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Stem cell derived monkey neural progenitor cells as a platform for translational research in Huntington's disease

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Abstract

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Richard Lee Carter III

Huntington's disease (HD) is a dominantly inherited, neurodegenerative disorder caused by the expansion of glutamine residues in the N-terminal region of the huntingtin (HTT) protein. The disease results in progressive neuronal loss, leading to motor, cognitive, and psychiatric impairment. HD is a fatal disorder for which there is no cure. Existing treatments are aimed at the alleviation of symptoms; however, therapies that slow or reverse disease progression have yet to be implemented. A major focus of HD research is directed towards understanding and modeling mechanisms contributing to the degeneration of neurons. Pluripotent cellular models have been explored as a powerful tool to understand disease related changes as neurons develop. Pluripotent cell models also show promise in providing opportunities for the discovery of novel therapies. Here we report the establishment of neural progenitor cell (NPC) lines derived from pluripotent stem cells (PSCs) of transgenic HD monkeys. NPCs are mutipotent, self-renewing neural precursors committed to the neuronal lineage. PSC derived monkey NPCs are capable to generate neurons upon in vitro neural differentiation, and following xenotransplantation into mice striatum. Additionally, we show that HD neural cells develop cellular features of HD, including the formation of nuclear inclusions, oligomeric mutant HTT aggregates, and increased apoptosis upon cell stress. These phenotypes are rescued by genetic suppression of HTT and pharmacological treatment, demonstrating the ability of our HD cell model to respond to therapeutic treatment. The development and reversal of HD associated phenotypes in neural cells from HD monkeys provides a unique non-human primate platform for modeling HD pathogenesis and evaluating therapeutics that could be assessed further in HD monkeys.

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List of Abbreviations

ASO: antisense oligonucleotides

BDNF: brain-derived neurotrophic factor

DARPP32: dopamine and cAMP-regulated neuronal phosphoprotein

DCX: doublecortin

EB: embryoid body

ESC: embryonic stem cell

FACS: Fluorescence-activated cell sorting

FGF: fibroblast growth factor

GABA: γ-aminobutyric acid

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GFP: green fluorescent protein

H_2O_2: hydrogen peroxide

HD: Huntington's disease

HTT: huntingtin

iPSC: induced pluripotent stem cells

MAP2: microtubule-associated protein 2

miRNA: micro RNA

MSI1: musashi-1

MSN: medium spiny neuron

NEP: neuroepithelial progenitor

NES: nestin

NeuN: neuronal nuclei

NHP: non-human primate

NMDAR: *N*-methyl-D-aspartate receptor

NPC: neural progenitor cell

Oct4: POU5F1 (POU class 5 homeobox 1)

Pax6: paired box gene 6

PSC: pluripotent stem cell

RNAi: RNA interference

SHH: sonic hedgehog homolog

shRNA: short-hairpin RNA

Sox2: SRY (sex determining region Y)-box 2

CHAPTER 1

General Introduction

This chapter contains sections that were published in two invited review articles:

Carter, R. L. Chan, A. W., 2012. Pluripotent Stem Cells Models for Huntington's Disease: Prospects and Challenges. J Genet Genomics. 39, 253-259.

Chen, Y., Carter, R. L., Cho, I. K.Chan, A. W. S., 2014. Cell-based therapies for Huntington's disease. Drug Discovery Today.

1.1 Huntington's Disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder that affects approximately 7-10 per 100,000 individuals, and is most prevalent in populations of European origin (Gil and Rego, 2008). Individuals with HD generally develop adult onset symptoms that include motor dysfunction classified as chorea, progressive cognitive impairment, and psychiatric disturbances (Zuccato et al., 2010). The disease is caused by an unstable expansion of CAG repeats in exon 1 of the Huntingtin gene *IT15* (MacDonald et al., 1993). In non-HD populations the repeat average is less than 36 units, however individuals with repeats of ≥ 40 units are at risk for developing HD as their lives progress (Li and Li, 2006; Gil and Rego, 2008) . The repeat expansion produces a stretch of glutamine residues spanning the N-terminus of the Huntingtin protein (HTT), consequently resulting in a protein associated with aggregation and a gain-of-function toxicity (Goldberg et al., 1994). Currently HD is a fatal diagnosis for which there is no cure. Existing treatments are aimed at the alleviation of symptoms, however therapies that slow or reverse disease progression have yet to be implemented. Studies suggest that mutant HTT (mHTT) impacts many cellular processes leading to neurotoxicity, specifically damaging cortical and striatal medium spiny neurons (MSNs) (Davies and Ramsden, 2001; Li and Li, 2006; Gil and Rego, 2008; Imarisio et al., 2008; Ross and Shoulson, 2009; Sassone et al., 2009; Johnson and Davidson, 2010). The mechanisms placing these specific neuron populations at risk is a critical question.

The hallmark of HD is neurodegeneration, predominantly in the striatum and cortex (Sapp et al., 1997). Nuclear inclusions of mHTT in striatal neurons actually precede symptom onset (Laforet et al., 2001). A loss of enkephalinergic neurons in the

external segment of the globus pallidus is also typical of pre-symptomatic HD (Deng et al., 2004). During the symptomatic phase of HD, the external segment of the globus pallidus, the substantia nigra pars reticulata, and the substantia nigra pars compacta are severely degenerated (Deng et al., 2004). GABAergic and parvalbuminergic striatal projection neurons are also severely affected (Hedreen and Folstein, 1995). Initial insight regarding the neuropathology of HD was identified in post-mortem brain tissue from human HD patients (DiFiglia, 1997; Sapp et al., 1997). This method of study is limited in that it represents the end-stages of the disease. Observations made from post-mortem brain tissue have identified key phenotype hallmarks associated with HD, however these may only uncover secondary phenotypes leaving the primary causes unidentified (Marchetto et al., 2011).

Genetics animal models of HD have also contributed to understanding disease neuropathology. Genetic HD models allow researchers to study the progression of disease longitudinally providing greater insight into alterations in the central nervous system at early and symptomatic time points (Zuccato et al., 2010; Pouladi et al., 2013). A number of genetic approaches have been employed to generate HD models in both mammalian and non-mammalian species. The phenotype outcome and severity observed is heavily influenced by a number of distinguishing factors. These factors include: constructs created using either a full-length or truncated *HTT* allele; the size of the CAG repeat mutation included in the construct; the promoter driving expression; and whether the animal is transgenic for human *HTT* or carries CAG mutations to the endogenous *HTT* copy (Zuccato et al., 2010; Pouladi et al., 2013). Together these factors combine to

produce much variability between models, however this can be useful in addressing specific questions in HD.

The versatility of HD mice models have made them the most widely used and studied animal system. HD mice mirror cortical and striatal neural atrophy that is observed in humans (Mangiarini et al., 1996; Li et al., 2005). In additional to reduced brain volumes and neuronal loss, HD mice also develop mHTT nuclear inclusions throughout affected brain regions (Davies et al., 1997). HD mice are well suited for the assessment of motor and cognitive HD phenotypes due to the large battery of established cognitive behavioral test that exist for rodents. These strengths highlight the utility of HD mouse models, however physiological differences between rodents and humans limit the types of investigation that can take place (Sasaki et al., 2009; Chan, 2013; Pouladi et al., 2013). HD mouse lines that have more severe phenotypes show a reduced life span, thus preventing the investigation of progressive age related phenotypes (Pouladi et al., 2013). Moreover, the difference in brain size and anatomical complexity, is not ideal for therapeutic studies that are sensitive to delivery methods (McBride et al., 2011; Hashimoto et al., 2012; Perrier and Peschanski, 2012; Pouladi et al., 2013). For example, studies that evaluate a compound or virus's ability to permeate multiple affected brain regions would translate poorly from mice to humans. For these types of studies large animal models may be better suited (McBride et al., 2011; van der Bom et al., 2011; van den Bogaard et al., 2012; Emborg et al., 2013).

Of the genetic HD animal models reported, NHPs are the most similar in genetic constitution and neurodevelopment to humans. Our research group reported the first transgenic HD NHP in rhesus macaques (Yang et al., 2008; Chan et al., 2014). HD

monkeys expressed exon 1 of the human *HTT* gene with expanded CAG repeats. These monkeys developed neuropathology similar to humans, including the development of nuclear inclusions, neuropil aggregates, and cortical atrophy. Alongside neuropathological features, HD monkeys also exhibited motor defects including chorea and dystonia (Yang et al., 2008). Another report from our group provided data suggesting that HD monkeys showed progressive HD related transcriptional changes in peripheral blood (Kocerha et al., 2013). Peripheral blood samples were collected from control and HD monkeys over a period of 39 months. From peripheral blood, mRNA transcriptional candidates were analyzed across time points and compared to transcriptional profiling data reported from human HD studies. This study revealed 11 candidate transcripts that were dysregulated in HD monkeys and correlated with human findings (Kocerha et al., 2013) Together these studies demonstrate the potential of transgenic NHPs to model disease progression and identify biomarkers comparable to human disease.

Similar to rodent models, HD NHPs models are useful in behavior studies due to a well-established battery of behavioral and motor test (Nemanic et al., 2004; Bachevalier and Nemanic, 2008; Bachevalier et al., 2011; Chan, 2013; Chan et al., 2014). In parallel with transcriptional profiling studies, HD monkeys also demonstrated development of learning and memory deficits, along with reduced striatal and hippocampal volume compared to wildtype (WT) monkeys (Chan et al., 2014). This study helps to highlight a number of advantages specific to NHP models of HD. Anatomical similarities to humans make NHPs better suited to replicate fine motor impairments associated with HD. Moreover, their longer life span compared to other animal models is advantageous for longitudinal studies (Chan, 2009; Chen et al., 2012; Chan, 2013). Another advantage

unique to NHPs is the opportunity for MRI imaging of the brain (Chan, 2013; Pouladi et al., 2013; Chan et al., 2014). This is an important feature for investigating neuropathology progression at pre-symptomatic and post-symptomatic time points non-invasively, as was demonstrated by Chan an colleagues (Chan et al., 2014). NHP studies are limited in the cost and time necessary to establish and maintain a monkey cohort. Due to small cohort size, HD NHPs studies may struggle to reach statistically significant conclusions (Pouladi et al., 2013). Nonetheless, HD NHPs are useful tools in a diverse set of genetic animal models of HD, allowing for a wide array of questions in HD to be interrogated.

1.2 Cellular pathology of Huntington's disease

HD is a monogenic disorder owing to a single mutation in a single gene. Despite the well characterized genetic etiology, mHTT is linked to the disruption of numerous cellular pathways (Labbadia and Morimoto, 2013) (**Figure 1-1).** mHTT aggregates were one of the first pathologic events described in HD (Davies et al., 1997; DiFiglia, 1997), and are considered a key hallmark of the disease. mHTT aggregates consist of β-sheet rich fibrils mediated by the aggregation of the poly glutamine stretch of HTT (Scherzinger et al., 1997). mHTT aggregates are observed as both neuropil aggregates and intranuclear inclusions in neuronal cells (DiFiglia, 1997). Evidence has suggested that aggregation of the protein confers both a gain-of-function, and simultaneously a loss-of-function toxicity in the cell (Goldberg et al., 1994; Zuccato et al., 2010). By sequestering transcription factors and other critical proteins, mHTT aggregates disturb numerous cellular processes. Additionally the toxic aggregates may impair HTT normal functions within the cell further exacerbating the aggregate mediated cellular toxicity

(Zuccato et al., 2010). This is a feature that is shared by a number of other neurodegenerative disorders including β-amyloid plaques in Alzheimer's disease, α-synuclein in Parkinson's disease, and TDP-43 in amyotrophic lateral sclerosis (Labbadia and Morimoto, 2013). Aggregation is also a feature of other polyglutamine disorders including dentato-rubral and pallido-luysian atrophy and several forms of spino-cerebellar ataxia (Ross and Poirier, 2004; Orr and Zoghbi, 2007). This shared aggregated protein pathology underscores the logic that similar mechanisms of toxicity may be shared by these neurodegenerative diseases, and furthermore suggest that advancements in reducing aggregates in disease cells may provide mutual therapeutic improvements across neurological disorders characterized by this phenotype.

Mitochondrial function and energy metabolism is a critical cellular pathway that is impaired in HD (Costa and Scorrano, 2012). Mitochondrial impairment was first linked to HD when selective degeneration of MSNs was observed following administration of 3-nitropropionic acid in rats (Beal et al., 1993). 3-nitropropionic acid is an inhibitor of succinate dehydrogenase leading to energy impairment and damage to the mitochondrial membrane (Beal et al., 1993; Garcia et al., 2002). This finding suggested that damage to mitochondria was somehow involved in replicating a phenotype similar to the loss of MSNs in HD. It is also suggested that mHTT binds to the outer membrane of mitochondria leading to the inhibition of mitochondrial complex II (Panov et al., 2002; Choo, 2004). This results in the depletion of ATP and an increase in reactive oxidative species (ROS) during cell metabolism due to electron transport chain impairment (Browne et al., 1997). Dysregulation of ROS causes numerous negative effects on cell health by increasing oxidative stress burden (Costa and Scorrano, 2012). In HD, inducing

oxidative stress conditions lead to increased apoptosis in cells expressing mHTT, and has been shown to produce somatic expansions of the CAG repeat, further exacerbating cytotoxicity (Coyle and Puttfarcken, 1993; Butterfield et al., 2001;
The Hd iPSC Consortium, 2012; Jonson et al., 2013). mHTT binding to the mitochondrial membrane is also associated with defects in calcium handling (Panov et al., 2002; Choo, 2004). In a study using isolated normal mitochondrial, exposure to mutant protein *in vitro* led to less resistance after calcium challenge and reduced calcium uptake capacity (Panov et al., 2003). The impact of mHTT on calcium homeostasis is an important driver of neuronal injury as elevated intracellular calcium levels activate apoptosis pathways (Arundine and Tymianski, 2003; Milakovic et al., 2006). Although other energy independent mechanisms are at play in HD pathogenesis, perturbations in mitochondrial function, oxidative stress handling, and calcium regulation have a clear role in the pathology of the disease.

Disturbances in synaptic physiology are a common pathology in a number of neurological disorders including HD. Excitatory neurotransmission through N-methyl-D-aspartate receptors (NMDAR) are important for healthy brain functions and neuron viability (Raymond et al., 2011). In HD, alterations in the activity of NMDARs play a role in gluatmatergic excitotoxicity, consequently leading to selective degeneration of medium spinal neurons (MSN) in the striatum (Okamoto et al., 2009; Milnerwood et al., 2010; Marco et al., 2013). NMDAR mediated toxicity is hypothesized to be a major contributor to the enhanced vulnerability of MSNs due to intrinsic properties of glutamate signaling and NMDAR expression in this particular subset of neurons (Raymond et al., 2011; Labbadia and Morimoto, 2013). Evidence for this theory was first shown in

chemical induced lesion models of HD. Glutamate agonist including kainic acid and quinolinic acid led to a pattern of striatal MSN degeneration closely resembling that in HD (Coyle and Schwarcz, 1976; Schwarcz and Coyle, 1977; Schwarcz et al., 1983; Sanberg et al., 1989) . Further studies found that excitotoxicity closely correlated with alterations in NMDAR trafficking in the neuron, in addition to receptor stability and expression levels (Coyle and Puttfarcken, 1993; Raymond et al., 2011). These findings were further substantiated by the rescue of HD phenotypes using NMDAR antagonist, thus supporting the role of NMDAR activity in HD neuronal pathology (Bogdanov et al., 2001). It is clear that glutamatergic excitotoxicity is associated with mHTT, however the extent to which this pathway influences phenotype, and how this critical synaptic mechanism can be repaired is under much investigation. NMDAR activity normally plays a role in pro-survival cellular pathways, however extrasynaptic NMDAR activity leads to activation of apoptotic signaling proteins as well as mitochondrial energy failure and Ca^{2+} overload (Arundine and Tymianski, 2003; Hardingham and Bading, 2010). Considering the impact of NMDAR activity in HD, models that recapitulate this phenotype would be instrumental in identifying therapeutic compounds that reduce cytotoxicity, and could prove useful in developing practical therapies for a spectrum of neurological disorders that are impacted by a similar disease pathway.

There are many complex cellular pathways that are impacted by mHTT in HD. Although no one pathway disturbance can fully account for the myriad of pathogenic features associated with neurodegeneration, many of these pathways are shared amongst similar neurological disorders. As we attempt to bridge the connections and identify therapeutic targets that can ameliorate the dysfunction found in HD neurons, it is

important that tools are developed to provide insight into these complex pathologies. The study of human post-mortem brains and HD animal models, has provided insight and advances in HD research, however these studies are more adept at investigating the gross neuropathology and behavior impairments of the disease. To fully uncover and target intercellular pathways, *in vitro* models derived from both HD patient and animal models are needed to elucidate the cell-autonomous impacts of mHTT. Using *in vitro* and molecular techniques to investigate intracellular alterations may provide a more focused view of pathogenesis and may lead to a better understanding of the intricate mechanisms that play a part in HD neurodegeneration.

