrich, 251291), 10 mM NaF (Sigma Aldrich, S7920), 1X Roche complete protease inhibitor (Roche, 05892791 001) specifications. 40 to 100 μg of total protein was then resolved on 10% low-bis acrylamide gels [Resolving layer: 8% Acrylamide (BioRad 161-040), 0.04% BIS (BioRad, 161-1042), 0.375M Tris pH 8.8, 0.075% TEMED, 0.075% APS; Stacking layer: 4% Acrylamide-Bis 29:1 (BioRad, 161-0157), 0.156 M Tris pH 6.8, 0.075% TEMED, 0.075% APS] as in Mattis et al. (2015) or Mini-Protean TGX 4-15% gel (BioRad, 450-1085). In order to determine band size a ladder

was loaded into the first lane (Biorad Precision Plus Protein ™ Dual Color Standard, 161-0374). Briefly, lysates were transferred onto PVDF membrane (BioRad Turbo transfer, 170-4157) for 7 min at 1.3 A. For ECL detection, the membrane was blocked in 6% dry non-fat milk in Tris-buffered saline plus 0.1% Tween 20 (Sigma-Aldrich, p9416; 1:1,000) for 1 h at room temperature, and then exposed to primary antibody against HTT (Millipore, MAB5374) in block for 1.5 h at room temperature. Anti-mouse secondary antibodies conjugated to peroxidase (Jackson Labs, 715-035-150; 1:10,000) was applied in block for 1 h at room temperature, followed by exposure to chemiluminescence kit (Super Signal West Femto Maximum Sensitivity Substrate, Thermo Scientific, 34095). A separate blot was run using the same lysate and probed for GFP (1:1,000) as above (secondary: Jackson labs, anti-rabbit 711-0350152; 1:10,000). Both blots were then stripped per manufacturer's instructions (Restore Western Stripping Buffer, ThermoFisher Scientific, 21059) and reprobed for rabbit anti-actin as a loading control (Sigma, D05060; 1:300) as above. For fluorescence detection, the membrane was blocked in Odyssey blocking buffer (LiCor, 927–50,000) for 1 h at room temperature. Primary antibodies were applied as above. Anti-mouse 680 (LiCor, 926–68,072; 1:10,000) or anti-rabbit 800 (LiCor, 926–32,213; 1:10,000) secondary antibodies were used for their respective primaries and detected on a LiCor Odyssey CLx.

Filter Trap Assay

Neural progenitor sphere pellets were lysed by incubation in 20–40 μl SDP buffer for 30 min on ice with vortexing every 5 min. Cellulose acetate membrane (Whatman, 10404180) was equilibrated in 0.1% SDS for at least 10 min. After dot blotter (BioRad, 170-6545) was assembled, 30 μg sample was diluted in 200 μL 2% SDS and boiled for 5 min. Samples were added to each well and vacuum was used to filter the samples through the membrane. After washing with 0.1% SDS, the membrane was blocked for 1 h in 5% milk before immunoblotting using a mouse anti-polyQ antibody (Sigma 3B5H10, P1874; 1:1,000), as above. Anti-mouse secondary antibodies conjugated to peroxidase (Jackson Labs, 715-005-150; 1:10,000) was applied in block for 1 h at room temperature, followed by exposure to chemiluminescence kit (Super Signal West Femto Maximum Sensitivity Substrate, Thermo Scientific, 34095).

Cell Titer-Glo Assay

Relative intracellular ATP values in neural progenitor cell extracts were measured using the CellTiter-Glo Luminescent Assay (Promega, G7571) according to manufacturer's instructions, using 100 μl of Cell-Titer Glo reagent and 100 μl of media containing 9,000 cells. This was plated in 96 well plates and luminescence was measured after 10 min using an Envision system (ThermoScientific, 2104 Multilabel Reader). Data for two separate clones for the HD180 line (CS97iHD180n1 and CS97iHD180n3) were pooled and presented as "HD," as they were not significantly different via one-way ANOVA. Two separate control lines were similarly pooled (CS83iCTR33n1 and CS14iCTR28n5).

BDNF Withdrawal

iPSC-derived neural progenitors were differentiated toward a striatal fate for 42 days and then transferred into basic NIM or NIM plus 100 ng/mL BDNF for 24 h. dbcAMP and VPA were removed from the medium in the above experiments, as they increase endogenous BDNF transcription, but are not critical for cell survival (Bredy et al., 2007; Pruunsild et al., 2011). After 24 h, cells were fixed in 3.6% PFA. Effects of BDNF withdrawal were assessed by quantifying TUNEL incorporation per total DAPI-stained nuclei, according to manufacturer recommendations (ThermoFisher, Click-iT® TUNEL Alexa Fluor® 594 Imaging Assay, C10246), using Metamorph software. All experiments were performed at least three separate times. As in the Cell Titer-Glo Assay, the data for the two separate clones for the HD180 line (CS97iHD180n1 and CS97iHD180n3) or two separate control lines (CS83iCTR33n1 and CS14iCTR28n5) were respectively pooled, as they were not found significantly different via one-way ANOVA.

Statistical Analyses

Group means were tested by one-way ANOVA, with *post-hoc* Bonferroni testing to adjust for multiple comparisons. All results are expressed as mean ± standard error of the mean. In the figures, asterisk indicate p-values as indicated ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). All experiments were performed at least three times.

RESULTS

In the development of the original PTM that targeted replacement of HTT exon 1, few modifications were made to optimize the *trans*-splicing efficiency (Rindt et al., 2012). Therefore, the primary objective in this report was to enhance *trans*-splicing by targeting different regions within the HTT RNA. In this new series of PTMs, a non-disease encoding HTT exon 1 with 21 CAG repeats was fused to a synthetic stretch of RNA that functioned as the targeting sequence or the "tether" (**Figure 1A**). Additionally, to enhance splicing to the endogenous HTT pre-mRNA, three tandem repeats of a previously identified ISE element were incorporated into the PTM, as well as the HTT intron 1-derived branch point. PTMs were targeted to the 5′ or 3′ end of intron 1, with T9 overlapping the branch point upstream of exon 2 (**Figure 1B**). To initially examine the efficiency of *trans*-splicing, a previously developed mini-gene system was utilized that comprises a sub-genomic fragment of the HTT gene spanning exons 1, 2, and 3, separated by shortened introns 1 and 2, respectively. Fourty-two CAG tandem repeats were engineered into the exon 1 mini-gene sequence. Following co-transfection of the mini-gene with the PTMs into HEK293 cells, RT-PCR was used to specifically detect the *trans*-splicing product (**Figure 1C**). As expected, the original T7 PTM, which tethered to the 5′ end of intron 1, resulted in low but detectible levels of *trans*-splicing. Similarly, low levels of *trans*-splicing were also observed using the similarly targeted T8 PTM. In contrast, T4 and T9, the PTMs that were proximally positioned to exon 2 and disrupted the *cis* branch point within the mini-gene pre-mRNA, resulted in greater levels of *trans*-splicing (**Figure 1C**).

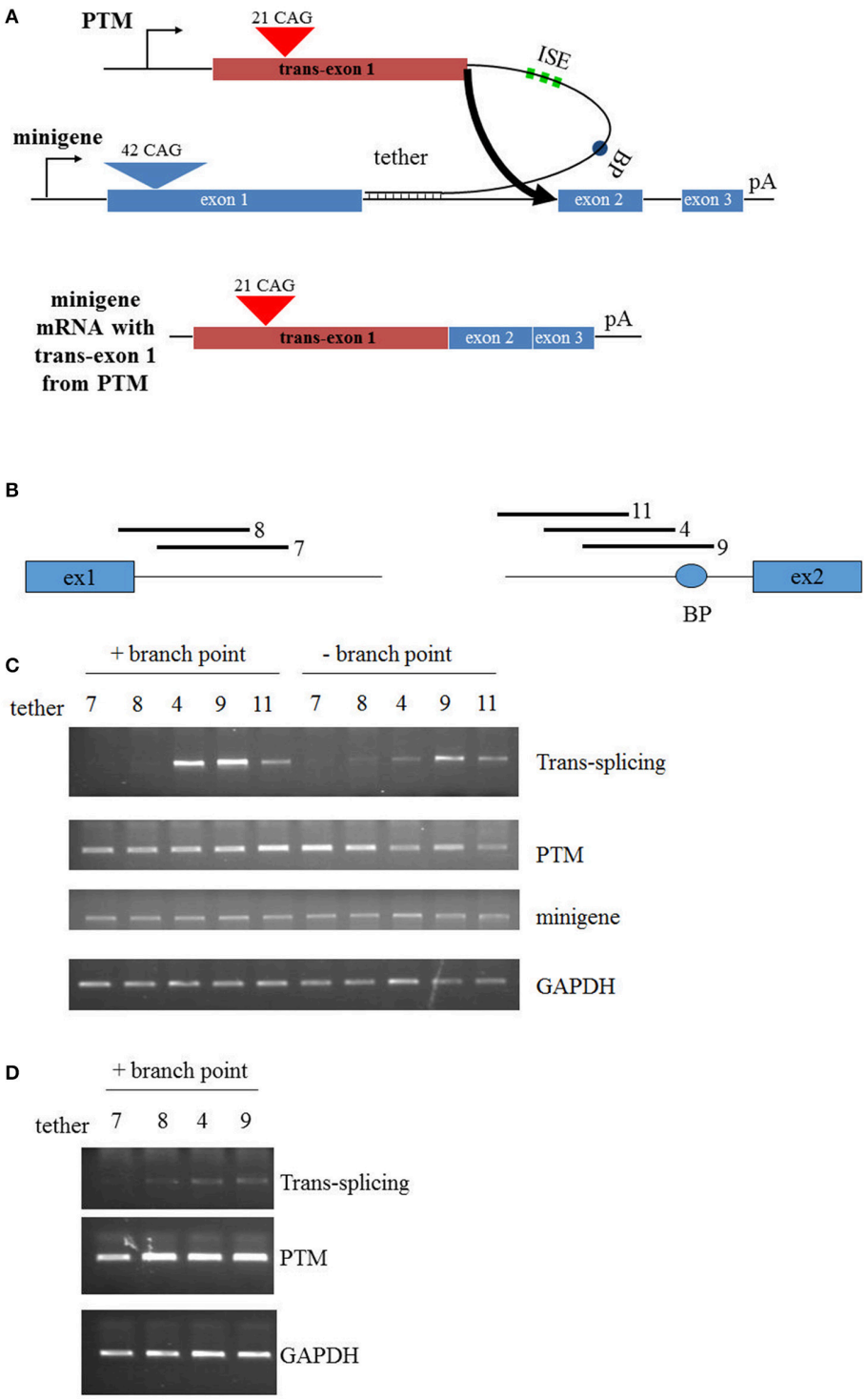

FIGURE 1 | *Trans*-splicing in HEK293 cells. **(A)** Schematic of PTM targeting. The PTM is driven by the CMV promoter and contains 21 CAG repeats within HTT exon 1. The PTM contains intronic splice enhancers (ISE), a branch point (BP), and a "tether" that targets the PTM to specific sequences within the HTT or mini-gene pre-mRNA. The HTT mini-gene is driven by the CMV promoter and consists of exon1-2-3 with intervening introns and a polyA signal. **(B)** Relative position of the PTM tethering elements for the PTMs. T7 and T8 target the 5′ end of intron 1; T4, T9, and T11 target the 3′ end of intron 1. **(C)** Mini-gene *trans*-splicing in HEK293 cells. RT-PCR of total RNA isolated from HEK293 cells transiently transfected with the indicated PTMs and the HTT mini-gene. PTMs contained a splicing branch point (+branch point) or had the branch point removed with flanking restriction sites (−branch point). *trans*-splicing RNA, PTM expression, mini-gene expression, and endogenous GAPDH expression is shown. **(D)** Endogenous *trans*-splicing in HEK293 cells. RT-PCR of total RNA isolated from HEK293 cells transiently transfected with the indicated PTMs. *trans*-splicing RNA, PTM expression, and endogenous GAPDH expression is shown.

Site-specific mutagenesis of the PTM branch point reduced *trans*-splicing efficiency in each instance, highlighting the importance of intact and functional splicing signals within the PTMs.

Having identified lead *trans*-splicing candidates using the mini-gene system, the branchpoint-containing PTMs were examined in a more challenging context: *trans*-splicing to endogenous HTT. HEK293 cells were transfected with PTM-expressing vectors and RNA was subsequently collected and analyzed via RT-PCR. The T4 and T9 PTMs were the most consistently active in this assay, resulting in high levels (10–15%) of *trans*-splicing product in multiple independent experiments (**Figure 1D**). Collectively, these results identify the T4 and T9 PTMs as *trans*-splicing RNAs that lead to enhanced replacement of the expanded HTT allele in a mini-gene system and with endogenous HTT in HEK293 cells. These therefore will serve as lead candidates in the following experiments that examine *trans*-splicing in disease-relevant contexts.

PTMs Can Be Expressed in Neural Stem Cells Derived from HD iPSCs

It has been previously demonstrated that PTMs can be used to successfully replace the mtHTT exon 1 in primary neural tissue from HD mice (Rindt et al., 2012). However, PTMs had yet to be expressed in human HD neural tissues. Therefore, the two PTMs identified with the greatest *trans*-splicing activity (T4 and T9) were over-expressed in neural progenitors derived from HD or control iPSC-derived neural progenitors (Ebert et al., 2013) via lentiviral infection. Post lentiviral infection, transgene incorporation into the progenitors was confirmed via live GFP expression, which was seen in the majority of cells (**Figure 2A**).

Next, HTT protein expression in the iPSC-derived neural progenitors was examined. First, Western blot analysis was performed in order to evaluate if reduction in endogenous mtHTT protein levels and/or the expression of the PTMs could be detected in the HD180 cells. As previously reported (Miller et al., 2011), the HTT antibody used to probe the blot preferentially recognize the CAG-expanded HTT protein expressed by the HD180 samples (EM48; **Figure 2B**, **Supplementary Figure 2**). While no significant decrease was seen in the high molecular weight band of mtHTT in the presence of the PTM (∼350 kDa, chevron), there was an additional band detected (∼47 kDa) in all samples overexpressing a PTM (**Figure 2B**, arrow). Interestingly, when probing the blot using an antibody that recognizes GFP, in addition to detecting a band at the predicted 27 kDa (**Figure 2C**, arrow-head), a band of similar size to the low molecular weight HTT isoform (∼47 kDa) was also detected in samples expressing a PTM [**Figure 2B** (arrow), **Supplementary Figure 2F**]. In the GFP-infected alone cells only the predicted 27 kDa band was detected. One possible explanation of a band of this size would be that in addition to PTM *trans*-splicing to endogenous HTT, there may also be some fusion or *cis*-splicing within the PTM transgene to generate a GFP:PTM product.

To further examine the HTT protein produced by cells expressing the PTM, lysates were analyzed for aggregate formation via a filter trap assay. Protein lysate from PTM-expressing iPSC-derived neural cultures was filtered through a membrane that specifically traps and retains large protein aggregates, while small species, including protein monomers, pass through. As previously described, the HD iPSC-derived neural progenitors had little to no HTT aggregation detected (The HD iPSC Consortium, 2012). However, using an antibody specific to polyQ repeats, it was demonstrated that all lines overexpressing the PTMs had increased polyQ-containing aggregate retention (**Figure 2C**).

To determine if PTM expression induced the production of visibly detectable HTT aggregates, the EM48 *HTT* antibody was used for immunocytochemistry on iPSC-derived neural progenitors. HTT aggregates were not large enough to be detected, nor was there a different sub-cellular localization between the HD or control progenitors, regardless of the absence/presence of PTMs or GFP alone (**Figure 2D**). Therefore, these data demonstrate that while aggregates can be detected via extremely sensitive measures, they are not large enough to be considered a visible polyQ inclusion.

PTMs Demonstrate HD Phenotypic Reversal in Neural Cultures from HD iPSCs

A common concern for trans-splicing reactions relates to translational fidelity and the production of functional protein following the trans-splicing reaction. To determine whether functional HTT was produced following trans-splicing, two HD clones (180 CAG repeats) and two control iPSC-derived neural progenitor lines (33 or 28 CAG repeats) expressing the T4 or T9 PTMs were examined for HD phenotypic reversal. Uninfected cells, or those infected with a lentivirus encoding GFP alone, were used as a control.

HD iPSC-derived neural progenitors derived have previously been shown to have a decreased metabolic rate (An et al., 2012; The HD iPSC Consortium, 2012; Ring et al., 2015), consistent with the energy deficiencies observed in HD patients (Mochel and Haller, 2011). Therefore, in order to establish whether PTM expression has an effect on this established HD phenotype, ATP levels were examined in the progenitors. As anticipated, both clones of the uninfected HD180 iPSC-derived neural progenitors demonstrated significantly lower ATP levels than the unaffected control neural progenitors (**Figure 3A**, **Supplementary Figure 3**). Expression of either the T4 or T9 PTM in the HD180 progenitors significantly increased ATP levels, compared to ATP levels in untreated cells. Expression of GFP alone did not impact ATP levels in the HD180 progenitor cells.

As the primary brain region affected in HD is the striatum, its generation is of particular interest for HD iPSC modeling. It has been previously demonstrated iPSC-derived HD striatal-like cultures exhibit a CAG-length dependent susceptibility to withdrawal of Brain Derived Neurotropic Factor (BDNF), resulting in a cell death phenotype (An et al., 2012; The HD iPSC Consortium, 2012; Lu et al., 2014; Mattis et al., 2015). To determine whether PTM expression could correct this, cell death was monitored in the HD iPSC-derived striatal cultures following a 24 h withdrawal of BDNF, after 42 days of differentiation.

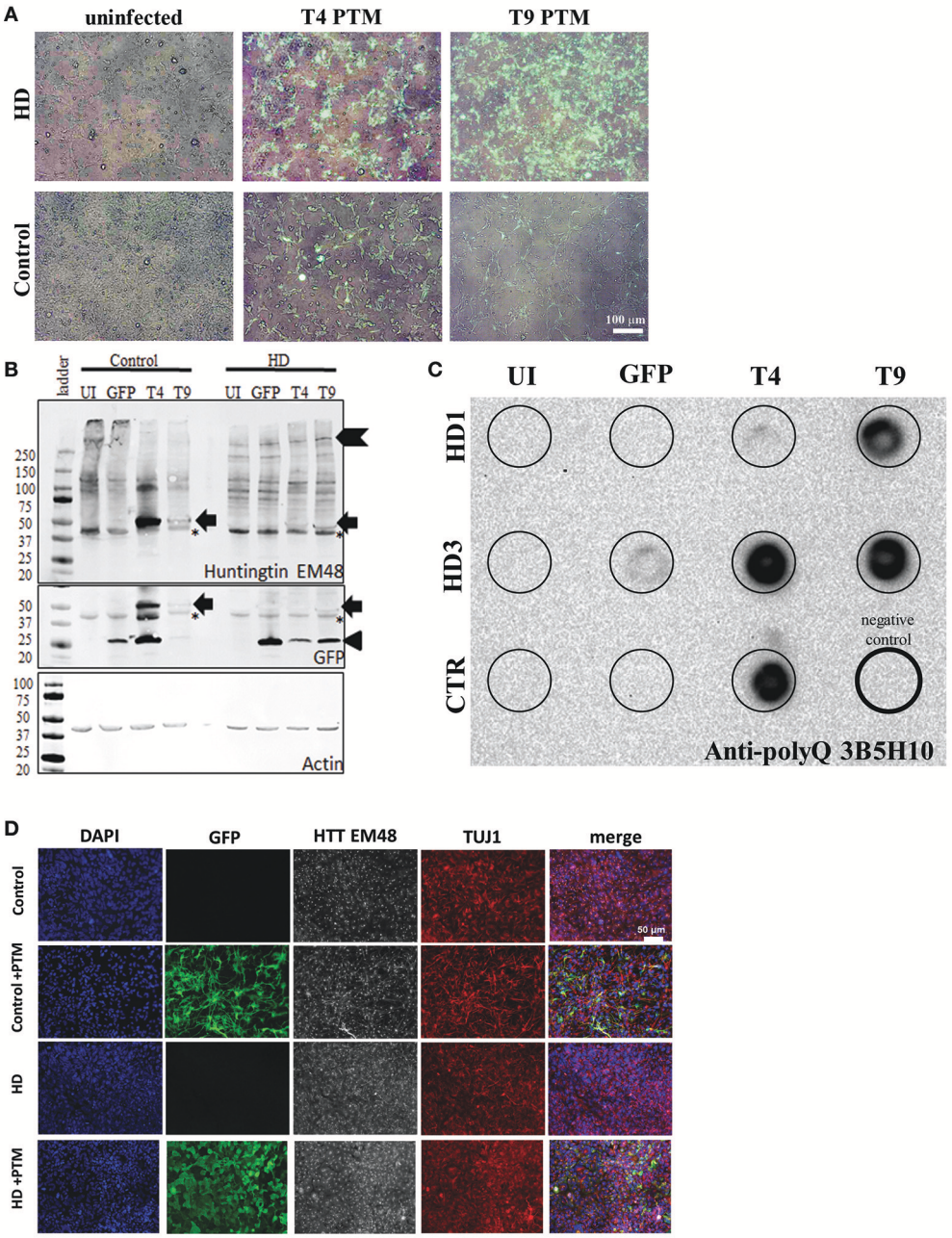

FIGURE 2 | PTMs can be expressed in human HD iPSC-derived NPCs. **(A)** Overlays of GFP and bright-field live images of HD or control NPCs after infection with lentivirus. Uninfected cells do not express GFP. Cells infected with PTM-encoding lentivirus have GFP expression in the majority of cells. White scale bar length represents 100 μm. **(B)** HD and control iPSC-derived NPCs infected with PTM-encoding lentivirus express the predicted high molecular weight HTT (indicated by chevron) as well as a specific low molecular weight N-terminal HTT fragment (indicated by arrow). Actin was used as an internal loading control. HD and control iPSC-derived NPCs infected with PTM-encoding lentivirus express two detectible isoforms of GFP via Western blotting. The lower molecular weight band corresponds to the expected size for GFP (indicated by arrowhead), whereas the higher molecular weight band is at the same size as the band found in the PTM-expressing cells via an anti-HTT antibody (indicated by arrow). Huntingtin (EM48) gave an additional a non-specific band (found in both + and – PTM) around 37 kDa (* indicates non-specific bands). **(C)** Filter trap assay detects that HD and control iPSC-derived NPCs infected with PTM-encoding lentivirus have more polyQ aggregation. The lower right lane (thick outside border) was loaded with lysis buffer only to serve as a negative control. **(D)** NPCs derived from HD and control iPSCs express similar levels and localization of HTT and β3-tubulin/TUJ1 (an immature neuron marker) regardless of presence of PTM (indicated by expression of GFP). White scale bar length represents 50 μm.

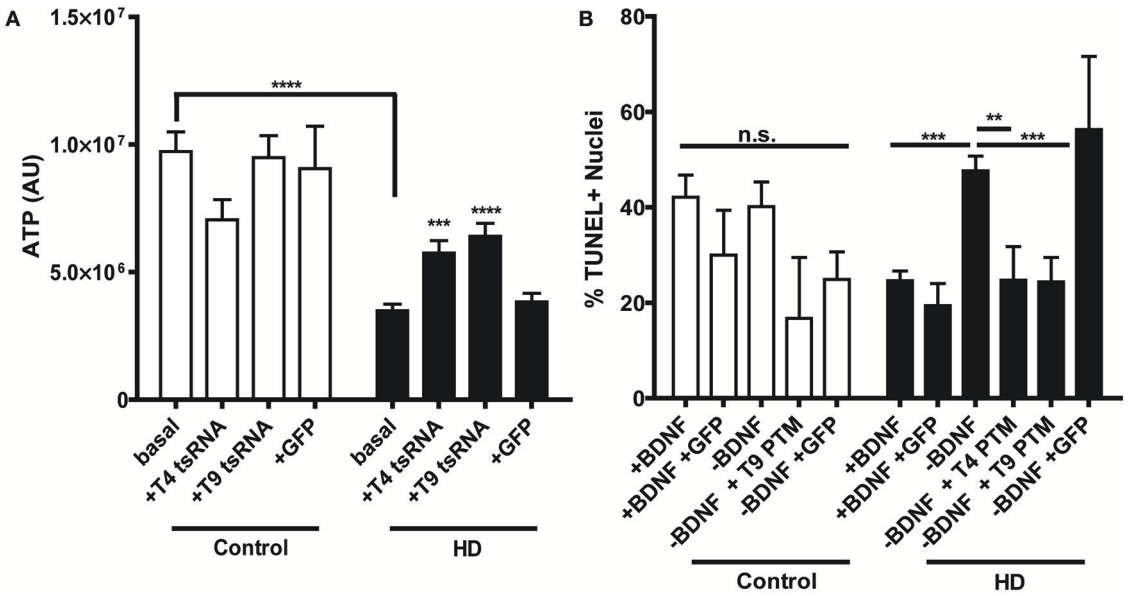

FIGURE 3 | PTM expression in HD180 iPSC-derived neural cultures reverses established mtHTT phenotypes. **(A)** ATP levels in HD and control NPCs. Cells were dissociated and ATP levels were assayed in equal numbers of cells. Control NPCs had significantly more ATP than HD NPCs. However, expression of PTMs significantly increased ATP levels in the HD, but not control, NPCs. Expression of GFP did not significantly affect ATP levels. $^{***}p < 0.001$, $^{****}p < 0.0001$, one-way ANOVA. Data are plotted as means and SEM. At least three separate passages of NPCs were examined. **(B)** HD and control iPSCs, with or without PTMs, were differentiated toward a striatal fate for 42 days before BDNF was withdrawn for 24 h. Cells were fixed, assayed for TUNEL, and counter stained with Hoechst. The percentage of TUNEL-positive nuclei was calculated as a measure of toxicity. The HD180 iPSC-derived striatal cells showed more cell death after BDNF withdrawal than did control iPSC-derived striatal cells. However, BDNF withdrawal in the presence of PTMs, but not GFP, reversed this toxicity. $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA. Data are plotted as means and SEM. At least three fields per coverslip (three coverslips per experiment) were counted at random during at least three separate differentiation experiments.

As each cell line has different basal levels of cell death, which is not HD-related, cell death after withdrawal with or without PTMs was compared within each cell line to the with BDNF condition. As expected, the control iPSC-derived cultures did not have increased cell death after withdrawal, regardless of the presence/absence of PTMs or GFP alone (**Figure 3B**). As previously described, the HD180 iPSC-derived cultures had significantly increased cell death after a 24 h withdrawal of BDNF, which was not affected by the expression of GFP alone. However, in the presence of either the T4 or T9 PTM, the amount of BDNF withdrawal-dependent cell death in HD180 lines was rescued, as determined by significantly reduced cell death in PTM-treated cultures. Collectively, these data indicated that the expression of a PTM for exon 1 mtHTT replacement via trans-splicing is a potential mechanism to reduce the well-defined HD pathology in iPSC-derived neural cultures.

DISCUSSION

This study provides further validation for the use of *trans*-splicing as a powerful potential therapy tool for genetic disorders such as HD. In this report, the objective was to determine whether targeting the PTM tether to different regions within the HTT pre-mRNA would lead to increased *trans*-splicing activity. In a variety of disease-relevant experimental contexts, targeting the *cis* branch point within the HTT pre-mRNA improved efficiency of the PTM in a variety of disease contexts.

As HD is a monogenic disorder, gene-based correction of mtHTT is a compelling prospective therapeutic tool. There are several paradigms for "gene therapy," each with important benefits as well as potential drawbacks (for a complete review please see Keiser et al., 2016; Yang et al., 2016). RNA interference (RNAi) and antisense oligonucleotides (ASOs) are single-stranded nucleotide sequences that work by binding specifically to the target mRNA, thereby knocking down gene expression. ASOs in particular do hold great promise as a treatment for HD. They have been demonstrated to efficiently knock-down mtHTT in an allele-specific manner (Østergaard et al., 2013; Skotte et al., 2014; Southwell et al., 2014) and are currently in HD patient clinical trials (Rollnik, 2017). One drawback however, includes the inability of ASOs to cross the blood-brain-barrier, therefore requiring direct administration to the brain, and the need for repeatedly administration over the course of a lifetime, as they are not made in the cells. Short-hairpin RNAs and microRNAs act in a similar fashion to RNAi and ASOs to knock-down gene expression, but can be virally delivered. This allows for continued gene expression after a single dose, however dosage control is more challenging. Another method to reduce/eliminate mtHTT expression at the protein level is the use of virally-delivered intracellular antibodies (intrabodies). However, proper intrabody folding and low solubility in the reducing cytoplasmic

environment are issues of functionality and stability for this proposed therapy (Biocca et al., 1995). New technologies have emerged in the past decade to alter gene expression or even permanently edit the mutant genes themselves, including zinc finger nucleases, transcriptional activator-like effector nucleases (TALENs), or clustered regulatory interspaced short palindromic repeat/CRISPR associated protein 9 (CRISPR/Cas9) (for review see Merkert and Martin, 2016). While these technologies can lead to the genetic correction of mtHTT at the DNA level, the current *in vivo* technology is limited, primarily due to the relatively low efficiency of genetic correction and the need to correct the expanded allele in many tissues throughout the body (Kolli et al., 2017; Yang et al., 2017). Future studies utilizing these technologies will hopefully continue to improve upon what is already demonstrated to be promising techniques, the current limitations lead us to optimize the strategy of *trans*-splicing as a potential addition to the therapy toolkit for HD.

Trans-splicing also has its own set of strengths and weaknesses. One positive attribute is the inherent control of gene expression: *trans*-splicing cannot lead to gene overexpression, since the maximal amount of the *trans*-splicing event relies upon the endogenous HTT promoter. This is important in terms of appropriate gene "dosing." A temporary knock-down of all HTT (both mutant and wild-type), termed a "*HTT* holiday" (Lu and Yang, 2012), has been shown to be tolerated in animals (McBride et al., 2011) and to reverse some symptoms of disease (Boudreau et al., 2009; Drouet et al., 2009; Kordasiewicz et al., 2012). However, other disease contexts demonstrate the complexity of HD, since a 50% decrease in murine Htt expression induces behavioral and cognitive abnormalities in ∼1/3 of the mice, coupled with neurodegeneration in adulthood (Nasir et al., 1995; O'Kusky et al., 1999). Additional studies have demonstrated that targeted inactivation of Htt in the adult mouse brain results in neurodegeneration, further demonstrating that a delicate balance is required when envisioning HD therapeutics (Dragatsis et al., 2000). These studies would implicate the need for balance between reducing mtHTT and maintaining some functional level of wildtype HTT. However, studies using a cre-lox system to deplete neural (using the Nestin or CamKII promoters) Htt expression in adult mice (>4 months) demonstrated no changes in total brain volume or expression of neuronal proteins (Wang et al., 2016). With these conflicting studies there is obviously still work to be done examining the necessity of HTT expression in the adult brain. *trans*-Splicing avoids the haploinsufficiency issues as the molecular reaction converts mtHTT to wildtype HTT and any off-target *trans*-splicing to the non-pathogenic allele still results in wildtype HTT expression. While this report does not address the potential of allele-specific targeting, the net effect of non-allele specificity is a steady-state level of HTT expression, with at least some mutant to wild-type HTT conversion. Ideally, in the future, further modifications could be made to allow for allele-specific *trans*-splicing by targeting the tethering region to SNPs in the mtHTT, as has been done for HD ASO therapy (Skotte et al., 2014). In moving forward toward *in vivo* studies the CMV promoter would ideally be replaced with something that has shown sustained and robust *in vivo* expression within the CNS, such as the CBA promoter (Gray et al., 2011).

Perhaps one of the most significant hurdles to *trans*-splicing involves *in vivo* efficiency (Berger et al., 2016). While efficiencies have been reported ranging from <1 to ∼40%, here we demonstrate that the efficacy of *trans*-splicing to the HTT mini-gene is ∼10–15%. However, as wildtype HTT has been demonstrated to have an anti-apoptotic effect (Rigamonti et al., 2000, 2001; Gervais et al., 2002), the small reduction in mtHTT, paired with the increase in corrected HTT, may be sufficient to confer a therapeutic benefit. Consistent with this notion, *trans*-splicing mediated induction of HTT protein lead to the phenotypic reversal within neural cells derived from HD patient iPSCs. Another potential problem lies with recent reports of toxic RNA mtHTT species, in addition to the toxic protein (Bañez Coronel et al., 2012, 2015). It is currently unknown what effect *trans*-splicing would have on these transcripts. Ideally, the endogenous exon 1 pre-mRNA that was not spliced into the mature mRNA would be targeted for degradation, a likely path as this transcript does not possess a polyA signal and should be rapidly degraded through normal cellular processes. However, if there were additional toxic transcripts produced from the trinucleotide repeat (Bañez Coronel et al., 2012, 2015), these products would most likely not be targeted by this technology. Lastly, there are potential issues of non-disease gene disruption via off-target effects of the PTM or integration of the viral cassette into the genome. This is a consideration that will need to be examined for all gene therapy paradigms in the future. Therefore, in moving forward toward a translational product, the PTMs would most likely need to use a non-integrating viral vector, such as AAV. While direct administration of virus to the CNS is an option, using a viral vector able to cross the blood-brain-barrier after peripheral administration would be ideal (Deverman et al., 2016). To date, *trans*-splicing to spurious genomic sites has not been detected in our hands, however, a variety of "-omics" platforms, including RNA- and whole genome sequencing should be utilized before advancing further toward the clinic.

One interesting finding in this study was a ∼47 kDa band, detected via both a GFP and a HTT antibody. This potential GFP-HTT fusion product was only detected in iPSC-derived neural progenitors that expressed PTMs. Theoretically, the reversal of the energy and cell death phenotypes seen in the HD iPSC-derived neural progenitors or striatal cultures, respectively, is due to the exon 1 replacement of mtHTT with the PTMs. However, we cannot exclude the possibility that the PTM expression of an exogenous HTT exon 1 fused with GFP is not contributing to the phenotype reversal. While it is known that HTT has pro-survival roles, the speculation presented here that a non-CAG expanded exon 1 alone could be of benefit in the presence of mtHTT in its self is extremely interesting and worthy of future pursuit as a potential avenue of therapy.

Trans-Splicing has been proposed for a potential therapeutic agent in other genetic disorders (Supplementary Table 2). These studies have all demonstrated efficacy of *trans*-splicing *in vitro,* and some *in vivo* (for a complete review please see Berger et al., 2016). Presently, this study builds upon our previous publication (Rindt et al., 2012) to demonstrate that modification of the binding modality of the PTM increases *trans*-splicing efficacy from 1–5% (Rindt et al., 2012) to 10–15% by altering

the tethering region to specifically target the branch point of the endogenous intron 1. One interesting finding of this study is the increased aggregate retention seen via filter-trap assay, possibly as a result of the N-terminal exon 1 fragment seen via Western blotting analyses. This increased aggregate retention was seen in all samples expressing a PTM, to varying degrees which likely correlate with the original infection efficiency. This increase in aggregate retention did not result in increased aggregate formation or localization seen via immunocytochemistry. While historically HTT aggregate formation has been thought of as pathogenic (Khoshnan et al., 2002; Colby et al., 2004; Takeuchi et al., 2014), more recent papers have questioned this paradigm (Arrasate et al., 2004). Evidence for each side exists. For example, aggregate-suppressing therapeutic candidates can suppress toxicity (Warrick et al., 1999; Heiser et al., 2000; Lecerf et al., 2001; Kazantsev et al., 2002; Yoshida et al., 2002; Wang et al., 2005). Alternatively, many cell types that do not undergo degeneration (Kuemmerle et al., 1999) express even more aggregates than those that do (Vonsattel et al., 1985). Additionally, the soluble HTT population has instead been implicated in disease pathology (Arrasate et al., 2004). A recent study demonstrates that the aggregation of mtHTT may be protective against apoptosis, but may ultimately lead to a slow eventual cellular necrosis (Ramdzan et al., 2017). The findings presented here from the reversal of known patient iPSC-derived neural phenotypes potentially support the hypothesis that increased aggregation may be protective in HD, at least in the short-term. Longer-term studies of the effect of *trans*-splicing PTMs as a potential HD therapeutic, including those in mouse models, will therefore need to be performed.

ETHICS STATEMENT

The iPSC lines used in this study were approved by the IRB/SCRO at CSMC. All fibroblasts have been de-identified for sharing with the research community prior to generation of iPSCs, and no personal health information is available to any investigators beyond those who were involved in depositing them.

AUTHOR CONTRIBUTIONS

HR, CT, CL, and VM had substantial contributions to the conception or design of the work, the acquisition, analysis, or interpretation of data for the work. HR, CT, CL, and VM all drafted the manuscript and/or contributed critical revisions for important intellectual content and approved the final version. HR, CT, CL, and VM agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Clive Svendsen for consultation, Dr. Jacqueline Gire O'Rourke for assistance with the dot blotting technique and Dr. Nur Yucer for assistance with fluorometric Western Blot. This work was supported by a Faculty Research grant from the University of Missouri College of Veterinary Medicine, the Huntington Disease Foundation of Canada, and the National Institutes of Health (1R21NS070072).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2017.00544/full#supplementary-material

Supplementary Figure 1 | Schematic outlining both *cis*- and *trans*-RNA splicing. Introns (lines) are removed from the pre-mRNA by the spliceosomal complex, including: a "GU" donor site at the 5′ end of the intron; an "A" branchpoint near the 3′ end of the intron; and a poly-pyrimidine tract with an "AG" splice acceptor site at the 3′ end of the intron. *cis*-Splicing occurs when introns are spliced out of a pre-mRNA and the two flanking exons (blue boxes) of the gene are ligated together. *trans*-Splicing occurs when exons from two different genes [or a gene and a PTM (red box)] are ligated together. In this schematic *trans*-splicing is represented by the PTM bound to the intron via the tether region which recognizes the intron sequence surrounding the branch point.

Supplementary Figure 2 | Supplemental Western blots of PTM expression in NSCs. **(A–D)** HD and control iPSC-derived NPCs infected with PTM-encoding lentivirus express the predicted high molecular weight HTT (∼350 kDa), as well as a specific low molecular weight N-terminal HTT fragment (indicated by arrow; anti-HTT EM48 antibody). Actin was used as an internal loading control. Huntingtin (EM48) blots picked up a non-specific band (found in both + and − PTM) around 37 kDa. **(E)** Quantification of ∼47 kDa band. Bands were quantified in the linear range and graphed as a ratio of detected intensity (AU) to the intensity of the UI cells within the same HD or control. **(F)** HD and control iPSC-derived NPCs infected with PTM-encoding lentivirus express two detectible isoforms of GFP via Western blotting. The lower molecular weight band corresponds to the expected size for GFP (indicated by arrowhead), whereas the higher molecular weight band is at the same size as the band found in the PTM-expressing cells via an anti-HTT antibody (indicated by arrow). Actin was used as an internal loading control.

Supplementary Figure 3 | PTM expression in HD180 iPSC-derived neural cultures reverses established mtHTT ATP phenotypes. ATP levels, expressed relative to the basal levels of control cells, in HD and control NPCs. Cells were dissociated and ATP levels were assayed in equal numbers of cells. Control NPCs had significantly more ATP than HD NPCs. However, expression of PTMs significantly increased ATP levels in the HD, but not control, NPCs. Expression of GFP did not significantly affect ATP levels. $^{***}p < 0.001$, $^{****}p < 0.0001$, one-way ANOVA. Data are plotted as means and SEM. At least at least three separate passages of NPCs were examined.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer UB and handling Editor declared their shared affiliation.

frontiers in Neuroscience

REVIEW
published: 24 October 2017
doi: 10.3389/fnins.2017.00591

Neuroprotective Potential of Cell-Based Therapies in ALS: From Bench to Bedside

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Motor neurons (MN) degeneration is a main feature of amyotrophic lateral sclerosis (ALS), a neurological disorder with a progressive course. The diagnosis of ALS is essentially a clinical one. Most common symptoms include a gradual neurological deterioration that reflect the impairment and subsequent loss of muscle functions. Up-to-date ALS has no therapy that would prevent or cure a disease. Modern therapeutic strategies comprise of neuroprotective treatment focused on antiglutamatergic, antioxidant, antiapoptotic, and anti-inflammatory molecules. Stem cells application and gene therapy has provided researchers with a powerful tool for discovery of new mechanisms and therapeutic agents, as well as opened new perspectives for patients and family members. Here, we review latest progress made in basic, translational and clinical stem cell research related to the ALS. We overviewed results of preclinical and clinical studies employing cell-based therapy to treat neurodegenerative disorders. A special focus has been made on the neuroprotective properties of adult mesenchymal stromal cells (MSC) application into ALS patients. Finally, we overviewed latest progress in the field of embryonic and induced pluripotent stem cells used for the modeling and application during neurodegeneration in general and in ALS in particular.

Keywords: stem cells, neurodegeneration, neuroprotection, clinical trials

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *01 September 2017*
Accepted: *09 October 2017*
Published: *24 October 2017*

Citation:
Forostyak S and Sykova E (2017) Neuroprotective Potential of Cell-Based Therapies in ALS: From Bench to Bedside. Front. Neurosci. 11:591. doi: 10.3389/fnins.2017.00591

HISTORY, GENETICS, AND CLINICS OF ALS

In 1848, Aran described for the first time a malady later become known as an amyotrophic lateral sclerosis (ALS). He reported 11 cases of the disease featuring a focal wasting and paresis, weakness and cramps in the upper extremities, and fatal end within 2 years (Aran, 1848). Aran proposed that the disease had been inherited from the parents. In 1873, Jean-Marie Charcot reported that ALS was never inherited, and that was the main reason for delineating ALS from muscular atrophy (Charcot, 1881). The view that ALS is rarely connected with family history persisted for almost 100 years. A new era in the field has started when several genes were linked with familial (FALS) and sporadic ALS (SALS) cases. It is thought that around 5–10 percent of all ALS incidents have a family history, whereas the rest are sporadic (Bento-Abreu et al., 2010; van Es et al., 2010; Andersen and Al-Chalabi, 2011). The mutations in the following genes have been found to result in FALS: superoxide dismutase 1 (*SOD1*), *TARDBP, Ubiquilin 2, Alsin, Senataxin, FUS, Angiogenin, SIGMAR1* (Rosen et al., 1993; Hadano et al., 2001; Hand et al., 2002; Sapp et al., 2003; Chen et al., 2004; Nishimura et al., 2004; Gitcho et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008; Vance et al., 2009; Elden et al., 2010). ALS has been recently associated with frontotemporal dementia, (FTD, ALS/FTD). A

GGGGCC hexanucleotide repeat in the intron of protein C9ORF72 has been demonstrated to cause an alternative splicing of this protein that is leading to similar pathological events in two diseases (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Another pathological features of ALS and FTD are TDP-43 and p62 positive cytoplasmic depositions in the hippocampus and cerebellum (Achi and Rudnicki, 2012; Mahoney et al., 2012). The main differences between ALS/FTD patients and classical FTD cases are presences of psychiatric signs and the distribution of TDP-43 inclusions: SALS primarily features TDP-43 pathology in the spinal cord, patients with FTD primarily affect the cortex, while FTD-ALS patients have TDP-43 pathology in both areas (Geser et al., 2009; Neumann et al., 2009). The genetic screening of US population demonstrated that TDP-43 expansion occurs in 12% of familial FTD and 22.5% of FALS (DeJesus-Hernandez et al., 2011), while European population screening showed higher prevalence in FALS (46%), followed by familial FTD (29%) and SALS (21%) (Renton et al., 2011).

Despite diverse genetic backgrounds, SALS and FALS are clinically indistinguishable, 95% of all ALS cases are sporadic, and the other five percent have a genetic background. The clinical hallmark of both types of ALS is a progressive deterioration of neurological functions correlated (clinically and pathologically) with loss of primary and secondary MN, sparing of the oculomotor and the spinal Onuf's nuclei, coexistent neurogenic atrophy, weakness, and fasciculations caused by secondary MN degeneration, together with hyperactive deep tendon reflexes, pyramidal tract signs, and increased muscle tone (Borasio and Appel, 2003). Patients present a wide range of diverse clinical outcomes regarding disease onset, rate of progression and survival (Burkhardt et al., 2013). Disease's symptoms are typically asymmetrical. Some 20–30% of all cases have bulbar onset, with more than 50% of bulbar symptoms in older women. In FALS minor pathological changes could be diagnosed in the spinocerebellar tracts, typically without accompanying symptoms. Most commonly, the disease strikes people between the ages of 40 and 70, although the early onset is not exceptional. Unlike other neurodegenerative maladies, ALS is not age-related disease. However, aging is one of a many risk factors. Incidence of ALS is fairly uniform 1–2 per 100,000 individuals, except for an elevated incidence in Kii peninsula of Honshu island and Guam (Kuzuhara and Kokubo, 2005; Steele, 2005). A lifetime risk of ALS development approaches 1/400-1/700 with a somewhat more frequent occurrence in males than in females (ratio is $\approx$ 1.5; Johnston et al., 2006; Bento-Abreu et al., 2010).

CURRENT TREATMENT AND MANAGEMENT OF ALS

Considering a great diversity of genetic and clinical forms of ALS, every therapeutic attempt could be regarded as experimental. Nevertheless, as for now a standard therapy for ALS includes an antiglutamatergic agent Riluzole that, however, does not alter the natural history of the disease (Lacomblez et al., 1996). Riluzole (100 mg/day), which reduces the presynaptic release of glutamate, remains the only effective drug that slows disease progression and extends the patients lifespan by 2–3 months (Lacomblez et al., 1996; Sykova et al., 2017). Additionally, all patients receive palliative or symptomatic therapy such as a non-invasive positive pressure ventilation (Hardiman, 2011), prescription of anticholinergic drugs (such as trihexyphenidyl, amitriptyline, or atropine) or the use of a portable suction machine if drooling is troublesome. Baclofen or diazepam might be used to deal with spasticity (Mustfa et al., 2006). Dysphagia could be managed by modifying food and fluid consistencies, postural advice, and in extreme cases of bulbar involvement, by gastrostomy or cricopharyngomyotomy. The results of such therapy are unsatisfactory, current clinical management is still extremely limited and novel therapeutic approaches are in an active search. The presymptomatic or at least the early diagnosis of ALS could offer wider possibilities for prevention and for the treatment of this devastating disease. Therefore, in the future screening of patients with FALS for mutations in the SOD1, TARDBP, FUS, and a several other genes might offer benefits in the diagnosis and treatment of ALS. Sadly, over 60 per cent of ALS patients die within 2–5 years of presentation mostly from pulmonary insufficiency with infections, with $<$10 per cent survive rate longer than 8 years (Kiernan et al., 2018). Considering that the above therapies just improve patient's quality of life, but do not extend his/her survival the main task of treatment in the terminal stages is to keep patients as comfortable as possible (McGeer and McGeer, 2005).

NEUROPROTECTIVE STRATEGIES IN ALS

Considering that the disease affects MN at different levels of the central nervous system (CNS), a neuroprotective strategy should aim to restore affected tissue homeostasis throughout the entire nervous system. Numerous attempts have focused on antiglutamatergic, antioxidant, antiapoptotic, anti-inflammatory, and neurotrophic molecules, as well as on gene therapy and stem cell application. These and other molecules are able to reach the MN after systemic (intravenous, intraarterial), local (intrathecal, intraspinal, intracerebral, etc.) or combined application, as it has been shown that the blood brain barrier (BBB) in ALS is also compromised (Garbuzova-Davis et al., 2007).

Antiglutamatergic Therapy

Antiglutamatergic Therapy has currently shown the best results in clinical trials. As already mentioned, the only anti-ALS medicine approved for the treatment of patients is Riluzole. The kynurenine pathway (KP), a major route for the metabolism of tryptophan, has been shown to play role in ALS. The KP excitotoxic catabolites such as N-methyl-D-aspartate receptor agonist quinolinic acid and the neuroprotective NMDA receptor antagonist kynurenic acid are involved into crosstalk between the CNS and immune systems, by modulating cell proliferation. Few companies such as Sanofi-Aventis or Teva Neuroscience developed KP inhibitors (Teriflunomide and Laquinimod, respectively), have entered clinical trials (Chen et al., 2009). Memantine, novel anti-excitatory drug is a non-competitive excitotoxic N-methyl-D-aspartate (NMDA)-receptor antagonist,

has been shown to delay the loss of hind limb motor activity and extend the survival of $SOD1^{G93A}$ mice (Wang and Zhang, 2005).

Antioxidant Therapy

Antioxidant Therapy aimed at ameliorating oxidative stress, could provide a possible healing effect in ALS patients. However, clinical trials examining the application of vitamin E, acetylcysteine, methylcobalamine, glutathione, or coenzyme Q10 (CoQ10) indicate that these drugs are ineffective in ALS patients (Levy et al., 2006; Kaufmann et al., 2009). An antioxidant peptide called SS-31 has been shown to improve mitochondrial dynamics, resulting in a significant extension of survival, better motor activity, decreased MN loss and reduced immunostaining for oxidative stress markers in G93A mice (Petri et al., 2006).

Immunotherapeutic Strategies

Immunotherapeutic Strategies to combat ALS also could be an attractive therapeutic approach. Active vaccination with misfolded mSOD1 in the G37R SOD1 mouse model of FALS has been tried, resulting in the reduced loss of spinal cord neurons and a modest but statistically significant increase in life expectancy (Urushitani et al., 2007; Brody and Holtzman, 2008). However, much work remains to be done before clinical trials could be started.

The discovery of neurotrophic factors (NTF), their anti-apoptotic effect and the ability to promote the MN survival during development, made these molecules attractive candidates for the treatment of neurodegenerative disorders affecting motor system (Appel, 1981; Gould and Oppenheim, 2011). Increasing number of studies implicate an impaired production of vascular endothelial growth factor (VEGF) by MN, rather than a lack of functional receptors, is associated with ALS. The exogenous VEGF has been shown to cause a direct neuroprotective effect via the expression of VEGF-receptors in MN (Van Den Bosch et al., 2004). Various routes of insulin-like growth factor-1 (IGF-1) or VEGF delivery, was reported to slow disease progression and improved lifespan in animals studies (Kaspar et al., 2003; Nagano et al., 2005; Wang et al., 2007). Interestingly, clinical trials utilizing the subcutaneous delivery of IGF-1 failed to show a beneficial effect, mainly due to a reduced bioavailability of IGF-1 (Sorenson et al., 2008; Howe et al., 2009). The combined usage of IGF with IGF-binding protein 3 (IGFBP), also called IPLEX, significantly increased the serum half-life time of IGF-1 and proceeded toward an early-phase clinical trial. Despite a great deal of debate surrounding the effectiveness of IPLEX (Bedlack et al., 2009; Gould and Oppenheim, 2011), clinical trial employing a delivery of VEGF protein into ALS patients CSF is currently in progress (http://clinicaltrials.gov, identifier # NCT01384162 and NCT008005501).

One should also consider that MN exhibit trophic heterogeneity, that is they respond to a distinct type/s of NTFs during their development (Kanning et al., 2010). Studies on glial cell line-derived neurotrophic factor (GDNF) knockout mice showed a dramatic and restricted loss of small ventral lumbar myelinated axons (γ-MN) and spared large myelinated axons (α-MN) (Gould et al., 2008). Hence, a monotherapeutic strategy might bring some improvements in motor activity or even extend survival, but this demands distinct NTFs that will target specific types of MN. This concern might be resolved by using cocktails of NTFs delivered in such a way so that they would be able to pass through the BBB in an efficient and controlled way. Alternatively, application of stem cells, which are well-known to have paracrine properties, could serve as a vehicle for the delivery of NTFs. Stem cells research has reached a stage when unlimited number of non-engineered and engineered neuroglia could be produced and used for therapeutic purposes aiming at protection, repair, or replacement of affected cells in disorders affecting the brain and spinal cord, thus bringing a new hope for patients.

CELL-BASED THERAPIES

A new avenue has been opened for basic research and regenerative medicine with the discovery of stem cells (SC) and their regenerative capacities. By using a SC terminology one would expect to deal with a cell with a pluri- or multipotent features and unlimited self-renewal capacities. Pluripotency means that the cell is capable to differentiate into any mature cell type in the organism originating from all three germ layers (Nistor et al., 2005; Lee et al., 2007). SC classification considers the tissue of origin, the developmental stage when cells appear and could be isolated in the organism. Current research is dealing with the following types of SC: (1) embryonic (ESC), a pluripotent cells that could give rise to any cell in the body; (2) fetal (FSC), a multipotent cells that could give rise to any cell of certain germ layer; (3) somatic (adult-derived), multi- or oligopotent cells found in different tissues of the fully developed organisms; and 4) induced pluripotent (iPS), similar to ESC, but generated from mature somatic cells after the artificial introduction of transcriptional factors (Takahashi and Yamanaka, 2006).

Mesenchymal Stromal Cells (MSC)

MSCs are attractive and accepted target for use in cell-based therapies (autologous application). MSC are oligopotent cells that could be isolated by a relatively simple procedure (Forostyak et al., 2016a) from bone marrow (BMSC), umbilical cord (UMSC), fat tissue (AMSC), Wharton's jelly and other fetal tissues. To meet the "mesenchymness" criteria every cell should meet following criteria: ability for extensive *in vitro* growth on plastic; differentiate *in vitro* into chondrocytes, osteocytes and adipocytes; express "mesenchymal" surface markers (Mezey et al., 2000; Krause, 2002; Dominici et al., 2006; Forostyak et al., 2016a). It is interesting to note that despite great similarities in features and properties, MSCs of different origin display certain differences in growth rate, surface markers expression and even physiological activities (Forostyak et al., 2016b). Several groups also reported MSC differentiation toward functional neural phenotype (Tropel et al., 2006).

A therapeutic potential of MSC is very broad and still hides many unknown features to be exlored. However, it is generally accepted that despite the MSC's origin, they primarily act via the growth factors (GF) secreted either into the growth medium or within the recipient's tissue (paracrine

function). A GF misbalance in the organism triggers cascades of intracellular changes that may result into various pathological states. Therefore, MSC ability to secrete a cocktail of growth factors brought attention for their use in regenerative medicine in general, and is very promising to use for the purpose of neuroprotection and neuroregeneration. We earlier reviewed available information about growth factors and corresponding genes that have been reported to be secreted by MSC (Forostyak et al., 2013b). Here, we will just name GFs that are known to contribute to ALS and were successfully tested *in vivo* for the purpose of neuroprotection or disease modification, those are: glia cell-line derived neurotrophic factor (GDNF), insulin growth factor type-1 (IGF-1), brain-derived neurotrophic factor (BDNF), neural growth factor (NGF), VEGF and others that play less significant role in ALS pathology. Apart from a paracrine effect, we have recently reported that after an intrathecal or combined application of MSC a level of apoptosis and inflammation decreases leading a better survival of host MN in host tissue (Forostyak et al., 2014). Moreover, MSC has been shown to repair a defective extracellular matrix (ECM) structure called perineuronal net (PNN) surrounding these MN in SOD1 rat transgenic model of ALS, by up-regulation of some chondroitin sulfate proteoglycan levels (CSPG) typically up-regulated at the end of a critical period of PNN development (Carulli et al., 2010; Forostyak et al., 2014). We could speculate that this finding could be indicating a reactivation of adult neural plasticity within a recipient's organism after MSC application. Notably, MSC do not stimulate alloreactivity, are able to pass a MHC barrier, and thus enabling appliocation between HLA-mismatched individuals (Le Blanc, 2003; Aggarwal and Pittenger, 2005; Rice and Scolding, 2008).

The above properties make these cells a very attractive target for neuroprotective and neuroreparative therapy. Increasing number of reports has led to the preclinical trials. These studies demonstrated positive effects of MSC on motor activity and survival after being delivered via various routes (mostly intrathecal, but also intravenous, intraspinal, intramuscular, or combined) using rodents' models of ALS (Mazzini et al., 2004; Garbuzova-Davis et al., 2008; Kim et al., 2010). Our preclinical study employing intrathecal (cisterna magna) injection of human MSC into symptomatic $SOD1^{G93A}$ transgenic rats, demonstrated prolonged survival of MSC-treated animals by more than 2 weeks compared with the vehicle-treated group, and also demonstrated decreased markers of inflammation in host CSF (Forostyak et al., 2014). Another studies using a combined (intraspinal and intravenous) BMSC transplantation, a part of an increased motor activity and survival and attenuated proliferation of microglial cells (Boucherie et al., 2009; Forostyak et al., 2011). Interestingly, we have noticed that despite a broad application of MSC in animal studies and in clinical trials involving patients, little has been known about MSC's physiology. The stem cells physiology may enable a better control over the graft and even further improvement of regenerative potential of cells prepared for human application. This knowledge may change and further improve effect of cell-based therapies of neurodegenerative and other types of diseases. These questions have been raised in several of our studies, where we have studied stem cells physiology with a help of Ca^{2+} signaling, ICC, electrophysiological methods and other methods of molecular biology. Ca^{2+} signals play a crucial role in the differentiation, proliferation and survival of stem cells starting from the early stages and later on mature cells (Forostyak et al., 2013a, 2016b). Dysregulation of calcium ($[Ca^{2+}]_i$) homeostasis is also crucial in the pathophysiology of ALS, and impaired $[Ca^{2+}]_i$ in the cytoplasm of neurons is a potential mechanism of decreased cell survival (Dafinca et al., 2016). MSCs effects are dose- and passage-dependent, and we showed that MSC from earlier passages (up to the fifth) are more suitable therapeutic application due to their stability, anti-inflammatory and neuroprotective effects (Choi et al., 2010; Forostyak et al., 2016a). Similar outcomes were achieved by the administration of 10^6 cells in asymptomatic SOD1 animals, while 10^5 cells failed to change the prognosis of the disease (Habisch et al., 2007; Kim et al., 2010).

Embryonic and Induced Pluripotent Stem Cells

The advances in stem cell research demonstrated a great potential of cell-based approach in the treatment of currently incurable diseases and this brings hope for patients and their families, especially in the case of neurodegenerative disorders or neurotrauma. The Nobel Prize in Physiology or Medicine for "The discovery that mature cells can be reprogrammed to become pluripotent" highlights the importance of SC research in general and particularly iPS technology (Takahashi and Yamanaka, 2006).

At the moment several groups are working on substitution of defective neurons with donor stem cell-derived neuronal progenitors. The idea behind is that the grafted cell will integrate after transplantation into existing neural circuits and take over the functions of defective cells (Kallur et al., 2006; Lindvall and Kokaia, 2006). It has been reported that MN generated from ESC are not just able to maintain typical motor neuronal phenotype *in vitro*, but also to functionally incorporate into host CNS (Wichterle et al., 2002; Papadeas and Maragakis, 2009). A glial restricted precursors (GRP) or human neural stem/progenitor cells (hNSC) seem to have a greatest potential in ALS research. These cell types were described to modify ALS prognosis, to reduce MN death and to establish functional synapses with structural integration into the motor circuitry (Lepore et al., 2008; Xu et al., 2009). In ALS functional integration of grafted cells is crucial. However, MN replacement would also expect an axonal outgrowth and neuromuscular junction (NMJ) formation to innervate muscle fibers that otherwise are dedicated to be atrophied. We found only few studies that have demonstrated formation of new functional connections between grafted SC and the recipients' muscles after the grafting (Deshpande et al., 2006; Yohn et al., 2008; Gowing and Svendsen, 2011).

Taking into the consideration above limitations, generation and grafting of protective cells that will support the remaining MN might be more effective. It is known that in ALS astrocytic reactivity with proinflammatory transcriptional and translational profiles exacerbates motor neurons (MNs) dysfunction (Sun et al., 2015). These specific pathological events could be targeted

by the application of either ES or iPS cells that were directed either toward progenitor or mature astrocytic phenotypes prior to transplantation (Popescu et al., 2013; Kondo et al., 2014).

The possibility of hyperproliferation and the formation of teratomas is always a risk related with ESC-/iPSC-derived neural progenitors (Seminatore et al., 2010). Latest preclinical studies demonstrate that this issue could be controlled, thus Food and Drug Administration (FDA) has permitted a phase I clinical study testing the safety of the intraspinal grafting of neural stem cells, into patients with confirmed ALS (http://clinicaltrials.gov, identifier #NCT01348451) (Gowing and Svendsen, 2011; Lunn et al., 2011). A transplantation of glial precursor cells or mature astroglia is another quit realistic approach to promote neuroprotection and trophic effects, that will support the failing motor neurons (Robberecht and Philips, 2013). These cells generated from somatic tissues, even from elderly patients, could be further reprogrammed to reach a pluripotent stage, and later differentiated toward neural/astroglial morphology of interest ready for delivery into the patients (Dimos et al., 2008; Hall et al., 2017). This alternative to embryonic/fetal cells, if isolated from affected individuals, could be used either as an *in vitro* model of neurodegenerative diseases, helping us to understand the mechanisms underlying the pathological processes, or could be used as therapeutic agents after avoiding the risk of graft rejection or the opposite, graft-vs.-host-disease if the donor and recipient is a same individual. The cutting-edge of modern cell biology is the generation of functional induced motor neurons or astrocytes from the patient's own fibroblasts, which after transplantation could protect the dying MN (Son et al., 2011; Hall et al., 2017).

Considering that neurodegenerative diseases often develop in elderly patients, there are some concerns related to the "quality" of stem cells if generated from an aged and sick organism and whether these cells might be used for cell replacement, neuroprotection and neuroregeneration. So far reports addressing this question are quit controversial. Dimos et al. by using the example of iPS cells generated from an 82-year-old woman with a FALS, showed that these cells possess the same properties as do ESC and that they could differentiate into MN (Dimos et al., 2008). However, in the case of iPS cells, which are generated using a cocktail of overexpressed transcriptional factors transferred to skin fibroblasts (or other somatic cells) by transfection of viral vector infection, it is necessary to note that we do not yet know whether the human use of these cells will not increase a risk of genetic modification of both donor and recipient cells (Wichterle and Przedborski, 2010). Concerns are also about the route of transplantation and the developmental stage of the pluripotent cells. It is necessary to keep in mind that the manipulation with the cells that are fully differentiated to the neural or neuronal phenotype is extremely difficult. Therefore, the cells at the precursor stage are potentially better candidates for the transplantation. According to the publicly available information, several clinical trials that are engaging human neural stem cells application to evaluate the safety and efficiency of such a therapy of ALS are currently recruiting patients or are on-going (Glass et al., 2012).

Clinical Trials of Stem Cell Therapy in ALS

In vivo experiments using rodent models of FALS formed a platform for clinical trials involving patients (Vercelli et al., 2008). We have analyzed supported past, current and future clinical trials from the clinicaltrial.gov website and summarized them into the overview table (**Table 1**). It is obvious that the number of phase 1/2 clinical trials is increasing annually. Mostly, these are safety studies involving small amounts of patients. Unfortunately, great majority of the trials does not describe details about the dosage, type of the cells, criteria for patients monitoring and does not report a feedback about the study outcomes. This complicates interpretation of the data and creates obstacles for further clinical application of various cell types. The majority of approved/available clinical trials employ mesenchymal stromal cells (MSCs) of different origin (mostly bone marrow) for the therapy of ALS. It could be explained mainly by the easiness of derivation and manipulation with autologous cells from patients, legal issues and long history of clinical application of bone marrow derived cells. On the other hand, this cells are quite unique, especially for their paracrine properties for detailed overview see (Forostyak et al., 2013b).

Unfortunately, majority of the granted trials did not perform patients' follow-up longer than 24 months. The first long-term follow-up after nearly 9 years of patients monitoring, showed that dorsal application of MSC is safe procedure with no clear clinical benefits, but also without any structural changes or deterioration in psychosocial status (Mazzini et al., 2003, 2010, 2011). Trial with the mononuclear CD133(+) fraction isolated from the peripheral blood after the frontal motor cortex grafting were reported to significantly extend life of ALS patients and to improve their lifestyle compared with patients that were not treated with the cells (Martinez et al., 2009). Deda et al. reported that one year after the implantation of hematopoietic progenitor stem cells significantly improved tested parameters in 70 percent of the treated patients, compared with their pre-operative status (Deda et al., 2009). A latest prospective, non-randomized, open label clinical trial has been completed in 2016 in Prague, Czech Republic (Sykova et al., 2017). Study evaluated the safety and the efficacy of autologous multipotent MSC in the patients with confirmed diagnosis of ALS (http://www.sukl.eu). The trial involved 26 patients with sporadic ALS, who received a single intrathecal (via a lumbar puncture) dose of autologous MSCs applied via a lumbar puncture into the cerebrospinal fluid. As compared to previous trials, this study included the largest group of ALS patients, had a longer pre-and post-treatment assessment period, and a relatively small dose of stem cells was used. A potential adverse reactions were assessed by clinical, laboratory and MR examination for 18 months. Patients underwent clinical evaluation using ALS functional rating scale (ALSFRS), Norris spinal and bulbar scale (NSS and NSB), forced vital capacity (FVC) and weakness scale (WS). This study showed that 30 percent of the patients experienced mild/moderate headache after MSCs application, not connected with the actual cell application. No suspected serious adverse reactions or new cerebrospinal pathology on MR examinations were observed. Eighty percent of patients preserved FVC values

TABLE 1 | An overview of clinical trials involving cell-based therapy to treat ALS (modified from www.clinicaltrials.gov).

Cell type (additional intervention)/dose	Stage of disease/place of cell delivery	Evaluation time after Tx	Country/Company/ Identifier (NCT and other)	Phase of the trial/stage/estimated trial end	Results	Side effects	PI/References
1. Intrathecal autologous bone marrow mononuclear cell transplantation/na	Na/intrathecal	2 years	India/Neurogen Brain and Spine Institute/NCT02242071	Phase 1/Completed/September 2016	na	na	Neurogen Brain and Spine Institute/na
2. Autologous intrathecal administration of hematopoietic stem cells/na	Na/intrathecal	During the procedure and at 1st, 2nd, 3rd, 6th, and 12th month after the procedure	Mexico/Hospital Universitario Dr. Jose E. Gonzalez/NCT01933321; NGBSI-10	Phase 2, phase 3/Completed/April 1, 2015	na	na	David Gomez Almaguer/na
3. Autologous bone marrow mononuclear cell transplantation on the survival duration	Na/intrathecal and intramuscular routes	Retrospective Control Study	India/Neurogen Brain and Spine Institute/NCT01984814; NGBSI-04	Phase 2/Completed/November 2013	na	na	Alok K Sharma/na
4. Mesenchymal stem cells/1 × 10^8 mesenchymal stem cells +10cc normal saline	Na/intraspinal injection of	Before transplatation and at 6 months, 12months, 18 months, and 24 months after transplantation	Iran/Isfahan neurosciences research center/NCT02116634;rokhsareh	Phase 1, 2/withdrawn/January 2016	**Withdrawn prior to enrolment**	na	Dr. Keivan Basiri /na
5. Human glial restricted progenitor cells (hGRPs; Q-Cells®)/na	Na/5-10 intraspinal grafts: **Cohort 1,** Unilateral lumbar surgical transplantation of Q-Cells dose level 1; **Cohort 2,** Unilateral cervical surgical transplantation of Q-**Cells** dose level 1; **Cohort 3,** Unilateral cervical surgical transplantation of Q-**Cells** dose level 2; **Cohort 4,** Unilateral cervical surgical transplantation of Q-Cells dose level 3; **Cohort 5,** Unilateral cervical surgical transplantation of Q-Cells dose level 4; **Cohort 6,** Unilateral cervical surgical transplantation of Q-Cells dose level 5	Before transplantation and during 9 months after Tx (Parallel Assignment). Following the 9-month study period, subjects who consent will continue to be followed for safety and efficacy long-term in a separate protocol.	/Q Therapeutics, Inc./NCT02478450; QALS-101	Phase 1/2a/not yet recruiting/April 2020/	na	na	Q Therapeutics, Inc./na
6. Autologous mesenchymal stem cells/ **Group 1**: single intrathecal dose of 1 × 10^7 cells; **Group 2:** single intrathecal dose of 5 × 10^7 cells; **Group 3:** one intrathecal dose of 5 × 10^7 cells followed one month later by a second intrathecal dose of 5 × 10^7 cells; **Group 4:** single intrathecal dose of 1 × 10^8 cells; **Group 5:** one intrathecal dose of 1 × 10^8 cells followed one month later by a second intrathecal dose of 1 × 10^8 cells	Five treatment groups of up to five patients each: **Groups 1, 2,** and **4** will receive a single dose of cells. **Groups 3** and **5** will receive 2 doses of cells separated by one month. Intrathecal injections into new subjects will be timed so that there is a minimum of 1 week between subject injections	Initial clinical follow-up will be weekly with scheduled blood, CSF and magnetic resonance imaging (MRI) evaluations. Regular assessment until death or for a minimum of 2 years after the final infusion. After 1 month, patients will have clinical evaluations at 3 month intervals, or earlier if indicated by clinical status.	US/Mayo Clinic/NCT01609283; 11-008415	Phase 1/ongoing, not recruiting/April 2018	na	na	Anthony Windebank, Mayo Clinic/na

(Continued)

TABLE 1 | Continued

Cell type (additional intervention)/dose	Stage of disease/place of cell delivery	Evaluation time after Tx	Country/Company/ Identifier (NCT and other)	Phase of the trial/stage/estimated trial end	Results	Side effects	PI/References
7. Autologous bone marrow-derived mesenchymal stem cells/na	Na/Intrathecal (via a standard lumbar puncture)	Every 2 months up to 1.5 year of the trial	Poland/University of Warmia and Mazury/NCT02881489; UWM/ALS-MSC.2015/002	Phase 1/Enrolling by invitation/April 2018	na	na	Wojciech Maksymowic/na
8. Autologous Bone Marrow Mononuclear Cells/ The average dose is 550 millions of cells (100–1,200 million) diluted in 2 ml saline. Placebo Comparator: Saline	Na/ Intramuscular infusion of autologous mononuclear **cells** (MNC) in TA muscle of one of the lower limb (randomly determined). Intramuscular infusion of 2 mL of saline (placebo) in the TA muscle of the contralateral limb (group control).	24 from baseline	Spain/Red de Terapia Celular/NCT02286011; TCIM/ELA2011-004801-25 (EudraCT Number)	Phase 1/ ongoing but not recruiting/December 2017	na	na	Joaquín A Gómez Espuch/na
9. Autologous bone marrow stem/progenitor cell/na	Na/intrathecally	1 year	Poland/Pomeranian Medical University Szczecin//NCT02193893; ZPO 02, ALS-BMSC #01 (Other Identifier: Department of General Pathology, PMU in Szczecin)	Phase 1/enrolling by invitation/December 2017	na	na	Boguslaw Machalinski, and Przemyslaw Nowacki/na
10. Adipose Derived Mesenchymal Stem Cell/2 × 10^6 per kilogram	Na/intravenous	2 months	Iran, Islamic Republic/Royan institute/NCT02492516; Royan-Nerve-008	Phase 1/Completed/April 2017	na	na	Leila Arab/na
11. Autologous mesenchymal stem cells/10 × 106	Na/intraspinal injection	2 years	US/Mayo Clinic/NCT01142856; 09-001995	Phase/Completed/April 2011	na	na	Anthony J. Windebank,/na
12. Human Neural Progenitor Cells Secreting Glial Cell Line-Derived Neurotrophic Factor (CNS10-NPC-GDNF)/ two sequential dosing groups	NA/Stereotactic surgical device	12 months	US/Cedars-Sinai Medical Center and California Institute for Regenerative Medicine/ NCT02943850; Pro00042350	Phase 1/2a/recruiting/ April 2019	na	na	Robert H. Baloh/na
13. Bone marrow-derived mesenchymal stem/stromal cells/15±4.5 x 10^6/patient	Na/single intrathecal injection (via standard lumbar puncture)	3, 6, 9, 12, and 18 months	Czechia/Bioinova Ltd./EudraCT No. 2015-000139-33	Phase 1/2/Completed/ December 2015	safe procedure; reduction in ALSFRS decline at 3 months after application; 80% of the patients, FVC values remained stable or above 70% for a time period of 9 months.	30% of the patients experienced a mild to moderate headache, resembling the headaches after a standard lumbar puncture	Eva Sykova/(Sykova et al., 2017)
14. Human allogeneic Wharton's jelly-derived mesenchymal stem cells	Na/Intrathecal (via a standard lumbar puncture)	6 months - first time ALSFRS) + and then every 2 months up to 1.5 year of the trial	Poland/University of Warmia and Mazury/NCT02881476; UWM/ALS-MSC.2015/001	Phase 1/Enrolling by invitation/ December 2018	na	na	Wojciech Maksymowicz/na
15. Autologous bone marrow-derived stem cells/na		3 months intervals for 12 months	US/TCA Cellular Therapy/NCT01082653; 2008-ALS-I	Phase 1/suspended recruiting/ May 2014	na	na	TCA Cellular Therapy/na
16. Autologous mesenchymal stem cell/2 intrathecal autologous MSCs infusions (1 × 10^8 cells)	Na/2 intrathecal autologous MSCs infusions	Intervalls 1, 3, 6, and 12 months	Brazil/ Hospital e Maternidade Dr. Christóvão da Gama/NCT02987413; HospitalMCG IEPSaoLucas	Phase 1/Completed/ August 2017	na	na	Leandro B Agati/na

(Continued)

TABLE 1 | Continued

Cell type (additional intervention)/dose	Stage of disease/place of cell delivery	Evaluation time after Tx	Country/Company/ Identifier (NCT and other)	Phase of the trial/stage/estimated trial end	Results	Side effects	PI/References
17. Efficacy of Transplantation of Autologous Mesenchymal Stem Cells Secreting Neurotrophic Factors (MSC-NTF)/	Na/Combined intramuscular and intrathecal placebo administration	24 weeks post-transplantation Time Frame: Visits 1, 2, 3, 5, 6, 7, 8, 9, 10	US/Brainstorm-Cell Therapeutics/ NCT02017912; BCT-001-US	Phase 2/Completed/July 2016	na	na	Merit Cudkowicz/na
18. HLA-haplo matched Allogenic Bone Marrow Derived stem cells("HYNRCS-Allo-ALS-02 inj")/1.0 × 10^6 cells/kg	Intrathecal	12 months	/Hanyang University Seoul Hospital/ NCT03214146; HYNR-CS-Allo-02	Phase 1/ recruiting/ November 2018	na	na	Seung Hyun Kim/na
19. Autologous peripheral blood mononuclear cells	Na/Subarachnoid Space	Week 1, week 2, week 4, week 12 after operation	China/The First Affiliated Hospital of Dalian Medical University/NCT03085706; DalianMU_003	Na/completed/March 2017	na	na	Jing Liu/na
20. Autologous CD4+ CD25+ regulatory T cells with concomitant subcutaneous IL-2 injections/Tregs (1 × 10^6 /kg) with concomitant subcutaneous IL-2 injections (2 × 10^5 IU/m^2) 25 days (±2 days) post leukapheresis	Na/4 infusions of autologous expanded Tregs with concomitant subcutaneous injections of IL-2	3 months post-treatment for a total of two years	US/ The Methodist Hospital System/NCT03241784; Pro00013616	Phase 1/ongoing, not recruiting/ February 2018	na	na	Stanley H. Appel/na
21. Autologous bone marrow-derived stem cells("HYNR-CS inj")/na	Intrathecal	Week 12, −8, −4, 0, 4, 8, 12, 16	Korea Rep./Corestem, Inc./NCT01363401; HYNR_CS_ALS201	Phase 1/2/Completed/ February 2017	na	na	Seung Hyun Kim/na
22. Induced Pluripotent Stem Cells From an Existing Collection of Human Somatic Cells/na	Derivation for research purposes	na	Israel/ Hadassah Medical Organization/ NCT00801333; 0511-08-HMO	/ recruiting/December 2020	na	na	Benjamin Reubinoff/na
23. Autologous mesenchymal stem cells/na	2 intrathecal injections	10 months	Brazil/University of Sao Paulo General Hospital/NCT02917681;401922/ 2014-6	phase 1/2/recruiting/ February 2019	na	na	Gerson Chadi/na
24. Autologous Purified Bone-Marrow-Derived Stem Cell/na	Combined intravenous and intrathecal	4 months	Jordan/ Stem Cells Arabia/NCT03067857;SCA-MND1	Phase 1/2/Ongoing, not recruiting/ January 2019	na	na	Stem Cells Arabia/na
25. Autologous mesenchymal stem cells/administration of MSC: 1 million MSC/kg, 2 million MSC/kg and 4 million MSC/kg.	Intravenous administration of MSC/ placebo	6 months	Spain/Andalusian Initiative for Advanced Therapies - Fundación Pública Andaluza Progreso y Salud/ NCT02290886; CeTMAd/ELA/2011	Phase 1/ 2/recruiting/February 2021	na	na	Óscar Fernández/na
26. Autologous Bone Marrow Stem Cells/unknown	>6 months and <36 months/I.S. (T3-4) and intrathecal via laminectomy injection of cell fraction (placebo, saline solution)	Every 3 months	Spain/Fundacion para la Formacion e Investigacion Sanitarias de la Region de Murcia /NCT01254539; Extension CMN/ELA 2006-003096-12 (EudraCT Number) EC07/90762 (Other Identifier: ISCIII)	Phase 1/2/Completed/November 2015	na	na	Jose María Moraleda Jiménez/na

(Continued)

TABLE 1 | Continued

Cell type (additional intervention)/dose	Stage of disease/place of cell delivery	Evaluation time after Tx	Country/Company/ Identifier (NCT and other)	Phase of the trial/stage/estimated trial end	Results	Side effects	PI/References
27. Mononuclear autologous bone marrow cells/unknown	After 6 months/2x I.S. ipsi/contralateral (posterior funiculus) after laminectomy	Every 3 months 1 year	Spain/ Fundacion para la Formacion e Investigacion Sanitarias de la Region de Murcia /NCT00855400	Phase 1 and 2/ completed/2010	Safe. Greater number of MNs in treated segments (neurotrophic effect). MNs surrounded with CD90+ cells and did not show degenerative ubiquitin deposits.	No severe transplant-related adverse event. Adverse events grade ≤2.	Jose Maria Moraleda Jiménez (Blanquer et al., 2012)
28. Umbilical Cord Mesenchymal Stem Cells/?	>6 months and <36 months/4x (every 3–5 days) intrathecally	1, 6, 12, and 24months	China/General Hospital of Chinese Armed Police Forces/NCT01494480/201 11207ALS	Phase 2/recruiting by invitations/2015	na	na	Dr. Yi Hua An/na
29. Human Spinal Cord Derived Neural Stem Cell/Group A: 3 ambulatory early-stage subjects with arm weakness but not paralysis, to receive bilateral C3 through C4 injections of 2×10^6 cells (10 injections × $2x10^5$ cells/ injection) • **Group B:** 3 ambulatory early-stage subjects with arm weakness but not paralysis, to receive bilateral C3 through C5 injections of 4×10^6 cells (20 injections × 2×10^5 cells/injection) • **Group C:** 3 ambulatory early-stage subjects with arm weakness but not paralysis, to receive bilateral C3 through C5 injections of 6×10^6 cells (20 injections × 3×10^5 cells/injection) • **Group D:** 3 ambulatory early-stage subjects with arm weakness but not paralysis, to receive bilateral C3 through C5 injections of 8×10^6 cells (20 injections × 4×10^5 cells/ injection) • **Group E:** 3 ambulatory early-stage subjects with arm weakness but not paralysis, to receive bilateral L2 through L5 injections of $8x10^6$ cells (20 injections of 4×10^5 cells/ injection) and then ∼4–12 weeks later to receive bilateral C3 through C5 injections of 8 × 10^6 cells (20 injections of 4 × 10^5 cells/ injection.	<24 months/I.S.	2 and 4 weeks, and then at 3, 6, 9, 12, 15, 18, 21, and 24 months, and then at every 6 months thereafter until death.	US/Neuralstem Inc./NCT01730716; NS2012-3	Phase 2/unknown/April 2012	na	na	Neuralstem Inc/na

(Continued)

TABLE 1 | Continued

Cell type (additional intervention)/dose	Stage of disease/place of cell delivery	Evaluation time after Tx	Country/Company/ Identifier (NCT and other)	Phase of the trial/stage/estimated trial end	Results	Side effects	PI/References
30. Human Spinal Cord Derived Neural Stem Cell/5 injection × 100,000 neural cells	<24 months/unilateral and bilateral intraspinal lumbar microinjection	1, 3, 6, 9, 12, 18, 24, 30, 36, 42, and 48 month follow-up/post-surgery visits	US/Neuralstem Inc./NCT01348451; NS2008-1	Phase 1/ongoing, not recruiting by invitations/March 2016	Twelve patients have received a transplant. By discharge, none had a documented motor function decrement. The procedural safety of unilateral and bilateral intraspinal lumbar microinjection was proved. Completion of phase I safety trial is planned by proceeding to cervical and combined cervical + lumbar microinjections in ALS patients	One instance of transient intraoperative somatosensory-evoked potentials depression. In the immediate postoperative period-—1 episode of urinary retention requiring Foley catheter reinsertion. 2 patients required readmission and reoperation for cerebrospinal fluid leak or suprafascial wound dehiscence ($n = 1$ each). Two deaths occurred at 8 and 13 months postsurgery; neither was related to the surgical transplant.	Neuralstem Inc./(Glass et al., 2012; Riley et al., 2012)
31. Human fetal neural stem cells/	<6months/I.S. microinjection	36 months	Italy/Azienda Ospedaliera Santa Maria, Terni and Università di Padova Italy /NCT01640067	Phase 1/completed/December 2015	• Safe procedure; • no increase of disease progression up to18 months; • transitory improvement of the subscore ambulation on the ALS-FRS-R scale (from 1 to 2) in 2 patients; • improvement of the MRC score for tibialis anterior in 1 patient (for 7 months); the latter and 2 additional patients refused PEG and invasive ventilation and died 8 months after surgery due to the progression of respiratory failure (confirmed by autopsies)	na	Angelo L Vescovi/(Gelati et al., 2013; Mazzini et al., 2015)

(Continued)

TABLE 1 | Continued

Cell type (additional intervention)/dose	Stage of disease/place of cell delivery	Evaluation time after Tx	Country/Company/ Identifier (NCT and other)	Phase of the trial/stage/estimated trial end	Results	Side effects	PI/References
32. HLA-haplo Matched Allogenic Bone Marrow Derived Stem Cell("HYNR-CS-Allo Inj")/0.25/0.5/1 × 10^6 cells/kg dose cohort	<5 years/2x Intrathecal injection with 28 days interval	4, 8, 12, 16 weeks	Korea/ Hanyang University Seoul Hospital; Corestem, Inc./NCT01758510; HYNR-CS-Allo-01	Phase 1/ ongoing/December 2017	na	na	Seung Hyun Kim/na
33. Bone Marrow Derived Mesenchymal Stem Cell/	<2 years/Intraventricular	1, 3, 6, and 12 months	Islamic Republic of Iran/Royan Institute/ NCT01759784	Phase 1/Withdrawn prior enrolment/December 2015	Withdrawn prior enrolment	na	Chair: Hamid Gourabi; Director: Nasser Aghdami/na
34. Bone Marrow Derived Mesenchymal Stem Cell/	<2 years/Intravenous	1, 3, 6, and 12 months	Islamic Republic of Iran/Royan Institute/NCT01759797; Royan-Nerve-005	Phase 1/Completed/January 2014	na	na	Chair: Hamid Gourabi Director: Nasser Aghdami, Director: Seyed Masoud Nabavi/na
35. Bone Marrow Derived Mesenchymal Stem Cell/na	<2 years/Intrathecal	1, 3, 6 and 12 months	Islamic Republic of Iran/Royan Institute/NCT01771640; Royan-Nerve-006	Phase 1/Completed/December 2015	na	na	Hamid Gourabi/na
36. Autologous cultured mesenchymal bone marrow stromal cells secreting neurotrophic factors (MSC-NTF)/ 94 × 10^6, 141 × 10^6, and 188 × 10^6 cells (depending on the groups)	<2 years/single Intrathecal plus multiple (24 sites) intramuscular	Monthly, than every 6 months	Israel/Hadassah Medical Organization; Brainstorm-Cell Therapeutics/NCT01777646; MSC-NTF-002-HMO-CTIL	Phase 2a/recruiting/2014	The treatment was found to be safe and well-tolerated over the study follow-up period; Of 14 patients, 13 (87%) were defined as responders to either ALS FRS-revised or forced vital capacity, having at least 25% improvement at 6 months after treatment in the slope of progression.	Most of the adverse effects were mild and transient	Dimitrios Karusis/(Petrou et al., 2016)
37. Autologous cultured mesenchymal bone marrow stromal cells secreting neurotrophic factors (MSC-NTF)/Intramuscular 24 × 10^6; Intrathecal 60 × 10^6	<2 years/single Intrathecal; intramuscular (24 sites)	Monthly, than every 6 months, 12 months	Israel/Hadassah Medical Organization/NCT01051882; MSC-NTF-001-HMO-CTIL	Phase 1 and 2/completed/January 2014	na	na	Dimitrios Karussis/(Petrou et al., 2016)

above 60 percent for 12 months. Fourteen patients (out of 26) with a remarkable pretreatment decline in functional scales, had significant reduction/stabilization in their total functional score decline at 3 months after application, which was less pronounced at 6 and 9 months.