Figure 1-1

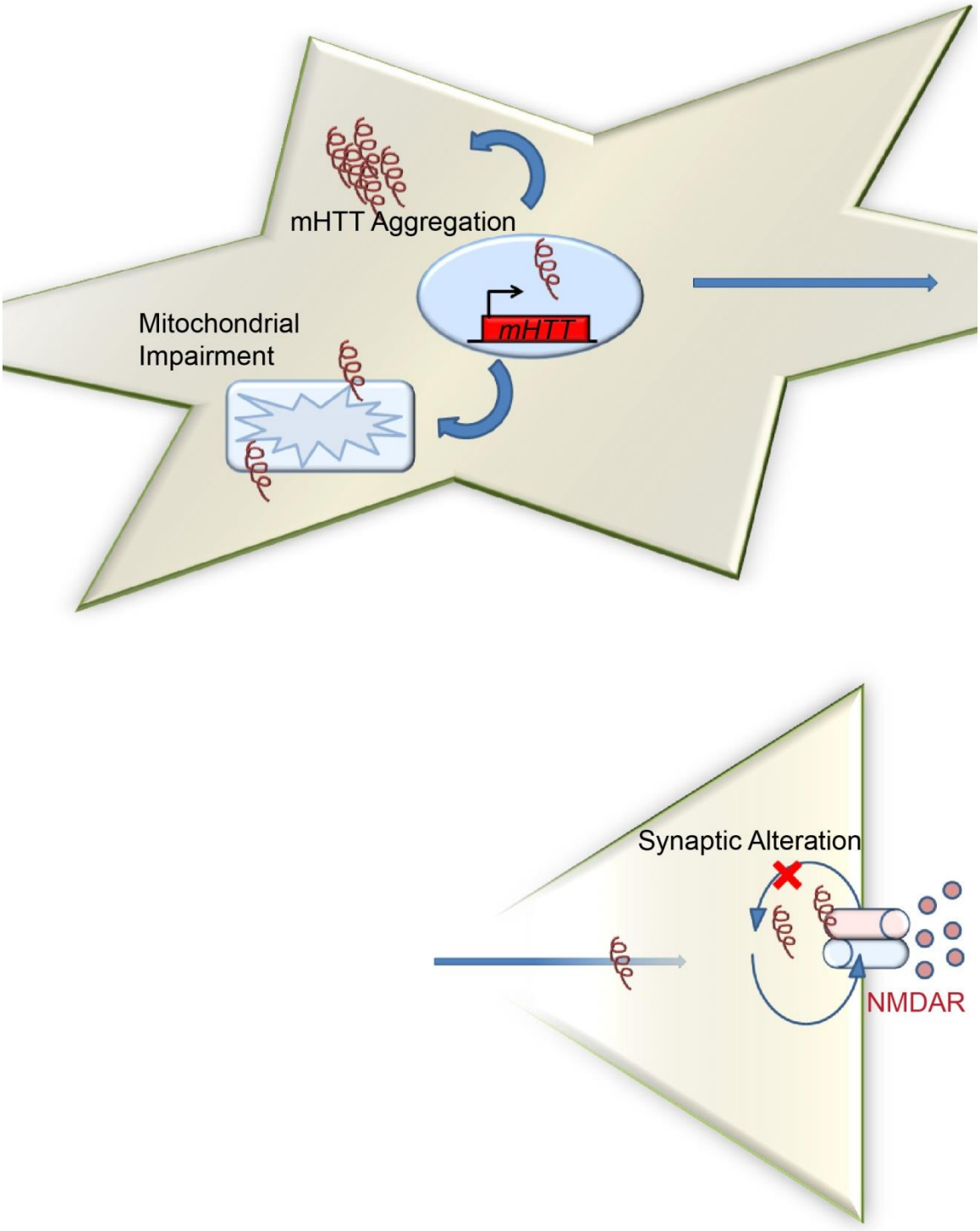

mHTT Aggregation
mHTT
Mitochondrial
Impairment
Synaptic Alteration
NMDAR

Figure 1-1. Cellular pathways disrupted in HD.

Mutant HTT (mHTT) forms protein aggregates that bind to multiple organelles in the cell. This results in disturbances to many key cellular processes having consequences on the function and health of affected neurons in the central nervous system. This schematic illustrates a neuron expressing mHTT. As mHTT is expressed, expanded CAG repeats lead to an aggregated conformation of the protein. mHTT binds to mitochondria, consequently impairing mitochondrial function and energy metabolism. mHTT is found at synaptic terminals and disrupts synaptic signaling, as well as receptor recycling. Other intracellular pathways including transcription, protein degradation, and calcium homeostasis (not shown here) are impaired in HD cellular pathology, and are suggested to further exacerbate pathogenesis in a cumulative manner.

1.3 Pluripotent stem cells as a model for neurodegeneration

Pluripotent stem cells (PSCs) have demonstrated much promise in investigating neurological and neurodegenerative diseases. Advancements in the generation, differentiation, and application of patient specific PSCs provide researchers with a powerful resource towards understanding complex cellular pathologies associated with disease. Studies in diseases including HD, amyotrophic lateral sclerosis, Parkinson's disease, and schizophrenia have shown evidence supporting the recapitulation of neuronal defects from patient specific PSCs (Grskovic et al., 2011; Bellin et al., 2012). Impaired synaptic activity, transcriptional dysregulation, and decreased energy metabolism have been revealed using PSC derived neural cultures (Kaye and Finkbeiner, 2013). By looking at these disease associated pathways *in vitro*, insights into the roles these phenotypes play in a cell-autonomous manner provide novel drug targets for therapy. PSCs are representative of their respective patient population, providing a platform to screen candidate compounds in cells that are of the same genetic background as the donor. By combining assays that robustly quantify phenotypic readouts with high-throughput technologies, PSCs provide a resource for the discovery of new therapies for neurological diseases.

PSCs can be broken down into two classes: embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs and iPSCs are biologically very similar, but originate from different sources, and are generated by different methods. ESC are pluripotent cells that are established from the inner cell mass of the blastocyst. In contrast, iPSCs are reprogrammed from somatic cells, typically fibroblast, to pluripotency through genetic manipulation, chemical induction, or small molecules

(Bellin et al., 2012; Yamanaka, 2012). Reprogramming is accomplished by forced expression of transcription factors, first described by Takahashi *et al.* (Takahashi and Yamanaka, 2006), that reestablish pluripotency. Although originally this was accomplished by viral incorporation of pluripotency genes into the genome, advances in this technology have led to other strategies of introducing these genes in a non-integrating manner (Robinton and Daley, 2012). Reprograming has also been accomplished using DNA-free techniques. Alternative strategies to avoid introduction of exogenous genes involve the delivery of reprogramming proteins, synthetic mRNAs, small molecules, and chemical induction (Zhou and Zeng, 2013). Despite the novelty and demonstration of success of DNA-free induction, the efficiency and reproducibility of these methods are still under investigation, and have not been widely adopted.

These two cell types, collectively called PSCs, have the potential to proliferate indefinitely and differentiate to any cell type in the body. This is an attractive feature, especially in the case with neurological disorders, where access to human neuronal populations is a major challenge (Lunn et al., 2011; Marchetto et al., 2011). Creating and optimizing protocols for neural differentiation to disease relevant neural cell types is an active area of study (Bellin et al., 2012; Gage and Temple, 2013). In HD, GABA MSNs derived from patient iPSCs have been used in studies to model phenotypes and assess therapeutic options (An et al., 2012; Chae et al., 2012; Jeon et al., 2012; Juopperi et al., 2012; The Hd iPSC Consortium, 2012). Differentiation to other disease relevant neural cell types have been reported including, dopaminergic neurons, motor neurons, and cholinergic neurons (Liu et al., 2013). Methods for *in vitro* differentiation involve carefully regionalizing cells toward a specific neuronal lineage from a pluripotent cell

stage (Conti and Cattaneo, 2010). This often involves the isolation of neuroepithelial progenitors (NEPs) from PSCs cultures (Sonntag et al., 2007). Following isolation of NEPs, specific combinations of growth factors and neural supplements are used in carefully defined growth medium to pattern cells towards their final neuron subtypes (Liu et al., 2013). The result is often a heterogeneous mixture of glia and immaturely differentiated cells that may require further selection (Pruszak et al., 2007; Yuan et al., 2011). Improvements towards generating more uniform neuronal cultures would benefit disease modeling in that more informed interpretations could be made about the specific neural cell types that develop phenotypes (Tiscornia et al., 2011; Bellin et al., 2012; Tabar and Studer, 2014). Furthermore, in late stage diseases such as Huntington's and Alzheimer's disease, therapeutic screening would require mature neuronal population that mirror the maturity and physiological properties of neurons found in the disease patient (Robinton and Daley, 2012; Tabar and Studer, 2014). .

Although methods for differentiation to relevant neural types continue to improve, techniques are highly variable among different groups. A major concern in comparing results from independent studies is the level of cell-line to cell-line variation (Bellin et al., 2012). This may arise from the diversity of cell culture procedures that have been published (Conti and Cattaneo, 2010). In order to improve reproducibility and account for technical variations between studies, more established and standardized protocols need to be in place.

Challenges also remain in developing methods to efficiently generate homogenous neural populations and overcome hurdles associated with PSC culture. The generation and maintenance of PSCs is still a technical and time demanding practice

(Reubinoff et al., 2001; Dhara et al., 2008). Moreover, neural differentiation form ESCs and iPSCs as a starting point gives rise to a multilineage population from three embryonic germ layers (Reubinoff et al., 2001; Gerrard et al., 2005; Itsykson et al., 2005; Dhara et al., 2008; Ruggieri et al., 2014). Therefore a uniform, enriched population of cells committed to the neuronal lineage is desired. Two strategies that may overcome these roadblocks include establishing neural progenitor populations and generating neurons by direct reprogramming from somatic cells. Neural progenitor cell (NPCs) represent an enriched, rapidly expandable progenitor cell population committed to the neuronal lineage, and is shown to generate neurons at much higher yield and purity compared to mutltilineage differentiation methods (Dhara et al., 2008; Conti and Cattaneo, 2010; Tabar and Studer, 2014). NPCs also hold advantages over PSCs as a donor cell type in cell replacement therapy. Undifferentiated PSCs are unacceptable in cell replacement applications due to high risk of tumor formation (Fong et al., 2010; Ruggieri et al., 2014). In contrast, transplantation of NPCs mitigate the risk of tumor formation, and retain capability to differentiate to neurons and glia (Ma et al., 2012; Emborg et al., 2013; Liu et al., 2013; Lu et al., 2014). NPCs also provide an *in vitro* platform to model neurogenesis and to uncover early pathogenic events in neurodegenerative diseases (Reubinoff et al., 2001; Carter and Chan, 2012; Chen et al., 2014).

In additional to establishing NPCs, directly reprogramming somatic cells to neurons has been explored. In this strategy, somatic cells are reprogrammed toward neuronal cells types by forced expression of neurogenic genes in a manner similar to the pluripotency factors used to reprogram iPSCs (Qiang et al., 2013; Ruggieri et al., 2014). These induced neurons may then be used in disease modeling and screening applications.

By skipping the derivation of iPSCs, this method circumvents the time consuming and labor intensive steps involved in iPSCs generation and maintenance. Despite its apparent utility in modeling neurological disease, the efficiency and cost of this strategy remains a limitation in its wide scale adoption (Qiang et al., 2013).

Patient specific iPSCs have rapidly emerging applications in disease modeling and drug discovery research. The 'disease in a dish' approach has the potential to model cellular pathology in neurological disorders, serve in high-throughput drug screening applications, and advance cell replacement therapy (Saha and Jaenisch, 2009; Inoue and Yamanaka, 2011; Bellin et al., 2012; Carter and Chan, 2012). Furthermore, applying this approach in PSCs derived from animal models provides opportunities to assess the safety and efficacy of therapeutic approaches. Methods for efficiently generating neuronal populations, either by deriving NPCs or induced neural reprogramming, are still developing. However, as techniques become more refined, PSCs may provide a powerful tool in neurodegenerative disease research.

1.4 Stem cell derived models of Huntington's disease

In the previous section the vast potential of PSCs in neurological disease was described. Evidence of that potential has been demonstrated in HD across a variety of different applications, as well as animal systems (**Table 1-1)**. A number of transgenic animal models have been successfully developed for the study of HD (Faber et al., 1999; Gunawardena et al., 2003; von Horsten et al., 2003; Miller, 2005; Yang et al., 2008; Jacobsen et al., 2010; Yang et al., 2010; Pouladi et al., 2013). As genetic HD animal have allowed researchers to study the progression of disease longitudinally, ESC and iPSCs

derived from these models provide further insight into causal disease mechanisms (Carter and Chan, 2012).

The R6/2 mouse is one of the most commonly used HD mouse models. R6/2 mice express a truncated N-terminal fragment of human mHTT with 144 CAG repeats driven by the human *HTT* promoter (Mangiarini et al., 1996). Using the R6/2 mice, Castiglioni *et al* reported the generation of 11 mouse HD-iPSC lines originating from fibroblast of R6/2 mice (Castiglioni et al., 2012). Mouse iPSCs were derived by reprogramming fibroblasts using Yamanaka factors. The authors report transcriptional alteration of genes involved in cholesterol biosynthesis and lysosome biogenesis. Their cellular models, however, did not show any differences when compared to wild type cells in regards to differentiation potential and proliferation rate (Castiglioni et al., 2012). Dong *et al* (2011) reported an alternative approach to modeling HD in a rat *in vitro* model. The authors transfected rat NPCs with an *HTT* exon 1 transgene with expanded CAG repeats. From this system the authors observe mHTT aggregation and neuronal death paralleling neural development. The observed phenotypes were exacerbated in the cell line carrying a larger CAG repeat number. (Dong et al., 2011)

Due to the anatomical and developmental similarities between NHPs and humans, there is great interest in the development of NHP models for HD. Our group produced the first transgenic HD rhesus macaques (Yang et al., 2008) and have since then reported a number of stem cell derived *in vitro* studies (Chan et al., 2010; Laowtammathron et al., 2010; Snyder et al., 2011; Putkhao et al., 2012). Our group has used a number of different strategies to explore modeling HD in PSCs (Chan et al., 2010; Carter and Chan, 2012). iPSC were established from HD monkey fibroblast using Yamanka reprograming. HD-

iPSCs expressed exon 1 of the human *HTT* gene with 72 CAG repeats. Upon neural differentiation, cells developed hallmark features of HD including nuclear inclusions and oligomeric aggregates of mHTT (Chan et al., 2010). Similar phenotypes were observed in an HD monkey hybrid cell line. In this study HD monkey fibroblast were fused to WT oocytes to generate hybrid HD-ESCs. Using this approach HD-ESCs developed nuclear inclusions and oligomeric aggregates during neural differentiation (Laowtammathron et al., 2010).

In addition to using fibroblast as a source for reprograming, our group established HD dental pulp stromal cells (DPSCs) from tooth germ of HD monkeys. HD monkey DPSCs retained multipotent differentiation capabilities, expressed mHTT, and similarly developed mHTT aggregation phenotypes (Snyder et al., 2011). Of the studies published by our group, all of the stem cell derived models show enhanced expression and aggregation of the mHTT proteins as well as the development of nuclear inclusions at the advent of neuronal differentiation. Work has also been done to characterize a number of iPSC lines derived from HD monkeys currently being monitored in a longitudinal study (Putkhao et al., 2012; Kocerha et al., 2013; Chan et al., 2014). Together these studies describe an *in vitro* platform derived from HD monkey PSCs that could potentially be used to identify novel cellular phenotypes and explore cell therapies.