Despite an increasing number of clinical trials proving the safety of the procedure there is a great need for bigger multicentre trials. Even though some small series of experiments involving patients showed an improvement of motor and sensory functions after the administration of stem cells, there is a need for bigger multicentre studies with placebo group of patients. The above trials that resulted with a neuroprotective effect after cell-based therapy have employed various routes of application, different type of cells, and not same ways of clinical evaluation, therefore there is a need for unification of future clinical trials design. One could also speculate that the combination of different routes of cell delivery might bring even better results related to survival and motor functions. We would like to stress once again, that current research should also employ physiological characteristics of all cells types that are supposed to be delivered into patients. This could give us more homogenous data between the trials as well as develop an algorithm that will enable prognosis of cell-based therapy in the future (Forostyak et al., 2016b). Finally, specific markers, which will enable early disease diagnosis, are of a great importance for the successful cell-based therapy, mainly because at the beginning of neurodegeneration stem cells might bring more benefits in rescuing neurones from inevitable death, if compared with the therapy at the terminal-stage of ALS.

AUTHOR CONTRIBUTIONS

SF and ES - data collection and literature overview, manuscript writing.

ACKNOWLEDGMENTS

This work has been funded by the Grant Agency of the Czech Republic (GACR 10504P, GACR 17-21146S and GACR 15-06958S). We would like to acknowledge the Ministry of Education, Youth and Sports (MEYS, MŠMT) of the Czech Republic (Centre of Reconstruction Neuroscience—NEURORECON, CZ.02.1.01/0.0/0.0/15_003/0000419).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 10 November 2017
doi: 10.3389/fnins.2017.00628

Induced Pluripotent Stem Cell-Derived Neural Stem Cell Transplantations Reduced Behavioral Deficits and Ameliorated Neuropathological Changes in YAC128 Mouse Model of Huntington's Disease

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OPEN ACCESS

Edited by:
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Reviewed by:
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Jeremy Michael Van Raamsdonk, Van Andel Institute, United States
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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *31 July 2017*
Accepted: *27 October 2017*
Published: *10 November 2017*

Citation:
Al-Gharaibeh A, Culver R, Stewart AN, Srinageshwar B, Spelde K, Frollo L, Kolli N, Story D, Paladugu L, Anwar S, Crane A, Wyse R, Maiti P, Dunbar GL and Rossignol J (2017) Induced Pluripotent Stem Cell-Derived Neural Stem Cell Transplantations Reduced Behavioral Deficits and Ameliorated Neuropathological Changes in YAC128 Mouse Model of Huntington's Disease. Front. Neurosci. 11:628. doi: 10.3389/fnins.2017.00628

Huntington's disease (HD) is a genetic neurodegenerative disorder characterized by neuronal loss and motor dysfunction. Although there is no effective treatment, stem cell transplantation offers a promising therapeutic strategy, but the safety and efficacy of this approach needs to be optimized. The purpose of this study was to test the potential of intra-striatal transplantation of induced pluripotent stem cell-derived neural stem cells (iPS-NSCs) for treating HD. For this purpose, we developed mouse adenovirus-generated iPSCs, differentiated them into neural stem cells *in vitro*, labeled them with Hoechst, and transplanted them bilaterally into striata of 10-month old wild type (WT) and HD YAC128 mice. We assessed the efficiency of these transplanted iPS-NSCs to reduce motor deficits in YAC128 mice by testing them on an accelerating rotarod task at 1 day prior to transplantation, and then weekly for 10 weeks. Our results showed an amelioration of locomotor deficits in YAC128 mice that received iPS-NSC transplantations. Following testing, the mice were sacrificed, and their brains were analyzed using immunohistochemistry and Western blot (WB). The results from our histological examinations revealed no signs of tumors and evidence that many iPS-NSCs survived and differentiated into region-specific neurons (medium spiny neurons) in both WT and HD mice, as confirmed by co-labeling of Hoechst-labeled transplanted cells with NeuN and DARPP-32. Also, counts of Hoechst-labeled cells revealed that a higher proportion were co-labeled with DARPP-32 and NeuN in HD-, compared to WT- mice, suggesting a dissimilar differentiation pattern in HD mice. Whereas significant decreases were found in counts of NeuN- and DARPP-32-labeled cells, and for neuronal density

measures in striata of HD vehicle controls, such decrements were not observed in the iPS-NSCs-transplanted-HD mice. WB analysis showed increase of BDNF and TrkB levels in striata of transplanted HD mice compared to HD vehicle controls. Collectively, our data suggest that iPS-NSCs may provide an effective option for neuronal replacement therapy in HD.

Keywords: neural stem cells, Huntington's disease, cell transplantations, YAC128, iPSCs, iPS-NSCs

INTRODUCTION

Huntington's disease (HD) is a progressive, neurodegenerative, genetic disorder characterized by choreic movements, behavioral and cognitive disturbances, and dementia (Craufurd et al., 2001). The disease is caused by an autosomal dominant mutation in the *huntingtin* gene (*HTT*), and the mode of inheritance is dominant with almost full penetration (with 40 or more CAG repeats). The genetic basis of HD was discovered in 1993 (MacDonald et al., 1993), and it was found to be caused by an elongated Cystosine-Adenine-Guanine (CAG) repeat on the short arm of chromosome 4p16.3 in the *HTT* gene. HD symptoms include psychiatric, motor, and cognitive deficits, and are variable among patients during early stages of the pathology. However, as the disease progresses, symptoms become predictable, with all patients eventually developing same characteristic pathologies (Walker, 2007). The life expectancy for HD patients ranges between 15 and 20 years after the appearance of motor symptoms (Landles and Bates, 2004; Walker, 2007). The most apparent and earliest damage is seen in the neostriatum, which is composed of the caudate nucleus and putamen (Walker, 2007). Medium spiny neurons (MSNs) in the striatum appear to be the most vulnerable neurons to the damage in HD (Albin et al., 1990).

Different animal models, either chemically or genetically induced, have been developed to study various aspects of HD. One of these models, theYAC128 HD mouse, contains the full-length human mutant *HTT* (m*HTT*) inserted into its genome which results in the expression of m*HTT* with 128 CAG repeats (Slow et al., 2003). YAC128 mice show selective, age-dependent, striatal and cortical atrophy and neurodegeneration, and develop progressive deterioration of motor and cognitive functions (Van Raamsdonk et al., 2005; Gray et al., 2008; Ehrnhoefer et al., 2009). The decline in motor abilities manifests as progressive deficits in accelerating rotarod performance that correlates with the loss of neurons in the striatum (Slow et al., 2003).

Experimental approaches used to treat HD aim to decrease the levels of the mHTT protein, improve the survivability of neurons, as well as to replace the affected neurons. Stem cell therapy holds significant promise for treating HD, and includes the transplantation of stem cells into the affected regions of the brain (Cundiff and Anderson, 2011). Owing to the promising outcomes in animal models of HD, the use of stem cell transplants in human clinical trials have been performed to test for efficacy (Clelland et al., 2008). Transplants of fetal tissue, embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), and neuronal stem cells (NSCs; both adult and differentiated from ESCs) have been used as experimental treatments for HD, and all possess different properties that may be beneficial for use as a therapy. Although some promising results have been found following the transplantation of any of these cell sources, the immune rejection of the graft (Bernreuther et al., 2006; Cicchetti et al., 2014) as well as some serious adverse events have been reported. Specifically, tumor formation has been found following ESC transplants (Aubry et al., 2008), and hemorrhage with multiple solid and cystic lesions in the brain was found following fetal tissue grafting (Keene et al., 2009). While the anti-inflammatory properties and ability for autologous transplantations result in a superior survivability of MSC transplants (Rossignol et al., 2009, 2011; Dey et al., 2010; Lin et al., 2011; Sadan et al., 2012; Serrano Sánchez et al., 2014), these cells do not readily differentiate into neurons (Przyborski et al., 2008), limiting their utility as a source of cellular replacement. Unlike transplants of MSCs, NSC transplants have the potential for replacing neurons that have degenerated within the targeted region (Vazey et al., 2006; Yang and Yu, 2009), yet the survivability of NSCs post-transplantation remains poor with signs of immune rejection (Johann et al., 2007; Rossignol et al., 2014). Similarly, the accessibility to NSCs is limited, and met with technical and ethical complications caused by the demand for using embryonic tissue for isolation.

A new source of pluripotent stem cells emerged when Takahashi and Yamanaka (2006) generated pluripotent stem cells by reprogramming somatic cells through inserting 4 genes (*OCT4, SOX2, Klf4*, and *c-Myc*) into fibroblasts obtained from the skin. These cells, defined as induced pluripotent stem cells (iPSCs), were able to differentiate into any cell type in the body (Takahashi and Yamanaka, 2006). iPSCs appear to have much of the same characteristics as ESCs, including morphology and differentiation capabilities, as well as the capacity to form teratomas containing cells of all three germ lineages when transplanted into severe combined immune deficient mice

Abbreviations: ANOVA, analysis of variance; BCA, bicinchoninic acid assay; BDNF, brain derived neurotrophic factor; bFGF, basic fibroblast growth factor; DARPP32, Dopamine- and cAMP-regulated phosphoprotein of 32 kDa; DMEM, Dulbecco's Modified Eagles medium; EDTA, Ethylene-di-amino-tetra-acetic-acid; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; HBSS, Hank's balanced salt solution; HD, Huntington's disease; HRP, Horseradish peroxidase; iNSC, induced neural stem cells; iPSC, Induced pluripotent stem cells; iPS-NSCs, induced pluripotent stem cells-derived neural stem cells; Klf4, Kruppel-like factor 4; MSN, Medium spiny neuron; NEAA, non-essential amino acids; NeuN, Neuronal nuclei; NSC, Neural stem cell; OCT4, Octamer-binding transcription factor 4; OD, Optical density; PBS, Phosphate buffer saline; PFA, Paraformaldehyde; PVDF, Polyvinylidene fluoride; QA, Quinolinic acid; RIPA, Radio immunoprecipitation assay; SDS, Sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SOX2, SRY (sex determining region Y)-box 2; TBS, Tris buffer saline; TrkB, Tropomyosin-related kinase B; YAC, Yeast artificial chromosome.

(Takahashi and Yamanaka, 2006). However, there are genetic and epigenetic differences between them (Robinton and Daley, 2012). The use of iPSCs offers a viable alternative to ESCs and circumvents the issues of availability and ethical concerns surrounding the use of embryos (Verma and Verma, 2011). Work in our laboratory indicated that transplanting rat-derived iPSCs into the striata of rats given 3- nitropropionic acid to model HD, revealed improvements in the motor function, and differentiation of transplanted cells into region-specific neurons in the striatum (Fink et al., 2014a). However, the transplantation of pluripotent stem cells may pose a risk of over-proliferation if the transplants occur without prior pre-engagement down a given germ layer, such as the neuro-ectoderm (Miura et al., 2009). Subsequently, NSCs that were derived from iPSCs obtained from a patient with juvenile onset HD, were transplanted into the striata of YAC128 mice, and showed significant improvement in motor functions (Jeon et al., 2014). This study demonstrated a proof-of-principle that therapeutic efficacy can still be derived following the transplantation of pre-engaged iPSCs, which may reveal to be a safer method of therapy.

Overall, accumulating evidence suggests that stem cell transplantations hold significant promise for use as a treatment for HD. However, there are many technical challenges that need to be addressed before this approach can be safely translated as a treatment in clinical cases of HD. This study aimed to further characterize the use of iPS-NSCs as a treatment for HD by utilizing behavioral, histological, and protein analyses as outcome measures for therapeutic efficacy.

We hypothesized that iPS-NSCs will provide a useful alternative for ESCs and NSCs for HD treatment because these cells can be generated in adequate amounts from somatic cells, and form a more personalized treatment that will promote integration of the transplanted cells, with less risk of immune rejection. Also, as iPS-NSCs are more restricted to the neuronal lineage compared to iPSCs, we believe they will confer less of a risk for unwanted proliferation and tumor formation *in vivo*.

This study tested the efficacy of transplanting of iPS-NSCs as a therapy for HD after intrastriatal transplantation in YAC128 HD mice by (1) assessing their effects on motor function; (2) the survivability and differentiation capabilities 10 weeks after transplantation; and (3) the histological and protein analyses 10 weeks after transplantation.

MATERIALS AND METHODS

Cell Generation and Culture

iPSC Culture

iPSCs used in this study, were generated and characterized as described in a previously published protocol in our laboratory (Fink et al., 2014b). In brief, iPSCs were generated from fibroblasts that were isolated from tails of adult wild type mice. These fibroblasts were reprogrammed into iPSCs by using two adenoviruses (ADs): one contains *Oct4*, *Sox2*, and *Klf4* and another contains *c-Myc* which are all considered pluripotent factors. The recombinant ADs were developed in our laboratory and described previously in a published protocol (Fink et al., 2014b). The generated iPSCs were confirmed to express pluripotent markers using immunocytochemistry (ICC) and flow cytometry. The cells were then cryopreserved in freezing media containing 10% dimethyl sulfoxide (DMSO) [Medium is composed of 45% knock out serum and 45% of Dulbecco's Modified Eagles Media (DMEM), and 10% DMSO]. The generated iPSCs were thawed and plated on 0.1% gelatin coat, and cultured in iPSC media [DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% knock-out serum, β-mercaptoethanol (Life Technologies, Carlsbad, CA), 1% 1X non-essential amino acids (NEAA; Life Technologies, Carlsbad, CA), 20 ng/mL basic fibroblast growth factor (bFGF; Life Technologies, Carlsbad, CA), 2 μM L-glutamine (Sigma, St. Louis, MO), 5 mg/mL streptomycin and 5 UI/mL penicillin, and 10 ng/mL leukemia inhibitory factor (LIF; Life Technologies, Carlsbad, CA)]. Cells were passaged by dissociating them in Accutase (Sigma, St. Louis, MO), centrifuging at 250 g for 5 min at 4°C, and plating them on 0.1% gelatin coat.

iPS-NSCs Generation

The iPS-NSCs were generated by differentiation of iPSCs following the first stage in a published protocol with some modifications (Niclis et al., 2013). Briefly, iPSCs were expanded to 80% confluency, and then, the iPSC media were replaced by neuronal induction media [Neurobasal-A (Life Technologies, Carlsbad, CA) supplemented with 1X B27-A (Life Technologies, Carlsbad, CA), 1X N2 (Life Technologies, Carlsbad, CA), 1X NEAA (Life Technologies, Carlsbad, CA), 1X Glutamax (Life Technologies, Carlsbad, CA), and 5 mg/mL streptomycin and 5 UI/mL penicillin]. Half of the media was changed every 3 days, and the cells were kept in culture until they detached and formed neurospheres. The media containing detached cells were centrifuged at 100 g for 5 min at 4°C, and the pellet was dissociated in 1 mL Accutase (Sigma, St.louis, Mo) for 5 min at 37°C, then suspended in 5 mL phosphate buffered saline (PBS) and centrifuged another time. Cells were then re-plated in neural stem cell media [Neurobasal-A supplemented with 1X B27-A, 1X N2 (Life technologies, Carlsbad, CA), 1X NEAA, 1X Glutamax (Life Technologies, Carlsbad, CA), 20 ng/mL epidermal growth factor (EGF; Life Technologies, Carlsbad, CA) and 10 ng/mL bFGF (Life Technologies, Carlsbad, CA), and 5 mg/mL streptomycin and 5 UI/mL penicillin] (**Figure 1**).

Characterization of iPS-NSCs

The iPSC-derived neurospheres were passaged every week for 3 weeks and then characterized through ICC for neural lineage specific protein expression (Nestin, Sox2, β-tubulin-III and NeuN). The cells were grown on poly-L-lysine-coated, 25 mm glass coverslips for 2 days, after which cells were washed with PBS (0.01 M at pH 7.4) three times and fixed using 4% paraformaldehyde for 10 min at 4°C. Then a blocking solution (10% normal goat serum in PBS) was added to the coverslips and incubated for 1 h at room temperature. After that, the primary antibodies [Nestin (mouse monoclonal), Sox2 (rabbit polyclonal), and NeuN (rabbit monoclonal); 1:500; Abcam, Cambridge, U.K, β-Tubulin III (chicken polyclonal antibody); 1:300; Aves Labs Inc., Tigard, OR] diluted in PBS containing 0.1% Triton X-100 were added to the assigned wells and incubated at

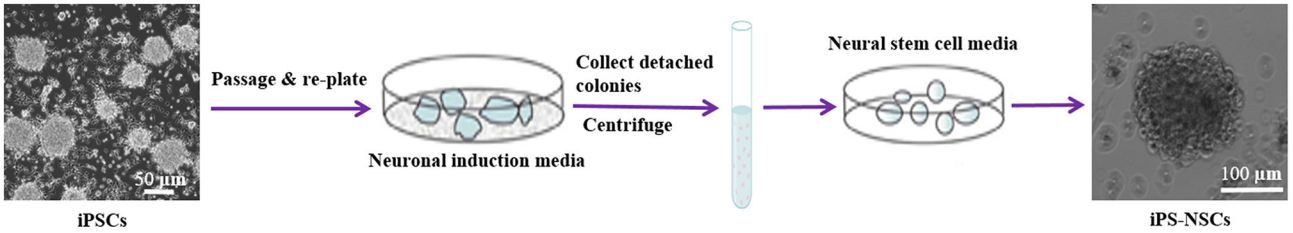

FIGURE 1 | Differentiation of iPSCs into iPS-NSCs. iPSCs were expanded to 80% confluency, and then, the iPSC media were replaced by neuronal induction media (Neurobasal-A, 1X B27-A, 1X N2, 1X NEAA, 1X Glutamax, and 5 mg/mL streptomycin and 5 UI/mL penicillin). The cells were kept in culture until they detached and formed neurospheres. The media containing detached cells were centrifuged, and the pellet was dissociated in Accutase. Cells were then re-plated in neural stem cell media (Neurobasal-A, 1X B27-A, 1X N2, 1X NEAA, 1X Glutamax, 20 ng/mL EGF, 10 ng/mL bFGF, and 5 mg/mL streptomycin and 5 UI/mL penicillin). Cells were passaged three times and then characterized using ICC.

4°C overnight. The primary antibodies were then aspirated and the coverslips were rinsed 3 times in PBS. Secondary antibodies with either AlexaFluor488 (1:500; goat anti-chicken IgG, or goat anti-rabbit IgG; Invitrogen, Carlsbad, CA), or AlexaFluor594 (1:500; goat anti-mouse IgG; Invitrogen, Carlsbad, CA) were then added and incubated at room temperature for 1 h. After that, the coverslips were rinsed 3 times in PBS. Hoechst-33342 (1:1,000; Sigma, St. Louis, MO) was added to each coverslip for 5 min at room temperature, and then, the coverslips were rinsed 3 times and mounted onto glass slides using Fluoromount reagent (Sigma, St louis, MO). Slides were visualized under fluorescent microscopy (Leica, Germany).

Animals

All procedures involving animals that were used in this study are approved by the Central Michigan University Institutional Animal Care and Use Committee. Twenty eight, 10-month-old male and female wild type and YAC 128 mice were randomly assigned to groups and housed in cages on a continuous 12-h day/night cycle (from 11:00 to 23:00 h). Mice had access to water and food, *ad libitum*, and they were kept at the same conditions of temperature and humidity.

Transplantation

Preparation of iPS-NSCs for Transplantation

On the day of surgery, iPS-NSCs were pre-labeled with 5 µg/mL of Hoechst 33342 (Sigma, St Louis, MO), and re-suspended at a density of 200,000 cells/µL in Hanks' Balanced Salt Solution (HBSS).

Surgeries

Surgeries were performed on all mice in the study at 10 months of age. Mice were randomly assigned into one of the following groups ($n = 7$): HD+HBSS, HD+iPS-NSCs, WT+HBSS, and WT+iPS-NSCs. The surgery was conducted under aseptic conditions. Mice were anesthetized using 2.0% isoflurane with 0.8 L/min oxygen maintenance throughout the procedure. The mice were continuously monitored throughout surgery, and adjustments of isoflurane and oxygen supply were made as needed. The back of the head of each mouse was shaved from the line between ears to the frontal part. After that, each anesthetized mouse was placed into the stereotaxic device (Kopf Instruments, Tujunga, CA), and the surgical site of the head was cleaned with chlorhexidine (Molnycke Healthcare, Norcross, GA). Then, a midline incision was made on the scalp, and skin was retracted. Two burr holes were made over the neostriatum (coordinates relative to bregma: anterior +0.5 mm; lateral ± 1.75 mm; with the tooth bar set at −3.3 mm). The iPS-NSCs or HBSS were loaded into a 10 µL Hamilton micro-syringes and every mouse received bilateral injections of cells and/or vehicle at a constant rate of 0.33 µL/min. Each hemisphere was injected with 200,000 cells at 2.5 mm ventral to the dura. After a 3-min rest period, the micro-syringe was moved 0.1 mm dorsally and another 200,000 cells were injected, followed by another 3-min rest period. The syringe was withdrawn slowly and re-positioned over the contralateral hemisphere and the procedure was repeated. Each hemisphere received total of 400,000 cells, while the vehicle control group received 2 µL HBSS. Incisions were closed by using 7-mm sterile wound clips, and analgesic ointment was applied to the incision site. Following surgeries, mice were monitored in recovery cages and transferred to their home-cages when they were fully recovered.

Postoperative care over a 5-day period included monitoring of vital signs, weight, movement, amount of food, water ingested as well as the status of the tissue at the incision site. Intra-peritoneal injections of physiological saline were given for mice showing signs of dehydration during the second post-surgical day. Clips were removed 10 days following the surgery.

Accelerating Rotarod Testing

The motor activity of the mice was assessed using the accelerating rotarod (San Diego Instruments; San Diego, CA). Mice were first trained at increasing speed starting from 5 rpm/s and accelerated at 0.5 rpm/s up to 40 rpm on five consecutive trials for 5 days before receiving the treatment. For testing, the mice were placed on the rod, which started rotating at 5 rpm/s and accelerated at 0.5 rpm/s until they fell. Mice were given 5 trials with a 45-s inter-trial interval. Baseline measurements were performed 1 day before the transplantation, and then, testing was done once each week, for 10 weeks after surgery. Motor function was measured by latency to fall (sec) from the accelerating rotarod.

Histology

Immunohistochemistry

Four mice from each group were anesthetized with sodium pentobarbital by intraperitoneal injection, and then, were transcardially perfused first with 0.01 M cold PBS (pH 7.4), followed by 4% paraformaldehyde for fixation of the brains and their brains were extracted and kept in 4% paraformaldehyde for 24 h at 4°C. The brains were then transferred to 30% sucrose in PBS for 48 h at 4°C and then flash-frozen in 2-methylbutane (Sigma, St. Louis, MO) on dry ice for 3 min and stored at −80°C until processing. The brains were sectioned coronally on a cryostat at 40 μm thickness and placed in 6 serial wells.

For immuno-histochemical (IHC) analysis, primary antibodies were used for double labeling of (1) mature neurons (mouse anti-NeuN clone A60, 1:500; Millipore, Billerica, MA) and (2) medium spiny neurons (rabbit monoclonal [EP720Y] to DARPP32, 1:500; Abcam, Cambridge, U.K). Equally spaced sections from each brain was used for labeling of astrocyte reactivity (Rabbit polyclonal to GFAP, 1:500; Abcam, Cambridge, U.K). Tissue sections were blocked using 10% normal goat serum in PBS for 1 h at room temperature, and then transferred to wells containing the primary antibodies in PBS with 0.1% Triton X-100, and incubated at 4°C overnight with continuous agitation. On the following day, the brain sections were rinsed three times in Tris-buffer saline with 0.1% Tween-20 (TBST) and transferred to wells containing the appropriately conjugated secondary antibodies [AlexaFluor488 (goat anti-mouse IgG), or AlexaFluor594 (goat anti-rabbit IgG); 1:1,000); Invitrogen] for 1 h at room temperature. Finally, the sections were rinsed in TBST and mounted onto positively charged glass slides, using Fluoromount media (Sigma, St. Louis, MO).

Imaging and Analysis

For NeuN and DARPP-32 slides from each mouse, both striatum from 4 randomly selected tissue sections were imaged under fluorescent microscopy, while maintaining a consistent exposure time and fluorescent intensity for each slide. Exposure settings were maintained at 360-, 460-, and 380-ms, respectively, for images obtained from Hoechst, Alexafluor-488, and Alexafluor-594 labeled sections. A 20-μm Z-stack of images was collected from seven individual depths, spaced 3 μm apart. Each striatum was imaged in its entirety by tiling each individual region under a 20-x objective. Following image acquisition, the complete Z-stack from each image was processed using ZEN 2.3 (Carl Zeiss AG; Oberkochen, Germany) through the extended depth of focus to flatten the acquired images, and the tiled image was stitched. Each color channel was exported as individual TIFFfiles and subsequently analyzed, and estimation of total neuronal profiles and area were calculated using MBF Stereo Investigator software (MBF Bioscience; Williston, VT). Counts of DARPP-32 and NeuN labeled cells were done within 200 × 200 counting frames spaced evenly throughout the striatum (grid size was 1,000 × 1,000 mm). Random confocal images were captured using confocal laser microscope (Zeiss; Thornwood, NY) to look at the co-localization of Hoechst labeled cells with DARPP-32 and NeuN. For GFAP, transplants site from each mouse was imaged using a fluorescence microscope (Leica, Germany).

Western Blot

Three brains from each group were used for Western blot (WB) analysis at 54-weeks of age. Mice were sacrificed by cervical dislocation and their brains were extracted and dissected. For every brain, striata were isolated, and lysed in cold radio-immuno-precipitation assay (RIPA) buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 0.1% SDS, 140 mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100, with protease inhibitors (Sigma, St. Louis, MO)]. The homogenate was centrifuged at 20 *g* at 4°C for 30 min. The supernatant was taken and aliquoted in PCR tubes and stored at −80°C until use. Protein concentrations for each sample were determined using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL). Samples were mixed with equal amount of 2X SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol and 0.2% bromophenol blue) and boiled for 2 min. For assessment, equal amount of protein of each sample was loaded and separated on gradient gel (4–20% SDS-PAGE). The SDS-PAGE was run at 100 V with running buffer (25 mM Tris-Base, 192 mM glycine, 0.1% SDS, and 1 mM EDTA). The proteins from gel were transferred overnight to the PVDF membrane (Millipore, Billerica, MA) in an ice cold buffer containing 25 mM Tris–Base, 192 mM glycine and 10% methanol. Following transfer, the blots were rinsed three times in TBST, and the membranes were blocked with 5% fat-free milk in TBST for 1 h. Then, the blots were incubated with primary antibody rabbit anti-BDNF (1:1,000; Sigma, St. Louis, MO) or rabbit anti-TrkB (1:1,000; Cell Signaling Technology, Danvers, MA), and rabbit anti- β-tubulin, (1:1,000; Abcam) in 5% fat-free milk powder dissolved in TBST for overnight at 4°C. Membranes were then rinsed three times with TBST, and incubated with the respective horse radish peroxidase (HRP) conjugated secondary antibodies (goat anti-rabbit IgG; diluted 1:10,000) in 1.5% fat-free milk powder in TBST for 1 h. The membranes were then washed three times with TBST. The blots were then developed with Immobilon^{TM} Western Chemiluminescent HRP-substrate (Millipore, Billerica, MA), and scanned. The optical density of each lane of the blot was measured using ImageJ software (NIH, Bethesda, MD).

Statistics

All statistical analyses were performed using SPSS v24. Accelerating rotarod data was analyzed using repeated measures analysis of variance (ANOVA). Fall latency, including the baseline and 10 weeks following transplantation, was used for statistical comparisons between wild type and YAC128 mice treated with iPS-NSCs, or received HBSS. One way ANOVA was performed to analyze differences of weekly fall latency amongst all groups. Histological and WB data were analyzed using one way ANOVA. Tukey's Honest Significant Difference (HSD) *post-hoc* test, was performed when the omnibus *F*-values were significant. The alpha level is set at $p \leq 0.05$ for all analyses.

RESULTS

iPS-NSC Characterization

To test the efficacy of iPS-NSC transplantations, we generated iPSCs from WT mice and differentiated them to NSCs. After differentiation of iPSCs to NSCs, ICC was performed to characterize the cells to confirm the expression of neural stem markers. Results of characterization confirmed that neurospheres showed positive expression of SOX2 and Nestin (M = 46.1%, SD = 18.7; M = 61.9%, SD = 14.3, respectively; **Figures 2A,C**). Similarly, cells from the neurospheres showed positive expression of the immature neuronal marker β-Tubulin-III (M = 11.8%, SD = 2.9; **Figures 2B,C**), confirming the immature status of neuronal cells derived from the iPS-NSCs.

Accelerating Rotarod

To determine if the transplantation of iPS-NSCs could improve motor function in YAC128 mice, we tested motor coordination using the accelerating rotarod. Repeated-measures ANOVA of accelerating rotarod (accelerod) data demonstrated a significant between-group effect in the performance of the mice over the course of the study [$F_{(3,24)}$ =8.461, p = 0.001]. Tukey *post-hoc* revealed a significant between-group difference in performance on the accelerod, with WT groups demonstrating longer latencies to fall than mice in the HD vehicle control group ($p = 0.001$), thus confirming the motor deficits of this animal model. No significant differences were found between WT group and iPS-NSCs-treated HD mice ($p = 0.077$), nor between HD vehicle-control and the iPS-NSCs-treated HD mice group (p = 0.336) (**Figure 3** and Tables S1, S2).

One-way ANOVA of weekly accelerod data showed a significant difference at baseline between WT and iPS-NSCs-treated HD mice ($p = 0.017$), but by week 4 after transplantation, the performance of iPS-NSCs-treated HD mice was not significantly different from that of the WT groups (p = 0.158). At 10 weeks following transplantation, the HD iPS-NSCs treated HD group was still performing similarly to WT mice ($p = 0.605$), however there was not a significant difference between their performance and that of the HD control animals (p = 0.393) suggesting an intermediate treatment effect at this time point. (Table S2).

Histological Results

Survivability and Differentiation of Transplanted iPS-NSCs

Hoechst-labeled cells were found in both WT and HD brains 10 weeks post-transplantation. However, fewer transplanted cells (Hoechst-labeled cells) in iPS-NSCs-treated WT mice were

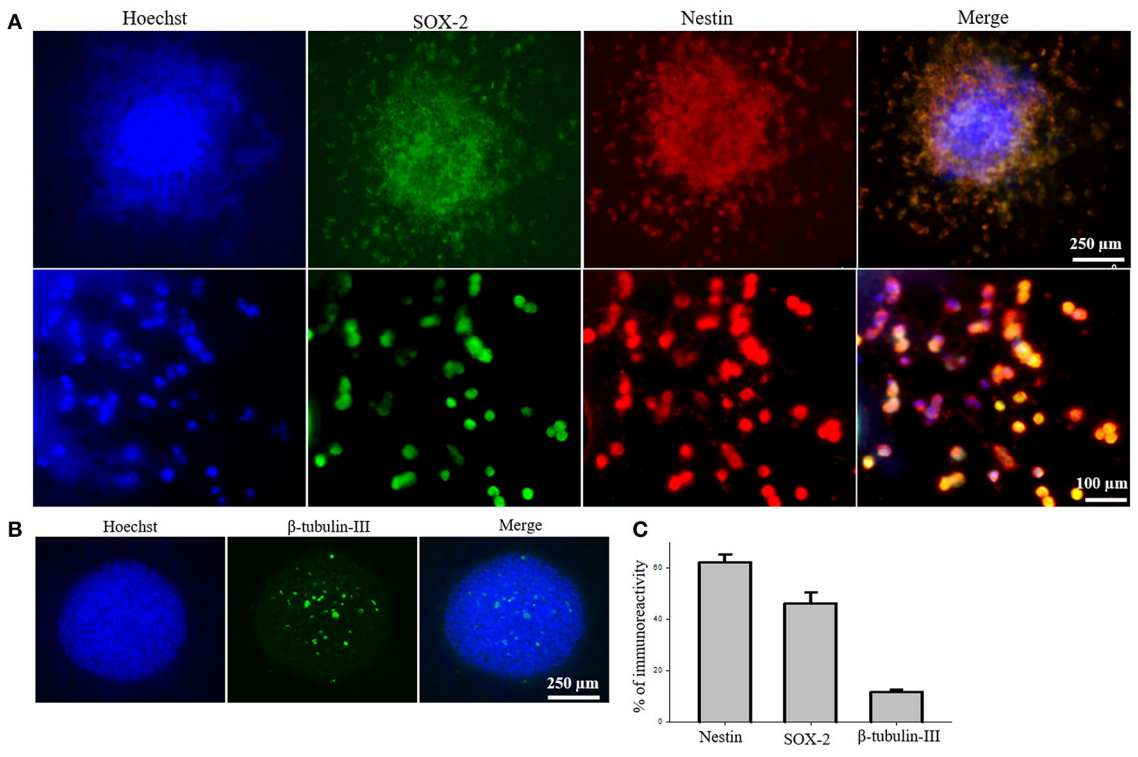

FIGURE 2 | Characterization of iPS-NSCs through ICC. **(A)** Hoechst-labeled iPS-NSCs (blue) showed positive expression of neural stem cells markers; SOX2 (green) and Nestin (red). Upper row shows a neurosphere, and lower row shows individual cells. **(B)** Few cells in the neurosphere showed positive expression of immature neuronal marker, β-tubulin-III (green). **(C)** Percentage of cells expressing Nestin (M = 61.9%, SD = 14.3), SOX2 (M = 46.1%, SD = 18.7), and β-tubulin-III (M = 11.8%, SD = 2.9).

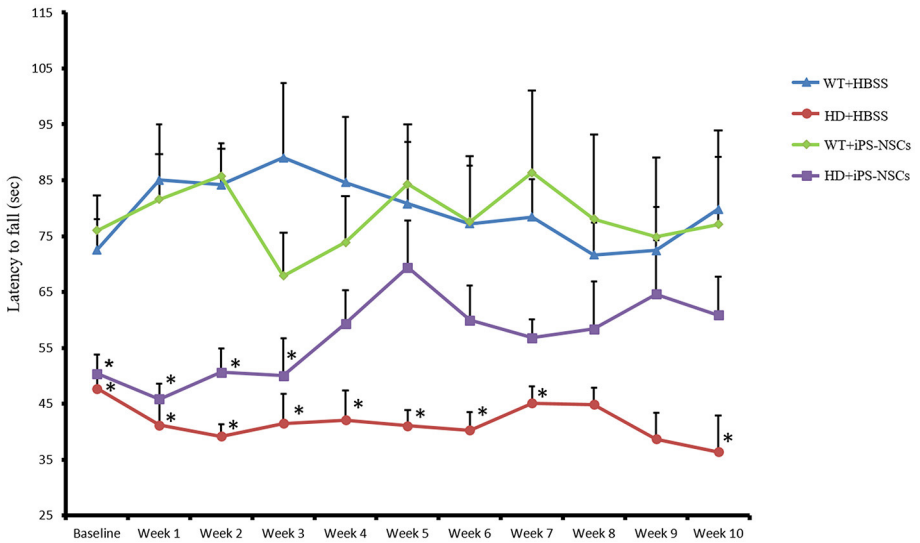

FIGURE 3 | Accelerating rotarod testing. Accelerod testing showed a significant decrease of the fall latency in HD vehicle control compared to WT mice ($p = 0.001$) but no significant decrease in the iPS-NSCs treated HD mice were found compared to WT mice ($p = 0.077$). At the same time, there was no overall significant difference between HD vehicle controls and iPS-NSCs treated HD mice ($p = 0.336$). Weekly testing on the accelerod revealed a significant difference between iPS-NSCs treated HD mice and WT mice at baseline ($p = 0.017$). However, iPS-NSCs treated HD mice showed no significant difference from WT mice starting from Week 4 after transplantation ($p = 0.159$) and up to 10 weeks after transplantation ($p = 0.605$). Also, there was no significant difference between iPS-NSCs treated HD mice and HD vehicle controls ($p = 0.393$) suggesting an intermediate treatment effect at this time point. *significant different from WT+HBSS, $p < 0.05$.

observed in the striatum compared to iPS-NSCs-treated HD mice (**Figure 4A**).

We observed that Hoechst-labeled cells co-localized with NeuN and DARPP-32 in both WT and HD mice. However, a higher proportion of Hoechst-labeled cells were co-labeled with DARPP-32 and NeuN in HD- ($M = 18.8\%$, $SD = 1.52$; $M = 46.3\%$, $SD = 3.56$, respectively) compared to WT-mice ($M = 9.00\%$, $SD = 6.63$; $M = 27.88\%$, $SD = 13.39$, respectively; **Figures 5A–C**).

Analysis of the stereologically acquired cell-count data revealed significant between-group differences in DARPP-32 and NeuN labeled cells in the striatum [$F_{(3, 12)} = 9.512$, $p = 0.002$, $F_{(3, 12)} = 8.573$, $p = 0.003$, respectively]. Specifically, Tukey *post-hoc* analysis showed that there was a significant difference between WT and HD vehicle control groups in DARPP-32 and NeuN stained cells ($p = 0.002$). However, there was no significant difference between WT mice and the iPS-NSCs-treated HD group ($p = 0.573$ for DARPP-32, $p = 0.793$ for NeuN). Furthermore, there was a significant difference between HD vehicle control and iPS-NSCs-treated HD mice ($p = 0.014$ for DARPP-32, $p = 0.011$ for NeuN; **Figures 4A,B,D**).

In addition, results of stereologically acquired density measures revealed significant between-group differences in the density of NeuN and DARPP-32 in striata [$F_{(3, 12)} = 5.031$, $p = 0.017$, $F_{(3, 12)} = 7.392$, $p = 0.001$]. Tukey *post-hoc* analysis revealed a significant reduction in NeuN- and DARPP-32 labeled cell densities in HD vehicle-control mice in comparison to the WT mice ($p = 0.012$, $p = 0.002$, respectively), but these differences were not seen between iPS-NSCs-treated HD and WT control groups ($p = 0.35$, $p = 0.29$, respectively), Moreover, the density of the DARPP-32 labeled cells was significantly elevated in iPS-NSCs-HD treated mice compared to HD vehicle controls ($p = 0.047$; **Figures 4A,C,E**).

Astrocyte Response at the Transplant Site

Reactive astrocytes were observed at the transplantation site. Higher astrocytic (GFAP; red) responses were observed in iPS-NSCs (Hoechst labeled cells; blue) transplanted brains in comparison to vehicle controls. Also, higher astrocytes response was observed in iPS-NSCs-transplanted WT mice in comparison to iPS-NSCs-transplanted HD mice (**Figure 6**).

Western Blotting of Striatal BDNF and TrkB

Analysis of WB data showed that there were significant between-group differences in BDNF and TrkB levels in striata [$F_{(3, 8)} = 4.250$, $p = 0.045$ & $F_{(3, 8)} = 4.739$, $p = 0.035$, respectively]. Tukey *post-hoc* analysis showed that there was a significant reduction in BDNF and TrkB in HD vehicle control mice compared to WT mice ($p = 0.041$, $p = 0.02$, respectively). However, there was no significant difference between iPS-NSCs-treated HD mice and WT group ($p = 0.59$, $p = 0.31$, respectively; **Figure 7**). Although there was no significant difference between iPS-NSCs treated HD mice and HD vehicle controls in levels of BDNF ($p = 0.24$), and TrkB ($p = 0.31$), levels were also not significantly different in iPS-NSCs-treated HD mice when compared to WT mice, suggesting an intermediate treatment effect.

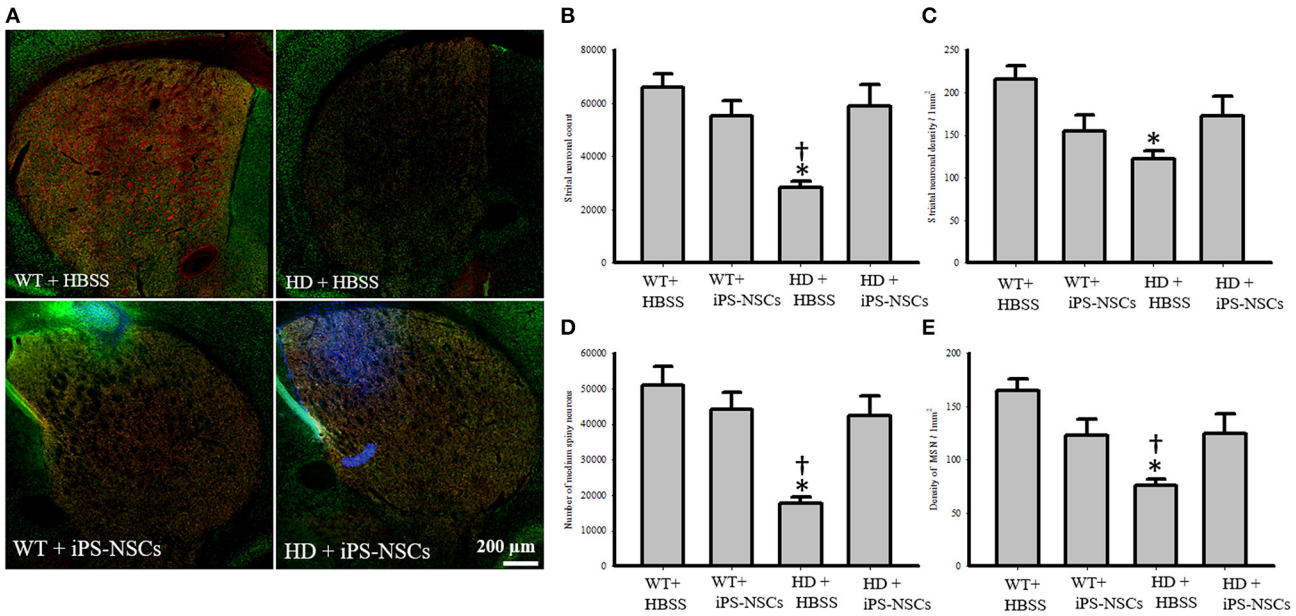

FIGURE 4 | Striatal neuronal and medium spiny neurons counts and densities. **(A)** Stitched images for striatum in each group showing markers of iPS-NSCs (Hoechst labeled cells; blue), mature neurons (NeuN; green) and medium spiny neurons (DARPP-32, red). **(B,D)** Analysis of NeuN and DARPP-32 labeled cells (mature neurons and medium spiny neurons, respectively) in striata showed that HD+HBSS mice are significantly different from WT+HBSS mice ($p = 0.002$), but HD+iPS-NSCs are not significantly different from WT+HBSS mice (NeuN, $p = 0.79$; DARPP-32, $p = 0.57$). Moreover, HD+iPS-NSCs mice are significantly different from HD+HBSS ($p = 0.01$; $p = 0.01$). **(C,E)** Analysis of NeuN and DARPP-32 labeled cells densities (mature neurons and medium spiny neurons, respectively) in striata showed that HD+HBSS mice are significantly different from WT+HBSS mice ($p = 0.01$, $p = 0.003$, respectively), but HD+iPS-NSCs are not significantly different from WT mice ($p > 0.05$). Also, DARPP-32 labeled cells densities were significantly increased in iPS-NSCs-HD treated mice compared to HD vehicle controls ($p = 0.047$). *Significant different from WT+HBSS, †significant different from HD+iPS-NSCs, $p < 0.05$.

DISCUSSION

The histological and motor deficits that are seen in the YAC128 mouse model recapitulates the HD changes in human HD patients, which makes this model ideal for assessing therapeutic interventions (Slow et al., 2003). The onset of motor symptoms in HD patients correlates with the striatal neuronal degeneration, and typically appear before the onset of neurodegeneration (Vonsattel et al., 1985). Similarly, in the YAC128 model, motor deficits on the accelerod start at 6 months of age before the onset of neuronal loss, and progress to hypokinesis by 12 months of age. Neuronal degeneration is seen in the striata as early as 9 months of age, with significant neuronal loss developing by 12 months of age (Van Raamsdonk et al., 2005). In this study, we transplanted iPS-NSCs into 10 months old mice and followed them for 10 weeks after transplantation. This age of YAC128 mice is equivalent to the middle age in human (Flurkey et al., 2007), which corresponds with the time of the evident motor deficits in HD patients, and pathological changes in HD brains (Walker, 2007). The defined progressive neuronal degeneration that is accompanied with a deterioration in accelerating rotarod performance in YAC128 mice is crucial in predicting the phenotype which helps in assessing therapeutic interventions.

The primary findings of the present study were that: (1) iPS-NSC transplants improved motor abilities in HD-treated mice; (2) iPS-NSCs survived for at least 10 weeks after transplantation in both WT and HD mice brains; (3) iPS-NSCs differentiated into mature neurons and region-specific neurons (medium spiny neurons); (4) increases in protein levels of BDNF and TrkB were found in iPS-NSCs-treated HD mice; and (5) differentiation patterns of the transplanted iPS-NSCs were dissimilar between HD and WT mice.

Consistent with previous studies in YAC128 mice, this study found significant motor deficits in the performance of HD mice on the accelerating rotarod, as evidenced by a decreased latency to fall relative to WT mice. Decreases in motor abilities, such as those necessary for performance on the accelerod, are related to striatal dysfunction that is usually preceded by neuronal loss (Van Raamsdonk et al., 2005). This study utilized iPS-NSCs as a treatment to ameliorate these motor deficits, and indicated that HD mice that received iPS-NSC transplantation showed behavioral sparing of motor performance on the accelerod. Additionally, significantly more neurons were found within the striatum of treated YAC128 mice compared to HD vehicle controls. Prior to our study, Jeon et al. (2014) transplanted iPSC-derived neuronal precursors generated from an HD patient into YAC128 mice, and found a significant increase in the latency to fall during the accelerating rotarod compared to HD vehicle controls. However, iPSCs generated from HD patients were found to show the HD phenotypes both *in vitro* (Hd iPSC Consortium, 2012) and *in vivo* (Jeon

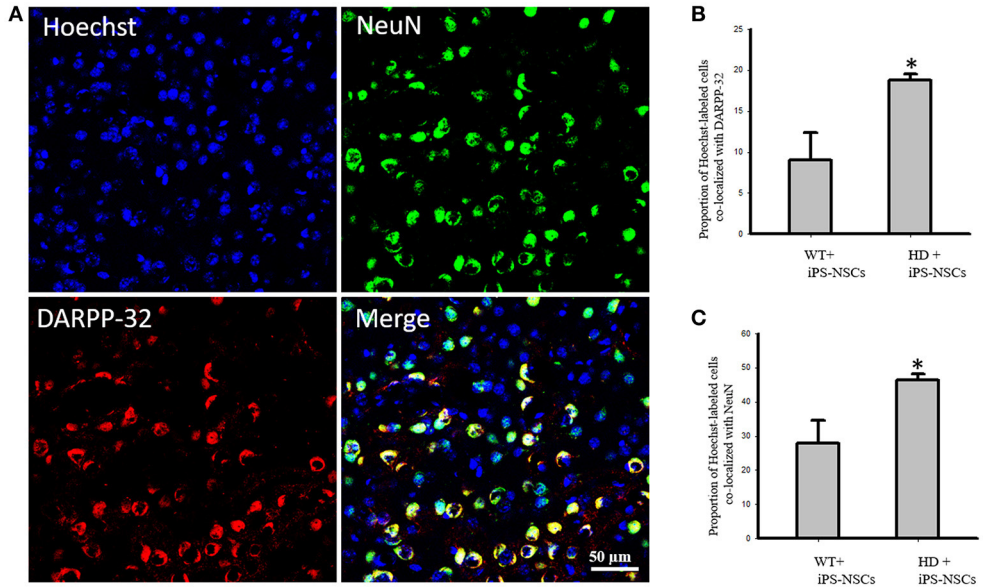

FIGURE 5 | (A) Transplanted iPS-NSCs survived and differentiated into mature neurons and medium spiny neurons in HD mice. Confocal images were captured from iPS-NSCs treated HD mouse. Hoechst labeled iPS-NSCs (blue) were found in striata 10 weeks post transplantation. Also, Hoechst labeled iPS-NSCs (blue) show co-expression of mature neurons marker (NeuN; green) and region specific neurons (DARPP-32; red). **(B)** Proportion of Hoechst labeled iPS-NSCs that are co-labeled with DARPP-32 is significantly higher in HD+iPS-NSCs mice compared to WT+iPS-NSCs ($p = 0.029$). **(C)** Proportion of Hoechst labeled iPS-NSCs that are co-labeled with NeuN is significantly higher in HD+iPS-NSCs mice compared to WT+iPS-NSCs ($p = 0.038$). *Significant different, $p < 0.05$.

et al., 2012). In this study, we transplanted NSCs derived from iPSCs that were generated from WT mice. Although the transplantations in our study were allogenic, the use of immunosuppressants could help integrate the transplanted cells in host tissue more efficiently, and lead to even better outcomes in motor performance.

In iPS-NSCs-treated HD mice, Hoechst-labeled cells were found co-localized with DARPP-32 and NeuN within the striatum, which suggests a regional specificity to the patterns of differentiation *in vivo*. The results from this study found a significant reduction of both total neurons, as well as medium-spiny neurons, in HD vehicle controls mice at 54 weeks of age. This is consistent with previous findings and confirms the progressive neuronal degeneration attributed to HD (Slow et al., 2003). However, in HD mice treated with iPS-NSCs, more neurons, including more medium-spiny neurons were found within the striatum, with a significant portion of these co-labeling with Hoechst. Although both the higher neuronal count, and the appropriate regional-specific pattern of differentiation could be detected following the transplantation of iPS-NSCs into HD mice, it is difficult to determine whether the transplanted cells were able to functionally integrate into the neuronal architecture without further investigations using methods in electrophysiology. Therapeutic benefits from cell transplantations can be derived from both cellular replacement, such as neuronal differentiation and integration, or by the release of trophic factors such as BDNF that can support the survival of neurons in compromised neurodegenerative conditions such as HD (Dey et al., 2010; Serrano Sánchez et al., 2014; Pollock et al., 2016). In this study, it may also be possible that the transplantation of these iPS-NSCs functioned to protect endogenous neurons from further degeneration by releasing neurotropic factors such as BDNF.

The role of BDNF in promoting neuronal survival and function has been demonstrated across several neuropathological conditions including HD (Strand et al., 2007; Zuccato and Cattaneo, 2007). In HD, BDNF and the BDNF receptors (TrkB) are substantially reduced as a consequence of epigenetic and transcriptional regulation (Zuccato et al., 2008), and BDNF-TrkB signaling is thought to be a major contributor to the progressive neuronal degeneration (Ginés et al., 2006). Medium-spiny neurons are a particularly sensitive population of neurons to BDNF dysfunction due to the necessity of maintaining its elaborate architecture (Zuccato and Cattaneo, 2007). This study also found a deficit in BDNF levels in HD mice, but following the transplantation of iPS-NSCs, a trend toward increased BDNF was found in striata of HD mice. It is possible that this increase in BDNF could be derived either from the transplanted cells, from preservation of endogenous neurons, or most likely a combination of transplant-derived BDNF that helped preserve endogenous neurons, leading to additional endogenous-derived BDNF. In either event, the increase of BDNF within the striatum of iPS-NSCs treated HD mice supports the utility of this treatment to improve the microenvironment of the HD brain. We also found a trend toward increased total TrkB levels in striata of iPS-NSCs transplanted HD mice compared to HD vehicle controls. There are two major TrkB isoforms; the full-length and the truncated isoforms (Fryer et al., 1996). The full length isoform contains an intracellular tyrosine kinase domain and it is activated by BDNF, while the truncated form lacks the

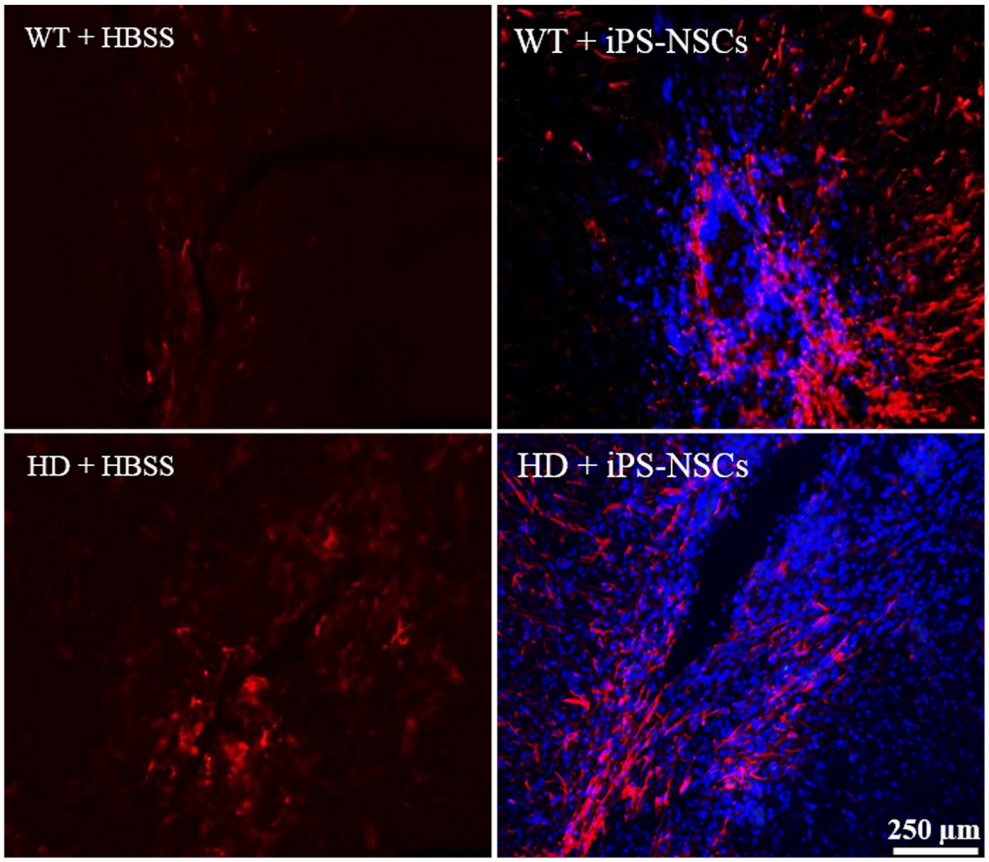

FIGURE 6 | Astrocyte response at the injection site following transplantation. Greater activation of astrocytes (GFAP; red) was observed in iPS-NSCs (Hoechst labeled cells; blue) transplanted brains in comparison to vehicle controls and in iPS-NSCs transplanted WT mice in comparison to iPS-NSCs transplanted HD mice.

intracellular kinase activity and contains a unique C-terminal (Eide et al., 1996). The function of truncated TrkB receptors is still not well described, but it was reported that they act as modulators of BDNF responsiveness; they sequester and translocate BDNF, and induce cascades of intracellular signaling. In addition, they play a role in enhancing neurite growth and modifying cytoskeletal structures (Carim-Todd et al., 2009; Fenner, 2012). The ultimate effectiveness of BDNF depends on the distribution and expression of TrkB receptors including the full-length and truncated isoforms (Fenner, 2012). The increase in total TrkB receptors in iPS-NSCs treated HD mice compared to HD vehicle controls in this study could be as a result from the increases in neurons, as well as BDNF levels that are found in straita of transplanted HD mice. This increase in total TrkB levels may have played a role in modulating BDNF-TrkB signaling and led to preservation of endogenous neurons.

With regards to the migration of iPS-NSCs post-transplantation, a dissimilar pattern of migration was observed between WT and HD mice. In HD-treated mice, Hoechst-labeled cells were found predominately within the striatum. However in WT-iPS-NSCs-treated mice, Hoechst-labeled cells were found predominately surrounding the needle track, with some labeled cells identified within the corpus callosum and cortex. This represents an interesting finding that suggests a differential pattern of migration of these cells post-transplantation in the HD condition. NSCs such as iPS-NSCs are known to express chemokine receptors such as CXCR4 (Stewart et al., 2017). The progressive neurodegeneration found in HD results in a substantially increased inflammatory response, which is accompanied by reactive microglia as well as increased levels of cytokines and chemokines (Träger et al., 2015). An increase in chemokines within the sriata of HD mice may function to facilitate the distribution of the transplanted iPS-NSCs within the striatum. WT mice on the other hand, lack this inflammatory response and could reasonably promote a less specific distribution of these cells throughout the brain, with fewer total cells enticed to leave the initial site. Similar to promoting migration of transplanted iPS-NSCs, an increase in chemokines and cytokines in HD mice may also encourage altered patterns of differentiation and survival, which could explain why a greater number of transplanted iPS-NSCs could be found in HD mice compared to WT mice in this study.

Although not explicitly quantified in this study, an increase in reactive astrogliosis was observed surrounding the needle track in iPS-NSCs treated WT compared to iPS-NSCs HD treated mice. The reason for this discrepancy is unknown,

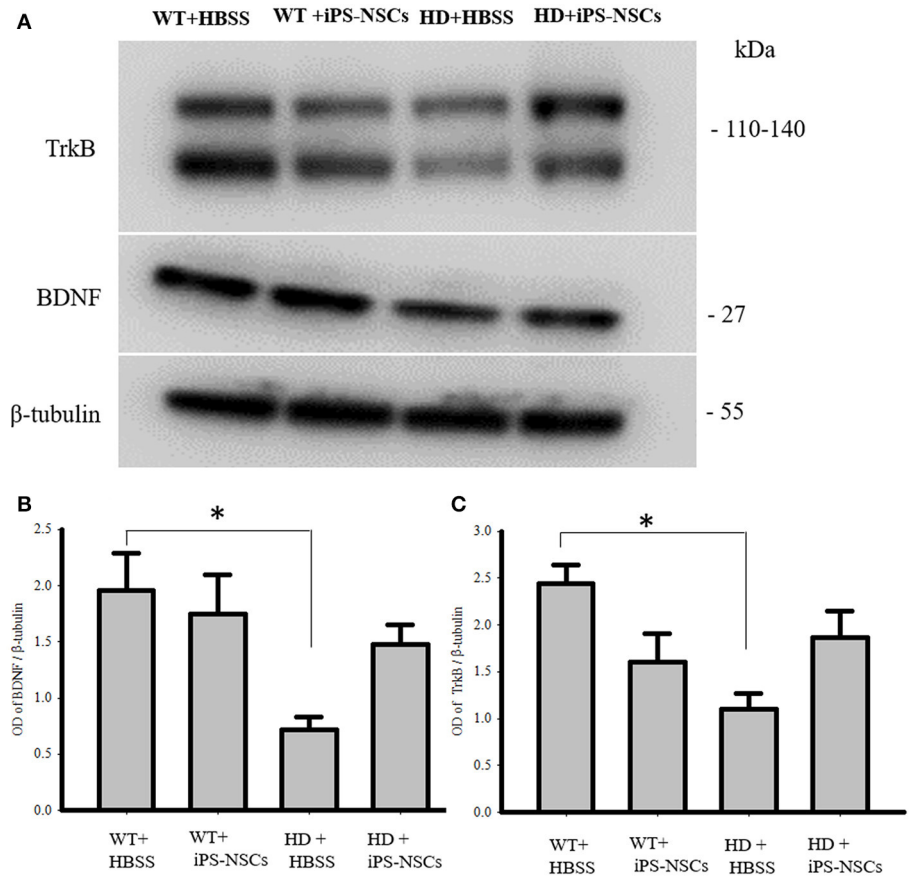

FIGURE 7 | Western blot analysis of BDNF and TrkB. **(A,B)** Western blot analysis showed a significant decrease of BDNF in striata of HD+HBSS compared to WT+HBSS mice ($p = 0.04$), but HD+iPS-NSCs were not significantly different from WT mice ($p = 0.59$), and HD+HBSS mice ($p = 0.25$) suggesting intermediate treatment effect. **(A,C)** Western blot analysis showed a significant decrease of total TrkB in striata of HD+HBSS compared to WT+HBSS mice ($p = 0.02$), but HD+iPS-NSCs were not significantly different from WT+HBSS mice ($p = 0.31$), and HD+HBSS ($p = 0.31$) suggesting intermediate treatment effect. *Significant different from WT+HBSS, $p < 0.05$.

however, several factors might account for this. First, it may be a function of an altered and dysfunctional capacity for glial scar formation in HD compared to WT mice. This could be due to co-morbid pathologies existing in HD, such as a weakened blood-brain barrier (Drouin-Ouellet et al., 2015). The increase in reactive gliosis may also be a function of transplanted iPS-NSCs differentiating more into astrocytes in WT mice compared to HD mice, with more integration surrounding the immediate transplant site. If HD mice possess a decreased ability for astrocyte function, then this may have important implications for the role of astrocyte dysfunction in supporting and protecting neurons in the HD condition (Maragakis and Rothstein, 2006). In either regard, further characterization of both the role of inflammation in transplant survival, differentiation, and migration, as well as the disturbance of astrocyte function in HD needs to be further studied.

Of important note, as iPSCs are generated through viral incorporation of oncogenes, there are concerns of insertion and subsequent mutagenesis, or of a permanent expression of the oncogenes, that could transform the transplanted cells into tumors (Rossignol et al., 2014). No tumors were found in this study, which is likely a result of pre-engaging the cells to a committed neuro-ectodermal lineage. The lack of tumors found in this study supports the approach of pre-differentiating of pluripotent cell lines prior to transplantation, however, the risk of having heterogeneous population containing some pluripotent stem cells within the transplanted cells is still possible (Miura et al., 2009). The need for optimizing the differentiation protocols, and sorting cells before transplantations to avoid undifferentiated cells is critical for the safety when translating iPS-NSCs for clinical applications. Also, many studies have shown that induced neural stem cells (iNSCs) can be generated directly by reprogramming somatic cells and bypassing the pluripotent stem cell stage (Lujan et al., 2012; Yu et al., 2015). This could also offer an alternative means of obtaining NSCs with less risk of tumor formation (Hemmer et al., 2014). However, since the protocols for direct differentiation of somatic human cells into NSCs use exogenous genes, tumor formation is still a concern when using induced cells for transplantations (Choi et al., 2017). Whether adopting the

iPS-NSCs or iNSCs approach, optimizing of protocols for reprogramming cells is needed to reduce the risk of tumor formation, and to provide a safe and effective treatment with clinical utility.

The current study provides an additional step in a long line of research supporting the clinical relevance of iPS-NSC transplantation in HD. We observed survival of transplanted iPS-NSCs up to 10-weeks following transplantation, with differentiation of transplanted cells into region specific neurons, and a behavioral sparing of motor performance. Collectively, these findings support the hypothesis that iPS-NSCs may prove to be a viable cell-replacement strategy with a high potential for therapeutic benefit. In order to fully assess the utility of transplantation of iPS-NSCs for cell replacement therapies in HD, future work will focus on long-term transplantation while analyzing behavioral function in HD mice.

AUTHOR CONTRIBUTIONS

AA-G: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing. RC: participated in the design of the study, collection and/or assembly of data. AS: collection and/or assembly of data, manuscript writing. BS, KS, LF, NK, DS, LP, and SA: collection and/ or assembly of data. AC and RW: generation of iPSCs. PM: design, administrative support, data analysis and interpretation, manuscript writing. GD and JR: conception and design, administrative support, data analysis and interpretation, manuscript writing, final approval of manuscript.

FUNDING

Support for this study was provided by the Field Neurosciences Institute, and the College of Humanities and Social & Behavioral Sciences, College of Medicine, and the John G. Kulhavi Professorship in Neuroscience at CMU.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2017.00628/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 28 November 2017
doi: 10.3389/fnins.2017.00647

The Evidence for the Spread and Seeding Capacities of the Mutant Huntingtin Protein in *in Vitro* Systems and Their Therapeutic Implications

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Neurodegenerative disorders are not only characterized by specific patterns of cell loss but the presence and accumulation of various pathological proteins—both of which correlate with disease evolution. There is now mounting evidence to suggest that these pathological proteins present with toxic, at times prion-like, properties and can therefore seed pathology in neighboring as well remotely connected healthy neurons as they spread across the brain. What is less clear, at this stage, is how much this actually contributes to, and drives, the core pathogenic events. In this review, we present a comprehensive, up-to-date summary of the reported *in vitro* studies that support the spreading and seeding capacities of pathological proteins, with an emphasis on mutant huntingtin protein in the context of Huntington's disease, although *in vivo* work remains to be performed to validate this theory in this particular disease. We have further reviewed these findings in light of their potential implications for the development of novel therapeutic approaches.

Keywords: Huntington's disease, Parkinson's disease, Alzheimer's disease, prions, tau, β-amyloid, α-synuclein, cell culture

OPEN ACCESS

Edited by:
Dustin R. Wakeman,
RxGen (United States), United States

Reviewed by:
Nolwen Lydiane Rey,
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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *19 September 2017*
Accepted: *07 November 2017*
Published: *28 November 2017*

Citation:
Masnata M and Cicchetti F (2017) The Evidence for the Spread and Seeding Capacities of the Mutant Huntingtin Protein in in Vitro Systems and Their Therapeutic Implications. Front. Neurosci. 11:647. doi: 10.3389/fnins.2017.00647

ARE "PRIONIC" PROTEINS PATHOGENIC AND FOUND IN ALL NEURODEGENERATIVE DISEASES?

The discovery of Lewy-body pathology within fetal ventral mesencephalic cells grafted in patients with Parkinson's disease (PD) years earlier has radically changed our views on the potential pathogenic mechanisms underlying sporadic neurodegenerative disorders of the central nervous system. This observation, initially reported by two independent teams (Kordower et al., 2008; Li et al., 2008), has led to the theory that the pathogenic α-synuclein protein can spread from the diseased brain to healthy tissue and cause protein aggregation and cellular dysfunction in a prion-like fashion (Olanow and Prusiner, 2009; Brundin et al., 2010; Soto, 2012). Indeed, it has been demonstrated both *in vitro* and *in vivo* that α-synuclein, the main component of Lewy bodies, can be released into the extracellular space and then be internalized by neighboring neurons (Desplats et al., 2009; Hansen et al., 2011), acting as a toxic agent that could seed pathology in the process. Furthermore, intracerebral inoculation of brain homogenates derived from aged α-synuclein transgenic mice, or injections of synthetic α-synuclein preformed fibrils, accelerates the formation of protein aggregates and precipitates neurological dysfunction in small animals (Luk et al., 2012a,b). It is also now known that there is pathology remote from the injection sites in

these types of studies, which further supports an intercellular transneuronal spread of protein, as has also been demonstrated in rodent allografts placed in animals expressing human α-synuclein (Angot et al., 2012). In the latter study, human α-synuclein was shown to co-localize with markers of endosomes and exosomes (Angot et al., 2012), which could represent one of the routes by which the protein is transferred (Goedert et al., 2010; Angot et al., 2012). This mode of protein spread and disease propagation has been shown experimentally with several other proteins including amyloid, tau, SOD1, TDP-43 and FUS (Soto, 2012; Jucker and Walker, 2013; Guo and Lee, 2014). However, these studies have proposed a number of other putative mechanisms for protein spread which include nanotubes (Costanzo et al., 2013; Abounit et al., 2016a,b), vesicular transport (Angot et al., 2012; Lee et al., 2012), endocytosis (Hansen et al., 2011; Wu et al., 2013; Ruiz-Arlandis et al., 2016) or even direct penetration of the plasma membrane (Ren et al., 2009).

What is emerging from all this work is that a number of these processes may be common to all neurodegenerative disorders, not just sporadic but also monogenic diseases such as Huntington's disease (HD) (Brundin et al., 2010; Soto, 2012; Cicchetti et al., 2014). In HD, recent evidence has added weight to the idea that the mutant huntingtin protein (mHTT)—the genetic product that defines the disease—can propagate from cell-to-cell. *In vivo* observations for the ability of mHTT to travel across synapses has been collected in embryonic human stem cells differentiated into neurons and implanted in R6/2 mice, a transgenic mouse model of HD (Pecho-Vrieseling et al., 2014). It has also been demonstrated by expressing the human HTT gene (138 CAG) within olfactory receptor neurons which was subsequently found in the synaptically connected large posterior neurons in the brain of drosophila (Babcock and Ganetzky, 2015). Various cell types (HEK), including neuron-like cells (Cos-7 and PC-12), have been shown to be capable of internalizing synthetic mHTT aggregates from the extracellular milieu (Yang et al., 2002; Ren et al., 2009). The aggregates can be either translocated to the nucleus where their pathogenic effects on transcription can be exerted leading to cell dysfunction and death (Yang et al., 2002) or act as seeds for further protein aggregation within the cell itself (Herrera et al., 2011). Prion-like spread has also been suggested to take place following the phagocytosis of mHTT aggregates by glial cells in a *Drosophila* model of HD. The engulfed aggregates gained access to the cytoplasm of microglia where they interacted with soluble huntigtin (HTT), initiating a prion-like dissemination of pathology (Pearce et al., 2015). Although there are no other reports on the role of immune cells in mHTT propagation (Weiss et al., 2012), this is a likely scenario that may, at least partly, contribute to HD pathology and that certainly cannot be dismissed at this stage.

The notion that neurodegenerative disorders, including HD, share several features with classical prion diseases is gaining momentum—although this concept remains more debated for this disease and more *in vivo* work is clearly needed to substantiate the initial *in vitro* claims. Notwithstanding, at least two fundamental questions remain to be answered: What are the routes by which the pathological proteins, such as mHTT, propagate and therefore spread and seed their pathogenic effects? And can any of these routes be blocked to prevent disease dissemination? The aim of this manuscript is to review the *in vitro* evidence for the spreading and seeding capacities of mHTT and what implications this has for the development of novel therapeutic approaches.

IS mHTT EQUALLY TOXIC TO STRIATAL AND CORTICAL NEURONS?

One of the most striking pathological features of HD is the massive loss of striatal projection neurons. However, the degeneration is not confined to this structure and cortical areas as well as a number of other sites all show cell loss early on in the disease process. However, cells in both of the cortical and striatal areas are tightly connected synaptically. They both express mHTT but their vulnerability to early degeneration in HD remains elusive, as does the chronology in which they degenerate with respect to one another. *In vitro* models in which mHTT expression is induced by high capacity adenoviral (Dong et al., 2012) or lentiviral vectors (Zala et al., 2005), have revealed that cortical neurons accumulate a significant number of inclusion bodies, but with no clear toxic consequences. In contrast, striatal neurons develop major morphological changes that are accompanied by the loss of neurofilaments and ultimately cell death, despite the fact that mHTT aggregates are rarely seen within these cells (Zala et al., 2005; Dong et al., 2012). The reasons for this differential vulnerability have been further investigated in the BACHD mouse model by genetically inhibiting mHTT production in the striatum and cortex simultaneously, or in either structure alone. Inhibition of mHTT synthesis in both the striatum and cortex combined had the most significant beneficial impact on motor and psychiatric HD-related behavioral phenotypes (Wang N. et al., 2014) with an abrogation of striatal degeneration. This may imply that the content of mHTT within cortical cells changes the cell properties; i.e. by producing more glutamate which creates excitotoxicity within their target structures such as the striatum. However, it may also indicate that preventing the propagation of mHTT via the cortico-striatal circuit can have a considerable impact on the disease.

TRANSNEURONAL/TRANSYNAPTIC PROPAGATION: HOW NEURONAL CIRCUITS ENABLE mHTT SPREAD

The validity of the transynaptic propagation theory (**Figure 1**) has gained additional support with the work of Pecho-Vrieseling et al. (2014). In this study, three distinct models were used to investigate this concept. In one set of experiments, the authors generated mixed-genotype (R6/2-wild-type) cortico-striatal cultures, more specifically combining R6/2 striatal neurons with wild-type cortical neurons or wild-type striatal neurons with R6/2 cortical neurons. Functional R6/2 cortical-wild-type striatal networks were created and used to study long-distance mHTT propagation from the cortex to wild-type DARPP-32+ medium spiny neurons. However, this was not seen

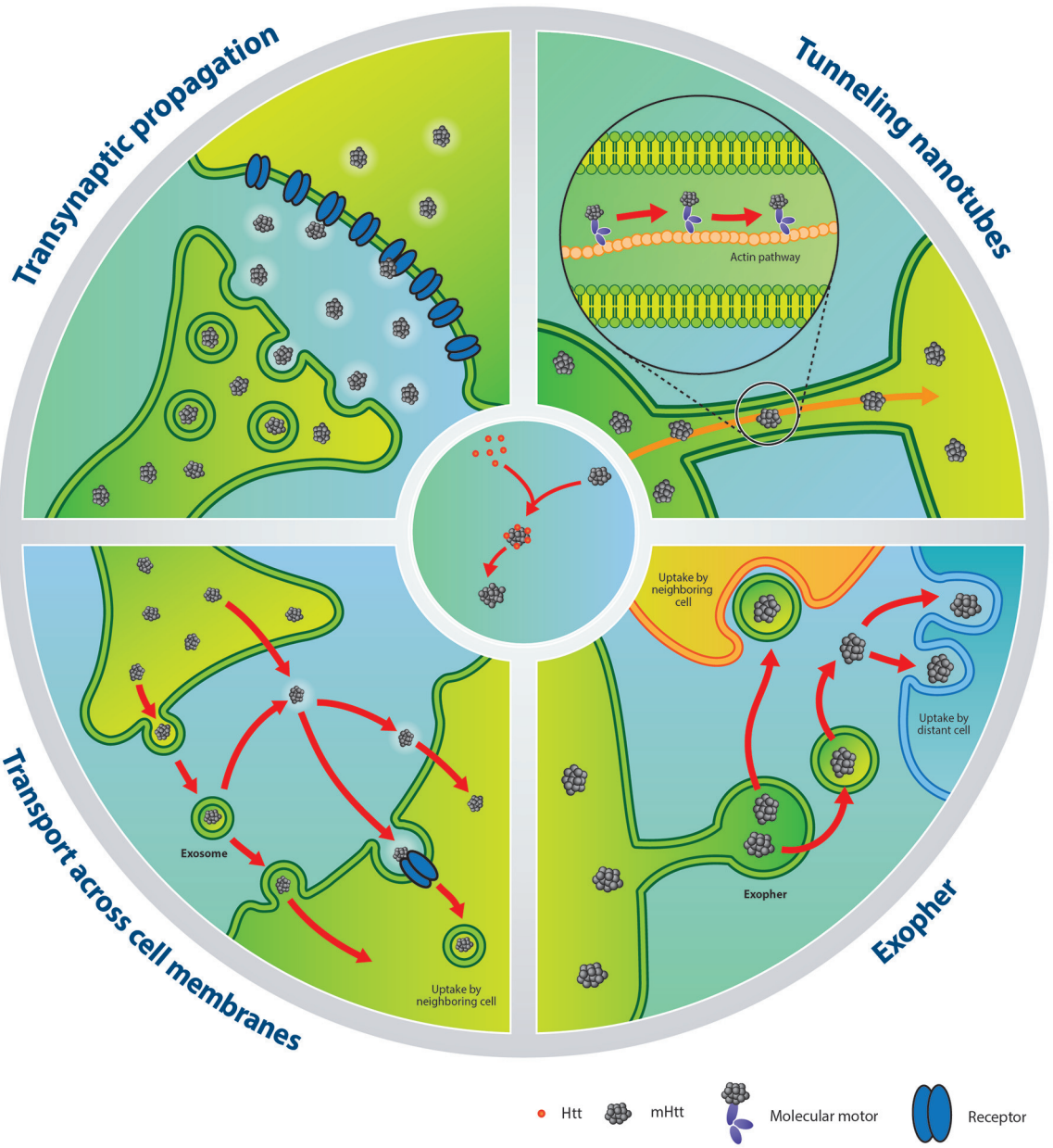

FIGURE 1 | Putative mechanisms of mHTT spreading and seeding capacities**. Left upper panel**: illustration of transynaptic propagation of mHTT. **Right upper panel**: transport mechanism of mHTT via tunneling nanotubes. **Left lower panel**: mHTT can be released within exosomes or in a free form. After extrusion, exosomes carrying mHTT can fuse with the plasma membrane of a neighboring cell. Alternatively, mHTT can escape from the exosomal-vesicle into the extracellular compartment, with the same fate as the counterpart released directly as a free form. Finally, mHTT can be internalized by a recipient cell via receptor-mediated endocytosis or directly penetrate the plasma membrane. **Right lower panel**: In neurons of C. elegans, mHTT has been shown to be contained within exophers, an entity which resembles mammalian exosomes. Released exophers may be incorporated by adjacent or distant cells or secrete their contents into the milieu. **Central panel**: schematic of the seeding process of mHTT. The misfolded protein recruits HTT in an elongation process creating toxic aggregates. HTT, huntingtin; mHTT, mutant huntingtin.

in the R6/2 striatum-wild-type cortical circuit as no significant amounts of mHTT deposits were detected in the wild-type cortex.

In a second set of experiments, the authors pursued the transynaptic propagation theory using embryonic human stem cells differentiated into neurons and tagged with GFP (hGFP) which they then transplanted into organotypic brain slices derived from R6/2 mice. Cell inoculations were performed within the cortex and striatum and the identity of the transplanted cells was confirmed by immunostainings of mature and structure-specific cellular markers such as Tbr1 (cortex) and DARPP-32 (striatum). Two major observations were made relating to the propagation of mHTT: from endogenous neurons of the R6/2

mice or to the freshly transplanted human cells. Firstly, two waves of mHTT accumulation were seen between the mouse neurons: one wave occurred at 3–4 weeks and a second took place between 6 to 8 weeks following the initiation of cultures. In the human transplanted cells, mHTT accumulation progressively increased within the striatum between 4 and 8 weeks at which point it plateaued, while in the cortex, the pattern of propagation was identical to that seen in mouse neurons. The impact of mHTT propagation was seen primarily on neurites, which became atrophied. This was accompanied by a concomitant loss of DARPP-32+ neurons, one of the main features of HD pathology. Finally, the location of mHTT aggregates was first identified in the cytoplasm and subsequently in the cell nucleus. It should be noted that the authors repeated the experiment using human pluripotent stem cells differentiated into neurons and also showed the transynaptic spread of mHTT from R6/2 host tissue to human grafts, indicating that this was common to different cell types.

A final set of experiments was performed *in vivo* where wild-type mice were injected with Q72-HTT-Exon1 and synaptophysin-GFP viral vectors into cerebral cortical layers. In this case, mHTT aggregates were detected predominantly in medium spiny neurons expressing GFP which were innervated by cortical projection neurons previously transduced with the viruses, suggesting an active role for cortical projections in mHTT propagation to striatal neurons. Post-mortem analyses of grafted hGFP-neurons in cortical areas of 4-week-old R6/2 mice further confirmed these observations.

The seminal work of Pecho-Vrieseling et al. (2014) confirmed the hypothesis brought forward by Cicchetti et al. (2014) that mHTT could propagate transynaptically between disease and healthy tissue. But exactly how mHTT is transported whithin neurons remains unanswered. To understand this, microfluidic culture devices have been employed in which neuronal somata could be isolated from their processes and other cell types. With this system, it was shown that synthetic α-synuclein fibrils can be transported both anterogradely and retrogradely (Volpicelli-Daley et al., 2011; Freundt et al., 2012; Brahic et al., 2016), as well as to be released and taken up by second order neurons (Freundt et al., 2012). Similar observations have been made with Aβ42 (Freundt et al., 2012; Brahic et al., 2016), tau (Wu et al., 2013; Brahic et al., 2016) and HTTExon1 fibrils (Brahic et al., 2016). However, HTTExon1 fibrils showed limited anterograde transport, although retrograde transport efficiency was similar to that seen with α-synuclein fibrils (Brahic et al., 2016; **Table 1**).

It has now been demonstrated, *in vitro*, *in vivo* and in post-mortem human samples, that mHTT is able to disrupt vesicular and mitochondrial trafficking by recruiting and sequestering key elements of the axonal trafficking machinery, such as normal HTT, within aggregates (Trushina et al., 2004). This could explain the differences observed in the axonal transport between mHTT and other types of protein fibrils, in particular the fact that, after retrograde transport, HTTExon1 fibrils accumulate intracellularly, while more than half of the α-synuclein and Aβ42 fibrils are released into the media (Brahic et al., 2016). It is clear that the properties of mHTT do not simply allow for it to be transferred between cells but that it is also capable of triggering aggregate formation using endogenous proteins within the recipient cell - and by so doing display prionic characteristics.

TUNNELING NANOTUBES: A CHANNEL FOR DISEASE DISSEMINATION

Tunneling nanotubes (TNTs) are small entities that serve as a communication bridge between cells (Abounit and Zurzolo, 2012). Using adhesion proteins and fusion molecules (SNARE or viral fusion proteins), TNTs can change their configuration to merge to other cellular surfaces (Marzo et al., 2012). Neurons and various other cell types have the ability to produce these temporary and retractable protrusions which are made of F-actin strains and lipid bilayers containing organelles, plasma membrane components and ions such Ca^{2+} which are key to cell signaling (Smith et al., 2011). In normal physiological conditions, they have been noted to participate in cell development (Gurke et al., 2008), contribute to immune responses (Watkins and Salter, 2005), engage in regeneration processes (Wang et al., 2011) as well as facilitating electrical conduction between cells (Smith et al., 2011).

Within the F-actin path forming the TNTs, molecular motors can be hijacked to transport pathogens and prion-like proteins (Gousset et al., 2009; Abounit and Zurzolo, 2012; **Figure 1**). This has been demonstrated in mouse catecholaminergic neuronal cells for α-synuclein (Abounit et al., 2016a) and in both mouse catecholaminergic neuronal cells and mouse cerebellar granule neurons for mHTT (Costanzo et al., 2013). Furthermore, HTT and mHTT have been shown to form strong interactions with phospholipid bilayers suggesting that they can drift on F-actin streams and lipid surfaces (Marzo et al., 2012). More specifically, α-synuclein fibrils taken up from the media are almost exclusively found embedded in endolysosomal vesicles (Abounit et al., 2016a) within TNTs. In contrast, mHTT fibrils are identified in a free form state within the cell cytoplasm (Ren et al., 2009; **Table 1**) and their colocalization with vimentin hints to potential transport within aggresome-like structures (Costanzo et al., 2013). Although TNT formation can offer a defense mechanism for expelling material that the cell cannot digest/degrade—for example fibrillar amyloids—this system is not sufficient to completely restore the cell's health. Additionally, cells that contain pathological proteins produce more TNTs (Costanzo et al., 2013; Abounit et al., 2016a,b), creating opportunities for pathological protein transfer and potentially facilitating the seeding process in naïve cells.

EXOSOMES AND EXOPHERS AS mHTT CARGO CARRIERS

It has now been clearly shown that misfolded proteins can be found within extracellular vesicles and that they can be carried and delivered to a recipient cell using this means (**Figure 1**). The demonstration that this applies to mHTT as well has also been shown using exosomes extracted from fibroblasts derived from a severe juvenile HD case harboring 143 CAG repeats (HD143F) and which were exposed to differentiated neuronal

TABLE 1 | *In vitro* evidence of mHTT spreading capacities.

Mechanism	Protein form	Cell model	Observations	References
Transynaptic propagation	Endogenous mHTT from R6/2 mice	*Ex vivo* mixed cortico-striatal cultures from R6/2 or wild type mice	Propagation of mHTT from R6/2 cortical to wild-type striatal neurons Significant vulnerability of striatal neurons in comparison to cortical neurons	Pecho-Vrieseling et al., 2014
	Endogenous mHTT from R6/2 mice	Human ESCs and human iPSC differentiated into neurons transplanted into organotypic brain slices of R6/2 mice	Propagation of endogenous mHTT from murine host tissue to grafted hGFP neurons followed by progressive neurodegeneration of recipient hGFP neurons	
TNTs	Transfection with GFP-480-68Q (donor); mCherry (acceptor)	Co-culture of CAD transfected cells (68Q or mCherry) Co-culture of transfected primary CGNs (68Q or mCherry)	Transfer of GFP-480-68Q to both CAD and CGN neuronal cells via TNTs	Costanzo et al., 2013
Vesicular transport				
Exosome	HD143F-derived exosomes	Co-culture of HD143F and NSCs	Spread of mHTT from HD143F to NSCs	Jeon et al., 2016
		NSCs exposed to HD143F-derived exosomes	Spread of exosomes-containing mHTT in NSCs	
Exopher	Genetically-engineered expression of Q128	*C. elegans*	Q128 gene expression increases the production of exophers Exopher content, including organelles, protein and mHTT, is found in remote cells of the *C. elegans*	Melentijevic et al., 2017
Endocytosis	Fibrillar Alexa488-HTTExon1Q44 and/or polyQ44	Undifferentiated and differentiated mouse and human neuroblastoma cells (N2A and SH-SY5Y)	Internalization and intracellular localization of HTTExon1Q44 and PQ44 fibrils in both types of neuroblastoma cells Fibrillar HTTExon1Q44 uptake via clathrin-dependent endocytosis No mechanisms evaluated for PQ44 fibrils	Ruiz-Arlandis et al., 2016
Direct penetration of plasma membrane	Synthetic K2Q44K2 fibrils	HEK; HeLa; Cos-7; CHO; N2A	Breach plasma membranes by K2Q44K2 fibrils in all cell types tested	Ren et al., 2009
	Transfection with ChFP-HTTQ25 and synthetic K2Q44K2 fibrils	HEK	Recruitment of soluble HTT forms into IBs by synthetic K2Q44K2 fibrils in transfected HEK cells	
	Chemically synthesized Q42, NLS-Q42 and NLS-Q20 fibrils	Cos-7; PC-12	In the nuclei, smaller aggregates are more toxic than larger ones in both cell types tested	Yang et al., 2002
Unknown	Transfection with 25/103QHTT-V1 and 25/103QHTT-V2	Co-culture of H4 cells expressing 103QHTT-V1 and HEK cells expressing 103QHTT-V2	Polymerization and cell-to-cell transmission of HTT oligomers	Herrera et al., 2011
	Exposure to conditioned medium derived from GFP-mHTT-Q19 or GFP-mHTT-Q103 transfected HEK cells	SH-SY5Y cells	Presence of exogenous mHTT protein (Q19 and Q103) within recipient SH-SY5Y cells	Jeon et al., 2016

CAD, mouse catecholaminergic neuronal cell line; CGNs, cerebellar granule neurons; CHO, epithelial-like cell line from Chinese hamster ovary; Cos-7, fibroblast like cell lines derived from monkey kidney; ESCs, embryotic stem cells; H4, human brain neuroglioma cells; GFP, green fluorescent protein; HD, Huntington's disease; HD143F, human fibroblast derived from Huntington's disease patient carrying 143 polyglutamine repeats; HEK, human embryonic kidney cells; HeLa, human uterine cervical carcinoma cells; hGFP neurons, human GFP positive neurons; HTT, huntingtin; IBs, inclusion bodies; iPSC, induced pluripotent stem cells; mHTT, mutant huntingtin; NSCs, neural stem cells; N2A, mouse neuroblastoma cell line; NLS, nuclear localization signals; PC-12, cell line from pheomochromocytoma of the rat adrenal medulla; PolyQ, polyglutamine; SH-SY5Y, human neuroblastoma cell line; TNTs, Tunneling nanotubes; V1, Venus protein half; V2, Venus protein half 2.

stem cells derived from embryonic cortical mouse tissue. After 4 days of contact between the exosomes and the cultured cells, the internalization of mHTT was detectable. Similar results were achieved when HD143F-derived exosomes were incubated with SH-SY5Y cells, confirming the spreading capacity of mHTT via exosomes in various cell types (Jeon et al., 2016; **Table 1**). Most strikingly, HD143F exosomes injected intraventricullarly into new-born wild-type animals led to the development of motor and cognitive HD-related behavioral phenotypes (Jeon et al., 2016). This study provided evidence that exosomes carrying mHTT could participate in disease induction/manifestation both *in vitro* and *in vivo*.

More recently, the *C. elegans* model has served to identify a novel vesicular entity capable of incorporating and extruding

dysfunctional organelles as well as protein aggregates. This transporter was named an "exopher" (Melentijevic et al., 2017). Despite their larger size and the fact that they are born from a different genesis process, exophers resemble, in many ways, exosomes found in mammalian cells. They are released in a multiple-step process which includes protrusion, elongation and separation (**Figure 1**), emerging from the soma of different neurons, independently of cell division or cell death (Melentijevic et al., 2017). Their production is triggered by cellular stress, disruption of physiological conditions or, importantly, by the presence of inclusion bodies such as mHTT aggregates (Melentijevic et al., 2017). For example, the expression of aggregable HTT-Q128 in the genome of the nematode causes impairments of the normal functions of chaperones, autophagy pathways, ubiquitin-proteasome systems and provokes the collapse of the integrity maintenance machinery in cellular elements. The accumulated stress increases the production of exophers in touch-responsive ALMR neurons expressing Q128 in this animal which in fact serves as defense mechanisms to restore the cell's equilibrium (Melentijevic et al., 2017). However, the downside of exopher production is that they may, like exosomes, enable cell-to-cell delivery of insoluble pathogenic proteins (Melentijevic et al., 2017; **Table 1**).

ACCESSING THE MEMBRANE OF NEIGHBORING CELLS BY ENDOCYTOSIS

Endocytosis is a cellular mechanism that controls various cellular functions such as internalization and recycling of plasma membrane components/ligands as well as the uptake and degradation of macromolecules and extracellular particles. The process begins with modifications of the plasma membrane, the generation of endocytic vesicles, which then matures into *early* and *late* endosomes, and finally leads to the degradation of the vesicle content through fusion with lysosomes (Miaczynska and Stenmark, 2008).

There is now compelling evidence that proteins, such as α-synuclein, can be incorporated into cells via clathrin-dependent endocytosis (Oh et al., 2016; **Figure 1**). Using undifferentiated and differentiated N2A cells exposed to HTTExon1Q44 and treated with various pharmacological compounds, clathrin-dependent endocytosis was identified to participate in mHTT uptake. In this particular study, HTTExon1Q44 fibrils were found in early endosomes of undifferentiated cells. This was observed as early as 6 h post-exposure to the toxic material or in both early endosomes and lysosomes 24 h post-exposure. At 48 h, concentrations of fibrils were unchanged within the lysosomes, but were found at lower concentrations in the early endosomes (Ruiz-Arlandis et al., 2016; **Table 1**). It was speculated that mHTT concentrations inside endosomes was lower because mHTT had been delivered to the lysosomes. In contrast, the stable concentrations of mHTT inside the lysosomes may have resulted from an equilibrium between the quantities of ingested vs. degraded fibrils. It is also feasible, according to the authors, that this observation reflects the saturation of the lysosome degradation machinery. It has been noted that the fate of the same fibrils differed in differentiated N2A, where co-localization within early endosomes could not be firmly established. Rather, mHTT fibrils were present within lysosomes, perhaps testifying to the cell's attempt to eliminate toxic mHTT through lysosomal degradation.

The idea that the turnover of HTT and mHTT aggregates is dependent on the endosomal–lysosomal system finds supports in another study in which it has been reported that following transfection, free HTT accumulated in the cytoplasm and inside autophagosome-like vacuoles, while mHTT localized both to the cytoplasm and nucleus. The presence of mHTT in vacuoles modified the morphology of the cells, which appeared atrophied, further implying that endosomal-lysosomal-vacuolar pathway activation may be responsible for this type of cell death (Kegel et al., 2000). HTT and mHTT have also be shown to co-localize with late endosome and lysosome markers and this has led to speculation that this was due to the lysosome's capacity to mediate the secretion of proteins fusing with the plasma membrane through an active calcium dependent process (**Figure 1**). Endosome ablation, calcium chelator or silencing synaptotagmin 7 (lysosome-specific calcium sensor) inhibited mHTT secretion, providing evidence for the involvement of late endosomal/lysosome pathway in secreting mHTT and, in smaller amounts, wild-type HTT (Trajkovic et al., 2017).

mHTT TRANSMISSION BY DIRECT PENETRATION OF PLASMA MEMBRANES

Release and uptake are the basic elements of a prion-like cell-to-cell protein propagation process. In order to dissect the mechanisms of transit between cells, artificially manufactured polyQ proteins were incorporated to the media of different mammalian cell cultures to verify and monitor their potential internalization, location after uptake and consequent toxic effects. Internalization of liposome-coated (4 h post-infection) and uncoated fibrils (24–48 h post-infection), characterized by 42 CAG repeats, were detected in both Cos-7 and PC-12 cells (**Table 1**). In this context, the presence of synthetic aggregates within the cytoplasm did not interfere with the physiological functions of the acceptor cells. To mimic the physiological expression of aggregates in the nucleus (Saudou et al., 1998)—which has been suggested to induce greater toxicity—the penetrance of the nuclear membrane was facilitated by the modification of the polyQ peptides by adding nuclear localization signals (NLS). While the F-Q42 was detected in proximity to the nuclear membrane, F-NLS-Q42 was easily delivered to the nucleus. Cell death was frequent in Cos-7 and PC-12 cells administered with F-LNS-Q42 or F-LNS-Q20 and was strictly associated with smaller sized fibrils (Yang et al., 2002), suggesting that the presence of polyQ into the nucleus is associated with high toxicity, independent of the polyQ length (**Table 1**).

A more elaborate study conducted in a wide variety of mammalian cells (Cos-7, HEK, N2A, CHO, and HeLa) revealed that only 1 h after contact, synthetic K2Q44K2 peptides were found within the cytoplasm, co-localizing with cytosolic quality control components in all cell types. Although their location was

expected within the endosomal compartment (Lee et al., 2008), no co-localization with endosome, lysosome and autophagosome markers was revealed suggesting that fibrils were found free within the cytoplasm and were thus available to aggregate with other proteins including HTT. Transmission electron microscopy further uncovered that K2Q44K2 could reach the intracellular compartment by physically breaching plasma membranes (**Figure 1**; **Table 1**).

The hypothesis of free Q44 fibrils interacting with HTT was more specifically investigated by assessing the aggregation state of cyan fluorescent protein (CFP)-tagged HTTExon 1 Q25 (CFP–HTTQ25) in HEK cells after exposure with K2Q44K2. CFP fluorescence in cells not yet exposed to polyQ fibrils showed a diffuse nucleocytoplasmic distribution, as expected of the soluble HTTQ25 fragment. Following incubation with K2Q44K2 fibrils, CFP florescence co-localized with the fibrils in distinct puncta, suggesting the recruitment of the Q25 soluble fraction into the aggregates. Using other forms of fibrils (non-fibrillar HTTQ18 and fibrillar HTTQ51) in the same cell culture conditions, cytosolic nucleation was induced by fibrillar polyQ peptides (Ren et al., 2009; **Figure 1**; **Table 2**). This technique was used in several studies providing evidence for mHTT seeding capacities in different cell lines such as HeLa (Trevino et al., 2012) or human and murine neuroblastoma cells (Ruiz-Arlandis et al., 2016). Additionally, exposure of PC-12—that can inducibly express truncated exon1—with cerebrospinal fluid derived from postmortem samples of HD patients or living BACHD transgenic rats, showed that mHTT could trigger the aggregation process (Tan et al., 2015; **Figure 1**; **Table 2**).

mHTT PROPAGATION TAKES PLACE BY UNKNOWN MECHANISMS

The seeding process of synthetic mHTT fibrils has been studied in greater detail with bimolecular fluorescence complementation assays (BiFC) alongside time-lapse microscopy, allowing for the visualization of HTTExon1 oligomer formation using halves of Venus fluorescent proteins (Herrera et al., 2011). With this approach, the reconstruction of a functional fluorophore testifies to the dimerization of mHTT fragments. A strong fluorescence signal was detected in Q103HTT-Venus transfected human glioma cells (H4), while much lower signals were measured in H4 cells expressing Q25HTT-Venus. Dimers of 103QHTT-Venus constructs appeared 30–45 min after exposure, and some cells began to show larger aggregates after only 1 h. Analyses at later time points, however, showed that all cells eventually died, indicating that both oligomers and inclusion bodies induced irreversible toxic effects. The cell-to-cell transmission potential of pathological mHTT exon1 fragment was further tested in H4 or HEK cells transfected with 103QHTT-V1 or 103QHTT-V2 plasmids and co-cultured. After 3 days, diffuse fluorescence revealed trafficking and, once again, the cell-to-cell transmission capacity of 103Q (Herrera et al., 2011; **Table 1**). One important point to take into consideration is that the mHTT propagation does not always require cell-to-cell contact. For example, the media of HEK cells overexpressing GFP-mHTT-Q19 or GFP-mHTT-Q103 triggers spreading of non-pathological (Q19) and pathological length (Q103) polyQ in SH-SY5Y cells after 5 days of incubation (Jeon et al., 2016; **Table 2**). This may indicate that, in *in vivo* contexts, mHTT could spread over extended distances and thereby exercise a toxic effect on remote cells.

Tau, α-synuclein and HTTQ50 fibrils can all be taken up by C17.2 cells and they are able to seed aggregation of intracellular tau RD-CFP/YFP, α-synuclein-CFP/YFP and HTT(Q25)CFP/YFP respectively (**Table 2**). In particular, internalization of tau and α-synuclein fibrils has been shown to be mediated by heparan sulfate proteoglycans (HSPGs)-binding, which stimulates cell uptake via macropinocytosis. This internalization can be inhibited by heparin, chlorate and heparinase III (Holmes et al., 2013). It should be noted, however, that such uptake has not been reported for HTTQ50 fibrils, which suggests it may use a different pathway (Holmes et al., 2013). Similarities and differences in uptake mechanisms between proteins will have to be carefully taken into account when designing treatment approaches.

TARGETING mHTT PROPAGATION MECHANISMS AS A TREATMENT OPTION

Taken together, there is now compelling evidence, with a large part emerging from *in vitro* studies, that mHTT, as for other proteins linked to neurodegenerative diseases, may behave in a prion-like fashion. However, given that HD is driven by a single gene that leads to the expression of mHTT in every cell of the body, is this of any relevance to the disease burden/onset? And can these propagation mechanisms be used for therapeutic benefits?

In many disorders, including HD, transynaptic/transneuronal spreading may be the favored route of protein transfer. Exocytosis, which occurs at the synaptic terminal, is regulated by a number of proteins, some of which interact pre- or post-synaptically with HTT. Consequently, mHTT can interfere with normal synaptic transmission by sequestering the wild-type protein, a phenomenon that is exacerbated as the polyQ length increases (Smith et al., 2005). The work of Pecho-Vrieseling and colleagues has provided evidence that mHTT itself can circulate between the pre- and post-synaptic membrane. Administration of BoNT—which works by cleaving and inactivating SNARE proteins (SNAP25 and VAMP-2)—blocked exocytosis and consequently mHTT transynaptic propagation (Pecho-Vrieseling et al., 2014).

mTOR inhibitors have also demonstrated to successfully rescue cortico-striatal degeneration in organotypic-striatal cultures derived from R6/2 and $Hdh^{(CAG)150}$ HD mouse models. mTOR regulates a wide variety of cell functions—including autophagy downregulation—and the mTOR inhibitor AZD8055 has been shown to decrease the size of mHTT aggregates and the amount of insoluble mHTT in medium-spiny neurons in both the models cited above (Proenca et al., 2013). mTOR inhibitiors have also been found to reduce mHTT accumulation and alleviate toxicity in fly and mouse models of HD (Ravikumar

TABLE 2 | *In vitro* evidence of mHTT seeding capacities.

Protein form	Cell model	Observations	References
Co-transfection with poly25Q EGFP and poly104Q c-Myc	Cos-1	Co-aggregation of normal length and extended polyQ tracts into cell cytoplasm	Kazantsev et al., 1999
Co-transfection with poly25Q nucleolin EGFP and poly104Q c-Myc	Cos-1	Heterogeneous aggregates of 104Q c-Myc and 25Q-nucleolin-EGFP within cell nuclei Appearance of homogenous cytoplasmic aggregates with the expression of 104Q c-Myc only	
Transfection with 104Q nucleolin EGFP and 104Q c-Myc	Cos-1	Extended polyglutamine colocalization in both the cytoplasm and nucleus with the coexpression of 104Q nucleolin EGFP and 104Q c-Myc Interactions between polyglutamines causes relocation 104Q c-Myc into the nucleus	
Co-transfection of HA-HDQ20 and/or HA-HDQ32 with GFP-HDQ72	Cos-1	Elongated HTT polyQ fragments can recruit wild-type HTT	Busch et al., 2003
Transfection with CFP-Q25HTTExon1 and exposure to K2Q44K2 fibrils	HEK	Co-localization of CFP-Q25HTTExon1 with K2Q44K2 induces amyloid nucleation in a sequence-specific manner	Ren et al., 2009
Transfection with ChFP-HTTExon1Q25 and exposure to positive, neutral or negatively charged FITC-labeled Q44 fibrils	HEK	Induction of nucleation within the cytoplasm of ChFP-HTTExon1Q25 transfected cells by all three types of fibrils (i.e., positive, neutral, and negative net charges)	Trevino et al., 2012
Transfection with ChFP-HTTExon1Q25 and exposure to non-fibrillar K2Q44K2 aggregates	HEK	Reduced internalization of non-fibrillar K2Q44K2 and nucleation of cytoplasmic ChFP-HTTExon1Q25 in comparison to fibrillar forms	
Transfection with ChFP-HTTExon1Q25 and exposure to HTTExon1Q44 or Q44 fibrils	HeLa	Internalization of HTTExon1Q44 and Q44 fibrils into HeLa cells and nucleation within the cytoplasm ChFP-HTTExon1Q25 transfected cells HTTExon1Q44 fibril internalization is less efficient than for the Q44 fibrils	
Transfection with HTT(Q25)CFP/YFP and exposure to HTT Q50 fibrils	C17.2	Heparan sulfate proteoglycans-independent internalization of Q50 fibrils and nucleation with exogenous HTT(Q25)CFP/YFP	Holmes et al., 2013
Transfection with ChFP-HTTExon1Q25 and exposure to HTTExon1Q44 fibrils	Undifferentiated and differentiated N2A	Seeding capacity of transfected HTTExon1Q44 fibrils with ChFP-HTTExon1Q25 in undifferentiated and differentiated N2A cells	Ruiz-Arlandis et al., 2016
PolyQ (KKQ30KK or KKQ40KK) oligomers	HTT14A2.6	Seeding of polyQ oligomers in HTT14A2.6 cells	Tan et al., 2015
Exposure to media and lysates from induced HTT14A2.6 cells	Naïve	Media and lysates from induced HTT14A2.6 cells can seed aggregation in naïve cells	
CSF from deceased HD patients	HTT14A2.6	CSF obtained from HD patients postmortem increase aggregate number in HTT14A2.6 cells	
CSF from BACHD rats	HTT14A2.6	CSF from living BACHD rats can seed aggregation	
Expression of PrDQ19, PrDQ54 and PrDQ92	GT17	Soluble Sup35 protein converts into insoluble aggregates following expression of PrDpolyQ pathogenic (≥54 glutamines) proteins	Goehler et al., 2010
Transfection with Rnq1Q19, Rnq1Q54 and Rnq1Q91	GT17	Pathogenic PolyQ tracts convert soluble Rnq1 into insoluble aggregates	
Transfection with HTT25Q-/103Q-GFP	74-D694	HTT103Q induce insoluble aggregates of Def1, Pub1, Rpn10, Bmh2, Sgt2, and Sup35 proteins	Nizhnikov et al., 2014
Transfection with HTT25Q-/103Q-GFP	BY4742 and 74-D694	HTTQ103 promotes it own aggregation and that of Sup35 and Def1 in different yeast strains Deletion of Def1, which normally enhances mHTT aggregation and toxicity, decreases selectively the amount of polymerized HTTQ103 and its cytotoxic effect in BY4742 cells	Serpionov et al., 2017

BACHD, bacterial artificial chromosome (BAC) transgenic rat model of HD; Bmh2, protein BMH2; BY4742, yeast strain; C17.2 cells, murine C17.2 neural precursor cells; c-Myc, c-Myc tag peptide; CFP, cyan fluorescent protein; ChFP, mCherry fluorescent protein; Cos-1, fibroblast-like cell lines derived from monkey kidney; Cryo-ET, cryo-electron tomography; Cryo-FLM, cryogenic-fluorescent light microscopy; CSF, cerebrospinal fluid; Def1, RNA polymerase II degradation factor 1; EGFP, enhanced green fluorescent protein; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GT17, yeast strain; HA-tag, human influenza hemaglutinin tag; HD, Huntington's disease; HEK, human embryonic kidney cells; HeLa, human uterine cervical carcinoma cells; HTT14A2.6:PC12 cells, (cell line from pheomochromocytoma) that inducibly express a fragment of mHTT (truncated exon 1) epitope tagged with enhanced green fluorescent protein (mHTTex1-GFP) in the presence of ponasterone A; N2A, murine neuroblastoma cell line; PolyQ, polyglutamine; PrD, prion domain; Pub1, nuclear and cytoplasmic polyadenylated RNA-binding protein; Rnq1, yeast prion protein; Rpn10, proteasome regulatory particle base subunit RPN10; Sgt2, small glutamine-rich tetratricopeptide repeat-containing protein 2; Sup35, yeast eukaryotic release factor 3; YFP, yellow fluorescent protein.

et al., 2004), prevent levo-dopa induced dyskinesias in mouse models of PD (Santini et al., 2009) and ameliorate cognitive deficits in various AD animal models (Wang C. et al., 2014).

Transneuronal propagation of pathological proteins is not a unique characteristic of mHTT. It has been demonstrated for tau and β-amyloid in AD (de Calignon et al., 2012; Lee et al., 2012;

Wu et al., 2013) and α-synuclein in PD (Desplats et al., 2009; Angot et al., 2012; Freundt et al., 2012; Luk et al., 2012a,b; Rey et al., 2016). Of great relevance is the fact that trans-neuronal spread of α-synuclein can be blocked by the monoclonal antibody 1H7 *in vitro* (Games et al., 2014) and *in vivo* (Spencer et al., 2017), ameliorating axonal transport and synaptic trafficking, raising the possibility of also treating HD through passive immunization. While the release of vesicles containing tau, α-synuclein and TDP-43 is increased by overexpression of the co-chaperone DnaJC5—possibly through non-canonical SNAP23 exocytosis—this is not the case for mHTT, indicating that not all pathological proteins may behave in the same manner under the same circumstances (Fontaine et al., 2016). We therefore may have to tailor therapies to specifically address the different mechanisms for propagation adopted by each protein, while other treatment options may be applicable to a range of proteins.

Previous reports have also indicated that tunneling nanotubes represent an efficient means for cells to communicate and share material. As we described above, F-actin is the principal component of the nanotube trafficking paths and its depolymerization by latrunculin or by cytochalasin B (Bukoreshtliev et al., 2009) abolishes the formation of TNTs, or at least reduces the number formed. If depolymerizing actin may be an attractive target by which to prevent bridge formation between cells—hence mHTT spreading—its implications for microfilaments and microtubules within the cytoskeleton organization would need to be known before applying such methodologies, given the detrimental consequences this may have on the cell integrity.

TNT formation is also promoted by oxidative stress, as shown with H_2O_2 administration and serum starvation (Wang et al., 2011). However, interventions designed to control oxidative stress are too non-specific to allow for the generation of a meaningful agent. Additionally, modulations of the M-Sec promoter protein (Hase et al., 2009), tumor suppressor p53, epidermal growth factor receptor (EGFR) and EGFR-regulated Akt, PI3K and mTOR (Wang et al., 2011) could be considered as targets given they all have a direct effect on nanotube growth. Unfortunately, reduction of physiological levels of any of these transcription factors could have devastating effects on the cell's vital functions.

If aiming to block the synthesis of F-actin, oxidative stress or various proteins involved in TNT formation is not a viable approach, the motor molecules that can carry mHTT could be a target option. For example, it has been reported that the transfer of endosome-related organelles is actomyosin dependent (Gurke et al., 2008) and therefore molecular motor myosin-X (Myo10) expression increases the number of TNTs and the transfer of vesicles between co-cultured cells (Gousset et al., 2013). In particular, a specific sequence of Myo10 is required for the formation and function of TNTs (Gousset et al., 2013), and its deletion does not affect filopodia involved in cell-to-cell communication (Bohil et al., 2006). However, it is important to note that TNT formation is beginning to be understood as a process that is cell-specific, in other words different cell lines can induce TNTs via different mechanisms (Gousset et al., 2013). This is a characteristic that is extremely important to take into consideration if we aim to inhibit TNT production in a specific cell population.

Endocytosis is also a very efficient pathway for mHTT internalization (Ruiz-Arlandis et al., 2016) and therefore could be considered as a process to target to halt propagation and disease dissemination. For example, chlorpromazine, monodansylcadaverine and dynasore can suppress mHTT uptake in N2A cells by a clathrin-dependent endocytosis mechanism (Ruiz-Arlandis et al., 2016). Although chlorpromazine has been used to treat psychotic disorders since the fifties, it is known to induce side-effects which include tardive dyskinesia, dystonia, motor restlessness and akathisia, which limits its use in a debilitating movement-disorder such as HD. Other pharmacological approaches could be employed to specifically inhibit clathrin-dependent endocytosis but a complete screening should be undertaken to identify such compounds, test their efficacy and monitor their potential side-effects as blocking this pathway could affect the well-being of the cells/neurons and only partially prevent mHTT propagation.

However, blocking mHTT uptake without targeting its release would not provide a fully efficient therapy and in order to address this issue, neutral sphingomyelinase and PI3-kinase inhibitors have been investigated and shown to be capable of reducing the secretion of mHTT–more specifically of 72Q and 25Q in transfected N2A cells, full length mHTT expressing lentiviruses were transduced in rat primary cortical neurons and endogenous mHTT of HdhQ111/Hdh$^+$ and HdhQ111/HdhQ111 murine striatal cells. This event occurred simultaneously with the depletion of intracellular mHTT sequestered in vesicles, highlighting, once again, the role of late endosomes/lysosomes activity in mHTT secretion. Surprisingly, the knockdown of mHTT secretion by neutral sphingomyelinase inhibitors did not increase the amount of mHTT in the intracellular compartment, neither did it induce cell toxicity, raising the possibility to target release and propagation of free forms of mHTT. The side-effects of PI3-kinase inhibitors were not reported (Trajkovic et al., 2017). The absence of intracellular mHTT accumulation may indicate the existence of other release mechanisms (Jeon et al., 2016). Indeed, mHTT is very often detected outside the cell boundary, notably in the cerebrospinal fluid (Tan et al., 2015; Wild et al., 2015) within the extracellular matrix (Cicchetti et al., 2014), in blood vessels (Drouin-Ouellet et al., 2015) and possibly in plasma (Jeon et al., 2016). mHTT found in the extracellular milieu is also likely to emerge from cell death driven by mHTT toxicity (Cicchetti et al., 2014).

The plasma membrane constitutes a major barrier to external toxins. To preserve the homeostasis of its internal compartments, it selectivity permits compounds to cross or bind to the surface. Interactions between proteins and cell membranes can be physical, as determined by electrostatic attractions, or chemical, through specific or non-specific lipid, protein or carbohydrate bindings. In particular, binding of the HTT-N-terminus with the lipids of biological membrane has been shown to dependent on electrostatic interactions (Kegel et al., 2005). mHTT carries variable repetitions of polyglutamine residues at the N-terminus, and increasing the number of polyQ offers more insertion sites into the lipid bilayer. This has been

demonstrated to occur via vesicles that simulate the biological membrane, corrupting its integrity (Kegel et al., 2009). Several other studies have demonstrated plasma membrane structural modifications following interactions with tau, Aβ or α-synuclein (Zhu et al., 2003; Flach et al., 2012). These interactions are well described in studies which compared the properties of α-synuclein and mHTT fibrils. They have revealed that soluble α-synuclein fibrils have differing binding capacities depending on their charge, while HTTExon1Q41 does not express this specificity, at least as demonstrated in synthetic vesicles (Pieri et al., 2012). It was also shown that HTTExon1 binding is dependent on the number of interaction sites, which is not the case for α-synuclein (Monsellier et al., 2016). Despite some dissimilarities, α-synuclein and mHTT fibrils do share common properties such as being able to modify the permeability of the lipid membrane by Ca^{2+} influxes (Monsellier et al., 2016). Furthermore, cholesterol concentration in the phospholipid bilayer impacts on its rigidity and stability, challenging the fibrils' capacity to alter the membrane's permeability (Pieri et al., 2012). In this regard, polyunsaturated fatty acid ethyl-eicosapentaenoic acid (ethyl-EPA) available in specific foods can dampen motor deficits in a HD mouse model (Clifford et al., 2002) probably due to its capacity to positively influence the plasma membrane properties, by preventing oxidative stress (Puri et al., 2005) or inhibiting apoptotic pathways (Murck and Manku, 2007). As its metabolite docosahexaenoic acid (DHA) (Wu et al., 2008), EPA was shown to modulate synaptic plasticity in *ex vivo* hippocampal slices from rats following the administration of ethyl-EPA for a period of 8 weeks (Kawashima et al., 2010). This compound has already completed a phase III clinical trial with some evidence of efficacy as shown by changes in the total motor scores in human patients with >45 CAG repeats (Puri et al., 2005).

PERSPECTIVES

In this review, we have presented the main findings from *in vitro* models, with a particular emphasis on mHTT propagation and seeding capacity, as it is becoming increasingly apparent that these mechanisms play a significant role in disease onset and manifestation and that targeting them may offer viable treatment options—keeping in mind that additional *in vivo* work must be conducted to go forward with the development of procedures that target this aspect of the disease. In the last few years, the focus of novel therapeutic approaches has been shifted and the aim is now to decrease the amounts of mHTT using approaches such as gene silencing (Lu and Yang, 2012) and editing (Yang et al., 2017). However, considerable challenges remain which relate to the capacity of mHTT to propagate between cells. Approaches designed to block propagation would not interfere with the primary aim of gene silencing/editing methodologies. This is particularly true in light of the fact that abnormalities in HD also lie outside the CNS (Sassone et al., 2009; Carroll et al., 2015). Future therapeutic perspectives will need to converge and tackle this genetic disorder from several fronts, including both pre-manifest and manifest patients, using combinations of agents that inhibit mHTT synthesis; restrict the damaging effects of the protein portion that is then secreted and by so doing prevent the accumulation and spread of this pathogenic protein.

AUTHOR CONTRIBUTIONS

MM was responsible for conducting the literature search as well as designing tables and figures. FC conceptualized and wrote the manuscript.

ACKNOWLEDGMENTS

FC is a recipient of a Research Chair from the Fonds de Recherche du Québec en Santé (FRQS, 35059) providing salary support and operating funds, and receives funding from the Canadian Institutes of Health Research (CIHR, MOP136883) to conduct her HD-related research. The authors would like to thank Mr. Gilles Chabot for artwork.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 13 December 2017
doi: 10.3389/fnins.2017.00671

Current Advances and Limitations in Modeling ALS/FTD in a Dish Using Induced Pluripotent Stem Cells

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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are two age-dependent multifactorial neurodegenerative disorders, which are typically characterized by the selective death of motor neurons and cerebral cortex neurons, respectively. These two diseases share many clinical, genetic and pathological aspects. During the past decade, cell reprogramming technologies enabled researchers to generate human induced pluripotent stem cells (iPSCs) from somatic cells. This resulted in the unique opportunity to obtain specific neuronal and non-neuronal cell types from patients which could be used for basic research. Moreover, these *in vitro* models can mimic not only the familial forms of ALS/FTD, but also sporadic cases without known genetic cause. At present, there have been extensive technical advances in the generation of iPSCs, as well as in the differentiation procedures to obtain iPSC-derived motor neurons, cortical neurons and non-neuronal cells. The major challenge at this moment is to determine whether these iPSC-derived cells show relevant phenotypes that recapitulate complex diseases. In this review, we will summarize the work related to iPSC models of ALS and FTD. In addition, we will discuss potential drawbacks and solutions for establishing more trustworthy iPSC models for both ALS and FTD.

Keywords: amyotrophic lateral sclerosis, frontotemporal dementia, iPSC, neurodegeneration, motor neuron

OPEN ACCESS

Edited by:
Dustin R. Wakeman, RxGen (United States), United States

Reviewed by:
Smitha Karunakaran, Indian Institute of Science, India
Renata Santos, UMR8197 Institut de biologie de l'Ecole Normale Supérieure (IBENS), France

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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *01 September 2017*
Accepted: *20 November 2017*
Published: *13 December 2017*

Citation:
Guo W, Fumagalli L, Prior R and Van Den Bosch L (2017) Current Advances and Limitations in Modeling ALS/FTD in a Dish Using Induced Pluripotent Stem Cells. Front. Neurosci. 11:671. doi: 10.3389/fnins.2017.00671

INTRODUCTION

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease caused by the selective death of both upper and lower motor neurons (Renton et al., 2014). The disease is also known as Lou Gehrig's disease, as it was named after the American baseball player who was diagnosed with ALS in 1939 and died as a result of the disease (Taylor et al., 2016). Typically, the onset of this disease is in late midlife and it is mostly fatal within 3–5 years after the detection of the first symptoms (Renton et al., 2014; Taylor et al., 2016). Currently, there are no effective treatments for ALS. The first FDA-approved drug for the treatment of ALS was riluzole, which is thought to interfere with glutamate metabolism. However, riluzole only increases survival by a few months (Petrov et al., 2017). Recently, edaravone was approved by FDA after it was shown that intravenous injection of this anti-oxidant slows down the disease progression in a subpopulation of ALS patients (Hardiman and van den Berg, 2017).

The incidence of ALS varies between 0.3 and 3.6 new cases per 100,000 per year depending on the demographics (Henry et al., 2015). In 90% of ALS cases, there is no familial history and these patients are considered as sporadic ALS patients. In ∼10% of patients, the disease is inherited

and these are classified as familial ALS (Renton et al., 2014; Henry et al., 2015). The genetic etiology of sporadic ALS and of ∼30% of familial ALS (European descent) is still unknown (He et al., 2015). The major genetic pattern of inheritance of familial ALS is autosomal dominant, but other hereditary patterns have been reported. The most prevalent mutated genes are *superoxide dismutase 1* (*SOD1*), *chromosome 9 open reading frame 72* (*C9ORF72*), *TAR DNA-binding protein 43* (*TARDBP*) and *fused in sarcoma* (*FUS*) (Renton et al., 2014).

Frontotemporal Dementia

Frontotemporal dementia (FTD) is comprised of a group of disorders caused by progressive neurodegeneration in the frontal and/or temporal lobes of the brain (Bang et al., 2015). It is also called Pick's disease after the physician Arnold Pick, who first described the disease in 1892 (Boxer and Miller, 2005). FTD accounts for up to 10–15% of all dementia cases and is considered the second most common form of early-onset dementia in people younger than 65 years of age, after Alzheimer's disease (Karageorgiou and Miller, 2014; Lashley et al., 2015). Patients show changes in social and personality behavior, apathy, blunting of emotions, and/or deficits in both expressive and receptive language (Bang et al., 2015; Burrell et al., 2016). Unfortunately, there is no effective treatment for FTD yet. The exact cause of FTD is still unknown, although in 40–50% of FTD cases there is a family history (Fong et al., 2012; Ng et al., 2015). Several gene mutations were linked to FTD. The most frequently mutated genes include *Microtubule Associated Protein Tau* (*MAPT*), *Progranulin* (*GRN*), and *C9ORF72* (Bang et al., 2015; Burrell et al., 2016).

ALS and FTD, the Extremities of a Disease Spectrum

Although ALS and FTD mostly occur as separate diseases, there are more and more common aspects identified for both disorders. Clinically, a number of patients diagnosed with ALS show signs of FTD such as behavioral, cognitive, or language dysfunctions (Ling et al., 2013; Ng et al., 2015). The frequency of FTD clinical features in ALS varies amongst different reports, but it was claimed that some FTD-related symptoms can be detected in up to 50% of ALS patients (Ferrari et al., 2011). Additionally, the opposite also occurs, in that some FTD patients develop motor symptoms during the disease process. Moreover, ALS and FTD can occur within the same family (Ferrari et al., 2011; Ling et al., 2013; Ng et al., 2015). In terms of genetics, hexanucleotide repeat expansions in *C9ORF72*, as well as mutations in the *Valosin-Containing Protein* (*VCP*) gene are found in both ALS and FTD (Ling et al., 2013). Furthermore, the repeat expansions in *C9ORF72* cause the disease in a high proportion of families in which both FTD and ALS occurs (Cooper-Knock et al., 2015). Interestingly, FTD and ALS also share some pathological hallmarks; the most important being the presence of neuronal inclusions of TDP-43, the gene product of the *TARDBP* gene (Ling et al., 2013). Similarly, FUS inclusions are observed in a limited number of ALS, as well as FTD patients (Ling et al., 2013). In addition, nuclear repeat-containing RNA foci as well as inclusions containing dipeptide repeat proteins (DPRs), both specific pathological characteristics of patients with *C9ORF72* expansions, are detected in *post mortem* material of these ALS and FTD patients (Ling et al., 2013; Cooper-Knock et al., 2015).

Multiple Pathological Mechanisms Have Been Associated with ALS/FTD

A large number of biological processes have already been suggested to be involved in ALS and some of these could also play a role in FTD. Amongst others, these include excitotoxicity, hyperexcitability, astrocytosis, neuroinflammation, mitochondrial dysfunction, axonal abnormalities, dysregulated autophagy, abnormal RNA metabolism, problems with stress granule dynamics, and nucleocytoplasmic transport defects.

The pathogenic mechanism for which there is ample evidence that it indeed plays a role in ALS patients is excitotoxicity (Van Den Bosch et al., 2006). Excitotoxicity is the process of neuronal degeneration caused by overstimulation of glutamate receptors. One of the major arguments for an involvement of excitotoxicity in the ALS disease process is that riluzole has anti-excitotoxic properties (Miller et al., 2003).

More recently, at least part of the therapeutic effect of riluzole was linked to a (transient) effect on axonal and cortical hyperexcitability (Vucic et al., 2013). Peripheral and central hyperexcitability mirrors clinical features (fasciculations) and neurophysiological findings (increased intracortical excitability). Although there are no treatments approved by the FDA for FTD until now, one transgenic mouse model carrying human TDP-43^{A315T} showed hyperactive somatostatin interneurons that disinhibited pyramidal neurons, which contributed to excitotoxicity (Zhang W. et al., 2016). In addition, another transgenic mouse model with a human Tau^{A152T} mutation showed excitotoxicity mediated by NR2B-containing NMDA receptors due to enhanced extracellular glutamate (Decker et al., 2016).

It is well-known that astrocytes as well as microglia are activated during the disease process. The activated astrocytes release toxic factors, of which the identity is largely unknown. Furthermore, they seem to be soluble in nature, as mutant astrocyte-conditioned medium is also toxic for primary motor neurons of embryonic stem cell (ESCs) derived motor neurons (Nagai et al., 2007). In addition, mutant SOD1 expressing astrocytes fail to protect neurons from excitotoxicity. Astrocytes can increase the excitability and Ca^{2+} influx in motor neurons by increasing the Na^{+} current or by decreasing the neuronal GluR2 expression leading to a higher Ca^{2+} permeability of the AMPA receptor, ultimately leading to neuronal excitotoxicity (Van Damme et al., 2007; Fritz et al., 2013). When microglia are activated during the disease process, the cell body enlarges and the processes get thicker. Microglia mimic properties of antigen-presenting cells and start to interact with T-cells, which infiltrate in the spinal cord and cortex (Engelhardt et al., 1993; Alexianu et al., 2001). This microglial activation starts before disease onset and the number of activated microglia and infiltrated T-cells increases with disease progression (Hall et al., 1998; Alexianu et al., 2001). Similarly, when GRN was injected in mouse brain,

an increase of Iba1-positive microglia around the injection site was observed. This indicates that there could be a link between FTD and microglial phagocytosis (Pickford et al., 2011).

Both in animal models and in ALS/FTD patients, dysfunctional mitochondria are observed. Mitochondria are not only crucial for ATP synthesis, but are also involved in intracellular Ca^{2+} homeostasis and induction of apoptosis. As a consequence, abnormal functioning of mitochondria can cause cell death. In the mutant SOD1 mouse model, swollen and vacuolated mitochondria were observed in motor neurons before the first signs of motor neuron degeneration appear (Wong et al., 1995; Kong and Xu, 1998; Higgins et al., 2003). From analysis of the mitochondrial functionality, a decrease of mitochondrial membrane potential was observed in fibroblasts from ALS/FTD patients carrying mutant *TARDBP* (Onesto et al., 2016). Interestingly, increased oxygen consumption and mitochondrial hyperpolarization were observed in fibroblasts from ALS/FTD patients carrying mutant *C9ORF72* (Onesto et al., 2016). Similar abnormalities are found in sporadic ALS/FTD patients (Sasaki and Iwata, 2007). In general, dysfunctions in mitochondrial respiration and ATP synthesis, axonal transport of mitochondria, mitochondrial dynamics, Ca^{2+} buffering (which could lead to excitotoxicity) and induction of apoptosis are all seen in SOD1 mouse models (Kawamata and Manfredi, 2010). Moreover, these abnormalities are not restricted to the mutant SOD1 pathogenesis, also mice overexpressing human TDP-43 have abnormal distribution of mitochondria and changes in mitochondrial dynamics (Xu et al., 2010; Wang et al., 2013), while neuronal cultures expressing ALS-linked FUS mutants contain smaller mitochondria (Tradewell et al., 2012).

Neurons in general and motor neurons in particular are highly dependent on axonal transport mechanisms along their very long axons to bring proteins, organelles and other cargoes to their required sites. Microtubules form tracks along axons on which different cargoes are transported with the help of motor proteins. Since axonal swellings containing neurofilaments were observed in sporadic and familial ALS patients (Okamoto et al., 1990; Sasaki and Maruyama, 1992), axonal transport defects have been intensively studied in ALS (De Vos and Hafezparast, 2017).

ALS is also characterized by the accumulation of ubiquitinated proteins. In the majority of ALS cases, these accumulations contain ubiquitinated TDP-43 even when there are no mutations in the *TARDBP* gene (Neumann et al., 2006). Both autophagy and the ubiquitin-proteasome system (UPS) systems are responsible for the proteostasis in eukaryotes and the prevention of protein aggregation. After deletion of two different essential genes for autophagy (Atg5 or Atg7), each of these transgenic mice develop a neurodegenerative phenotype which suggests an essential role of autophagy in neurons (Hara et al., 2006; Komatsu et al., 2006). Ubiquitin-positive immunoreactive inclusions have been reported as the neuropathological hallmark of FTD patients (Brun et al., 1994).

Disturbances in the RNA metabolism were linked to ALS/FTD after the discovery of mutations in *TARDBP* and *FUS*, encoding two different RNA-binding proteins (TDP-43 and FUS) (Ling et al., 2013). Under normal conditions, both TDP-43 and FUS are localized in the nucleus. In brain and spinal cord of ALS patients, TDP-43 and FUS are in the cytoplasm of the neurons and sometimes also of the glial cells. In response to stress, both TDP-43 and FUS localize to the stress granules present in the cytoplasm. In these stress granules, mRNA is translationally inactive. Both TDP-43 and FUS contain low-complexity domains which are essential for their localization in these stress granules. After periods of stress, the stress granules resolve and mRNA becomes available again to be translated. FUS mutations seem to accelerate the transition from the liquid to the aggregated state in these stress granules (Patel et al., 2015). Interestingly, FUS inclusions have also been reported in FTD patients which do not carry FUS mutations (Lagier-Tourenne et al., 2010). A number of recent papers link the toxicity of the hexanucleotide repeats in *C9ORF72* to an interference with the nucleocytoplasmic transport process (Boeynaems et al., 2016). This could also explain the cytoplasmic mislocalization of TDP-43 in patients with hexanucleotide repeats in *C9ORF72*.

Despite our understanding of the genetic causes and of the pathological processes responsible for ALS/FTD, many potential therapies were unsuccessful in human clinical trials. However, most of these therapeutic strategies were based on preclinical research on animal models. Successful modeling of ALS and FTD by using human materials could deepen our knowledge of these complex diseases, aid in the identification of therapeutic targets and overall, have a significant impact on ALS/FTD research (Picher-Martel et al., 2016; Sances et al., 2016; Lee and Huang, 2017).

iPSC-DERIVED MODELS FOR ALS/FTD

Until now, rodent models are the most widely used ALS/FTD models both to study disease mechanisms and to test potential treatments with newly developed compounds (Petrov et al., 2017). With the advancement of genetic and technological approaches, a wide range of additional model systems became available to study these diseases. These model systems differ from *in vitro* to *in vivo*, from invertebrates to vertebrates, from yeast to human iPSCs. It is clear that all these model system have their advantages and disadvantages (Van Damme et al., 2017).

In recent years, several publications identified a number of pathogenic molecular mechanisms of ALS/FTD using human iPSC models (**Table 1**). Most of these studies focused on familial types of these diseases because the known mutations could give a clue to the underlying mechanism. We will summarize the results obtained with these iPSC models based on the different mutated genes. In addition, we will discuss the results obtained starting from iPSCs from sporadic ALS/FTD patients (**Figure 1**).

Advantages of iPSCs as a Model System

iPSCs are pluripotent stem cells that are generated directly from adult cells. The iPSC technology was developed by Shinya Yamanaka's lab by introducing four specific transcription factors into adult cells and converting these cells into pluripotent stem cells (Takahashi et al., 2007). As one of the newest ALS model systems, this technology allows for the generation of iPSCs from patient-derived somatic cells from ALS patients and opens new

TABLE 1 | Summary of published iPSC models of ALS and FTD.

Disease	Gene mutation	Reprogramming strategy	iPSC-derived cell subtype	ALS/FTD phenotype	References
ALS	$SOD1^{A4V}$ $SOD1^{D90A}$	Retroviral/Sendai viral	Motor neurons	Neurofilament misregulation, Neurite degeneration, Mutant SOD1 aggregates	Chen et al., 2014
ALS	$SOD1^{A4V}$	Retroviral	Motor neurons	Survival reduction, Transcriptional change, Mitochondrial defects, Activation of ER stress, Unfolded protein response	Kiskinis et al., 2014
ALS	$SOD1^{A4V}$	Retroviral	Motor neurons	Hyperexcitability, Loss of voltage-gated currents (K^+)	Wainger et al., 2014
ALS	$SOD1^{A272C}$	Episomal	Motor neurons	Transcriptional changes	Wang et al., 2017
ALS	TDP-43^{Q343R} TDP-43^{M337V} TDP-43^{G298S}	Retroviral	Motor neurons	Transcriptional changes, TDP-43 aggregates, Neurite degeneration, Vulnerability to oxidative stress	Egawa et al., 2012
ALS	TDP-43^{M337V}	Retroviral	Motor neurons	Survival reduction, TDP-43 aggregates, Vulnerability to PI3K inhibition	Bilican et al., 2012
ALS	TDP-43^{M337V}	Retroviral	Astrocytes/Motor neurons	Astrocytes: Survival reduction, Increased insoluble TDP-43, Cytoplasmic mislocalization of TDP-43 Motor neuron-astrocyte-coculture: No effect on motor neuron survival	Serio et al., 2013
ALS	TDP-43^{A90V} TDP-43^{M337V}	Retroviral	Neurons	Cytoplasmic mislocalization of TDP-43, Decreased miR-9 expression	Zhang et al., 2013
ALS	TDP-43^{M337V}	Retroviral	Motor neurons	Hyperexcitability (early stage), Hypoexcitability (late stage), Loss of synaptic activity, Loss of voltage-gated currents (Na^+)	Devlin et al., 2015
ALS	FUS^{M511FS} FUS^{H517Q}	Retroviral	Motor neurons	Hyperexcitability	Wainger et al., 2014
ALS	FUS^{R521L} FUS^{R521C}, $FUS^{R495QfsX527}$	Retroviral	Motor neurons	Hypoexcitability, Decreased synaptic activity, Lower sodium to potassium (Na^+/K^+) ratios	Naujock et al., 2016
ALS	FUS^{P525L}	Episomal	Motor neurons	Cytoplasmic FUS localization, FUS aggregation	Liu et al., 2015
ALS	FUS^{H517D}	Episomal	Motor neurons	Cytoplasmic FUS localization, FUS aggregation, Cell death, Aberrant gene expression and splicing	Ichiyanagi et al., 2016

(Continued)

TABLE 1 | Continued

Disease	Gene mutation	Reprogramming strategy	iPSC-derived cell subtype	ALS/FTD phenotype	References
ALS	FUS^{R521C} $FUS^{R495Qfs527}$ $FUS^{Asp502Thrf*27}$	Lentiviral	Motor neurons	Cytoplasmic FUS localization, FUS aggregation, Cell death	Higelin et al., 2016
ALS	FUS^{R514S} FUS^{R521C} FUS^{P525L}	Lentiviral	Motor neurons	Cytoplasmic FUS localization, FUS aggregation, Cell vulnerability	Lenzi et al., 2015
ALS	FUS^{R521H} FUS^{P525L}	Sendai viral	Motor neurons	Cytoplasmic FUS localization, Hypoexcitability, Decreased synaptic activity, Axonal transport defects	Guo et al., 2017
ALS	C9ORF72	Sendai viral	Motor neuorns	Cell death, Abnormal protein aggregation, and stress granule formation, Increased ER stress, Reduced mitochondrial membrane potential, p62 accumulation	Dafinca et al., 2016
ALS	C9ORF72	Episomal	Motor neurons	Cell vulnerability, DNA damage	Lopez-Gonzalez et al., 2016
ALS	C9ORF72	Retroviral	Motor neurons	Hyperexcitability	Wainger et al., 2014
ALS	C9ORF72	Retroviral	Motor neurons	Hyperexcitability (early stage), Hypoexcitability (late stage), Loss of synaptic activity, Loss of voltage-gated currents (K^+/Na^+)	Devlin et al., 2015
ALS	C9ORF72	Retroviral	Motor neurons	Transcriptional changes	Kiskinis et al., 2014
ALS	C9ORF72	Retroviral	Neurons	RNA foci, Transcriptional changes, RAN translation products, Vulnerability to glutamate toxicity	Donnelly et al., 2013
ALS	C9ORF72	Episomal	Motor neurons	RNA foci, Transcriptional changes	Sareen et al., 2013
ALS	C9ORF72	Retroviral	Neurons	RNA foci, RAN translation products, Vulnerability to inhibition of autophagy, p62 accumulation	Almeida et al., 2013
ALS	C9ORF72	Not mentioned	Motor neurons	DPRs, Cell-to-cell transmission of DPRs	Westergard et al., 2016
ALS	C9ORF72	Retroviral	Motor neurons	Modulation of actin dynamics	Sivadasan et al., 2016
ALS/FTD	C9ORF72	Lentiviral	iNeurons	Autophagy impairment	Webster et al., 2016

(Continued)

TABLE 1 | Continued

Disease	Gene mutation	Reprogramming strategy	iPSC-derived cell subtype	ALS/FTD phenotype	References
ALS	C9ORF72	Lentiviral/Episomal	Astrocyte/Motor neurons	Abnormal protein aggregation, p62 accumulation	Madill et al., 2017
FTD	C9ORF72	Episomal	Cortical neurons	Nucleocytoplasmic transport defect	Freibaum et al., 2015
ALS	C9ORF72	Retroviral	Neurons	Nucleocytoplasmic transport defect	Zhang et al., 2015
ALS	$VAPB^{P56S}$	Retroviral	Motor neurons	Reduced expression levels of VAPB	Mitne-Neto et al., 2011
ALS	Sporadic ALS	Unknown	Motor neurons	TDP-43 aggregates	Burkhardt et al., 2013
ALS	Sporadic ALS	Lentiviral	Motor neurons	Aberrant gene expression	Alves et al., 2015
ALS	Sporadic ALS	Sendai viral	Astrocytes	Disorganized neurofilaments, Aggregated ubiquitin, Synaptic defects	Qian et al., 2017
FTD	TAU^{A152T}	Retroviral	Neurons	Increased tau fragmentation, Increased tau phosphorylation	Fong et al., 2013
FTD	GRN^{S116X}	Retroviral	Neurons /Microglia	PGRN haploinsufficiency, Vulnerability to ER stress, Vulnerability to inhibition of proteasome	Almeida et al., 2012
FTD	GRN null	Retroviral	Cortical neurons /Motor neurons	Inefficient cortical neuron formation PGRN haploinsufficiency Transcriptional changes	Raitano et al., 2015

opportunities. Compared with other model systems, there are a number of advantages of the iPSC model system. First, it gives the researcher the opportunity to model ALS with no need to overexpress the transgene with the disease-causing mutation. Second, gene editing technologies, such as CRISPR/Cas9, make it possible to correct these ALS-causing mutations in the same patient-derived iPSCs. This creates an isogenic control with exactly the same genetic background as the patient and makes it also possible to search for disease modifiers in ALS/FTD (Van Damme et al., 2017). Third, the iPSC technology enables researchers to investigate sporadic ALS cases in the laboratory. This has never been possible with other model systems before. Fourth, it allows researchers to investigate human diseases by using patient material to create different cell types. Especially for neurodegenerative diseases, patient-derived neurons are a new research tool which was unavailable before the invention of the iPSC technology. Finally, the constant improvements of differentiation protocols gives the possibility to differentiate iPSCs into many different cell (sub)types (lower motor neurons, upper motor neurons, astrocytes, oligodendrocytes, cortical neurons, . . .), which allows researchers to investigate the different cell types involved in the disease process or to study the dynamics between the different cell types (Emdad et al., 2012; Maury et al., 2014; Douvaras et al., 2016). The major drawback of this model system is the *in vitro* approach. As a consequence, cross-validation in other *in vivo* systems and/or in patient material remains crucial (Van Damme et al., 2017). Moreover, the costs of the iPSC model system are high compared to some other model systems. However, the valuable output of this relatively new model system is promising and could be very useful in basic research.

Modeling of ALS in a Dish

SOD1

In 2014, two back-to-back studies were published which used novel *in vitro* models for ALS by generating iPSC-derived motor neurons from patients carrying a $SOD1^{A4V}$ or a $SOD1^{D90A}$ mutation (Chen et al., 2014; Kiskinis et al., 2014). In each study, the authors used genetic correction of both of these mutations as isogenic controls (Chen et al., 2014; Kiskinis et al., 2014). Both studies reported a high ratio of electrophysiologically active motor neurons differentiated from these iPSCs (Chen et al., 2014; Kiskinis et al., 2014). These motor neurons also recapitulated the spontaneous and progressive decrease in cell viability observed in humans (Chen et al., 2014; Kiskinis et al.,

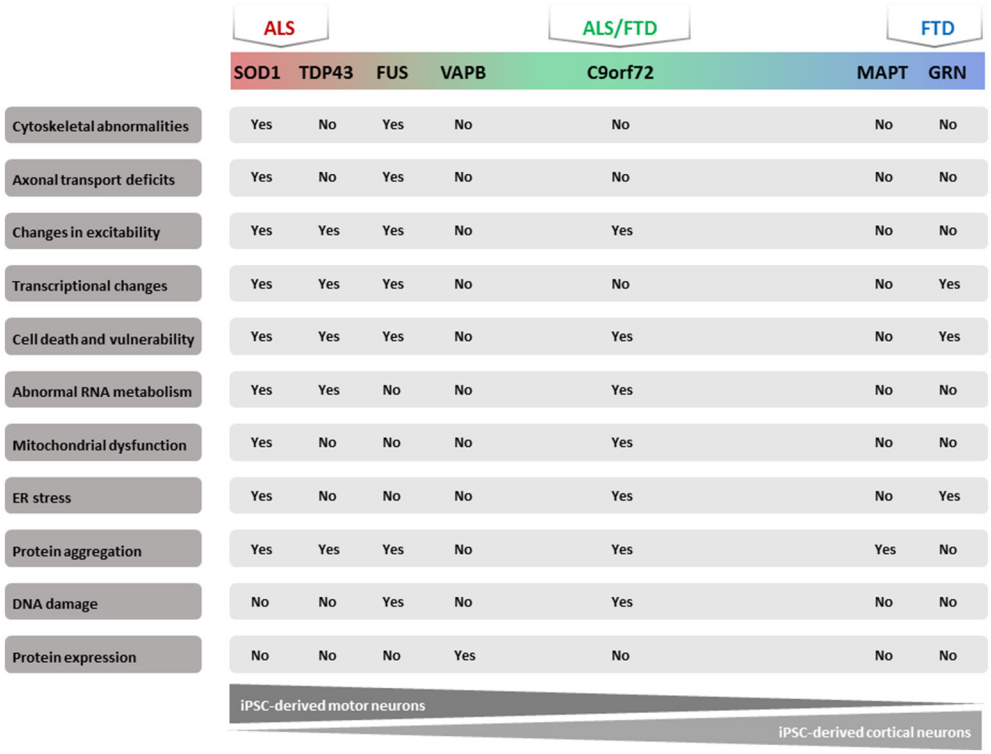

FIGURE 1 | Major phenotypes in ALS/FTD iPSC models. Summary of observed phenotypes from motor neurons or cortical neurons derived from ALS/FTD patients.

2014). Additionally, ALS-related morphological changes were observed *in vitro*, including a reduction in soma size and an altered dendrite length (Chen et al., 2014). This phenotype was linked to the dysregulation and aggregation of neurofilaments, an event that preceded the occurrence of neuronal apoptosis (Chen et al., 2014). RNA sequencing showed that several genes were misregulated in the patient-derived motor neurons in comparison to isogenic controls. These included transcripts related to cytoskeletal organization, mitochondrial function and structure, and protein translation (Kiskinis et al., 2014). Furthermore, the unfolded protein response was activated (Kaus and Sareen, 2015). Moreover, ER stress occurred in $SOD1^{A4V}$ motor neurons (Kiskinis et al., 2014). In another study, Wainger and colleagues used the same iPSC lines carrying the $SOD1^{A4V}$ mutation and demonstrated that patient-derived motor neurons showed consistent hyperexcitability (Wainger et al., 2014). The authors used this disease-specific phenotype as a tool for drug screening. Retigabine, a clinically approved anticonvulsant, blocked hyperexcitability in these patient-derived motor neurons by activating subthreshold Kv7 currents. Moreover, a significantly increased survival of motor neurons was observed in this study after treatment with retigabine (Wainger et al., 2014). This study provided the rationale to start a clinical trial using retigabine in ALS patients (Noto et al., 2016). However, hypoexcitability in combination with lower Na^+/K^+ ratios were observed in motor neurons from an ALS patient carrying the $SOD1^{D90A}$ mutation (Naujock et al., 2016). In addition, this hypoexcitability could be improved by the FDA-approved drug 4-aminopyridine (4AP) which is targeting K^+ currents (Naujock et al., 2016). Although it is still a debate whether hyper- or hypoexcitability plays a crucial role in ALS, it is evident that electrophysiological changes comprise a major phenotype in iPSC models, which may be a therapeutic avenue worth exploring.

TARDBP

All published studies using iPSCs derived motor neurons from mutant *TARDBP* patients reported some significant ALS-related pathological features, including motor neuron degeneration and accumulation of insoluble TDP-43 protein (Egawa et al., 2012).

The research group of Chandran first described a human motor neuron model derived from iPSCs from an ALS patient carrying the $TDP\text{-}43^{M337V}$ mutation (Egawa et al., 2012). The functional maturation of motor neurons was not affected by the mutation. However, the survival of these neurons was significantly decreased. Furthermore, these $TDP\text{-}43^{M337V}$ motor neurons also showed an increased level of soluble and detergent-resistant TDP-43 compared to healthy control lines. In addition, they also demonstrated that these neurons were more susceptible to inhibition of phosphoinositide 3-kinase (PI3K), while no abnormalities in response to inhibition of mitogen-activated protein kinase (MAPK) or ER stress induction were observed (Egawa et al., 2012). As some neurotropic factors rely on the PI3K pathway, this result supports the importance of neuronal trophic support. In a later publication, this research group used the same iPSC line ($TDP\text{-}43^{M337V}$) to generate almost pure astrocytes in order to investigate the astrocyte pathology in ALS

(Serio et al., 2013). Astrocytes from TDP-43^{M337V} iPSCs showed cytoplasmic mislocalization of soluble TDP-43 (Serio et al., 2013). In comparison to wild type astrocytes, TDP-43^{M337V} astrocytes showed a cumulative risk of death under basal conditions. However, these cells did not show increased levels of detergent resistant TDP-43, which is different from iPSC-derived motor neurons (Serio et al., 2013). Moreover, the authors used an isogenic control to confirm the observations linking TDP-43 cytoplasmic localization to survival in astrocytes (Serio et al., 2013). In addition, coculturing motor neurons and astrocytes showed that mutant TDP-43 iPSC-derived astrocytes had no effect on motor neuron survival (Serio et al., 2013). Conversely, murine glia overexpressing mutant SOD1 were shown to be toxic to cocultured human iPSC-derived motor neurons (Hedlund and Isacson, 2008).

Egawa and colleagues generated several iPSC lines from three ALS patients with different TDP-43 mutations (Q343R, M337V, and G298S). The authors did not observe that TDP-43 mutations impaired motor neuron maturation (Egawa et al., 2012). However, insoluble TDP-43 inclusions were found in these neurons (Egawa et al., 2012). In contrast to a previous report from Bilican et al. (2012), TDP-43 mRNA levels were higher in mutant cell lines compared to control lines. To explain this discrepancy, the authors suggested that the variation between different iPSC lines and the different purity of motor neurons could account for this difference (Bilican et al., 2012). The study also identified decreased neurite lengths and a higher vulnerability to oxidative stress in the mutant motor neurons. Interestingly, in their drug screening assay, anacardic acid, which is a histone acetyltransferase, was demonstrated as an efficient drug candidate which could reverse the disease-related phenotypes (Egawa et al., 2012).

Serio et al. focused on the physiological aspect of motor neurons carrying the TDP-43^{M337V} mutation by using the same iPSC lines used for generating astrocytes. Hyperexcitability at early stages followed by a progressive loss in action potential output and synaptic activity were shown in the patient-derived motor neurons. As mentioned above, this hyperexcitability phenotype is shared with iPSC-derived motor neurons from patients with SOD1 mutations (Serio et al., 2013).

The group of Gao derived neuronal cultures from iPSCs of an FTD patient, who also showed ALS symptoms, containing an A90V mutation in TDP-43 (Zhang et al., 2013). The A90V mutation is a rare mutation which was identified as a risk factor for both ALS and FTD (Zhang et al., 2013). The disease duration in a patient with this mutation can be more than two decades from the time of diagnosis, which is a particularly long disease duration for ALS. Mislocalized cytoplasmic TDP-43 and decreased expression of total TDP-43 after treatment with staurosporine were observed in the neurons derived from these patient iPSCs (Zhang et al., 2013). Interestingly, the neurons generated from a patient carrying the M337V mutation showed increased TDP-43 aggregates in the absence of any stress (Bilican et al., 2012). This suggested a cell-autonomous toxicity in TDP-43^{M337V} lines. In addition, decreased miR-9 expression was shown in neurons with A90V, as well as with the M337V mutation in TDP-43 (Zhang et al., 2013). Since miRNA misregulation has also been reported in FUS-ALS, this could reveal a common downstream process in different disease subtypes that are caused by mutations in different ALS-associated genes (Bilican et al., 2012; Zhang et al., 2013).

FUS

Over the past few years, several FUS-iPSC models have been generated. One publication described motor neuron cultures derived from two FUS-iPSC lines carrying a frameshift mutation at residue 511 (M511FS) or a H517Q point mutation. These motor neurons were shown to be hyperexcitable (Wainger et al., 2014). Conversely, another research group reported hypoexcitability in FUS lines carrying a point mutation (R521L or R521C) and a frame shift mutation (R495QfsX527) (Naujock et al., 2016).

Another study demonstrated cytoplasmic mislocalization and the formation of FUS aggregates in differentiated motor neurons from a patient carrying a P525L mutation (Liu et al., 2015). Similarly, motor neurons derived from ALS patients carrying a H517D mutation in FUS showed cytoplasmic FUS localization and stress granule formation under stress conditions. Moreover, exon array analysis combined with CLIP-seq data revealed aberrant gene expression and/or splicing patterns in patient-derived motor neuron precursor cells (Ichiyanagi et al., 2016). The cytoplasmic FUS mislocalisation was also observed in motor neurons derived from patients with a benign R521C mutation and the more severe R495Qfs527 and Asp502Thrfs*27 frameshift mutations (Higelin et al., 2016). Moreover, the amount of cytoplasmic FUS accumulation correlated with the clinical severity of the underlying FUS mutation (Higelin et al., 2016). Furthermore, the severity of the FUS mutation, as well as neuronal aging, also induced spontaneous formation of cytoplasmic FUS inclusions (Higelin et al., 2016). These aggregates showed typical characteristics of FUS-ALS including the presence of methylated FUS (Higelin et al., 2016). Additionally, higher vulnerability and increased DNA damage were observed in patient-derived motor neurons after irradiation (Higelin et al., 2016).

Lenzi et al. used iPSCs derived from patient fibroblasts carrying a R514S or a R521C mutation in combination with iPSC cells, in which the P525L mutation was introduced using transcription activator-like effector nucleases (TALENs)-directed mutagenesis (Lenzi et al., 2015), to differentiate into motor neurons. The presence of mutant FUS resulted in the aberrant localization and recruitment of FUS into stress granules (SGs). However, this only occurred upon induction of stress and the incorporation into SGs was proportional to the amount of cytoplasmic FUS (Lenzi et al., 2015).

We recently generated and characterized iPSCs from ALS patients with R521H and P525L mutations in FUS. Patient-derived motor neurons showed typical cytoplasmic FUS pathology, hypo-excitability, as well as progressive axonal transport defects. Cytoplasmic mislocalization of FUS was most pronounced in the P525L mutant line and was not specific for motor neurons. Both cytoplasmic FUS pathology and axonal transport defects were rescued by "clustered regularly interspaced short palindromic repeats" (CRISPR)/Cas9-mediated

genetic correction of the *FUS* mutation in patient-derived iPSCs. Furthermore, we could rescue the defects pharmacologically by histone deacetylase 6 (HDAC6) inhibition and by genetic silencing of HDAC6, which suggests that this could become a new therapeutic strategy for ALS (Guo et al., 2017).

C9ORF72

The immense expansion of G_4C_2 repeats in *C9ORF72* makes it particularly difficult to develop good animal models. As a consequence, iPSCs could offer the ideal solution for modeling *in vitro* the effect of the hexanucleotide expansion in *C9ORF72*. Three potential mechanisms have been proposed to explain the pathogenic role of the hexanucleotide expansion in *C9ORF72*. First, a reduction in the expression level of *C9ORF72* observed in some *C9ORF72* patients has led to the hypothesis that a loss of *C9ORF72* may contribute to the disease (Waite et al., 2014). Second, the accumulation of RNA foci containing the hexanucleotide expansion found in the brain and spinal cords of patients suggested a disease mechanism, involving a toxic gain of function (DeJesus-Hernandez et al., 2011). This is thought to be mediated by repeat-containing RNA that can potentially bind RNA-binding proteins (DeJesus-Hernandez et al., 2011). Third, both the sense and antisense repeat RNAs can undergo repeat-associated non-ATG (RAN) translation, resulting in the formation of a series of potentially toxic DPRs (Gendron et al., 2013; Zu et al., 2013). RAN translation can occur from both sense and antisense expansion transcripts, resulting in the expression of six RAN proteins (antisense: Pro-Arg, Pro-Ala, Gly-Pro; and sense: Gly-Ala, Gly-Arg, Gly-Pro). These DPRs form neuronal cytoplasmic and intranuclear inclusion in patients carrying the expansion (Gendron et al., 2013; Zu et al., 2013). However, the exact contribution of each of these mechanisms to neuronal death is not yet clear.

Several studies on *C9ORF72* patient iPSCs have shown the potential of iPSCs in recapitulating the major pathological signatures of the disease. In 2013, three groups generated iPSCs from ALS/FTD patients carrying the *C9ORF72* mutation (Almeida et al., 2013; Donnelly et al., 2013; Sareen et al., 2013), in which several aspects of *C9ORF72*-related pathology were observed. RNA foci were detected in patient-derived neurons and the sequestration of RNA binding proteins, including ADARB2, hnRNPA1, and Pur-α, by the expanded RNA repeat was observed (Donnelly et al., 2013; Sareen et al., 2013). Dipeptide RAN pathology was also described in neurons derived from *C9ORF72* patients (Almeida et al., 2013; Donnelly et al., 2013). Interestingly, Westergard and collaborators demonstrated, by using *C9ORF72* iPSCs-derived spinal motor neurons (sMNs), evidence for cell-to-cell spreading of DPRs in a co-culture system of control and *C9ORF72* iPSC-derived sMNs (Westergard et al., 2016). In terms of haploinsufficiency, *C9ORF72* expression was observed to be reduced in patient-derived neurons compared to controls (Almeida et al., 2013; Donnelly et al., 2013), whereas in the study of Sareen et al., no change in *C9ORF72* expression was found in patient-derived motor neurons (Sareen et al., 2013). From a therapeutic point of view, two studies investigated antisense oligonucleotides (ASOs) in *C9ORF72* iPSCs-derived neurons (Donnelly et al., 2013; Sareen et al., 2013). Interestingly, after ASO treatment, the toxicity associated with the endogenous *C9ORF72* mutation was abrogated (Donnelly et al., 2013; Sareen et al., 2013). Moreover, the *C9ORF72* knockdown with ASOs had no impact on iPSCs-derived neuron survival, leading the authors to argue against a loss of function mechanism as the major cause of *C9ORF72* pathology and toxicity seen in iPSCs (Donnelly et al., 2013; Sareen et al., 2013).

Aside from recapitulating the major pathological hallmarks of the disease, iPSC-based platforms have also revealed novel mechanisms of neurodegeneration. Two independent studies showed impaired nucleocytoplasmic transport in patient-derived neurons (Freibaum et al., 2015; Zhang et al., 2015). Zhang and colleagues showed that RanGAP1, a key regulator of nucleocytoplasmic transport, physically interacted with the expanded RNA foci and was mislocalized in the cytoplasm of *C9ORF72* iPSC-derived neurons. In addition, the authors also showed that nuclear import of proteins and RNAs was impaired in patient iPSC-derived neurons, and these deficits were rescued by antisense oligonucleotides targeting the *C9ORF72* RNA (Zhang et al., 2015). Similarly, Freibaum and colleagues observed impaired nucleocytoplasmic transport in *C9ORF72* iPSC-derived neurons. Furthermore, investigation of the total RNA distribution revealed increased nuclear RNA retention in *C9ORF72* iPSC-derived cortical neurons (Freibaum et al., 2015).

Vulnerability to ER and oxidative stress was also investigated in *C9ORF72* iPSCs-derived motor neurons. Dafinca and colleagues showed that *C9ORF72* iPSC-derived motor neurons exhibited loss of Ca^{2+} homeostasis, which was associated with a decrease in mitochondrial potential and an increase in ER stress (Dafinca et al., 2016). Moreover, an increase of yH2AX, a marker of DNA damage, was observed in *C9ORF72* iPSCs-derived motor neurons in an age-dependent manner (Lopez-Gonzalez et al., 2016). Interestingly, the ectopic expression of the DPR protein $(GR)_{80}$, but not $(GA)_{80}$, increased DNA damage in iPSC-derived control neurons. Pharmacological or genetic reduction of oxidative stress partially reduced DNA damage in *C9ORF72*-derived motor neurons suggesting that oxidative stress could play an important role in the disease mechanism (Lopez-Gonzalez et al., 2016).