Perhaps the most clinically relevant work being done in the field is the establishment of human iPSC lines from patients with HD mutations. By deriving pluripotent cells directly from patients it is possible to capture and investigate gene mutations that directly reflect the disease population. Efforts toward this goal in HD were first described by Verlinsky (2005) followed by Mateizel (2006) using embryos identified

in preimplantation genetic diagnosis (PGD). Human ESC lines were established from donated mutant embryos and characterized to show properties of pluripotency, genomic integrity, and presence of expanded CAG repeat size (Mateizel, 2005; Verlinsky et al., 2005; Niclis et al., 2009; Bradley et al., 2011). These lines were made available for research, however follow up study was limited and no disease relevant phenotypes were reported. The first human iPSC line was described by Park *et al* (2008), however initial phenotypic characterization was not reported and a follow-up study only observed a slight increase in caspase activity in iPSC derived neurons after the withdrawal of growth factors from the culture medium (Park et al., 2008; Zhang et al., 2010). In a more recent study, additional iPSC lines derived from patients with homozygous and heterozygous *HTT* mutations were reported to show increased caspase activity, vulnerability to cell death, impaired energy metabolism, and altered electrophysiology recordings (The Hd iPSC Consortium, 2012). Meanwhile, by employing homologous recombination gene targeting, An *et al.* reported the reversal of cell death and transcriptional phenotypes by corrected the *mHTT* allele in HD patient iPSCs (An et al., 2012). These reports show key phenotypes replicated *in vitro*, however other hallmark HD feature including oligomeric mHTT aggregation and nuclear inclusion were not observed. Together these reports highlight the utility of ESC and iPSC models in HD, yet there is much room for improvement on not only the recapitulation of robust phenotypes, but also the production of phenotypes that are captured in neural populations in which the phenotype is most dramatic.

Table 1-1. Stem cell derived models of Huntington's disease.

Mammalian Species	Stem Cell Class	Source	Promoter	HTT Mutation	Phenotype Observed	Reference
Human	ESC	Embryos	Human HTT	Human HTT; 37,51 CAG repeats	Transcriptional changes; Increased cell death; Altered mitochondrial activity	Niclis *et al.* (2013)
Human	iPSC	Fibroblast	Human HTT	Human HTT; 72 CAG repeats	EM48 aggregates	Cheng *et al.* (2013
Human	iPSC	Fibroblast	Human HTT	Human HTT; 50 CAG repeats	Vacuolation in astrocyte	Juopperi *et al.* (2012)
Human	ESC	Embryos	Human HTT	Human HTT; various CAG size	Transcriptional changes	Feyeux *et al.* (2012)
Human	iPSC	HD patient fibroblast	Human HTT	Human HTT; various CAG size	Transcriptional changes; Increased cell death; vulnerable to stress and toxicity	An *et al.* (2012)
Human	iPSC	HD patient fibroblast	Human HTT	Human HTT; various CAG size	Altered cell, energetics, function; Increased cell death; vulnerable to stress and toxicity	HD iPSC Consortium (2012)
Human	iPSC	HD patient fibroblast	Human HTT	Human HTT; various CAG size	Increase in lysosomal activity	Camnasio *et al.* (2012)
Human	ESC	Embryos	Human HTT	Human HTT; 44 CAG repeats	None reported	Mateizel *et al.* (2005)
Human	ESC	Embryos	Human HTT	Not reported	None reported	Verlinsky *et al.* (2005)

Human	iPSC	HD patient fibroblast	Human HTT	Human HTT; 72 CAG repeats	Elevated caspase activity	Park *et al.* (2008) Zhang *et al.* (2010)
Human	ESC	Embryos	Human HTT	Human HTT; 40-48 CAG repeats	None reported	Bradley *et al.* (2011)
Rhesus macaque	ESC	Embryos	Human polyubiquitin	Human HTT exon-1 fragment; 72 CAG repeats	Oligomeric mutant HTT aggregation; formation of nuclear inclusions	Laowtammathron *et al.* (2010)
Rhesus macaque	iPSC	HD monkey fibroblast	Human polyubiquitin	Human HTT exon-1 fragment; 72 CAG repeats	Oligomeric mutant HTT aggregation; formation of nuclear inclusions	Chan *et al.* (2010)
Mouse	iPSC	R6/2 mouse fibroblast	Human HTT	Human HTT 5' fragment; 144 CAG repeats	Transcriptional alteration in lysosome biogenesis and cholesterol biosynthesis	Castiglioni *et al.* (2012)

ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; HTT, huntingtin

Table 1-2. List of reported cellular HD phenotypes in stem cell derived models of Huntington's disease.

Reference	HTT Mutation	Aggregation	Apoptosis	Transcriptional changes	Morphology Defects	Energy Metabolism	Lysosomal Storage/ Vacuoles
Carter *et al. (2014)* Chan *et al.* (2010)	Human HTT exon-1 fragment; 72 CAG repeats	Yes	Yes	Yes	Yes	No	No
Niclis *et al.* (2013)	Human HTT; 37,51 CAG repeats	No	No	No	No	No	No
Cheng *et al.* (2013	Human HTT; 72 CAG repeats	Yes	No	No	No	No	No
Juopperi *et al.* (2012)	Human HTT; 50 CAG repeats	No	No	No	No	No	Yes
Feyeux *et al.* (2012)	Human HTT; various CAG size	No	No	Yes	No	No	No
An *et al.* (2012)	Human HTT; various CAG size	No	Yes	Yes	No	Yes	No
HD iPSC Consortium (2012)	Human HTT; various CAG size	No	Yes	Yes	Yes	Yes	No
Camnasio *et al.* (2012)	Human HTT; various CAG size	No	No	No	No	No	Yes
Park *et al.* (2008) Zhang *et al.* (2010)	Human HTT; 72 CAG repeats	No	Yes	No	No	No	No

1.5 Stem cell-based therapy for Huntington's disease

Stem cell-based therapy shows promise as an emerging strategy towards the treatment of HD. Advances in the generation of patient specific iPSC opens new avenues to explore autologous stem and progenitor cell transplantation into the brain regions that are most affected by HD neuropathology. Although there are many exciting applications of this approach, a critical barrier must first be addressed. In order for stem cell-based therapy to be considered as a viable approach to HD treatment, methods to suppress the expression of *mHTT* in grafted cell populations must be investigated (Carter and Chan, 2012; Matsui and Corey, 2012; Chen et al., 2014). The monogenetic etiology of HD provides a key opportunity for the suppression of the mutant protein. Antisense oligonucleotides (ASOs) and RNA interference (RNAi) mediated strategies have shown much promise as a potential therapy, and have provided a means to mitigate the neuropathology caused by the mutant form of the protein.

There is evidence that supports the feasibility and efficacy of reducing mHTT levels in HD cells. Earlier studies successfully demonstrated the efficacy of *mHTT* reduction through RNAi. Using mouse models of HD, these studies report amelioration of key HD phenotypes correlating with the knock-down of both mutant and wildtype alleles of the *HTT* transcript (Harper, 2005; DiFiglia et al., 2007; McBride et al., 2008; Drouet et al., 2009). These therapeutic RNAi studies provided a foundation for reducing *HTT* transcript levels, moreover the Davidson group published an investigation comparing the safety and efficacy between short-hairpin RNA (shRNA) and micro-RNA based methods for RNAi silencing of *HTT* (McBride et al., 2008). The authors reported that shRNA, although more potent, produced higher levels of cell toxicity compared to

miRNA, which showed better safety profiles in mice models of HD (McBride et al., 2008)

Studies demonstrating the therapeutic potential of reducing *mHTT* expression levels are encouraging and lay the groundwork for potential strategies to correct HD cells and mitigate disease associated phenotypes. On the other hand RNAi approaches that non-specifically target the normal allele as well as the *mHTT* allele are met with serious safety concerns (Sah and Aronin, 2011; Lu and Yang, 2012; Matsui and Corey, 2012). Normal HTT plays an important role in neurogenesis and neuron function (Dragatsis et al., 2000; Godin et al., 2010), therefore approaches that specifically target and reduce the *mHTT* allele, while maintaining clinically safe levels of the normal allele are desirable (Carroll et al., 2011; Hu et al., 2012; Matsui and Corey, 2012).

ASOs have been instrumental in developing strategies to specifically target the *mHTT* allele for suppression. ASOs are single-stranded oligodeoxynucleotides that suppress the synthesis of the targeted protein (Bennett and Swayze, 2010). An alternative approach to achieving *mHTT* allele specific inhibition is to target the CAG nucleotide repeat expansion. The driving principal supporting this strategy is that the expanded repeat region provides more binding sites for complimentary oligomers, thus leading to preferential inhibition of the mutant allele (Hu et al., 2012; Yu et al., 2012). This approach has been investigated using ASOs as well as RNAi mediated mechanisms (Bilsen et al., 2008; Carroll et al., 2011; Hu et al., 2012; Yu et al., 2012). Additional strategies to genetically correct the mutant allele include homologous recombination mediated gene targeting (An et al., 2012), and new strategies that use zinc-finger nucleases, transcription activator-like effector nucleases (TALEN), and clustered

regulatory interspaced short palindromic repeats (CRISPR/Cas) DNA endonucleases for allele specific silencing (Joung and Sander, 2012; Mussolino and Cathomen, 2012; Gaj et al., 2013; Mali et al., 2013).

The advancement in developing ASOs and RNAi therapies to inhibit *mHTT* expression provides a promising framework for correcting the underlying driver in patient derived HD cells. By silencing *mHTT* in stem cells populations for cell therapy, corrected populations may serve as a source for autologous transplantation in brain regions highly affected by HD.

1.6 Study Proposal

HD is a devastating disease that currently lacks effective therapy and strategies to slow down or prevent onset of disease. Despite well documented characterization of the gross neuropathology and cellular pathways revealed to be associated with HD, more research is need to understand the complex mechanisms by which mHTT impacts neurons leading to neurodegeneration. This dissertation will focus on the development of an *in vitro* model of HD generated from transgenic HD rhesus macaques. Chapter 2 will describe the *in vitro* derivation of NPCs and GABA forebrain neurons from monkey iPSCs and ESCs. Chapter 3 will examine the impact of mHTT in HD monkey neural cells. Chapter 4 will examine the reversal of HD associated cellular phenotypes in HD monkey neural cells. Altogether this study describes a stem cell derived *in vitro* platform that recapitulates key HD associated cellular phenotypes, and is capable to respond to genetic and pharmacological therapy **(Figure 1-2)**. This body of works sheds light onto cell autonomous pathologies contributing to neurodegeneration, establishes methods for

GABA neuron directed differentiation in NHP iPSCs, and provides the foundation towards a pre-clinical platform for drug screening and cell therapy evaluation.

Figure 1-2

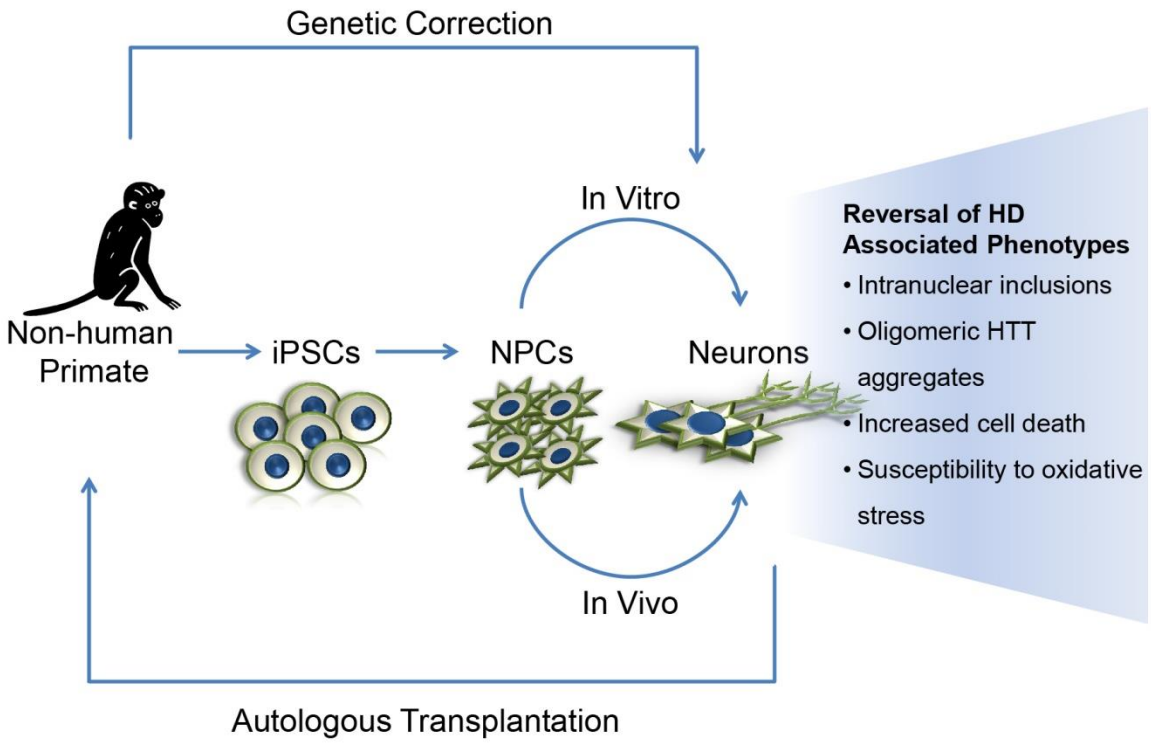


Figure 1-2. Graphical abstract of study design, findings, and implications to further work.

Pluripotent stem cells (PSCs) were derived from HD and WT rhesus monkeys. Neural differentiation of PSCs generated neural progenitor cells (NPCs) and neurons *in vitro* and *in vivo*. HD neural cells recapitulate HD associated cellular phenotypes that could be reversed by therapeutic treatment. This study opens possibilities for gene therapy and cell replacement investigations in HD monkeys

CHAPTER 2

Derivation and characterization of neural cells from non-human primates

This chapter will be published in **Stem Cell Reports:**

Carter, R.L., Chen, Y., Kunkanjanawan, T., Xu, Y., Putkhao, K., Moran, S.P., Yang, J., Huang A.H.C., Parnpai, R., and Chan A.W. Reversal of cellular phenotypes in neural dells derived from Huntington's disease monkey induced pluripotent stem cells. *Stem Cell Reports*. 2014 (Under review)

2.1 Abstract

Differentiation of pluripotent stem cells (PSCs) into neural populations has promising applications in neurodegenerative disease research. Non-human primates (NHPs) are physiologically and phylogenetically similar to humans, and therefore offer a clinically relevant animal model to investigate Huntington's disease (HD) and stem cell-therapy applications. In this study we derived neural progenitor cells (NPCs) from monkey induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs). NPCs were stably expanded over multiple passages, and were capable to differentiate to neural cell populations both *in vitro* and *in vivo*. These results demonstrate that monkey NPCs display characteristics of a progenitor population and can be efficiently induced to differentiate along the neural lineage

2.2 Introduction

The pluripotent properties of ESC and iPSCs are desirable for modeling neurological diseases. iPSCs mirror the genetic background of the donor source potentially leading to the recapitulation of disease phenotypes in the cells (Tiscornia et al., 2011; Bellin et al., 2012; Robinton and Daley, 2012). Although PSCs have the potential to generate neurons and glial cell types, there is need of improvement in methods to efficiently derive disease specific neuron sub-types. A major limitation is the sub-optimal yield of mature functional neurons at an adequate level of homogeneity to make high-throughput assays feasible (Gerrard et al., 2005; Li et al., 2005; Dhara et al., 2008). Furthermore, PSCs would not be an appropriate cell population for cell replacement therapy due to their increased risk of forming tumors post transplantation

(Fong et al., 2010; Ruggieri et al., 2014). To resolve these issues, an increasing number of studies are focused on the establishment NPCs to serve as an neural lineage restricted precursor in neurological disease studies (Kirkeby et al., 2012; Gage and Temple, 2013; Lu et al., 2013; Tabar and Studer, 2014). Additional work has been done to better define a neuronal restricted population of NPCs in order to reduce contamination of unwanted cell types resulting from non-specific, mutilineage differentiation protocols (Yuan et al., 2011; Kirkeby et al., 2012; Liu et al., 2013; Lu et al., 2013). NPCs are a stable, self-renewing progenitor cell population that give rise to neural cell lineages during embryonic and adult neurogenesis (Conti and Cattaneo, 2010). In contrast to PSCs, NPCs are committed to the neuronal lineage and can be used to generate neurons at a much higher yield and purity compared to non-specific differentiation methods (Reubinoff et al., 2001; Gerrard et al., 2005; Itsykson et al., 2005; Dhara et al., 2008; Koch et al., 2009). NPCs are easier to maintain in long term culture, compared with the labor intensive methods used to propagate primate ESC and iPSC cultures. Together these properties make NPCs a useful progenitor population for downstream research applications in modeling neurodegenerative disease, and for development of cell-therapies.

NPCs originate from neuroepithelial cells that are generated with the induction of the neuroectoderm (Götz and Huttner, 2005; Conti and Cattaneo, 2010). During *in vitro* differentiation, careful introduction of neuronal patterning growth factors are added to the cell culture medium to induce progressive neural lineage restriction similar to what occurs during neurogenesis *in vivo.* This is accomplished by inhibition of SMAD signaling usually by increased concentrations of noggin, and results in the organization of

cell into neuroepithelial rosettes structures. This preliminary neural population is highly sensitive to factors that direct regionalization of NPCs that will influence differentiating into specific sub-classes of neurons (Gage and Temple, 2013). In HD, γ-aminobutyric acid (GABA) medium spiny neurons (MSNs) and other interneurons that make up the striatum and cortex are the most severely affected (Reiner et al., 1988; Raymond et al., 2011). Sonic hedgehog homolog (SHH) acts to regionally define the medial ganglionic eminence regions of the cortex that form the striatum and give rise to MSNs (Liu et al., 2013). By mimicking these neurodevelopmental cues *in vitro*, it is capable to generate disease relevant neural sub-types from NPCs derived from ESCs and NPCs (Aubry et al., 2008; Liu et al., 2013).

Most reported *in vitro* methods to derive NPCs from PSCs follow either a monolayer, single cell protocol, or an embryoid body (EB), neurosphere differentiation protocol (Dhara and Stice, 2008; Erceg et al., 2009). The monolayer approach involves establishing a feeder free adherent culture of PSCs. Once dissociated to single cells, the PSCs are induced to form NPCs by introducing neuronal patterning factors, such as fibroblast growth factor (FGF2) (Gage and Temple, 2013). FGF2 promotes cell proliferation and neuralization of differentiating cells, and may act synergistically with noggin as an antagonist to SMAD signaling (Götz and Huttner, 2005; Dhara and Stice, 2008).

EB differentiation methods involve forming micro aggregates of PSCs that are patterned as free-floating clusters of cells. EBs are then attached in culture dishes to form neuroepithelial rosette structures that are isolated and either cultured in suspension as neuropheres, or expanded as a monolayer neural progenitors (Dhara and Stice, 2008;

Erceg et al., 2009). Both FGF2 and noggin are used in patterning neurospheres followed by SHH for regionalization (Gage and Temple, 2013). Although both methods yield NPCs populations, monolayer methods require lower concentrations of patterning factors, and are easier to monitor cell morphology. Conversely EB methods are more widely accepted and defined, also this methods more closely mirrors normal neuroectodermal differentiation (Erceg et al., 2009).

Once established, NPC lines are characterized by a number of parameters to evaluate maturation and homogeneity. NPC markers including nestin (NES), Sox2, Pax6, and Musashi-1 (MSI1) are used to identify NPC populations using cell staining, gene expression, and flow cytometry analysis (Dhara et al., 2008).

Further differentiation of NPC to neural cells involves the introduction of additional growth factors including fibroblast growth factor 8 (FGF8), SHH, glial cell line-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF) that promote pro-neural gene expression and support neuronal cell survival (Aubry et al., 2008; Gage and Temple, 2013). Alongside *in vitro* differentiation methods, cells are transplanted back into *in vivo* environments by stereotaxic injection, to assess the capability to form neurons in the natural central nervous system milieu (Parmar and Björklund, 2012).

Here we show the establishment of NPCs derived from monkey iPSCs and ESCs. We found that established monkey NPC lines were stable for more than 30 passages, were expandable for downstream assays, and were competent in differentiating into disease relevant neuronal populations both *in vitro* and *in vivo.* This work not only provides a renewable, neural committed progenitor population for the study of HD, but

also establishes defined methods for the differentiation and sub-culture of monkey neural cells on par with methods of more familiar culture systems using human and rodent PSCs.

2.3 Methods

Reprogramming HD monkey iPSCs and culture

HD monkeys were generated as described by Yang et al. Briefly, exon 1 of the human *HTT* gene with expanded CAG repeats was inserted into a lentiviral vector. An additional lentiviral vector carrying EGFP was created for co-infection. Both vectors were under regulation of the human poly ubiquitin-C promoter (Yang et al., 2008). HD dental pulp stromal cells and fibroblasts were harvested from the dental pulp and skin of HD monkeys as described previously (Chan et al., 2010; Snyder et al., 2011). Harvested DPSCs and fibroblasts were infected by retrovirus expressing rhesus macaque Oct4, Sox2, and Klf4. At approximately 2-3 weeks post-retroviral transfection, a primate ES cell-like colony was selected based on morphology and mechanically passaged onto mouse fetal fibroblast (MFF) feeder cells with primate ES media. Cell lines are further described in **Table 2-2**.

Cytogenetic analysis/G-Banding analysis

Cytogenetic analysis was performed by Cell Line Genetics LLC (Madison, WI). A total of 20 metaphases were analyzed, and images were captured using the CytoVision® digital imaging system (Applied Imaging).

Derivation and culture of monkey NPCs

NPCs were derived from monkey PSCs using monolayer and EB protocols.

Monolayer differentiation: PSCs were adapted to feeder free culture conditions by removal of MFF feeder layer and manual passaging of colonies on 1μg/cm^2 laminin (Sigma) coated 35mm tissue culture dishes supported by MFF conditioned ES cell medium. After several feeder free passages, ESCs and iPSCs were trypsinized (0.25% trypsin in EDTA) and seeded at approximately 3 x 10^5 cells per dish. After 24 hr (Day 1), the ES cell medium was replaced with derivation medium [DMEM/F12 (with 1x N2 (Invitrogen), 4 ng/ml bFGF (R&D), 2mM L-glutamine, and 1 x P/S (Invitrogen)]. During this period cells cultured without passage with medium refreshed every two days. On day 15 cells were passaged and expanded in neural proliferation medium [Neurobasal medium (Life Technologies) supplemented with 1 x P/S (Invitrogen) and 1x B27 (Life Technologies), 2mM L-glutamine, 20ng/ml bFGF (R&D), and 10 ng/ml mLIF (Chemicon)]. Cells were expanded in neural proliferation medium for 7 days. Following 7 days NPC were subcultured in P/L coated [1μg/cm^2 laminin (Sigma) and 20ug/mL Poly-L-ornithine (Sigma)] 35 mm cell culture dishes.

EB differentiation: PSCs were mechanically dissociated from MFF feeder and were cultured in low-attachment petri dishes supported by MFF-conditioned ES cell medium without bFGF (R&D). After 7 days ES cell medium was replaced with derivation medium [DMEM/F12 (with 1x N2 (Invitrogen), 4 ng/ml bFGF (R&D), 2 mM L-glutamine, and 1x P/S (Invitrogen)]. During this period cells were cultured without passage with medium refreshed every 2 days. After 7 days EBs were plated onto P/L-coated cell culture dishes and expanded in neural proliferation medium [Neurobasal medium (Life Technologies) supplemented with 1 x P/S (Invitrogen) and 1x B27 (Life Technologies), 2 mM L-glutamine, 20 ng/ml bFGF (R&D), and 10 ng/ml mLIF

(Chemicon)]. After 7-10 days neural rosettes were manually picked and seeded onto a fresh P/L-coated cell culture dish. Neural rosettes were allowed to proliferate as neuroepithial progenitors (NEPs) until approximately 90% confluency. After reaching confluence NEPs were enzymatically dissociated (1x Accutase; Life Technologies) into single cells and seeded onto P/L coated dishes in neural proliferation medium at a density of $3x10^4$ cells/cm^2.

Rhesus monkey NPC *in vitro* neural differentiation

For *in vitro* differentiation of NPCs to the neuronal lineage, NHP NPCs were seeded at density of $5x10^4$ cells/cm^2 on a P/L-coated glass chamber (Fisher Scientific) in neural proliferation medium. The following day medium was replaced with differentiation medium [DMEM/F12 (Life Technologies), P/S (Invitrogen), 2 mM L-glutamine, 1x N2 (Invitrogen), and 1x B27 (Life Technologies)]. At 4 days of neural induction, SHH (R&D, 0.2 μg/mL) and FGF8 (R&D, 0.1 μg/mL) were added to medium. At day 8, 200 μM ascorbic acid (Sigma) was added in combination with SHH and FGF8. At the end of 21 days, cells were either fixed for immunocytochemistry or harvested for RNA isolation.

Rhesus monkey NPC *in vivo* neural differentiation

All protocols involving animal care and handling were approved by Emory University's Institutional Animal Care and Use Committee. For *in vivo* differentiation to the neuronal lineage, WT-2 NPCs and HD-14 NPCs were dissociated and suspended at 50,000 cells/μL in artificial cerebrospinal fluid solution. By stereotactic injection, cell suspension was infused into the right and left hemispheres of the striatum (Anterior-Posterior = +0.74, Medial-Lateral +/-1.7, dorsal/ventral = -3.8, relative to Bregma). Cell

transplantation was performed in Fox River SCID® mice (CB17/lcr-, Charles River Laboratories).

Immunocytochemistry

NPCs and neurons cultured on P/L-coated glass chamber slides were fixed in 4% paraformaldehyde (PFA) followed by permeabilization and blocking with 3% bovine serum albumin (Sigma) in phosphate buffered saline (PBS) solution. Fixed slides were incubated overnight at 4°C with primary antibodies. Slices were washed in PBS followed by incubation with secondary antibody for 1 hr at room temperature. Primary and secondary antibodies used included: Nestin (1:1000; AB5922, Millipore), SOX2 (1:500; AB5603, Millipore), MSI1 (1:500; AB5977, Millipore), PAX6 (1:300; PRB-278P, Covance, Atlanta, GA), GABA (1:300; A2052, Sigma), βIII-tubulin (1:300; MAB1637, Millipore), doublecortin (DCX; 1:500; AB18723, AbCam), microtubule-associated protein (MAP2; 1:500; MAB3418, Millipore), and mEM48 (1:50). Secondary antibodies included Alexa Fluor 594 (1:1000; A-21205, Life Technologies), and Hoechst 33342 (5 mg/mL) was for DNA staining. Samples were examined using a microscope (Olympus BX51) equipped with an epifluorescent device.

Histological analysis of NPC transplantation

At 12.5 and 15 weeks following transplantation, mice were perfused transcardially with PBS followed by 4% PFA prepared in PBS. Brains were dissected and postfixed in the 4% PFA overnight. Fixed brains were cryopreserved with 15% sucrose overnight and switched to 30% sucrose for 8 hr to reach equilibrium. The brains were then embedded in OCT compound and cryosectioned coronally to 30-μm thick slices. Free-floating brain slices were incubated in blocking buffer (5% serum and 2% BSA) for 30 min, and then

incubated overnight at 4°C with primary antibodies, including DCX (1:500; AB18723, AbCam), NeuN (1:500; MAB377 Chemicon), GABA (1:300; A2052, Sigma), dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP32; 1:200; 11365, Santa Cruz), and mEM48 (1:50). Brain slices were washed with PBST (PBS + 0.2% Triton X-100) 3 times (10 min/time). Secondary antibodies (Alexa Fluor 594, A-21205; or Alexa Cy5, 81-6716) (1:500; Molecular Probes) were applied for 1.5 hr at room temperature. Slices were washed with PBST 2 times (10 min/time), and then incubated with Hoechst for 20 min. Subsequently, slices were mounted onto glass slides. Images were acquired using a Zeiss LSM 510 NLO META confocal microscope (Oberkochen, Germany).

Real-time quantitative PCR (RT-qPCR)

Total RNA from cell samples was prepared using TRIzol® (Life Technologies), and genomic DNA was removed from the RNA sample using Turbo DNA-free (Applied Biosystems) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using a RNA-to-cDNA kit (Applied Biosystems). RT-qPCR was performed using Gene Expression Master Mix (Applied Biosystems) and TaqMan® gene expression primers on a CFX96 Real-Time Detection System (Bio-Rad). RT-qPCR primer sequences are listed in **Table 2-2.** PCR conditions followed manufacturer's instruction (Applied Biosystems): Initial 95°C TaqMan activation step for 10 min followed by amplification cycles 95°C for 15 sec and 60°C for 60 sec for 40 cycles.

Fluorescence-activated cell sorting (FACS) analysis

Cells were dissociated using 1x Accutase (Life Technologies) and fixed in 1x BD FACS Permeabilizing Solution (BD Biosciences). Cells were washed in following permeabilization. Cells were incubated with primary antibody for 1 hr, followed by 3

wash steps (5min/wash) in 0.5% BSA/PBS. Cells were incubated with fluorochrome-conjugated secondary antibody for 1 hr in Falcon 5mL round-bottom polystyrene tubes (BD Biosciences) and quantified on FACSCalibur flow cytometer (BD Biosciences). All samples were gated to assess only single cells as determined by forward scatter area vs. side scatter area. Background fluorescence was subtracted using unlabeled cells, and channel compensation was performed using fluorochrome labeled compensation beads (BD Biosciences). A total of 100,000 events were recorded. Quantification was analyzed using FlowJo analysis software (TreeStar).

Whole cell patch clamp recordings

Electrophysiology recordings were measured similar to methods described by (Minami et al., 2011). Individual brain slices were transferred to a submersion-type recording chamber mounted on the fixed stage of a Leica DMLFS microscope (Leica Microsystems Inc.), and continuously perfused by gravity-fed oxygenated 32°C artificial cerebrospinal fluid solution. Slices were viewed using differential interference contrast (DIC) optics and infrared (IR) illumination with an IR sensitive CCD camera (Orca ER, Hamamatsu). Thin-walled borosilicate glass patch electrodes (WPI, Sarasota) were filled with 130mM K-gluconate, 2mM KCl, 10mM HEPES, 3mM MgCl2, 2mM K-ATP, 0.2mM NaGTP, and 5mM phosphocreatine, adjusted to pH 7.3 with KOH, and having an osmolarity of 280–290 mOsm. Individual neurons were visualized *in situ* using DIC microscopy in combination with a 40x water immersion objective and displayed in real time on a computer monitor. Neurons were identified according to their characteristic size and shape, as well as expression of GFP. Data acquisition and analysis were performed using a MultiClamp700B amplifier in conjunction with pClamp10.0 software and a DigiData

1320A AD/DA interface (Molecular Devices). Whole cell patch clamp recordings were obtained and recorded voltages were low-pass filtered at 5 kHz and digitized at 10–20 kHz.

2.4 Results

NHP and human PSCs share many similar characteristics including differentiation potential, marker expression, and morphology (Tibbitts et al., 2006), therefore establishing PSC-derived models from NHPs is a valid approach for translational studies in neurodegenerative disease. Monkey DPSCs and fibroblasts were reprogrammed by introducing rhesus genes encoding Oct4, Sox2, and Klf4 through retroviral gene transfer following protocols described in our previous report (Chan et al., 2010; Snyder et al., 2011). HD-iPSC lines (HD-3, and HD-14) were generated from DPSCs and fibroblast, respectively, from two transgenic HD monkeys **(Table 2-2)**. HD-2 are ESCs derived from transgenic embryos from an HD monkey (Putkhao et al., 2012). WT cells lines (WT-2, WT-14, and WT-28) were generated from non-transgenic, wild-type (WT) monkeys with normal *HTT* alleles **(Table 2-2)** derived from ESCs (WT-2 and WT-28) or by cellular reprogramming from fibroblast (WT14). All iPSC and ESC lines expressed pluripotent stem cell markers, including Oct4, SSEA4, Tra-1-60, and alkaline phosphatase **(Figure 2-1 A-B)** (Chan et al., 2010; Putkhao et al., 2012). In addition, cytogenetic analysis revealed a normal diploid karyotype matching gender to that of the monkey donor **(Figure 2-1A-B)**.

To overcome technical hurdles associated with long term stem cell culture, we derived stable NPC lines. Monkey NPCs were derived from PSCs using monolayer **(Figure 2-2A)** and EB methods (**Figure 2-2C)**. NPCs displayed neuroectodermal rosette-

like structures and exhibited characteristic NPC morphology **(Figure 2-2B and 2-2D)**. NPC populations were expandable for over 30 passages, and can be cryopreserved with high viability, maintaining their neural differentiation competency. NPCs expressed canonical neural precursor markers, including NES, MSI1, Sox2, and Pax6 confirmed by immunocytochemistry **(Figure 2-3)**. Expression of neural precursor markers in NPCs was further confirmed by RT-qPCR **(Figure 2-4)**. Expression analysis showed increased expression of Sox2, NES, MSI1 and Pax6 compared to ESC samples. Pluripotency marker Oct4 was reduced in NPCs indicating that NPCs no longer possessed pluripotent cell characteristics.

A specific goal in deriving NPCs from PSCs is to generate a uniform, enriched progenitor population for neural differentiation and transplantation applications (Reubinoff et al., 2001). To assess whether monkey PSCs differentiation to NPCs resulted in uniform populations, NPCs were analyzed by fluorescence-activated cell sorting (FACS) **(Figure 2-5 and summarized in Table 2-3)**. FACS analysis demonstrated that NPCs homogenously expressed neural progenitor markers at percentages near or exceeding 90% of the total population. Together these immunostaining, RT-qPCR, and FACS results demonstrate that NPCs were successfully derived from HD and WT PSCs, and uniformly expressed progenitor markers.