Several studies performed electrophysiology on *C9ORF72* iPSC-derived neurons to investigate possible impairments in excitability, as observed in *C9ORF72* patients (Williams et al., 2013; Geevasinga et al., 2015). Using multi-electrode array and patch clamp recordings, Wainger and colleagues showed network hyperexcitability in *C9ORF72* iPSC-derived motor neurons (Wainger et al., 2014). In direct contrast, another group showed a diminished capacity to fire continuous spikes upon depolarization in *C9ORF72* iPSC-derived motor neurons compared to control motor neurons (Sareen et al., 2013). Moreover, *C9ORF72* iPSC-derived motor neurons showed altered expression of genes involved in membrane excitability, including the delayed rectifier potassium channel (KCNQ3) which is consistent with hypoexcitability (Sareen et al., 2013). As indicated before, these divergent findings can possibly be explained by the temporal analysis of *C9ORF72* iPSC-derived motor neuron excitability as performed by Devlin and colleagues (Devlin et al., 2015). This research group demonstrated that

patient iPSC-derived motor neurons displayed an intrinsic hyperexcitability at early time points (21–28 days in culture), followed by a loss in action potential output and synaptic activity in cells maintained for up to 70 days in culture. This loss of functional output was correlated to a progressive loss of both voltage-activated Na^+ and K^+ currents (Devlin et al., 2015). Further experimental evidence is required to determine whether the altered excitability observed in *C9ORF72* motor neurons plays indeed a pathogenic role in ALS or whether it is related to a homeostatic response to specific culture conditions.

Non-cell autonomous toxicity mechanism were also investigated using patient iPSCs-derived cells. Mayer and colleagues showed that *C9ORF72* iPSC-derived astrocytes were toxic when co-cultured with motor neurons. This strongly indicates that astrocytes play an important role in the *C9ORF72*-mediated pathology (Meyer et al., 2014). Similarly, oligodendrocytes from *C9ORF72* patients, obtained through different reprogramming protocols, induced motor neuron death both through conditioned media as well as in co-cultures (Ferraiuolo et al., 2016).

Several studies provided evidence for a role of *C9ORF72* in important cellular functions suggesting that a loss of function of *C9ORF72* could eventually contribute to the disease process. *C9ORF72* is homologous to members of the DENN (differentially expressed in normal and neoplastic cells) domain containing protein family and is predicted to function as a guanine-nucleotide exchange factor for several Rab proteins (Levine et al., 2013). Consistent with the prediction of its function, *C9ORF72* protein was found to colocalize with Rab proteins and to be involved in endosomal trafficking and autophagy (Farg et al., 2014). Subsequently, several studies showed a role of *C9ORF72* in the induction of autophagy (Sellier et al., 2016; Sullivan et al., 2016; Webster et al., 2016; Yang et al., 2016) and reduction in the basal autophagy levels in *C9ORF72* patient-derived neurons (Webster et al., 2016). Moreover, increased level of p62, an autophagy marker, were observed in *C9ORF72* iPSCs-derived neurons (Almeida et al., 2013; Dafinca et al., 2016), reminiscent of the p62 pathology observed in *C9ORF72* patients (Mackenzie et al., 2014). Recently, a study in human iPSC-derived neurons revealed that *C9ORF72* modulates cytoskeletal actin dynamics via phosphorylation of cofilin and shRNA mediated knockdown of *C9ORF72* resulted in axonal outgrowth deficits (Sivadasan et al., 2016).

Taken together, the above studies used iPSC technology to establish *in vitro* models for *C9ORF72*-ALS. Apart from providing novel insights into the disease mechanisms, antisense strategies were suggested as a promising treatment option to counteract the negative impact of the hexanucleotide repeat expansions in *C9ORF72*.

VAPB

A mutation in the "*vesicle-associated membrane protein-associated protein B and C" (VAPB*) gene is a rare cause of ALS (subtype 8), and has an autosomal dominant mode of inheritance. Mitne-Neto and colleagues generated iPSCs from an ALS patient with a P56S mutation in *VAPB*, as well as from their non-carrier siblings. These cells could be differentiated into mature motor neuron. No obvious alterations in VAPB distribution were detected in patient-derived cells. Similarly, no aggregates were found in patient cells, even after inducing stress in the cells using the proteasome inhibitor MG132. However, a significant decrease in VAPB protein level was observed in patient-derived motor neurons and the reduction was ∼50% compared to controls (Mitne-Neto et al., 2011).

Sporadic ALS

Although sporadic forms of ALS account for 90% of total ALS cases, it is much more difficult to model this form of the disease as the exact causes are unknown. As already indicated, iPSC technology could give us the unique opportunity to mimic some pathological and electrophysiological phenotypes *in vitro*.

The first publication on a sporadic ALS iPSC model was from Burkhardt and colleagues. Skin fibroblasts were reprogrammed from 16 sporadic ALS patients, 8 familial ALS patients and 10 healthy persons as controls. Nearly 100 iPSC clones from 34 patients were analyzed. Spontaneous TDP-43 pathology was observed in iPSC-derived motor neurons from 20% of the sporadic ALS patients (Burkhardt et al., 2013). These TDP-43 aggregates were hyperphosphorylated, but no ubiquitination was observed. Additionally, the TDP-43 aggregates in motor neurons differentiated from iPSC were validated in anterior horn neurons of spinal cord and cortical neurons from one of the patients who donated the fibroblasts. Interestingly, this was the first time that a pathological phenotype found in iPSC-motor neurons could be linked to *post mortem* tissue from the same ALS patient, thus validating iPSC-derived models as a powerful tool.

iPSC-motor neurons from one sporadic ALS patient were also used for a drug screening assay (Burkhardt et al., 2013). In total, 1757 bioactive compounds were tested on these neurons and the percentage of neurons containing TDP-43 aggregates was used as the criterion to analyze the efficiency of these compounds. After two rounds of selection, four classes of compounds, including cyclin-dependent kinase inhibitors, Digoxin, Lanatoside C, and Proscillaridin A, were obtained that reduced in a dose-dependent manner the percentage of cells with TDP-43 aggregates (Burkhardt et al., 2013). These findings rely on a prominent role of protein aggregation in the progression of neuronal degeneration in ALS (Burkhardt et al., 2013). Another large gene profiling experiment starting from differentiated motor neurons from sporadic ALS patients showed that most dysregulated genes were related to mitochondrial function. As a consequence, this result highlights mitochondrial participation in motor neuron degeneration and indicates that cell autonomous mechanisms could be associated with sporadic ALS (Alves et al., 2015).

Modeling FTD Using iPSCs

MAPT

From a pathological point of view, there are three main subtypes of FTD: FTD-TDP, FTD-FUS, and FTD-tau. Tau pathology is also a hallmark of Alzheimer's disease and progressive supranuclear palsy (Xia and Dickerson, 2017). FTD-tau is caused by mutations in the *MAPT* gene which encodes the tau protein (Rovelet-Lecrux et al., 2010; Ling et al., 2013). MAPT transcripts are diverse and

widely expressed in the nervous system. The expression level of these transcripts depends on the neuronal maturation stage and the neuronal subtype. Tau is a microtubule-associated protein which is highly expressed in neurons (Rovelet-Lecrux et al., 2010).

Fong and colleagues generated iPSC from an individual carrying a heterozygous A152T mutation in tau (Fong et al., 2013). By using zinc-finger nuclease-mediated gene editing, they developed two isogenic iPSC lines: one with the point mutation corrected, and one with a homozygous point mutation. These iPSCs were successfully differentiated into neurons which was confirmed by a "microtubule-associated protein 2" (MAP2) staining (Fong et al., 2013). The survival of these neurons was affected by the point mutation (Fong et al., 2013). Axonal degeneration and caspase-cleaved tau fragmentation increased in severity from heterozygous to homozygous mutant lines, while corrected isogenic control lines remained normal (Fong et al., 2013). Furthermore, the amount of phospho-tau-positive neurons also increased in a dose-dependent manner. Taken together, this iPSC-derived model mimicked the tau pathology *in vitro*, confirmed the causal link between the tau mutation and tau pathology and helped to elucidate some of the key underlying pathogenic mechanisms.

GRN

The *GRN* gene encodes a progranulin precursor protein which is cleaved into different Granulins (Grns) that are glycosylated and secreted. Progranulin as well as GrnE have neurotrophic actions both *in vitro* and *in vivo* (Bhandari and Bateman, 1992; Van Damme et al., 2008). Autosomal dominant mutations in the *GRN* gene have been implicated in up to 25% of familial FTD cases and these mutations seem to cause the disease due to haploinsufficiency (Guven et al., 2016; Rainero et al., 2017).

To investigate the pathogenic mechanism of *GRN* in FTD, two research group created iPSCs from FTD patients with different mutations in *GRN*. Almeida and colleagues differentiated iPSCs containing a non-sense mutated *GRN* (S116X) into neurons and microglia (Almeida et al., 2012). The mixed postmitotic neurons expressed MAP2, vesicular glutamate transporter 1, glutamate decarboxylase and tyrosine hydroxylase. Compared to controls, no differences were observed in the neuronal differentiation of mutant cells (Almeida et al., 2012). Grns mRNA levels were significantly decreased in fibroblasts, iPSCs and iPSC-derived neurons from the GRN^{S116X} FTD patient compared to normal control and sporadic FTD patients (Almeida et al., 2012). In addition, GRN^{S116X} neurons were more sensitive to specific protein kinase inhibitors which indicates that the PI3K/Akt and MEK/MAPK signaling pathways could be involved in the molecular pathogenesis of FTD. Neither mitochondrial nor oxidative stressors increased the sensitivity of FTD patient derived neurons, implicating that these two pathway were not affected by the GRN level (Almeida et al., 2012). Both neurons from GRN^{S116X} FTD patient and sporadic FTD patient showed vulnerability to ER stress in a GRN-independent way (Almeida et al., 2012).

We were involved in the creation of iPSCs from FTD patients carrying the $GRN^{IVS1+5G>C,null}$ mutation and haploinsufficiency of *GRN* was observed in these FTD-iPSCs (Raitano et al., 2015). The iPSCs were further differentiated into cortical neurons, which are affected in FTD patients. Although neuroprogenitors could be generated without any problem, the generation of "COUP-TF-interacting protein 2" (CTIP2), forkhead box protein P2 or "T-box, brain, 1" (TBR1) positive cortical neurons was dramatically lower in FTD patient lines compared to lines from their normal family members. This was specific for cortical neurons as the FTD-iPSCs could be normally differentiated into motor neurons. After introduction of *GRN* cDNA into the AAVS1 locus of the FTD-iPSC line, the cortical neurogenesis defect was restored. RNA-seq data showed that genes related to the Wnt/β-catenin signaling pathway were higher expressed in FTD-iPSCs compared to a corrected control line or an ESC line (Raitano et al., 2015). After treatment with IWP2, an inhibitor of active β-catenin levels, more brain lipid-binding protein- and CTIP2-positive cells were generated from the FTD patient lines. All together, these results highlight the Wnt signaling pathway as a potential therapeutic target in FTD (Raitano et al., 2015).

C9ORF72

The first iPSCs from FTD patients with *C9ORF72* hexanucleotide repeats were created by Almeida and colleagues (Almeida et al., 2013). They developed several iPSC lines from two patients with *C9ORF72* repeats (G_4C_2 > 1,000 repeats) belonging to the same FTD family. Similar to the effect of other mutations causing ALS/FTD, the maturation process of neurons was not impaired. RNA foci were observed in fibroblasts, iPSCs, iPSC-derived neurons from the patient cell line, but not in healthy controls. RAN translational products were also found in the iPSCs-derived neurons from patients. Decreased cell viability and increased caspase-3 activation were observed in human neurons carrying G_4C_2 repeat expansions after treatment with two different autophagy inhibitors (Webster et al., 2016). Furthermore, p62 significantly accumulated in neurons with *C9ORF72* repeats, but not in neurons derived from control iPSCs of healthy persons or neurons derived from iPSCs of an FTD patient with a *GRN* mutation (Webster et al., 2016). These results strongly suggest an involvement of autophagy in *C9ORF72*-FTD.

CURRENT LIMITATIONS TO iPSC TECHNOLOGY

Although the iPSC technology has been used for modeling human diseases and drug screenings worldwide, there are still a number of limitations that should be solved. In the research area of ALS and FTD, more specific questions have to be answered in order to validate and improve this emerging tool (**Figure 2**).

Dissimilarity among iPSC Lines

As more and more iPSC lines are established and compared between different laboratories, large variations are discovered among different iPSC lines and, surprisingly, even between iPSC lines originating from the same donor sample. As a consequence, it is extremely important to standardize the different procedures involved in reprogramming in order to minimize these differences.

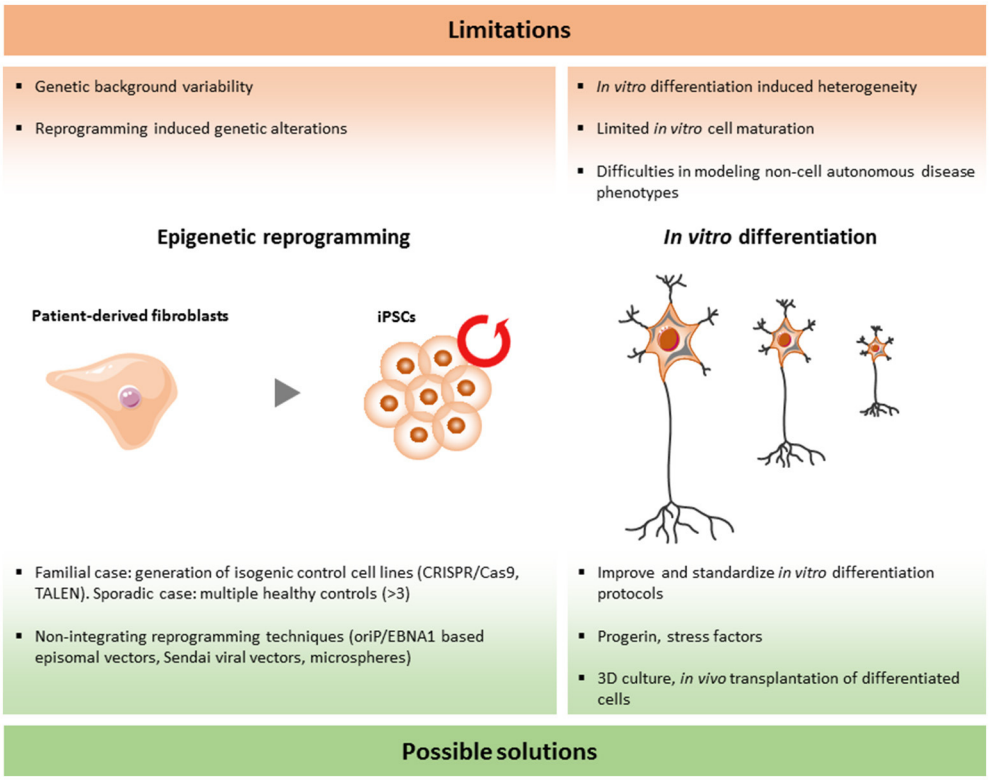

FIGURE 2 | Limitations and current solutions to iPSC technology in ALS/FTD.

Setting Back the Clock: The Reprogramming Process

The initial work of Shinya Yamanaka in 2007 drew global interest to iPSC disease modeling. In their first description of the reprogramming method of somatic cells, four transcription factors (Oct3/4, Sox2, Nanog, cMyc) were delivered to human fibroblasts by integrating retroviral vectors (Takahashi et al., 2007). This method is still widely used to develop iPSC lines. However, this will result in the random insertion of these transgenes into the genome. The exact effect of this random integration is unknown and genomic modifications could have an unexpected and unwanted impact on the iPSC lines. For instance, transcriptional changes of certain genes could interfere with the analysis of the phenotypes. To avoid this drawback, Cre/loxP recombination and piggyBac transposon systems have been proposed to remove the transgenes, but both of these methods are time consuming and have a low efficiency (Karow et al., 2011; Zhou and Zeng, 2013). The introduction of integration free vectors is a milestone to avoid this problem in the reprogramming process. The first integration free reprogramming method was the cloning of the reprogramming factors into oriP/EBNA1 based episomal vectors, which are gradually lost during cell proliferation (Yu et al., 2009). Another method is the use of inactivated Sendai viral vectors. These are RNA virus based vectors with a high transfection efficiency, without the capability to integrate into the genome and they are also easily removed after a few cell passages. Another concern which was raised from research with mouse iPSCs is that the selection of reprogramming factors can influence the differentiation potential of iPSCs (Buganim et al., 2014). The use of the standardized integration free "Sendai Reprogramming Kit" with well-characterized reprogramming factors and protocols may help to avoid these unwanted effects (Trott et al., 2017). In addition, a novel approach was proposed recently by using microspheres conjugated with reprogramming factors for delivery into human fibroblasts (Unciti-Broceta et al., 2015). If this is a success, the use of small molecules to reprogram could be the next important step.

Pluripotency

A critical step in the procedure to obtain iPSCs is the characterization of the pluripotency potential. The final goal of the reprogramming process is to obtain iPSCs that are comparable to ESCs with respect to their pluripotency. The traditional status quo for pluripotency characterization is a combination of gene and antigen expression analysis, morphology, and capacity to differentiate *in vivo*. With respect to this procedure, several questions could be raised. In addition, the random inactivation of one of the X chromosomes (lyonization) is also a factor that could cause variation amongst female iPSC lines (Tomoda et al., 2012; Dandulakis et al., 2016). This can change the gene expression of some neuronal genes located on the X chromosome.

Analysis of teratomas has always been the golden standard for the confirmation of pluripotency (Nelakanti et al., 2015).

However, it has been reported that even partially reprogrammed colonies can form teratomas *in vivo* (Baker, 2012). Therefore, more landmarks have been proposed to identify a fully reprogrammed iPSC. In theory, and to avoid these problems, a whole genome transcription and methylation analysis could provide more reliable information. In practice, this technology is expensive and is not available to every lab. As a consequence, the ideal situation would be the establishment of an international consortium collecting and storing all fully characterized iPSC lines containing all possible information for each of these established iPSC lines.

Controls

For iPSC disease modeling, the ultimate identification of disease phenotypes largely depends on the availability of reliable controls. It was shown in numerous situations that genetic background could play an important role in the disease manifestation and disease progression (Turner et al., 2017). For ALS and FTD, some causative genes have already been identified, but even for these there are huge variations in the disease process (Régal et al., 2006). It is not clear what causes these differences. However, unknown environmental factors and genetic risk factors may play a role in these dissimilarities. In order to avoid the influence of this genetic background on the disease-related phenotypes, proper controls are crucial. For the familial cases, genome editting is a promising strategy to correct point mutations or to remove pathological repeats in iPSC lines in order to create isogenic controls. Zinc finger nucleases, TALENs, as well as the more recently discovered CRISPR guided Cas9 nuclease system are the widely used technologies to perform these modification (Carroll, 2011; Beurdeley et al., 2013; Ran et al., 2013). A method based on piggyBac transposons allows seamless genome modification in patient derived iPSCs (Xie et al., 2014). The isogenic control keeps exactly the same genetic background as the mutant iPSCs. As there is still the risk of inducing off-targets effects or unwanted mutations by using these gene engineering technologies, exon sequencing and the choice of unique target sequences is crucial to minimize these drawbacks (Cho et al., 2014). In conclusion, corrected isogenic lines can be considered as the best controls for the familial type of ALS/FTD patient lines.

Differences between Differentiation Methods

Numerous neuronal subtypes exist and selective vulnerability of these different neurons to the disease process is typical for the various neurodegenerative disorders. Motor neurons are mainly degenerating in ALS, while 70% of the affected cells in FTD patients are pyramidal cortical neurons (Rowland and Shneider, 2001; Boxer and Miller, 2005; Ling et al., 2013). By using different combinations of small molecules, several methods were developed to differentiate iPSCs into motor neurons or cortical neurons, but with varying efficiencies.

Identity of Motor Neurons

According to developmental studies, motor neuron specification goes through multiple steps and results in different subtypes of motor neurons. The inner cell mass first differentiates into ectoderm. Later, the inhibition of TGF-β/Smad signaling in combination with the enhancement of FGF and Wnt signaling triggers the specification from ectoderm to neuroectoderm (Vallier et al., 2009; Davis-Dusenbery et al., 2014; Kiecker et al., 2016). In the next step, a retinoic acid (RA) gradient and the activity of FGFs and "growth differentiation factor 11" (Gdf11) promote the neural tube pattern which results in the initial status of the central nervous system (Davis-Dusenbery et al., 2014; Maury et al., 2014). By the different regulation of *CDX* and *HOX* gene expression, motor neuron can be further specified into different subtypes (Davis-Dusenbery et al., 2014; Kiecker et al., 2016). For spinal cord motor neuron specification, "sonic hedgehog" (Shh), "bone morphogenic protein" (BMP)/TGFβ signaling, and the floor plate in the neural tube function together to trigger this specific differentiation pattern (Ribes et al., 2010).

In vitro differentiation from ESC/iPSC to motor neurons is also based on this process by triggering the different gene expression in every stage of the differentiation. The best way to generate motor neurons *in vitro* is to use small chemicals with different dosages at specific intervals to mimic every step of specification that takes place during development. The first description of motor neuron differentiation from human ESCs was published in 2005 and was based on RA inducing neuronal rosette formation and Shh inducing spinal cord motor neuron specification (Shin et al., 2005). This protocol results in Hb9/Islet1 positive, functional motor neurons with about 20% efficiency. Moreover, it is a time consuming protocol which requires 33 days to achieve initial motor neurons and a much longer time in order to gain functional mature motor neurons (Shin et al., 2005). Subsequently, several modified protocols were optimized based on the original one (Sances et al., 2016). They commonly share three key phases: neuronal induction, motor neuron specification, and motor neuron maturation. SB-431542, LDN-193189, and dorsomorphin were used for inhibition of TGFβ/BMP signaling in order to trigger neuronal differentiation (Patani et al., 2011; Amoroso et al., 2013; Gouti et al., 2014; Kiskinis et al., 2014; Maury et al., 2014; Du et al., 2015). In addition, the FGF pathway and Wnt signaling were also used to promote this differentiation stage (Dimos et al., 2008; Hu et al., 2009; Patani et al., 2011; Gouti et al., 2014). Nestin, PAX6 and Sox1 expression are used to check the quality of the neuronal progenitors (Yuan et al., 2011; Tian et al., 2013; Li et al., 2017). RA is used to link the neuroprogenitor phase to the motor neuron specification phase and a combination of RA, Shh, purmorphamine or SAG were used to initiate the Shh signaling pathway which contributes to the ventralization (Lee et al., 2007; Dimos et al., 2008; Amoroso et al., 2013; Kiskinis et al., 2014; Maury et al., 2014; Qu et al., 2014). The motor neuron progenitors should be positively stained for Olig2 (Masahira et al., 2006; Li et al., 2008; Hu and Zhang, 2009; Lee et al., 2014). It was reported that γ-secretase inhibition can significantly increase the motor neuron production upon SHH pathway activation (Maury et al., 2014). The expression of Hb9 and Islet1 expression are used as the criteria for the success of the initial motor neuron differentiation (Davis-Dusenbery et al., 2014). For the final motor neuron maturation, neurotrophic factors are applied to promote the formation of mature functional motor neurons which form

functional synapses, that are electrophysiologically active and that can form neuromuscular junctions when co-cultured with myoblasts (Chen et al., 2014; Davis-Dusenbery et al., 2014; Sances et al., 2016).

As more and more protocols are reported, it is crucial that the criteria used to obtain and to characterize motor neurons are harmonized. This harmonization is also important for drug screening and to increase the success of translation of the results to the clinic. This could also help to obtain more convincing and reproducible results, as some findings obtained with motor neurons derived from iPSCs couldn't be replicated in different labs. This could, at least partially, be due to the different protocols using different molecules at different concentrations. For instance, the level of antioxidants present in the culture media could affect ALS-related phenotypes, as oxidative stress seems to be involved in triggering the disease process (Barber et al., 2006; Mattson and Magnus, 2006; Niedzielska et al., 2016). In addition, the amount and/or type of neurotrophic factors could also affect the appearance of ALS-related phenotypes, as some neurotrophic factors could affect ALS (Henriques et al., 2010). This should also be taken into account. As indicated previously, checking specific motor neuron markers (Islet1, Hb9, ...) in combination with functional analysis is crucial to identify motor neurons in culture (Davis-Dusenbery et al., 2014; Sances et al., 2016).

By definition, both upper and lower motor neurons are affected in ALS patients (Nijssen et al., 2017). Although most of the protocols are focused on making spinal cord motor neuron with a high efficiency, only one study optimized a protocol to induce around 52% of upper motor neurons, characterized by PHOX2B and TBX20 upregulation (Maury et al., 2014). This was achieved by using lower concentrations of RA and Wnt agonist (Maury et al., 2014). This opens new perspectives as it gives the opportunity to study different motor neuron types, which could also help to gain more insights into ALS.

Another important drawback of iPSC modeling is that the motor neurons are not in their natural environment. Even co-cultures of neurons with other cell types will never be able to model the complexity of the *in vivo* environment. One possible solution could be to inject iPSCs into the central nervous system in animal models to study their integration, survival, and behavior in the *in vivo* enviroment.

Reliability of Cortical Neuron Cultures

During development, mammalian cortical neurons start specialization in the rostral dorsal part of the neural tube (Pierani and Wassef, 2009; O'Leary et al., 2013). The neuroepithelial cells near the ventricular zone (VZ) and subventricular zone (SVZ) develop into the pyramidal neurons, interneurons, and glial cells in the cortex (Noctor et al., 2004). The early postmitotic neurons subsequently migrate away to form the cortical plate (CP) and then separate into a marginal zone (MZ, Layer I) and a deep subplate (SP) (Noctor et al., 2004). Cajal-Retzius cell are developed from the layer I (Noctor et al., 2004). Other specific cortical layers (II-VI) are formed from the CP (Noctor et al., 2004). The SP is formed by migrating through the intermediate zone (IZ), which is a layer that finally contains the axonal tracts of the cortex. At the same time, the SVZ appears between the VZ and the IZ (Noctor et al., 2004; Clinton et al., 2014). The SVZ mainly produces glia (Noctor et al., 2004; Clinton et al., 2014). The glia function as scaffolds to support and direct the migration of new neurons from the VZ to the CP (Noctor et al., 2004; Clinton et al., 2014). Maturing neuronal axons migrate out toward their targets and form functional synapses (Noctor et al., 2004).

The cortical neuron differentiation *in vitro* seems to be also very complicated. There are two main reasons for this. First, the cortex contains plenty of neuronal subtypes, which work together in a very complex way. Second, the architecture of the different cortical layers is very difficult to achieve *in vitro*.

In order to address the differentiation in the different cortical subtypes, a protocol was developed without the addition of morphogens (Espuny-Camacho et al., 2013). Only the BMP inhibitor, Noggin, was used to increase the neuronal differentiation from human iPSCs (Espuny-Camacho et al., 2013). Furthermore, pyramidal neurons from different layers were obtained at different time points (Espuny-Camacho et al., 2013). Pax6 and Otx1/2 were expressed after 10–19 days of differentiation, which resembles early dorsal forebrain (Espuny-Camacho et al., 2013). The first neurons are positive for TBR1, calretinin, and reelin which are the markers for Cajal-Retzius neurons (Espuny-Camacho et al., 2013). Around 24–28 days, FOXP2 positive and TBR1/CTIP2 positive neurons of layer VI started to appear. At 37 days, CTIP2 positive neurons were present (Espuny-Camacho et al., 2013). From 40 days on, the TBR1-positive neurons were increasing, while calretinin positive neurons decreased (Espuny-Camacho et al., 2013). This resembles the TBR1+/calretinin- deep layer neurons (VI and V) (Espuny-Camacho et al., 2013). At the last time point (61–72 days), STATB2 positive neurons resembling the layer V and upper layer appeared in culture. In addition, CUX1/BRN2 positive neurons of the upper layer appeared at the end (Espuny-Camacho et al., 2013). At the same time, deep layer markers were downregulated. Electrophysiological properties were confirmed by patch clamp experiments. After transplantation into mouse brain, the *in vitro* derived cortical neurons could integrate properly and formed axonal projections and dendritic patterns (Espuny-Camacho et al., 2013). Another study reported the differentiation of cortical neurons by using different morphogens in a 2D system (Shi et al., 2012). In this 2D model, neuronal subtypes that belong to different cortical layers appeared at staggered time points, much like during development, and integrated amongst each other.

Another major challenge is to mimic the complex structure of the cortex *in vitro*. Some initial spatial patterns are observed in 2D culture. For instance, neuronal progenitors form rosettes which have typical apicobasal polarity and interkinetic nuclear migration (Espuny-Camacho et al., 2013). These features are typical in the VZ during cortical development (Eom et al., 2013). However, long term 3D cultures of hESC/iPSC derived neocortex could solve, at least partially, the problem of the complex cortical

organization (Lancaster and Knoblich, 2014; Zhang Z.-N. et al., 2016). These 3D cultures started from hESC/iPSC (Lancaster and Knoblich, 2014). Wnt inhibitor and TGFβ inhibitor combined with low cell adhesion culture plate were used to trigger cortical generation during day 0 to day 18 (Lancaster and Knoblich, 2014). From day 18 onwards, further long term cortical neuroepithelial cultures were started by controlling the environment (proper substrate and 40%O_2/5%CO_2; Lancaster and Knoblich, 2014). These cortical neuroepithelial cells could be kept in culture for more than 13 weeks and formed multilayer structures including three neuronal zones (SP, CP, and Cajal-Retzius cell zones) and three progenitor zones (VZ, SVZ, and IZ), which were similarly organized as in the human fetal cortex (Lancaster and Knoblich, 2014). Although it is a complicated protocol, this breakthrough will certainly contribute to a better modeling of FTD *in vitro* and has the potential to be used for future drug testing.

Relevance of Phenotypes to ALS/FTD

Aging

Both ALS and FTD are age-related neurodegenerative diseases with symptoms occurring late in life. As a consequence, the question arises whether relevant phenotypes can be observed in cultured motor neurons that can be kept in culture for several weeks or at most a few months. When ALS/FTD patient are sick, their neurons have already suffered the consequence of the disease process for many years. In contrast, the iPSC-derived neurons are much younger and could more closely resemble the fetal stages, rather than an adult stage. In the best case, these young neurons could mimic the presymptomatic phase of ALS/FTD (Inoue, 2010). Despite the fact that proteomic data indicate that there are clear resemblances between motor neurons derived from endogenous spinal motor neurons (Toma et al., 2015), it is reasonable to expect that aging could play a crucial role in the development of the disease-related phenotypes. To properly mimic late stages of ALS/FTD *in vitro*, inducing aging and setting landmarks of aging could be necessary. Nine features of mammalian aging are proposed as typical hallmarks, including genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (López-Otín et al., 2013). One approach of inducing aging in iPSC-derived neurons is to use progerin (Miller et al., 2013). Progerin is an aberrant form of the nuclear architectural protein lamin A and can cause the premature aging disease Hutchinson-Gilford Progeria via inducing DNA damage (Musich and Zou, 2011; Miller et al., 2013). It is also observed in physiological aging. By treating neurons with progerin for a few days, multiple aging-related characteristics were observed including genomic instability, telomere attrition, and reactive oxygen species (Miller et al., 2013). Although this study only tested this approach in dopaminergic neurons, it is worth attempting this strategy in other neuronal types.

Another recent approach is to directly convert fibroblasts into neurons by transfecting transcription factors with a cocktail of small molecules (Hu et al., 2015; Liu et al., 2016). These directly induced neurons, also called iNeurons, show an age-specific transcriptional background and an age-related reduction of the nuclear transporter RanBP17 (Mertens et al., 2015; Gopalakrishnan et al., 2017). These iNeurons could present with more relevant phenotypes as these induced neurons did not undergo a complete reprogramming which might repair the macromolecular damage which could contribute to ALS/FTD related phenotypes (Mertens et al., 2015; Gopalakrishnan et al., 2017). One disadvantage of these iNeurons is that the fibroblasts used as starting material are not an unlimited resource of cells.

Environmental Factors

Apart from aging, environmental factors could contribute to ALS/FTD, although convincing evidence for this is still scarce (Ingre et al., 2015). These environmental factors could eventually include malnutrition, hypoxia and psychological stress (Ingre et al., 2015). All these factors could lead to oxidative stress. Clinically, oxidative stress biomarkers in cerebrospinal fluid, plasma, and urine are elevated in ALS/FTD patients, suggesting that oxidative stress could play a pivotal role in motor neuron or cortical neuron degeneration (D'Amico et al., 2013; Mao, 2013; Turner et al., 2013). Moreover, the recently approved drug, edaravone, is thought to exert its therapeutic effect by counteracting oxidative stress (Hardiman and van den Berg, 2017). In addition, oxidative stress is also a major event during aging (López-Otín et al., 2013). Induction of oxidative stress *in vitro* could be helpful to model ALS/FTD associated phenotypes. Arsenite treatment and hydrogen peroxide are two examples to induce stress (Henkler et al., 2010). Stress granules and FUS/TDP-43 protein aggregations were reported to appear after adding oxidative stress (Dewey et al., 2012; Carrì et al., 2015; Lenzi et al., 2015). In addition, shortage of certain growth factors in ALS (e.g., VEGF, BDNF, GDNF, CNTF, …) were observed and could contribute to ALS disease progression *in vivo* (Bogaert et al., 2010; Henriques et al., 2010). Therefore, removal of growth factors can serve as a strategy to induce phenotypes in *in vivo* models. Moreover, based on the discovery of physiological changes in ALS iPSC models (Wainger et al., 2014; Devlin et al., 2015; Naujock et al., 2016), addition of excitotoxins could also be a method to induce phenotypes.

CONCLUSIONS

In this review, we systematically summarized the published iPSC models of ALS/FTD, along with their advancements in our knowledge of these complex diseases, but as well as their current drawbacks and discrepancies. Indeed, there are many advantages to modeling ALS/FTD using iPSCs, however, one always has to keep in mind that this is just another model for ALS/FTD. It remains an *in vitro* model with all the disadvantages that come with it. Neuronal cultures could provide results that might not be relevant to the *in vivo* disease process. As a consequence, translating results from iPSC-related *in vitro* work into clinical trials without prior confirmation in additional models or validation in patient material should be avoided.

AUTHOR CONTRIBUTIONS

WG contributed to the writing and organization of the whole manuscript. LF contributed to the *C9ORF72* part and the figures of this review. RP contributed to the general improvement of the manuscript. LVDB contributed to the organization and quality control of this manuscript.

ACKNOWLEDGMENTS

Research of the authors is supported by KU Leuven (C1 and "Opening the Future" Fund), VIB, the "Fund for Scientific Research Flanders" (FWO-Vlaanderen), the Agency for Innovation by Science and Technology (IWT; SBO-iPSCAF), the Belgian Government (Interuniversity Attraction Poles Programme P7/16 initiated by the Belgian Federal Science Policy Office), the Thierry Latran Foundation, the "Association Belge contre les Maladies neuro-Musculaires" (ABMM) and the ALS Liga België ("A Cure for ALS"). WG is supported by the China Scholarship Council (CSC). RP was supported by grants from the Central Remedial Clinic (CRC) Ireland and the National University of Ireland (NUI) and is currently supported by the FWO-Vlaanderen.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 05 February 2018
doi: 10.3389/fnins.2017.00736

Altered Expression of Matrix Metalloproteinases and Their Endogenous Inhibitors in a Human Isogenic Stem Cell Model of Huntington's Disease

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OPEN ACCESS

Edited by:
Dustin R. Wakeman, RxGen, United States

Reviewed by:
Julien Rossignol, Central Michigan University, United States
David Schaffer, University of California, Berkeley, United States

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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *11 September 2017*
Accepted: *18 December 2017*
Published: *05 February 2018*

Citation:
Naphade S, Embusch A, Madushani KL, Ring KL and Ellerby LM (2018) Altered Expression of Matrix Metalloproteinases and Their Endogenous Inhibitors in a Human Isogenic Stem Cell Model of Huntington's Disease. Front. Neurosci. 11:736. doi: 10.3389/fnins.2017.00736

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by a progressive movement disorder, psychiatric symptoms, and cognitive impairments. HD is caused by a CAG repeat expansion encoding a stretch of polyglutamine residues in the N-terminus of mutant huntingtin (mHTT) protein. Proteolytic processing of mHTT yields toxic fragments, which cause neurotoxicity and massive neuronal cell death predominantly in the striatum and cortex. Inhibition of mHTT cleavage reduces neuronal toxicity suggesting mHTT proteolysis contributes to HD pathogenesis. A previously conducted unbiased siRNA screen in our lab for known human proteases identified matrix metalloproteinases (MMPs) as modifiers of mHTT proteolysis and toxicity. To further study MMP activation in HD, isogenic HD, and control corrected (C116) neural stem cells (NSCs) prepared from HD patient-derived induced pluripotent stem cells were used to examine the role of MMPs and their endogenous inhibitors in this highly relevant model system. We found altered expression of MMP-2 and MMP-9 (gelatinases), MMP-3/10, and MMP-14, activity in HD-NSCs when compared to control C116-NSCs. Dysregulation in MMP activity was accompanied with concomitant changes in levels of endogenous inhibitors of MMPs, called tissue inhibitors of matrix metalloproteinases (TIMPs). Specifically, we observed decreased levels of TIMP-1 and TIMP-2 in HD-NSCs, suggesting part of the altered expression and activity of MMPs is due to lower abundance of these endogenous inhibitors. Immunofluorescence analysis revealed increased MMP/TIMP localization in the nucleus or aggregates of HD-NSCs, suggesting potential interaction with mHTT. TIMP-1 was found to associate with mHTT aggregates in discrete punctate structures in HD-NSCs. These events collectively contribute to increased neurotoxicity in HD. Previous characterization of these NSCs revealed transforming growth factor beta (TGF-β) pathway as the top dysregulated pathway in HD. TGF-β was significantly upregulated in HD-NSCs and addition of TGF-β to HD-NSCs was found to be neuroprotective. To determine if TGF-β regulated MMP and TIMP activity, C116- and HD-NSCs were exogenously treated with recombinant TGF-β. TIMP-1 levels were found to be elevated in response to TGF-β treatment, representing a

potential mechanism through which elevated TGF-β levels confer neuroprotection in HD. Studying the mechanism of action of MMPs and TIMPs, and their interactions with mHTT in human isogenic patient-derived NSCs elucidates new mechanisms of HD neurotoxicity and will likely provide novel therapeutics for treatment of HD.

Keywords: Huntington's disease, neural stem cells, matrix metalloproteinases, tissue inhibitors of metalloproteinases, transforming growth factor-β

INTRODUCTION

Primarily characterized as a movement disorder, Huntington's disease (HD) is an autosomal-dominant neurodegenerative disorder with cognitive decline, chorea, and emotional disturbances as the disease progresses. Marked striatal atrophy accompanied with selective degeneration of medium spiny neurons represent classical pathological hallmarks of HD. HD is caused by an expanded CAG triplet repeat in the huntingtin (HTT) gene that encodes a stretch of polyglutamine residues which renders the mutant huntingtin (mHTT) protein prone to aggregate formation. mHTT is susceptible to proteolysis at the N-terminus with the resulting short N-terminal fragments contributing to cellular toxicity by inducing apoptotic cell death. Truncated N-terminal mHTT fragments have been reported in human HD postmortem tissue and mouse models of HD (Mende-Mueller et al., 2001; Wellington et al., 2002; Wang et al., 2008). Furthermore, inhibition of mHTT cleavage reduced toxicity both *in vitro* and *vivo* (Wellington et al., 2000; Gafni et al., 2004; Graham et al., 2006), indicating an important role for mHTT proteolysis in HD pathogenesis. In order to identify critical proteases that directly cleave mHTT, an unbiased western blot-based siRNA screen for 514 known human proteases was conducted (Miller et al., 2010). This screen confirmed 11 proteases that, when silenced, reduced toxic N-terminal HTT fragment formation. Interestingly, three of these eleven modifiers of HTT proteolysis and toxicity belonged to the matrix metalloproteinase (MMP) family (MMP-10, -14, and -23B).

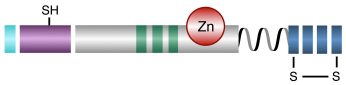
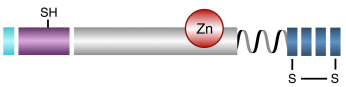
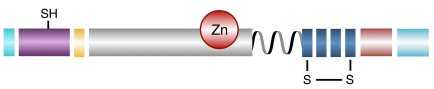
MMPs are Ca^{2+} dependent, zinc-containing proteolytic enzymes. At least 25 members of the MMP family have been identified in humans so far, and they exhibit different substrate specificity and domain organizations categorized into collagenases, stromelysins, gelatinases, membrane-type MMPs (MT-MMPs), matrilysins, and other MMPs (Nagase et al., 2006; **Table 1**). MMPs are mostly secreted into the extracellular space, except for MT-MMPs that are transmembrane proteases. MMPs are first produced as inactive zymogens and are activated by other proteases (or MMPS) or free radicals (Ra and Parks, 2007). They occupy central roles in several normal physiological processes, including, stem cell differentiation, proliferation, migration, wound repair, angiogenesis, and apoptosis (Malemud, 2006). Although altered MMP expression has been observed in several neurodegenerative diseases (Brkic et al., 2015), including Alzheimer's disease (AD) (Lorenzl et al., 2003b; Lim et al., 2011), Parkinson's disease (PD) (Lorenzl et al., 2002), and amyotrophic lateral sclerosis (ALS) (Lim et al., 1996; He et al., 2013), the exact contribution of MMPs to the pathogenesis of diseases remains unclear. MMP activity is tightly regulated *in vivo* by endogenous inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) (Brew and Nagase, 2010). The mammalian TIMP family presently consists of four members (TIMP-1 to -4). TIMPs inhibit active forms of MMP by binding to the Zn^{2+} cation in the MMP catalytic domain. Studies indicate that TIMPs also serve MMP-independent functions that help modulate cell proliferation, apoptosis, and synaptic plasticity (Brew and Nagase, 2010).

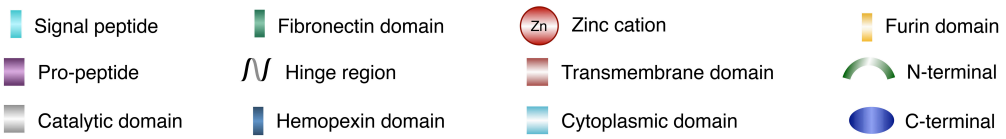
Previous studies from our lab demonstrated an upregulation of MMP-10 and MMP-14 in $Hdh^{111Q/111Q}$ mouse striatal cells compared to the $Hdh^{7Q/7Q}$ cells (Miller et al., 2010). Additionally, MMP-10 was shown to directly cleave HTT into toxic N-terminal fragments. siRNA mediated knockdown of MMP-10 and MMP-14, or overexpression of TIMPs, in $Hdh^{111Q/111Q}$ blocked caspase-3/7 activity, a direct measure of cellular apoptosis. Furthermore, treatment with NNGH, a broad spectrum MMP inhibitor, resulted in a dose-dependent inhibition of caspase-3/7 activity. These data unequivocally show that MMP inhibition ameliorates mHTT-induced striatal toxicity. Elevated MMP activity was also observed in the striatum of older R6/2 and YAC128 HD mouse models, suggesting that MMPs exhibit specific spatial and temporal patterns of expression. Finally, reducing MMP activity significantly improved motor performance in the HD Drosophila model. These studies underscore the need for further investigation into the role of MMPs and TIMPs in HD in order to design effective therapies.

To this end, we characterized MMP and TIMP expression in an induced pluripotent stem cell (iPSC)-derived neural stem cell (NSC) model developed previously in our lab (An et al., 2012). Briefly, patient-derived HD-iPSCs (72Q/19Q) were genetically corrected by replacing the expanded CAG repeat (72Q/19Q) with a normal repeat using targeted homologous recombination. The corrected "C116-iPSCs" (21Q/19Q) retained characteristics of pluripotent stem cells. The merit of this approach is that both the C116- and HD-iPSC lines are isogenic (thus, differences in gene expression can be solely attributed to the length of the CAG repeat) and allow for patient-specific modeling of HD. Previous characterization using differential gene expression and pathway analysis revealed that more than 4,000 genes were differentially expressed in HD-NSCs in contrast to only about 400 genes in HD-iPSCs. These findings indicated that HD-associated cellular and molecular phenotypes were evident only in differentiated HD-NSCs but not in HD-iPSCs (Ring et al., 2015), making NSCs an attractive human HD cellular model.

Our results demonstrate altered MMP expression and cellular localization in HD-NSCs as compared to C116-NSCs. Both TIMP-1 and TIMP-2 were found to be downregulated in HD-NSCs. Previous genomic analysis of HD-and corrected

TABLE 1 | Classification of MMPs and TIMPs.

	Name	Location	Main substrates / Proteases inhibited*	Structure
MMP-2	Gelatinase A	Secreted	ECM: collagens, gelatin, elastin, fibronectin, etc. Non-ECM: pro-IL-1b, plasminogen, other MMPs	
MMP-9	Gelatinase B	Secreted		
MMP-3	Stromelysin-1	Secreted	ECM: collagens, gelatin, elastin, fibronectin, laminin, aggrecan Non-ECM: pro-IL-1b, plasminogen, pro-MMP-1, -8, -9, -13, MMP/TIMP complex, antithrombin III, fibrinogen, plasminogen, IGFBP	
MMP-10	Stromelysin-2	Secreted		
MMP-14	Transmembrane type 1 MMP	Membrane -bound	ECM: collagens, gelatin, elastin, laminin, vitronectin Non-ECM: pro-MMP-2 and -13	
TIMP-1	-	Secreted	Inhibits all MMPs, except MMP-14	
TIMP-2	-	Secreted	Inhibits all MMPs tested, required for pro-MMP-2 activation	



ECM, extracellular matrix. *References: 1. Crocker et al. (2004). 2. Kapoor et al. (2016).*

C116-NSCs revealed increased upregulation of TGF-β expression in HD-NSCs. This upregulation was shown to be neuroprotective, thereby reflecting a possible compensatory attempt to re-establish CNS homeostasis in HD (Ring et al., 2015). However, the mechanism remains unknown. In this study, we show that upregulation of TIMP-1 expression in response to TGF-β represents a potential mechanism through which TGF-β confers neuroprotection in HD-NSCs.

MATERIALS AND METHODS

RNA-Seq Analysis

RNA-Seq analysis was performed as described previously and **Figure 1** was generated using this data set (Ring et al., 2015). Briefly, total RNA was purified from iPSCs and NSCs using the RNeasy Mini kit (Qiagen). A Qiacube instrument was used to extract the RNA using the RNase Micro protocol. Subsequent RNA-Seq by Illumina library preparation was performed on an Illumina Hiseq 2000 sequencer. Resulting data was analyzed using enrichment and networking analysis and hierarchical clustering analysis. The accession number for the RNA-Seq data used is GEO: GSE74201.

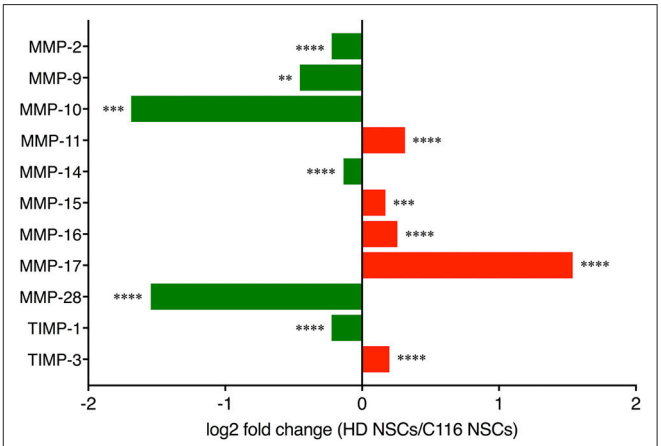


FIGURE 1 | RNA-Seq analysis. RNA-Seq analysis of MMP and TIMP expression in HD- vs. corrected C116-NSCs via IPA analysis reveals a dysregulated MMP/TIMP axis in HD-NSCs (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Data mined from Ring et al. (2015).

Targeted Correction of the Expanded HTT Gene in HD-iPSCs

Patient-derived HD-iPSCs (72Q/19Q) were corrected using targeted homologous recombination, resulting in the reduction of the expanded HTT gene to normal 21 polyglutamine repeats, as described previously (An et al., 2012). Correctly targeted clones (21Q/19Q) were selected using G418 selection and verified using western and Southern blot analysis. The corrected C116-iPSCs comprised of a homogeneous cell population and retained characteristics of pluripotent stem cells (An et al., 2012).

Cell Culture

NSCs were generated from C116- and HD-iPSC lines using STEMdiff Neural Induction Medium (Stem Cell Technologies) (Ring et al., 2015) and characterized (An et al., 2012) as described previously. 6 cm dishes or 6-well plates (Corning, Nunclon Delta Surface) were coated with Matrigel (1:60; BD Corning) for 1 h. NSCs were plated and cultured in Neural Proliferation Medium (NPM) in humidified incubator under 37°C, 5% CO_2. NPM was prepared using Neurobasal medium supplemented with 1X B-27 supplement (Life Technologies), 2 mM L-Glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 ng/mL human Leukemia Inhibitory Factor (LIF) (Peprotech, 300-05), and 25 ng/mL human basic Fibroblast Growth Factor (bFGF) (Peprotech, 100-18B).

TGF-β Treatment

C116- and HD-NSCs were treated with recombinant human TGF-β1 (Peprotech, AF-100-21C) reconstituted in NPM at 10 or 20 ng/mL concentration for 1 or 24 h. Untreated NSCs served as controls.

Western Blot Analysis

NSCs were harvested in M-PER Mammalian Protein Extraction Reagent (Pierce) with cOmplete Mini EDTA-free protease inhibitor (1 tablet/10 mL) (Roche). Whole-cell lysates from NSCs were sonicated with a 5 second pulse followed by a 5 second rest (X 5 times) at 40% amplitude. Samples were centrifuged at 14,000 rpm at 4°C for 20 min, and supernatant was collected and stored at −20°C. Protein concentrations were estimated using the BCA assay (Pierce). Cell lysates were denatured under reducing conditions by boiling 10–20 μg total protein with 1 μL of 1M DTT and 4X LDS sample buffer (Invitrogen) at 95°C for 10 min. SDS-PAGE was performed using NuPage 4-12% Bis-Tris gels (Invitrogen). Gels were run in 1X MES or 1X MOPS running buffer containing 500 μL of antioxidant (Life technologies), and then transferred to 0.45 μm PVDF membrane using 1X NuPage transfer buffer at 20 V for 14 h. Membranes were blocked with 5% non-fat milk in TBS with 0.1% Tween 20 (TBS-T) for 1 h at room temperature (RT), incubated with primary antibody reconstituted in 5% non-fat milk overnight at 4°C, followed by incubation with secondary antibody for 2 h at RT. Blots were washed in TBS-T for 10 min (3X), and developed using Pierce ECL (Thermo Scientific). β-actin or α-tubulin served as loading controls. Densitometry analysis was performed using ImageQuant TL v2005. Western blot analysis was performed to study MMP/TIMP expression at basal levels in C116- and HD-NSCs, and additionally in presence or absence of exogenously added TGF-β. Biological replicates (BR) were used for each experiment. Primary and secondary antibodies used are listed in Supplementary Table 2.

Gelatin Zymography

C116- and HD-NSCs were harvested in lysis buffer (50 mM Tris-HCL (pH 7.6), 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Birj-35, 1% Triton X-100, 0.02% NaN_3), and sonicated as described above. For each sample, 500 μg of sonicated cell lysate was suspended in 500 μL of lysis buffer and 50 μg of Gelatin Sepharose 4B beads (GE healthcare) and placed on rotator for 1 h at 4°C for MMP binding. Suspensions were centrifuged, supernatant was removed, and affinity-bound MMPs were eluted by resuspending the bead complexes in 50 μL elution buffer (10% DMSO in PBS). 20 μg of elute (with 4X LDS sample buffer) was then loaded on a 8% zymogram gel containing 0.1% gelatin (Resolving gel: 1.5 M Tris-HCl (pH 8.8), 0.4% SDS, ddH_2O, 40% acrylamide/bis-acrylamide, tetramethylethylenediamine (TEMED), 10% ammonium persulfate (APS), and 0.1% gelatin. Stacking gel: 1.0 M Tris-HCl (pH 6.8), ddH_2O, 40% acrylamide/bis-acrylamide, TEMED, 10% APS). Zymogram gels were run in 1X Tris-Glycine SDS buffer under non-reducing conditions at 150 V for 1 h. Gels were then washed with renaturation buffer (2.5% Triton X-100 in ddH_2O) for 40 min at 25°C, briefly rinsed with ddH_2O, and incubated in 50 mL of incubation buffer (50 mM Tris HCl, 0.15 M NaCl, 10 mM $CaCl_2$) for 20-72 h at 37°C. Finally, gels were briefly washed with ddH_2O, stained with 0.05% Coomassie Brilliant Blue solution for 1 h, and de-stained for 1 h at RT. Gels were scanned using an Epson Scanner.

For conditioned media, fresh medium was added to near-confluent cultures and the media was harvested after 3 days of incubation at 37°C and 5% CO_2. Five hundred microliters of conditioned media was loaded onto Amicon Ultra-0.5 Centrifugal Filter Devices (EMD Millipore, UFC501008) with a molecular weight cut-off of 10 kD. Conditioned media was concentrated to 20 μL by centrifuging the devices at 14,000 × g for 30 min. Concentrated media was recovered by turning the devices upside down in clean tubes and centrifuging the tubes at 1,000 × g for 2 min. 20 μg of elute (with 4X LDS sample buffer) was then loaded on a 8% zymogram gel containing 0.1% gelatin, and gelatin zymography was performed as described above.

Immunocytochemistry and Fluorescence Microscopy

C116- and HD-NSCs were plated in Matrigel-coated 8-well glass chamber slides (BD Falcon) (80,000 cells per well). Cells were fixed in 4% paraformaldehyde for 15 min at RT and washed three times with PBS. Cells were permeabilized and blocked with 0.1% Triton X-100, 10% donkey serum in PBS for 30 min at RT. The cells were washed once with PBS and incubated with primary antibody (suspended in 1% BSA in PBS) for 24 h at 4°C in a humid chamber. Slides were then washed with PBS (10 min, 3X), and incubated with secondary antibody (suspended in 1% BSA in PBS) at RT for 90 min in the dark. Finally, slides were washed with PBS (5 min, 3X), and coverslips were mounted with ProLong Gold with DAPI antifade reagent (Life Technologies). Slides were cured 24 h in the dark at RT. Imaging was performed on Nikon Eclipse Ti-U microscope using the Plan Apo λ 20X/0.75 objective. Z-stacks for Supplementary Figure 2 were acquired on the Zeiss LSM 780 confocal microscope using the 63X Zeiss plan-apochromat oil, 1.4 NA, DIC objective. Primary and secondary antibodies used are listed in Supplementary Table 2.

RT-PCR Analysis

Total RNA was isolated from NSCs using ISOLATE II RNA Mini Kit (Bioline). cDNA was prepared from 1 μg of RNA in a total reaction volume of 20 μl using the SensiFAST cDNA synthesis kit (Bioline). RT-PCR reactions were setup in a 384-well format using 2X SensiFAST Probe No-ROX kit (Bioline) and 1 μl cDNA per reaction in a total volume of 10 μl. RT-PCR was performed on the Roche LightCycler 480 instrument. For quantification, the threshold cycle, Ct, of each amplification was determined by using the second derivative maximum method. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression levels of each gene normalized against the house-keeping gene β-Actin. Primer-probes used are listed in Supplementary Table 3.

Statistical Analysis

Student's paired *t*-test and ANOVA with Tukey's multiple comparison test was used to study differences in MMP/TIMP expression in C116- and HD-NSCs in presence or absence of recombinant TGF-β. All statistical analysis and graph plotting was performed using PRISM 7 by GraphPad Software (La Jolla, CA, USA). $p < 0.05$ was considered as statistically significant.

RESULTS

RNA-Seq analysis revealed altered expression of MMPs and TIMPs in HD-NSCs compared to C116-NSCs (**Figure 1**) indicating a dysregulation of the MMP/TIMP axis in HD-NSCs.

MMP Expression Is Modulated in HD

MMP-3/10 and MMP-14 Levels Are Altered in HD

Our previous studies showed elevated levels of MMP-14 and proteolytically processed active MMP-10 form in mouse striatal $Hdh^{111Q/111Q}$ cells compared to $Hdh^{7Q/7Q}$ cells (Miller et al., 2010). Additionally, MMP-10 was reported to directly cleave HTT. Another recent study reported a direct correlation between increasing MMP-3 levels in cerebrospinal fluid (CSF) and worsening of disease in HD patients (Connolly et al., 2016). To determine if MMP levels are also dysregulated in our human iPSC-derived NSC model, control C116- and HD-NSC lysates were subjected to western blot analysis. MMP-3 and MMP-10 are both stromelysins and share the highest sequence homology (86%) among all MMPs (Bertini et al., 2004), potentially serving redundant biochemical functions. Consistent with our previous findings, MMP-3/10 levels were found to be elevated in HD-NSCs compared to C116-NSCs [**Figure 2A** (1.6-fold increase), Supplementary Figure 1A (3.6-fold increase), Supplementary Figure 1B (1.4-fold increase)]. Immunofluorescence analysis not only confirmed these findings but also revealed altered localization of MMP-3/10 between the two genotypes (**Figure 2B**). While MMP-3/10 is predominantly expressed in the cytoplasm in C116-NSCs, robust nuclear expression is also observed in HD-NSCs, which appears to be associated with apoptotic cells (degenerating nuclei) as well (white arrowheads in **Figure 2B**). Interestingly, MMP-14 levels were decreased in HD-NSCs compared to C116-NSCs (**Figure 3**). Despite the reduced overall levels detected by western blot analysis (**Figure 3A**, 1.7-fold decrease) and RT-PCR analysis (Supplementary Figure 1C, 15.4-fold decrease), MMP-14 expression was found to be strongly nuclear in HD-NSCs where it possibly associates with nuclear HTT immunoreactivity (**Figure 3B**).

MMP-2 and MMP-9 (Gelatinases) Expression in HD

Altered levels of MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B), potentially contributing to disease pathogenesis, have been shown to be altered in several neurodegenerative diseases including AD (Lim et al., 2011; Hernandez-Guillamon et al., 2015; Chowdhury, 2016; Weekman and Wilcock, 2016), and ALS (Lim et al., 1996; Fang et al., 2010; Kaplan et al., 2014). Furthermore, increased CSF MMP-9 levels directly correlate with disease severity in HD patients (Connolly et al., 2016). To determine if these changes in MMP-2 and MMP-9 expression levels can be recapitulated in our iPSC-derived NSC lines, gelatin zymography was performed on cell lysates (**Figure 4A**) and conditioned media (**Figure 4B**) obtained from C116- and HD-NSCs. Although modest changes were observed in pro-MMP-2 levels, the zymogram demonstrated a marked decrease in active MMP-2 levels in HD-NSCs when compared to control C116-NSCs, a result further confirmed by immunofluorescence analysis (**Figure 4C**). Strong upregulation of MMP-2 expression was observed especially in association with apoptotic cells (punctate degenerating nuclei) (white arrowheads in **Figure 4C**), suggesting a role for MMP-2 in cellular apoptosis. Increased expression of pro-MMP-9 was observed in samples derived from HD-NSCs compared to those obtained from C116-NSCs (**Figures 4A,B**). Furthermore, immunofluorescence analysis again revealed strictly nuclear localization of MMP-9 in HD-NSCs, in contrast with the diffuse nuclear and cytoplasmic expression in C116-NSCs (**Figure 4D**).

TIMP Levels Are Modulated in HD

TIMPs are endogenous inhibitors of MMPs, and thus, altered MMP levels are often associated with concomitant changes in TIMP levels. Elevated levels of TIMPs have been reported in several neurodegenerative diseases, including PD, AD, HD, and ALS (Lorenzl et al., 2003a), where they have been ascribed roles in neuroprotection (Tan et al., 2003; Magnoni et al., 2007; Fujimoto et al., 2008; Tejima et al., 2009; Walker and Rosenberg, 2009; Kim et al., 2010; Ashutosh et al., 2012; Lee and Kim, 2014; Gibb et al., 2015), remyelination (Jiang et al., 2016) and maintaining CNS homeostasis (Gardner and Ghorpade, 2003). To determine if TIMP expression is modulated in HD, western blot analysis (and RT-PCR analysis) was performed on cell lysates prepared from C116- and HD-NSCs. Our results demonstrate a decreased expression of both TIMP-1 [**Figure 5A** (3.3-fold decrease), Supplementary Figure 1D (1.4-fold decrease)] and TIMP-2 [**Figure 5B** (1.6-fold decrease), Supplementary Figure 1E (1.2-fold decrease)] in HD-NSCs. The downregulation of these endogenous MMP inhibitors likely accounts for the unabated MMP activity in HD-NSCs. Immunocytochemistry analysis further revealed that nuclear TIMP-1 is found in association with nuclear HTT (**Figure 5C**) suggesting that TIMP-1 regulates MMP activity in the nucleus and potentially plays a role

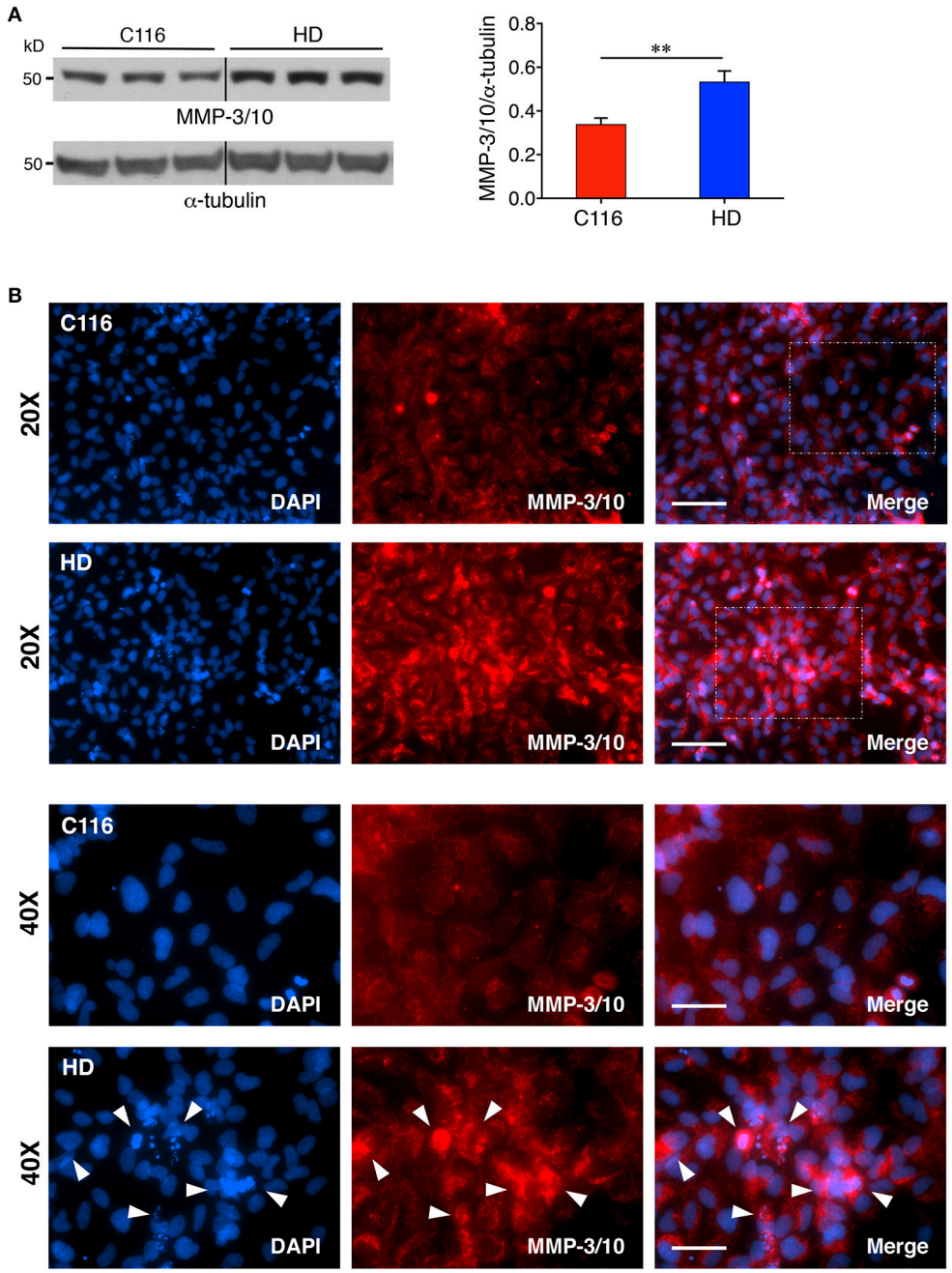


FIGURE 2 | MMP-3/10 expression is elevated in HD-NSCs. **(A)** Western blot analysis of MMP-3/10 reveals a robust increase in expression in HD-NSCs. MMP-3/10 immunoreactivity was quantified by densitometry and normalized for α-tubulin immunoreactivity ($n = 3$ BR, $^{**}p < 0.01$; *t*-test). Error bars represent *SD*. **(B)** Immunofluorescence analysis confirms increased expression of MMP-3/10 in HD-NSCs, and also shows elevated nuclear presence of MMP-3/10 in HD-NSCs (40X magnified insets) as compared to C116-NSCs. Additionally, increased MMP-3/10 immunoreactivity is observed in apoptotic cells with degenerating nuclei in HD-NSCs (40X magnified inset, white arrowheads). Scale Bars: 20X = 100 μm, 40X = 50 μm.

in inhibiting cellular apoptosis (Mannello and Gazzanelli, 2001). HD-NSCs readily form cytoplasmic perinuclear mHTT aggregates as detected by anti-huntingtin antibody (a.a. 115-129, MAB5490). Interestingly, there appears to be an interaction between TIMP-1 and mHTT aggregates in HD-NSCs (white arrowheads in **Figure 5C**, Supplementary Figure 2).

TGF-β Regulates TIMP-1 Expression

Our previous studies on differential gene expression and Ingenuity Pathway Analysis (IPA) of HD-NSCs and corrected C116-NSCs highlighted TGF-β as the top dysregulated pathway (Ring et al., 2015). HD-NSCs expressed significantly higher levels of TGF-β in all its forms (precursor, monomer, and

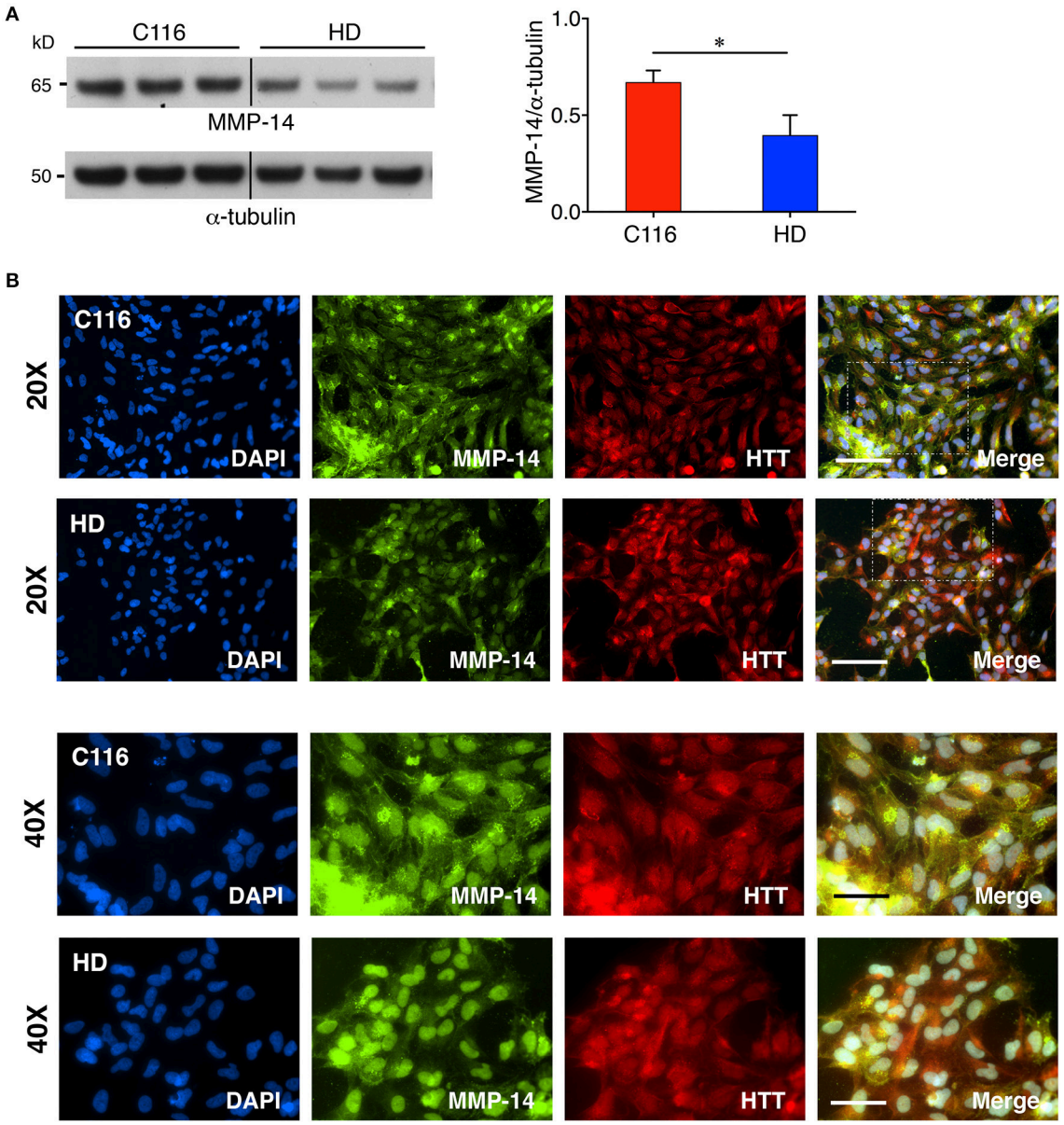


FIGURE 3 | Decreased MMP-14 expression in HD-NSCs. **(A)** Western blot analysis of MMP-14 shows a significantly decreased expression of MMP-14 in HD-NSCs. MMP-14 immunoreactivity was quantified by densitometry and normalized for α-tubulin immunoreactivity ($n = 3$ BR, $^*p < 0.05$; *t*-test). Error bars represent *SD*. **(B)** Immunofluorescence analysis of MMP-14 not only confirms decreased expression in HD vs. C116-NSCs, but also reveals predominantly nuclear expression of MMP-14 in HD-NSCs which possibly associates with nuclear mHTT expression (40X magnified insets). Scale Bars: 20X = 100 μm, 40X = 50 μm.

dimer) compared to C116-NSCs. Exogenous addition of TGF-β conferred neuroprotection in HD-NSCs as indicated by amelioration of elevated caspase-3/7 activity, a direct measure of cellular apoptosis, and by rescuing mitochondrial deficits. Altered levels of TGF-β have been reported in HD mouse models and peripheral blood of HD patients as well (Battaglia et al., 2011). Although very little is known about TGF-β-mediated regulation of MMP/TIMP activity, recent studies have shown that TGF-β modulates the homeostasis between MMPs and TIMPs through putative signaling pathways (Hall et al., 2003; Gomes et al., 2012; Kwak, 2013). To determine if TGF-β regulates MMP and TIMP expression in C116- and HD-NSCs, cells were treated with 10 ng/mL TGF-β for 24 h. Western blot analysis revealed that both cell lines expressed the TGF-β receptor at basal conditions (**Figure 6A**), and that TGF-β treatment did not alter the expression levels of its cognate receptor. Interestingly, treatment with TGF-β resulted in significantly increased expression of TIMP-1 in HD-NSCs, but not in C116-NSCs (**Figure 6B**). No significant change in expression of TIMP-2 (**Figure 6C**), or MMP-3/10 (**Figure 6D**)

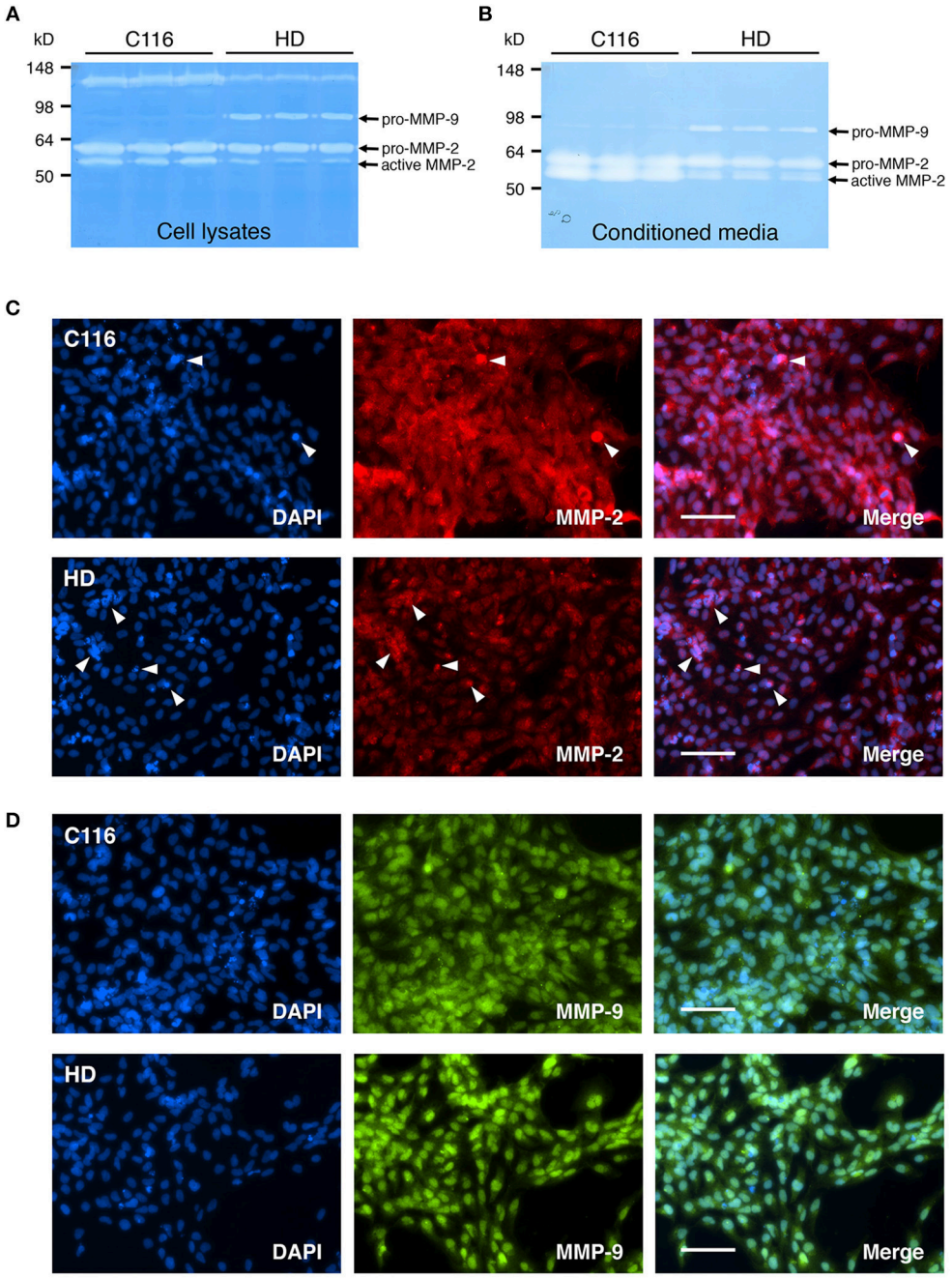


FIGURE 4 | Expression of MMP-2 and MMP-9 (Gelatinases) is altered in HD-NSCs. Gelatin zymogram of C116- and HD-NSC lysates **(A)** shows increased activity of pro-MMP-9 in HD-NSCs, which is also extracellularly secreted into the conditioned media **(B)**. In contrast, decreased production of active-MMP-2 is observed in samples prepared from HD-NSCs when compared to C116-NSCs. Immunofluorescence analysis using anti-MMP-2 **(C)** and anti-MMP-9 **(D)** antibodies confirms these findings and reveals strictly nuclear localization of both MMP-2 and -9 in HD-NSCs. Strongly increased MMP-2 expression was also found in association with apoptotic cells (white arrowheads in **C**). Scale Bars: 100 μm.

was observed. A very modest increase in MMP-14 expression in HD-NSCs (**Figure 6E**) was observed in response to TGF-β. To further confirm these finding, C116- and HD-NSCs were treated with a higher dose of 20 ng/mL TGF-β for 24 h. A robust upregulation of TIMP-1 expression was observed not only in HD-NSCs, but also in C116-NSCs (Supplementary Figure 3A). These results together indicate that corrected C116-NSCs have a higher threshold for TIMP-1 induction. Consistent with previous findings, very little modulation of TIMP-2 expression was observed at the higher TGF-β dose in across both genotypes (Supplementary Figure 3B). These data suggest that one of the possible mechanisms by which TGF-β exerts its neuroprotective

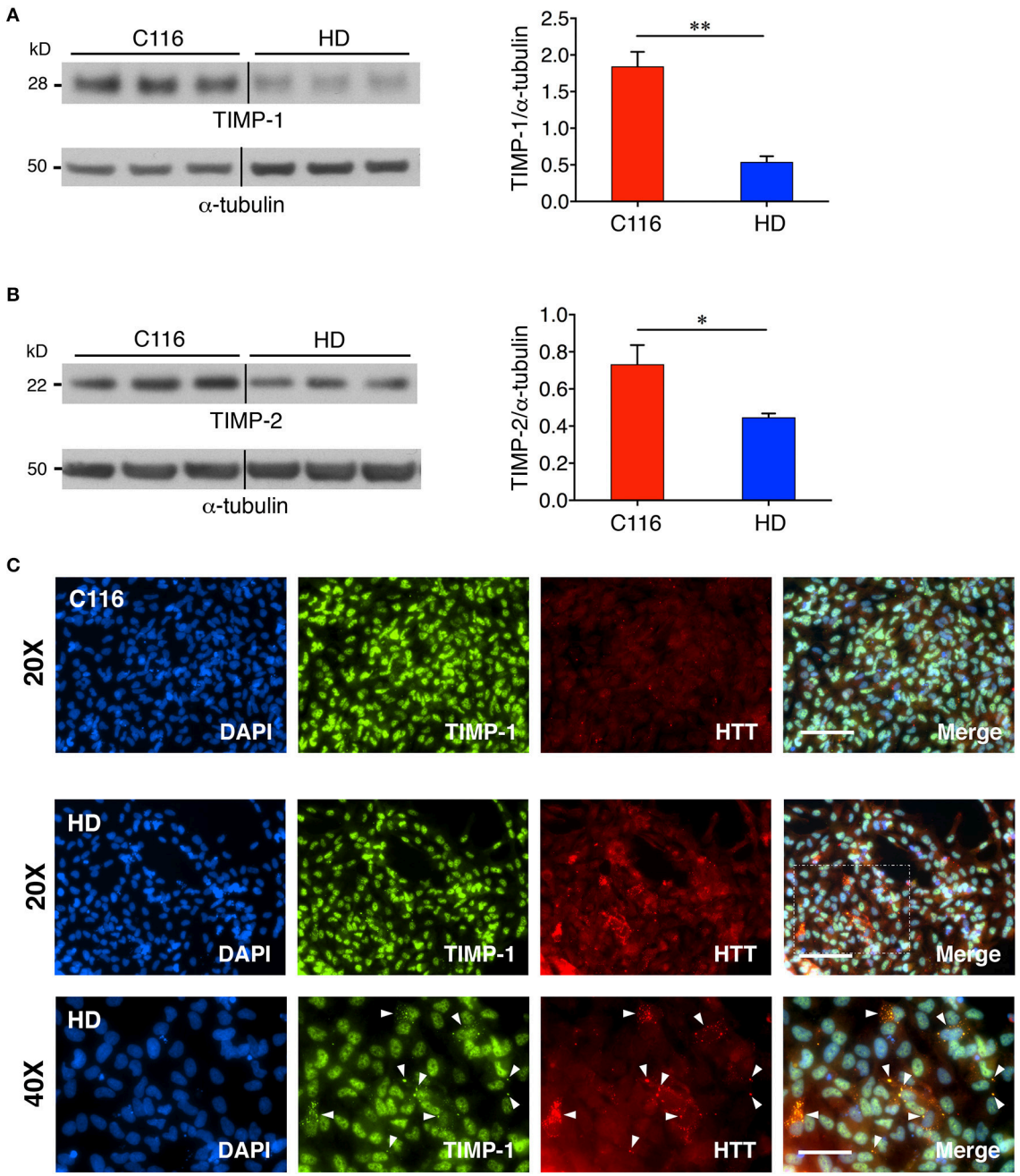


FIGURE 5 | TIMP expression is downregulated in HD-NSCs. Western blot analysis of TIMP-1 **(A)** and TIMP-2 **(B)** expression reveals significantly decreased levels in HD-NSCs compared to C116-NSCs ($n = 3$ BR, $^{*}p < 0.05$; $^{**}p < 0.01$; *t*-test). Error bars represent *SD*. Immunofluorescence analysis using anti- TIMP-1 antibody **(C)** demonstrates nuclear localization of TIMP-1 and suggests a direct association between TIMP-1 and mHTT aggregates in HD-NSCs (40X magnified inset, white arrowheads). Scale Bars: 20X = 100 μm, 40X = 50 μm.

effects is through the upregulation of TIMP-1 expression. Finally, altered localization of TIMP-1 expression was observed as a function of time in response to treatment with 20 ng/mL TGF-β, with TIMP-1 expression shifting from being strictly nuclear at 1 h post-treatment (**Figure 7A**), to completely cytoplasmic at 24 h post-treatment (**Figure 7B**), as revealed by immunofluorescence analysis.