As a model for studying HD pathogenesis, specifically neural associated phenotypes, it was important that the HD-NPCs were capable of differentiating into neuronal cell types. GABA MSNs in the striatum are the most severely affected neuronal population in HD (Zuccato et al., 2010; Raymond et al., 2011), therefore we were interested in differentiating NPCs towards GABA neurons. Monkey NPCs were cultured

for 21 days on polyornithine/laminin (P/L)-coated glass slides supported by neural differentiation medium **(Figure 2-6A)**. After 21 days differentiation, immunostaining showed that neurons derived from NPCs expressed structural neural markers, MAP2, β-tubulin III, and DCX as well as neurotransmitter marker GABA **(Figures 2-6B and 2-6C)**.

We further assessed neural differentiation potential for monkey NPCs *in vivo*. WT-2 and HD-14 monkey NPCs were transplanted into the striatum of 10-week-old SCID mice. While HD-NPCs carry both m*HTT* and *EGFP* transgenes, WT-NPCs were infected with lentivirus expressing *EGFP* under the control of the human polyubiquitin-C promoter for identification. At 12.5 and 15 weeks post-transplantation, the brains of the transplanted mice were collected, cryosectioned, and stained for neuronal markers. Confocal imaging revealed both WT-2 and HD-14 cells expressed GFP and were also positive for neuronal markers DCX **(Figure 2-7; A and B)**, GABA **(Figures 2-7 E/F and G)**, and striatal neuron marker DARPP-32 **(Figures 2-7 H and I)**. NeuN-positive cells from HD-14 and WT-2 were sparse **(Figure 2-7 C and D)**. A possible explanation for this observation is that only 50,000 NPCs were grafted into the mouse striatum. This number is relatively low compared to other studies that report injections ranging from 100,000-500,000 cells (Nori et al., 2011; Ma et al., 2012; Liu et al., 2013; Nicholas et al., 2013). Furthermore, in this study NPCs were transplanted as single cells, potentially leading to lower survival of transplanted cells compared to other transplantation studies that graft neurospheres (Nori et al., 2011).

We also investigated functional properties of *in vivo* differentiated neurons by whole cell patch clamp. We found that at 30 weeks post transplantation grafted cells

integrated into mouse striatum and showed spontaneous synaptic activity (**Figure 2-8)**. WT neurons co-labeled with GFP (green) and biocytin filling (red) show positive for glutamate receptor (GluR1) **(Figure 2-8A)**. Representative spontaneous synaptic activity was recorded from grafted WT-2 neurons **(Figure 2-8B)**. Synaptic activity was abolished by application of glutamate receptor antagonist DNQX demonstrating glutamate mediated signaling in WT-2 neurons **(Figure 2-8B)**. We did not observe spontaneous firing of action potentials, suggesting that transplanted neurons were had not yet developed full functionality.

2.5 Discussion

In this study we have established a system for differentiating monkey ESC and iPSC to NPCs, and further to GABA neurons, using both *in vitro* and *in vivo* methods. These methods began with neuroectodermal specialization of ESCs and iPSCs via FGF2, followed by neuronal patterning towards GABA positive neurons via SHH, and FGF8. We established PSCs from both WT and HD monkeys that express canonical pluripotency makers. From there using both a monolayer or EB approach, we derived stable NPC lines expressing hallmark progenitor markers Nestin, Sox2, MSI1, and PAX6 demonstrated through immunocytochemistry, RT-qPCR, and FACS analysis. We generated competent NPCs line with high homogeneity supported by FACS data showing 90% of total cells positive for NPC specific markers. Monkey NPCs were capable of differentiating into GABA neurons *in vitro* demonstrated by positive immunofluorescence staining for GABA, and mature neural markers. Expression of GABA and other neural markers were also observed following striatal transplantation of NPCs into mice, however, neurons examined after *in vivo* differentiation did not show

spontaneous or induced action potential firing suggesting that transplanted neurons had not gained functional properties.

This work contributes significantly to a popular field of study that has not yet fully taken advantage of a NHP system. The majority of existing protocols for neuronal differentiation from PSCs, mainly are purposed for rodent and human systems. Our study using rhesus monkey PSCs provides a foundation for future studies using NHP models of neurological disease. Moreover, the establishment of NHP NPC lines overcomes technical challenges associated with long term iPSC and ESC culture, and provides an enriched, expandable progenitor population for generating neurons. Though efforts to directly differentiate somatic cells to target cells of interest are ongoing (Qiang et al., 2013), establishing potent NPC lines from PSCs provides an advantage by allowing easy maintenance of a progenitor cell population committed to the neuronal lineage, thus enhancing homogeneity in subsequent neural differentiation (Dhara et al., 2008; Koch et al., 2009).

This study also supports the potential of NPCs as a source for cell replacement studies. Transplanted NHP NPCs survived transplantation into mice striatum, and did not form tumors. These results lay the foundation for future studies that include autotransplantation of NPCs into monkey disease models of HD. NHPs are well suited to investigate the safety and efficacy of cell transplantation for cell therapy, and more closely mirror the size and anatomical structure of the human brain compare to rodents (Chen et al., 2012; Chan et al., 2014). At this time, there are numerous reports that describe behavior improvements following cell replacement therapy in rodent models (Kim et al., 2008; Maucksch et al., 2013). However, for this line of research to advance,

pre-clinical studies in NHP models of neurological disease would be needed before these finding could translate to human applications.

Methods used in this study to derive NPCs have been shown to pattern neural progenitors towards a forebrain regional identity, however markers that more narrowly define regional specification of neural cell populations have been reported (Kirkeby et al., 2012; Liu et al., 2013; Lu et al., 2013). We acknowledge that NPCs generated by our current methods require additional characterization using markers that rule out progenitors fated towards hindbrain neural fate, as well as glial progenitors. By more narrowly defining NPCs we may improve differentiation to specific neuronal lineages, thus providing a homogenous neuron population. Additional steps to further purify our neuronal population may include cell sorting strategies that take advantage of cell surface markers to delineate specific neural cell types, although the effects of neuronal sorting on viability and synaptic function have not been studied (Pruszak et al., 2007; Yuan et al., 2011).

In our *in vivo* differentiation efforts, we noticed an extensive loss of cells transplanted into the striatum. Compounded by a low yield of cells that were positive for mature neuronal markers, these results presented hurdles for the study of disease neuron function. Improvements to our approach such as grafting a greater number of NPCs into the striatum, in combination with neuroprotective neurotrophins, such as BDNF, may improve the survival and differentiation potential of transplanted NPCs. Proof-of-concept for this approach was demonstrated in a hypoxic-ischemic stroke mouse model (Rosenblum et al., 2014). In this study neural stem cells were pretreated with BDNF, and

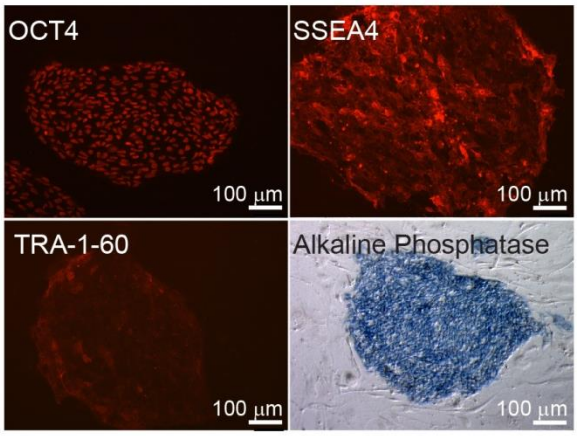
subsequently co-injected with BDNF into stroke mice. This resulted in a greater survival, engraftment, and differentiation potential in transplanted cells.

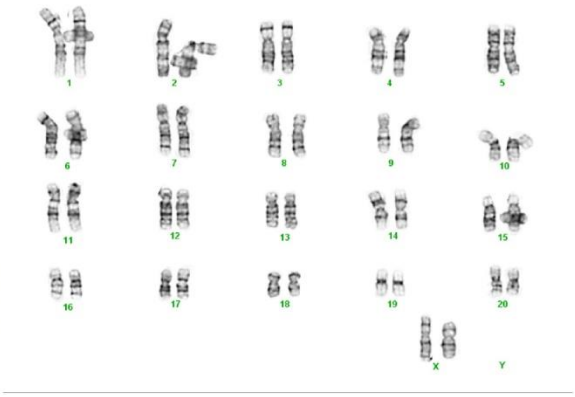
In summary, this study demonstrates the establishment and neural differentiation of NPCs from HD monkey PSCs. These finding not only support the potential for cell transplantation applications in NHP, but provide a resource for the investigation of HD neuronal phenotypes *in vitro*.

Figure 2-1

A

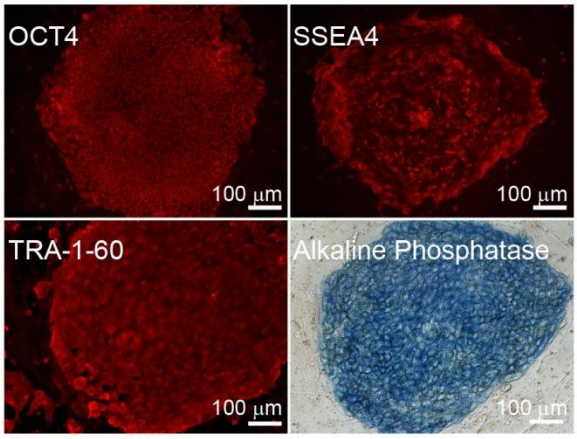
HD-14 Pluripotency





B

WT-2 Pluripotency



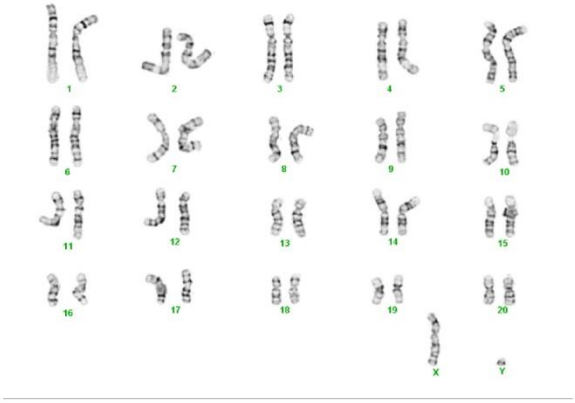


Figure 2-1. Characterization of PSCs from HD and WT monkeys.

(A) Reprogrammed HD-14 cells from HD monkey form colonies and express stem cell pluripotency markers Oct4, SSEA4, Tra-1-60, and alkaline phosphatase. G-banding analysis show normal diploid karyotype (42; XX).

(B) ESC line WT-2 from WT monkey form colonies and express stem cell pluripotency markers Oct4, SSEA4, Tra-1-60, and alkaline phosphatase. G-banding analysis shows normal diploid karyotype (42; XY). Scale bars: 100μm.

Figure 2-2

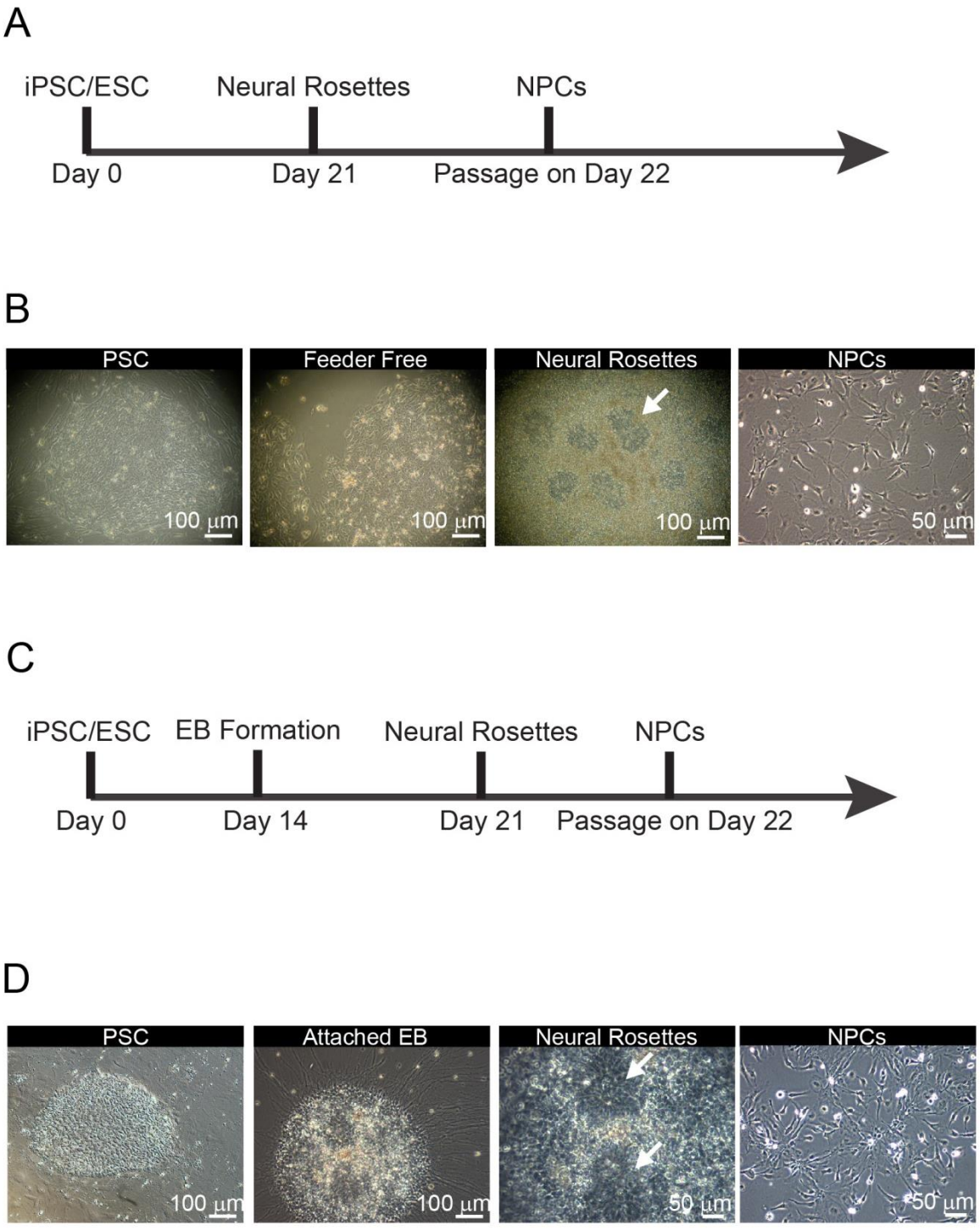

A
iPSC/ESC
Neural Rosettes
NPCs
Day 0
Day 21
Passage on Day 22
B
PSC
Feeder Free
Neural Rosettes
NPCs
100 μm
100 μm
100 μm
50 μm
C
iPSC/ESC
EB Formation
Neural Rosettes
NPCs
Day 0
Day 14
Day 21
Passage on Day 22
D
PSC
Attached EB
Neural Rosettes
NPCs
100 μm
100 μm
50 μm
50 μm

Figure 2-2. Derivation of NPC lines from monkey PSCs.

(A) Schematic diagram depicting monolayer NPC derivation from monkey PSCs. PSCs were patterned toward neural ectoderm progenitors for 21 days. Neural ectoderm progenitors were then passaged and expanded as NPCs for characterization.

(B) Bright field images of HD-14 NPCs at progressing stages of differentiation. Neural induction of stem cells progress to neural rosette formation. Arrows indicate neural rosettes. NPCs sub-cultured as single cell monolayer population show NPC morphology. Scale bars: 100μm and 50μm.

(C) Schematic diagram depicting EB NPC derivation from monkey PSCs. Stem cell colonies were manually passaged and grown as free floating clusters to from EBs. During this time EBs were patterned towards neural ectoderm progenitors. After 14 days EBs were seeded to P/L coated dishes to develop neural rosettes structures. Neural rosettes were then manually passaged and re-seeded as single cells for expansion.

(D) Bright field image of WT-28 NPCs at progressing stages of differentiation. Following neural patterning EBs were attached to P/L coated dish and develop neural rosettes during neural induction. Arrows indicate neural rosettes. NPCs sub-cultured as single cell monolayer population show NPC morphology. Scale bars: 100μm and 50μm.

Figure 2-3

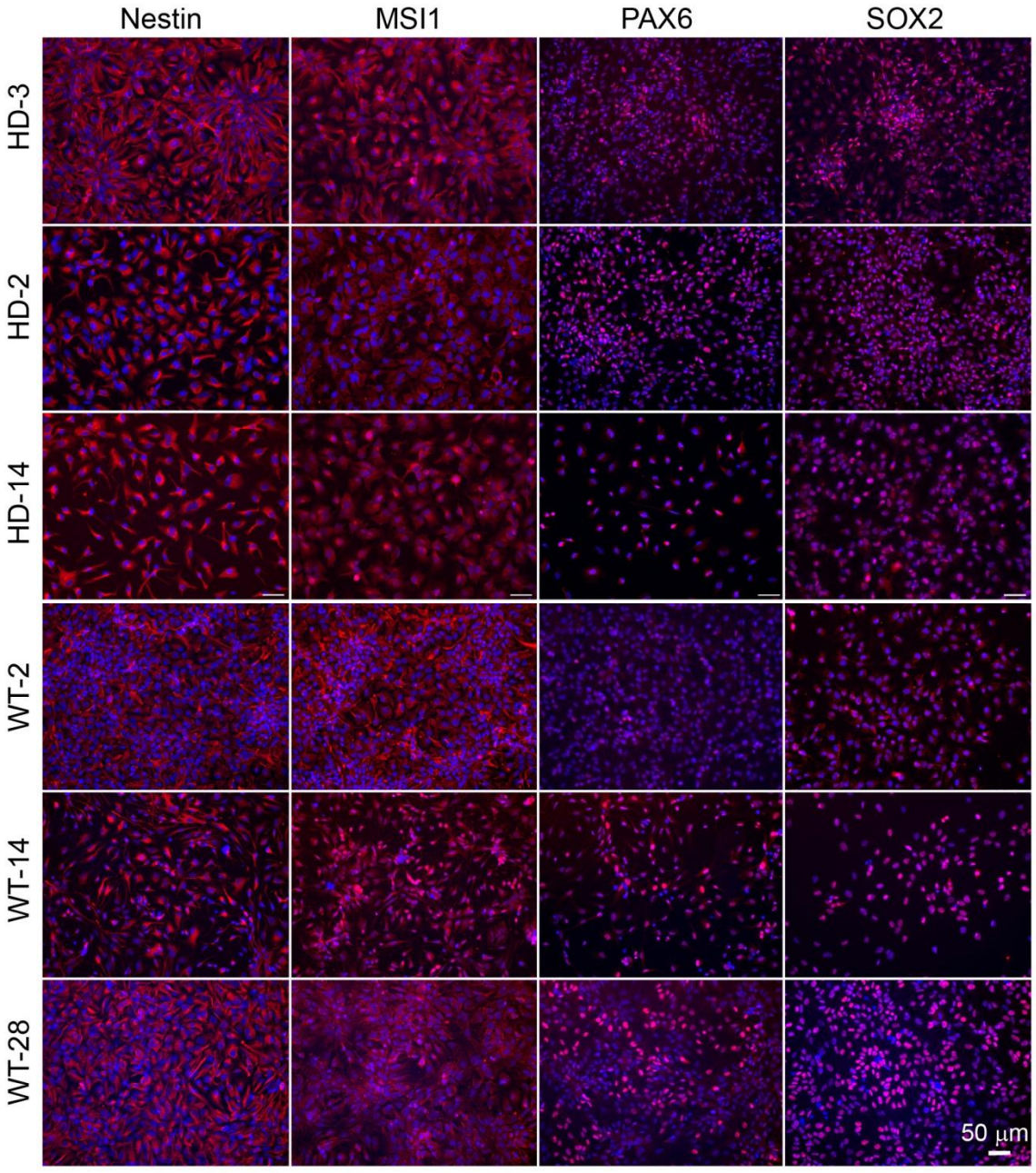


Figure 2-3. NPCs derived from monkey PSCs express neural precursor markers. Immunocytochemistry of NPCs derived from monkey PSCs reveal positive staining in all cell lines (HD-3, HD-2, HD-14, WT-2, WT-14, WT-28) for NPC markers Nestin, MSI1, Pax6, and Sox2 (red). Nuclear staining shown using Hoechst (blue). Scale bars: 50μm.

Figure 2-4

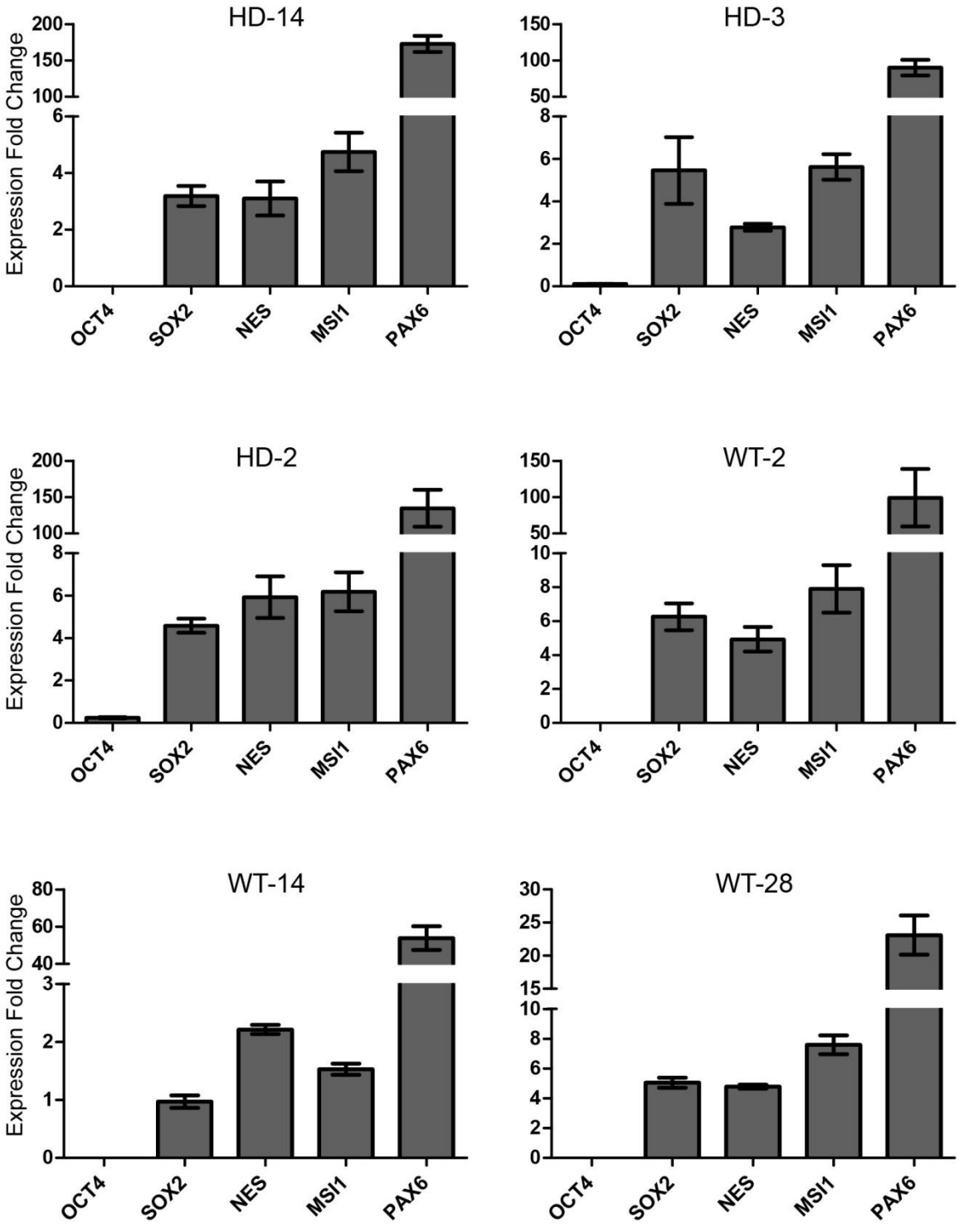

HD-14
HD-3
HD-2
WT-2
WT-14
WT-28
Expression Fold Change
OCT4
SOX2
NES
MSI1
PAX6

Figure 2-4. Neural precursor markers are expressed in NPCs derived from monkey PSCs.

RT-QPCR analysis show increased expression of NPC markers Sox2, Nestin, MSI1, and Pax6 in monkey NPCs. Pluripotency marker Oct4 expression was reduced in NPCs. Expression values were normalized to GAPDH. All graphs are relative to expression values in WT-2 ES cells and plotted as delta-delta CT method. Data are represented as mean +/- SEM.

Figure 2-5

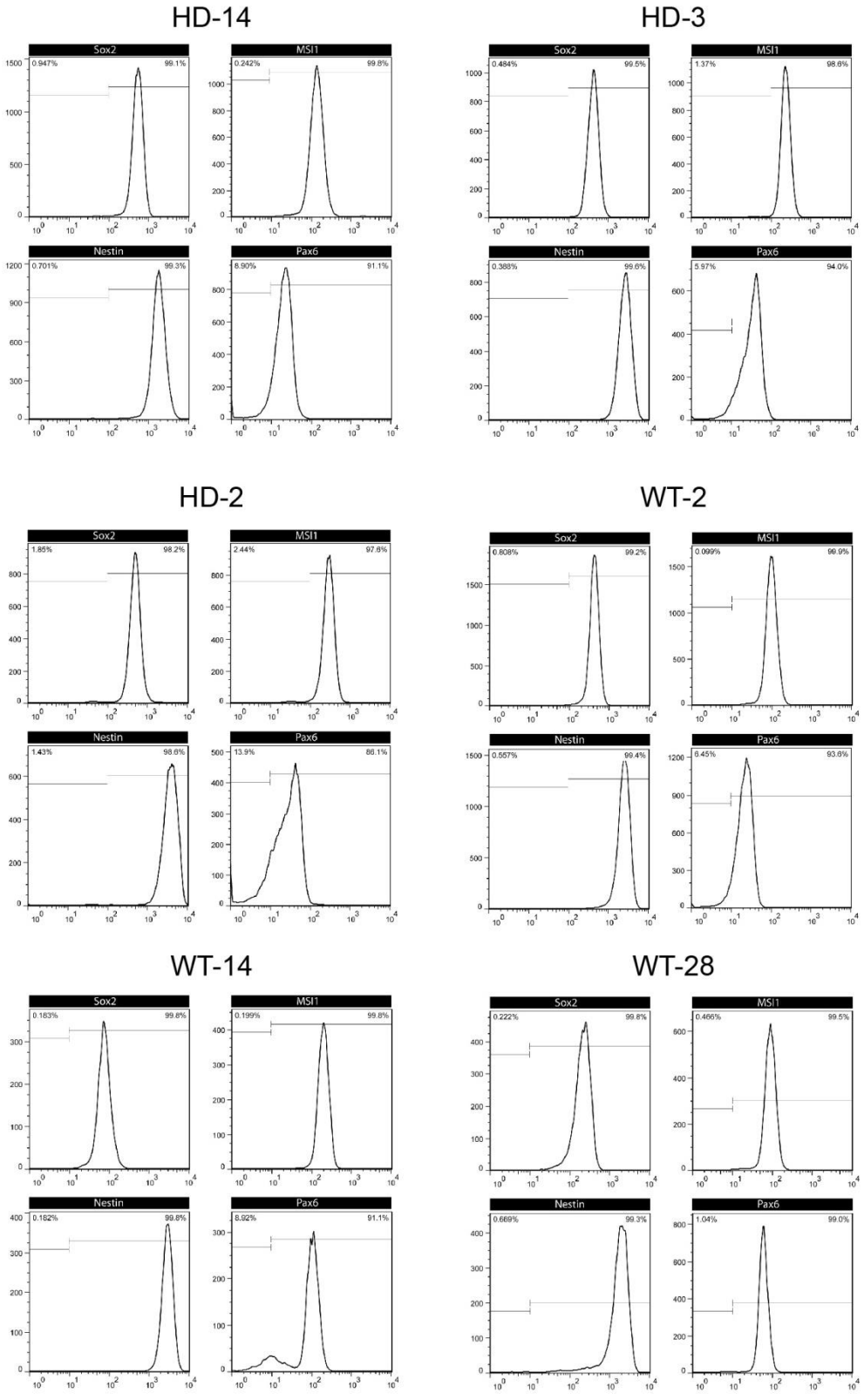

HD-14
Sox2
0.947%
99.1%
MSI1
0.242%
99.8%
Nestin
0.701%
99.3%
Pax6
8.90%
91.1%
HD-3
Sox2
0.484%
99.5%
MSI1
1.37%
98.6%
Nestin
0.388%
99.6%
Pax6
5.97%
94.0%
HD-2
Sox2
1.85%
98.2%
MSI1
2.44%
97.6%
Nestin
1.43%
98.6%
Pax6
13.9%
86.1%
WT-2
Sox2
0.808%
99.2%
MSI1
0.099%
99.9%
Nestin
0.557%
99.4%
Pax6
6.45%
93.6%
WT-14
Sox2
0.183%
99.8%
MSI1
0.199%
99.8%
Nestin
0.182%
99.8%
Pax6
8.92%
91.1%
WT-28
Sox2
0.222%
99.8%
MSI1
0.466%
99.5%
Nestin
0.669%
99.3%
Pax6
1.04%
99.0%

Figure 2-5. FACS analysis demonstrates uniform population of NPCs derived from monkey PSCs. FACS analysis of cells immunostained for NPC markers Sox2, Nestin, MSI1, and Pax6 reveal robust homogeneity was achieved in all monkey NPC cell lines. Histogram plots represent quantification of cells positive for respective markers over the total population of cells counted. Percentages reflect the negative and positive frequency of cells. FACS data for all cell lines is tabulated in **Table 2-2**.

Figure 2-6

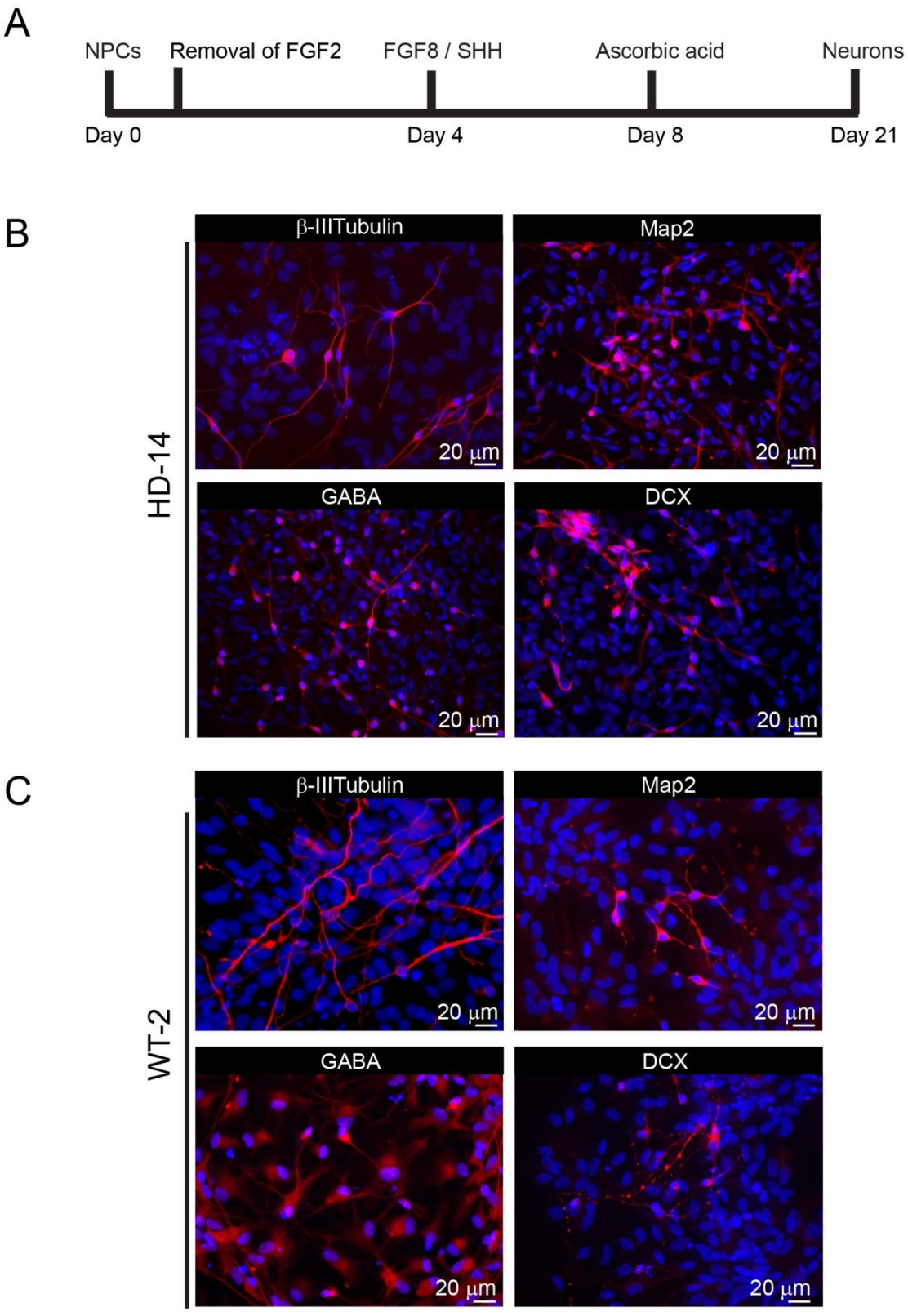

A
NPCs
Removal of FGF2
FGF8 / SHH
Ascorbic acid
Neurons
Day 0
Day 4
Day 8
Day 21
B
HD-14
β-IIITubulin
Map2
GABA
DCX
20 μm
C
WT-2
β-IIITubulin
Map2
GABA
DCX
20 μm

Figure 2-6. Monkey NPCs derived GABA neurons *in vitro.*

(A) Schematic diagram depicting neural differentiation from monkey NPCs. At the start of neural differentiation FGF2 was removed from culture medium. After 4 days FGF8 and SHH were added to culture medium to induce neural differentiation. Ascorbic acid was added to culture medium at day 8 of differentiation. After 21 days neurons were characterized.