DISCUSSION

Our study successfully demonstrates altered expression of MMPs and TIMPs across the corrected C116- and HD-NSC lines. Previous studies in our lab demonstrated that MMP-10 colocalized with mHTT aggregates in discrete punctate structures and that MMP-10 directly cleaves mHTT into N-terminal toxic

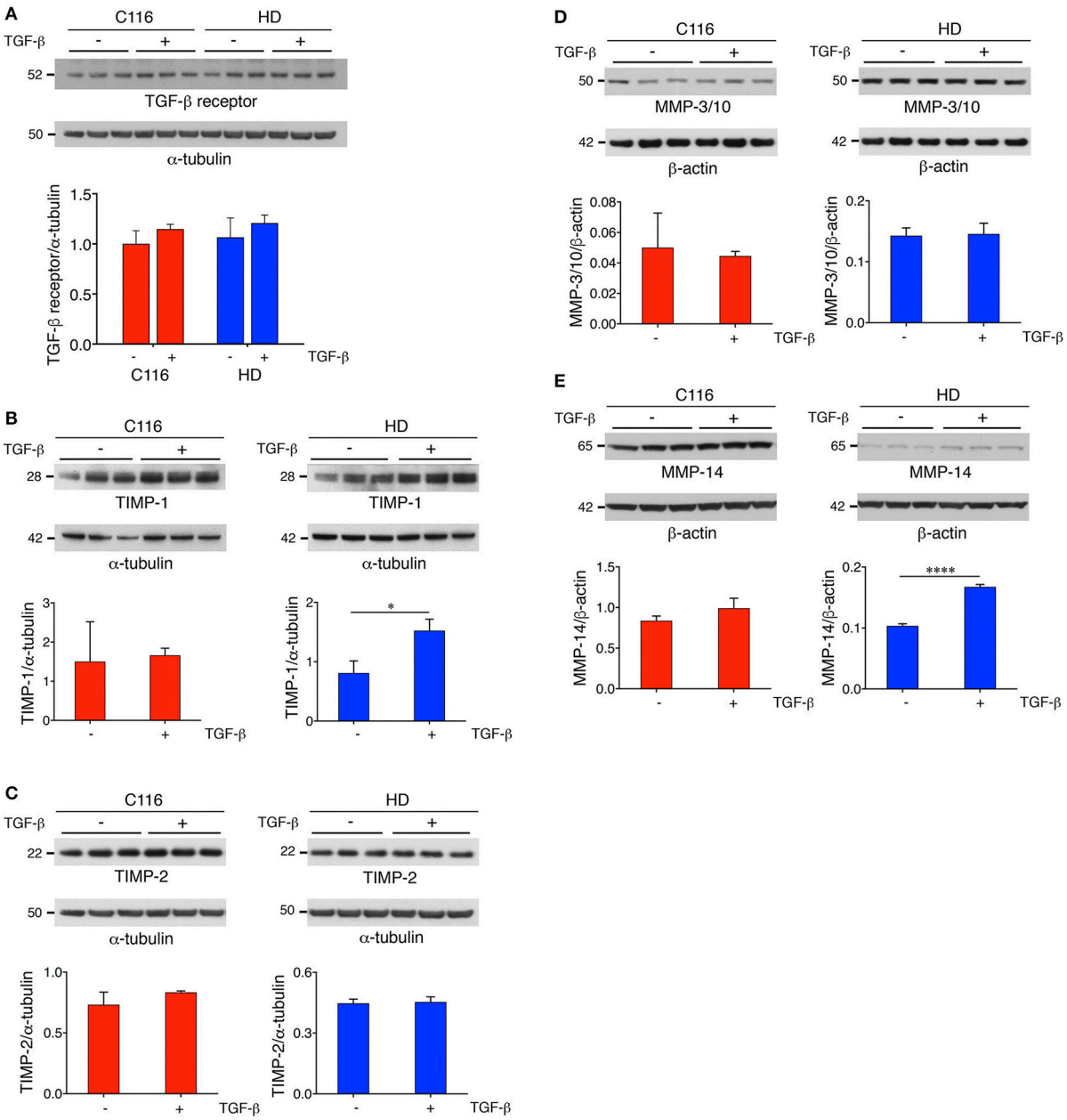


FIGURE 6 | TGF-β treatment regulates TIMP-1 expression. C116- and HD-NSCs were treated with 10 ng/mL TGF-β for 24 h. **(A)** Immunoblot analysis demonstrates that both corrected C116- and HD-NSCs express the TGF-β receptor at equal levels, and that exogenous TGF-β administration does not modulate expression of its cognate receptor (ANOVA with Tukey's multiple comparison test). Western blot analysis reveals that TGF-β treatment resulted in increased expression of TIMP-1 in HD-NSCs **(B)**, but not in C116-NSCs. No changes are observed in the expression levels of TIMP-2 **(C)** and MMP-3/10 **(D)** in response to TGF-β, however, a very modest increase is observed in MMP-14 expression in HD-NSCs **(E)** (*$p < 0.05$; ****$p < 0.0001$; *t*-test). Error bars represent *SD*.

fragments (Miller et al., 2010). Since MMP-10 shares a very high degree of homology with MMP-3 (86% identical catalytic domains) (Bertini et al., 2004), MMP-3 may very well be implicated in contributing to mHTT cleavage and toxicity as well. Our results showing a significant increase in MMP-3/10 activity in HD-NSCs suggests that mHTT toxicity occurs in early stages of neuronal cell development in HD. A study has shown that neurons that are under cellular stress release the active form of MMP-3, which in turn triggers microglial activation and production of pro-inflammatory cytokines including Tumor necrosis factor α (TNF-α), interleukin-6 (IL-6) and IL-1 (Kim et al., 2005), thereby contributing to the neuroinflammatory response in the HD brain (Bjorkqvist et al., 2008), and worsening of disease (Connolly et al., 2016). Furthermore, nuclear presence of MMP-3 (as shown in **Figure 2B**) was found to be associated with caspase-3 mediated cellular apoptosis (Mannello et al., 2005;

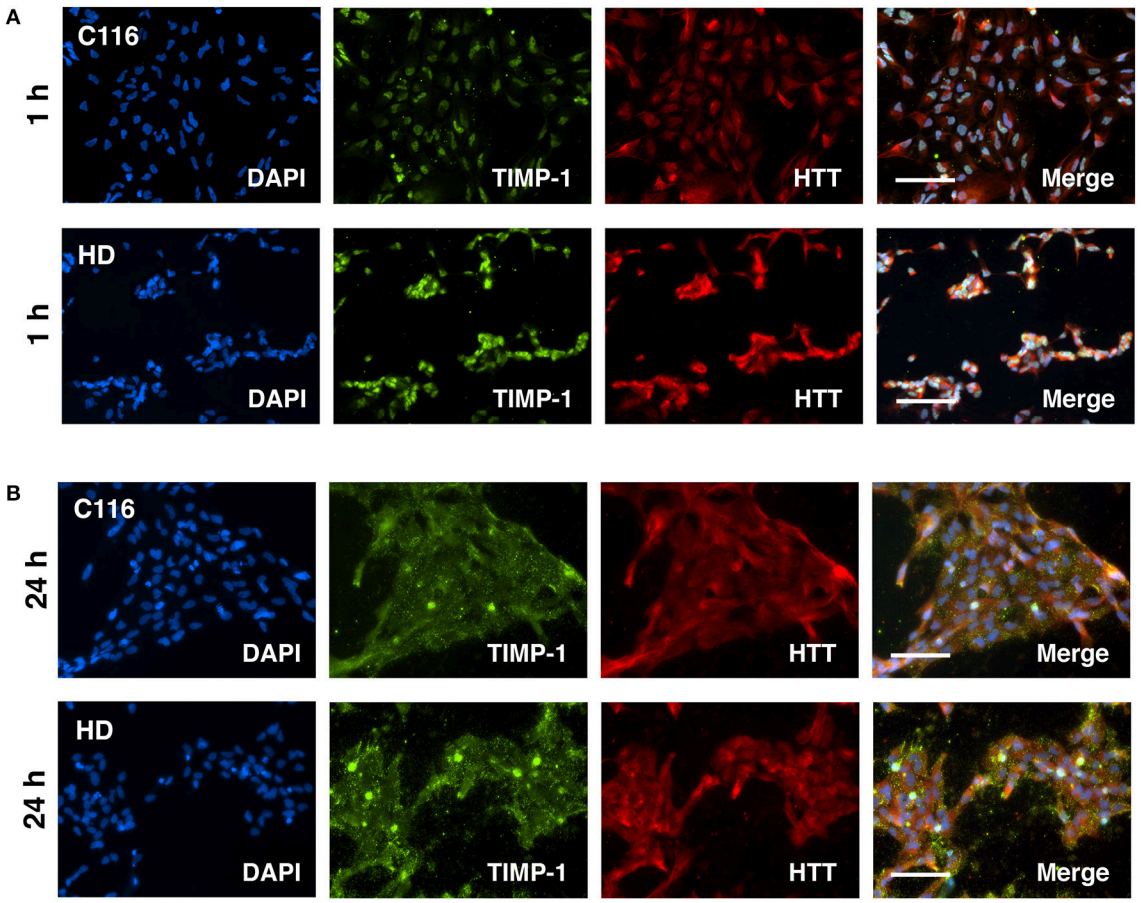


FIGURE 7 | Altered localization of TIMP-1 in response to TGF-β treatment as a function of time. C116- and HD-NSCs were treated with 20 ng/mL TGF-β. **(A)** Immunofluorescence analysis shows robust induction of TIMP-1 levels in HD-NSCs. Strong nuclear expression of TIMP-1 is observed in both C116- and HD-NSCs. However, the expression becomes predominantly cytoplasmic after 24 h of TGF-β treatment **(B)** across both genotypes. Scale Bars: 100 μm.

Si-Tayeb et al., 2006; Choi et al., 2008), thus contributing to neuroinflammation and neuronal damage (Kim and Hwang, 2011). These studies put together indicate that specific inhibition of MMP-3 and MMP-10 activity may prove to be of therapeutic value in treating HD.

Contrary to our previous findings that MMP-14 levels were elevated in $Hdh^{111Q/111Q}$ (Miller et al., 2010), diminished expression of MMP-14 was observed in HD-NSCs. This can be attributed to change in steady-state levels of MMP-14 during activation and processing of this enzyme or to difference in cell type or species since previous studies involved immortalized mouse striatal cell lines vs. human NSCs in this study. In fact, elevated MMP-14 levels in HD-NSCs may be beneficial as indicated by a study showing that elevated MMP-14 levels found in association with reactive astrocytes surrounding amyloid deposits exhibited Aβ-degrading activity *in vitro* (Liao and Van Nostrand, 2010). Thus, boosting MMP-14 levels may help facilitate Aβ clearance and be of therapeutic benefit in AD. Similarly, it would be interesting to explore whether MMP-14 directly interacts with mHTT containing aggregates as well, thereby facilitating mHTT clearance and diminishing associated toxicity in HD.

Our gelatin zymography studies clearly demonstrate altered activation of MMP-2 and -9 in HD-NSCs. While increased expression of MMP-9 was observed in HD-NSCs, activation of MMP-2 was diminished. Since a ternary complex of pro-MMP-2, TIMP-2, and MMP-14 is required for activation of MMP-2 (Nagase, 1998; Itoh and Seiki, 2004) (**Figure 8**), decreased expression of MMP-14 and TIMP-2 in HD-NSCs possibly accounts for decreased levels of active MMP-2.

Our immunofluorescence analysis indicated that increased nuclear MMP-2 and -9 expression is associated with degenerating nuclei in apoptotic cells. Much like caspase-3 activity, intranuclear MMP-2 and -9 have been shown to have pro-apoptotic functions in neurons by hindering DNA strand break repair through inactivation of poly-ADP-ribose-polymerase-1 (PARP-1), a DNA repair enzyme (Mannello et al., 2005; Hill et al., 2012). Intranuclear MMP activity in neurons has also been associated with neuroinflammation (Kimura-Ohba and Yang, 2016).

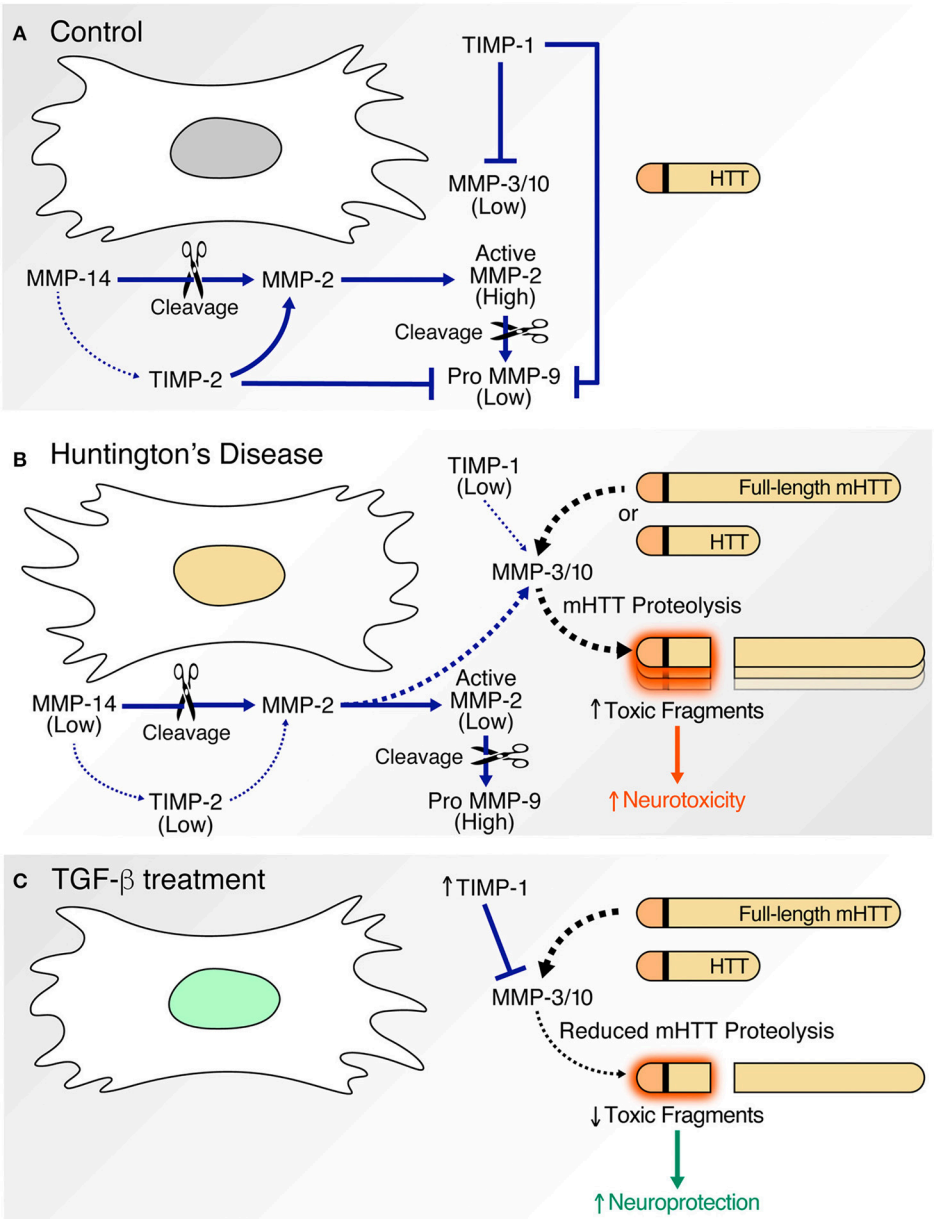


FIGURE 8 | Proposed model. In comparison to corrected C116-NSCs **(A)**, HD-NSCs express altered levels of MMPs and TIMPs **(B)**. **(A)** Under basal conditions, MMP-3/10 activity is kept in check by TIMP-1. MMP-14, MMP-2, and TIMP-2 form a trimolecular complex that facilitates activation of MMP-2, which in turn induces MMP-9. MMP-9 activity is greatly inhibited by TIMPs. **(B)** Decreased expression of TIMPs in HD-NSCs result in unregulated MMP activity as indicated by elevated MMP-3/10 expression. MMP-3/10 directly cleaves full-length mutant HTT (mHTT) leading to the production of toxic N-terminal polyQ fragments, consequently contributing to increased cell death in HD-NSCs. Decreased expression of TIMP-2 and MMP-14 result in reduced activation of MMP-2. Increased MMP-9 activity is observed possibly as a result of decreased TIMP expression. **(C)** TGF-β treatment upregulates TIMP-1 expression, which in turn inhibits MMP-3/10 activity. This inhibition prevents MMP-3/10-mediated cleavage of full-length mHTT, thereby decreasing the production of toxic N-terminal fragments and amelioration of cell death. Thus, TGF-β conferred neuroprotection is likely mediated through increased expression of TIMP-1.

The biology of the MMP-2 and -9 is truly complex. Contrary to the above studies, these gelatinases were also shown to have beneficial roles in AD. When incubated with Aβ40 and Aβ42, MMP-2, and -9 sequentially degraded them into highly soluble, non-toxic truncated fragments, thereby facilitating Aβ clearance (Hernandez-Guillamon et al., 2015). Thus, it would be interesting to study potential interactions of expanded mHTT with recombinant MMP-2 and MMP-9.

MMP-2 and -9 have been widely implicated in pathogenesis of several neurodegenerative diseases including AD, HD, and ALS. Decreased levels of plasma MMP-2 (Lim et al., 2011) and elevated levels of MMP-9 in post-mortem cortical tissue

(Bruno et al., 2009), CSF samples (Adair et al., 2004), and plasma samples (Lorenzl et al., 2003b) of AD patients have been reported, and shown to bear direct correlation with worsening Mini-Mental State Examination (MMSE) scores (Bruno et al., 2009; Lim et al., 2011). These studies clearly underscore the clinical utility of these MMPs as valuable prognostic biomarkers of neurodegenerative disease progression. Furthermore, MMP-9 inhibition was reported to improve Aβ-mediated cognitive impairment and neurotoxicity in mice (Mizoguchi et al., 2009). On similar lines, studies by Kaplan et al. demonstrated that MMP-9 is a prospective marker for motor neuron loss in late-stage ALS, and that reduction of MMP-9 function through gene ablation, viral gene therapy, and pharmacological inhibition significantly delayed muscle denervation (Kaplan et al., 2014). Additionally, a positive correlation was found between significantly increased MMP-9 CSF levels and disease severity in HD (Connolly et al., 2016). Thus, MMP-9 appears to be a good candidate therapeutic target for treatment of several neurodegenerative disorders including HD.

Western blot analysis further demonstrated that both TIMP-1 and TIMP-2 expression was downregulated in HD-NSCs compared to C116-NSCs, thereby directly accounting for the altered MMP levels in HD-NSCs. However, TIMPs are pleiotropic and also serve MMP-independent functions in the central nervous system (CNS) (Stetler-Stevenson, 2008; Moore and Crocker, 2012). Both TIMP-1 and TIMP-2 are anti-apoptotic (Mannello and Gazzanelli, 2001; Brew and Nagase, 2010) and have direct roles in conferring neuroprotection (Kim et al., 2010; Ashutosh et al., 2012). Nuclear localization of TIMPs, especially TIMP-1, has been previously reported (Li et al., 1995; Zhao et al., 1998), inviting the speculation that they have important roles in nuclear functions such as replication and transcription (Mannello and Medda, 2012). Furthermore, association of TIMP-1 with mHTT aggregates in discrete punctate structures as revealed by immunofluorescence analysis suggests a direct physical interaction of TIMP-1 with mHTT. Presence of TIMP-1 in these mHTT aggregates possibly serves to directly inhibit MMP-10 activity, which has been reported to strongly colocalize with mHTT aggregates as well (Miller et al., 2010).

Studies by Moore et al. show that compact myelin formation is significantly delayed in TIMP-1 knockout mice, and that astrocytic TIMP-1 promotes oligodendrocyte differentiation and enhances CNS myelination (Moore et al., 2011). Furthermore, transplantation of immature iPSC-derived astrocytes or administration of their conditioned medium promoted recovery following brain injury in a TIMP-1 dependent manner (Jiang et al., 2016). TIMP-1 overexpression also ameliorates MMP-9 mediated blood-brain barrier leakage in models of stroke, traumatic brain injury (Tejima et al., 2009) and cerebral ischemia (Fujimoto et al., 2008). Thus, astrocytic TIMP-1 plays an important role in CNS homeostasis and disease and may have significant therapeutic relevance (Gardner and Ghorpade, 2003).

TIMP-2 plays an equally important role in the CNS. TIMP-2 is expressed in spinal motor neurons, and is necessary for development and maintenance of the neuromuscular junction, as indicated by the presence of motor deficits in TIMP-2 deficient mice (Jaworski et al., 2006). TIMP-2 also has a potent anti-inflammatory role. Overexpression of TIMP-2 in LPS-stimulated microglia inhibited the production of reactive oxygen species and proinflammatory cytokines, including TNF-α and IL-1β, while simultaneously increasing production of anti-inflammatory IL-10 (Lee and Kim, 2014). TNF-α and IL-6 were found to be significantly elevated in postmortem HD patient samples and in mouse models of HD (Bjorkqvist et al., 2008), and thus, boosting TIMP-2 levels in the HD brain could attenuate the neuroinflammatory response, thus providing therapeutic benefit.

As with MMPs, TIMPs can also be valuable biomarkers for neurodegenerative diseases (Lorenzl et al., 2003a). Studies by Mroczko et al. showed that simultaneous examination of MMP-2, -9, and TIMP-1 in the CSF allowed for differential diagnosis between AD and other types of dementia (Mroczko et al., 2013). Elevated CSF levels of TIMP-1 and TIMP-2 in HD patients, which probably represent a compensatory attempt at controlling unabated MMP activity, could also be potential biomarkers for HD (Lorenzl et al., 2003a; Connolly et al., 2016).

A proposed mechanism for our findings illustrates the contribution of altered MMP and TIMP expression to HTT proteolysis and neuronal cell death (**Figure 8**). Under basal conditions (**Figure 8A**), MMP activity is tightly regulated by TIMPs. MMP-2, MMP-14, and TIMP-2 form a ternary complex (Nagase, 1998; Itoh and Seiki, 2004). MMP-2 zymogen is proteolytically processed into its active form by MMP-14, and MMP-2 further activates MMP-9. TIMPs inhibit the activity of MMP-3/10 (Batra et al., 2012), MMP-2, and MMP-9 (Fujimoto et al., 2008; Tejima et al., 2009). In HD (**Figure 8B**), decreased TIMP-2 levels result in reduced MMP-2 activation. MMP-2 further activates MMP-9 and potentially also activates MMP-3/10. Reduced TIMP levels in HD-NSCs simultaneously contribute to unabated MMP-3/10 and MMP-9 activity. Previous work in the lab has shown that MMP-3/10 directly cleaves full-length mHTT into smaller N-terminal fragments which associate with increased neurotoxicity. Thus, elevated CSF levels of TIMPs (TIMP-1 and TIMP-2) in neurodegenerative diseases, including HD (Lorenzl et al., 2003a), represent a compensatory attempt at regulating already elevated and uncontrolled MMP activity. Exogenous administration of TGF-β (**Figure 8C**) increases TIMP-1 expression in HD-NSCs, thereby inhibiting MMP-3/10 expression and concomitant proteolysis of mHTT into toxic fragments. In conclusion, upregulation of TIMP-1 expression represents a potential mechanism for TGF-β conferred-neuroprotection in HD (Ring et al., 2015). Thus, boosting TGF-β signaling could potentially offer therapeutic benefit in HD. In fact, a recent study demonstrated that deficiency in neuronal TGF-β signaling promoted PD-related pathologies and motor deficits (Tesseur et al., 2017). Increasing TGF-β signaling in the substantia nigra through adeno-associated virus expressing a constitutively active type I receptor significantly reduced MPTP-induced dopaminergic neurodegeneration and motor deficits, and thus, represents a potential therapy for PD. This study also indicates that boosting TGF-β signaling through constitutively activating TGF-β receptor in a cell-type specific manner is more beneficial compared to *in vivo*

delivery of TGF-β ligands, which has yielded contradictory results (Krieglstein and Unsicker, 1994; Poulsen et al., 1994; Krieglstein et al., 1995; Sanchez-Capelo et al., 2003), possibly due to global activation of TGF-β signaling. Thus, we speculate that augmenting TGF-β signaling specifically in the striatum potentially represents a feasible strategy for treatment of HD as well.

Our study also indicates that MMP inhibition can provide therapeutic benefit in HD. However, multiple MMPs are expressed in all cell types throughout the body, with some having crucial multifaceted roles in normal physiology (Brkic et al., 2015), thereby emphasizing the need for developing very specific MMP inhibitors. This has been exemplified by drug discovery efforts in the cancer field showing that more than 50 MMP inhibitors investigated have failed in clinical trials, owing to lack of inhibitor specificity (Vandenbroucke and Libert, 2014). For instance, administration of broad-spectrum MMP inhibitors resulted in musculoskeletal syndrome manifesting as stiffening of joints, hypothesized to be due to inhibition of MMPs crucial to maintaining connective tissue homeostasis, namely MMP-1 and MMP-14 (Becker et al., 2010). In the light of these findings, efforts are now focused on developing highly specific MMP inhibitors that spare off-target isozymes. These studies underscore the need to develop targeted MMP inhibition therapies for treatment of HD.

In conclusion, elucidating the mechanism of action of MMPs and TIMPs, their interactions with mHTT, and their regulation by other cytokines or neurotrophic factors will shed light on their contribution to HD pathology, and help develop novel therapies for treatment of HD. Findings of this study will be broadly applicable to other neurodegenerative diseases and normal aging as well.

AUTHOR CONTRIBUTIONS

LME: designed the project, organized the entire research, and provided the financial support; AE, SN, KM, and KR: conducted the experiments; SN and LME: performed data analysis and interpretation; AE, SN, and LME: wrote the manuscript; SN and LME: revised the manuscript; LME approved the final manuscript; All authors discussed the results and reviewed the manuscript.

FUNDING

This research was supported by CIRM EDUC2-08391 (AE), T32 training grant AG000266 (SN, LME), and NIH R01 NS074408 (LME) and RO1 NS100529 (LME). Support was also provided by "The Taube Family Program in Regenerative Medicine Genome Editing for Huntington's Disease" to LME.

ACKNOWLEDGMENTS

We thank John Carroll for designing the summary figure schematic (**Figure 8**).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2017.00736/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

MINI REVIEW
published: 06 February 2018
doi: 10.3389/fnins.2018.00047

Open Science Meets Stem Cells: A New Drug Discovery Approach for Neurodegenerative Disorders

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Neurodegenerative diseases are a challenge for drug discovery, as the biological mechanisms are complex and poorly understood, with a paucity of models that faithfully recapitulate these disorders. Recent advances in stem cell technology have provided a paradigm shift, providing researchers with tools to generate human induced pluripotent stem cells (iPSCs) from patient cells. With the potential to generate any human cell type, we can now generate human neurons and develop "first-of-their-kind" disease-relevant assays for small molecule screening. Now that the tools are in place, it is imperative that we accelerate discoveries from the bench to the clinic. Using traditional closed-door research systems raises barriers to discovery, by restricting access to cells, data and other research findings. Thus, a new strategy is required, and the Montreal Neurological Institute (MNI) and its partners are piloting an "Open Science" model. One signature initiative will be that the MNI biorepository will curate and disseminate patient samples in a more accessible manner through open transfer agreements. This feeds into the MNI open drug discovery platform, focused on developing industry-standard assays with iPSC-derived neurons. All cell lines, reagents and assay findings developed in this open fashion will be made available to academia and industry. By removing the obstacles many universities and companies face in distributing patient samples and assay results, our goal is to accelerate translational medical research and the development of new therapies for devastating neurodegenerative disorders.

Keywords: neurodegenerative diseases, drug discovery, induced pluripotent stem cells, Open Science, iPSC-derived neurons, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis

OPEN ACCESS

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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *08 September 2017*
Accepted: *19 January 2018*
Published: *06 February 2018*

Citation:
Han C, Chaineau M, Chen CX-Q, Beitel LK and Durcan TM (2018) Open Science Meets Stem Cells: A New Drug Discovery Approach for Neurodegenerative Disorders. Front. Neurosci. 12:47. doi: 10.3389/fnins.2018.00047

INTRODUCTION

Neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), are incurable and debilitating conditions characterized by progressive degeneration of specific neurons within the brains of affected individuals. According to the World Alzheimer Report 2016, there are 46.8 million people living with dementia in the world (Prince et al., 2016). The total estimated worldwide cost of dementia in 2015 was $818 billion US, and is forecast to rise to over a trillion dollars by 2018. The Parkinson's Disease Foundation estimates seven to 10 million people worldwide are living with PD. Medication costs an average of $2,500 per year per patient (Parkinson's Foundation, 2017) and associated costs markedly increase over time (Martinez-Martín et al., 2015; Bovolenta et al., 2017); therapeutic surgery can cost up

to $100,000 (Parkinson's Foundation, 2017). ALS Worldwide reports more than 500,000 people around the world currently suffer from ALS, with an average life expectancy of about 2 to 5 years from the time of diagnosis (Naqvi, 2017). Moreover, the average cost of ALS to a family over the course of the disease can be $150,000 to $250,000 (Arthur et al., 2016). Since the incidence of neurodegenerative conditions increases significantly with age, and world populations are rapidly ageing, the number of people with dementia or PD is expected to reach up respectively to 131.5 million and 8.7 million by 2040 (Kowal et al., 2013), while the number of people with ALS is estimated to increase to 377,000 (Arthur et al., 2016). Thus, for countries throughout the world, neurodegenerative diseases have become an enormous economic burden that is projected to grow significantly over the next few decades in the absence of any new therapeutic interventions.

Despite massive investments in drug discovery and growing numbers of molecules in development, there are still no cures or disease-modifying therapies for neurodegenerative diseases. Currently available therapies only help manage symptoms of these disorders, and none identified to date can halt or prevent progression of these disorders. Only four drugs are approved and currently used in symptomatic treatment for AD: acetylcholinesterase inhibitors, including donepezil (1997), rivastigmine (2000), and galantamine (2001), to ameliorate the clinical manifestations of AD by enhancing cholinergic neurotransmission in relevant parts of the brain (Birks et al., 2000; Olin and Schneider, 2002; Cacabelos, 2007); and memantine (2003), a N-methyl-D-aspartate receptor antagonist for improving AD behavioral symptoms (van Marum, 2009). For PD, L-dihydroxyphenylalanine (L-DOPA), combined with peripheral inhibitors of L-amino acid aromatic decarboxylase (carbidopa and benserazide) is still the gold-standard of care (LeWitt, 2015) but unfortunately, the beneficial effects of L-DOPA are not permanent and motor fluctuations and dyskinesia occur after a few years of treatment (Guridi et al., 2012). Also, none of the current anti-parkinsonian agents, including L-DOPA, has shown convincing activity as a disease modifier. Rilutek (also known as riluzole), was the first medication that the FDA approved specifically for the systemic treatment of ALS. Although it helps slow down the progression of ALS/motor neuron disease and prolongs survival, it does not cure ALS nor reverse nerve damage or muscle weakness (Petrov et al., 2017). Edaravone, a free radical scavenger approved by the FDA in May 2017, is only effective in specific well-defined types of early stage ALS and there is no evidence showing it can prolong survival (Hardiman and van den Berg, 2017).

THE CLASSICAL DRUG DISCOVERY PIPELINE

Hence, only a limited number of drugs are currently available for treatment of neurodegenerative disorders, and despite increased investment in R&D for the past seven decades, the number of new drugs brought to market by pharmaceutical companies has not increased accordingly (Munos, 2009). The classical drug discovery pipeline comprises different stages, with the first step using a target or phenotype-driven drug screen to identify one or more small molecules. Candidate molecules with the largest effect are next directed into medicinal chemistry programs, to modify their structures and enhance specificity, efficacy and stability. One or two lead compounds are tested in animals to determine the molecule's toxicity, and optimal dose and delivery route. Following success in cell and animal models, the lead molecule is brought forward to a phase I trial to test the safety of the molecule in humans, before being tested for efficacy in an increased number of patients in phase II and III clinical trials. After completion of phase III, the candidate drug must be approved by relevant regulatory agencies such as the Food and Drug Administration (FDA) in the US, the Health Products and Food Branch in Canada, or the European Medicines Agency in European Union, before being released to the market. According to the Pharmaceutical Research and Manufacturers of America, developing a new medicine costs $2.6 billion on average from drug discovery to FDA approval. Drug discovery and development is inherently risky, with recent figures indicating that less than 11% of new pharmaceutical agents that entered clinical development reached the marketplace across all therapeutic areas (DiMasi et al., 2003). Drugs for the central nervous system, including neurodegenerative diseases, that entered clinical development, have a considerably lower probability of reaching the marketplace (7%) than the industry average across other therapeutic areas (15%), and require a longer time for development and regulatory approval (average of 12.6 years) compared with most other diseases (e.g., 6.3 years for cardiovascular and 7.5 years for gastrointestinal indications) (Kola and Landis, 2004; Pangalos et al., 2007). In AD, for example, the cost of developing a disease-modifying therapy, including the cost of failures, is currently estimated at $5.7 billion (Scott et al., 2014). However, over 100 compounds tested as potential therapies were either abandoned in development or failed in clinical trials, e.g., a negative Phase III trial of the once-promising AD therapy solanezumab (Doody et al., 2014), and a halted late-stage trial on the drug verubecestat for AD (Hawkes, 2017).

CHALLENGES IN DRUG DISCOVERY

Developing new therapies requires a deep understanding of the genes and targets that drive neuronal death. While our understanding of these disorders has advanced significantly, the complexity of the brain and a lack of access to human tissue has hindered progress. Consequently, current models, including cell and animal models, may not predict whether a drug candidate is likely to modify disease progression or improve patient behavior. Another barrier in current drug development is the lack of transparency in communicating and sharing of data and reagents. Most studies, including clinical trials, keep their data and biospecimens behind restrictive firewalls and material transfer agreements (MTAs) and only publish positive results, leaving large amounts of negative, but potentially meaningful data lying dormant. Hence, it is essential to improve the current drug development process for neurodegenerative diseases, to efficiently share clinical samples and research data, and to find

strategies that lower the cost, time, and risk in delivering new therapies.

CLASSICAL CELLULAR AND ANIMAL MODELS

While we are beginning to understand the cellular pathways involved in neurodegenerative diseases, many experimental studies and drug trials have been based on results from laboratory-grown cell lines and experimental animal models. Attempts at translating "cures" from mice to humans have been largely unsuccessful for neurodegenerative diseases, due to fundamental species-specific differences. The relatively short lifespan of rodents may not allow for development of clear-cut neurodegenerative phenotypes, while acute models may not accurately represent the mechanisms underlying chronic neurodegeneration. Although results from animal models may predict drug efficacy for symptomatic treatment, they are less helpful for identifying drugs that potentially act as disease modifiers. Thus, many clinical trials arising from preliminary work in animal models have failed (Becker, 2007; Mehta et al., 2017). In term of cellular models, most groups use immortalized fibroblasts, nervous system tumors, or immortalized neuronal progenitor cell lines for *in vitro* assays to identify potent agents with desired selectivity profiles. Although these cell lines grow readily at a relatively low cost, they generally cannot fully represent critical features of endogenous neural cells, and often fail to reflect relevant disease pathways. One strategy to overcome this hurdle would be drug screening in the most relevant cell-types (e.g., cholinergic basal forebrain neurons for AD, dopaminergic neurons for PD, and motor neurons for ALS) obtained from patients afflicted with the disease. In cancer, access to such patient material (via tumor resections or biopsies) has led to a revolution in new therapies, with many patients now bypassing toxic chemotherapy regimens for newer targeted personalized therapies, based on their tumor biology (Goodspeed et al., 2016). Unfortunately, access to relevant patient-derived cells has been a major hurdle in neurodegenerative diseases, as biopsies or resections to obtain neurons are rarely, if ever, carried out in patients afflicted with these diseases.

INDUCED PLURIPOTENT STEM CELLS AS A NEW DRUG DISCOVERY MODEL FOR NEURODEGENERATIVE DISEASES

The discovery of the Yamanaka factors more than a decade ago (Takahashi and Yamanaka, 2006), has led to a paradigm shift in stem cell biology, providing the tools to efficiently generate human induced pluripotent stem cells (iPSCs) using skin (Takahashi et al., 2007), blood (Loh et al., 2009) or urine-derived cells (Zhou et al., 2011). Under the appropriate conditions, iPSCs can be differentiated into any cell type, including neurons. This technology has opened a new avenue for research, allowing scientists access to human neurons and other cell types involved in neurodegenerative diseases, such as astrocytes, microglial cells and oligodendrocytes, in an unlimited manner (**Figure 1**). Disease-related phenotypes in patient iPSC-derived neurons are undoubtedly helpful for understanding disease mechanisms and pursuing potential treatments, and to bridge the gap between current pre-clinical research and clinical testing by giving us a better predictive value than current animal and cellular models.

The development of iPSC technology makes it possible to acquire disease-specific cell lines from patients carrying familial mutations and these cell types show exciting promise for the elucidation of neurodegenerative disease etiology. A few clinical trials have been initiated based on results obtained by using iPSC technology. Bright and colleagues generated iPSCs from patients with sporadic or presenilin-1-mutant AD. By comparing the cortical neurons derived from these AD patients and age-matched controls, they discovered the AD-derived neurons secreted a specific form of Tau and they developed BMS-986168 as a specific antibody for the Tau fragments (Bright et al., 2015). In 2017, BMS-986168, licensed by Biogen, entered Phase II clinical trials for AD, and progressive supranuclear palsy. In another study, iPSC-derived motor neurons from ALS patients were found to be hyperexcitable compared to controls, and Retigabine, an approved drug for epilepsy, could rescue this hyperexcitability phenotype in motor neurons derived from patients with different ALS-associated mutations (Wainger et al., 2014). Presently, Retigabine is under a placebo-control Phase II clinical trial with 192 ALS patients in collaboration with GlaxoSmithKline. Moreover, iPSC-derived neurons also create opportunities to study sporadic forms of neurodegenerative diseases, which are the vast majority, and in which the causes remain largely unknown. There is a growing number of iPSC lines derived from patients with sporadic AD, PD, or ALS (Qian et al., 2017; Zhang et al., 2017). These cell lines, which carry different genetic risk variants, will help us obtain better insights into the pathogenesis of sporadic disorders, and will be useful *in vitro* cellular models for drug discovery. For example, motor and cortical neurons differentiated from sporadic ALS patients show *de novo* TDP-43 aggregation, which is one of the observed pathologies in postmortem tissue from ALS patients (Burkhardt et al., 2013). Using the TDP-43 aggregation phenotype as readout in a high-content chemical screen in lower and upper motor neuron-like cells, the authors also identified previously approved drugs with known targets that could modulate TDP-43 aggregation. Moreover, iPSC-derived disease models are starting to be used for drug discovery for other neurological diseases including spinal muscular atrophy (Ando et al., 2017), multiple sclerosis (Miquel-Serra et al., 2017) and autism spectrum disorders (Mokhtari and Lachman, 2016). With the technology to reprogram and generate selected types of functional neurons, iPSCs are also widely considered to have good potential for cell replacement therapy in neurodegenerative diseases. Intriguingly, neural precursor cells differentiated from reprogrammed iPSCs were reported to migrate into various brain regions upon transplantation, to differentiate into glia and neurons, including dopaminergic neurons, and to improve behavior in both rodent and primate models of PD (Wernig et al., 2008; Kikuchi et al., 2017). However, it is important to note that key questions related to safety and efficacy of such therapy

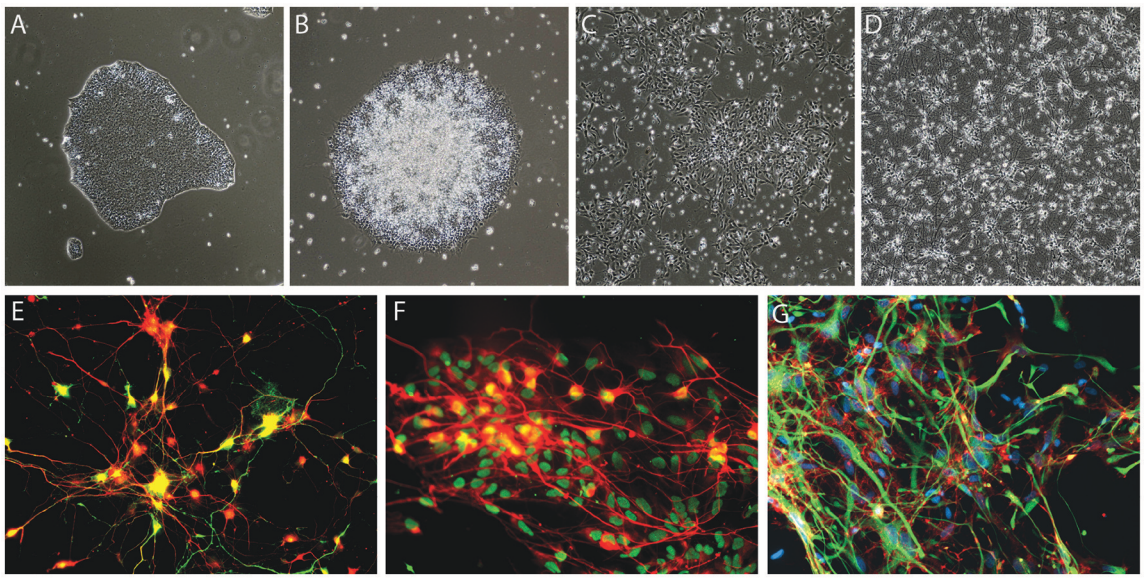

FIGURE 1 | Examples of iPSC-derived cells. **(A–E)** Generation of dopaminergic neurons from human iPSCs based on the protocol developed by Kriks et al. (2011). Brightfield images of iPSC neural differentiation, **(A)** iPSCs, **(B)** neural rosettes, **(C)** dopaminergic neural precursor cells, **(D)** dopaminergic neurons, and **(E)** tyrosine hydroxylase (TH)-expressing dopaminergic neurons derived from iPSCs, TH in green and pan-neuronal marker beta-III tubulin in red **(F)** example of motoneurons generated based on the protocol of Du et al. (2015), HB9 in green and pan-neuronal marker beta-III tubulin in red **(G)** iPSCs-derived glial fibrillary acidic protein (GFAP)-expressing astrocytes generated using Krencik and Zhang's protocol (Krencik and Zhang, 2011), GFAP in green and CD44 in red.

still need to be addressed before clinical trials of stem cell-based transplantation for PD (Barker et al., 2017).

With the advent of iPSCs, a burgeoning pharmaceutical and biotechnology field has emerged (Passier et al., 2016). In early years, several start-ups were founded, focused on using iPSCs for deriving human cells for safety studies, small molecule screens and *in vitro* disease modeling. Several companies proved successful at deriving neurons and other cell types to sell to pharmaceutical industries and to other users for drug toxicity testing or for further research. Other start-ups focused on developing new drug discovery platforms for neurodegenerative diseases using iPSC-derived neurons. More companies are now starting to take advantage of iPSCs to generate new clinical products, using iPSC-based disease models to bring new therapies into clinical trials. IPSC technology has advanced significantly in the last 5 years, reducing both the time and cost involved. In parallel, new genes and pathways have been identified that can be harnessed to develop disease-relevant assays. We foresee that using iPSC technology to probe disease mechanisms and screen for new drugs will effectively usher in a new era of therapeutics and personalized medicine for devastating neurodegenerative diseases.

However, under the current model of MTAs and legal agreements, accessing iPSCs can be cumbersome, with complex legal agreements required before researchers can access cell lines. Moreover, researchers are often heavily restricted in their use of these lines, and even in how they can disseminate their findings. All these obstacles increase the time and efforts required to go from the bench to clinic, adding to the already long drug development pipeline. Thus, we believe that removing these restrictions and streamlining the process in a more "open" manner, and making iPSCs and all data generated from these lines openly available to the research community, will help to accelerate the drug discovery process.

OPEN SCIENCE, A NEW PATH FOR RESEARCH AND DEVELOPMENT

The goal of Open Science is to accelerate research and discovery by encouraging collaborations and partnerships. The term "Open Science" embraces different levels of openness, from "open data" which implies sharing results with the scientific community, to an "open access" model in which every step of the research process should be transparent to the community. Such a model would mandate that results, publications, reagents, compounds and even clinical trials results are accessible to the public and all groups without restriction. Using iPSC technology to study neurodegenerative diseases will lead to an increased number of biological samples collected and amount of data generated. Adopting an Open Science policy is one strategy to build an efficient infrastructure to support the exploration, integration and utilization of existing data and biological samples resources to accelerate drug discovery in neurodegenerative diseases. Among the most famous Open Science initiatives are the Human Genome Project and the Allen Institute. A formal agreement to encourage free distribution of research data, technology and resources created by the Human Genome Project has already had a major input on research across the life sciences. Especially, it brings important genetic clues to understanding diseases in

terms of human biology and pathology, which is "starting to have profound impact on biomedical research and promises to revolutionize the wider spectrum of biological research and medical medicine" (Kelavkar, 2001). The Allen Institute, founded in 2003, quickly became a powerful resource for brain scientists worldwide by freely sharing gene-expression maps for human and mouse brains (Siegle et al., 2017).

The Open Science era is also expected to be of great benefit to drug development by increasing partnerships between academia and pharmaceutical companies, and eliminating barriers between the different stages of drug development. A successful example is the "Pathogen Box," which is an open-access collection comprised of 400 compounds with demonstrated biological activity against specific pathogenic organisms that cause tropical and neglected diseases. Upon request, researchers around the world will receive a Pathogen Box of molecules to help catalyze neglected disease drug discovery. In return, researchers are asked to share any data generated in the public domain, creating an open and collaborative forum for neglected diseases drug research (Duffy et al., 2017). One of the successes from "Pathogen Box" has been published with the identification of *Candida albicans* biofilm inhibitor (Vila and Lopez-Ribot, 2017). Another precedent is the sharing of the chemical probe JQ1. JQ1 is a small molecule targeting bromodomain proteins that regulate protein-histone association and chromatin remodeling. After the discovery of JQ1's effect on specific cancer cells (Filippakopoulos et al., 2010), the researcher released all the information on JQ1 and distributed samples of JQ1 to academic and industrial laboratories worldwide. This open-access manner considerably accelerated drug discovery for this class of compound, not only in the field of cancer, but also for other diseases, including neurodegenerative diseases (Scott, 2016). According to the data from ClinicalTrials.gov, there are 21 Phase I clinical trials, 2 Phase I/II trials, and 1 Phase III trial for bromodomain inhibitors (Xu and Vakoc, 2017). There are more and more initiatives sharing well-characterized preclinical compounds with the whole research community. Boehringer Ingelheim recently launched a platform opnME portal to share nearly 20 high-quality chemical probes, without intellectual property restrictions (Mullard, 2017). The Structural Genomic Consortium (SGC) is another outstanding example of an organization involved in advancing the Open Science model. SGC, founded in 2004, represents a worldwide partnership between universities and pharmaceutical companies. The key to making this model work is the combination of different principles, including a full commitment by scientists in exchange for predictable funding, as long as they meet their milestones, and a requirement for data sharing and increasing reproducibility (e.g., by using electronic lab notebooks) (Edwards, 2016a). Currently, SGC scientists from six universities are collaborating with scientists from nine large pharmaceutical companies to test the effects of compounds and chemical probes in primary human cells from patients with different diseases, such as cancer and inflammatory and auto-immune diseases (Edwards, 2016b). Recently, SGC started a collaboration with the MNI, as part of the Neuro Open Science initiative, to screen their compounds on iPSC-derived cells from patients with PD and ALS.

As discussed above, the Open Science model can help the pharmaceutical industry and academics to work together to advance the discovery and development of medicines. However, intellectual property is a key concern in this model. In the pharmaceutical industry, with multibillion dollar investments in molecules that can easily be recreated by competitors, investors require proof of protection of their assets. It is therefore natural to assume that open access may jeopardize this, as it is a widely held belief that an exclusivity period is required for an organization to profit from a new drug. However, in the case of open access to JQ1, there has been a clear increase in research activity around the bromodomain proteins, leading to more than 100 filed patents. These patents are not for JQ1 itself, but for other molecules that target bromodomains; the development of many of these was guided by the use of JQ1 as a research tool (Arshad et al., 2016). With a wider, multidisciplinary research community contributing to higher impact research into the molecule itself, the initial free availability of the JQ1 molecule led to increased downstream patenting. This offers evidence that open access is a commercially viable model for drug discovery with the potential to lead to improved commercial gain for drug developers in the long run. By allowing the initial stages of drug development to be carried out in an Open Science model, many researchers can benefit from the availability of information regarding drug candidates during a time of high risk and attrition. The Open Science environment would allow this high risk to be distributed among different stakeholders, all the while facilitating downstream patenting, allowing inventors to benefit from their inventions at a later and more commercially viable stage of drug translation. This could in fact lead to greater profit for an industry that has been suffering from declining reimbursement in the past few decades.

OPEN SCIENCE MEETS iPSCs

Seeking to accelerate the generation of knowledge and to develop novel effective treatments for brain disorders, the MNI is adopting an institutional Open Science policy that includes five aspects: open access, open data, open intellectual property, open sharing of biological samples and other resources, and open commercialization (Poupon et al., 2017). As the first academic research institution to develop an Open Science framework, a robust cyberinfrastructure platform plays a critical role in allowing sharing of data and materials. The MNI implemented its own cyberinfrastructure, using the LORIS and C-Brain platforms developed at the MNI by Dr. Evans, and have made a vast amount and variety of data easily accessible (Das et al., 2016). Beyond cyberinfrastructure, two other key components to stimulate drug discovery are the MNI Open Clinical Biological Imaging and Genetic Repository (C-BIGR), and the Open Drug Discovery Platform (ODDP). The C-BIGR is a freely-shared source of information linked to biomedical specimens, based on the strategy that deep phenotypic information about each patient will be obtained, as well as a variety of biological samples. Under the supervision of the Research Ethics Board, C-BIGR created an information and consent form for patients, outlining

how their biological material and data will be used and stored; and also developed an encrypted system to protect all related personally identifiable information. Within this infrastructure, C-BIGR is designed to curate brain imaging, clinical, demographic, genetic (DNA), and cell data, along with biological samples from patients with neurological disorders, all of which will be made openly available to users upon request. The Open Drug Discovery Platform includes the MNI iPSC/CRISPR platform, Neuro-SGC (assay development), and Neuro-CDRD (Center for Drug Research and Development) (automation and screening). These combined platforms will use iPSCs derived from C-BIGR samples to create disease-relevant assays that should facilitate accurate therapeutic target identification, and bring new drugs more rapidly to market (**Figure 2**).

Combining iPSC technology and Open Science infrastructure will be advantageous for accelerating and disseminating developments in disease-modifying therapies. First, through collaborations between C-BIGR and the iPSC/CRISPR platform, we have direct access to neurons generated from patient-derived iPSCs to study mechanisms of neurodegenerative diseases. These studies will greatly enhance our knowledge and provide valuable information on potential drug targets. Secondly, with

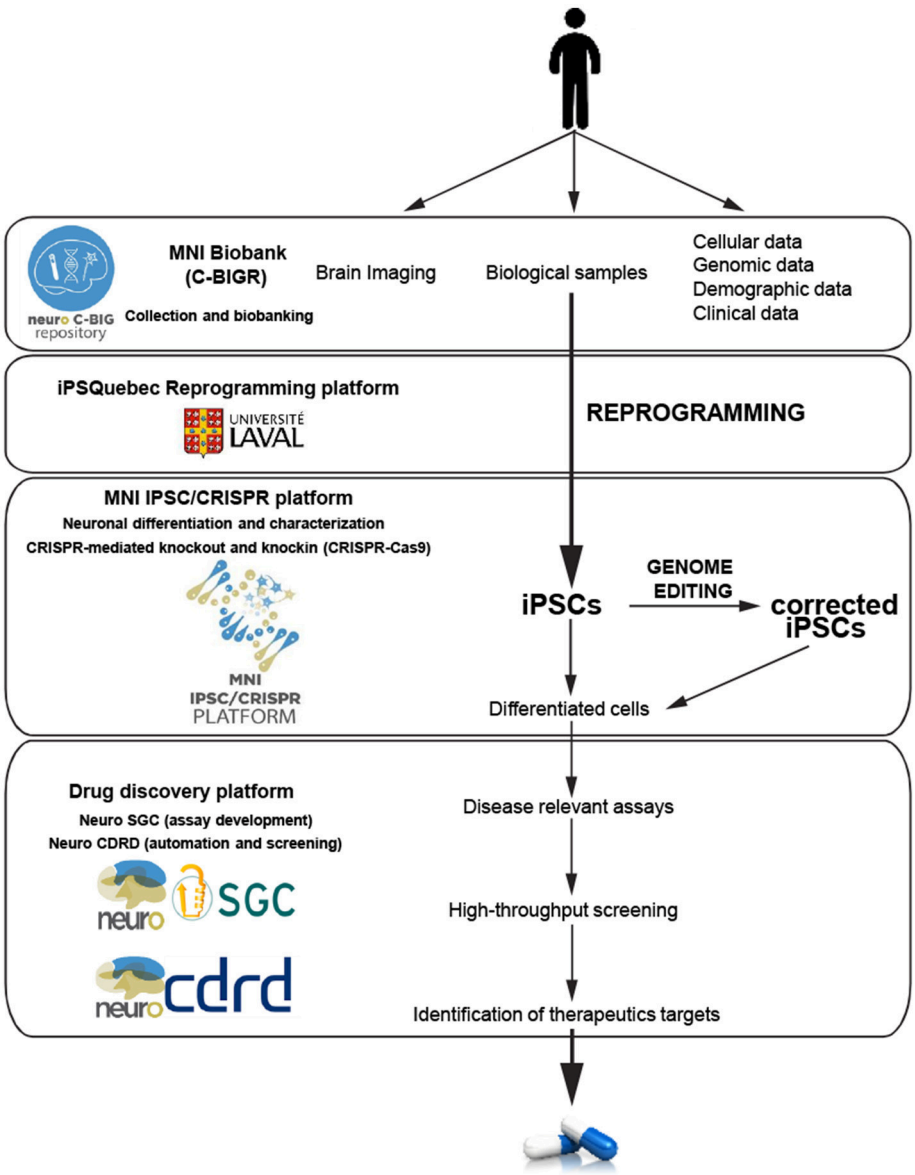

FIGURE 2 | Schematic of the hiPSC neurodegenerative disease modeling for drug discovery at the MNI. Data and biological samples from patients with neurodegenerative diseases are collected and banked by the C-BIGR. hiPSC derived from somatic cells from patients are characterized and isogenic controls are created by the iPSC-CRISPR platform. IPSC are differentiated into specific cells including different types of neurons, astrocytes and glial cells. Relevant differentiated cells are then used by the drug discovery platform to develop disease-relevant assays to screen for therapeutic targets.

an "Open Access" policy, all research results and observations will be published on MNI Open Research, a science publishing platform (https://mniopenresearch.org/), in a nearly-immediate and no-restriction way, including negative results. This policy will provide support for research integrity, reproducibility and transparency, which are the foundations for success of translational medical research. Sharing data openly can also bring about the opportunity to explore existing data in a worldwide collaborative efficient manner, which should directly accelerate neurodegenerative disease drug discovery.

Developments in iPSC technology and other rapid advances in cellular and molecular neurobiology, wide collaboration between industry/pharma, clinicians and academic researchers, and commitment to an Open Science philosophy will be the future driving forces to accelerate development of disease-modifying therapies for neurodegenerative diseases, and to spark further discovery and development, including commercialization. By working together in this open manner, we are hopeful this innovative approach will accelerate the development of new treatments for the millions of people with neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

CH, MC, CC, LB, and TD participated in conception and design. CH, MC, and CC drafted the manuscript. CH, MC, LB, and TD revised the manuscript for important intellectual content.

ACKNOWLEDGMENTS

We wish to thank Michael Sundstrom and Aled Edwards (SGC), Tom Pfiefer (CDRD) and Edward A. Fon for rewarding conversations on the merits of open drug discovery with iPSCs. This work was made possible through the financial support of Brain Canada, the Consortium Québécois sur la Découverte du Médicament (CQDM), Mr. Sebastian Van Berkom, the Ellen Family Foundation, the Sandra and Alain Bouchard Foundation for Intellectual Disabilities, the Structural Genomics Consortium, the Centre for Drug Research and Development. The MNI's Tanenbaum Open Science initiative has been made possible with the support of the Larry and Judy Tanenbaum family. TD is supported by a Michael J Fox research grant (Grant ID: 12241) and a Parkinson's Canada Young investigator award.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 09 February 2018
doi: 10.3389/fnins.2018.00056

Representing Diversity in the Dish: Using Patient-Derived *in Vitro* Models to Recreate the Heterogeneity of Neurological Disease

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OPEN ACCESS

Edited by:
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Reviewed by:
Gerardo Morfini, University of Illinois at Chicago, United States
Savina Apolloni, Fondazione Santa Lucia (IRCCS), Italy

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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *13 October 2017*
Accepted: *23 January 2018*
Published: *09 February 2018*

Citation:
Ghaffari LT, Starr A, Nelson AT and Sattler R (2018) Representing Diversity in the Dish: Using Patient-Derived in Vitro Models to Recreate the Heterogeneity of Neurological Disease. Front. Neurosci. 12:56. doi: 10.3389/fnins.2018.00056

Neurological diseases, including dementias such as Alzheimer's disease (AD) and fronto-temporal dementia (FTD) and degenerative motor neuron diseases such as amyotrophic lateral sclerosis (ALS), are responsible for an increasing fraction of worldwide fatalities. Researching these heterogeneous diseases requires models that endogenously express the full array of genetic and epigenetic factors which may influence disease development in both familial and sporadic patients. Here, we discuss the two primary methods of developing patient-derived neurons and glia to model neurodegenerative disease: reprogramming somatic cells into induced pluripotent stem cells (iPSCs), which are differentiated into neurons or glial cells, or directly converting (DC) somatic cells into neurons (iNeurons) or glial cells. Distinct differentiation techniques for both models result in a variety of neuronal and glial cell types, which have been successful in displaying unique hallmarks of a variety of neurological diseases. Yield, length of differentiation, ease of genetic manipulation, expression of cell-specific markers, and recapitulation of disease pathogenesis are presented as determining factors in how these methods may be used separately or together to ascertain mechanisms of disease and identify therapeutics for distinct patient populations or for specific individuals in personalized medicine projects.

Keywords: IPSC, iNeuron, ALS, FTD, Alzheimer's disease, Parkinson's disease, Huntington's disease

INTRODUCTION

Despite recent, rapid advancement in our understanding of biology and genetics, neurological disease remains a persistent and fatal threat to human health. The World Health Organization (WHO) estimates that the fraction of worldwide deaths attributable to neurological conditions increased 89% from 2000 to 2015 (WHO, 2017). Alzheimer's disease (AD) and other dementias make up the vast majority of these fatalities, but Parkinson's disease (PD), epilepsy, Huntington's disease (HD), and motor neuron diseases such as amyotrophic lateral sclerosis (ALS) are each responsible for tens of thousands of deaths per year. The variety of symptoms and heterogeneity of affected individuals make diseases of the central nervous system (CNS) difficult to diagnose, study, and treat (Kim and Jeon, 2016; Van Cauwenberghe et al., 2016; Van Damme et al., 2017).

Staining of post-mortem tissue remains the only means of officially diagnosing several neurological diseases, and studying the mechanisms behind defining proteinopathies is difficult in this static state. The postmitotic nature of neurons and difficulty of tissue access make biopsies of the CNS infeasible, and the blood-brain barrier isolates many biochemical signals of disease progression from peripheral biofluids. Thus, in order to properly study pre-symptomatic disease or disease progression mechanisms in patients, we must rely on human *in vitro* models.

For genetically inherited disease, where a specific causal mutation has been identified, cellular models of disease can be created by genetic manipulations (overexpression, siRNA knockout, CRISPR/Cas9 knock-in, or knock-out) of cells lines or primary rodent cell cultures. Alternatively, primary rodent cell cultures can be generated from existing animal models of disease (e.g., mutant SOD1 mouse model of ALS, R2/6 mouse model of Huntington's disease). These approaches do come with limitations. For example, overexpression models can simultaneously mask subtle disease phenotypes while falsely exaggerating or creating entirely non-biological pathology. While these models may be used for early studies into disease mechanisms, true biological representation, especially for potential therapeutic development, requires endogenous expression of known genetic mutations and their genetic interactors, which emphasizes the need for patient-derived models.

Patient-derived models become even more necessary for studying sporadic disease, as there is no way of definitively recapitulating the disease in a non-diseased organism. The genetic and epigenetic heterogeneity of sporadic patients may make specific causative mutations statistically undetectable, or a confluence of small changes may be required to cause disease phenotypes. Such complexity would be impossible to recapitulate through genetic manipulation of a non-diseased cell, even if the full spectrum of contributory expression was identified. Since sporadic patients make up the majority of patients in diseases such as ALS, Frontotemporal Dementia (FTD), and AD (Kim and Jeon, 2016; Van Cauwenberghe et al., 2016; Van Damme et al., 2017), sporadic patient-derived cells offer the most widely applicable tool for studies of disease mechanisms and therapeutic screens.

Two primary methods for generating patient-derived cells have emerged. Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) and then differentiated into the desired cell type or directly converted (DC) into the desired cell type (**Figure 1**). Each method has been expanded over the last decade for differentiation into a wide variety of CNS cells used to study and treat neurological disease. Here, we review how these models have developed over the last 10 years, the methods used to differentiate them, the specific cell types those methods produce, and how disease has been studied in these models. We also highlight how human culture models are providing us with new mechanistic insights that were previously unattainable with postmortem patient tissue analysis or animal models of disease, and discuss the current limitations of *in vitro* modeling of CNS disease.

IPSC OVERVIEW

Early experiments in using stem cells to model neurons were largely developmental and relied upon the differentiation of embryonic stem cells (ESCs) (Thomson et al., 1998). Because these cells were not patient-derived, they were either cultured from mutant mouse embryos (Di Giorgio et al., 2007) or were healthy ESCs transfected to overexpress known mutants (Karumbayaram et al., 2009). These models encounter the same setbacks as all over-expressions, and thus offer little improvement over simpler models. Recently, preimplantation genetic diagnosis (PGD) has offered an opportunity for ESCs to be generated from embryos known to carry disease causing mutations, namely the hexanucleotide expansion of C9orf72 (C9) (Cohen-Hadad et al., 2016). Methylation analysis of motor neurons differentiated from these ESCs compared to adult C9 patient-derived iPSCs revealed hypermethylation of the iPSCs, which would suggest that ESCs provide a better representation of patient epigenetics. However, ethical concerns, the limited availability of endogenously mutated ESCs, and their inability to be used to model sporadic disease calls for the ability to generate human-derived *in vitro* models from adult patients. Experiments which showed that ESC fusion could confer pluripotency to fibroblasts suggested that certain cellular products might induce pluripotency even in an adult somatic cell (Cowan et al., 2005).

Since the initial, groundbreaking discovery of four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) capable of converting adult mouse (Takahashi and Yamanaka, 2006) and human (Yu et al., 2007) fibroblasts into pluripotent stem cells, a number of new methods for reprogramming somatic cells have been developed, including the use of various viral and chemical transfection methods to express optimized pluripotency factors. iPSCs can be generated from a wide variety of somatic cells, including skin, peripheral blood mononuclear cells, and hair, which enables non- or minimally-invasive collection of patient samples to generate models of their disease (Egusa et al., 2010).

IPSC DIFFERENTIATION METHODS

Prior to the choice of which specific neural cell to generate, the basic method of differentiation must be chosen. Many protocols rely on the addition of small molecules and growth factors to first induce embryoid bodies followed by neural rosettes (Elkabetz et al., 2008), or procede directly from iPSCs to neuroprogenitor cells (NPCs) via dual-SMAD signaling inhibition (Chambers et al., 2009). NPCs can be maintained and further differentiated and matured using growth factors. NPCs can be infinitely expanded, frozen down, and thawed to provide a mid-differentiation starting point, which reduces derivation time by ∼30% (Brafman, 2015).

Some differentiation protocols combine small molecules and growth factors with transfection of lineage-specific transcription factors, in a manner similar to direct conversion. For example, Wen et al. used transcription factors established by Son et al. for direct conversion to generate iPSC induced motor neurons for the study of repeat associated non-ATG (RAN) translated dipeptide repeat proteins (DPRs) in C9 ALS (Son et al., 2011;

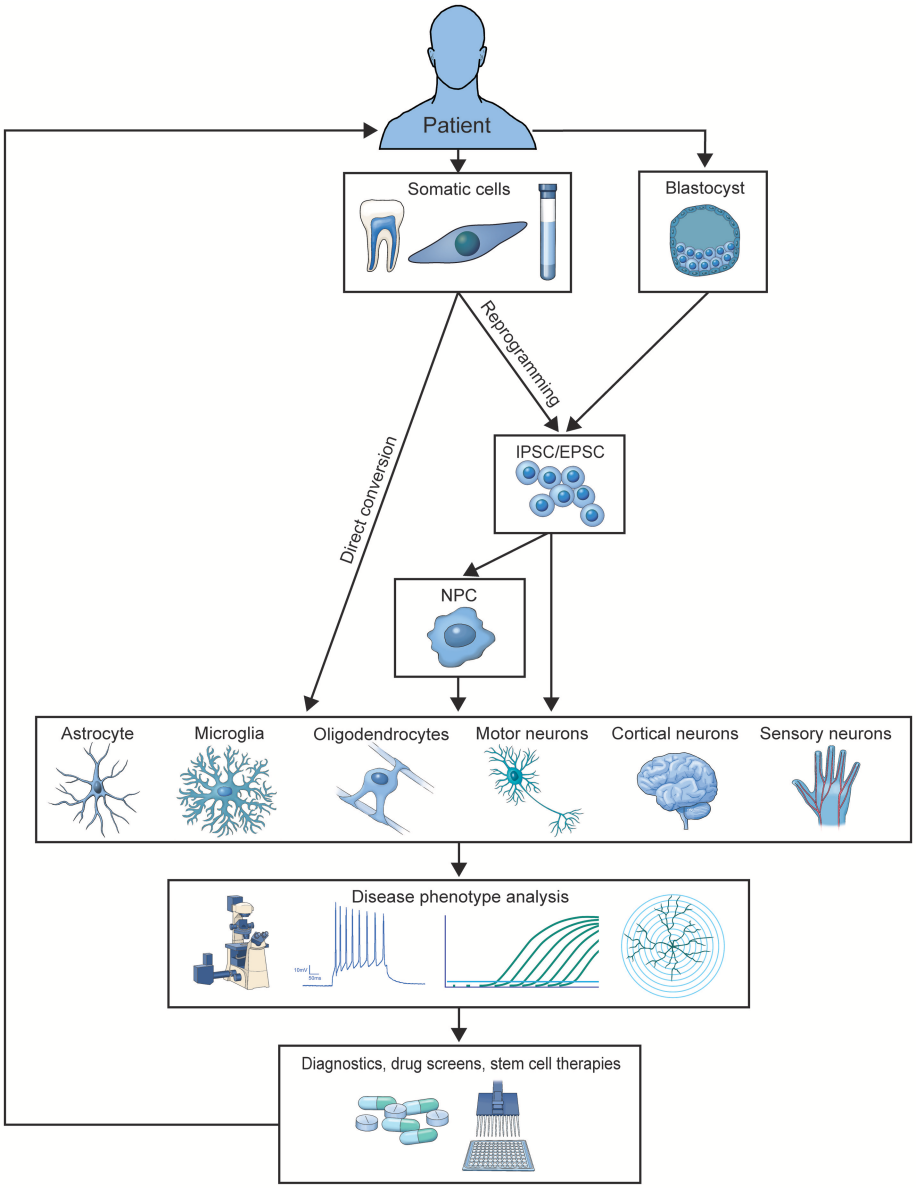

FIGURE 1 | Illustration of the generation and application of patient-derived models. Patient cells are used to generate iPSC or DC-based *in vitro* models of familial or sporadic disease. These models are studied to elucidate disease-contributing mechanisms and screen preclinical therapeutics, which can be translated into treatments for donor patient populations.

Wen et al., 2014). Other groups have reported that transcription factors may be used to more quickly generate very pure cultures of dopaminergic and GABAergic neurons (Theka et al., 2013; Yang et al., 2017). Neural progenitor cells (NPCs) have been derived using only small molecules (Reinhardt et al., 2013), and Li et al. discovered that microRNA 199a could induce angiogenesis in iPSCs, which may indicate that small-molecule only and microRNA based differentiation protocols, which have been used more widely in direct conversion, could be developed for iPSCs (Li Z. et al., 2015).

Another differentiation alternative attempts to recreate the physics of the biological environment by differentiating iPSCs in structured organoids. Unique basal hydrogels, custom culture chambers, and physical agitation can be used to encourage iPSCs to differentiate into a specific three-dimensional structure (Lindborg et al., 2016). This may lead to greater representation of brain-layer formation, which may improve cell maturity, stimulate expression of disease phenotypes, and provide a more accurate model for drug screens (Raja et al., 2016). In addition, organoids can provide a method of modeling diseases which are

difficult to recreate in animal models, such as microencephaly (Lancaster et al., 2013).

IPSC DIFFERENTIATED CELL TYPES

Human iPSCs have been successfully differentiated into a number of disease-relevant cell types, as verified by morphology, gene expression profiles, and cell-specific protein expression. Many differentiation protocols begin with the development of (NPCs). These have been used primarily for the development of therapeutic neuron replacement (Nicaise et al., 2017). Similarly, oligodendrocyte precursors have been used to drive implanted cells to an oligodendrocyte fate for remyelination therapies (Douvaras et al., 2014; Kawabata et al., 2016). Differentiation beyond precursors yields neurons and glia with varying efficiencies and purity. Variations in small molecule and growth factor selection, maturation duration, and culture conditions continue to be optimized.

iPSC Brain-Native Neuronal Subtype Differentiation

iPSCs have been differentiated into a number of brain-native neuronal subtypes. Cortical glutamatergic neurons can be generated by initializing neuralization using retinoids and SMAD inhibition. This generates cerebral cortex stem cells, which mature over 60 days into neurons representing the cortical layers (Shi et al., 2012). Viral expression of LMX1A in NPC cultured on PA6 stromal cell yields dopaminergic (DA) neurons (Sanchez-Danes et al., 2012). The combined effect of virally expressed ASCL1, NURR1, and LMX1A can generate iPSC DA neurons without the need for PA6 co-culture (Theka et al., 2013). Inhibitory GABA-ergic neurons can also be derived via viral induction of ASCL and DLX2 (Yang et al., 2017). These are but a few examples of the wide variety of neuronal subtypes created from iPSCs from an even wider array of protocols.

iPSC Spinal Motor Neuron Differentiation

Neurons native to the spinal cord and periphery, including motor and sensory neurons, can be generated readily from iPSCs. iPSC motor neurons (iPSCMNs) are generated by treatment with retinoic acid and sonic hedgehog (SHH), which is required for floor-plate differentiation (Ericson et al., 1996). iPSCMNs express HB9, Islet-1, and ChAT, which indicate a mature cholinergic maturation (Dimos et al., 2008). Peripheral nerves have also been successfully modeled using iPSCs. Nociceptor precursor cells can be generated from stem cells in 24 days, transfected to overexpress Neurog1, and matured into capsaicin-sensitive nociceptors to study nerve pain disorders (Boisvert et al., 2015).

iPSC Glial Differentiation

Glia can also be recreated from iPSCs, including astrocytes, oligodendrocytes, and microglia. These can be studied as mono-cultures or co-cultured with neurons to study non-cell autonomous effects on disease, be they contributory or compensatory.

Astrocytes are necessary to provide neurotrophic support and maintain healthy synaptic conditions. Krencik et al first reported the differentiation of iPSCs into astrocytes (Krencik et al., 2011). The resulting cells were GFAP positive, S100B positive, and took 180 days to differentiate, because astrocyte differentiation occurs after neuronal differentiation (Emdad et al., 2012). Differentiating iPSCs into neural stem cells before astrocyte differentiation reduced the generation time to 6 weeks (Shaltouki et al., 2013). Fluorescence-activated cell sorting (FACS) of differentiated iPSCs virally transfected to express GFP under a GFAP promoter allows for the selection of a purely astrocytic culture and reduces "noise" from neurons and other glia remaining from early periods of differentiation (Zhang et al., 2016).

In addition to the use of oligodendrocyte precursors (OPCs) in therapeutic transplantation, mature iPSC-derived oligodendrocytes have been generated. Early reports suggested that iPSCs might contain some inherent factor which prevented oligodendrocyte maturation (Tokumoto et al., 2010), however, a low yield, high purity, three-month protocol was soon developed (Hu et al., 2009). These mature oligodendrocytes were capable of generating myelin sheaths. Oligodendrocyte precursors can be matured by co-culture with dorsal root ganglion (DRG) neurons and will myelinate them *in vitro* (Czepiel et al., 2011). Douvaras et al. used a protocol similar to iPSMN differentiation, including adherent dual SMAD inhibition and early caudalization and ventralization, to increase OPC yield and shorten differentiation time to 75 days (Douvaras et al., 2014).

Microglia derive from macrophages but do not gain certain distinct features until they develop in concert with the nervous system. Early attempts to generate microglia from iPSCs resulted in cells called microglia-like macrophages, and while they did not express typical microglial morphology, they were positive for IBA1, CD45 and CD11b (Muffat et al., 2016). When microglia-like macrophages were co-cultured with iPSCNs, they exhibited a more mature microglial morphology and phagocytic behavior (Haenseler et al., 2017a). Recently, Douvaras et al. reported a method to develop mature, ramifying microglia without co-culturing (Douvaras et al., 2017).

iPSC Differentiation into Neural-Interfacing Cell Types

Non-neuronal cells relevant to neurological disease have also been derived. iPSCs can be differentiated into myoblasts, which form contracting, electrically and chemically responsive myotubes that are capable of forming neuromuscular junctions (NMJs) (Demestre et al., 2015). Additionally, endothelial cells can be generated to model the blood-brain-barrier, which is suggested to be compromised in neurodegeneration (Lippmann et al., 2012).

With the ability to differentiate into the full complement of neurons, glia, and neural-interfacing cells, iPSCs provide a powerful model for the study of disease in individual cells, in co-culture, and in three-dimensional reconstructions of complex tissues. Many examples of subtype-specific differentiation methods focus on the expression of specific marker proteins. While this is an important indicator of an individual cell type, more parameters can and should be considered when evaluating

differentiation protocols. For example, positive expression of marker proteins should be correlated with the lack of expression of specific marker proteins of similar, yet distinct, cell types, e.g., cortical forebrain neurons should lack markers of motor neurons. In addition, gene expression profiles should be compared to data from either primary murine cultures of the same cell type or isolated cell types from postmortem brain tissues. Similarly, morphology and electrophysiological activity should be included in the cell characterization. This will increase confidence in claims of cell-type specificity and also provide more experimental readouts to use as standards for maintaining consistent models across multiple rounds of differentiation.

Additional steps may be employed to reduce differentiation variability and to ensure disease relevance of observed deficits. While the use of multiple disease and control cell lines controls for the variability between individual human subjects, multiple differentiations of the same cell line accounts for variability between differentiations. In addition, CRISPR-Cas9 mediated gene editing allows for the correction of disease-causing mutations (Cong et al., 2013). This provides the possibility of creating isogenic control iPSC lines, which genetically and epigenetically match patient derived lines, except for the corrected mutation. If this correction alleviates deficits, it supports the association between the gene mutation-dependent phenotypes and actual disease pathology. This can be strengthened further by using the same technique to introduce known mutations in healthy control iPSCs, creating a positive control. Unfortunately, isogenic controls cannot be created to study sporadic disease which, as mentioned above, represents the majority population of most neurodegenerative diseases.

IPSC MODELS OF DISEASE

Amyotrophic Lateral Sclerosis/Frontotemporal Dementia

iPSCs have been used extensively to model neurological disease (**Figure 2**). ALS, a fatal motor neuron disease, and FTD, a neurodegenerative dementia, appear to exist on a genetic spectrum of disease, with several shared genetic causes and identifying pathologies. It is in this spectrum that the widest variety of iPSC modeling has been employed. iPSCMNs are the most widely used, and have been shown to demonstrate the specific pathology and physiology of ALS subtypes. Dimos et al. were the first to report the generation of iPSCMNs from an ALS patient that expressed the motor neuron-specific transcription factors HB9 and Islet 1 (Dimos et al., 2008). It was not until four years later that actual disease phenotypes were first shown in TDP-43 ALS patient-derivedmotor neurons. Egawa and colleagues differentiated iPSCMNs from three ALS patients carrying three distinct TDP-43 mutations and showed that, among other deficits, the diseased motor neurons exhibited hallmark TDP-43 aggregates similar to those found in postmortem ALS brain tissues (Egawa et al., 2012). The authors were able to rescue these phenotypes with a histone acetyltransferase inhibitor, providing first evidence that patient-derived iPSCMN culture models could be used for drug screening purposes. iPSC modeling of FTD soon followed when Almeida and colleagues generated iPSC neurons and microglial cells from FTD patients carrying a mutation in progranulin (PGRN) (Almeida et al., 2012). The differentiated human cells confirmed the presence of PGRN haploinsufficiency, in addition to aiding in the new discovery of mutant PGRN-specific deficits of certain protein kinase pathways. When the C9orf72 hexanucleotide expansion was discovered as the most common shared mutation in ALS/FTD disease spectrum in 2011(DeJesus-Hernandez et al., 2011; Renton et al., 2011), the fastest way to study disease mechanisms was via iPSCs, as the generation of the repeat expansion in mouse models proved to be difficult. Several laboratories shortly thereafter provided the first evidence of the three major disease mechanisms of mutant C9orf72 using iPSC neurons: haploinsuffiency, RNA foci formation, and repeat-associated non-ATG (RAN) translation (Almeida et al., 2013; Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Sareen et al., 2013). These studies also revealed disease-mediated aberrant gene expression and neuronal function in patient iPSCs. Numerous follow up studies examining both neuronal and glial iPSC culture models have since been used to further investigate how mutant C9orf72 leads to neuronal degeneration, ranging from studies on nucleocytoplasmic trafficking deficits (Freibaum et al., 2015; Zhang et al., 2015; Lopez-Gonzalez et al., 2016; Westergard et al., 2016) to the toxic potential of RAN dipeptides, as recently reviewed (Selvaraj et al., 2017).

Other familial ALS mutation phenotypes, such as valosin-containing protein (VCP) (Hall et al., 2017), TAR DNA-binding protein 43 (TDP-43) (Serio et al., 2013; Barmada et al., 2014), and superoxide dismutase (SOD1) (Bhinge et al., 2017), have been recapitulated in iPSCs showing characteristic proteinopathies, and endoplasmic reticulum (ER) and oxidative stress. To determine which disease pathologies in the model are caused primarily by the mutation, isogenic corrections of known genetic causes, such as SOD1, have been generated, and were shown to successfully rescue the mutant phenotypes, such as previously described hyperexcitability in SOD1 mutant iPSCMNs (Wainger et al., 2014; Bhinge et al., 2017).