(B and C) Immunocytochemistry of neurons derived from HD-14 and WT-2 NPCs differentiated *in vitro* for 21 days show positive staining for neural markers β-III Tubulin, MAP2, DCX, and GABA (red). Nuclear staining shown using Hoechst (blue). Scale bars: 20μm

Figure 2-7

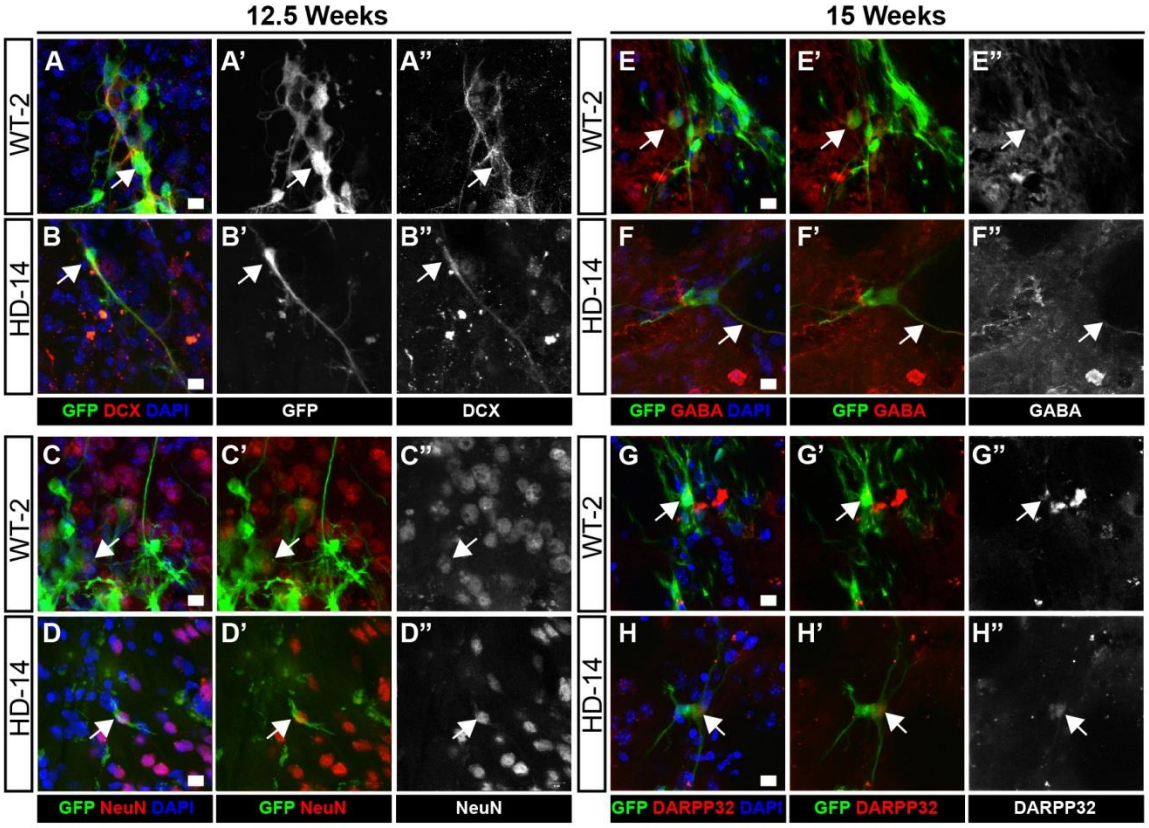

12.5 Weeks
15 Weeks
WT-2
HD-14
A
A'
A"
B
B'
B"
E
E'
E"
F
F'
F"
C
C'
C"
D
D'
D"
G
G'
G"
H
H'
H"
GFP DCX DAPI
GFP
DCX
GFP GABA DAPI
GFP GABA
GABA
GFP NeuN DAPI
GFP NeuN
NeuN
GFP DARPP32 DAPI
GFP DARPP32
DARPP32

Figure 2-7. Survival and neural differentiation of transplanted NPC in mouse striatum.

Immunohistochemical staining showed monkey NPCs transplanted into the striatum of mouse survived and were capable to differentiate to neurons.

(A, B) After 12.5 weeks grafting, GFP-expressing WT-2 NPCs and HD-14 NPCs are positive for DCX (Red) as arrows indicate. A', A", B', and B" are separation of GFP (green) and DCX (red) for clarity. (C, D) Grafted WT-2 NPCs are expressing mature neuron marker, NeuN (red), as arrow indicates, but HD-NPCs are NeuN-negative. C' and D' show the co-localization of GFP/NeuN. C" and D" are separated for clarity.

(E) Grafted WT-2 NPCs express GABA staining (red). E' and E" show separated GFP (green) and GABA (red) for clarity. (F) After 15 weeks grafting, GFP-expressing WT-2 NPCs and HD-14 NPCs show positive for GABA (red) as arrows indicate. F', F" are separated for clarity.(H, I) WT-2 NPCs and HD-14 NPCs show positive for DARPP32 staining (red). H', H", I', I" are separated for clarity. Scale bars: 10μm

Figure 2-8

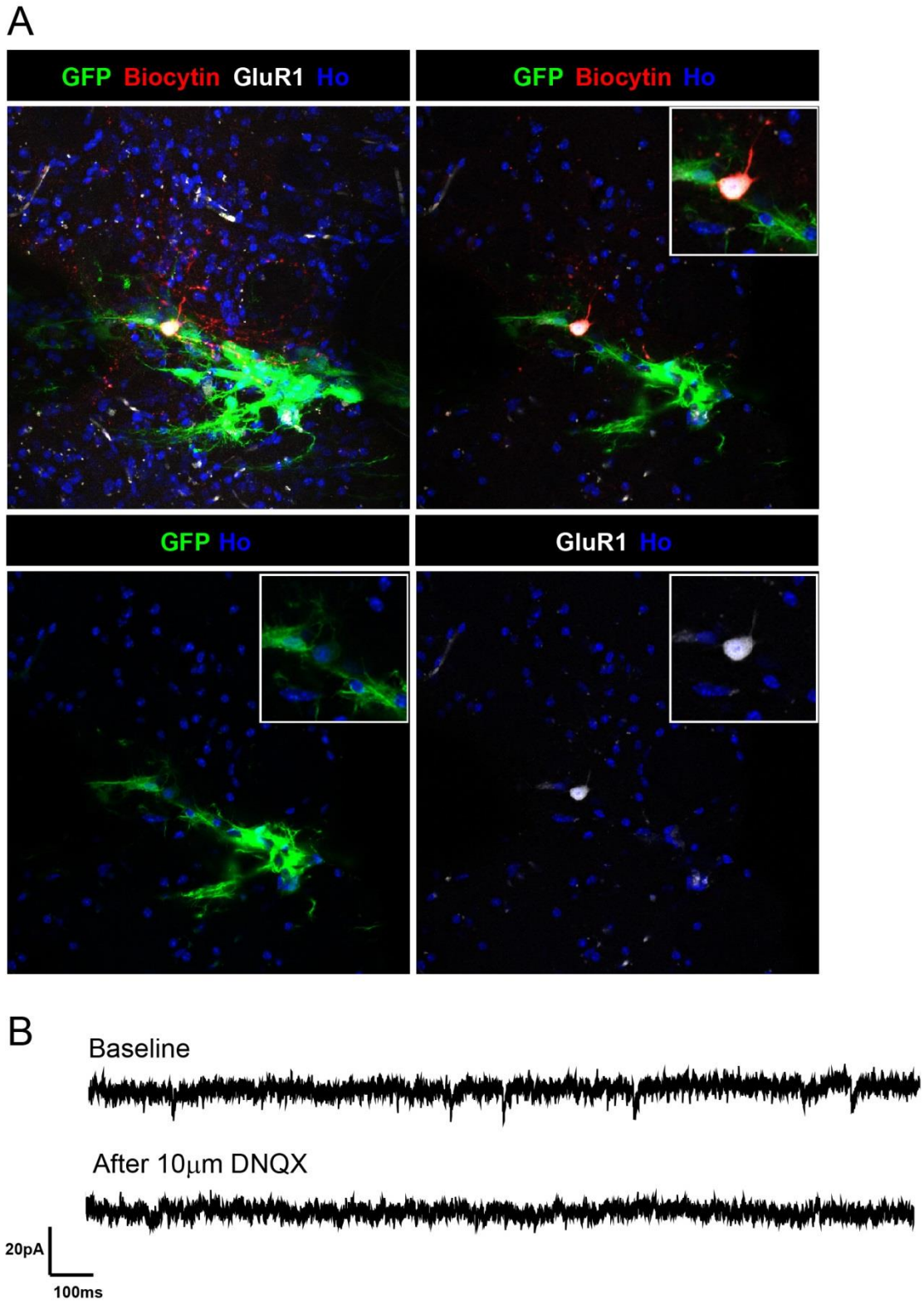

A
GFP Biocytin GluR1 Ho
GFP Biocytin Ho
GFP Ho
GluR1 Ho
B
Baseline
After 10μm DNQX
20pA
100ms

Figure 2-8. Transplanted monkeys neurons show spontaneous synaptic activity.

At 30 weeks post transplantation WT-2 cells integrated into mouse striatum. Whole cell patch recording showed spontaneous synaptic activity at resting membrane potential. (A) Neurons co-labeled by GFP (green) and biocytin filling (red) show positive staining for glutamate receptor (GluR1) (white). (B) Representative spontaneous synaptic activity was recorded from transplanted WT neuron. Synaptic activity was abolished after application of glutamate receptor antagonist DNQX.

Table 2-1. RT-qPCR primers used in gene expression analysis

Gene Symbol	TaqMan® Primer Context Sequence	
GAPDH	TCCAGGAGCGAGATCCCTCCAAAAT	
MSI1	TTTGAGCAGTTTGGGAAGGTGGACG	
NES	CCACGTACAGGACCCTCCTGGAGGC	
PAX6	ATGCAGAACAGTCACAGCGGAGTGA	
POU5F1 (OCT4)	CCCTGGGGGTTCTATTTGGGAAGGT	
SOX2	GGCCCCGGCGGAAAACCAAGACGCT	
	SYBR Forward Primer	SYBR Reverse Primer
HTT Exon 1	GCGACCCTGGAAAAGCTGAT	CTGCTGCTGCTGGAAGGACT
Ubiquitin C	CCACTCTGCACTTGGTCCTG	CCAGTTGGGAATGCAACAACTTTA

Table 2-2. Summary of HD and WT monkey cell lines included in this study

Cell Line	HD-14	HD-3	HD-2	WT-2	WT-14	WT-28
Monkey Donor Reference	Yang *et al*., 2008	Chan *et al*., 2010	Putkhao *et al*., 2012			
Phenotype	HD	HD	HD	WT	WT	WT
Source	DPSC	Fibroblast	ICM/ESC	ICM/ESC	Fibroblast	ICM/ESC
CAG size	27, 65	72	29	NP	NP	NP
Mutant *HTT* Expression	X	X	X	ND	ND	ND
ICC Characterization	X	X	X	X	X	X
RT-qPCR Characterization	X	X	X	X	X	X
FACS Analysis	X	X	X	X	X	X
***In Vitro* Differentiation**	X	X	X	X	X	X
***In Vivo* Differentiation**	X	NP	NP	X	NP	NP

Table 2-3. Summary of FACS analysis

Cell Line	FACS Results (% Positive)			
	Nestin	**SOX2**	**PAX6**	**MSI1**
HD-14	99.3	99.1	91.1	99.8
HD-3	98.6	98.2	86.1	97.6
HD-2	99.6	99.5	94	98.6
WT-2	99.4	99.2	93.6	99.9
WT-14	99.8	99.8	91.1	99.8
WT-28	99.3	99.8	99.0	99.5

CHAPTER 3

Huntington's disease monkey neural cells develop classic neuropathologic phenotypes

This chapter will be published in **Stem Cell Reports:**

Carter, R.L., Chen, Y., Kunkanjanawan, T., Xu, Y., Putkhao, K., Moran, S.P., Yang, J., Huang A.H.C., Parnpai, R., and Chan A.W. Reversal of cellular phenotypes in neural dells derived from Huntington's disease monkey induced pluripotent stem cells. *Stem Cell Reports*. 2014 (Under review)

3.1 Abstract

Pluripotent stem cells (PSCs) are a powerful system for investigating cellular pathology in Huntington's disease (HD). Capable of generating disease relevant neural populations, HD PSCs mirror the genetic background of the donor, and recapitulate the pathologic events leading to neurodegeneration in HD. As these studies reveal the potential for pluripotent HD stem cell models as a tool for drug discovery and therapeutic development, the need arises for a preclinical non-human primate (NHP) model of HD that will be ready to assess the safety and efficacy of new therapeutics. In this study we show HD monkey neural cells develop HD associated phenotypes. Upon differentiation to neurons, HD neural cells developed cellular features of HD including the formation of nuclear inclusions and progressive accumulation of oligomeric mutant huntingtin (mHTT) aggregates. HD neural cells also show susceptibility to cell stress demonstrated by increased apoptosis. Together these findings highlight the utility of PSCs derived neural cells from HD monkeys in providing a unique NHP platform for studying HD pathogenesis, drug discovery study, and pre-clinical research for therapeutics that can be validated in HD monkeys.

3.2 Introduction

The hallmark of HD is neurodegeneration, predominantly in the striatum and cortex (Reiner et al., 1988). Nuclear inclusions of mHTT in striatal neurons precede symptom onset, however the specific cause of neural degeneration is not well understood (Sapp et al., 1997; Labbadia and Morimoto, 2013). Proposed disease mechanisms include the activation of proteases leading to apoptosis, transcriptional dysregulation,

mitochondrial and proteosome dysfunction, as well as increased susceptibility to oxidative stress (Zuccato et al., 2010; Labbadia and Morimoto, 2013). Many of these cellular pathologies are potential targets for developing therapies that may halt neurodegeneration of affected neurons, subsequently slowing or reversing disease progression. Recently the attention of HD research has turned towards establishing PSC models from HD patients and animal models. The ability to direct PSC differentiation to specific neuronal sub-types provides opportunities for both mechanistic studies and the discovery of new disease therapies. Published works using this approach have reported a degree of success, nonetheless these studies are limited in that induced pluripotent stem cells (iPSCs) derived from human patients have not shown complete recapitulation of HD neuronal phenotypes (Zhang et al., 2010; An et al., 2012; Camnasio et al., 2012; The Hd iPSC Consortium, 2012). In addition, human studies are limited in the opportunity for *in vivo* longitudinal follow up to evaluate the safety and efficacy of candidate treatments (Perrier and Peschanski, 2012).

As these studies reveal the potential for pluripotent HD stem cell models as a tool for drug discovery and therapeutic development, the need arises for a preclinical large animal model of HD that may facilitate assessment of safety and efficacy of new therapeutics (Perrier and Peschanski, 2012; Emborg et al., 2013). Since safety concerns limit the direct translation of findings from human HD-iPSC studies, incorporating large animal models such as NHPs provides a specific advantage for long-term *in vivo* assessment, especially in the case of genetic correction and cell transplantation studies. Our research team developed the first transgenic NHP model of HD (Yang et al., 2008). HD monkeys were generated by injecting oocytes with lentiviruses expressing exon 1 of

the human *HTT* gene carrying 84 CAG repeats, under the control of human polyubiquitin-C promoter. HD monkeys demonstrated clinical features of HD including dystonia and chorea. Post-mortem neural pathological analysis revealed the presence of nuclear inclusion and neuropil aggregates in the cortex and striatum, demonstrating a neuropathology consistent with that observed in human patients (Yang et al., 2008).