Importantly, considering sporadic disease represents 90% of ALS patients, sporadic iPSCs have also been differentiated into neurons and were shown to exhibit TDP-43 pathology (Burkhardt et al., 2013; Qian et al., 2017). Furthermore, gene expression profiling of sporadic ALS iPSCMNs suggested deficits in mitochondrial function (Alves et al., 2015). More studies are required to better understand the pathogenesis of sporadic disease, and human cell culture models are currently the only way to gain insight into what triggers and propagates sporadic ALS and FTD.

Limited studies have addressed the role of glial cells in ALS/FTD, although both ALS and FTD patient-derived iPSC astrocytes have been employed to study the non-cell autonomous mechanisms of neurodegeneration. Interestingly, while studies using SOD1 mutant mouse models and primary cultures generated from these mice unequivocally demonstrated that mutant astrocytes trigger motor neuron degeneration, iPSC astrocytes differentiated from ALS patients carrying a TDP-43

		Neurons	Astrocytes	Microglia
Amyotrophic Lateral Sclerosis/Fronto-Temporal Dementia	iPSC	RNA foci & glutamate excitotoxicity[1] Nuclear stress caused by G-quadruplex in C9[2] TDP43 aggregation in sporadic[3] DPR expression & decreased survival in C9[4] TDP43 mis-localization, ER stress, mitochondrial dysfunction, & cell death in VCP ALS[5]	Increased TDP43 expression, mis-localization, & decreased survival in TDP43 mutant[6] Stimulating autophagy increases survival in TDP43 mutant[7] Sporadic iPSC astrocytes cause neurodegeneration when transplanted in vivo[8]	Progranulin haploinsufficiency in GRN FTD[9]
	DC	Cytoplasmic retention of FUS[10,11] Failure to form functional NMJs. shrunken somas, & susceptibility to cell death[12] Poly(GP) & Poly(PR) inclusions, RNA foci[13]	$SOD1^{A4V}$ & C9orf72 repeat expansion toxicity[14]	
Alzheimer's Disease	iPSC	PSEN2 mutation increased Aβ42: Aβ40[15] Aβ oligomer accumulation & oxidative stress[16] Increase in total tau & p-tau[17] Decreased neurite length & hyperexcitability[18] PSEN1 mutation increased Aβ42: Aβ40 & changes in gene expression[19]	Nuclear retention of S100B[20] APOE E4/E4 astrocytes less effective at supporting neurotrophic functions[21] Intracellular Aβ oligomer accumulation, ER & oxidative stress[16]	Microglial gene expression changes in response to tau & Aβ phagocytosis[22]
	DC	Increase in extracellular levels of Aβ40 & Aβ42[23,24]		
Huntington's Disease	iPSC	Intracellular inclusions of aggregated mutant Htt protein[25] Disruption in nuclear cytoplasmic transport[26] Susceptibility to cell death & loss of electrical activity[27] Ca^{2+} dys-homeostasis[25,27]	Abnormal cellular vacuolation[28] Blocking TNF signaling protects HD astrocytes from cytokine-induced toxicity[29] Increased expression in VEGF-A[30]	
	DC	Nuclear mutant Htt inclusions, abnormal neuritic branching, & decreased cell survival[31] Higher vulnerability to DNA damage[24]		
Parkinson's Disease	iPSC	Increase in alpha-synuclein expression[32] Increased oxidative stress, Lewy-body formation, & abnormal mitochondria morphology[33] Impaired recruitment of Parkin & increased mitochondria count[34]		*SNCA* Triplication mutation causes phagocytic deficits in a microglia-like macrophage model[35]
	DC	Disrupted mitochondrial quality control by PTEN-induced putative kinase 1 mutation[36]		

FIGURE 2 | Examples of patient-derived *in vitro* models of neurological disease. Select examples of how patient-derived iPSC and DC neurons, astrocytes, and microglia have been used to model ALS/FTD, AD, HD, and PD. Numbers correspond to the following references: 1. Donnelly et al., 2013; 2. Wang et al., 2014; 3. Burkhardt et al., 2013; 4. Wen et al., 2014; 5. Hall et al., 2017; 6. Serio et al., 2013; 7. Barmada et al., 2014; 8. Qian et al., 2017; 9. Almeida et al., 2012; 10. Liu et al., 2016; 11. Lim et al., 2016; 12. Son et al., 2011; 13. Su et al., 2014; 14. Meyer et al., 2014; 15. Yagi et al., 2011; 16. Kondo et al., 2013; 17. Muratore et al., 2014; 18. Balez et al., 2016; 19. Sproul et al., 2014; 20. Jones et al., 2017; 21. Zhao et al., 2017; 22. Abud et al., 2017; 23. Hu et al., 2015; 24. Hou et al., 2017; 25. Nekrasov et al., 2016; 26. Grima et al., 2017; 27. Mattis et al., 2015; 28. Juopperi et al., 2012; 29. Hsiao et al., 2014; 30. Hsiao et al., 2015; 31. Liu et al., 2013; 32. Devine et al., 2011; 33. Imaizumi et al., 2012; 34. Seibler et al., 2011; 35. Haenseler et al., 2017b; 36. Puschmann et al., 2017.

mutation, while exhibiting TDP-43 mislocalization themselves, did not affect motor neuron survival in a co-culture model (Serio et al., 2013). On the other hand, iPSC astrocyte-conditioned media derived from C9orf72 patients leads to impairments of iPSC motor neuron autophagy (Madill et al., 2017). Similarly, iPSC astrocytes from mutant VCP patients affect motor neuron survival *in vitro* (Hall et al., 2017). In addition, transplanting sporadic ALS iPSCs into the spinal cord of WT mice resulted in differentiation into astrocytes and triggered a loss of neurons in the spinal cord, with accompanying movement deficits (Qian et al., 2017).

Even fewer studies to date have generated and characterized iPSC-derived oligodendrocytes. Ferraiuolo and colleagues used both sporadic and fALS patient-derived oligodendrocytes to show that, similar to astrocytes, diseased oligodendrocytes affect motor neuron survival (Ferraiuolo et al., 2016). At the same time, Livesey and colleagues studied the maturation and electrophysiological properties of C9orf72 patient-derived iPSC oligodendrocytes and did not observe any obvious impairments or alterations of these cells when grown in mono-cultures (Livesey et al., 2016).

The only study describing iPSC microglial cells from ALS or FTD patients was reported by Almeida et al. who showed that PGRN FTD patient microglial cells exhibit progranulin haploinsufficiency, but do not show the deficits in serine/threonine kinase S6K2 observed in iPSC neurons from the same patients (Almeida et al., 2012).

Alzheimer's Disease

The most common dementia, Alzheimer's disease (AD), has been examined extensively in patient-derived iPSCs, and these studies been thoroughly reviewed (Devineni et al., 2016; Arber et al., 2017; Robbins and Price, 2017; Tong et al., 2017). One of the first reports using patient-derived iPSC neurons to study mechanisms of AD examined the pathogenesis of familial Presenilin 1 (PS1) and Presenilin 2 (PS2) (Yagi et al., 2011). The mutant iPSC neurons exhibited increased amyloid β 42 secretion, which was inhibited by a γ-secretase inhibitor. This study was followed by a report from Israel and colleagues who found an increase in Aβ and phosphorylated tau (p-tau) in familial amyloid-β precursor protein (APP) duplication (APP(Dp)) and sporadic iPSC cortical neurons (Israel et al., 2012). Treating patient neurons with β-secretase inhibitor, but not γ-secretase inhibitors, significantly reduced the documented disease phenotypes in the cells, providing new knowledge on the mechanisms of APP pathogenesis.

These early studies were followed by reports using both familial and sporadic AD patient iPSCs differentiated into cortical neurons or, to a limited degree, glial cells to better understand mechanisms of neurodegeneration in AD and for novel therapeutic target investigation. For example (Duan et al., 2014), used sporadic AD patient iPSCs with ApoE3/E4 genotypes and reported not only an increased ratio of Aβ42/40 and responsiveness to γ-secretase inhibitors, but also increased susceptibility to glutamate toxicity with a concomitant increase in free intracellular calcium levels, suggesting that actual neuroprotection could be examined using these patient-derived neurons. Similarly, iPSCs from AD patients carrying an APP mutation (V717I) showed increased expression levels of APP and Aβ, in addition to aberrant β and γ secretase cleavage of APP and increased levels of total and phosphorylated tau (Muratore et al., 2014). Interestingly, the authors were able to show that treatment with specific Aβ antibodies early during the differentiation process rescued the mutant APP phenotypes. This new paradigm of testing drugs in patient-derived cells was strengthened by a study in which apigenin has been tested as a potential anti-inflammatory therapeutic in AD patient-derived neurons co-cultured with activated murine microglial cells. The treatment was successful in reversing morphological deficiencies, reducing hyper-excitability, and protecting against apoptosis (Balez et al., 2016).

Sproul and colleagues studied iPSC derived neural precursor cells (NPC) to understand whether, even at a pre-neuronal stage of development, human cells show AD phenotypes (Sproul et al., 2014). Differentiating iPSCs from mutant PS1 patients into NPCs, the authors observed an increased Aβ42/40 ratio and an aberrant gene expression profile after transcriptome analysis. A select number of gene aberrations were confirmed to be similarly altered in postmortem AD brain tissues. Similar findings were confirmed in PS1 patient iPSC neurons (Mahairaki et al., 2014). Interestingly, generating isogenic iPSC lines using TALENs (TAL Effector Nucleases) technology to insert PS1Δ E9 mutations in otherwise healthy control patient lines lead to the discovery that this mutation represents a toxic gain of function mutation that impairs PS1-dependent γ secretase activity, but not unrelated γ secretase functions (Woodruff et al., 2013).

Few studies thus far have used patient-derived iPSC astrocytes to model Alzheimer's disease. Kondo et al showed that iPSC astrocytes from sporadic AD patients and patients with an (APP)-E693delta mutation displayed ER and oxidative stress, which was alleviated with treatment of docosahexaenoic acid (DHA) (Kondo et al., 2013). A more recent study showed that iPSC astrocytes from familial and sporadic AD patients display significant morphological phenotypes with an overall atrophic profile and mislocalization of astroglial marker proteins, including S100B (Jones et al., 2017). IPSCs astrocytes from normal individuals with APOE ε4/ε4 genotypes were less effective in promoting neuronal survival and synaptogenesis when compared to iPSC astrocytes from normal individuals with APOE ε3/ε3 genotypes (Zhao et al., 2017), illustrating that patient-derived culture models could aid in exploring the role of individual apoE isoforms in AD disease pathogenesis. Finally, iPSC astrocytes differentiated from patients carrying the PSEN1 delta E9 mutation exhibited typical AD pathology—increased β amyloid, altered cytokine release, defective calcium homeostasis, increased oxidative stress and reduced lactate secretion (Oksanen et al., 2017). None of these phenotypes were present in healthy control or gene-corrected isogenic iPSC astrocytes.

Similar to ALS above, only one study to date has used patient-derived microglial cell to study AD disease mechanisms. Abud and colleagues generated iPSC microglial-like cells and showed altered gene expression in response to Aβ fibrils and aberrant phagocytic activity when exposed to brain-derived tau oligomers (Abud et al., 2017). Further work is required to determine whether the observed AD iPSC-derived glial phenotypes contribute to the neuronal disease pathogenesis in a non-cell autonomous manner.

To overcome the observed inability of 2 dimensional iPSC neuron cell culture models to generate extracellular protein aggregations, the AD field has quickly tried to model AD in 3 dimensional (3D) human cell culture systems (reviewed by and Choi et al., 2016; Lee et al., 2017). One of the first published studies using 3D iPSC differentiated neuron cultures showed that only in the 3D environment did the AD neurons display p21-activated kinase-mediated sensing of Aβ oligomers, in addition to the presence of F-actin associated protein phenotypes (Zhang et al., 2014). Choi and colleagues developed a 3D iPSC neuron culture model from familial AD patients (mutant APP and mutant presenilin), which, for the first time, represented a disease model that displayed hallmarks of AD pathology: extracellular deposition of amyloid-β, including amyloid-β plaques, and aggregates of phosphorylated tau, as well as filamentous tau (Choi et al., 2014; Kim et al., 2015). More recent studies have applied the 3D culture model to generate high throughput models for drug screening against tau aggregation (Medda et al., 2016), or to compare efficacy of drug candidates (β or γ secretase inhibitors) in 2D vs. 3D culture systems (Lee et al., 2016). Finally, the generation of brain organoids, initially developed to model human brain development (Lancaster et al., 2013), has been adopted to study mechanisms of AD as well. Raja and colleagues reported that brain organoids from familial AD patients recapitulate AD disease phenotypes and pathologies including amyloid aggregation, hyperphosphorylated tau, and endosome abnormalities, all of which were reduced by treatment with secretase inhibitors (Raja et al., 2016).

Huntington's Disease

Zhang and colleagues were the first group to characterize HD patient-derived iPSC neuronal cells (Zhang et al., 2010). The authors differentiated iPSCs into striatal neurons expressing cell-specific markers such as DARPP-32. The neurons maintained the CAG repeat expansion and showed increased susceptibility to growth factor removal as shown by enhanced caspase activity. The same research team also generated the first gene-corrected isogenic cell lines, which resulted in the rescue of disease phenotypes, including the susceptibility to cell death and mitochondrial abnormalities (An et al., 2012). A large consortium of Huntington's disease researchers reported increased cell death, sensitivity to stressors, increased glutamate toxicity, and reduced

sporadic electrical firing in HD patient-derived iPSC striatal-like neurons as characterized by the expression of Map2a/b and Bcl11B (iPSC Consortium, 2012). Interestingly, the severity of several of these phenotypes increased in proportion to the patient's CAG-expansion length in the *HTT* gene. Similar findings were reported by Mattis and colleagues, who reported increased cell death following BDNF withdrawal, but also showed that increased susceptibility to glutamate toxicity could be blocked by NMDA and AMPA receptor inhibitors (Mattis et al., 2015), suggesting novel therapeutic approaches for HD.

Several other potential Huntington's disease pathways have been identified using patient derived iPSC neurons, such as miR196a dysregulation (Cheng et al., 2013), MAPK and Wnt signaling (Szlachcic et al., 2015), the protective potential of A2A adenosine receptor activation (Chiu et al., 2015), and astrocyte-mediated cytokine-induced neuronal cell death (Hsiao et al., 2014). CRISPR-Cas9 gene-corrected isogenic HD patient lines showed rescue of HD phenotypes, such as deficits in mitochondrial respiration (Xu et al., 2017). Most interestingly, though, these isogenic lines showed that gene expression differences between HD and healthy control iPSCs were not present when HD lines were compared to their isogenic control, suggesting that general differences in genetic background could be falsely identified as transcriptomic abnormalities triggered by the disease-causing mutation. Neskarov et al. reported that HD iPSC neurons show increased numbers of lysosomes/autophagosomes and exhibit increased cell death alongside nuclear indentation (Nekrasov et al., 2016). Further nuclear deficits, including nucleoporin aggregation and impaired nucleo-cytoplasmic transport, have recently been demonstrated in HD patient-derived neurons (Grima et al., 2017); similar to what has been described in ALS (Kim and Taylor, 2017). One of the most recent studies showed that previously reported repression of peroxisome proliferator-activated receptor delta (PPAR-δ)—a ligand-gated transcription factor that promotes mitochondrial biogenesis and oxidative metabolism—in an HD mouse model is also found in HD patient-derived iPSC neurons (Dickey et al., 2016, 2017). Indirect activation of PPARγ via the small molecule compounds bexarotene or KD3010 significantly rescued impaired oxidative metabolism in HD neurons.

There have been few reports of iPSC differentiations into non-neuronal cells for HD. Huntington's iPSC astrocytes were shown to express higher levels of vascular endothelial growth factor-A (VEGF-A) (Hsiao et al., 2015) and formed electron-clear vacuoles, which increased during the differentiation period but appeared independently of cellular stressors (Juopperi et al., 2012). To date, patient derived microglia and oligodendrocytes have not been studied in Huntington's disease, but differentiation of HD iPSCs into brain microvascular endothelial cells revealed abnormalities in angiogenesis and blood brain barrier properties (Lim et al., 2017).

In summary, the use of patient-derived iPSC cultures in HD has provided new insight regarding disease pathogenesis and also provided a novel model to perform pre-clinical drug discovery (Tousley and Kegel-Gleason, 2016). A potential additional application of these patient-derived cells lies in the transplantation of stem cells or their derivatives to replace diseased or lost neurons. While not discussed in this review, numerous efforts have been made toward successful transplantation of cells at the iPSC stage or the neural precursor stage in rodents with the hope to create a therapeutic path toward HD patient transplantations (Golas and Sander, 2016; Connor, 2017; Choi et al., 2018).

Parkinson's Disease

Parkinson's disease (PD) has been extensively modeled using patient-derived iPSCs (Cobb et al., 2017). PD is modeled chiefly by the differentiation of iPSCs into dopaminergic (DA) neurons. Park et al. created the first Parkinson's disease iPSC line, together with other neurodegenerative disease lines (Park et al., 2008). Patient-derived DA neurons exhibit the typical pathology of PD: the presence of Lewy bodies and their main component, α-synuclein. This pathology is present in iPSC DA neurons from patients with mutations in the SNCA gene and in patients with mutations in leucine-rich repeat kinase 2 (LRRK2) (Devine et al., 2011; Nguyen et al., 2011) and, rarely, in patients with mutations in parkin (Imaizumi et al., 2012), but not in patients with a mutation in PTEN-induced putative kinase 1 (PINK1) (Jiang et al., 2012). This reflects the pathology as it has been observed in postmortem patient brain tissue. Other disease phenotypes previously observed in either mouse models or autopsy tissue were confirmed in patient-derived familial and sporadic iPSC DA neurons, including mitochondrial deficits and oxidative stress (Byers et al., 2011; Cooper et al., 2012; Hsieh et al., 2016) and lysosomal and autophagy deficits (Mazzulli et al., 2011, 2016; Sanchez-Danes et al., 2012; Schondorf et al., 2014; Fernandes et al., 2016).

Similar to the aforementioned neurodegenerative diseases, researchers are now using genetically corrected patient-derived iPSCs to study PD pathogenesis in order to reduce the potential for general patient-to-patient genetic variability to obscure disease-specific cellular and genetic alterations, and to pinpoint specific disease mechanisms linked to the genetic mutation examined, and not to potential cellular deficits arising from the actual cell differentiation process (Soldner et al., 2011; Arias-Fuenzalida et al., 2017; Qing et al., 2017).

Parkinson's disease has not been studied extensively in iPSC-derived glia; however, Haenseler et al. have recently shown that phagocytosis is impaired in SNCA triplication microglia (Haenseler et al., 2017b).

Neuroinflammation and Neuropsychiatric Diseases

Modeling disease with iPSCs extends beyond neurodegeneration. Although much research in de-myelinating disease, such as multiple sclerosis (MS), focuses on differentiating healthy oligodendrocyte precursors for transplantation replacement therapy (Kawabata et al., 2016; Sato et al., 2017), MS patient-derived iPSCs have been differentiated into neurons (Song et al., 2012), astrocytes (Song et al., 2012), oligodendrocyte precursors (Nicaise et al., 2017), and oligodendrocytes (Song et al., 2012; Douvaras et al., 2014). The only differences so far identified are an inability of MS iPSCNs to fire spontaneous action potentials (Song et al., 2012), and reduced neuroprotection by MS iPSC

oligodendrocyte precursors (Nicaise et al., 2017). Microglia, which have been sparingly used to model neurological disease, have also been used to model Rhett syndrome (Muffat et al., 2016). The use of iPSCs to model neuropsychiatric disorders is expanding rapidly, as reviewed by Ho et al. (2015).

Reprogrammed patient-derived cells have been used to model sporadic and familial neurodegenerative, neurodevelopmental, and neuropsychiatric disease. Much early work using iPSCs to study neurological disease focused on validating previously-observed phenotypes from animal models and postmortem autopsy tissues. Now that the ability of iPSC-derived CNS models to recreate disease pathology has been established, more recent studies are beginning to use iPSCs to tease out disease mechanisms and screen for pre-clinical therapeutics. These efforts are supported by continuing improvements in cell type-specific differentiation methods, the expansion of co-culture and 3D culture models, and the generation of genetically corrected isogenic patient lines to account for the diversity of patients' intrinsic genetic backgrounds.

Despite the advantages and advancements of patient-derived iPSCs, concerns about differentiation time, culture specificity and purity, teratoma formation in transplantation, and, most importantly, the difficulty for rejuvenated cells to model aging diseases has led to the development of a complimentary methodology: lineage-specific direct conversion of patient-derived somatic cells.

DIRECT CONVERSION OVERVIEW

One distinct advantage of direct lineage conversion, also known as transdifferentiation, is that there is no reprogramming step. The epigenetic identity of the starting cells remains after conversion, as opposed to the hypermethylation observed when somatic cells are rejuvenated into iPSCs (Cohen-Hadad et al., 2016). This retains "cellular memories." Most conversion protocols feature mesoderm to ectoderm conversions—meaning fibroblasts to neurons. Due to the infancy of this field, the definition of an induced neuron (iN) has many meanings (Yang et al., 2011). Direct conversion can occur from fibroblasts (Vierbuchen et al., 2010), blood (Lee et al., 2015), urine cells (Zhang S. Z. et al., 2016), hepatocytes (Marro et al., 2011), and adipocyte progenitors (Yang Y. et al., 2013), which provides a wide variety of non-invasive starting materials for modeling patient disease.

The first report of direct lineage reprogramming by Vierbuchen et al. (2010) used combinatorial expression of three neural lineage-specific transcription factors—Brn2, Ascl1, and Myt1L, known as the BAM factors—to induce neurons from mouse fibroblasts (Vierbuchen et al., 2010). The induced neurons (hereafter called iNeurons or iNs) that result from this method express pan-neuronal markers including Tuj1, NeuN, MAP2, and synapsin. These iNs also fire induced and spontaneous action potentials and form functional synapses. However, the BAM factors alone cannot convert human fetal or postnatal fibroblasts into functional neurons. The addition of the basic helix-loop-helix transcription factor NeuroD1 to the BAM factor conversion protocol was found to be necessary for iNs to be produced from human somatic cells (Pang et al., 2011).

Here, we will first describe the general methods used to directly convert somatic cells. We then specify conversion methods applied to the generation of neurons and glial cells, followed by a summary on how these directly converted cells have been studied in varying neurodegenerative diseases thus far.

DIRECT CONVERSION METHODS

There are three primary methods of directly converting human somatic cells into CNS cells. Transcription factor overexpression, microRNA overexpression, and small molecule treatments can, individually or in concert, generate a variety of cell types from the aforementioned starting materials.

Transcription Factor-Mediated Direct Conversion

Since Pang et al. first successfully created human iNs, a number of other groups have obtained similar results using some or all of the BAM transcription factors (Ambasudhan et al., 2011; Caiazzo et al., 2011; Pfisterer et al., 2011; Son et al., 2011). Furthermore, Marro et al. used the BAM factors to convert hepatocytes into functional neurons, providing the first proof that an endodermal cell can be converted into an ectodermal cell (Marro et al., 2011). Renal epithelial cells from urine can be directly converted into neurons via lentivirus-mediated expression of BAM factors plus NeuroD1 and c-Myc (Zhang S. Z. et al., 2016). Mitchell et al. (2014) showed the Oct4 expression alone was enough to generate NPCs from somatic cells. These are only a few examples of the variety of transcription factors used to generate neurons (Mitchell et al., 2014).

MicroRNA-Mediated Direct Conversion

Several groups have also achieved direct conversion via microRNAs, although concomitant expression of neural lineage-specific transcription factors is required to fully mature these iNs. Coexpression of miR-9/9* and miR-124 is sufficient to directly convert human fibroblasts into neurons (Yoo et al., 2011). While initial conversion efficiency using these microRNAs was very low, efficiency increased with the addition of the transcription factors NeuroD2, Ascl1, and Myt1. Furthermore, striatal medium spiny neurons or motor neurons can also be generated by co-expression of miR-9/9* and miR-124 in addition to the transcription factors BCL11B (CTIP12), DLX1, DLX2, and MYT1L or ISL1 and LHX3, respectively (Victor et al., 2014; Richner et al., 2015; Abernathy et al., 2017). Huh et al. also showed that direct conversion using this protocol preserves age-related epigenetic and cellular signatures from the donor, similar to established transcription factor-only methods (Huh et al., 2016). Primary dermal human fibroblasts can also be converted into functional neurons by expression of miR-124 in addition to transcription factors Brn2 and Myt1 (Ambasudhan et al., 2011). An additional method reported by Zhou et al. (2014) showed that depletion of p53 by zinc finger nuclease (ZFN) technology is sufficient to convert fibroblasts into neurons, oligodendrocytes, or astrocytes, depending on the selection media used (Zhou et al., 2014). It has

not yet been established whether p53 depletion generates neurons which recapitulate disease pathology.

Similar to microRNA-mediated approaches, expression of short interfering RNA (siRNA) inhibiting the polypyrimidine-tract binding (PTB) protein has been shown to be sufficient for transdifferentiation (Xue et al., 2013). The suppression of PTB allows for expression of various neuronal genes, including the neuron specific microRNA-124. In non-neuronal cells, the PTB protein inhibits microRNAs which act on various components of the RE1-silencing transcription factor complex. Relief of this PTB-protein inhibition leads to activation of an array of neuronal lineage-specific genes, thereby promoting neuronal induction.

Lau et al. report using a self-regulating viral vector which expresses BAM factors and has target sequences for microRNA-124. Once the cells have been converted into neurons, they will express miR-124, and then downregulate the transgenes (Lau et al., 2014). The microRNA-mediated downregulation of the neural conversion genes allow for more complete functional maturation in culture.

MicroRNAs, with their ability to both promote expression of lineage specific genes and suppress expression after conversion, offer a path to fine-tune conversion to best replicate endogenous expression of cell-type specific genes and more accurately recreate their effect on disease biology in the patient. This benefit must be weighed against the fact that most microRNAs have a variety of target genes, and overexpression may cause unintended dysregulation of disease-relevant genes.

Small Molecule-Mediated Direct Conversion

In addition to transcription factors, small molecules can convert human fibroblasts into functional neurons without the addition of exogenous genetic factors. The resulting neurons are called chemically induced neurons (CiNs). Small molecules have been shown to increase efficiency of conversions when combined with transcription factors (Ladewig et al., 2012; Liu et al., 2013; Lee et al., 2015; Mertens et al., 2015; Shi et al., 2016). SMAD, GSK3β, and ALK inhibitors, alongside cyclic AMP signaling enhancers, are common between CiN and iPSC differentiation. In an early study, (Ladewig et al., 2012) combined Ascl1 and Ngn2 with the small molecules SB-431542, noggin, and CHIR99021 (a GSK3β inhibitor). Reports of purely small molecule conversions include a combination of forskolin, isoxazole 9 (an inducer of neural stem cell differentiation), CHIR99021 and I-BET151 (a BET bromodomain inhibitor), which can convert fibroblasts into functional neurons that express pan-neuronal markers (Li X. et al., 2015), and a small molecule cocktail called VCR, consisting of Valproic acid, CHIR99021 and Repsox, which is capable of inducing NPCs (Cheng et al., 2014). Hu et al. expanded VCR and generated human CiNs using the cocktail named VCRFSGY, which consisted of Valproic acid, CHIR99021, Repsox, Forskolin, SP600125 (a JNK inhibitor) GO6983 (a PKC inhibitor) and Y-27632 (a ROCK inhibitor) (Hu et al., 2015). These hCiNs showed some spontaneous electrical activity and a train of action potentials when stimulated.

DIRECT CONVERSION CELL TYPES

Direct conversion has generated nearly as wide a variety of CNS cell types as have stem cell differentiation approaches. Proneural transcription factors without subtype specification largely result in glutamatergic and GABA-ergic iNs (Vierbuchen et al., 2010; Wang et al., 2014; Mertens et al., 2015). Success of specific cell type generation by direct conversion techniques are largely based on expression of cell type-specific markers, but the question remains if the final cell type generated mimics bona fide neuron subtypes. As in iPSC models, cell type confidence can be increased by considering subtype-specific morphology, gene expression, and electrophysiology in concert with marker proteins and in comparison to individual cell types of adult human tissue.

Direct Brain-Native Neuronal Subtype Conversion

Induced neural progenitor cells (iNPCs) can be generated via expression of pluripotency factors in possible combination with small molecules (Kim et al., 2011a; Mitchell et al., 2014; Lee et al., 2015; Hou et al., 2017). iNPCs are not post-mitotic, and can differentiate into various neuronal subtypes, oligodendrocytes, and astrocytes *in vivo* and *in vitro*. Pro-neural and serotonergic neuron-specific transcription factors, in addition to small molecules, can generate induced neurons that have characteristics of native serotonergic neurons such as release of serotonin, response to SSRIs, increased expression of specific serotonergic genes, and firing of spontaneous action potentials (Vadodaria et al., 2016; Xu et al., 2016). Dopaminergic neurons are of particular interest due to their clinical relevance in Parkinson's disease. It has been shown that induced DA neurons (iDAs) can be generated from human fibroblasts via a combination of transcription factors (Caiazzo et al., 2011; Kim et al., 2011b; Pfisterer et al., 2011; Liu et al., 2012; Torper et al., 2013; Dell'Anno et al., 2014). The resulting iDAs displayed characteristic DA uptake and release, expressed DA neuron-specific markers, and were electrically active.

Direct Motor Neuron Conversion

Transdifferentiation can also model nervous system disease outside of the brain. The small molecules Forskolin and Dorsomorphin, in addition to the transcription factors Neurogenin2 and SOX1, can generate human induced cholinergic neurons with mature electrophysiological properties, motor neuron-like features, and morphology (Liu et al., 2013). Human induced motor neurons (hiMNs), which form functional NMJs when cultured with primary mouse skeletal myotubes, can be generated via expression of Ngn2, SOX11, ISL1, and LHX3, in addition to the small molecules forskolin and dorsomorphin (Liu et al., 2016). Son et al. utilized a transcription factor-only (BAM factors plus motor neuron-specific transcription factors Lhx3, Hb9, and Isl1, and NeuroD1) approach to generate functional human spinal motor neurons (Son et al., 2011).

Direct Sensory Neuron Conversion

Induced sensory neurons can be generated by the transient co-expression of Brn3a with either Ngn1 or Ngn2 and exhibit properties of endogenous sensory neurons such as gene expression, Trk receptor expression, response to ligands, and morphology (Blanchard et al., 2015). Nociceptive sensory neurons with nociceptor-specific functional receptors can be converted from fibroblasts using BAM factors in addition to Isl2, Ngn1, and Klf7 (Wainger et al., 2015) or via OCT4 and a small molecule cocktail which inhibits SMAD and GSK-3β signaling (Lee et al., 2015).

Direct Glial Conversion

Direct conversion can also produce glia. The transcription factors NFIA, NFIB, and SOX9 can convert native brain embryonic and postnatal mouse fibroblasts into astrocytes (iAstrocytes) (Caiazzo et al., 2015). Induced oligodendrocyte precursor cells (iOPCs) can be generated from mouse and rat fibroblasts by direct lineage conversion using transcription factors such as SOX10, Olig2, and Zfp536, Nkx6.2, Egr2 (Najm et al., 2013; Yang N. et al., 2013; Mazzara et al., 2017). These iOPCs can differentiate *in vivo* and myelinate sections of axons that are not myelinated. To date, there have been no reports of direct conversion of somatic cells into microglia. Further development of glial cell protocols will be required to simulate non-cell autonomous neurological disease.

DIRECT CONVERSION MODELS OF DISEASE

The diversity of conversion methods and sub-type specific fates gives researchers considerable flexibility for modeling disease. Disease states have been repeatedly shown not to impede conversion into neurons. The use of iNs in studying disease is still new and somewhat limited (**Figure 2**).

Amyotrophic Lateral Sclerosis

Lim et al. generated iNeurons via the inhibition of PTB method to investigate three mutations in the nuclear localization signal region of the gene Fused in Sarcoma (FUS), a causative ALS gene (Lim et al., 2016). The ALS iNeurons showed increased cytoplasmic FUS localization when compared to controls. Another report studying mechanisms of mutant FUS generated hiMNs converted from the fibroblasts of three ALS patients with known FUS mutations. FUS was mislocalized into the cytoplasm in all cases; however, only one of the three ALS patients used in this study had a FUS mutation known to increase cytoplasmic retention of FUS (Liu et al., 2016), emphasizing that the use of patient-derived DC models aides in the discovery of novel disease mechanisms. These FUS ALS hiMNs also showed dramatically reduced electrical activity, failed to form functional NMJs when co-cultured with primary mouse skeletal muscles, had shrunken somas, and were more susceptible to cell death when compared to controls. Control iMNs co-cultured with glia from $SOD1^{G93A}$ mice undergo more cell death than those co-cultured with control glia, replicating previously reported non-cell autonomous effects (Son et al., 2011). Similarly, induced NPCs from one patient with a $SOD1^{A4V}$ mutation and three patients with the C9orf72 repeat expansion were differentiated into iAstrocytes and were found to be toxic to co-cultured primary mouse neurons (Meyer et al., 2014). iNs converted from C9orf72 ALS patients by PTB inhibition showed RNA foci, poly(GP) inclusions and poly(PR) inclusions (Su et al., 2014). These reports suggest that DC models can replicate pathological hallmarks of multiple ALS subtypes in ways similar to iPSC-derived models.

Alzheimer's Disease

CiNs from four Alzheimer's disease patients with mutations in APP (V717I) or PSEN1 (I167del, A434T, or S169del) showed higher extracellular levels of Aβ40 and Aβ42 when compared to controls. iNs from AD patients also showed an increase in total tau and phosphorylated tau (Hu et al., 2015). Similar results regarding extracellular levels of Aβ40 and Aβ42 have been reported in induced NPCs differentiated into neurons from three AD patients, one with APOE4/E4 mutation and two with mutations in the PSEN1 gene (Hou et al., 2017).

Huntington's Disease

Liu et al. (2014) investigated the use of induced neuron technology to directly convert human fibroblasts from patients with Huntington's disease. Human fibroblasts from HD patients were converted by inhibition of PTB (Liu et al., 2014). The HD iNs exhibited nuclear mutant-Htt inclusions, abnormal neuritic branching, and lower cell survival. In another study, iNPCs were generated from HD patient fibroblasts and then further differentiated into neurons which exhibited higher vulnerability to DNA damage than controls (Hou et al., 2017).

Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) patient fibroblasts converted into hiMNs saw reduction in neurite growth rate and, after 60 days *in vitro*, degeneration of the neurites (Zhang et al., 2017). Induced neurons from patients with mutations in the PTEN-induced putative kinase 1 (PINK1) pathway, a genetic risk factor for Parkinson's disease, show reduction of downstream targets of this pathway which are involved in regulation of mitochondrial quality control (Puschmann et al., 2017).

Direct conversion cells have demonstrated the ability to mimic disease pathology and physiology in a number of neurological disorders (**Figure 2**). What has not been established yet is whether or not the retention of epigenetic signatures allows DC models to recreate disease pathology more accurately than rejuvenated iPSC models and whether cells generated from the same patient using both differentiation methods would display different disease phenotypes.

CONCLUDING REMARKS

Patient-derived CNS models have expanded rapidly in the past decade. A variety of adult somatic cells can be reprogrammed into iPSC's and differentiated or directly converted into the cells necessary to model disease, test novel drug candidates, and be used for cell transplantations. As researchers today design new studies of familial and sporadic neurological conditions, they

must carefully consider which model and method most efficiently answers their scientific question (**Figure 3**).

Patient-derived CNS models are time intensive. While direct conversion decreases the time to generate neurons from somatic sample collection due to the lack of reprogramming, the time it takes to differentiate and mature functional neurons from fibroblasts or iPSCs is similar. iPSC-based models offer more efficient production of cells than direct conversion because cells are infinitely expandable in both the iPSC stage and the NPC stage. While the yield of the desired cell type (e.g., neuron vs. astrocyte) is high, sub type (e.g., ChAT+ vs. CTIP2+) yield can vary widely between different small molecule and growth factor-based differentiation methods. Virally-expressed transcription factors may increase specific cell subtype yield in iPSC differentiations. While fibroblasts used for direct conversion are infinitely expandable, the lack of an intermediate progenitor stage creates a maximum one-to-one ratio of starting fibroblasts to desired cell type. In addition, there are reports of desired neuronal subtype yields of fewer than 10% (Vierbuchen et al., 2010; Pfisterer et al., 2011). Given the time and expense of generating the cells, this low yield must be weighed against the scientific benefits of aging retention in the model.

One application for patient-derived cells which has not been addressed at length in this review, but has been recently reviewed by Marsh and Blurton-Jones (2017), Czarzasta et al. (2017), Wang et al. (2017), and Smith et al. (2017), is the ability to implant iPSC-derived or directly converted NPCs and differentiate them *in vivo* to perform neurotrophic roles or replace cells lost through degeneration.

While comparative analyses of epigenetics and gene expression (particularly age-related genes) in iNeurons and iPSNs have demonstrated the loss of aging signatures in reprogramming (Mertens et al., 2015; Huh et al., 2016), the effects of these expression changes on the ability to model disease is poorly understood. Whether or not certain disease hallmarks, including proteinopathies, morphological and functional changes, and differences in survival in response to stressors will manifest differently when epigenetic signatures are preserved remains to be seen. A recent comparison of methylation of the C9orf72 transcript in repeat expanded eSCs and iPSCs suggests that toxic RNA transcription, and thus presumably RAN DPR translation, is attenuated in iPSCs and may suppress the role these mechanisms play in disease (Cohen-Hadad et al., 2016). This supports the argument that reprogramming will have an effect on disease pathology.

The clonal nature of iPSCs allows for the generation of CRISPR-Cas9 correction of disease-causing mutations, which can help establish whether certain pathologies observed in the model are attributable to the mutation and provides an isogenic control. This technique is not currently available in direct conversion; however, there may be some advantage to generating model neurons from a genetically and epigenetically heterogeneous population of somatic cells rather than from copies of a single iPSC clone, especially given the heterogeneity of disease pathology between cells in patient tissue.

Both direct conversion and iPSC differentiation have produced glia and several neuronal subtypes. Specific differentiation methods, including transcription factor choice, small molecule and growth factor choice, and plating methods have been and will continue to be adjusted to create a higher yield of desired cell types in less time. While, due to its earlier discovery, iPSC differentiation technology is currently better explored and developed, it is likely that iNeurons and other direct conversion cell types will be equally fine-tuned in the near

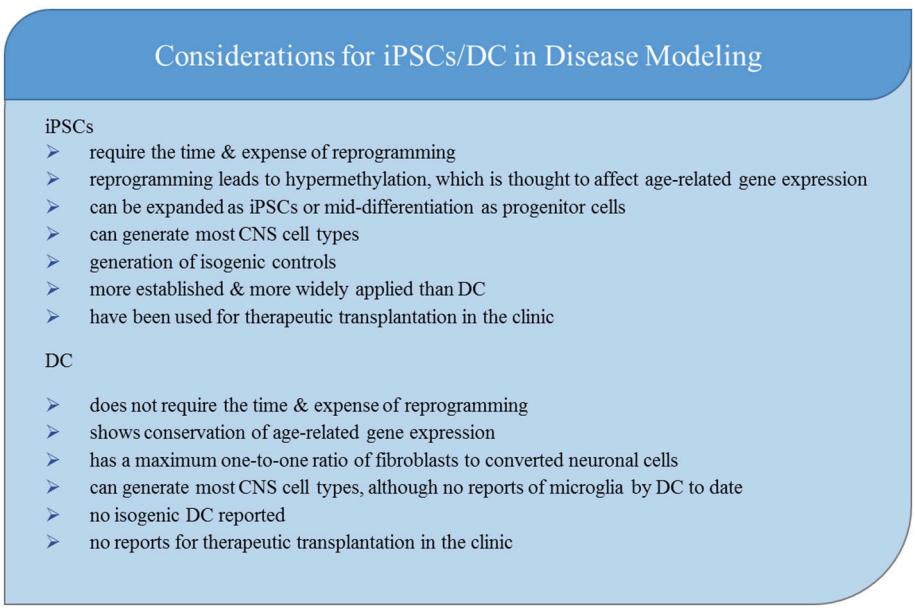

FIGURE 3 | Key considerations for choosing iPSCs or DC to model neurological disease.

future. With either method, care should be taken in regards to the true identity of generated cell types, given the extensive cellular conversion these cells undergo. Genetic and proteomic analyses of differentiated or converted cell types, along with comparisons to postmortem cell-type specific analyses, can address some of these concerns.

The extensive reports of disease pathologies and pathogenesis summarized in this review suggest that, despite the limitations of *in vitro* human cell culture models, they are capable of recreating disease-specific pathologies. In addition, new disease pathways have been discovered using patient-derived cell culture models which were then validated in postmortem patient tissue, such as the presence of nucleocytoplasmic transport deficits and nuclear pore dysfunction in ALS, FTD, HD and, likely, in other neurodegenerative diseases. It is therefore likely that other novel disease mechanisms will be revealed using these human cell culture models.

Patient-derived cells offer hope for an endogenous, human model of neurological disease, especially for sporadic cases which cannot be modeled any other way, and provide a platform for rapid therapeutic discoveries that are both personalized and clinically translatable.

AUTHOR CONTRIBUTIONS

LG, AS, AN, and RS: conceptualized and wrote the manuscript.

ACKNOWLEDGMENTS

This work was support by the NIH (RO1 NS085207), the Muscular Dystrophy Association (MDA 348086), the ALS Association (ALSA), the Robert Packard Center for ALS Research and the Barrow Neurological Foundation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 20 February 2018
doi: 10.3389/fnins.2018.00086

Modeling Protein Aggregation and the Heat Shock Response in ALS iPSC-Derived Motor Neurons

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Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder caused by the selective loss of the upper and lower motor neurons. Only 10% of all cases are caused by a mutation in one of the two dozen different identified genes, while the remaining 90% are likely caused by a combination of as yet unidentified genetic and environmental factors. Mutations in *C9orf72*, *SOD1*, or *TDP-43* are the most common causes of familial ALS, together responsible for at least 60% of these cases. Remarkably, despite the large degree of heterogeneity, all cases of ALS have protein aggregates in the brain and spinal cord that are immunopositive for SOD1, TDP-43, OPTN, and/or p62. These inclusions are normally prevented and cleared by heat shock proteins (Hsps), suggesting that ALS motor neurons have an impaired ability to induce the heat shock response (HSR). Accordingly, there is evidence of decreased induction of Hsps in ALS mouse models and in human post-mortem samples compared to unaffected controls. However, the role of Hsps in protein accumulation in human motor neurons has not been fully elucidated. Here, we generated motor neuron cultures from human induced pluripotent stem cell (iPSC) lines carrying mutations in *SOD1, TDP-43,* or *C9orf72*. In this study, we provide evidence that despite a lack of overt motor neuron loss, there is an accumulation of insoluble, aggregation-prone proteins in iPSC-derived motor neuron cultures but that content and levels vary with genetic background. Additionally, although iPSC-derived motor neurons are generally capable of inducing the HSR when exposed to a heat stress, protein aggregation itself is not sufficient to induce the HSR or stress granule formation. We therefore conclude that ALS iPSC-derived motor neurons recapitulate key early pathological features of the disease and fail to endogenously upregulate the HSR in response to increased protein burden.

Keywords: C9orf72, SOD1, OPTN, TDP-43, HspB8, HspB1, BAG3, stress granules

OPEN ACCESS

Edited by:
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RxGen (United States), United States

Reviewed by:
Gene Yeo,
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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *03 September 2017*
Accepted: *02 February 2018*
Published: *20 February 2018*

Citation:
Seminary ER, Sison SL and Ebert AD (2018) Modeling Protein Aggregation and the Heat Shock Response in ALS iPSC-Derived Motor Neurons. Front. Neurosci. 12:86. doi: 10.3389/fnins.2018.00086

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal, adult onset neurodegenerative disorder caused by the loss of the upper and lower motor neurons. Approximately 90% of all cases are sporadic (sALS), likely caused by a combination of as yet unidentified genetic and environmental factors. The remaining cases, familial ALS (fALS), are caused by a mutation in one of the two dozen different genes that have thus far been linked to ALS (Zufiria et al., 2016). Of these, mutations in *C9orf72*, *SOD1*, or *TDP-43* are the most common, together responsible for at least 60% of fALS (Zufiria et al., 2016). However, the identified genes have varied functions which, together with the inherent

heterogeneity of sALS, have made identifying convergent mechanisms of this disease challenging.

Despite this difficulty, a few similarities have been identified between all cases of ALS, including the presence of protein aggregates in the brain or spinal cord (Mizuno et al., 2006). These inclusions typically contain a core set of proteins, including SOD1, TDP-43, and optineurin (OPTN), to name a few. Given the known toxic effects of protein aggregates (Boya et al., 2003; De Kimpe et al., 2013; Yu et al., 2014), attempting to clear these aggregates represents a desirable therapeutic target that could be broadly applicable.

Heat shock proteins (Hsps) are cells' natural defense system against protein aggregation. These chaperone proteins recognize and bind misfolded proteins and either promote their refolding or degradation. Interestingly, it has been shown that Hsps are downregulated during natural aging and various neurodegenerative disorders (Brehme et al., 2014). In accordance with this, a decrease in Hsp70, Hsp40, and HSF1 protein levels in a mouse model of TDP-43-linked ALS and sALS post-mortem tissue has been reported (Chen et al., 2016). Additionally, the protective nature of Hsps in an ALS disease state is further supported by *in vitro* and mouse studies showing an amelioration of disease symptoms by upregulating the heat shock response (HSR) (Kieran et al., 2004; Kalmar et al., 2008; Lin et al., 2013, 2016). However, in contrast to these data, others report increased serum levels of Hsp70 and Hsp90 in ALS patients (Miyazaki et al., 2016). As previous studies have indicated that aberrant and sustained activation of the HSR diminishes its overall function (Roth et al., 2014), it is important to better understand what the expression levels of Hsps are in human ALS motor neurons under physiological conditions and whether these levels are consistent across multiple genetic backgrounds.

Previous reports have shown that motor neurons from ALS mice, postmortem human patients, and iPSCs exhibit protein aggregates (Mizuno et al., 2006; Mackenzie et al., 2007; Kalmar et al., 2008; Maruyama et al., 2010; DeJesus-Hernandez et al., 2011; Lin et al., 2013; Chen et al., 2016; Dafinca et al., 2016; Bhinge et al., 2017), which may contribute to motor neuron loss. As increased levels of insoluble protein triggers the HSR in an effort to degrade and/or refold aberrant proteins (Ananthan et al., 1986; Baler et al., 1992; Morimoto, 1998; Westerheide et al., 2012), we hypothesized that ALS motor neurons have minimal endogenous HSR activation thereby contributing to the presence of protein aggregates. Here, we used motor neurons derived from iPSCs generated from ALS patients expressing mutations in SOD1, C9orf72, and TDP-43. Consistent with previous reports, ALS iPSC-derived motor neurons showed accumulation of insoluble SOD1, TDP-43, and OPTN, but the specific content varied among the different genetic backgrounds. However, the presence of insoluble protein was not sufficient to induce a robust HSR or enhanced stress granule formation in ALS iPSC-derived motor neurons, despite the general ability to induce the HSR with heat stress. Therefore, these data suggest that ALS iPSC-derived motor neurons fail to endogenously upregulate the HSR in response to protein accumulation, which could adversely impact motor neuron health and survival.

MATERIALS AND METHODS

iPSC Culture

iPSCs were grown on Matrigel (Corning, cat. 354230) coated 6-well plates (VWR, cat. 82050-842) in either mTeSR1 (STEMCell Technologies, cat. 5850) or Essential 8 (Life Technologies, cat. A1517001). Colonies were split every 4–7 days with Versene (Life Technologies, cat. 15040-066) and routinely tested for mycoplasm using the Mycoalert Detection Kit (Lonza, cat. LT07-318). At the time of passage, the remaining wells were harvested to use for filter-trap experiments. For these studies, one SOD1 (N139K) line (Hosoyama et al., 2014), three C9orf72 lines (Sareen et al., 2013), one TDP-43 (M337V) line (Bilican et al., 2012), and three control lines (Ebert et al., 2009; McGivern et al., 2013; Riedel et al., 2014) were used.

Motor Neuron Differentiation

Motor neurons were generated by following the protocol described by Maury and colleagues with minor modifications (Maury et al., 2015). Briefly, embryoid bodies (EBs) were generated from monolayer iPSC colonies and transferred to a T25 culture flask. EBs were subjected to various patterning factors for 2 weeks before being dissociated and plated onto Matrigel coated plates for RNA and protein analysis, or Matrigel coated glass coverslips for immunocytochemistry. Motor neurons were allowed to mature for an additional 1–2 weeks before being used for further experiments. For heat shock experiments, plates were wrapped with parafilm and placed in a water bath at 42°C for 1 h. Cells were immediately harvested for RNA and protein analysis.

Immunocytochemistry

Motor neurons grown on glass coverslips were fixed with 4% Paraformaldehyde for 10 min at room temperature. Cells were permeabilized and blocked with 0.1% Triton and 5% donkey serum for 1 h, and incubated in primary antibody overnight at 4°C or 1 h at room temperature. Secondary antibody was added for 1 h at room temperature. Stained coverslips were blinded and mounted onto glass microscope slides (Fisher, cat. 22-230-891) using Fluoromount-G (Southern Biotech, cat. 0100-01). Antibodies used are listed in **Table 1**.

Microscopy

Images were taken on an upright Nikon E400 microscope and QCapture camera. Light source was a Lumen200 metal arc lamp (Prior Scientific). Pictures of puncta staining were taken with a 100x oil objective, pixel resolution of 0.06 μm/px. All others were taken with a 40x objective, pixel resolution of 0.16 μm/px. Exposure times varied with each target, but were kept consistent when imaging each differentiation. The exposure time ranges per channel are as follows: FITC 700 ms−1 s, TRITC 700 ms−1 s, Cy5 1 s−2 s, DAPI 40–100 ms.

Image Analysis

Quantification was performed in NIS Elements (Nikon). For cell count quantification, cells were counted by a blinded observer. HspB1 and HspB8 staining was quantified by selecting the region of interest, determining the total intensity and then dividing by the total area. Punctate expression was automatically detected in

TABLE 1 | Antibodies.

Antibody	Company	Catalog number
Rabbit α-BAG3	abcam	ab47124
Goat α-ChAT	Millipore	AB144P
Mouse α-G3BP	abcam	ab56574
Rabbit α-HSF1 Phospho S326	abcam	ab76076
Mouse α-HspB1	Cell Signaling	2402
Rabbit α-HspB8	abcam	ab151552
Mouse α-Islet1	DSHB	40.2D6
Rabbit α-Optineurin	abcam	ab151240
Rabbit α-SOD1	abcam	ab13498
Rabbit α-TDP-43	abcam	ab109535
Chicken α-Tuj1	GeneTex	GTX85469
Rabbit α-Tuj1	Covance	MRB-435P

TABLE 2 | Primer Sequences.

Target	Sequences	Citation
HSPB1 Forward	AAGCTAGCCACGCAGTCCAA	Wang et al., 2013
HSPB1 Reverse	CGGCAGTCTCATCGGATTTT	Wang et al., 2013
HSPB8 Forward	GCCAGAGGAGTTGATGGTGAAGACC	Ospelt et al., 2014
HSPB8 Reverse	CATGTTTGCCAGACACCTCCACG	Ospelt et al., 2014
BAG3 Forward	CATCCAGGAGT GCTGAAAGTG	Li et al., 2017
BAG3 Reverse	TCTGAACCT TCCTGACACCG	Li et al., 2017
GAPDH Forward	GTGGACCTGACCTGCCGTCT	Patitucci and Ebert, 2016
GAPDH Reverse	GGAGGAGTGGGTGTCGCTGT	Patitucci and Ebert, 2016

NIS Elements by utilizing the Object Counts module. Intensity and size thresholds were set and kept consistent for each target. Individual neurons were outlined as regions of interests to quantify the number of neurons with puncta as well as the number of puncta per neuron.

qPCR

Total RNA was isolated with RNeasy Mini Kit (Qiagen, cat. 74104) from cell pellets collected at 4 weeks of total differentiation. 2 μg of RNA was used to generate cDNA according to manufacturer instructions (Promega, cat. A3500). qPCR for HspB1, HspB8, BAG3, and GAPDH was performed using 20 ng cDNA and a Bio-Rad Connect96 Thermocycler with the following cycle conditions: 95°C for 10 min and 40 cycles of 95°C for 10 s and 60°C for 45 s for HspB1, HspB8, and GAPDH. The BAG3 qPCR was performed with the same cycle conditions but with an annealing temperature of 55°C. All signals were normalized to GAPDH and then compared to controls. Primer sequences are listed in **Table 2**.

Western Blot

Protein was isolated by resuspending cell pellets in 1x CHAPS Buffer (Cell Signaling, cat 9852S) followed by three freeze-thaw cycles. The protein concentration of the resulting lysate was determined by a Bradford Assay. 20 μg of protein was loaded into a 4–15% Mini-PROTEAN® TGX Stain-Free™ Protein Gel (Bio-Rad, cat. 4568083), run at 105 V for approximately 70 min, and subsequently transferred to a PVDF membrane (Li-COR, cat. 926-31099) at 105 V for 30 min. The membrane was allowed to dry immediately after transfer for at least 1 h to cross-link the protein before proceeding. REVERT Total Protein Stain (Li-COR, cat. 926-11010) was performed to confirm proper transfer and equal loading. Blots were blocked with a 1:1 dilution of Odyssey Blocking Buffer (Li-COR, cat. 927-50000) and TBS with gentle shaking. Primary antibody was diluted in the same 1:1 dilution plus 0.2% Tween-20 and incubated overnight at 4°C with gentle shaking. Signal was detected by incubating in secondary antibody, diluted in Odyssey Blocking Buffer, TBS, 0.2% Tween-20, and 0.02% SDS, in a light protected box for 30 min at room temperature. Blots were imaged with the Odyssey Scanner (Li-COR). All signals were quantified in Image Studio (Li-COR) and normalized to REVERT Total Protein Stain according to manufacturer instructions. Data are presented as fold change over control. Representative images were converted to grayscale for figures. All antibodies used are listed in **Table 1**.

Filter-Trap Assay

Fourty micrograms of protein was diluted in 1x PBS and 10% SDS, then diluted further in 1x PBS + 0.1% SDS. Protein was loaded onto a Cellulose Acetate Membrane (SterliTech, cat. CA023001) through a dot-blot apparatus and washed with 1x PBS + 0.1% SDS. The membrane was immediately blocked in 5% milk for 1 h and incubated in primary antibody overnight at 4°C with gentle shaking. Signal was detected by WesternBright ECL (Advansta cat. K-12045) after incubating in an HRP-conjugated secondary antibody for 1 h. Densitometry was performed in ImageJ. Representative images were converted to grayscale for figures. **Table 1** lists the antibodies used.

Statistical Analysis

A minimum of three independent experiments with a minimum of three biological replicates were performed per experiment. Data were analyzed by performing a one-way ANOVA with Tukey *post-hoc* test or Student's *t*-test, as appropriate. Significance was determined based on a $p < 0.05$.

RESULTS

Motor Neuron Cultures from Multiple ALS Genotypes Do Not Show Decreased Viability

While motor neuron loss is a key feature of fALS and sALS, previous *in vitro* studies have shown that iPSC-derived motor neurons do not exhibit reduced viability when grown in the absence of astrocytes (Egawa et al., 2012; Almeida et al., 2013; Chen et al., 2014; Devlin et al., 2015). Consistent with previous reports, there was no difference in the number of motor neurons in SOD1, C9orf72, or TDP-43 cultures compared to controls at 4 weeks in culture (**Figure 1**). Approximately 60% of cells in the cultures were Tuj1+ neurons, with the vast majority of neurons

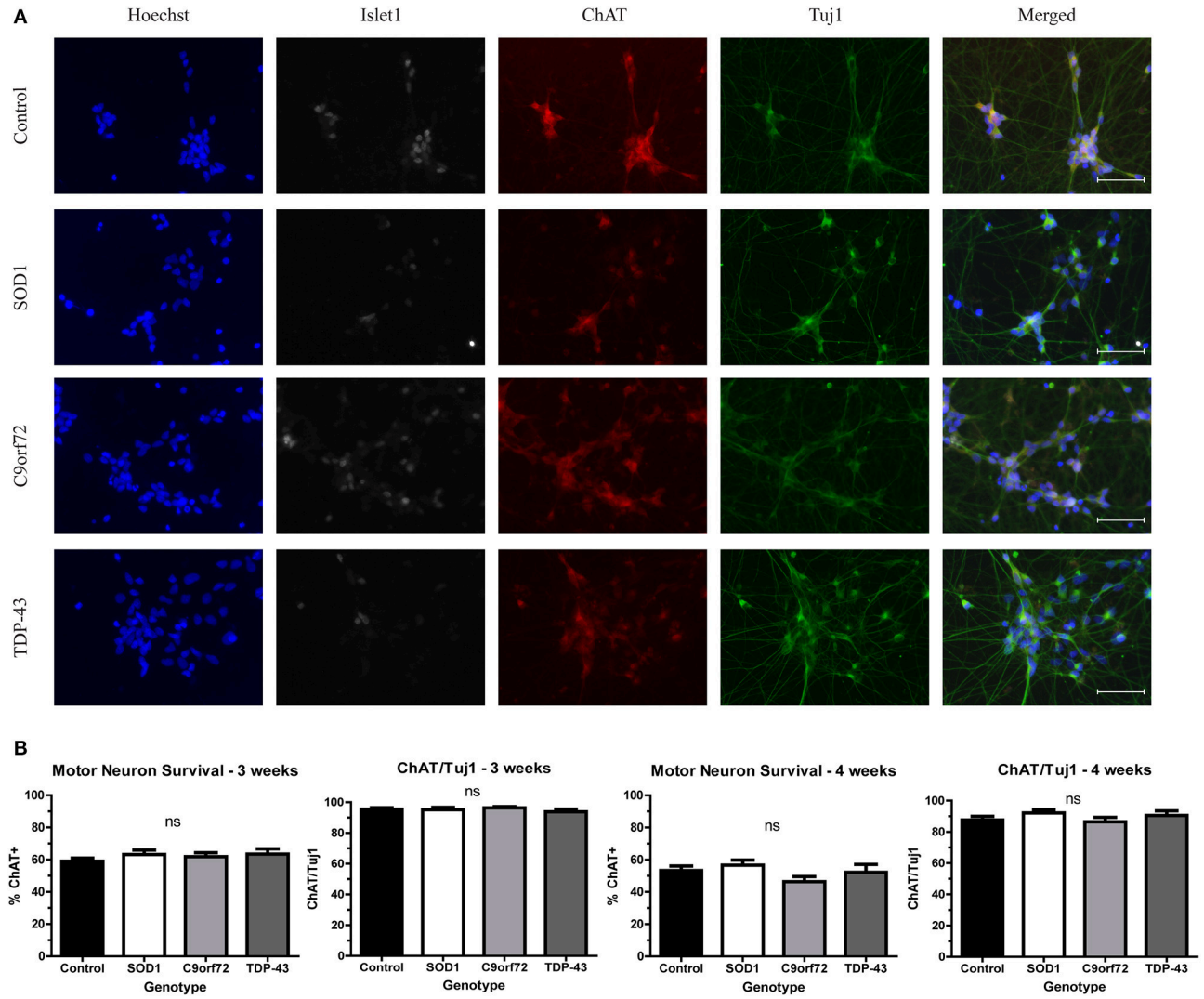

FIGURE 1 | iPSC-derived motor neurons from multiple ALS genotypes do not exhibit reduced viability in culture. **(A)** Representative images at 3 weeks of differentiation of control and ALS motor neurons immunostained for Tuj1 (green), ChAT (red), and Islet1 (white). Nuclei are labeled with Hoechst nuclear dye (blue). Scale bar = 50 μm. **(B)** Quantification shows no difference in the number of motor neurons at 3 or 4 weeks of differentiation. $n = 3$–4. Not significant by one-way ANOVA with Tukey *post-hoc* test.

(∼95%) expressing the motor neuron markers Islet1 and ChAT (**Figure 1**) with negligible expression of the astrocyte marker GFAP (data not shown; Maury et al., 2015).

Divergent Solubility of Aggregation-Prone Proteins in ALS Motor Neurons

We next sought to determine whether ALS iPSC-derived motor neuron cultures endogenously develop protein aggregates as this is an important phenotypic hallmark of ALS pathology. While protein aggregation has consistently been observed in mouse models of ALS (Kalmar et al., 2008; Lin et al., 2013; Chen et al., 2016), there has been variability in iPSC models (Devlin et al., 2015; Dafinca et al., 2016; Bhinge et al., 2017). We first asked whether SOD1 aggregates can be detected. Previous studies have reported SOD1+ aggregates in SOD1 iPSC-derived motor neurons (Chen et al., 2014; Bhinge et al., 2017), but TDP-43 and C9orf72 motor neurons have not been evaluated. We found that while there was no difference in the number of neurons with SOD1+ puncta across the different lines, C9orf72 and TDP-43 motor neuron cultures exhibited fewer puncta per neuron compared to control motor neurons (**Figure 2A**). SOD1 iPSC-derived motor neurons also exhibited a trend toward increased levels of insoluble SOD1 (**Figure 2C**) and a trend toward decreased levels of soluble SOD1 protein compared to control (**Figure 2B**). Interestingly, levels of insoluble SOD1 were also trending upwards in C9orf72 motor neurons (**Figure 2C**), although this phenotype varied among the three independent C9orf72 iPSC lines. These data contrast with the result that

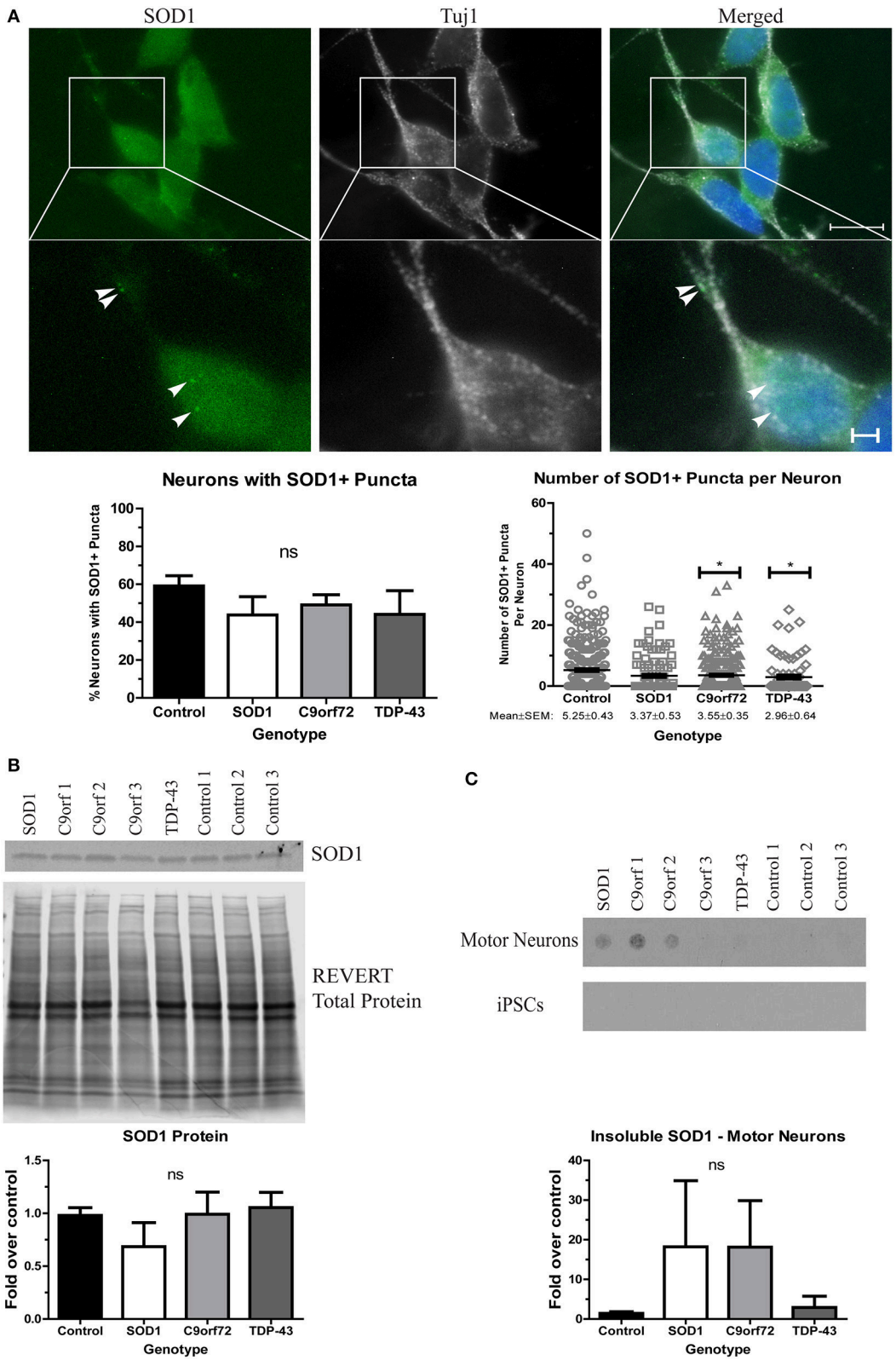

FIGURE 2 | Altered solubility of SOD1 in SOD1 and C9orf72 motor neurons. **(A)** Representative immunocytochemistry images of Hoechst nuclear stain (blue), SOD1 (green), and Tuj1 (white) at 4 weeks of differentiation shows no significant difference in the number of neurons with SOD1+ puncta in ALS motor neurons compared to

(Continued)

FIGURE 2 | control (ns, $p > 0.05$ by one-way ANOVA with Tukey *post-hoc* test). C9orf72 and TDP-43 motor neurons exhibited fewer SOD1+ puncta per neuron compared to control (*$p < 0.05$ by one-way ANOVA with Tukey *post-hoc* test). $n = 3$, scale bar = 10 μm (full image) or 2 μm (expanded). **(B)** Western blot for SOD1 shows no significant difference in ALS lines compared to control lines. Data are normalized to REVERT total protein stain and expressed as a fold change over control (ns, $p > 0.05$ by one-way ANOVA with Tukey *post-hoc* test, $n = 3$–6). **(C)** Filter-trap assay shows a trend toward increased levels of insoluble SOD1 in SOD1 and C9orf72 motor neurons but not in iPSC colonies (ns, $p > 0.05$ by one-way ANOVA with Tukey *post-hoc* test). Data are expressed as fold change over controls. $n = 3$.

C9orf72 motor neurons had fewer SOD1+ puncta per neuron, which suggests that observable puncta may not directly correlate with protein insolubility. Insoluble SOD1 was not detected in any of the iPSC colonies (**Figure 2C**), which indicates that SOD1 aggregation increases with neuron differentiation.

When evaluating TDP-43 aggregation, we found no significant difference in either the number of neurons with cytoplasmic TDP-43+ puncta or the number of puncta per neuron in any of the ALS iPSC lines compared to control (**Figure 3A**), which is in contrast to a previous report that showed more inclusions in TDP-43 iPSC-derived cultures (Egawa et al., 2012). While all lines showed a trend toward increased soluble levels of TDP-43 compared to control (**Figure 3B**), these data did not reach significance. However, the TDP-43 motor neurons did exhibit a strong trend toward increased insoluble TDP-43 compared to control (**Figure 3C**). As with SOD1 protein aggregation, the increase in insoluble protein was most pronounced in the motor neuron cultures (**Figure 3C**).

Finally, we asked whether OPTN aggregates in ALS iPSC-derived motor neurons. OPTN is an adaptor protein in autophagy, which, along with the HSR, is a component of the proteostasis network. OPTN can be found in aggregates in both sALS and fALS, and specific mutations have been linked to a small percentage of fALS cases (Maruyama et al., 2010). However, OPTN pathology has not yet been evaluated in iPSC-derived motor neurons. We found no difference in the number of neurons with OPTN+ inclusions in SOD1, TDP-43, or C9orf72 cultures compared to control (**Figure 4A**). Additionally, the number of OPTN+ puncta per neuron was similar in all lines (**Figure 4A**). However, whereas soluble protein levels were unchanged across the different lines (**Figure 4B**), insoluble levels trended higher in SOD1 and C9orf72 motor neuron cultures compared to control cultures, which exhibited a low basal level of insoluble protein (**Figure 4C**). While one C9orf72 iPSC cell line exhibited increased insoluble OPTN in the undifferentiated state, this phenotype was largely restricted to motor neurons (**Figure 4C**). Taken together, these data suggest that ALS iPSC-derived motor neurons exhibit accumulation of aggregation-prone proteins, but genetic background does influence protein content and solubility.

ALS Motor Neurons Fail to Fully Upregulate the HSR

Protein aggregation is normally prevented by the protein quality control network, which includes the HSR. As such, previous studies have investigated whether inducing the HSR reduces the toxicity of SOD1 or TDP-43 aggregates (Kieran et al., 2004; Lin et al., 2013, 2016; Chen et al., 2016). However, other data indicate that sustained activation of the HSR actually diminishes the function of the protein quality control network (Roth et al., 2014). Since there is an increase in insoluble protein in ALS iPSC-derived motor neurons (**Figures 2–4**), we next aimed to test whether this could be due to insufficient or aberrant HSR activation. As such, we examined the expression levels of HspB1, HspB8, and BAG3 in ALS iPSC-derived motor neuron cultures (**Figure 5**). These chaperone proteins were of interest because of previous data linking them to SOD1 (Crippa et al., 2010; Mateju et al., 2017) and TDP-43 pathology (Crippa et al., 2016; Ganassi et al., 2016) in both cell culture and *in vivo* models. Additionally, BAG3 has been shown to complex with HspB1 and HspB8 to promote protein clearance (Ganassi et al., 2016; Rauch et al., 2017). Whereas the transcript levels of HspB1, HspB8, and BAG3 were not significantly altered in C9orf72 motor neurons, we found a trend toward increased BAG3 transcript in SOD1 and TDP-43 motor neurons compared to control motor neurons (**Figure 5A**). This corresponded to a modest, but significant increase in BAG3 protein levels in TDP-43 motor neurons and a strong trend toward increased protein levels in SOD1 motor neurons compared to control cultures as detected by western blot (**Figure 5B**). HspB1 and HspB8 protein levels were evaluated by immunocytochemistry intensity quantification, which found no change in HspB1 protein levels in any of the ALS lines compared to control, but a significant increase in HspB8 protein only in SOD1 motor neurons compared to control (**Figure 5C**). These data indicate that ALS iPSC-derived motor neurons do not have endogenous activation of the HSR in response to increased protein burden.

Considering the modest and incomplete upregulation of the HSR in ALS iPSC-derived motor neurons, we hypothesized that upstream HSR signals may also be blunted. Therefore, we next assessed levels of phosphorylated HSF1 in ALS iPSC-derived motor neurons. HSF1 has been shown to be a master regulator of the HSR (Santoro, 2000; Westerheide et al., 2012). HSF1 is constitutively expressed and sequestered in the cytoplasm by Hsp70, Hsp90, and Hdj1 in its inactive, monomeric form (Morimoto, 1998; Shi et al., 1998). Upon stress and protein aggregation, the chaperones holding HSF1 are recruited to the site of misfolded proteins, releasing HSF1 (Morimoto, 1998; Shi et al., 1998; Santoro, 2000; Westerheide et al., 2012). HSF1 can then trimerize and translocate to the nucleus (Morimoto, 1998; Santoro, 2000). Additionally, HSF1 undergoes a number of post-translational modifications, including phosphorylation at Serine 326, which has been shown to be required for transcriptional activity (Guettouche et al., 2005). As such, expression of the S326 phosphorylated form can be used to determine whether the HSR is robustly activated. However, we detected no significant increase in HSF1 phosphorylation levels in any of the ALS iPSC-derived motor neurons compared to control (**Figure 5D**), consistent with an incomplete induction of HspB1, HspB8, and BAG3. Therefore, these data indicate that the observed increase

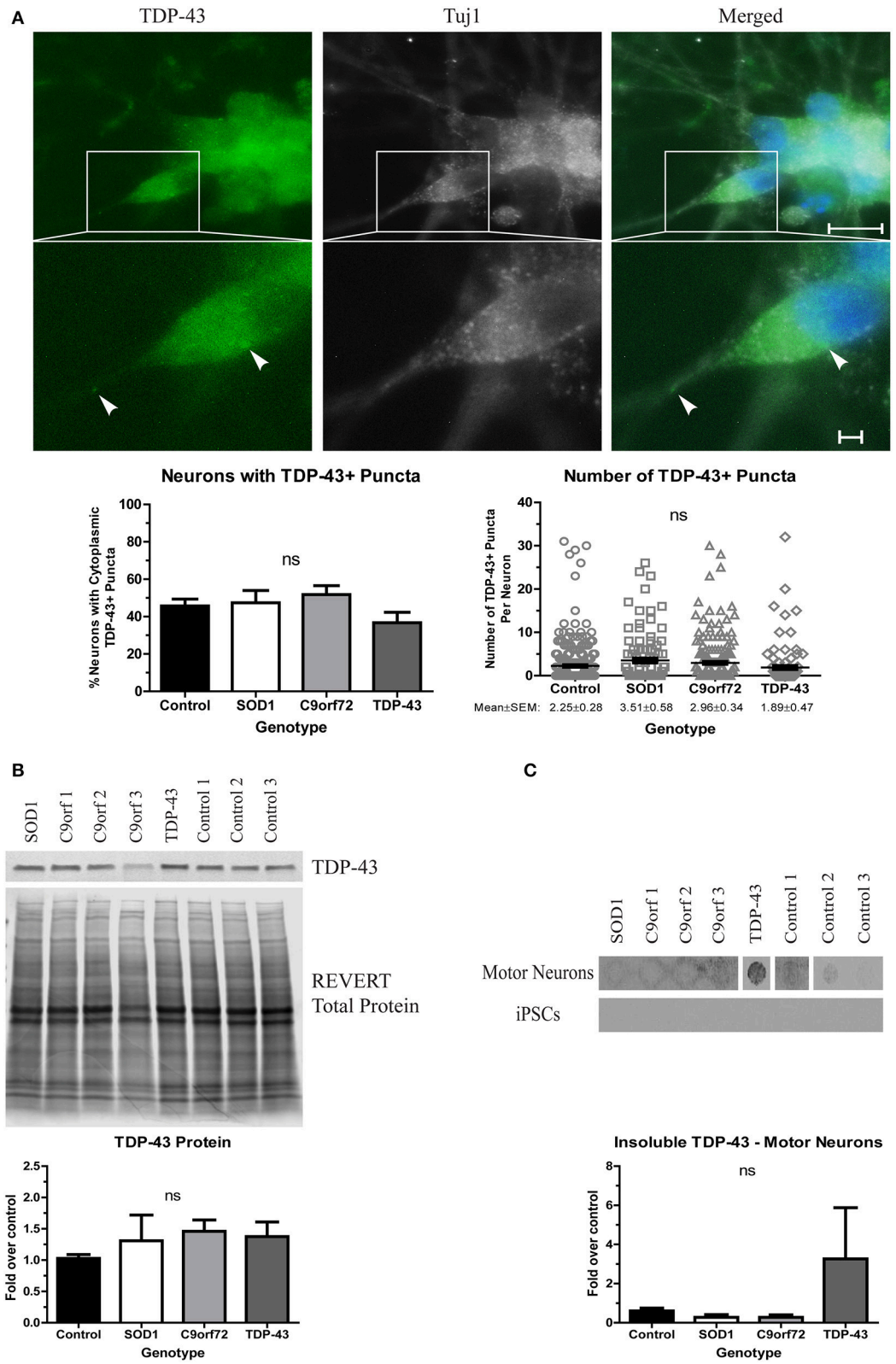

FIGURE 3 | Alterations in TDP-43 solubility in TDP-43 motor neurons. **(A)** Representative images at 4 weeks of differentiation show no change in the number of neurons with cytoplasmic TDP-43+ puncta or the number of TDP-43+ puncta per neuron in ALS motor neuron cultures (ns, $p > 0.05$ by one-way ANOVA). $n = 3$,

(Continued)

FIGURE 3 | scale bar = 10 μm (full image) or 2 μm (expanded). **(B)** Western blot for TDP-43 shows no significant difference in protein levels in ALS motor neuron cultures. Data are normalized to REVERT total protein stain and expressed as a fold change over control (ns, $p > 0.05$ by one-way ANOVA, $n = 3$–5). **(C)** Filter-trap assay of TDP-43 shows a trend toward an increase in insoluble levels in TDP-43 motor neurons but not in iPSC colonies (ns, $p > 0.05$ by one-way ANOVA with Tukey *post-hoc* test). Data are expressed as fold change over controls, $n = 3$.

in insoluble protein burden is not sufficient to activate the HSR in ALS iPSC-derived motor neurons.

Since ALS iPSC-derived motor neurons do not robustly activate the HSR in response to increased protein aggregation, we next sought to determine if ALS motor neurons were capable of upregulating the HSR at all. Therefore, we incubated ALS and control iPSC-derived motor neurons at 42°C for 1 h to heat stress the cells. With the exception of HspB8, control motor neurons showed robust induction of HspB1 and BAG3 transcript (**Figure 6A**) and phosphorylated HSF1 protein (**Figure 6B**) following heat stress. Similarly, heat stressed SOD1 motor neurons significantly induced transcript levels of HspB1, HspB8, and BAG3 (**Figure 6A**), as well as exhibited increased phosphorylated HSF1 protein levels compared to the unstressed state (**Figure 6B**). C9orf72 motor neurons also showed increased HspB1, HspB8, and BAG3 transcript levels following heat stress, although to a lesser extent than the control or SOD1 motor neurons (**Figure 6A**). Similarly, C9orf72 motor neurons exhibited increased levels of phosphorylated HSF1, although this increase was just below significance (**Figure 6B**). Interestingly, the TDP-43 motor neurons did not significantly induce any of the measured HSR components after heat shock (**Figure 6**), suggesting an intrinsic deficiency in the ability of these motor neurons to induce this protein quality control pathway. Together these data suggest that ALS iPSC-derived motor neurons may be able to activate the HSR under certain conditions, but that insoluble protein burden is not a sufficient signal to do so and genetic background may influence overall response.

ALS Motor Neurons Do Not Exhibit Enhanced Stress Granule Formation

Stress granules (SGs) traditionally sequester mRNAs and RNA-binding proteins, like TDP-43, during acute cellular stress into membrane-less compartments (Kedersha et al., 1999). After stress alleviation, SGs disassemble and translation resumes (Kedersha et al., 1999). However, under prolonged stress, misfolded proteins, including SOD1, can accumulate into SGs which can alter SG dynamics, leading to insolubility (Ganassi et al., 2016; Mateju et al., 2017). Additionally, several SG components, including eIF3 and TIA-1, have been found in aggregates in post-mortem ALS tissue (Liu-Yesucevitz et al., 2010), and a recent report has identified TIA-1 as a rare causative mutation for fALS (Mackenzie et al., 2017). We therefore sought to determine whether SGs spontaneously form in ALS iPSC-derived motor neurons as an indication of whether ALS motor neurons upregulate stress pathways as a response to increased insoluble protein. We found that while the staining pattern for the SG marker G3BP was primarily diffuse, the majority of neurons had at least one G3BP+ puncta, with the average number of G3BP+ puncta ranging ∼3–5 per neuron (**Figure 7A**). Interestingly, C9orf72 motor neurons exhibited a modest but significant decrease in the number of SGs per neuron (**Figure 7A**), which may indicate a deficiency in the ability of C9orf72 motor neurons to induce this protective mechanism. There was no overall change in the protein levels of G3BP in the ALS lines compared to control (**Figure 7B**), and following heat stress, we also did not detect a change in G3BP protein levels in any sample compared to the unstressed condition (**Figure 7B**). These data are in contrast to previous studies (Mateju et al., 2017) and suggest that acute heat stress may not be sufficient to induce stress granule formation in the iPSC system. Taken together, these data suggest that ALS iPSC-derived motor neurons lack the capabilities to fully activate stress response systems despite increased insoluble protein burden.

DISCUSSION

ALS is a highly complex neurodegenerative disease that has neither a cure nor an effective therapy. Despite first being described in the late 1800s, the cause of sALS is still unknown. Additionally, two dozen different genes with varying prevalence and protein functions have been identified as causative for fALS. Given this heterogeneity, it is imperative that any convergent mechanisms and phenotypes be identified in order to develop therapies that would be applicable to the greatest number of patients. Previous studies using *in vitro* and *in vivo* model systems have focused mainly on SOD1, TDP-43, and in recent years C9orf72-linked ALS, as mutations in these three genes represent the most prevalent causes of fALS. However, these studies have limitations. The SOD1 and some TDP-43 mouse models were generated by over-expressing the mutant form of the human proteins, which may not accurately represent the human condition. Multiple C9orf72 mouse models have been made to date, but results have been mixed as each mouse exhibits a different phenotype. A C9orf72 knock-out mouse showed no motor deficits, but did exhibit an immune phenotype and an enlarged spleen (O'Rourke et al., 2016). Alternatively, two BAC C9orf72 models did not develop ALS despite the presence of RNA foci and dipeptide repeat proteins that are characteristic of this mutation (O'Rourke et al., 2015; Peters et al., 2015), while a third BAC mouse did exhibit motor deficits and reduced survival, consistent with an ALS disease state (Liu et al., 2016). Numerous other studies use non-motor neuron cell types that rely on mutant protein over-expression in potentially non-disease-relevant cell types. In contrast, iPSCs offer a unique human model system to study endogenous cellular processes associated with ALS in disease-relevant cell types (Dimos et al., 2008). Additionally, iPSCs are more amenable to concurrently studying multiple genetic backgrounds, which will be crucial for identifying convergent disease mechanisms.

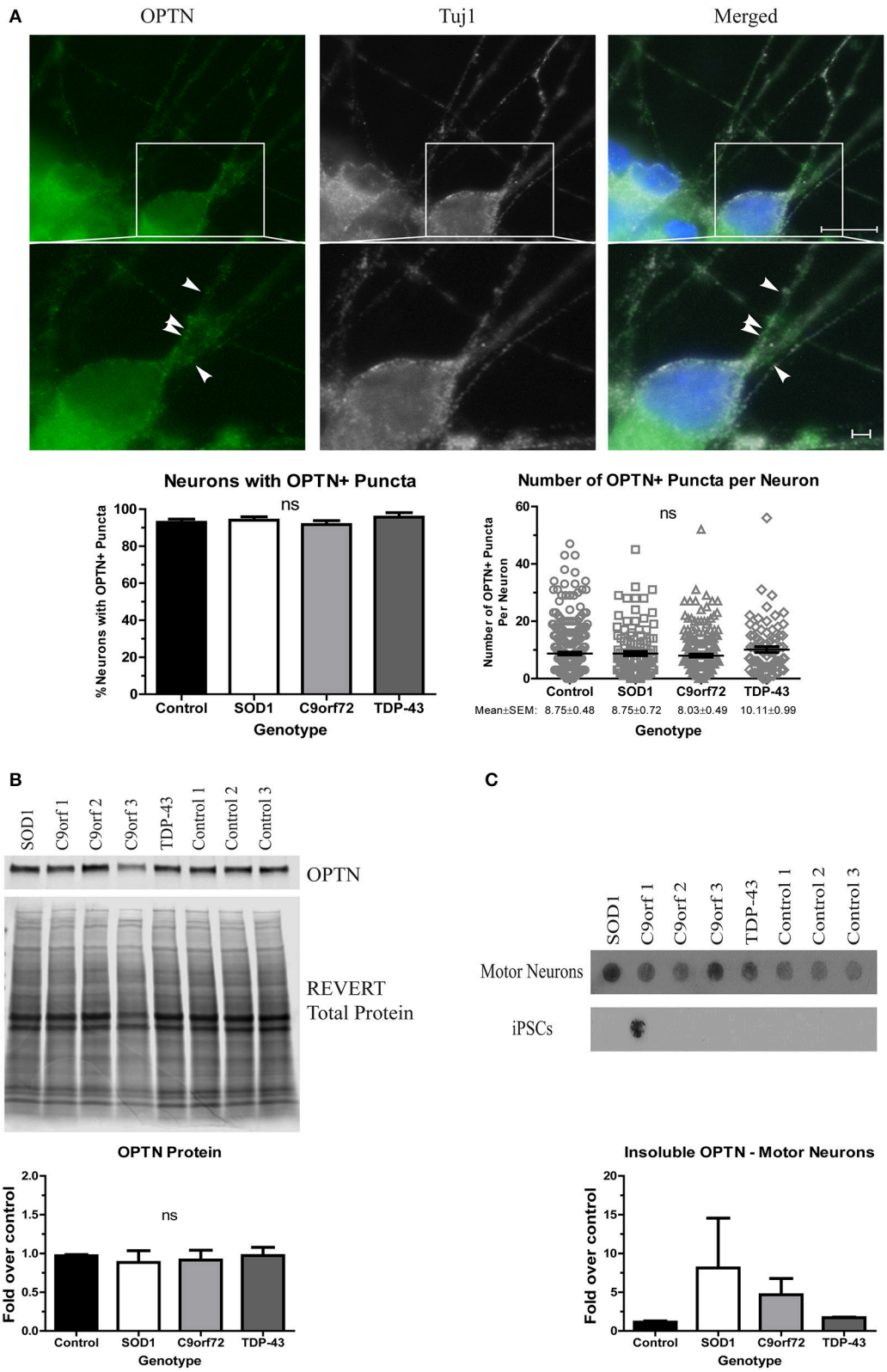

FIGURE 4 | OPTN is insoluble in SOD1 and C9orf72 motor neurons. **(A)** Representative immunocytochemistry images of Hoechst nuclear stain (blue), OPTN (green), and Tuj1 (white) at 4 weeks of differentiation. No difference is seen in the number of neurons with OPTN+ puncta (ns, $p > 0.05$ by one-way ANOVA with Tukey

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FIGURE 4 | *post-hoc* test). $n = 3$, scale bar = 10 μm (full image) or 2 μm (expanded). **(B)** Total protein levels of OPTN are unchanged by Western blot. Data are normalized to REVERT total protein and expressed as a fold change over control (ns, $p > 0.05$ by one-way ANOVA with Tukey *post-hoc* test). $n = 3$. **(C)** OPTN filter-trap assay shows a trend toward increased insoluble protein in SOD1 and C9orf72 motor neurons (ns, $p > 0.05$ by one-way ANOVA with Tukey *post-hoc* test). Insoluble OPTN was observed in the colonies from one C9orf72 iPSC line, but in no others. Data are expressed as fold change over controls, $n = 3$.

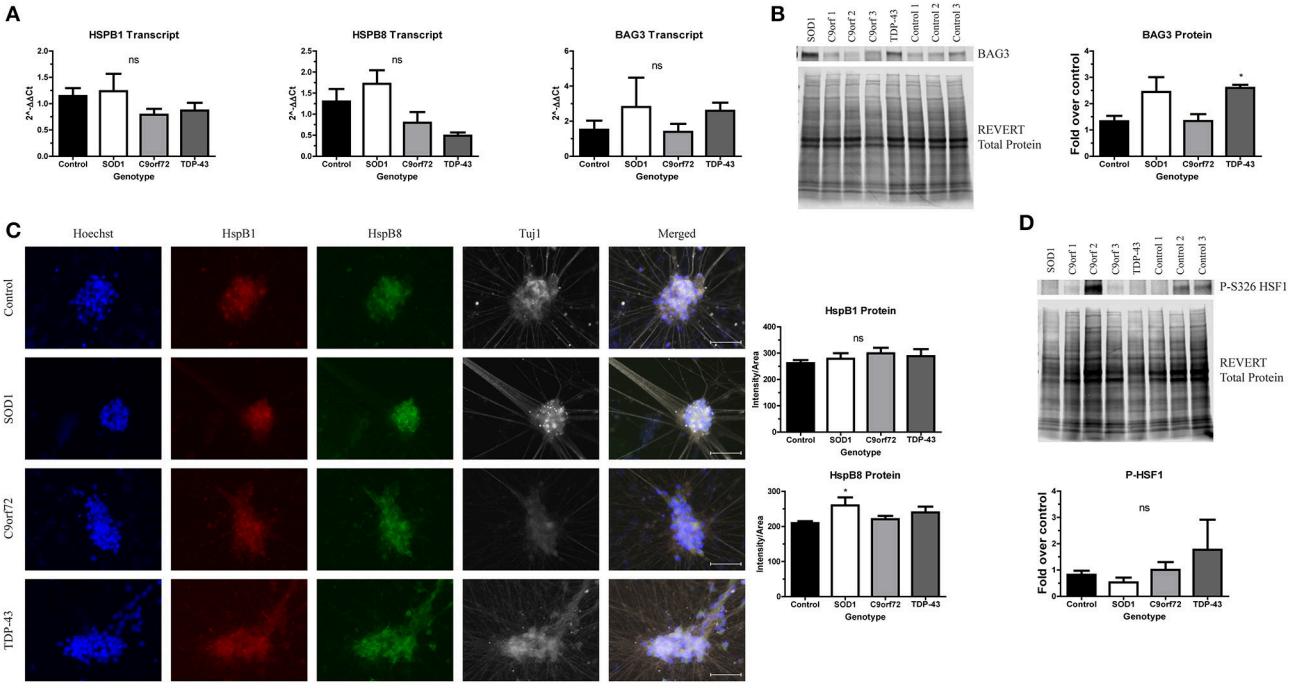

FIGURE 5 | ALS motor neurons have altered expression of HspB1, HspB8, and BAG3. **(A)** BAG3, HspB1, and HspB8 transcript levels are not significantly increased in ALS iPSC-derived motor neurons, $n = 3$–6. **(B)** Western blot for BAG3 shows an increase in TDP-43 motor neurons. Data are normalized to REVERT total protein and expressed as a fold change over control (*$p < 0.05$ by one-way ANOVA with Bonferroni *post-hoc* test, $n = 3$). **(C)** Quantification of HspB1 (green) and HspB8 (red) immunocytochemistry intensity shows a significant increase in protein levels of HspB8 in SOD1 motor neurons (*$p < 0.05$ by one-way ANOVA with Tukey *post-hoc* test, $n = 3$). Scale bar = 50 μm. **(D)** Western blot for phosphorylated HSF1 at S326 shows no significant HSR induction in ALS motor neurons. Data are normalized to REVERT total protein and expressed as a fold change over control (ns, $p > 0.05$ by one-way ANOVA with Tukey *post-hoc* test). $n = 3$.

Even though motor neuron loss is a key pathological feature of ALS, iPSC-derived motor neuron cultures do not exhibit a cell death phenotype when grown in the absence of astrocytes (**Figure 1**), (Dimos et al., 2008; Egawa et al., 2012; Almeida et al., 2013; Chen et al., 2014; Devlin et al., 2015). However, ALS iPSC-derived motor neurons do recapitulate other features of ALS. Specifically, we detected increased levels of insoluble SOD1, TDP-43, and OPTN protein levels in differentiated motor neurons (**Figures 2–4**) but, each genotype had different components and characteristics. For instance, the SOD1 and C9orf72 motor neurons, but not TDP-43 motor neurons, had increased insoluble SOD1 compared to control motor neurons, (**Figure 2C**). SOD1 protein aggregation was not observed in any of the iPSC lines in the undifferentiated state suggesting that protein burden increases with terminal differentiation. Although previous studies have shown that SOD1 motor neurons have an abundance of insoluble SOD1 protein (Chen et al., 2014; Bhinge et al., 2017), TDP-43 and C9orf72 iPSC-derived motor neurons had not previously been evaluated for SOD1 expression levels. On average the C9orf72 lines exhibited a trend toward increased insoluble levels of SOD1 (**Figure 2C**), yet this phenotype varied across the multiple lines. Since each line has approximately the same number of expanded repeats (Sareen et al., 2013), it is possible that there are other disease modifiers contributing to this phenotype that varies in the population. Additionally, it is important to note that only one SOD1 line and one TDP-43 line was used for these studies. As such, utilizing multiple iPSC-lines to take into account population variance will be necessary for future studies.

We also detected TDP-43 pathology in TDP-43 motor neurons. As evidenced by the increase in insoluble protein levels (**Figure 3C**), TDP-43 has seemingly aggregated in the TDP-43 motor neurons, which is consistent with a previous report (Egawa et al., 2012). Interestingly, while post-mortem tissue from C9orf72 patients show TDP-43+ aggregates (DeJesus-Hernandez et al., 2011), we do not observe an increase in insoluble TDP-43 levels in C9orf72 iPSC-derived motor neuron cultures (**Figure 3C**). This is perhaps because TDP-43 pathology in C9orf72 ALS is likely a secondary phenotype initiated by the dipeptide repeats that

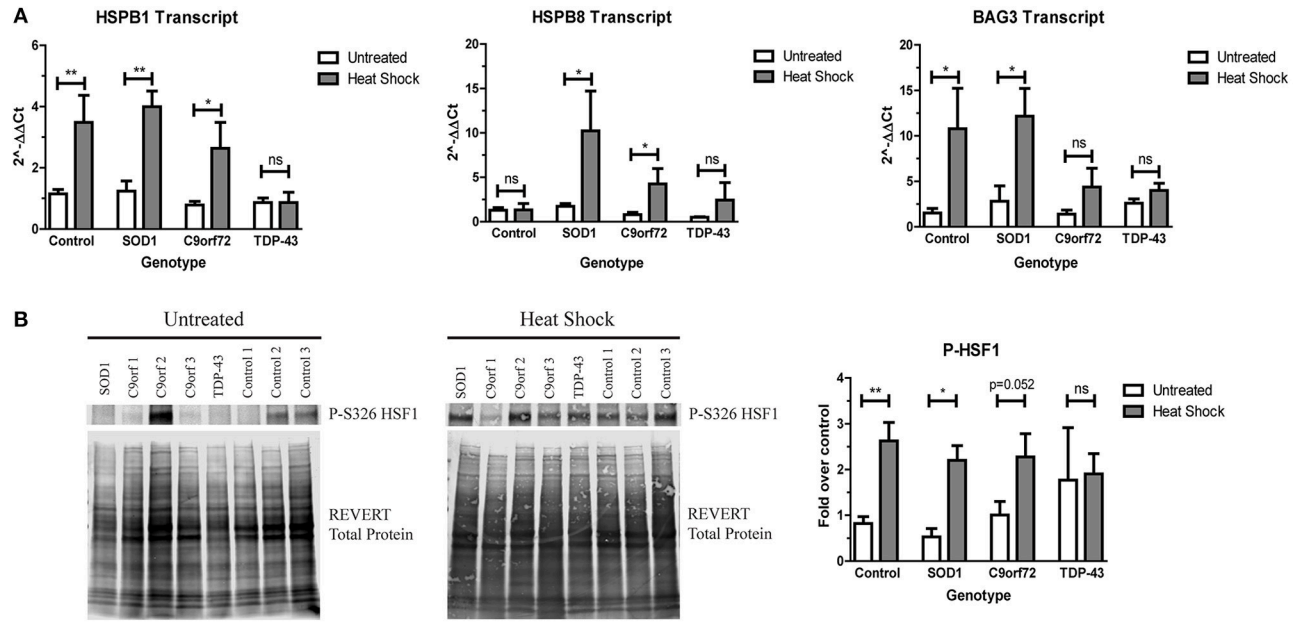

FIGURE 6 | SOD1 and C9orf72 motor neurons induce the HSR after acute heat stress. **(A)** SOD1 and C9orf72 motor neurons have increased transcript levels of HspB1, HspB8, and BAG3 compared to their untreated samples after 1 h of heat shock. Data are normalized to untreated controls (**$p < 0.01$, *$p < 0.05$ by Student's *t*-test). $n = 3$–4. **(B)** Western blot for phosphorylated HSF1 at S326 shows a significant induction in the HSR in control and SOD1 motor neuron cultures compared to their untreated samples after 1 h of heat shock. Untreated western blot from **Figure 5D** is shown again for ease of comparison. Data are normalized to REVERT total protein stain and expressed as a fold change over untreated control samples. (**$p < 0.01$, *$p < 0.05$ by Student's *t*-test). $n = 3$–4.

are characteristic of C9orf72-linked pathology (Khosravi et al., 2017). Although the iPSC-derived motor neurons were carried out to four weeks in culture, TDP-43 protein aggregation and pathology might become pronounced with extended times in culture.

OPTN can often be found in protein aggregates in post-mortem ALS samples and is one of the many rare mutations linked to fALS (Maruyama et al., 2010). Importantly, OPTN is an autophagic adaptor protein, and therefore a part of the proteostasis network. Thus, any alterations in OPTN levels, solubility, or localization could have deleterious effects on the neuron's ability to clear protein aggregates. However, OPTN pathology has not previously been evaluated in ALS iPSC-derived motor neuron cultures. Although there was no evidence of OPTN aggregation in TDP-43 motor neurons, increased insoluble OPTN protein levels were observed in SOD1 and C9orf72 motor neurons compared to controls (**Figure 4C**). Importantly, with the exception of one undifferentiated C9orf72 iPSC-line that showed insoluble OPTN, insoluble protein was most pronounced in differentiated motor neurons. Together, these data are supported by a recent report showing that SOD1, TDP-43, and OPTN are supersaturated in motor neurons, and therefore are especially susceptible to aggregation (Ciryam et al., 2017). As such, although genetic background may alter the specific content of aggregation-prone proteins, motor neurons may not be able to efficiently handle the protein load resulting in motor neuron malfunction and loss.

The proteostasis network, which includes the HSR, ubiquitin proteasome system, and autophagy, functions to prevent protein aggregation. Beyond motor neuron loss, protein aggregation is one of the only pathological similarities that has been identified across ALS patients. Therefore, it is possible that upregulating one or more components of the proteostasis network may be a potential therapy for many cases of ALS. As such, previous studies have determined that inducing the HSR by either overexpressing HSR components or utilizing pharmacological agonists reduces the number and toxicity of protein aggregates in both SOD1 and TDP-43 ALS cell culture and mouse models (Kieran et al., 2004; Kalmar et al., 2008; Crippa et al., 2010, 2016; Lin et al., 2013, 2016; Chen et al., 2016; Ganassi et al., 2016). However, previous work has also shown that aberrant activation of the HSR can diminish the overall efficacy of protein refolding and breakdown (Roth et al., 2014); interestingly human brain samples from patients with Parkinson's disease, Huntington's disease, and Alzheimer's disease all show aberrant HSR activation (Brehme et al., 2014) indicating that this could be an important feature of neurodegeneration. We found that SOD1, TDP-43, and C9orf72 motor neurons have minimal and/or incomplete HSR activation in response to physiological protein burden (**Figure 5**). HspB1 and HspB8 are both a part of the small chaperone protein family that recognizes misfolded proteins. Additionally, mutations in both genes have been linked to distal hereditary motor neuropathy (Echaniz-Laguna et al., 2017), further indicating their role in motor diseases. BAG3,

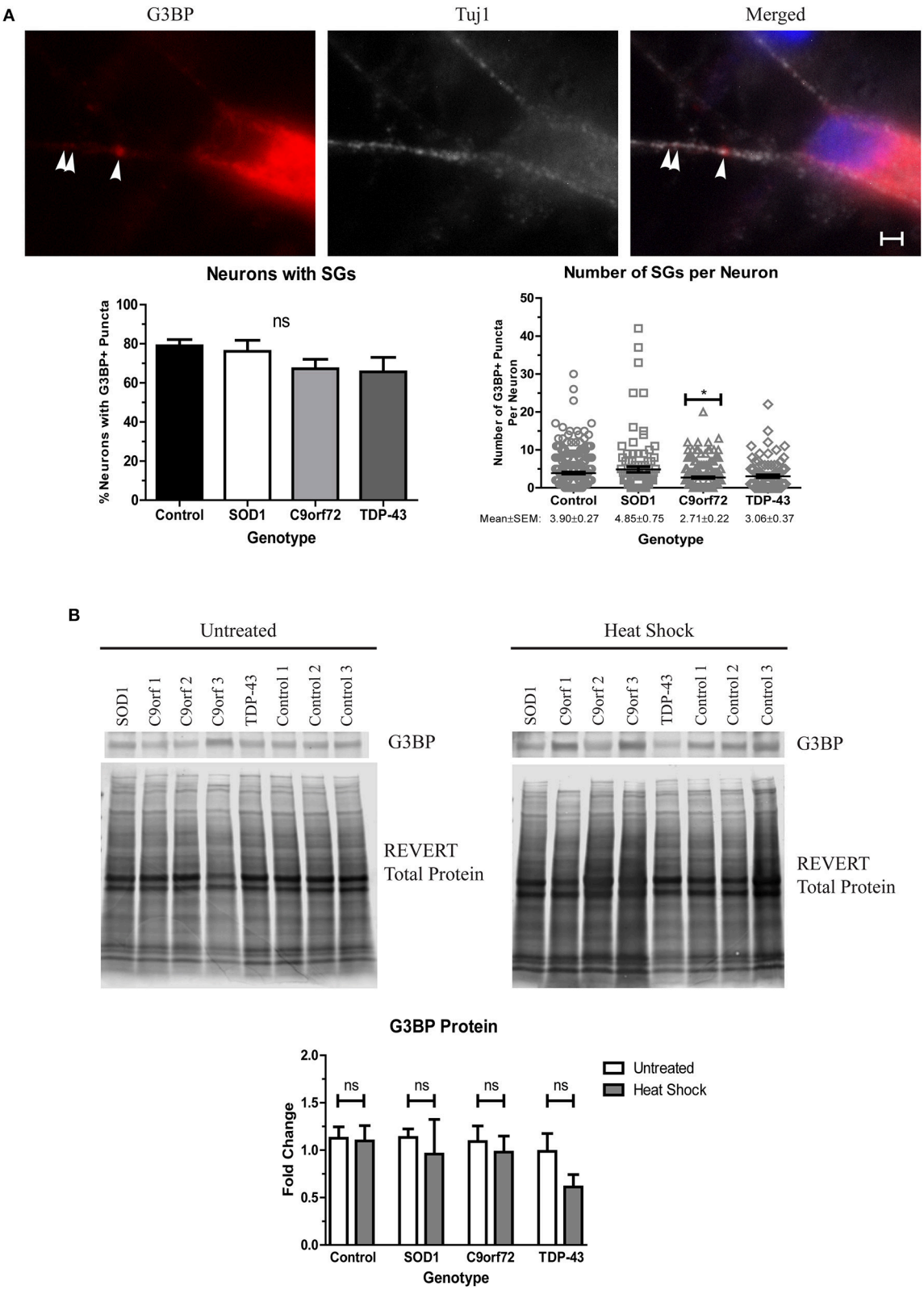

FIGURE 7 | ALS motor neurons do not show enhanced stress granule formation. **(A)** Immunocytochemistry of Tuj1 (white) and G3BP (red) shows no difference in the number of neurons with at least one G3BP+ puncta, but C9orf72 motor neurons exhibit fewer G3BP+ puncta per neuron (*$p < 0.05$ by one-way ANOVA with Tukey *post-hoc* test). $n = 3$, scale bar = 2 μm. **(B)** Western blot for G3BP shows no difference in protein levels in untreated or heat shocked motor neurons (ns, $p > 0.05$ by Student's *t*-test). Data are normalized to REVERT total protein stain and expressed as a fold change over untreated control samples.

a co-chaperone protein, can complex with both HspB1 and HspB8 to aid in protein clearance (Ganassi et al., 2016; Rauch et al., 2017). We found that although TDP-43 motor neurons have increased expression of BAG3 compared to controls (**Figures 5A,C**), there was not a concurrent increase in HspB1 or HspB8 (**Figures 5A,B**). Similarly, we found that SOD1 motor neurons showed an induction in HspB8 expression compared to control (**Figures 5A,B**), but only a trend toward higher BAG3 and no increase in HspB1 (**Figures 5A,B**). Interestingly, the C9orf72 iPSC-derived motor neurons did not show any significant increase in BAG3, HspB8, or HspB1 compared to control (**Figure 5**). These data, along with no significant change in phosphorylated HSF1 levels in any ALS line compared to controls (**Figure 5D**), suggest that increased insoluble protein burden is not sufficient to fully activate the HSR in ALS motor neurons.

Perhaps most interestingly, ALS motor neurons exhibited divergent induction of the HSR in response to heat shock. When cultured at 42°C for 1 h, SOD1 motor neuron cultures were able to robustly induce the HSR compared to the non-stressed condition (**Figure 6**). Given the apparent benefit of this pathway in clearing SOD1 aggregates (Kieran et al., 2004; Kalmar et al., 2008; Crippa et al., 2010; Lin et al., 2013), these data support the use of HSR agonists in the treatment of SOD1-linked ALS, such as the HSR inducing agent arimoclomol (Kieran et al., 2004; Kalmar et al., 2008, 2014). Although to a lesser degree than control and SOD1 motor neurons, C9orf72 iPSC-derived motor neurons were able to induce this pathway after heat shock (**Figure 6**). These results also suggest that HSR agonists may be beneficial for C9orf72-associated ALS, although a particularly effective agonist may be required given the more modest induction. TDP-43 motor neurons, however, did not induce the HSR after heat shock as evidenced by the lack of significant increase in HspB1, HspB8, or BAG3 transcript levels nor increased HSF1 phosphorylation (**Figure 6**). It is possible that TDP-43 motor neurons have an intrinsic deficit in their ability to activate the HSR and HSR agonists may not be an effective therapy for TDP-associated ALS, but more research is needed since only one TDP-43 iPSC line was used. Curiously, we did not observe an induction of HspB8 transcript levels in control motor neurons after heat shock (**Figure 6A**). Given that these qPCR experiments were performed on the same samples as those used for HspB1 and BAG3, it is unlikely that there was a problem with the heat shock itself. However, as HspB8 is temperature sensitive, it is unclear why it was not induced in any of the three control lines.

SGs have primarily been linked to FUS-linked ALS as FUS is an RNA-binding protein that can be found in normal granules. However, various SG components have previously been found in post-mortem protein aggregates that are immunopositive for TDP-43 (Liu-Yesucevitz et al., 2010), and HspB1 and HspB8 are recruited to aberrant SGs (Ganassi et al., 2016; Mateju et al., 2017). While dynamic SGs are a part of the normal stress response, prolonged exposure to misfolded proteins can cause SGs to lose this dynamism which in turn leads to more insoluble aggregates (Mateju et al., 2017). Interestingly, we saw no change in the number of neurons with at least one SG in ALS motor neuron cultures (**Figure 7A**). This is in contrast to previous reports that have shown increased numbers of SGs in *in vitro* models of SOD1-, TDP-43-, or C9orf72-linked ALS (Liu-Yesucevitz et al., 2010; Dafinca et al., 2016; Mateju et al., 2017). However, the majority of these previous studies demonstrating an increase in SG formation were observed under exogenous stress conditions, including heat shock. Yet, we did not see a significant increase in protein levels of G3BP after heat shock (**Figure 7B**). It is therefore possible that acute heat shock is not a sufficient stress to induce SG formation in ALS iPSC-derived motor neurons, and that aging or DNA damage may be necessary. This is corroborated by studies that have shown an induction in the number of SGs in FUS iPSC motor neuron cultures exhibiting significant DNA damage (Higelin et al., 2016).

iPSCs represent a valuable human motor neuron model system in order to further study the role of the HSR in ALS pathogenesis, as different genetic backgrounds and variable protein burden may influence the overall cellular response. These data show that despite maintaining viability, physiological levels of mutant proteins in SOD1, TDP-43, and C9orf72 iPSC-derived motor neurons induced insoluble protein accumulation, but that the protein burden varied across different genotypes. Moreover, insoluble protein may accumulate in ALS motor neurons over time due to a minimal endogenous activation of the HSR rather than aberrant overexpression. As such, these data lend further support to the use of HSR agonists as a potential therapeutic strategy for ALS. However, as there were also differences in expression of HSR components across the different ALS iPSC lines, more research is needed to parse out how the individual mutated proteins could impact the overall function of the protein quality control system in ALS.

AUTHOR CONTRIBUTIONS

ES and AE designed the experiments. ES performed experiments, analyzed data, and wrote the manuscript. SS assisted with data analysis and iPSC maintenance. AE assisted with data analysis and wrote the manuscript. All authors reviewed and approved of the final document.

FUNDING

Funding was provided by Advancing a Healthier Wisconsin (AE). ES is supported in part by the Quadracci Memorial Fellowship.

ACKNOWLEDGMENTS

We thank Dr. Matt Scaglione for advising on the filter-trap assay and kindly providing the cellulose acetate membrane and dot blot apparatus. We also thank Dr. Clive Svendsen and Dr. In-Hun Park for providing the C9orf72 and TDP-43 iPSC lines, respectively.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 09 March 2018
doi: 10.3389/fnins.2018.00144

"Till Death Do Us Part": A Potential Irreversible Link Between Aberrant Cell Cycle Control and Neurodegeneration in the Adult Olfactory Bulb

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OPEN ACCESS

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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *21 December 2017*
Accepted: *22 February 2018*
Published: *09 March 2018*

Citation:
Omais S, Jaafar C and Ghanem N (2018) "Till Death Do Us Part": A Potential Irreversible Link Between Aberrant Cell Cycle Control and Neurodegeneration in the Adult Olfactory Bulb. Front. Neurosci. 12:144. doi: 10.3389/fnins.2018.00144

Adult neurogenesis (AN) is an ongoing developmental process that generates newborn neurons in the olfactory bulb (OB) and the hippocampus (Hi) throughout life and significantly contributes to brain plasticity. Adult neural stem and progenitor cells (aNSPCs) are relatively limited in number and fate and are spatially restricted to the subventricular zone (SVZ) and the subgranular zone (SGZ). During AN, the distinct roles played by cell cycle proteins extend beyond cell cycle control and constitute key regulatory mechanisms involved in neuronal maturation and survival. Importantly, aberrant cell cycle re-entry (CCE) in post-mitotic neurons has been strongly linked to the abnormal pathophysiology in rodent models of neurodegenerative diseases with potential implications on the etiology and progression of such diseases in humans. Here, we present an overview of AN in the SVZ-OB and olfactory epithelium (OE) in mice and humans followed by a comprehensive update of the distinct roles played by cell cycle proteins including major tumors suppressor genes in various steps during neurogenesis. We also discuss accumulating evidence underlining a strong link between abnormal cell cycle control, olfactory dysfunction and neurodegeneration in the adult and aging brain. We emphasize that: (1) CCE in post-mitotic neurons due to loss of cell cycle suppression and/or age-related insults as well as DNA damage can anticipate the development of neurodegenerative lesions and protein aggregates, (2) the age-related decline in SVZ and OE neurogenesis is associated with compensatory pro-survival mechanisms in the aging OB which are interestingly similar to those detected in Alzheimer's disease and Parkinson's disease in humans, and (3) the OB represents a well suitable model to study the early manifestation of age-related defects that may eventually progress into the formation of neurodegenerative lesions and, possibly, spread to the rest of the brain. Such findings may provide a novel approach to the modeling of neurodegenerative diseases in humans from early detection to progression and treatment as well.

Keywords: adult neurogenesis, olfactory bulb, cell cycle control, cell cycle proteins, tumor suppressors, aging, neurodegeneration

INTRODUCTION TO ADULT NEUROGENESIS IN MAMMALS

Overview of Adult Neurogenesis as a Lifetime Developmental Process

Neurogenesis or "the birth of neurons" is the developmental process by which mature and functional neurons are generated in the brain from a pool of neural stem and progenitor cells (NSPCs). Although neurogenesis is primarily an embryonic process, the recent discovery that it persists throughout life in vertebrates including mammals (with distinct spatiotemporal variations and cell fate restrictions across species) forced a re-assessment of the adult brain's plasticity and regenerative capacity (Bonfanti and Peretto, 2011). It is now well established that adult neurogenesis (AN) is restricted to two neurogenic sites in the adult mammalian brain: the sub-granular zone (SGZ) of the hippocampus (Hi) and the sub-ventricular zone (SVZ) lining the lateral ventricles (LV) (Ming and Song, 2011). Rather than producing massive numbers of the different neuronal subtypes as is the case during development, AN provides a continuous but limited supply of specific subtypes of young neurons that allow for qualitative contribution to existing networks in the form of structural plasticity. In addition, unlike during embryonic neurogenesis, AN is precisely modulated by the level and forms of interaction with the environment and hence is largely activity-dependent. In humans, neurogenesis is ongoing throughout life in the adult hippocampus where it is associated with the formation of new memories and learning but ceases early in the olfactory bulb (OB) during the second year of infancy.

Massive investigation is under way trying to determine whether neurogenesis may potentially carry a regenerative or possibly a restorative role inside the adult brain besides its modulatory contribution to normal brain function. So far, studies have demonstrated that the radial glia-like NSCs population found in the SVZ is regionally specified and heterogeneous and is therefore capable of generating oligodendrocytes and astrocytes (albeit at low numbers) in addition to neurons *in vivo* (Ahn and Joyner, 2005; Menn et al., 2006; Codega et al., 2014; Mich et al., 2014) and *in vitro* (Ortega et al., 2013). Moreover, consistent with the embryonic origin of adult NSPCs (Fuentealba et al., 2015; Furutachi et al., 2015), many of the cellular and molecular mechanisms controlling adult neurogenesis are notably similar to those acting during development but often display contextual differences (for review; Lim and Alvarez-Buylla, 2016). Interestingly, studies have also shown that neurogenesis is stimulated or can be affected by brain injury and various brain pathologies e.g., psychiatric disorders as well as neurodegenerative diseases such as Alzheimer's disease and Parkinson's Disease (Winner and Winkler, 2015). Nonetheless, the nature of such interactions e.g., whether direct or indirect and/or based on cause-and-effect relationship or not, is still under investigation.

Adult Neurogenesis in the SVZ-OB in Mice: Cell Types, Key Regulators and Function

Adult neural stem cells (aNSCs) found in the SVZ are type B1 radial glia-like quiescent cells that express a number of glial markers including Glial Fibrillary Acidic Protein (GFAP), Glutamate-Aspartate Transporter (GLAST), and Brain Lipid-Binding Peptide (BLBP). They also display regional specification whereby distinct NSCs located in different compartments along the walls of the LV generate distinct subtypes of OB interneurons (Merkle et al., 2007, 2014). Once activated, type B1 cells express Nestin and give rise to transient-amplifying cells or type C, which generate neuroblasts or Type A that migrate to the OB where they differentiate into distinct subtypes of interneurons occupying the granule cell layer (GL; ∼95% of the total newborn neurons) and the periglomerular layer (PGL; ∼5%) (Codega et al., 2014; Bonaguidi et al., 2016). Many signaling molecules including Shh, BMP, Wnt, Notch, and, transcription factors such as *Sox2, Ascl1, Dlx2, Pax6, Tbr2, Prox1* as well as mitogens and growth factors e.g., FGF2, EGF are common regulators of both embryonic and adult neurogenesis and act in a developmentally similar context. Yet, significant differences exist about how these factors control NSPCs properties such as cell fate determination and maintenance at the molecular level (Urban and Guillemot, 2014; Gotz et al., 2016; Lim and Alvarez-Buylla, 2016). Notably, aNSCs have much longer cell-cycle length compared to their embryonic counterparts, possibly to avoid accumulation of genetic mutations and DNA damage, premature shortening of telomeres and/or pool exhaustion (Gotz et al., 2016).

From a functional perspective, addition of newborn neurons during AN is considered a dynamic form of neuronal plasticity allowing the brain to refine its structural organization and circuitry functions in response to constantly changing interactions with the environment. At the network level, this activity-driven plasticity translates into dynamic cellular changes that are primarily occurring at the synaptic contacts such as addition of new synapses, removal of existing ones and/or relocation of others which interestingly seems to allow a faster adaptation to environmental stimuli as it has been recently suggested (Hardy and Saghatelyan, 2017). As a result, a tight regulation of AN is required to maintain the proper balance between circuit stability and plasticity (associated with the addition of new neurons) in order to preserve normal brain function. In fact, while the absolute number of newborn neurons depends on the rate of proliferation in the SVZ, about 40–50% of these neurons are turned over in the rodent OB primarily by apoptosis while the rest survive for less than 18 months (Winner et al., 2002; Whitman and Greer, 2009). Importantly, this turnover mechanism by apoptotic death peaks during a "critical period" between 14 d and 28 d after cell birth (Yamaguchi and Mori, 2005). It is also a highly dynamic process that is largely influenced by environmental stimuli and sensory input such as experience-induced alterations or challenging associative sensory tasks. For instance, studies have shown that sensory deprivation by nostril occlusion following birth reduces the number of granule cells by increased apoptosis whereas exercise or olfactory enrichment triggers the opposite effect (Corotto et al., 1994; Petreanu and Alvarez-Buylla, 2002; Rochefort et al., 2002).

Two central questions are still the subject of extensive research concerning the contribution of AN to the OB circuitry: (1) is adult neurogenesis necessary for normal olfactory function or specific olfactory tasks only? (2) What are the cellular/molecular properties that distinguish newborn adult neurons from perinatal

mature neurons and thus render their influence on circuit function unique? Using variable genetic and chemical approaches to disrupt AN, several studies have attempted to answer these questions and showed that AN is likely dispensable for normal odor and threshold detection but can still accelerate discrimination learning e.g., during operant associative olfactory tasks and enhance long-term memory (reviewed in Lazarini and Lledo, 2011; Alonso et al., 2012). Moreover, adult-born neurons seem to differ from mature GCs born around birth by their degree of excitability and plasticity as well as their targets. This is directly linked to their synaptic properties including synaptic maturation and output, such as a resistance to synaptic depression mediated by the metabotropic GABAb receptor as well as synaptic refinement through re-location (Breton-Provencher and Saghatelyan, 2012; Valley et al., 2013; Hardy and Saghatelyan, 2017).

Adult Neurogenesis in the SVZ-OB in Humans Compared to Rodents

Adult neurogenesis in the OB is not restricted to rodents and has been described in other mammalian species including adult monkeys (Kornack and Rakic, 2001). But when compared to other mammals including primates, the adult human SVZ is occupied by a "ribbon" of GFAP-expressing astrocytes lining the ependymal layer, thus lacking the classical cellular organization observed in rodents and described above (types B, C, and A cells) (Sanai et al., 2004). Early studies have reported the presence of ongoing neuroblast migration between the LV and OB and the generation of newborn neurons in the adult human OB (Bedard and Parent, 2004). However, later reports argued for the lack of substantial neurogenic activity in the SVZ and the absence of high number of dividing neuroblasts along the RMS in the adult human brain. Instead, they revealed high proliferation only in the human fetal brain with cells co-expressing the migrating neuroblast markers Double-Cortin (DCX) and Polysialylated Neural Cell Adhesion Molecule PSA-NCAM (Sanai et al., 2011; Wang et al., 2011). In addition, Sanai et al. identified a second migratory stream of DCX+ cells heading to the ventromedial prefrontal cortex in the fetal brain and noted that the rate of proliferative and migratory activities in the SVZ and RMS respectively is greatly reduced after 8 months of age (Sanai et al., 2011). More recently, robust SVZ migration was further extended to the whole infant frontal lobe (up till 5 months of age), specifically the anterior cingulate cortex (Paredes et al., 2016a). Also, in favor of the lack of bulbar neurogenesis in humans is a ^{14}C birthdating analysis of OB neuronal DNA showing around 0.008% neuronal turnover in the human OB annually (less than 1% of neurons being replaced per 100 years) (Bergmann et al., 2012) compared to about 50% in rodents (Imayoshi et al., 2008). The discrepancy observed among the above studies could partially be due to the existence of resident NSCs in the adult human OB (Pagano et al., 2000). In terms of relative volume, the OB makes up around 0.01 and 2% of the human and mouse brain, respectively (McGann, 2017), which can also be accounted for by the negligible addition of newborn OB neurons in humans (Ernst and Frisen, 2015). Some researchers have argued that the longer migratory path i.e., 50–60 mm-long adult human RMS compared to 30 mm in the fetal brain on average and, the structural complexity of the adult brain reflect unique challenges facing immature neurons in order to successfully reach the adult OB (Paredes et al., 2016b). Others, however, are more skeptical about the proposed correlation between a higher number of OB neurons and more functional significance (Lledo and Valley, 2016), especially since the human OB has a higher glomeruli-to-olfactory receptor ratio (∼16) compared to mice (∼2) (Maresh et al., 2008) and is uniquely distinct of other brain regions with respect to its relative size (Finlay and Darlington, 1995). Indeed, the number of human OB neurons falls within the same order of magnitude of all mammals (around 10 million cells) and this would even explain why, contrary to a widespread misconception, human olfaction is as successful and efficient as that of other mammals (McGann, 2017).

As described above, the appearance of a hypocellular gap occupied by astrocytes but is devoid of neuronal cell bodies in the subventricular ependyma in adult humans (Quinones-Hinojosa et al., 2006) suggests a connection between loss of proliferation in this specific layer after the infancy period (around 18 months) and lack of adult bulbar neurogenesis (Paredes et al., 2016b). Interestingly, Ernst et al. established the striatum as a third site of adult neurogenesis that is unique to humans, by the detection of IdU-retaining interneurons in postmortem tissue from cancer patients treated with this analog. Using carbon dating, the same group also showed a 2.7% turnover rate in the previous subpopulation per year (Ernst et al., 2014) and later speculated that the newborn striatal neurons are SVZ-derived (Ernst and Frisen, 2015). The origin of these striatal interneurons remains uncertain though since other studies have reported different origin(s) of these cells such as the medial ganglionic eminence (Wang et al., 2014; Lepousez et al., 2015) or possibly local parenchymal astrocytes as reported after stroke in mice (Magnusson et al., 2014). Nonetheless, the implications of striatal neurogenesis having a SVZ-derived origin in psychiatric disorders have been discussed (Inta et al., 2016). In addition, a difference in SVZ-neuronal output, for example migration to the OB vs. the striatum, could account for shared functions associated with circuit plasticity in both regions such as cognitive flexibility involving the human striatum and associative operant learning in the OB (Sakamoto et al., 2014). In summary, human-specific variations associated with the SVZ neurogenic niche, especially during early postnatal neurogenesis, might explain the lack of bulbar neurogenesis with respect to other mammals.

Adult Neurogenesis in the Olfactory Epithelium of Rodents and Humans

Unlike the adult human OB, the olfactory epithelium (OE) in both rodents and humans is an active site of constitutive neurogenesis throughout life where new neurons are continuously generated in order to replace worn out ones under normal conditions or damaged cells after epithelial injury (Graziadei and Graziadei, 1979; Hinds et al., 1984; Hahn et al., 2005). The OE is a pseudo-stratified epithelium lining part of the nasal cavity along its apical side as well as the basal membrane

at its basal side, whereby its structure and function are vastly conserved among mammals (Lane et al., 2002; Nibu, 2002). The OE contains a single type of bipolar neurons, the olfactory sensory neurons (OSNs), which extend their dendrites expressing specific odorant receptors (OR) into the nasal cavity for odor detection. OSNs relay sensory signals to the OB by projecting their axons along the olfactory nerve layer (ONL) that synapse with dendrites of second-order neurons, the mitral and tufted cells (M/T cells), inside glomeruli in the periglomerular layer (PGL) (Buck, 2004). Two cell types, the globose basal cells (GBC) and the horizontal basal cells (HBC), reside in the basal layer of the OE and are responsible for retaining lifetime neurogenesis and regeneration in rodents and also in humans, albeit at much lower rate (Schwob et al., 2017). In mice, these two populations are clearly distinguished based on their distinct morphology and molecular content (Calof et al., 1998; Mackay-Sim, 2010). However, these differences are less clearly defined and require further investigation in humans (Chen et al., 2014). The GBCs are a multipotent and actively proliferating population of stem/progenitor cells capable of generating all cell types in the developing OE and carry the day-to-day replenishment of neurons in normal ongoing turnover and post-injury in the adult tissue. They are a heterogeneous population at the molecular level, marked by the expression of different types of transcription factors at distinct stages of development including common key regulators found in other neurogenic sites e.g., *Sox2, Pax6, Ascl1, NeuroD1* (reviewed in Schwob et al., 2017). In comparison, HBCs arise later in development and are suggested to exhibit multipotent capacity. However, they are primarily quiescent under homeostatic conditions and appear to contribute to epithelial reconstitution in response to severe lesions only e.g., death of sustentacular cells. Despite the ongoing constitutive OE neurogenesis in humans, the regenerative capacity of the OE declines with age due to a decrease in number and function of stem/progenitor cells (pool exhaustion) as described below (Doty and Kamath, 2014).

ROLE OF CELL CYCLE PROTEINS DURING ADULT NEUROGENESIS IN THE SVZ-OB AND OE

Under normal physiological conditions, AN is controlled by an array of cell-intrinsic and extrinsic factors that exhibit complex interactions inside the neurogenic niches and regulate stem/progenitor self-renewal, proliferation and differentiation. In the SVZ, such regulatory factors include mitogens, growth factors, transcription factors, chromatin modifiers and non-coding RNAs (for review; Lim and Alvarez-Buylla, 2016). In this context, the classical cell cycle machinery comprised of tumor suppressor genes, cyclins, cyclin–dependent kinases (Cdk) and cyclin-dependent kinase inhibitors (Cdki), is the master regulator of the proliferative properties of embryonic NSPCs including quiescence (G0), self-renewal/maintenance, cell cycle length and checkpoints (G1-S, G2-M) as well as spatiotemporal regulation of cell division. Besides cell cycle control, many cell cycle proteins were notably shown to carry out "*second careers*" implicating them in the regulation of progenitor specification, neuronal commitment, neuronal migration or terminal differentiation during development (Herrup, 2013). In addition, studies have uncovered conserved but also divergent functions attributed to cell cycle proteins during AN. Interestingly, only some of these proteins turn out to be involved in cell proliferation control in the adult neurogenic sites by displaying cell-type specific functions e.g., regulation of stem cell vs. progenitor cell proliferation or tissue-specific functions inside the SVZ compared with the SGZ. In some cases, they are differentially required at distinct developmental stages e.g., young adult vs. adult vs. aged adult (for in depth reviews on cell proliferation control in the embryonic and adult brain, see Beukelaers et al., 2012; Bartesaghi and Salomoni, 2013; Cheffer et al., 2013).

As during development, cell cycle proteins fulfill distinct non-cycling functions in the adult brain ranging from stem cell quiescence and senescence to progenitor commitment in the SVZ as well as neuroblast migration in the RMS and long-term survival of post-mitotic adult born neurons in the OB. Here, we expand and update these findings in the light of the latest data by re-constructing the distinct roles played by the cell cycle machinery according to the developmental context of each cell type found in the SVZ-OB (**Figure 1**). It is imperative to mention that most studies on cell cycle regulators relied on the use of germline knock-out (KO) or conditional KO mouse models which may not necessarily discriminate between primary vs. secondary effects as a result of gene deletion on adult neurogenic processes. For example, phenotypic consequences could result from developmental and/or early-postnatal alterations in the VZ-SVZ rather than adult-specific functions. Therefore, the use of inducible conditional KO or transgenic reporter lines or lineage tracing techniques e.g., labeling of adult NSPCs through stereotaxic injections of recombinant viruses into the LV will guarantee a more accurate characterization of adult-specific cycling versus non-cycling functions attributed to cell cycle proteins (Dhaliwal and Lagace, 2011; Enikolopov et al., 2015).

Control of Self-Renewal and Quiescence in Type B aNSC

Type B1 SVZ-aNSCs are radial glia-like cells that are regionally specified and capable of unlimited self-renewal throughout life. They are known to alternate between a quiescent state and an actively dividing state where they can self-renew through two modes of divisions, symmetric and asymmetric (Bonaguidi et al., 2016). Upon activation, they primarily gave rise to distinct neuronal lineages (Nestin-positive) destined to replenish the stock of adult inhibitory neurons in the OB. Using distinct lineage tracing methods, two recent studies have demonstrated that the majority of B1 cells become regionally specified early during development and as a result originate from a sub-population of embryonic NSCs between E13.5 and E15.5. They remain largely quiescent until post-natal re-activation (Fuentealba et al., 2015; Furutachi et al., 2015). Notably, the Cdki, p57, was identified as a key factor required for maintaining quiescence in this population and later on, the generation of most aNSCs. These two functions rely on its Cdk inhibitory domain, hence the

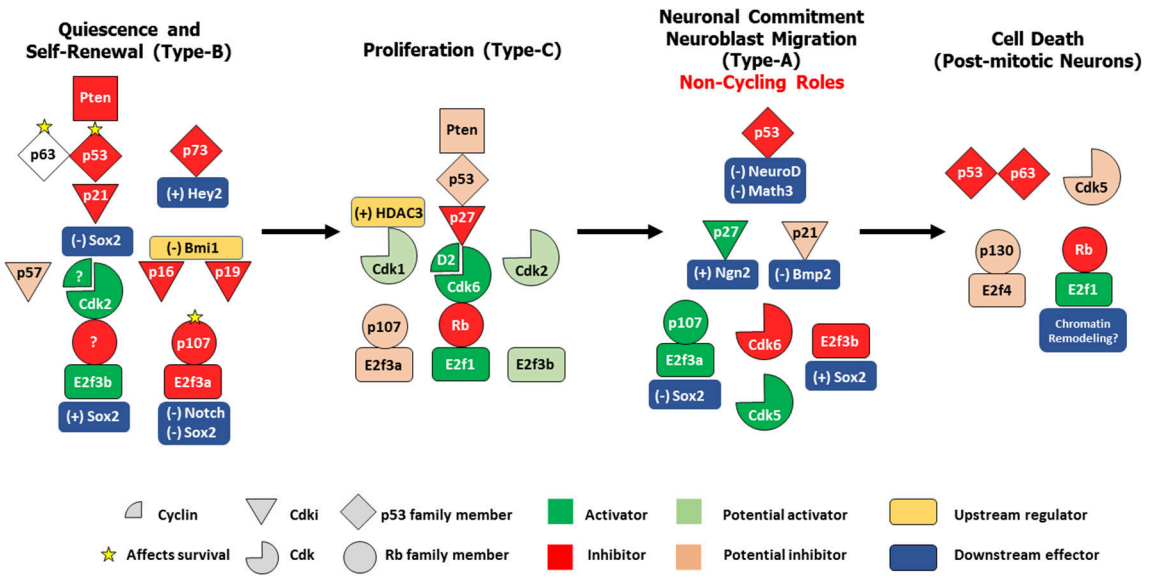

FIGURE 1 | Differential control of distinct stages in adult SVZ-OB neurogenesis by different cell cycle regulatory proteins. In response to the proper cues, quiescent type-B stem cells get activated to produce rapidly dividing type-C progenitors, which in turn preferentially commit to a neuronal lineage as neuroblasts that migrates along the RMS and differentiate into post-mitotic interneurons in the OB. These four populations are under the tight control of the cell cycle machinery. In both NSCs/type-B cells and NPCs/type-C cells, a classical/general signaling pathway can be constructed based on existing literature: Pten -> p53 -> Cdki -> Cdk/Cyclin -> Rb family member/E2f. However, distinct members of these protein families seem to play non-redundant roles during these two stages e.g., p21, Cdk2, and p107 act specifically in type-B maintenance while p27, Cdk6, and Rb preferentially regulate type-C proliferation. Interestingly, within the same population, family members can have non-overlapping functions e.g., p63 and p73 in controlling survival of type B cells, or even more, might have opposing roles e.g., E2f3a and E2f3b in regulating Sox2-dependent expansion of the NSCs pool. In neuroblasts, several cell-cycle proteins switch to non-cycling tasks as they act to maintain neuronal commitment e.g., p21 and Cdk6 and neuroblast migration e.g., Cdk5. Finally, following neuronal maturation, specific regulators e.g., Rb, p53, Cdk5 act to preserve cell cycle suppression by inhibiting cell-cycle re-entry (CCE), a process prone to deregulation in the aging OB, thus potentially leading to neurodegeneration. (+); positive control, (–); negative control.

regulation of the activity of the cyclin-Cdk complexes (Furutachi et al., 2015). Although a role of p57 in aNSC maintenance was not described, it is not surprising that several other Cdki and tumor suppressor genes were shown to control self-renewal and/or quiescence of aNSCs, two defining features linked to longevity and multipotentiality of these cells. Genetic studies primarily relied on the phenotypic analyses of null mutants or inducible/conditional KO animals (targeted gene deletion) *in vivo* and cell culture *in vitro* e.g., neurosphere assays.

On top of this regulatory hierarchy is Pten, an inhibitor of Akt phosphorylation by PI3K and upstream activator of p53 (Freeman et al., 2003), which negatively regulates aNSC self-renewal and subsequent progenitor proliferation without affecting neuronal production in the OB (Gregorian et al., 2009). Conditional deletion of *Pten* in a subpopulation of aNSCs leads to enhanced OB neurogenesis and olfactory function and surprisingly no sign of NSCs exhaustion in culture. Yet, it is not clear whether this is also true *in vivo*. An equivalent negative control of NSCs self-renewal is echoed with several members of the p53 family. Loss of p53 results in significant downregulation of its direct target gene, $p21^{Cip1}$ (Meletis et al., 2006), and gives a proliferative advantage to both slow- and fast-dividing cells in the SVZ along with their rapid differentiation (**Figure 1**) (Gil-Perotin et al., 2006). These findings are consistent with a previous study showing that loss of $p21^{Cip1}$ compromises aNSCs quiescence and eventually leads to NSC pool exhaustion in 16 month-old mice (Kippin et al., 2005). This p21 function was later shown to be mediated by a direct negative regulation of the expression of the pluripotency transcription factor Sox2 (Marques-Torrejon et al., 2013). The same group also revealed that BMP2, which is under the direct negative control of p21, induces premature terminal differentiation of multipotent NSCs into mature astrocytes in p21-null mice (Porlan et al., 2013). Within the p53 family, p73 maintains aNSCs self-renewal and the neurogenic capacity inside the niche independently of p53 (Gonzalez-Cano et al., 2010) and through direct transcriptional regulation of bHLH Hey2, which in turn prevents premature neuronal differentiation (Fujitani et al., 2010). Also, p73 can compensate for p63 haplo-insufficiency (in compound double heterozygotes) and divert it from inducing p53-dependent apoptosis in NSCs to trigger cellular senescence instead (Cancino et al., 2013b; Fatt et al., 2014). Furthermore, Bmi-1 was shown to promote NSC self-renewal by repressing the senescence pathways of two Cdkis, $p16^{Ink4a}$ and $p19^{Arf}$ (Molofsky et al., 2005). Despite this, the age-dependent increase in the expression of $p16^{Ink4a}$ was linked to the decline in NSPCs number and function in the SVZ-OB but not the SGZ in old mice (Molofsky et al., 2006). Downstream of all the above regulators is Cdk2 which is required for proper self-renewal and proliferation in the SVZ in young mice but not at earlier/perinatal stages. This is probably due to

transient compensatory mechanisms involving Rb inactivation by increased expression of Cdk4 (**Figure 1**) (Jablonska et al., 2007).

The Rb protein family member p107 negatively regulates the number of aNSCs in the developing and adult brain by directly inhibiting the Notch1-Hes1 pathway (Vanderluit et al., 2004), and is required for neural progenitor commitment to a neuronal fate (Vanderluit et al., 2007). A more recent report further demonstrated that p107 specifically associate with E2f3a isoform to form a transcriptional repressor complex while E2F3b recruits RNA polymerase II to create an activator complex. These two complexes aid to fine-tune the balance between neural precursor expansion and neurogenesis via direct Sox2 transcriptional regulation in the embryonic and adult brain (**Figure 1**) (Julian et al., 2013). Since the loss of many cell cycle regulators described above is often associated with the depletion of the aNSC pool, it would be intriguing to examine whether this could be due to reduced or lost Notch signaling which was demonstrated to be absolutely required for the maintenance of NSCs in both neurogenic sites in the adult brain (Imayoshi et al., 2010). Finally, it is worth mentioning that, unlike Nestin-positive SGZ-radial-glia like precursors, Nestin-expressing (activated) SVZ-NSCs cannot return to a quiescent state once activated, but rather undergo a fast clonal expansion (Bonaguidi et al., 2011; Calzolari et al., 2015), a fact that could also place a limitation on the long-term neurogenic potential inside the aging SVZ.

Control of Proliferation in Type C Transient-Amplifying Cells

NSPCs proliferation in the adult mammalian SVZ is subject to tight regulation by intrinsic factors such as transcription factors and epigenetic mechanisms, and extrinsic ones such as morphogens and growth factors. Equally relevant but less understood is the effect of environmental cues and activity on SVZ neurogenesis e.g., physical exercise and learning as well as that of pathological disorders e.g., epilepsy, stroke, and Alzheimer's disease (Ming and Song, 2011). Cell-cycle proteins can act to either initiate or mediate such regulatory mechanisms. Moreover, as described above, some cell-cycle regulators control cell-type or tissue-specific functions while others display broader developmental effects. For instance, $p27^{Kip1}$ was shown to selectively control the number of transient-amplifying progenitors or type C cells at the expense of neuroblasts produced in the SVZ without affecting stem cell proliferation (Doetsch et al., 2002). In contrast, genetic deletion of cyclin D2 but not cyclin D1 completely eliminates proliferation of all neuronal precursors (hence generation of newly born neurons) in both neurogenic zones in the adult brain (Kowalczyk et al., 2004). Furthermore, both E2F1 and E2F3 promote precursor proliferation in the SVZ and may have redundant functions. Yet, it is not clear whether this function affects NSCs and/or NPCs proliferation (Cooper-Kuhn et al., 2002). More recently, we have shown that Rb, the master regulator of the G1-S phase checkpoint, specifically regulates progenitor proliferation (but not stem cells) in the SVZ (Naser et al., 2016) and proliferation of late-born progenitors/immature neuroblasts in the SGZ (Vandenbosch et al., 2016). Hence, loss of Rb leads to enhanced prognitor proliferation in both regions. Given that both E2F1 and E2F3 are physiologically relevant Rb-interacting partners during brain development (Callaghan et al., 1999), similar interactions are likely to control progenitor proliferation during AN, although direct evidence was not established.

In return, Rb could be under the negative regulation of Cdk6, the only Cdk shown to promote NPC proliferation *in vivo* by controlling the duration of the G1 phase in committed progenitors (Beukelaers et al., 2011). Also, Rb could possibly be a downstream target of Cyclin D2 that positively regulates proliferation in the same type of cells in the SGZ (Kowalczyk et al., 2004). In comparison, HDAC3 controls progenitor proliferation by regulating the G2/M phase progression through post-translational stabilization of the G2/M kinase, Cdk1 (**Figure 1**) (Jiang and Hsieh, 2014). It is noteworthy that the cell cycle regulators required for maintenance, self-renewal or quiescence of aNSC population (described in the previous section) may also have an independent (direct) role in controlling NPC proliferation and not just a secondary effect. As a matter of fact, the increase in precursor expansion in $E2f3a^{-/-}$ adult mice was directly linked to Sox2 overexpression to enhance self-renewal of aNSC at the expense of neuroblast production in both the SVZ and SGZ (Julian et al., 2013). Yet, it is still unclear whether this phenotype also reflects an additional increase in progenitor cell proliferation or not. One way to resolve this issue is to generate inducible conditional KO-reporter mice under the control of specific promoters and/or regulatory elements of NPCs specific markers such as *Mash1*, *Dlx2*, and *Tbr2* (Ming and Song, 2011).

Control of Lineage Commitment and Migration of Neuroblasts

Although AN in the SVZ can produce a small population of non-myelinating and myelinating oligodendrocytes (Menn et al., 2006) in addition to astroglia in the RMS and the corpus callosum (Sohn et al., 2015), most Type-C cells end up committing to a neuronal fate where they give rise to neuroblasts that migrate along the RMS en route to the OB and differentiate into subtypes of inhibitory neurons (Whitman et al., 2009). It is at these stages of neuronal commitment and migration that some cell cycle regulators exhibit their second *careers*. Beukelaers et al. showed for instance that Cdk6 is needed for the switch between cell proliferation and neuronal differentiation in adult progenitors and its deficiency prevents this transition by lengthening the G1 phase duration (Beukelaers et al., 2011). Moreover, Gil-Perotin et al showed that p53 and $p27^{Kip1}$ play antagonistic effects on neuroblast production in the SVZ even though both genes negatively regulate precursor proliferation (but not synergistically) as described above. In specific, p53 suppresses neuroblast generation by the negative control of the pro-neural genes NeuroD and Math3 whereas $p27^{Kip1}$ promotes neuronal commitment by stabilizing the neurogenic transcription factor Ngn2 (Gil-Perotin et al., 2011). Again, the level of Sox2 expression critically affects the final output of neurogenesis whereby high Sox2 levels mediated by E2F3b trigger precursor

proliferation at the expense of neuronal differentiation while low Sox2 levels as induced by E2f3a/p107 lead to the opposite effect (Julian et al., 2013). Importantly, Cdk5, a neuronal protein kinase and non-conventional cell cycle protein (does not bind to cyclins), is required for proper neuroblast migration in the adult RMS. Its loss hence impairs the speed, directionality and leading process extension of neuroblasts in a cell-autonomous fashion (**Figure 1**) (Hirota et al., 2007).

Control of Terminal Differentiation and Survival of Adult-Born Neurons in the OB

It is estimated that 20,000–30,000 neuroblasts arrive at the rodent OB every day and, 4 weeks later, 50% of these immature adult-born granule cells are eliminated by natural turnover. The remaining interneurons survive up to 12 months in mice and 19 months in rats before getting turned over by another round of newborn neuroblasts (Breton-Provencher and Saghatelyan, 2012). Of note, it was believed for a long time that neurons are permanently post-mitotic and as a default state, they cannot re-enter the cell cycle. Recent evidence has strongly challenged this dogma and studies have shown that neuronal cell-cycle re-entry (CCE) could be triggered by many assaults e.g., accumulation of DNA damage with aging and oxidative stress (see next section). Thereafter, several cell-cycle proteins are crucially required to keep neurons in an arrested state at the G0 phase (cell cycle suppression) by carrying "non-cycling" functions (Herrup and Yang, 2007). The OB is no exception as we have recently shown a critical requirement for Rb in the long-term survival of adult-born OB neurons (1–4 month old). This is despite the fact that loss of Rb does not have any obvious effect on terminal neuronal differentiation or integration into existing networks (Naser et al., 2016) as seen during embryonic brain development (Ghanem et al., 2012).

In this context, Rb might act, as in NPC proliferation, to repress the function of classical cell cycle genes such as E2f1 and/or E2F3 as well as E2F-induced apoptotic genes (Cooper-Kuhn et al., 2002). However, many observations argue against this hypothesis. Instead, the resulting cell death due to loss of Rb may not depend on classical E2f-regulated apoptotic targets such as Puma and Apaf1 but rather on other mechanisms. In fact, induced deletion of Rb in post-mitotic cortical neurons was shown to trigger markers of DNA damage and DNA repair enzymes at a time the classical p53 and E2f-mediated responses do not appear to be initially activated (Andrusiak et al., 2012). Also, the Rb LXCXE binding domain is dispensible for cortical cell death, which instead is caused by E2f-responsive chromatin modeling (Andrusiak et al., 2013). In line with this, generating inducible conditional deletions of both Rb and p53 using Nestin-Cre^{ERT2}-$Rosa26^{YFP}$/double floxed mice does not rescue cell death in Rb-null adult-born neurons (Saliba A and Ghanem N, unpublished data). Moreover, loss of p53 alone also compromises long-term survival in the same neurons eventually leading to their apoptotic death through p53-independent mechanisms (Saliba A and Ghanem N, unpublished data). Consistently, Gil-perotin reported an increase in p53-independent cell death in the SVZ in p53-null mice (Gil-Perotin et al., 2006). Given the latency of cell death induced following the loss of Rb in the adult brain, it would be interesting to examine whether p130, an Rb-related family member and major regulator of neuronal survival, can compensate, at least transiently, for the absence of Rb as in the case of cultured cortical neurons where p130-E2F4 complex recruits the chromatin modifiers HDAC1 and Suv39H1 to promote gene silencing and neuron survival (Liu et al., 2005).

Cdk5 acts a potent cell cycle suppressor in post-mitotic neurons *in vivo* and in primary neurons in culture by forming a dimer with p35 and sequestering E2F1 to disrupt the E2F1-DP1 dimer (Zhang et al., 2010; Zhang and Herrup, 2011). In addition to Rb and p53, it would be interesting to determine whether Cdk5 plays a pro-survival role in adult-born OB neurons given that it is needed for maturation and survival of adult-born granule neurons in the DG where it is specifically activated by p35 (Jessberger et al., 2008; Lagace et al., 2008). p107 does not seem to play a role in survival of post-mitotic neurons although increased apoptosis was observed in the SVZ of p107-null mice. This is likely to offset the enhanced rate of stem cell self-renewal (Vanderluit et al., 2004). Alternatively, p63 protects against cell death in the OB (**Figure 1**) (Cancino et al., 2013b). Finally, given all the above, it should be emphasized that the dual role played by key cell cycle proteins in cell cycle suppression and neuronal survival warrant further investigation especially that CCE is fatal and often converges with neurodegeneration in many diseases (see next section).

Cell Cycle Proteins During OE Neurogenesis

Despite the considerable progress made in understanding the regulatory roles played by cell cycle proteins in the control of distinct phases of the cell cycle during AN in the SVZ and SGZ, cell cycle control in the developing and adult OE is still poorly investigated. p63, a p53 gene family member, was shown to be the main regulator to maintain the quiescent state of HBCs reserve pool in the OE. Following harsh OE injury, p63 expression is downregulated in order to induce the exit of HBC from quiescence and their subsequent activation to allow OE reconstitution (Schnittke et al., 2015). In a recent study, we have shown that Rb controls late progenitors' proliferation in the developing OE and the establishment of proper synaptic connections between OE-OB. Importantly, Rb is also required for terminal maturation and survival of OSNs, which is consistent with its role in proliferation control and neuronal survival in other brain regions (Jaafar et al., 2016). Whether Rb plays a similar role in the adult OE remains to be investigated.

CELL CYCLE CONTROL IN THE ADULT SVZ-OB, OLFACTORY DYSFUNCTION AND NEURODEGENERATION

Tumor Suppressor Genes and Neuro-Oncogenesis in the SVZ-OB

Brain tumors are among the most aggressive and fatal cancers in humans and are classified into subtypes based on histologic features (Kleihues et al., 2002; Louis et al., 2007; Fuller, 2008).

Gliomas are thought to originate from glial cells (due to expression of glial markers) and are divided into low-grade gliomas (LGG; type I and II) and high-grade gliomas (HGG; type III and IV) based on invasiveness and proliferation (Ghotme et al., 2017). Embryonic tumors, on the other hand, are thought to originate from neuronal progenitor cells, due to expression of neuronal markers and are classified based on their location: e.g., medulloblastoma occur in the posterior fossa and pineoblastoma arise in the pineal region. These are collectively termed "primitive neuro-ectodermal tumors" or PNETs. Type IV gliomas, primarily glioblastoma multiform, are the most aggressive brain tumors. Studies done in mice have uncovered important developmental aspects of these tumors however without reaching a consensus about their cell-of-origin. Moreover, the role of the SVZ neurogenic niche in the formation and propagation of brain tumors have been tackled and, as expected, SVZ-NSPCs transformation was correlated with loss of function of cell cycle proteins, typically tumor suppressor genes e.g., p53, Rb, Pten, and NF1. Thus, it was proposed that SVZ-NSCs/NPCs could be the initiating cells during gliomagenesis based on several shared features with tumor cells such as multipotency, expression of stem cell markers e.g., Sox2 and Nestin, and, responsiveness to extrinsic signals e.g., Sonic Hedgehog (Kusne and Sanai, 2015). For instance, following the exposure of $p53^{-/-}$ mice to the mutagen *n*-ethyl-*n*-nitrosourea (ENU), glioblastoma-like tumors can form in periventricular locations and are characterized by enhanced NSCs self-renewal, recruitment to the fast-proliferating progenitor population (type C) and impaired differentiation (Gil-Perotin et al., 2006). Consistently, other studies showed that conditional deletions of p53 in combination with NF1 (Neurofibromin I) and/or Pten in adult astrocytes or NSPCs primarily resulted in the formation of HGGs and, less frequently, LGGs and medulloblastomas (Zhu et al., 2005; Alcantara Llaguno et al., 2009; Wang et al., 2009).

Moreover, by generating various combinations of Rb, p53 and Pten deletions, Jacques et al. reported that GFAP-expressing NSCs, but not astrocytes, gave rise to brain tumors irrespective of their location. In addition, loss of Rb was essential for developing PNET whereas Pten and p53 induce glioblastoma formation through upregulation of Cdk4 (Jacques et al., 2010). More recently, Qi et al. showed that overexpression of PIKE-A (Phosphoinositide 3-kinase enhancer) and Cdk4 in p53-Pten double KO glioblastoma mouse model synergistically shortens the latency of tumor onset and survival compared to control mice (Qi et al., 2017). In contrast with the study by Jacques et al., another study showed that combinations of p53, Rb and Pten conditional inactivation induced by GFAP-Cre^{TM} gave rise to high-grade astrocytomas (HGAs; type III) in adult mice which is consistent with concurrent mutations of these pathways in human HGAs (Chow et al., 2011). The same study argued that some of these tumors may possibly originate from mature astrocytes, while others also suggested reactive astrocytes following brain repair (de Weille, 2014), although formal evidence for both is still lacking.

Oncogenic events in the SVZ can faithfully spread to the OB, albeit reportedly infrequent. For instance, following the inducible dual KO of Rb and p53 in GFAP-Cre^{TM} mice (Chow et al., 2011), low incidence of OB tumors was described, with a "strong resemblance to human olfactory neuroblastoma" (a rare malignant neuroectodermal tumor which leads to unilateral nasal obstruction and nosebleeds, Lubojemska et al., 2016). Moreover, human glioblastoma cells injected into the striatum of immune-deficient nude mice were shown to migrate to the SVZ and subsequently to the OBs while still being potentially tumorigenic (Kroonen et al., 2011) (for a recent review on the role of cell cycle proteins in neuroblastoma cell differentiation, refer to Partridge et al., 2017). Even more rarely diagnosed are nasal gliomas in adult humans (Xie et al., 2015).

Notably, all the above studies concluded that loss of one tumor suppressor gene was not sufficient to induce tumor formation in mice, while the accumulation of cooperative mutations (or induced inactivation) in two or more tumor suppressors is required for gliomagenesis in the adult brain (still with relatively late onset). Given this and the fact that loss of many of the above genes including Pten (Gregorian et al., 2009), Rb (Naser et al., 2016), and p53 (Gil-Perotin et al., 2006; Saliba A. and Ghanem N., unpublished data) is incompatible with long-term neuronal survival in the SVZ/OB or following cortical damage (as described above), we further emphasize the hypothesis that cancer and neurodegeneration may be operating along overlapping/convergent pathways at least partially. Accordingly, and depending on the cellular context, alteration of specific cell cycle genes may lead to either process e.g., tumorigenesis in proliferating cells or neurodegeneration in post-mitotic cells (Morris et al., 2010; Jabir et al., 2015). Further support to this comes at the clinical level where it was reported that tumor formation and neurodegenerative diseases seem to be mutually exclusive to a large degree. For instance, a recent meta-analysis conducted on nine published studies of AD concluded that patients diagnosed with Alzheimer's disease (AD) are 45% less likely to be at risk of cancer (Shi et al., 2015). A similar study has previously revealed a 27–38% reduced risk of cancer in Parkinson's disease (PD) patients (Bajaj et al., 2010). We therefore support the point of view that, in mature neurons including adult-born OB neurons in mammals, neurodegeneration may result from accumulation of genetic insults e.g., loss of cell cycle suppression, DNA damage, oxidative stress and/or following aging, all of which may predominantly lead to CCE (see next sections) although the potential mechanisms involved warrant further investigation.

From Olfactory Dysfunction to Neurodegeneration: One Fatal Journey Implicating the OB

At a time when cell cycle deregulation in SVZ-NSPCs is tightly linked to neuro-oncogenesis, olfactory dysfunction is strongly correlated with the initiation and progression of neurodegenerative mechanisms in the aging brain. Therefore, we propose that the OB may be a well suitable model to study age-related defects, many of which are hallmark features of neurodegenerative diseases. A growing body of evidence supports this hypothesis. First, olfactory dysfunction is a common and early symptom of major neurodegenerative

diseases, namely AD, PD, Huntington's disease, amyotrophic lateral sclerosis (ALS) and others (Attems et al., 2014). In fact, it was shown that PD progression is linked to an increasing impairment of olfactory function or hyposmia which can be a useful marker of early disease development (Berendse et al., 2011) while severe hyposmia anticipates the development of PD's dementia (Baba et al., 2012). Similarly, olfactory deficit assessment might be an adequate prognostic tool to predict the conversion from mild cognitive impairment (MCI) to Alzheimer's dementia (Conti et al., 2013). Interestingly, these observations are also corroborated at the level of the underlying pathophysiology, where the OB is among the early structures to display deposits of protein inclusions such as hyperphosphorylated tau starting at Braak's stage 0 and I in AD (Kovacs et al., 2001) and α-Synuclein in PD (Braak et al., 2003). Furthermore, it was proposed that the OB could be the initiation site for the spread of these pathologies within the brain, which could occur in a prion-like manner and could hence be used to investigate the network-driven underlying mechanisms i.e., from entry of pathogens through the OE to sensitivity of the OS to oxidative stress and inflammation (**Figure 2**) (Rey et al., 2016).

Second, aging and/or loss of cell cycle suppression (hence post-mitotic state) can lead to CCE in mature neurons which is believed to be mechanistically related to the onset of AD and PD pathophysiology and once again, places the OB at the early stages of this process as described below. Third, since aging is the major risk factor of most neurodegenerative diseases and affects all brain regions, alterations in the aged SVZ and/or OE can trigger pro-survival compensatory responses in the OB involving mechanisms that are strikingly similar to those observed in neuronal anomalies of neurodegenerative diseases (**Figure 2**). We propose that future studies should aim at investigating whether cell-cycle defects affecting the maintenance of the SVZ niche and/or the OB-OE circuitry may indeed translate into manifestation of protein inclusions that are characteristic of neurodegenerative diseases, specifically AD and PD. In the next sections, we will review the evidence supporting the above observations as well as the effect of aging and neurodegeneration on AN in both the OB and the OE.

Cell Cycle Re-entry in Mature Neurons and Neurodegeneration: Is It Likely in the OB?

Several cell cycle proteins including Rb, p130 and Cdk5 among others are known to maintain a post-mitotic state in differentiated neurons, a process that is disrupted in major neurodegenerative diseases (Arendt, 2008). Loss of cell cycle suppression in mature neurons or loss of DNA integrity (which may lead to CCE) promotes an apoptotic cascade event that mimics neurodegeneration in AD and PD as some have argued (**Figure 3**) (Folch et al., 2012). In fact, on a more mechanistic level, neuronal CCE activates DNA repair machinery through a non-homologous end joining (NHEJ) response (Chow and Herrup, 2015). However, if DNA damage is unrepairable, some have suggested that instead of triggering cell death, the cell can commit to the senescence-associated secretory phenotype (SASP)—a mechanism by which mitotic cells evade cell-cycling especially in response to aging. Moreover, these senescent-like neurons can promote induction of the same phenotype in surrounding cells thus driving age-related diseases (Fielder et al., 2017). Besides, aneuploidy can serve as an early molecular signature of AD onset or MCI, where a chromosomal malsegregation or tetraploid state evades subsequent cell death and can lead to neuropathogenic effects such as tau phosphorylation, abnormal conformational changes or accumulation of aggregates (Arendt, 2012). Then again, another perspective proposes a "two hit hypothesis" in AD, whereby a first *hit* of mitogenic signaling dysregulation induces CCE causing a "mitotic steady state" that is not sufficient to cause cell death. However, this comes at the expense of accumulating more insults whereby a second *hit* such as of oxidative stress then triggers neuronal degeneration (Zhu et al., 2007; Aliev et al., 2014; Khan et al., 2016). Both events are necessary and sufficient to cause the disease and may be common to other neurodegenerative diseases (**Figure 3**). Indeed, using three different mouse models of AD, Yang et al. demonstrated that cortical neurons undergo DNA replication 6 months before developing β-amyloid plaques (known to accumulate in AD patients) or displaying activated microglia, yet without committing to cell death. This faithfully reproduces the ectopic cell cycling seen in human AD (Yang et al., 2006). However, the temporal pattern of CCE with respect to Tau pathology may differ between animal AD model(s) and AD patients as reported using the 3xTg AD mouse (Hradek et al., 2015).

Interestingly, several studies reported that loss of cell-cycle regulators so as to trigger CCE can itself be mechanistically linked to the onset of neurodegenerative lesions. For instance, inhibition of Rb in primary cortical neurons by miR-26b, a microRNA highly expressed in pathological areas in the human brain in AD, leads to aberrant CCE with increased vulnerability to tau hyperphosphorylation (another hallmark of AD pathology) and subsequent cell death mediated by pro-apoptotic downstream targets including E2F genes and cyclin E1 (Absalon et al., 2013). Notably, the same study reported nuclear export and increased activity of Cdk5, a major kinase implicated in cell cycle suppression and previously shown to contribute to tau phosphorylation and death in post-mitotic neurons after translocating to the cytoplasm (Zhang and Herrup, 2011). Similarly, in two mouse models of aging and AD, p73 haplo-insufficiency led to early appearance of phospho-tau aggregates by increased tau phosphorylation with simultaneous activation of three tau kinases GSK3β, c-Abl, and Cdk5 (Cancino et al., 2013a). However, it is not clear whether this is a direct effect or not (**Figure 3**). In addition, using a stochastic simulation model, Proctor and Gray predicted that, during DNA damage, increased activity and interaction between p53 and GSK3β may anticipate the accumulation of tau aggregates overtime, which in turn causes increased levels of reactive oxygen species (ROS) and more DNA damage (Proctor and Gray, 2010). Alternatively, cell cycle activation in Alzheimer's disease could be associated with neuroplasticity whereby specific cell cycle regulators can participate in the control of non-mitogenic functions such as network stability and cytoskeletal dynamics as seen in healthy neurons (for review on this topic, van Leeuwen and Hoozemans,

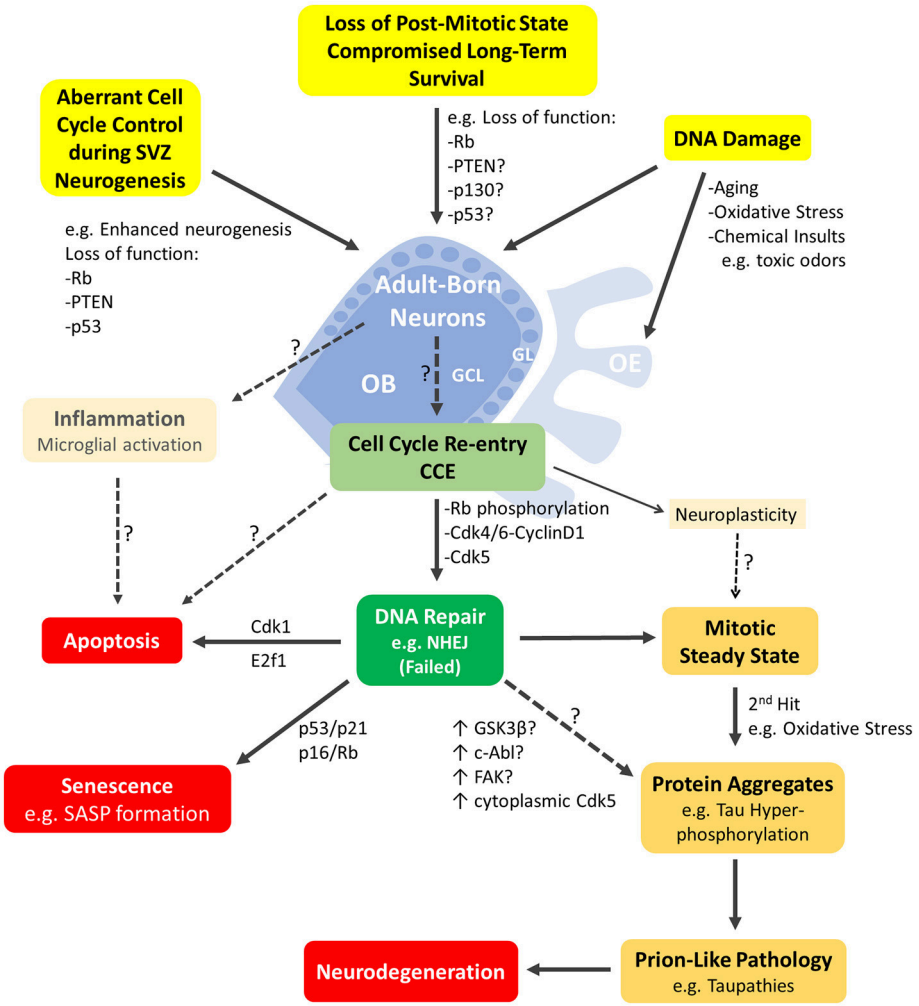

FIGURE 2 | Schematic representation of the major cellular events mediated by cell-cycle proteins and likely to be underpinning neurodegeneration in the adult olfactory bulb. Adult born neurons in the olfactory bulb (OB) can re-enter the cell cycle (CCE; light green box) following various cellular events (highlighted in yellow boxes). These include DNA damage or aberrant cell cycle control due to loss of function mutations in key cell cycle genes such as Rb, p130, p53, Pten or others. CCE (G1/S phase checkpoint) requires phosphorylation of Rb by Cdk4/Cyclin D1 among others mitogenic events. CCE primarily triggers DNA repair (dark green box) but may be possibly accompanied by inflammatory response of resident microglia and/or non-mitogenic signaling such as neuroplasticity. Neurons with failed DNA repair may proceed to the S-phase and undergo: (1) apoptosis e.g., mediated by E2F1, (2) senescence induced by p53 or p16 (with manifestation of senescence-associated secretory phenotype (SASP) (red boxes) or (3) a mitotic-steady state with accumulation of additional insults that will lead to protein aggregation(s)/pathology (yellow boxes) and neurodegeneration eventually (red box). The early steps of prion-like pathologies e.g., Tau phosphorylation may be triggered by several potential kinases with upregulated activities such as cytoplasmic Cdk5, GSK3β, c-Abl and/or others. Refer to text for references. OE, Olfactory epithelium; GCL, granule cell layer; GL, glomerular layer.

2015). This being said, all of the above described mechanisms following CCE are not mutually exclusive.

The implication of CCE in the formation of neurotoxic protein inclusions is yet to be established in the adult OB, but there is compelling evidence that this is likely possible. The tau kinase GSK3β (upregulated in models of AD) is abundantly expressed in the adult OB where it is essential in mediating spontaneous neural activity and odor habituation (Xu et al., 2013). Likewise, the focal adhesion kinase (FAK), thought to regulate cyclin D1 and being also involved in AD pathology (Caltagarone et al., 2007), is deregulated early in the OB of APP/PS1 mouse model prior to β-amyloid plaque formation (Lachen-Montes et al., 2016). In addition, as described earlier, loss of cell-cycle regulators in the OB leads to neuronal cell death on the long run in mice models such as Rb cKO (Naser et al., 2016), p53-null (Gil-Perotin et al., 2006) and Rb-p53 double cKO (Saliba and Ghanem, unpublished). This suggests an ability of OB neurons to better tolerate the loss of some cell cycle proteins as a *mitotic steady state* would indicate. It is noteworthy to mention that while CCE in the young adult OB may be directly linked to a neurodegenerative role, the aged OB is more adjusted to reduce neuronal loss which can reflect different roles

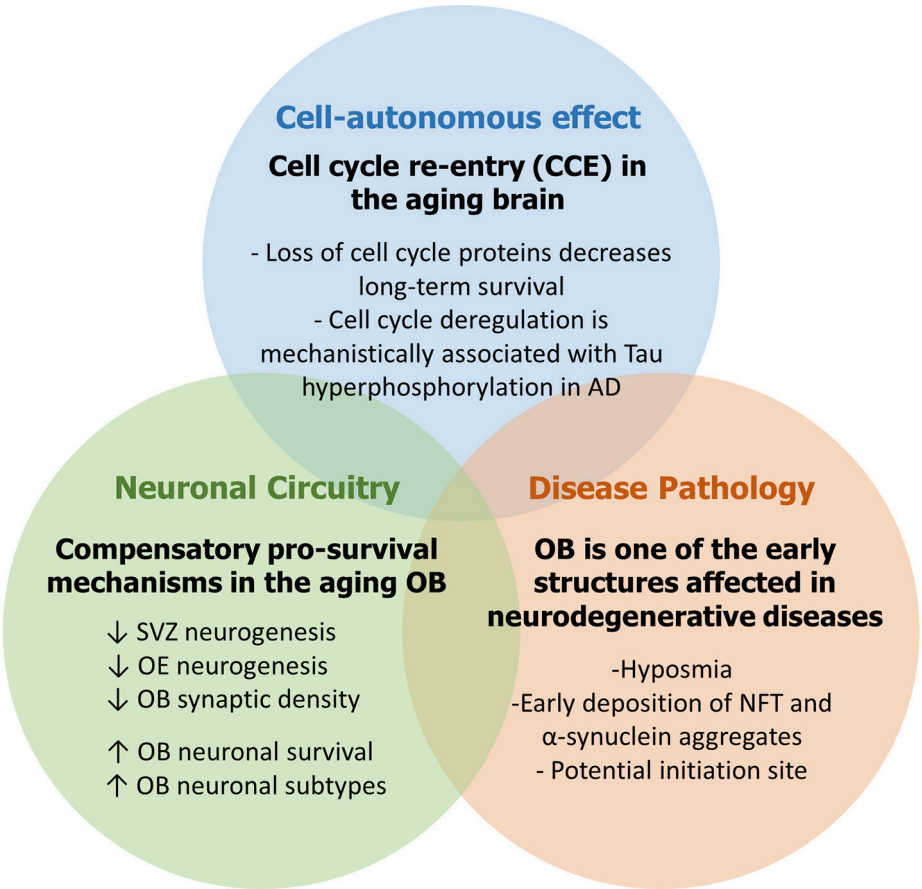

FIGURE 3 | The aging OB as working model to study neurodegenerative diseases.

of cell-cycle regulators during aging (Ohsawa et al., 2009). Finally, proteomic analysis of human postmortem OBs detected a specific deregulation of the DNA damage pathway in initial AD stages and that of the cell cycle in intermediate AD stages, albeit with high heterogeneity within the same AD stage (Zelaya et al., 2015).

Similar Pro-Survival Mechanisms Are Detected During Aberrant OB Neurogenesis and Neurodegenerative Diseases

The OB has been shown to adapt relatively well to aberrations affecting SVZ-derived neurogenesis and OE-based sensory input as well as age-related defects. For instance, during young adulthood, increased neuronal survival was reported in the OB following reduced SVZ proliferation in response to striatal dopaminergic denervation (Sui et al., 2012). However, this effect is likely to be only transient and will be eventually followed by a decrease in newborn OB neurons on the long run (Imayoshi et al., 2008). Nonetheless, some compensation of olfactory circuit functions can still be triggered (Breton-Provencher and Saghatelyan, 2012) e.g., retained olfactory learning following SVZ focal irradiation (Lazarini et al., 2009) and enhanced granule cell excitability following sensory deprivation by nostril closure (Saghatelyan et al., 2005). Surprisingly, despite the age-dependent decline in SVZ neurogenesis and OE deterioration (refer to next sections), the neuronal population (both projection neurons and interneurons subpopulations) remains stable in the aged OB overall, which explains the drop in adult-born neurons turnover in the OB with aging (Ohsawa et al., 2009). However, this is accompanied by a reduction of afferent synaptic input and local modulatory circuit synapses in OB glomeruli (Richard et al., 2010) as well as a deterioration of noradrenergic stimulation in the aged OB. All of these defects lead to an impairment in olfactory perceptual learning that is independent of the level of neurogenesis (Moreno et al., 2014).

Strikingly, the aged OB displays a higher caspase-9 expression (an initiator of apoptotic cell death) but not its downstream executioner active-caspase 3 that remains at normal level (Ohsawa et al., 2009). This observation may underline a unique strategy to avoid apoptosis in the aging OB through incomplete caspase signal propagation, as seen during the "abortosis" program in AD thought to promote neuronal survival in a similar manner (Raina et al., 2001). Moreover, the progressive decrease in cell death in the aging OB over time (Ohsawa et al., 2009) can lead to a buildup of some neuronal subtypes,

which resonates with an increased number of dopaminergic OB neurons detected in PD, AD and frontotemporal dementia (FTD) patients (Mundinano et al., 2011). Such increase could reflect a compensatory mechanism following the early degeneration of other neurotransmitter systems and could cause the symptomatic hyposmia of the disease (Huisman et al., 2004). Finally, in the near-zero levels of neurogenesis in the adult human OB (estimated to be <1% neuronal turnover after 100 years) (Bergmann et al., 2012), it is tempting to speculate that the human OB may rely on similar compensatory mechanisms among others to maintain a constant pool of perinatal neurons throughout life.

AGING, CELL CYCLE CONTROL AND NEURODEGENERATION IN THE OLFACTORY SYSTEM

Effect of Aging and Neurodegeneration on SVZ-OB Neurogenesis

Aging is an inevitable process that leads to major decline in homeostasis and regenerative capacity in many organs including the brain. Age-related aberrations manifest by various structural and functional changes at the level of specific neuronal networks thus contributing to cognitive deficit. For instance, in mature neurons, age-dependent DNA damage is due to accumulating stressors such as exposure to radiation, oxidative stress, neuronal activity, telomere dysfunction or loss of selective repair mechanisms, all of which affect genomic integrity and the so-called "neuronal health." These scars can develop independently in different subpopulations of neurons and lead to CCE or senescence-like phenotype or apoptosis as pointed earlier, but can also create a feed-forward degenerative cycle in the aging brain that is characteristic of neurodegenerative diseases (Sedelnikova et al., 2004; for recent reviews on genomic integrity and the aging brain, refer to Chow and Herrup, 2015; Fielder et al., 2017). Several studies have demonstrated that such insults do not spare the SVZ niche, hence causing major cytoarchitectural (atrophy) and proliferative changes inside the niche (Sahin and Depinho, 2010; for recent review on this topic; see Conover and Todd, 2017).

Specifically, in response to age-dependent insults or environmental changes inside the niche, SVZ-NSPCs can display various developmental aberrations such as decreased cell proliferation, reduced neuroblast number and RMS thinning, decreased expression of stage-specific markers (Luo et al., 2006; Ahlenius et al., 2009; Mobley et al., 2013), lengthening of the cell-cycle (Tropepe et al., 1997), permanent cell-cycle exit (cellular senescence) (Ahlenius et al., 2009), decreased telomerase activity (Ferron et al., 2009), increased type-B quiescence that could be rescued by intra-cerebro-ventricular infusion of growth factors such as FGF2 and HB-EGF (Jin et al., 2003; Bouab et al., 2011), as well as layer-specific loss of synaptic density inside the OB (Richard et al., 2010). As a result, the SVZ neurogenic potential sharply declines by 50–75% in the aged brain (Conover and Shook, 2011; Shook et al., 2012) despite the continuous production of few newborn neurons (Ahlenius et al., 2009; Mobley et al., 2013). In turn, this causes several behavioral deficits in fine olfactory discrimination (Enwere et al., 2004) and olfactory perceptual learning (Moreno et al., 2014) as well as short-term memory (Rey et al., 2012). Given that many of the above developmental processes are regulated, at least partially, by cell-cycle proteins in younger mice (as described earlier and **Figure 1**), it would be intriguing to assess the contribution of cell cycle machinery to the aging process in the SVZ-OB. While this information is still generally lacking, few studies have reported, in the aged SVZ, changes in expression of known senescence-markers (van Deursen, 2014; Zalzali et al., 2015) although their results remain controversial. For instance, Molofsky et al. associated the decrease in neuronal progenitor proliferation and subsequent neurogenesis with enhanced $p16^{INK4a}$ expression, which may be promoting a senescence phenotype in the aging SVZ (Molofsky et al., 2006). While this finding could not be reproduced in a later study, the authors reported an increase in $p27^{Kip1}$ levels with older age and higher expression of $p19^{ARF}$ in SVZ-derived primary neurosphere cultures compared with embryonic ones (Ahlenius et al., 2009). Likewise, a recent report further showed that the increased (but controlled) gene dosage of Ink4/Arf and p53 attenuates the age-dependent decline in SVZ neurogenesis and significantly improves the behavioral outcomes (Carrasco-Garcia et al., 2015). An increase in SVZ cell death, typically in neuroblasts, was also detected in old- but not middle-aged mice (Luo et al., 2006).

Finally, it is noteworthy that impaired AN was described in numerous animal models of neurodegenerative diseases and only few post-mortem studies. As highlighted earlier, alterations in neurogenesis impact pathophysiological mechanisms associated with these diseases. For instance, alpha-synuclein pathology caused decreased adult OB neurogenesis in A30P mutant mouse model of PD (Marxreiter et al., 2009) as well as following overexpression of human wild type alpha-synuclein (May et al., 2012). Cheng et al. showed a robust cell-autonomous degeneration in OSNs (via Aβ-independent mechanism) and olfactory dysfunction using mouse models expressing a humanized amyloid precursor protein (hAPP) and lacking the β-site APP cleaving enzyme 1 (Cheng et al., 2011, 2013, 2016). In summary, many of the molecular regulators of AD, PD and HD also modulate AN and display different effects on NSPCs fate by regulating cell proliferation, synaptic plasticity as well as spine and axonal morphology (for recent reviews on this topic, refer to Winner and Winkler, 2015; Horgusluoglu et al., 2017). Future studies should be aimed at investigating the molecular mechanisms affected during aging in the brain, particularly those implicating cell cycle regulators with common signaling pathways in neurodegenerative diseases.

Effect of Aging and Neurodegeneration on OE Neurogenesis

Studies have shown age-related changes affecting the cell dynamics and regenerative capacity during OE neurogenesis that parallel those observed following aging in the SVZ-OB. Indeed, advanced age is ultimately associated with dramatic decline in OE neurogenesis overall (up to 90% decrease between 3 and 16 months in rodents, Suzukawa et al., 2011) and

also with decreased olfactory sensitivity and impaired olfactory discrimination learning in rodents and primates (for recent reviews on this topic; see (Brann and Firestein, 2014; Broad, 2017). Such alterations are linked to reduced GBC proliferation (Watanabe et al., 2009), decreased number of differentiating basal cells as assessed by *Ascl1* (*Mash1*) expression, the main pro-neural gene required for OSN production (Guillemot et al., 1993), reduced EGF signaling (Enwere et al., 2004) and apoptosis (Robinson et al., 2002). In fact, Kondo et al. reported a decline in the rates of cell proliferation and cell death in the OE with increasing age (Kondo et al., 2010). Similarly, it was shown that OSN ablation following treatment with an olfacto-toxic drug triggers neurogenesis to a lesser extent in aged mice compared to young animals, suggesting an age-related decline in neuroepithelial proliferative capacity after injury (Suzukawa et al., 2011). Notably, the previous two studies detected no change in neuronal differentiation but a slower turnover rate of mature OSNs and extended OSN lifespan in the older OE compared to the younger one. The latter process is remarkably similar to the age-related compensatory mechanisms that extend survival of adult-born neurons in the aged OB (Ahlenius et al., 2009). On the other hand, with increasing age, the OSN population becomes more vulnerable to elevated levels of oxidative stress and accumulation of DNA damage, therefore rendering it more prone to neurodegeneration as might be the case inside the OB (Mattson and Magnus, 2006). In addition, telomere shortening, a common mechanism in aged tissues, was shown to impair the regenerative capacity of the OE post-lesion only by inhibiting cell cycle progression in a p21-dependent manner but without affecting OE homeostasis during mouse aging (Watabe-Rudolph et al., 2011). This is possibly justified by the compensatory regeneration that usually rescues proliferation in telomerase-deficient organ systems with low rate of cellular turnover (Brown et al., 1997; Lee et al., 1998; Rudolph et al., 1999; Kondo et al., 2010). In a related context, microarray analysis performed on senescence-accelerated mouse models (SAM) revealed dysregulated cell cycle gene expression in the OE during aging, whereby altered cell proliferation was associated with down-regulated expression of several genes involved in DNA synthesis, mitotic spindle formation and cell cycle progression such as cyclin B1 (Getchell et al., 2003).

In humans, aging also results in a "neurogenically exhausted" OE that is characterized by a gradual loss of OSNs and GBCs but not quiescent HBCs and supporting sustentacular cells. Moreover, the OE can be replaced by metaplastic respiratory epithelium (Holbrook et al., 2005, 2011; for recent review see Schwob et al., 2017). Interestingly, similar pathological changes with respect to neurogenic exhaustion were observed in rodents following bulbectomy (OB ablation) and chemical OE lesions (Kondo et al., 2009, 2010; Suzukawa et al., 2011). As described earlier, newborn OSNs extend their unmyelinated axons along the ONL and establish appropriate synaptic connections with existing neural circuits inside the OB (Cho et al., 2009). The impact of aging on this process is still poorly studied but it is likely that successful axonal targeting may be also compromised over time as seen in other tissues e.g., mouse retinal projection neurons (Samuel et al., 2011). As a matter of fact, the number of synapses is reduced in the aged OB glomeruli (Richard et al., 2010). Yet, it is not clear whether this is due to OSN loss and/or reduced axonal targeting (Mobley et al., 2014). In summary, the molecular mechanisms underlying age-induced GBCs proliferative decline and OE deterioration warrant further investigation in both animal models and humans.

While several factors contribute to age-associated decrease in olfactory function including OE damage resulting from pathogen infections, reduced expression of specific olfactory receptors and nasal engorgement (Attems et al., 2015), other functional deficits in the OE can be related to the role played by cell cycle regulators in the modulation of neurogenesis (Brann and Firestein, 2014). For instance, age-related decrease in GBC proliferation correlates with changes in gene expression of positive cell cycle regulators such as decreased expression in several cyclin-dependent kinases e.g., Cdk1, Cdk2, Cdk4 and, all D cyclins. In addition, the expressions of specific Cdkis including $p27^{Kip1}$, $p19^{Ink4d}$, $p18^{Ink4c}$, and $p21^{Cip1}$ change in a temporal pattern between young and adult stages, which may reflect changes in stem cell quiescence, cell cycle exit and neuronal differentiation with older age (Legrier et al., 2001). Yet, it is not clear whether these genetic changes are causative in nature or correspond to secondary effects associated with aging. Since aging is a common risk factor of neurodegenerative diseases, the OE, like the OB, is among the early structures to be affected by age-related alterations and disease pathology (Brann and Firestein, 2014; Mobley et al., 2014; Rey et al., 2016). A thorough examination of the molecular mechanisms involving cell cycle proteins in early stages of the neurodegenerative process and during aging is a pressing need.

CONCLUDING REMARKS

In this review, we examined the role played by the cell cycle machinery within each stage of the adult neurogenic SVZ-OB axis (as summarized in **Figure 1**). While this work serves as an update of previous comprehensive reviews (Beukelaers et al., 2012; Bartesaghi and Salomoni, 2013; Cheffer et al., 2013), our aim was to further implicate AN in the etiology and pathogenesis of human neurodegenerative disorders, especially in light of very few reviews that portray AN, both hippocampal and bulbar, as mediator and not simply the subject of these anomalies (**Figure 2**) (Gallarda and Lledo, 2012; Winner and Winkler, 2015; Hollands et al., 2016). In addition, we propose the OB as a potential candidate to model the early non-symptomatic pathophysiology of AD and PD (**Figure 3**) which is grounded in its remarkable susceptibility to aging at both the cellular level (as part of the whole brain's challenge to evade cycling in the post-mitotic state, Zhu et al., 2007; Chow and Herrup, 2015) and the network level (in an attempt to compensate for reduced AN in the aged SVZ and OE, Ohsawa et al., 2009; Mobley et al., 2014). More evidence in favor of this hypothesis comes from olfactory dysfunction being an effective predictor of the subsequent symptomatic features of most neurodegenerative diseases (Attems et al., 2014). Additionally, despite our focus here on cell-autonomous processes following cell cycle deregulation

(Herrup and Yang, 2007) whereby cancer and neurodegeneration are thought to share common mechanisms (Morris et al., 2010), other features of the OB mark its centrality in the disease process e.g., robust pro-inflammatory responses from resident microglia following sensory de-afferentation and traumatic brain injury (Lazarini et al., 2012; Siopi et al., 2012) as well as the age-dependent decrease in synaptic density and responsiveness to noradrenergic stimulation (Richard et al., 2010; Moreno et al., 2014).

Previous studies have discussed the diagnostic implications of this proposition, such as the detection of olfactory deficits in order to anticipate the conversion from MCI to AD (Conti et al., 2013). To ameliorate olfactory function, Broad KD recently reviewed a number of therapeutic interventions ranging from behavioral to dietary and pharmaceutical interventions, all of which are believed to act partially by increasing AN (Broad, 2017). Here, we emphasized the role played by cell cycle proteins in neurodegeneration in hopes to reiterate existing treatments to a less obvious process of neurodegenerative disorders, currently known for more drastic manifestations e.g., memory loss in AD and motor dysfunction in PD. In fact, a recent review addressed the possibility of repurposing cancer drugs for the treatment of AD, several of which proved to relieve amyloid burden and tau aggregation. However, permeability to the brain blood barrier and dose-dependent efficacy pose a limitation to their successful delivery (Monacelli et al., 2017). Interestingly, the olfactory system offers alternative mechanisms of delivery by the very means of which it is most vulnerable to environmental toxic factors, that is its direct exposure to the nasal cavity (Doty, 2008). In line with this, Kovács T reviews intranasal delivery of insulin and cholinesterase inhibitors as a promising therapeutic pathway to treat or delay AD pathology (Kovács, 2013). While more research is warranted, the olfactory system serves as a gateway for disease initiation and progression, while also an early and accessible window for therapeutic intervention.

AUTHOR CONTRIBUTIONS

SO and CJ wrote the manuscript. NG participated in writing, edited the manuscript and provided financial support.

ACKNOWLEDGMENTS

NG research laboratory is supported by grants from the University Research Board (URB), Kamal A. Shair Research Fund (KS) and Farouk Jabre Biomedical Research Grant (FJ) at AUB as well as grants from the Lebanese National Council for Scientific Research (LNCSR). Part of NG research is performed using common equipment available at Kamal A. Shair Central Research Science Laboratory (KAS CRSL) at AUB. We thank Dr. Amal Rahmeh and Dr. Firas Kobeissy for their critical review of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 09 April 2018
doi: 10.3389/fnins.2018.00199

Advancing Stem Cell Models of Alpha-Synuclein Gene Regulation in Neurodegenerative Disease

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Alpha-synuclein (*non A4 component of amyloid precursor, SNCA, NM_000345.3*) plays a central role in the pathogenesis of Parkinson's disease (PD) and related Lewy body disorders such as Parkinson's disease dementia, Lewy body dementia, and multiple system atrophy. Since its discovery as a disease-causing gene in 1997, alpha-synuclein has been a central point of scientific interest both at the protein and gene level. Mutations, including copy number variants, missense mutations, short structural variants, and single nucleotide polymorphisms, can be causative for PD and affect conformational changes of the protein, can contribute to changes in expression of alpha-synuclein and its isoforms, and can influence regulation of temporal as well as spatial levels of alpha-synuclein in different tissues and cell types. A lot of progress has been made to understand both the physiological transcriptional and epigenetic regulation of the alpha-synuclein gene and whether changes in transcriptional regulation could lead to disease and neurodegeneration in PD and related alpha-synucleinopathies. Although the histopathological changes in these neurodegenerative disorders are similar, the temporal and spatial presentation and progression distinguishes them which could be in part due to changes or disruption of transcriptional regulation of alpha-synuclein. In this review, we describe different genetic alterations that contribute to PD and neurodegenerative conditions and review aspects of transcriptional regulation of the alpha-synuclein gene in the context of the development of PD. New technologies, advanced gene engineering and stem cell modeling, are on the horizon to shed further light on a better understanding of gene regulatory processes and exploit them for therapeutic developments.

Keywords: alpha-synuclein, *SNCA*, transcriptional regulation, induced pluripotent stem cells, Parkinson's disease, Dementia with Lewy bodies

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *21 January 2018*
Accepted: *13 March 2018*
Published: *09 April 2018*

Citation:
Piper DA, Sastre D and Schüle B (2018) Advancing Stem Cell Models of Alpha-Synuclein Gene Regulation in Neurodegenerative Disease. Front. Neurosci. 12:199. doi: 10.3389/fnins.2018.00199

INTRODUCTION

Alpha-synuclein (*non A4 component of amyloid precursor, SNCA, chr4q21-22, NM_000345.3, MIM #163890*) was the first gene in which a causative mutation for Parkinson's disease (PD) was discovered in 1997 (Polymeropoulos et al., 1997; Nussbaum, 2017). The detection of alpha-synuclein protein as a constituent of Lewy bodies further strengthened the role of alpha-synuclein in PD and dementia with Lewy bodies (DLB) as a central player in the pathogenesis of neurodegeneration (Spillantini et al., 1997; Goedert et al., 2017). It is now well-established that rare point mutations and large genomic multiplications of the *SNCA* gene can cause autosomal-dominant parkinsonism which can present itself as a wide range of clinical and histopathological

features including typical PD (Langston et al., 2015), Parkinson's disease dementia (PDD), DLB (Beyer et al., 2009), multiple system atrophy (MSA) (Jellinger and Lantos, 2010), or even fronto-temporal dementia (FTD) (Polymeropoulos et al., 1997; Krüger et al., 1998; Singleton et al., 2003; Chartier-Harlin et al., 2004; Farrer et al., 2004; Ibáñez et al., 2004; Zarranz et al., 2004; Nishioka et al., 2006; Fuchs et al., 2007a; Deng and Yuan, 2014).

Genetic variants in the non-coding region of the *SNCA* gene, including single nucleotide polymorphisms (SNPs) were discovered by association and genome wide association studies (GWAS) (Nalls et al., 2014; Campelo and Silva, 2017) and small structural variants increase the risk for developing PD (Chiba-Falek, 2017), however the underlying mechanisms are still under investigation as will be reviewed herein.

The mere overexpression of wildtype alpha-synuclein in patients with *SNCA* multiplications is sufficient to cause parkinsonism and point mutations in the *SNCA* gene seem to increase alpha-synuclein inclusion formation or its interaction with acidic phospholipids suggesting that alpha-synuclein, when mutated, causes neurodegeneration and parkinsonism via a toxic gain-of-function mechanism (Rajagopalan and Andersen, 2001; Collier et al., 2016).

Interest in alpha-synuclein as a therapeutic target for PD has been growing not only due to the genetic link, but also because of the pathology of alpha-synuclein spreading through the nervous system and the cell-to-cell propagation of aggregated alpha-synuclein. These observations have led to consensus that lowering the alpha-synuclein content and/or eliminating toxic alpha-synuclein species in cells could be the key to slowing, reversing, or even preventing the disease and such alpha-synuclein lowering therapeutic strategies are currently being developed (Bergström et al., 2016; Brundin et al., 2017).

While advancements of genetic technologies have greatly increased our understanding of the genetics of the *SNCA* gene, alpha-synuclein aggregation, interaction, and post-translational modification has been intensively studied over the last two decades, there is still a gap in current knowledge about transcriptional and epigenetic regulation of *SNCA* gene and how it contributes to PD and related alpha-synucleinopathies. This has been in part due to the paucity of suitable methods, but also because of lack of models and complexity of the modeling of temporal and cell-specific transcriptional regulation of gene expression.

In addition, recent advances in our understanding of stem cell biology, nuclear reprogramming, and genome engineering offer new avenues for disease modeling of PD given the challenges of animal models for progressive neurodegenerative aspects of the disease. Nuclear reprogramming has been successful in modeling virtually any adult somatic tissue type using different delivery protocols of integrating (Takahashi et al., 2007) or non-integrating viruses (Yang et al., 2008), episomal vectors (Su et al., 2014), excisable vectors (Woltjen et al., 2009), mRNA (Mandal and Rossi, 2013), protein (Seo et al., 2017), or chemical compounds (Hou et al., 2013; Kimura et al., 2015; Silva et al., 2015). Regarding PD, patient-specific induced pluripotent stem cells (iPSCs) lay the foundation for differentiation into the tissue-type of interest, i.e., mid-brain dopaminergic neurons. As a result, there is a great excitement and an intensive effort within the PD community to derive iPSCs from patients with genetic and sporadic forms of PD to model disease and use these cells for exploring disease mechanisms and for drug discovery (Schüle et al., 2009; Imaizumi and Okano, 2014). Specifically, Mendelian forms of PD have been of particular interest including SNCA, which is the focus of this review, leucine-rich repeat kinase 2 (LRRK2), glucosidase, beta, acid (GBA), PTEN-induced putative kinase 1 (PINK1), or PARKIN (reviewed in Hartfield et al., 2012; Torrent et al., 2015). Furthermore, genome engineering allows for generation of isogenic cell lines that only differ by the introduced change in the genome which allows for the correction of mutations or introduction of genetic variants to understand their functional consequences (Hockemeyer and Jaenisch, 2016). Mutant forms of these nucleases can be utilized for complex transcriptional modulation, such as Clustered Regularly Interspaced Short Palindromic Repeats interference (CRISPRi; Du and Qi, 2016; Mandegar et al., 2016).

A working hypothesis for the importance of studying the physiological regulation of the *SNCA* gene is that a subtle to moderate overexpression of alpha-synuclein due to one or more genetic risk factors (or in combination with environmental triggers) over many decades can either predispose or even cause the neurodegenerative changes similar to *SNCA* gene multiplications. Neurons subjected to higher, non-physiological levels of alpha-synuclein might be more likely to be damaged by misfolding and aggregation of this protein, eventually leading to neuronal cell death.

This review focuses on genetic variants in the *SNCA* gene that predispose to PD and on how transcriptional regulation directed by the non-coding and epigenomic elements within the *SNCA* gene can lead to PD and neurodegeneration. Furthermore, we highlight emerging new technologies and stem cell models for studying gene regulation, e.g., CRISPRi screens will allow for rapid advancements in our understanding of transcriptional regulation of the non-coding genome and epigenome.

MULTIPLICATIONS OF THE *SNCA* GENE LEAD TO RAPID PROGRESSIVE PARKINSONISM

SNCA Genomic Triplications

Copy number variants (CNVs) of the *SNCA* genomic locus on chromosome 4q21 were first discovered in 2003 in a family with parkinsonism now termed the Iowa kindred (Singleton et al., 2003). The mutation is a genomic triplication of the *SNCA* gene and adjacent genes resulting in a size of ∼1.7 Mb. Patients with an *SNCA* triplication carry four functional copies of the *SNCA* gene, three copies from the mutant allele and one copy from the wildtype allele, resulting in a 2-fold overexpression of alpha-synuclein mRNA and protein. We recently performed a high-resolution comparative genomic hybridization array and determined that this *SNCA* triplication was derived in two steps with an underlying region of a slightly larger duplication (Zafar et al., 2018; **Figure 1A,** Supplementary Table 5).

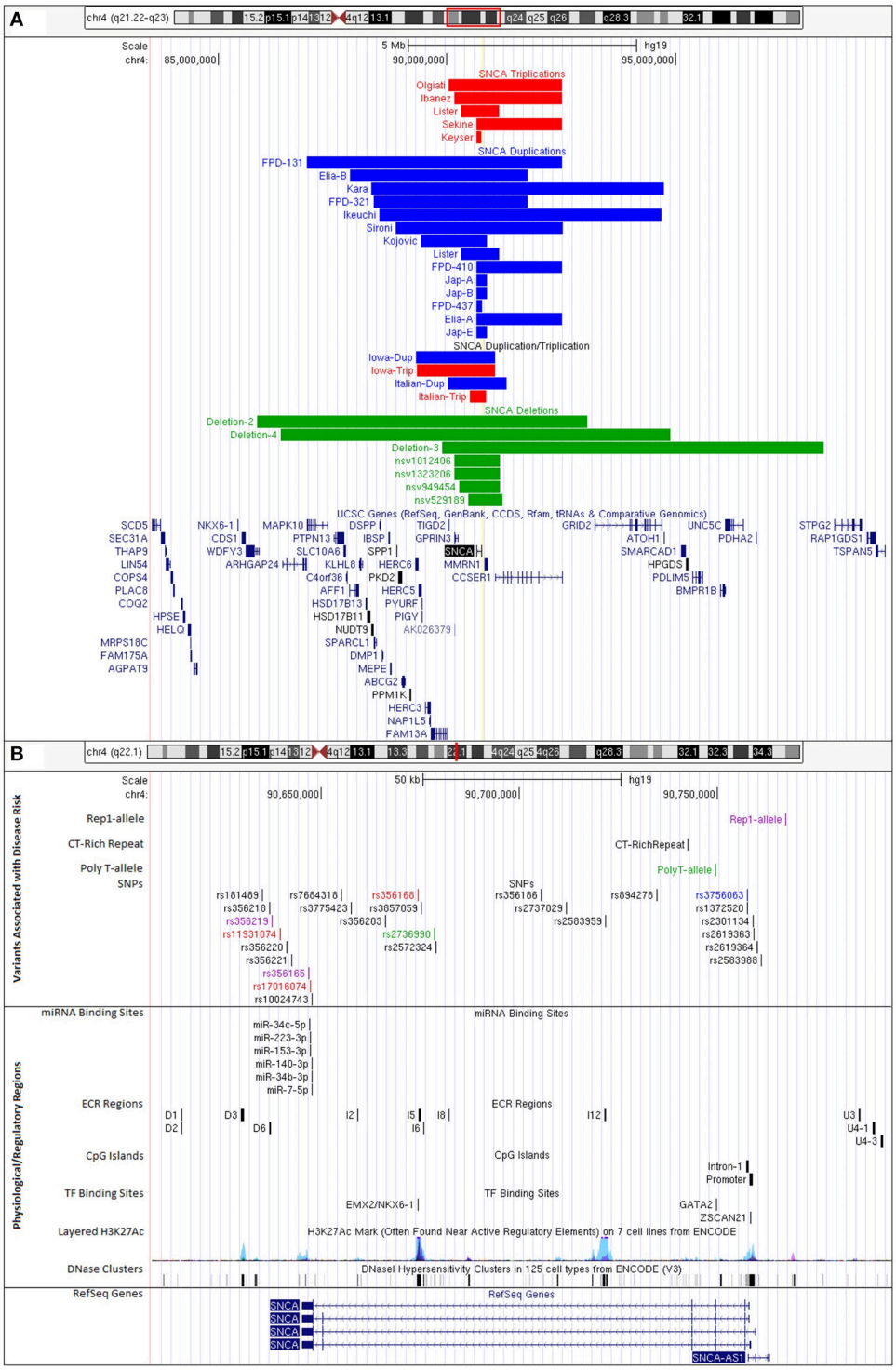

FIGURE 1 | UCSC Genome Browser custom tracks for PD-risk associated variants and regulatory regions impacting SNCA expression. **(A)** CNVs of SNCA locus on chromosome 4q21.23-q22.3 (GRCh37/hg19, chr4:84,239,011-98,739,011). Colors indicate gene copy numbers. Red: *SNCA* CNV triplications; blue: *SNCA* CNV duplications; combination: *SNCA* triplication (red) and duplication (blue); green: *SNCA* CNV deletions. IDs given to tracks were either based on family identifiers from literature or are first author's last name of publication where case/family has been reported. **(B)** Disease variants and regulatory regions of the SNCA genomic locus on chromosome 4q22.1 (GRCh37/hg19, chr4:90,608,984-90,793,984) (details in Supplementary Tables 2–4). Colors indicate functional changes related to variants. Red: total mRNA expression; blue: affects *SNCA* splice-isoform; green: *SNCA* methylation; pink: multiple associated functions. Additional tracks include microRNA and transcription factor binding sites, CpG islands, and integrated regulation from ENCODE (Layered H3K27Ac and DNase Clusters). (Supplementary Table 5 lists all positions to build custom tracks in UCSC Genome Browser).

SNCA Genomic Duplications

In addition, there are also families with *SNCA* genomic duplications (total of three *SNCA* gene copies) of chromosome 4q21 (Konno et al., 2015). The length of the genomic regions varies (Ross et al., 2008; Kara et al., 2014; **Figure 1A**), there are additional cases presenting a combination of duplications and triplications (Ferese et al., 2015). We mapped size and location of published cases with *SNCA* triplication and duplications (**Figure 1A**) and also identified through database searches CNV deletions of the *SNCA* locus. Interestingly, these CNV deletion cases were from large cohorts of children with intellectual disability/developmental delay, multiple congenital anomalies, and/or autism (Miller et al., 2010; Vulto-van Silfhout et al., 2013; Coe et al., 2014; Duyzend et al., 2016; **Figure 1A**). A common primary mechanism underlying the generation of such microdeletions/duplications is non-allelic homologous recombination (NAHR) that give rise to a number of disorders with such reciprocal rearrangements of specific chromosomal regions (Hastings et al., 2009; Watson et al., 2014). The SNCA/4q21 locus has been primarily recognized with various forms of parkinsonism as a duplication/triplication, but detailed clinical presentations of SNCA/4q21 microdeletions are pending.

Clinically, the *SNCA* duplications generally present with typical PD, whereas *SNCA* triplications have an earlier onset of motor symptoms (34.5 ± 7.9 years for triplication vs. 47.2 ± 10.6 years for duplication) and in addition present more frequently with cognitive decline at an earlier age (39.6 ± 5.5 years for triplication vs. 56.5 ± 9.6 years for duplication) (Book et al., 2017). The increasing severity of the clinical and histopathological phenotype with the number of *SNCA* gene copies clearly points to gene dosage effect for the *SNCA* gene.

SNCA Genomic Triplication Stem Cells Models Replicate Known Disease Mechanisms

The *SNCA* genomic triplication was also the first mutation for which patient pluripotent stem cells were derived and characterized (Byers et al., 2011; Devine et al., 2011; **Table 1** Supplementary Table 1). Undifferentiated patient-derived iPSCs carrying the *SNCA* triplication show a 2-fold increase in SNCA mRNA and protein expression according to the genomic copy number. Although alpha-synuclein is more highly expressed in differentiated neurons, the ratio of 2-fold increased expression of alpha-synuclein compared to sibling controls is maintained. The *SNCA* triplication neurons are more prone to oxidative stress, and show changes on neuronal maturation which is illustrated by a lower expression of tyrosine hydroxylase, the rate limiting enzyme for dopamine synthesis (Oliveira et al., 2015). Also, patient-derived neuroprecursors while showing normal cellular and mitochondrial morphology, exhibit substantial changes in growth, viability, cellular energy metabolism, and stress resistance when challenged by starvation or toxicant challenge. However, these phenotypic changes were reversible upon *SNCA* knockdown (Flierl et al., 2014). Another study has investigated the cell-to-cell transfer for alpha-synuclein and describes that the secretion of alpha-synuclein in the medium is increased in the *SNCA* triplication neurons and furthermore, secreted alpha-synuclein is taken up by co-cultured mouse neuroblastoma cells (Reyes et al., 2015).

iPSC-derived neurons from the *SNCA* triplication were also utilized for drug screening and repurposing. Using an unbiased screen targeting endogenous alpha-synuclein gene expression, b2-adrenoreceptor (b2AR) agonists were discovered as a regulator of the alpha-synuclein gene (*SNCA*). b2AR ligands were shown to downregulate *SNCA* transcription through inhibition of histone 3 lysine 27 acetylation by about 20% (Mittal et al., 2017). These findings were confirmed in neuroprecursors derived from the induced pluripotent stem cells of a patient carrying the *SNCA* triplication which demonstrated that b2AR agonist clenbuterol could normalize *SNCA* expression and reduce alpha-synuclein protein, and ameliorate mitochondrial function (Mittal et al., 2017).

Three additional studies should be mentioned that utilized the *SNCA* triplication line from Devine et al. (2011). The first study developed a CRISPR inhibition system to downregulate alpha-synuclein. The authors found one specific guide RNA that downregulated alpha-synuclein by 50% at the mRNA and protein level in healthy controls and neurons from the *SNCA* triplication carrier (Heman-Ackah et al., 2016).

Generating isogenic iPSCs that only differ by the introduced mutation is believed to greatly reduce biological noise. Two studies show independently the CRISPR/Cas9 targeting of exonic regions of the *SNCA* gene (exon 2 or exon 4) and create panels of isogenic lines with various functional copies of the *SNCA* gene (Heman-Ackah et al., 2017; Zafar et al., 2017). Interestingly, comparative expression analysis of neuronal cultures from the parental *SNCA* triplication and a 2-copy knockdown presented with an ER stress phenotype, marked by induction of unfolded protein response (UPR) and leading to terminal UPR activation (Heman-Ackah et al., 2017).

These patient-iPSC studies in *SNCA* genomic triplications emphasize that this *in vitro* cellular model reflects many aspects of neurodegeneration as described in human tissues and other models (**Table 1,** Supplementary Table 1).

MISSENSE MUTATIONS IN THE CODING REGION OF THE *SNCA* GENE

Polymeropoulos et al., identified the first causative mutation for PD, a missense mutation in exon 3 of the SNCA gene (p.A53T, NM_000345.3:c.157G>A; Polymeropoulos et al., 1997) which ignited the era of genetics in PD. However, the frequency of mutations in the *SNCA* gene remains low, at about 0.5% in familial and sporadic cases (Deng and Yuan, 2014). There are only a few point mutations that are considered clearly causative or pathogenic, p.A30P (NM_000345.3:c.88G>C; Krüger et al., 1998, 2001; Seidel et al., 2010), p.E46K (NM_000345.3:c.136G>A; Zarranz et al., 2004), p.H50Q (NM_000345.3:c.150T>G; Appel-Cresswell et al., 2013), p.G51D (NM_000345.3:c.152G>A; Kiely et al., 2013, 2015; Lesage et al., 2013), p.A53E (NM_000345.3: c.158C>A; Pasanen

TABLE 1 | Human iPSC models with mutations and genetic variants in *SNCA* gene elicit specific molecular and cellular phenotypes.

References	iPSC Source and clonal lines	Mutation type	Phenotype in human iPSC model
***SNCA* CNV**			
Byers et al., 2011	Skin fibroblasts: 1 patient (*SNCA* triplication from Iowa kindred, 42 yrs male), 1 control (46 yrs female, mutation-negative sibling)	CNV	*SNCA*-tri and control lines had similar pluripotency marker expression and neuronal differentiation patterns. Alpha-synuclein levels are higher in *SNCA*-tri iPSCs and neurons compared to controls. There is a 1.5- to 4-fold increased expression of oxidative stress and protein aggregation-related genes in *SNCA*-tri cultures.
Devine et al., 2011	Skin fibroblasts: 1 patient (*SNCA* triplication from Iowa kindred, 55 yrs, female), 1 control (first degree relative)	CNV	SNCA-tri and control fibroblasts do not have detectible protein levels of alpha-synuclein. Alpha-synuclein protein detected in all iPSC-derived neurons. Elevated *SNCA* expression found in SNCA-tri iPSCs which increased with differentiation. SNCA-tri -derived neurons showed 2-fold increase of *SNCA* mRNA compared to controls. For SNCA paralogous genes, *SNCG* expression was significantly lower in SNCA-tri neurons but *SNCB* was unchanged.
Flierl et al., 2014	iPSCs 1 patient (*SNCA* triplication, 42 yrs male), 2 healthy controls (46 yrs female sibling, 61 yrs old male Byers et al., 2011)	CNV	SNCA-tri NPCs had normal cellular and mitochondrial morphology but altered growth, viability, cellular energy metabolism, and stress resistance. Knockdown of alpha-synuclein by shRNA reversed phenotypic alterations.
Oliveira et al., 2015	iPSC-derived neural progenitors (NPCs): 1 patient (*SNCA* triplication, 42 yrs male), 2 controls (unaffected sister, 46 yrs; unrelated healthy control, 62 yrs, male Byers et al., 2011; Flierl et al., 2014)	CNV	SNCA-tri overexpresses alpha-synuclein and expression increases during *in vitro* neuronal differentiation. *SNCA*-tri neurons fail to develop complex networks and showed reduced neurite outgrowth. TH+ cell number was lower in *SNCA*-tri than control. Over-expression of alpha-synuclein impairs neuronal maturation. *SNCA*-tri neurons presented lower neuronal activity. Genes associated with neuronal differentiation and signal transduction were down regulated in *SNCA*-tri.
Reyes et al., 2015	Skin fibroblasts: 1 patient (*SNCA* triplication), 1 control (mutation negative family member Devine et al., 2011)	CNV	Differentiated neurons from *SNCA*-tri patient secrete higher levels alpha-synuclein compared to control neurons. Five-day co-cultures SNCA-tri neurons and N2a cells with restricted cell-to-cell contact showed alpha-synuclein puncta around and within the N2a cells.
Heman-Ackah et al., 2017	iPSCs: patient with *SNCA* triplication (ND34391G iPSCs, NINDS/Coriell Institute), 1 control (NCRM-5, NIH CRM), 13 CRISPR-edited isogenic clones	CNV	Alpha-synuclein mRNA and protein levels were reduced in CRISPR-edited isogenic iPSC clones (two functional SNCA gene copies). SNCA-tri has little effect on neuronal differentiation based on RNA-Seq. Ninety-fold overexpression of *SNCA* mRNA in SNCA-tri neurons were restored in isogenic controls. The three branches of UPR were upregulated in AST neurons. SNCA-tri showed ER stress phenotype, induction of IRE1a/XBP1 axis [unfolded protein response (UPR)] and UPR activation.
Mittal et al., 2017	iPSC-derived NPCs: 1 patient (*SNCA* triplication, 42 yrs male), 1 healthy control (46 yrs female sibling Flierl et al., 2014)	CNV	Beta-adrenoreceptor agonist clenbuterol reduces alpha-synuclein expression by 20% in SNCA-tri NPCs. Clenbuterol reduces mitochondria-associated superoxide in SNCA-tri and positively affects viability when exposed to rotenone. Alpha-synuclein downregulation by beta-adrenoreceptor agonists was shown to be mediated by a decrease in H3K27 acetylation in promoter and intron 4 enhancers of the *SNCA* gene.
***SNCA* POINT MUTATIONS**			
Chung et al., 2013	iPSCs (*SNCA*, p.A53T (female, AAO 49 yrs; Golbe et al., 1996) and *SNCA* triplication Byers et al. (2011), 1 male control (BG01)	CNV and Point mutation	In yeast, nitrosative stress is caused by alpha-synuclein and contributes to toxicity. There is also increased nitric oxide in A53T cortical neurons compared to corrected neurons. *SNCA* p.A53T alpha-synuclein leads to ERAD dysfunction. NAB2, an N-arylbenzimidazole, activates Rsp5/Nedd4 pathway and reduced nitric oxide levels in *SNCA* p.A53T neurons. NAB2 improves forward protein trafficking through ER in SNCA-tri neurons.
Soldner et al., 2011	Skin fibroblasts 1 patient (*SNCA* p.A53T mutation, Golbe et al., 1996 and Supplementary Table 1 Chung et al., 2013,) BG01 and WIBR3 hESCs	Point mutation	Several pairs of ZFN-isogenic hiPSC/hESCs were generated and characterized for neuronal differentiation: hESC—hESC$^{SNCAA53T/wt}$, hESC—hESC$^{SNCAE46K/wt}$, hiPSC SNCA p.53T—hiPSC corrected. *SNCA* p.A53T was inserted into *SNCA* gene via ZFN without drug selection. Increased efficiency of introducing a second mutation (*SNCA* p.E46K) via single-stranded oligodeoxynucleotides into hESCs. *SNCA* wild-type sequence containing donor vector and ZFNs genetically corrected *SNCA* p.A53T mutation in patient-derived hiPSCs.

(Continued)

TABLE 1 | Continued

References	iPSC Source and clonal lines	Mutation type	Phenotype in human iPSC model
Ryan et al., 2013	2 isogenic pairs: iPSCs (Soldner et al., 2011 *SNCA* p.A53T and paired mutation ZFN-corrected clone); hESC (BG01) line and paired ZFN-induced SNCA p.A53T mutation	Point mutation	iPSC-derived dopaminergic neurons from *SNCA* p.A53T carrier show alpha-synuclein aggregation resembling Lewy body-like pathology. *SNCA* p.A53T mutant neurons display variations in mitochondrial machinery and an increase in mitochondrial toxin susceptibility. ROS/RNS abundance leads to changes of MEF2C in *SNCA* p.A53T neurons.
***SNCA* RISK VARIANTS AND GENE REGULATION**			
Soldner et al., 2016	hiPSC line derived from fibroblast AG20446 (male, PD, 57 yrs) and 2 hESCs from Whitehead Institute Center for Human Stem Cell Research and NIH (WIBR3, BG01)	SNV and SSV	Generation of CRISPR-modified isogenic hESC allelic panels for *SNCA* gene risk variants rs356168 and NACP-Rep-1. CRISPR insertion of G-allele at rs356168 results in increased expression of *SNCA*. Sequence-specific binding of TFs EMX2 and NKX6-1 represses intron 4 enhancer activity, modulating *SNCA* expression. Allelic series of NACP-Rep1 (genotypes 257/261, 259/261 261/261 263/261) did not show expression differences for alpha-synuclein.
Heman-Ackah et al., 2016	Skin fibroblasts: a patient with *SNCA* triplication (ND34391G, iPSCs from NINDS/Coriell Institute), 1 control (NCRM-5, RUDCR Infinite Biologics)	*SNCA* gene regulation	Binding affinity between different sgRNAs and relative position to the TSS are critical for CRISPRi. dCas9 can be used for gene expression manipulations and gene contributions of neurodegenerative disease. CRISPR/dCas9-KRAB and TSS2-1 sgRNA expression reduced endogenous alpha-synuclein mRNA levels in SNCA-tri iPSC-derived neurons by 40%.
Tagliafierro et al., 2017	IPSCs from healthy patient (GM23280, Coriell Repository), iPSCs from *SNCA*-tri patient (ND34391, NINDS Repository)	miRNA expression	Differentiation into two different neuronal cell types, midbrain dopaminergic and cholinergic neurons, were developed. MiR-7-5p, miR-153-3p, and miR223-3p had higher levels in dopaminergic neurons while miR-140-3p was only slightly increased in cholinergic neurons. SNCA-tri miR-7-5p levels in neurons were 10-fold decreased compared to control neurons, other miRNAs showed similar trends as in control neurons.

et al., 2014; Martikainen et al., 2015), and p.A53V (NM_000345.3: c.158C>T; Yoshino et al., 2017).

Interestingly, while most of the *SNCA* missense mutations present with typical Lewy body Parkinson's disease (reviewed in Langston et al., 2015), some of these point mutations show additional Parkinson-plus clinical features. In a recent case report, two unrelated patients with *SNCA* p.A53T mutation presented with early-onset frontal-dysexecutive dysfunction with apathy and resembling frontotemporal dementia (FTD) followed by motor symptoms of PD. An MRI showed marked frontal, parietal and temporal, neocortical atrophy, and atrophy in the mesial temporal lobe. No autopsy data are presented (Bougea et al., 2017). The *SNCA* p.A53E was reported in a patient with atypical PD starting at the age of 36 which came to autopsy at the age of 60 with fulminant alpha-synuclein pathology characterized as MSA and PD (Kiely et al., 2013; Pasanen et al., 2014). Patients with *SNCA* p.G51D mutations show clinical signs of early-onset rapidly progressive levodopa responsive parkinsonism, but can also present with pyramidal signs and severe cognitive impairment and hallucinations (Lesage et al., 2013; Tokutake et al., 2014; Kiely et al., 2015). In a case with *SNCA* p.A51D mutation, unusual neuropathological findings were described, including fine, diffuse cytoplasmic inclusions containing phospho-alpha-synuclein at position 129 in superficial layers of the cerebral cortex and entorhinal cortex together with severe neuronal loss in the substantia nigra and locus ceruleus (Lesage et al., 2013).

In vitro experiments of mutant alpha-synuclein shows that several of the SNCA point mutations increase the propensity of alpha-synuclein to form fibrils, including p.A53T, p.E46K, and p.H50Q (Conway et al., 1998; Narhi et al., 1999; Greenbaum et al., 2005; Appel-Cresswell et al., 2013; Ghosh et al., 2013; Proukakis et al., 2013). On the other hand, the pathogenic point mutations p.A30P, p.G51D, and p.A53E seem to attenuate the fibrillation rate of alpha-synuclein (Li et al., 2001; Fares et al., 2014; Ghosh et al., 2014; Pasanen et al., 2014), which suggests that fibrillation rate is not directly involved in PD pathogenesis and other kinetics such as oligomerization should be tested and pursued (Ghosh et al., 2017).

SNCA p.A53T Point Mutation and Genetically Engineered Isogenic Controls in Human Stem Cells Models

Using zinc-finger technology, two point mutations, p.A53T, NM_000345.3:c.157G>A and p.E46K (NM_000345.3:c.136G>A) were genetically engineered into human pluripotent stem cells and the p.A53T mutation was genetically corrected (Soldner et al., 2011). Derived clonal lines were karyotypically normal and maintained neuronal differentiation potential (Soldner et al., 2011). Phenotypic differences were presented in a follow-up publication for the p.A53T point mutation which show increased thioflavin T

staining which detects amyloid aggregates increased alpha-synuclein phospho 129 immunofluorescence which represents a pathological form of alpha-synuclein found abundantly in Lewy bodies (Ryan et al., 2013). Also, mitochondrial function was impaired as shown by an increase in ROS species (Ryan et al., 2013).

In iPSC-derived cortical neurons from a patient with the *SNCA* p.A53T mutation and the *SNCA* triplication nitrosative stress, accumulation of ER-associated degradation (ERAD) substrates and ER stress was observed as an early pathogenic phenotype (Chung et al., 2013). Based on the discovery in a yeast screen, the authors identified a small molecule which activated E3 ubiquitin ligase neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4) and rescued the molecular phenotype in human iPSC-derived cortical neurons (Chung et al., 2013; **Table 1**, Supplementary Table 1).

REPEAT VARIANTS IN THE *SNCA* GENE PREDISPOSE TO PD

Rep-1 Allele

Probably the most studied polymorphism in the 5′ region of the *SNCA* gene is the non-A-beta component of Alzheimer disease amyloid, precursor (NACP)-Rep1 polymorphism of the *SNCA* promoter. The Rep1 dinucleotide repeat is located 9.8 kb upstream of the transcriptional start site of the *SNCA* gene and exhibits five alleles of different sizes. Some studies showed an association of the Rep1 allele with PD (Krüger et al., 1999; Farrer et al., 2001; Tan et al., 2003; Pals et al., 2004; Mellick et al., 2005; Hadjigeorgiou et al., 2006), but other studies could not reproduce this association (Parsian et al., 1998; Khan et al., 2001; Spadafora et al., 2003). There could be multiple reasons for non-replication such as differences in populations, sample size, methodological differences for standardization of sampling and measurements. Only a large collaborative effort for 2,692 cases and 2,652 controls showed the association of the long Rep-1 allele with PD (odds ratio, 1.43) (Maraganore et al., 2006; **Figure 1B**).

The Rep-1 repeat acts as a modulator of *SNCA* transcription and shows a 4-fold increase in promoter activity (Touchman et al., 2001). The longest repeat (263bp allele, risk allele for PD) resulted in a 2.5-fold increase in luciferase activity over the shortest repeat. The 261bp allele (major allele) showed only a 1.5-fold increase over the shortest repeat, whereas the 259 bp allele increased expression by 3-fold (Chiba-Falek et al., 2003). Furthermore, in human brain tissue from 228 PD cases and 144 normal cases, the protective 259 bp (homozygous) allele presented with a 40–50% *SNCA* mRNA reduction in the temporal cortex and substantia nigra (Linnertz et al., 2009).

Modeling of a Genetically Engineered Rep1 Allelic Series in Human Pluripotent Stem Cells

Recent work in human embryonic stem cell (ESC)/iPSC-derived neuronal models in which the four haplotypes (alleles 257/261, 259/261, 261/261, and 263/261) were genetically engineered by CRISPR, the increase in *SNCA* mRNA expression was not confirmed in 25-day neuronal cultures from two different human embryonic stem cell lines (WIBR3 and BGO1; Soldner et al., 2016). However, when comparing these data with the data from human brain, only human brains that carried the homozygous protective allele (259/259) showed a significant downregulation of SNCA mRNA expression (Linnertz et al., 2009). Alternatively, the lack of differentiation expression of the Rep-1 alleles in the model could be due to the neuronal subtypes that were neuronally differentiated. The iPSC-neuronal cultures primarily consist of glutamatergic neurons and *SNCA* gene might not be regulated through the Rep-1 allele like the effect seen in human brain regions from the frontal cortex where no change in *SNCA* mRNA expression was detected in any of the Rep-1 haplotypes (Linnertz et al., 2009). In addition, it could also have a technical explanation with respect to primer design and quantification of the various *SNCA* isoforms and transcripts (Chiba-Falek, 2017; **Table 1,** Supplementary Table 1).

SNCA CT-Rich Haplotype

Recently, an intronic polymorphic CT-rich region was identified in the *SNCA* gene through a genome-wide screen for short structural variants (SSV) (Saul et al., 2016; Chiba-Falek, 2017). The SSV evaluation system focuses on GWAS regions and prioritizes SSVs in close proximity to the GWAS signal based on repeat context, conservation tracks, epigenetic histone marks, transcription factor binding sites, and miRNA binding sites (Saul et al., 2016). The intron 4 *SNCA* CT-rich SSV has four different haplotypes and the risk haplotype is associated with Lewy body pathology in Alzheimer disease. In addition, the *SNCA* CT-rich risk haplotype leads to a higher mRNA expression of *SNCA* in human brain. This suggests that the *SNCA* CT-rich haplotype acts as an enhancer region in the *SNCA* gene (Lutz et al., 2015; **Figure 1B**). An iPSC model for haplotypes of this variant is underway (Chiba-Falek, 2017).

Intron 2 Poly-T

An intronic poly-T polymorphism upstream of exon 3 (rs149886412) comprises three alleles (5T, 7T, and 12T). The most common genotypes are 7T/7T (47.0%) and 7T/12T (34.9%), whereas less frequent alleles are 5T/7T and 12T/12T (8.7% each), and 5T/12T is very rare (0.7%) (Beyer et al., 2007). Interestingly, the length of the poly-T polymorphism is associated with expression of the *SNCA* splice isoform *SNCA* 126. *SNCA*126 levels are lower in blood from carriers with the 5T allele and increased when 12T allele is present (Beyer et al., 2007). Comparing different age groups for the presence of the poly-T allele, the shorter allele 5T/7T was more frequent at a younger age, whereas the 12T allele was more frequent with increasing age in control subjects (Beyer et al., 2007). The length of the poly-T allele could affect the distance to splicing enhancers or silencer, thus modulating alternative splicing for *SNCA* exon 3 (**Figure 1B**).

This polymorphism is another candidate for generating an isogenic panel of pluripotent stem cells similar to Soldner et al. for the Rep-1 allele (Soldner et al., 2016).

GWAS HITS IN THE *SNCA* GENE INCREASE PD RISK

More than 50 studies have been conducted investigating whether certain SNPs within the *SNCA* gene are associated with parkinsonism either as case-control studies or within GWAS (Mueller et al., 2005; Mizuta et al., 2006; Ross et al., 2007; Winkler et al., 2007; Nalls et al., 2014; Campelo and Silva, 2017). Many SNPs have been identified and confirmed throughout the *SNCA* gene encompassing the 5′ region/promoter, intron 4, and 3′ region/3′UTR (**Figure 1B**). Exploring the effects and functional relevance of these SNPs is becoming a new focus of research and we are beginning to unravel the underlying phenomena and mechanisms that lead to increased disease risk for PD and related alpha-synucleinopathies. These functional effects of SNPs can be categorized as (i) changes in levels of total alpha-synuclein mRNA/protein expression, (ii) changes in *SNCA* isoform expression, (iii) changes in DNA methylation of *SNCA* CpG oligodeoxynucleotide regions, and (iv) changes in transcription factor binding/enhancer/repressor sites or miRNA binding sites. Out of 25 PD-associated SNPs, seven have been reported to influence alpha-synuclein levels, however, some findings are controversial (Supplemental Table 2). Six SNPs within intron 4 and 3′ region were studied for total alpha-synuclein mRNA levels in post-mortem brain samples, blood, or *in vitro* with variable findings for alpha-synuclein expression (Fuchs et al., 2007a, 2008; Westerlund et al., 2008; Sotiriou et al., 2009; Mata et al., 2010; Hu et al., 2012; Rhinn et al., 2012; Cardo et al., 2014; Glenn et al., 2017).

One study evaluated whether disease-associated SNPs affect *SNCA* splicing. There are four *SNCA* isoforms that can arise due to alternative splicing of exons 3 and 5: alpha-synuclein 98 (missing exon 3 and 5), alpha-synuclein 112 (missing exon 5), and alpha-synuclein 126 (missing exon 3) in addition to the full length alpha-synuclein 140 (Beyer and Ariza, 2013). Interestingly, alpha-synuclein 126 has been shown to be reduced in DLB whereas isoform 112 is overrepresented in DLB (Beyer et al., 2006). Three SNPs (rs2736990, rs356165, rs356219) facilitate expression of the alpha-synuclein 112 isoform in frontal cortex. The location of the SNPs were predicted to lie within splice enhancer domains which could explain the effect on 112 isoform over total alpha-synuclein 140 expression (McCarthy et al., 2011).

A SNP in intron 1 (rs3756063, CpG19) which is located within the differentially methylated region of the *SNCA* gene, has been consistently described to mitigate hypomethylation in PD cases in blood and brain (Pihlstrøm et al., 2015; Schmitt et al., 2015; Wei et al., 2016).

Conceptually, the alteration of a transcription factor binding site by a SNP is an attractive hypothesis to study. One publication describes a 3′ region SNP (rs356219, A-allele, protective) to preferentially bind the transcription factor yin yang 1 (YY-1). While alpha-synuclein expression was unchanged, the antisense non-coding RNA RP11-115D19.1 was stimulated by YY1 overexpression, whereas knockdown of RP11-115D19.1 increased alpha-synuclein expression.

In addition to gene engineering the Rep1 allele in human iPSCs, Soldner et al. also identified a SNP in intron 4 which is located within an enhancer region (Soldner et al., 2016). By CRISPR/Cas9 mediated genome editing, an allelic series of this SNP was generated in human pluripotent stem cells and the G-allele showed increase in alpha-synuclein expression in neuroprecursor cells and differentiated neurons. Furthermore, in electrophoretic mobility shift assays (EMSA) SNP-dependent binding of empty spiracles homeobox 2 (EMX2) and homeobox protein NKX6-1 with preference for the protective lower expressing A-allele at rs356168 was observed. This is the first study, that used iPSC modeling to interrogate functional effects of PD associated SNPs. The same SNP was subsequently tested for changes in *SNCA* expression in frontal and temporal cortex of a cohort of 134 healthy individuals. That study concluded the opposite effect for the G-allele with an ∼20% decrease in alpha-synuclein mRNA in individuals homozygous for the G-allele (Glenn et al., 2017).

While it is exciting to witness the advancements toward a functional understanding of disease-associated non-coding SNPs, more work is needed to successfully address the complexity of transcriptional regulation in a disease context.

While it is necessary to directly study target tissue, in this case with a human brain, in which transcriptional changes are expected, there are at this point limitations to this approach which may influence the results, such as sample size per genotype, confounding factors which add biological noise, post-mortem interval, mixture of neuronal and glial cell populations, and finally the fact that in post-mortem tissue from patients with neurodegenerative disease, there is usually a moderate to severe neuronal loss of the cell type of interest.

Human stem cell models might be able to mitigate some of these challenges. Gene engineering of human pluripotent stem cells allow for specific generation of genotypes and allelic series of genotypes which should reduce experimental variability. Differentiation protocols allow for high yield of specific cell types which can be further enriched by cell sorting. Lastly, these models can be further manipulated by toxins to address environmental triggers and gene-environment interactions.

METHYLATION IN THE INTRON 1 IS RELATED TO PD PATHOGENESIS

DNA methylation at CpG sites is considered an epigenetic regulation mechanism to fine-tune transcriptional regulation of genes (Li and Zhang, 2014). In general, DNA methylation is considered to repress gene expression. New findings also include hydroxymethylation which seems to be specific for gene regulation in the central nervous system (Wen and Tang, 2014).

The *SNCA* gene contains a large CpG island in the promoter region comprising 70 CpGs, which does not seem to be differentially methylated in PD. However, there is another smaller CpG region in intron 1 of the *SNCA* gene that has been identified to be methylated in cancer cell lines (Matsumoto et al., 2010).

Studies of DNA methylation in PD can be divided into global DNA hydroxy-/methylation (Desplats et al., 2011; Masliah et al.,

2013; Stöger et al., 2017), specific methylation of *SNCA* intron 1 in human brain regions (Matsumoto et al., 2010; de Boni et al., 2011, 2015; Desplats et al., 2011; Guhathakurta et al., 2017) and peripheral tissues (Song et al., 2014; Tan et al., 2014; Eryilmaz et al., 2017; Funahashi et al., 2017; Supplemental Table 3).

Overall, the *SNCA* intron 1 region is hypomethylated with 0.5–3% methylation in brain (de Boni et al., 2011; Guhathakurta et al., 2017) and 6–10% methylation in peripheral blood cells (Song et al., 2014; Tan et al., 2014) and comparison between cohorts of PD and controls show controversial results, possibly due to experimental techniques, small sample size, or mixture of tissue types in samples. None of the studies showed an effect of *SNCA* expression due to hypomethylation of the region.

The studies for global methylation are similarly controversial in their findings with one study found hypomethylation in a PD sample set, whereas another study did not report a difference in global methylation (Desplats et al., 2011; Masliah et al., 2013). Nevertheless, hydroxymethylation was reported to be lower in the cerebellum in PD samples vs. controls (Stöger et al., 2017).

Human pluripotent stem cell models would present a unique model to interrogate DNA methylation changes introduced by environmental toxins or stressors and cell types of interest can be selected or enriched by cell sorting. On the other hand, CRISPR/Cas9 techniques can selectively methylate CpG sites via deactivated Cas9 nuclease and catalytic domain of the DNA methyltransferase DNMT3A to experimentally study effects of DNA methylation on gene expression (Vojta et al., 2016).

SNCA GENE REGULATION BY TRANSCRIPTION FACTORS

Transcription factors are a group of proteins that bind to specific DNA sequences or motifs and are critical for the regulation of gene expression in a temporal and spatial fashion throughout life (Mitchell and Tjian, 1989). The transcriptional regulation of the *SNCA* gene by trans-acting transcription factors is not entirely understood. One of the first reports of transacting factors regulating the *SNCA* gene was poly-(ADP-ribose) transferase/polymerase-1 (PARP-1), a DNA-binding protein and transcriptional regulator, which was shown to bind to the Rep-1 repeat by EMSA, CHIP, and mass spectrometry. When PARP-1 was inhibited, it increased *SNCA* mRNA levels in SH-SY5Y cells (Chiba-Falek et al., 2005). In luciferase assays, PARP-1 binding to the NACP-Rep1 element reduced the transcriptional activity of the REP1/*SNCA* promoter construct (Chiba-Falek et al., 2005).

In *in vitro* studies in SH-SY5Y cells, overexpression of transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) also increases alpha-synuclein expression and eight C/EBPβ interaction motifs CCAAT were predicted within the *SNCA* promoter region (Gomez-Santos et al., 2005).

Next, it was shown that the hematopoietic transcription factor GATA-binding factor 1 (GATA-1) activates *SNCA* transcription in GIE-ER-GATA-1 cells and GATA-1 occupies a specific region in the *SNCA* promoter (Scherzer et al., 2008). The neuronal counterpart GATA-2 is highly expressed in dopaminergic neurons and knockdown of GATA-2 in SH-SY5Y cells resulted in a decrease of alpha-synuclein expression (Scherzer et al., 2008).

Transcription factor zinc finger and SCAN domain containing 21 (ZSCAN21) which is expressed in different brain areas, regulates alpha-synuclein expression (Dermentzaki et al., 2016). Depending on the cell type and maturation level, ZSCAN21 can have antagonistic effects on transcription of alpha-synuclein. Silencing of ZSCAN21 leads to an increase in alpha-synuclein expression in mature cortical cultures of rats. On the contrary, when ZSCAN 21 is knocked down in neurosphere stem cell cultures, alpha-synuclein expression is reduced, but no alterations are observed in postnatal and adult hippocampus. When ZSCAN21 is overexpressed in cortical neurons only *SNCA* mRNA is increased, but not alpha-synuclein protein expression which suggests that ZSCAN21 is not a master regulator and additional regulatory mechanisms are involved (Dermentzaki et al., 2016).

Brenner et al. (2015) was able to detect GATA2 and C/EBPβ in frontal cortex, cingulate gyrus and medulla oblongata and ZSCAN, although expressed at low levels, was detected by IP assays. *In silico* analysis revealed 17 putative binding sites for these transcriptions factors. By ChIP analysis, only two predicted transcription factor binding sites could be confirmed for ZSCAN21 in intron 1 GATA-2 for intron 2 of the *SNCA* gene (**Figure 1B,** Supplemental Table 4). No direct interaction for C/EBPβ was determined (Brenner et al., 2015).

Recently, p53 was shown to have a binding site CATG in murine *SNCA* promoter at positions −970 to −967, and a feedback loop between alpha-synuclein and p53 has been postulated where upon depletion of p53 alpha-synuclein is downregulated (Duplan et al., 2016; Alves da Costa et al., 2017). Follow-up studies in human models should confirm these very interesting findings that could link neurodegeneration and cancer (Alves da Costa et al., 2017).

Human pluripotent stem cell models are ideal to interrogate these questions due to the human genetic background, ability to derive specific cell types and ease of *in vitro* studies with relevant phenotypes.

NON-CODING RNAS IN THE *SNCA* REGION REGULATING *SNCA* EXPRESSION

MicroRNAs (miRs, miRNAs) are a class of single-stranded non-coding RNAs of 18–22 nucleotides and play a key post-transcriptional role in gene expression. miRNAs function by base-pairing with complementary sequences of the target mRNA molecule most commonly within the 3′ untranslated (UTR) region. This binding activates the RNA-induced silencing complex (RISC), leading to downregulation of the gene by cleavage of target mRNAs by RISC, translational repression, or mRNA decapping and decay (Karnati et al., 2015).

There is now growing evidence that alterations of miRNAs play a role in the pathogenesis of PD (Qiu et al., 2015). The first publication that linked miRNA regulation to PD found

that miR-133b is specifically expressed in post-mitotic midbrain dopaminergic neurons and regulates maturation and function via negative feedback loop by paired-like homeodomain transcription factor Pitx3. In human midbrain samples expression of miR-133b is significantly reduced in PD cases (Kim et al., 2007). To date, additional miRNAs have been implicated PD including some that have shown to directly bind to and negatively regulate alpha-synuclein expression (Recasens et al., 2016). Several studies confirmed that both miR-7 and miR-153 are highly expressed in the brain and have been shown to bind to the 3′ untranslated region of *SNCA* and can downregulate *SNCA* expression levels. Interestingly, these two miRNAs have an additive effect on *SNCA* downregulation (Junn et al., 2009; Doxakis, 2010; **Figure 1B**). In addition, miR-34b and 34c as well as miR-140 and miR-223 have been shown *in vitro* assays or have been predicted to bind to the SNCA 3′UTR (Lim and Song, 2014; Kabaria et al., 2015; Wang et al., 2015; Tagliafierro et al., 2017).

Differential miRNA Expression in iPSC-Derived Midbrain and Cortical Neurons

The first study of miRNA assessment in human iPSC models was published by Tagliafierro et al. (2017). Differentiation into two different neuronal cell types, midbrain dopaminergic neurons and cholinergic neurons, were developed to understand regulation of miRNAs in neurons that exhibit histopathological features of PD and DLB. MiR-7-5p, miR-153-3p, and miR223-3p showed higher levels in dopaminergic neurons while miR-140-3p was slightly increased in cholinergic neurons (Tagliafierro et al., 2017) from *in vitro* cultures of a healthy control. When comparing miRNA levels in neuronal cultures from a *SNCA* genomic triplication, a 10-fold decrease in expression levels of miR-7-5p was shown compared to neurons from a healthy iPSC control, while other miRNAs showed similar trends as in control neurons (Tagliafierro et al., 2017). It will be interesting to understand why miR-7-5p levels are downregulated and how this might contribute to neurodegeneration of dopaminergic neurons (**Table 1,** Supplementary Table 1).

DISCOVERY OF NOVEL REGULATORY ELEMENTS BY COMBINING *IN SILICO* COMPARISON WITH *IN VITRO* VALIDATION

Non-coding conserved genomic regions (ncECRs) within a certain gene, or even up to several hundred kilobases away, can serve as enhancers, silencers, or modifiers to ensure the accurate temporal and spatial expression of a gene by recruiting transcription factors that bind to them.

Comparative genomics is based on the prioritization of a genomic region by searching for highly conserved non-coding sequences between several species to identify potential functional regulatory elements. The rationale behind this approach is that functional or regulatory sequence elements are under a selection pressure and do not diverge as rapidly as "neutral" sequences. By comparative analyses between different species (e.g., human-mouse) or multiple comparisons including fish, non-coding conserved regions can be recognized, which could harbor gene regulatory function (Ahituv et al., 2004; Bird et al., 2006). Examples that this comparative genomics approach is useful for showing the identification of functional variants in non-coding regulatory regions in otherwise mutation-negative families (Marlin et al., 1999; Lettice et al., 2003; Sabherwal et al., 2007), understanding of regulatory elements within disease genes such as Ret proto-oncogene (RET) and Methyl-CpG binding protein 2 (MECP2) (Grice et al., 2005; Liu and Francke, 2006), or identification of a functional SNP responsible for Warfarin toxicity (Rieder et al., 2005). In a pair-wise comparison of the *SNCA* genomic region, 32 evolutionary conserved DNA sequences with high homology to mouse were identified and 11 conserved sequence elements showed an increase or reduction of luciferin activity which indicates cis-regulatory function on gene expression which need to be further evaluated to be linked to alpha-synuclein expression (Sterling et al., 2014).

NEW HIGH-THROUGHPUT TECHNOLOGIES TO DISSECT TRANSCRIPTIONAL REGULATION

Even though many mechanisms of transcriptional regulation of the *SNCA* gene at the physiological level and in context of disease have been studied, many areas still remain controversial and need further evaluation and confirmation. Human pluripotent stem cell models could become a new benchmark for success as they can be more tightly controlled via new gene engineering technologies allowing the creation of allelic series of human stem cell lines with defined genotypes. In addition, novel high-throughput technologies could pave the way to identify regulatory elements at a much larger scale.

Recent advancements in technology such as CRISPR gene manipulation allows us now to directly interrogate genomic regions in the context of endogenous genes and in the target tissue which makes the results easier to interpret. Two groups identified non-coding regulatory elements by high-throughput CRISPR screens (Fulco et al., 2016; Sanjana et al., 2016). These pooled CRISPR screens utilize CRISPR interference which can alter chromatin state through a Krüppel associated box (KRAB) effector domain fused to catalytically dead Cas9 (dCas9), but do not introduce permanent mutations. This allows for the characterization of the regulatory effects of up to one megabase of genomic sequence at a given locus (Sanjana, 2016; Wright and Sanjana, 2016) and several thousand guide RNAs can be simultaneously tested. Fulco et al. assessed 1 Mb sequence around myelocytomatosis oncogene cellular homolog (MYC) and GATA-binding factor 1 (GATA1) and identified 9 enhancers (Fulco et al., 2016), whereas Sanjana et al. targeted ∼700 kb of sequence around the genes neurofibromatosis type 1 (NF1), neurofibromatosis type 2 (NF2), and cullin 3 (CUL3) and found non-coding regions that modulate drug resistance (Sanjana et al., 2016). This CRISPR interference screen might be a tool to identify and confirm regulatory elements in the *SNCA* genomic locus.

To better understand the functional effects of non-coding SNPs, a new pipeline was developed which includes fine-mapping, epigenomic profiling, and epigenome editing and then interrogation for causal function by using genome editing to create isogenic pluripotent stem cell lines (Spisák et al., 2015). A very similar approach was successfully implemented by Soldner et al. (2016) who assessed histone acetylation, generated panel of isogenic lines with series of genotypes, and then tested the expression of the *SNCA* gene (Soldner et al., 2016).

COMBINING HUMAN PLURIPOTENT STEM CELL MODELS WITH POST-MORTEM TISSUES TO DECIPHER THE ROLE OF SNCA TRANSCRIPTIONAL REGULATION IN NEURODEGENERATIVE DISEASE

To further advance the field of studying functional effects of disease-associated risk variants, it is critical to contrast and compare different models and tissues. This review focuses on the use of human tissues (primarily brain and blood samples) and how human ESC/iPSC models can complement such case-control series.

Studies of human brain are very valuable, however, there are several challenges when assessing human brain, such as biological differences and confounding factors like age, gender, environmental exposure/lifestyle, disease duration, concomitant disease. Next, circumstances of death, post-mortem interval, preservation methods, and dissections protocols of the tissue vary and influence quality of samples. Since transcriptional regulation is cell type-specific, therefore the assessment of a mixed neuronal population can mask changes in rarer cell types, contrariwise the isolation of cellular subtypes in frozen brain using e.g., laser-capture technique or cell sorting is technically demanding. Lastly, the cell type of interest in a brain area might have been already degenerated during the disease process, thus the target cell type of interest is no longer present.

Human iPSC models on the other hand can address some of the challenges of studying human brain. Human iPSCs have a human genetic background and are derived from patients with or without known disease-causing mutations. These iPSC cultures can be genetically engineered to create isogenic cell lines or allelic series that only differ by the introduced genetic variant thus greatly reducing biological noise. What should be considered when studying *SNCA* transcriptional regulation in human iPSC-differentiated cultures is the temporal manipulation of *SNCA* gene expression or introduction of mutations *in vitro* e.g., by CRISPR. As described for the SNCA CNVs, a CNV deletion resulting in presumably 50% reduction in gene expression can potentially contribute clinically to developmental delay or autism. Depending on the introduction of *SNCA* transcriptional modulation, there might be effects in differentiation efficiency, phenotypic stability and outcomes. Furthermore, these iPSCs can be differentiated into many cell and tissue types of the human body. *In vitro* differentiation protocols are constantly being improved to faithfully mimic the target cell types of interest not only for expression of cell markers, but also for physiological function, e.g., dopaminergic neurons or organoids for PD (Jo et al., 2016; Kirkeby et al., 2017), cholinergic neurons for DLB/Alzheimer-related DLB (Duan et al., 2014), enteric nervous system (Workman et al., 2017), or oligodendrocytes for MSA (Djelloul et al., 2015). Side-by-side comparison of human primary tissues with evolving human iPSC models can inform and complement the current experimental approaches for transcriptional regulation.

CONCLUSION

Transcriptional regulation is complex and requires both cis-acting genomic elements as well as trans-acting transcription factors in a temporal and tissue-specific manner. Alterations introduced by mutations or epigenetic changes could disturb this tightly regulated system and lead to diseases such as PD, LBD/Alzheimer-related Lewy body disease and other alpha-synucleinopathies. These diseases share alpha-synuclein protein conformational changes and aggregation as a common denominator; however, the distribution, progression, and severity differ. Understanding the temporal and cell-type specific regulation of alpha-synuclein might provide insights in the development and distribution of alpha-synucleinopathies as the disruption of transcriptional regulation could be the underlying culprit ultimately driving shared pathological processes in these diseases.

AUTHOR CONTRIBUTIONS

BS, DP, and DS: Contributed to the conception and design of the study; DP: Prepared UCSC custom tracks and reviewed iPSC publications; BS: Wrote the first draft of the manuscript; DP and DS: Wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This project was supported by the California Institute of Regenerative Medicine Quest—Discovery Stage Research Projects (DISC2-09610) and CIRM Bridges II (EDUC2-08394).

ACKNOWLEDGMENTS

We acknowledge Cath Tyner from UCSC for assisting on the design of the UCSC Genomic Browser custom tracks, Kim Christina Krieg for careful review of the manuscript, and Cindy Gamble, who is an invaluable sounding board for new ideas.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00199/full#supplementary-material

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