The HD cells included in this work are derived from HD monkeys. Here we show that HD monkey neural cells develop classic HD cellular phenotypes. Elevated expression of mHTT was observed upon induction to the neuronal lineage, along with the development of nuclear inclusions and oligomeric aggregation. Furthermore we demonstrate vulnerability of HD neural cells to oxidative stress and removal of growth factors. These results provide a unique cellular platform for studying HD pathogenesis to gain further insight into cellular disease mechanisms, and serve as a resource for the screening and evaluation of drug and gene based therapies.

3.3 Methods

Rhesus monkey NPC *in vitro* neural differentiation

For *in vitro* differentiation of neural progenitor cells (NPCs) to the neuronal lineage, NHP NPCs were seeded at density of $5x10^4$ cells/cm^2 on a P/L-coated glass chamber (Fisher Scientific) in neural proliferation medium. The following day medium was replaced with differentiation medium [DMEM/F12 (Life Technologies), P/S (Invitrogen), 2 mM L-glutamine, 1x N2 (Invitrogen), and 1x B27 (Life Technologies)]. At 4 days of neural induction, SHH (R&D, 0.2 μg/mL) and FGF8 (R&D, 0.1 μg/mL) were added to medium. At day 8, 200 μM ascorbic acid (Sigma) was added in combination with SHH and FGF8.

At the end of 21 days, cells were either fixed for immunocytochemistry or harvested for protein extraction.

Real-time quantitative PCR (RT-qPCR)

Total RNA from cell samples was prepared using TRIzol® (Life Technologies), and genomic DNA was removed from the RNA sample using Turbo DNA-free (Applied Biosystems) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using a RNA-to-cDNA kit (Applied Biosystems). RT-qPCR was performed using SsoAdvanced Universal SYBR Green Suppermix (Bio-Rad) and SYBR green primers on a CFX96 Real-Time Detection System (Bio-Rad). RT-qPCR primer sequences are listed in **Table 2-1.** PCR conditions followed manufacturer's instruction (Bio-Rad): Initial 95°C polymerase activation step for 30 sec followed by amplification cycles 95°C for 10 sec and 55°C for 30 sec for 40 cycles.

Western Blot Analysis

Cell samples were lysed in RIPA buffer including protease inhibitors at 4^{o}C for 30 min followed by sonification. Homogenates were centrifuged at 14,000 g at 4^{o}C for 15 min. Protein supernatant was collected and protein samples were quantified using Bio-Rad DC Protein Assay (Bio-Rad). Equal amounts of protein extract (100μg) were loaded and separated by electrophoresis in 9% SDS-PAGE gel. Proteins were transferred to a PVDF membrane overnight at 4^{o}C run at 30V. Blots were probed with primary antibodies: mEM48 (1:100) and γ-tubulin (1:500, Sigma), and secondary antibody: peroxidase-conjugated AffiniPure donkey anti-mouse IgG (1:1000; Jackson ImmunoResearch). mEM48 antibody recognizes mHTT with expanded poly glutamine.

Immunocytochemistry

NPCs and neurons cultured on P/L-coated glass chamber slides were fixed in 4% paraformaldehyde (PFA) followed by permeabilization and blocking with 3% bovine serum albumin (Sigma) in phosphate buffered saline (PBS) solution. Fixed slides were incubated overnight at 4°C with primary antibodies. Slices were washed in PBS followed by incubation with secondary antibody for 1 hr at room temperature. Primary and secondary antibodies used included βIII-tubulin (1:300; MAB1673,Millipore), doublecortin (DCX; 1:500; AB18723, AbCam), microtubule-associated protein (MAP2; 1:500, Millipore), and mEM48 (1:50). Secondary antibodies included Alexa Fluor 594/488 (1:1000; A21203, Molecular Probes), and Hoechst 33342 (5 mg/mL) for DNA staining. Samples were examined using a microscope (Olympus BX51) equipped with an epifluorescent device.

Cell Stress and Apoptosis Assays

Hydrogen Peroxide (H_2O_2): NPCs were treated with 5 mM H_2O_2 in culture medium for 24 hr. Following incubation cells were harvested and stained to assess caspase 3/7 activity using Muse™ Caspase-3/7 Kit (Millipore) according to the manufacturer's instructions. Cell counts were analyzed using Muse Cell Analyzer (Millipore).

Growth factors removal: Growth factors were removed from base culture medium for 24 hr. Following 24 hr withdrawal of growth factors, cells were harvested and stained to assay caspase 3/7 activity using a Muse™ Caspase-3/7 Kit (Millipore) according to the manufacturer's instructions. Cell counts were analyzed using Muse Cell Analyzer (Millipore).

TUNEL: Effects of H_2O_2 were further assessed by TUNEL analysis. Samples were prepared on P/L-coated coverslips. Following H_2O_2 treatment for 24 hr, samples were

fixed in 4% PFA and stained using an In Situ Cell Death Detection Kit (Roche) to quantify the percentage of TUNEL-positive cells. For each replicate, 6 images were taken randomly and quantified using CellSens software (Olympus).

Statistical analysis

One-way ANOVA with Tukey's posttest was used for the comparison of multiple groups. All experimental data using cell cultures represent three biological replicates, and samples were run in duplicate or triplicate. Statistical significance was defined as $P < 0.05$, and all graphs are expressed as mean ± SEM.

3.4 Results

Methods to derive NPCs and neuronal populations from HD monkey iPSC and ESCs were described in Chapter 2. HD cell lines carry exon 1 of human *HTT* transgene with expanded CAG repeats driven by the human polyubiquitin-C promoter and green fluorescent protein (GFP) **(Figure 3-1)**. HD-14 is estimated to have two copies of the transgene with 27 and 65 CAGs, HD-3 is estimated to have 72 CAGs, and HD-2 is estimated to have 29 CAG repeats. All WT cell lines are derived from non-transgenic, wild-type (WT) monkeys.

Aggregation of mHTT and the formation of intranuclear inclusions are classic HD associated neuronal phenotypes (DiFiglia et al., 1997) that have been described in human patients as well as in animal models, including HD monkeys (Yang et al., 2008) and HD rodent models (Wang et al., 2008; Pouladi et al., 2013). Although *HTT* is expressed in all cell types (Li et al., 1993), neuronal cell types are most susceptible to the toxicity caused by mHTT (Johnson and Davidson, 2010). RT-qPCR analysis of HD-NPCs during neural

differentiation revealed elevated expression of total *HTT* transcripts in HD neural cells compared to WT neurons **(Figure 3.2)**.

We next looked to determine if increased expression in neural cell also led to increase oligomeric aggregation in HD neurons. After 21 days *in vitro* differentiation, protein samples were isolated for western blot. Using mEM48, an antibody reactive to mHTT with expanded glutamine repeats (Gutekunst et al., 1999; Chan et al., 2010), western blot analysis of HD and WT cells at different stages of differentiation revealed increased accumulation of oligomeric mHTT at high molecular weight (>250kDa) in HD-3 and HD-14 **(Figure 3.3)**. Interestingly, the increased oligomeric aggregates are only seen in HD neural cells and are absent in HD-iPSCs. These findings are consistent with a prior study showing that the expression of *mHTT* and the accumulation of oligomeric mHTT aggregates increase during *in vitro* neural development (Chan et al., 2010; Laowtammathron et al., 2010)

Along with oligomeric aggregates, the development of nuclear inclusions is a key hallmark of HD (Davies et al., 1997; DiFiglia, 1997). Aggregates were shown to be especially toxic when directed to the cell nucleus (Yang et al., 2002), and studies in HD mice suggest that the formation of inclusions may correlate with disease progression (Zuccato et al., 2010). Nuclear inclusions develop in the cortex and striatum of HD monkeys, therefore we asked if neural cells derived from HD monkeys recapitulate this phenotype. Consistent with western blots results, immunostaining with mEM48 to detect mHTT aggregates revealed HD neural cells developed mHTT nuclear inclusions, shown co-labeled with neuronal markers DCX and MAP2 **(Figure 3.4)**. In addition to nuclear inclusions, cytosolic mHTT aggregates were found in HD neurons. These results

demonstrate the recapitulation of a hallmark cellular features of HD that correlates with neural differentiation in monkey HD cells.

In addition to the development of classic HD cellular phenotypes, we also investigated whether HD-NPCs were more susceptible to oxidative stress compared to WT-NPCs, as previously shown in human HD-iPSCs and neurons (An et al., 2012; The Hd iPSC Consortium, 2012). Oxidative stress is thought to play a role in a number of age related disorders including HD (Butterfield et al., 2001; Rotblat et al., 2014). mHTT interacts with the mitochondria and may impair pathways that protect the cell from reactive oxygen species, leading to mitochondrial and DNA damage (Rotblat et al., 2014). Oxidative stress was induced by exposing cells to H_2O_2 added to cell culture medium. Following incubation for 24 hr, TUNEL assay was performed to measure cell death in response to H_2O_2 exposure. TUNEL is a widely used methods for detecting DNA fragmentation resulting from apoptosis. TUNEL analysis demonstrated a significant increase in cell death in HD-NPCs compared to WT-NPCs following H_2O_2 exposure **(Figure 3.5 A-B)**.

We next took a population based approach to assess caspase mediated apoptosis in response to oxidative stress. Caspase 3/7 activity was used in combination with flow cytometry to measure the percentage of apoptotic cells after H_2O_2 exposure. Consistent with TUNEL results, analysis of caspase 3/7 activity revealed a significant increase in apoptotic HD-NPCs compared to WT-NPCs after exposure to H_2O_2 **(Figure 3.6A)**. Cell stress can also be induced by withdrawal of growth factor support in the culture media. Removal of growth factors has been shown to induce cell death in cellular models of HD (Zhang et al., 2010; An et al., 2012). To test this in monkey HD-NPCs, growth factors

were withdrawn from culture medium for 24 hours. Analysis of caspase 3/7 activity showed a significant increase in apoptotic HD-NPCs compared to WT-NPCs. (**Figure 3.6B)**. Together, these findings demonstrate that HD-NPCs are more vulnerable cell stress and recapitulates HD associated cell death phenotypes.

3.5 Discussion

HD monkeys recapitulate clinical features of HD that open up a new opportunity for future pre-clinical studies in a higher primate model. Given the advances in iPSCs for modeling human diseases and developments towards personal medicine, specifically cell replacement therapy, we now need a preclinical model that could help assess therapeutic efficacy prior to clinical translation (Perrier and Peschanski, 2012).

As a potential platform to model HD cellular pathogenesis, we focused on the expression pattern of the *mHTT* transgene in our model. An important goal of this study was to assess whether our cellular model recapitulates disease associated pathologic events. We found that an increase of intranuclear inclusions and the accumulation of oligomeric, insoluble mHTT aggregates were correlated with neuronal differentiation. This observation mirrors a key neural pathology seen in human patients' brains, as well as in HD animal models (Mangiarini et al., 1996; DiFiglia et al., 1997; Yang et al., 2008). Aggregation of mHTT is suspected to impair a number of important cellular processes. Evidence suggest mHTT may disrupt cellular homeostasis by overloading protein cleavage pathways (Li et al., 2010). Other studies show that mHTT interacts with a host of other proteins in the cell, and that aggregates may sequester transcription factors and structural proteins, leading to alterations in metabolism, transcription, axonal trafficking (Li et al., 2000; Choo, 2004; Bae et al., 2005).

In addition to mHTT aggregation, HD-NPCs show increased apoptosis after exposure to H_2O_2 as a means of inducing oxidative stress. HD-NPC also showed increased apoptosis following withdrawal of growth factors. This disease dependent difference in vulnerability to exogenous stress recapitulates phenotypes characterized by studies using human pluripotent stem cells (Nguyen et al., 2011; An et al., 2012; The Hd iPSC Consortium, 2012). Understanding cellular pathways that contribute to apoptosis in HD is of major importance. Finding increased apoptosis during oxidative stress conditions, suggest mitochondrial function may also be impaired in our HD-NPCs. Moreover apoptosis resulting from grow factor withdrawal may correlate to alterations in transcription and metabolite regulation in HD-NPCs.

Together these findings suggest that our *in vitro* system is comparable to other previously described cellular models that HD-NPCs and derivative neural cells develop pronounced HD cellular phenotypes. Furthermore, these HD phenotypes develop without requiring proteasome inhibition, which in some reports was necessary to induce HD associated phenotypes (Jeon et al., 2012; Cheng et al., 2013). We suspect, unlike human iPSCs, our HD-NPCs express multiple copies of truncated *mHTT* gene with expanded polyglutamine tract regulated by human ubiquitin-C promoter, which may lead to more pronounced phenotype and impact neural differentiation.

HD has also been shown to negatively alter synaptic function of neurons (Miller et al., 2008; Walker et al., 2008; The Hd iPSC Consortium, 2012). Due to limitation discussed in Chapter 2, we were unable to evaluate electrophysiological changes in HD neurons. This may likely be attributed the aggressive neuropathology of our HD cell lines. We found that HD cells show defects in neuronal morphology, and struggled to

survive long differentiation protocols that are necessary for full neuronal maturity. Nonetheless, recapitulation of phenotypes in HD-NPCs and early neurons provides a paradigm to investigate early cellular consequences of mHTT. This work may also be further expanded to investigations in other cell types that may influence neurodegeneration. Evaluation of HD associated phenotypes in astrocytes and microglia, could provide valuable insight into disease mechanisms that are attributed to non-neural cells.

In summary, we generated an *in vitro* platform derived from HD monkey PSCs. The replication of hallmark features of HD *in vitro* allow for deeper investigation into cellar processes that may be involved in neurodegeneration and are potential targets for therapy.

Figure 3-1

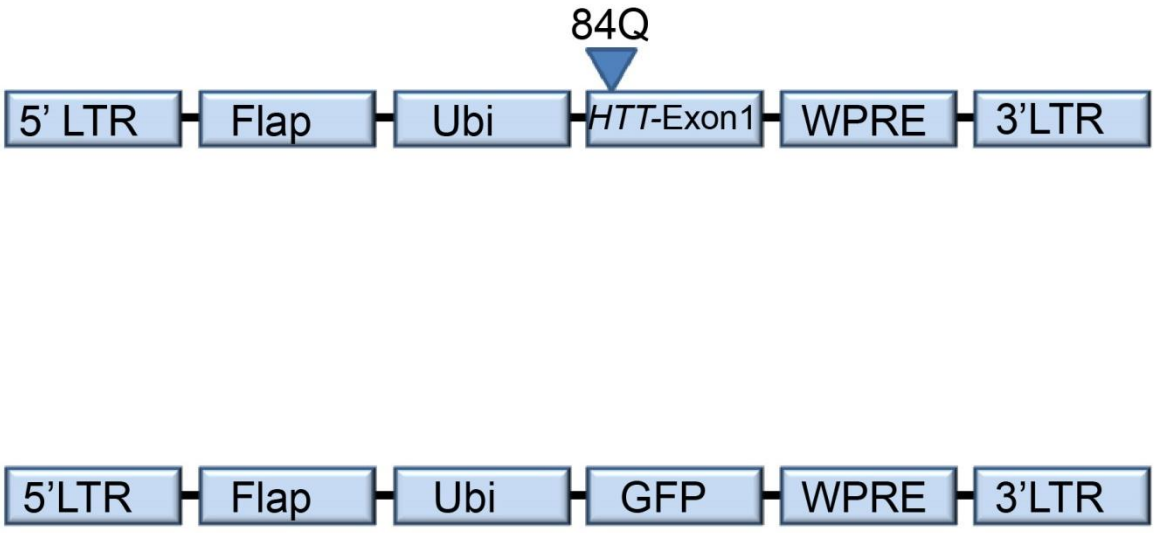


Figure 3-1. HD monkey cells carry mutant *HTT* transgene.

HD monkey cell lines are derived from transgenic HD monkeys carrying *mHTT* transgene. This schematic illustrates the lentiviral vector carrying exon 1 of the human *HTT* gene with expanded CAG repeats. Cells derived from HD monkeys also carry *GFP*. Both vectors regulated by human poly-ubiquitin-C promoter. LTR, long terminal repeats; Flap, HIV-flap sequence; GFP, green florescent protein; *HTT*, human huntingtin exon 1; Ubi, ubiquitin promoter; WPRE, woodchuck post-transcriptional regulatory element.

Figure 3-2

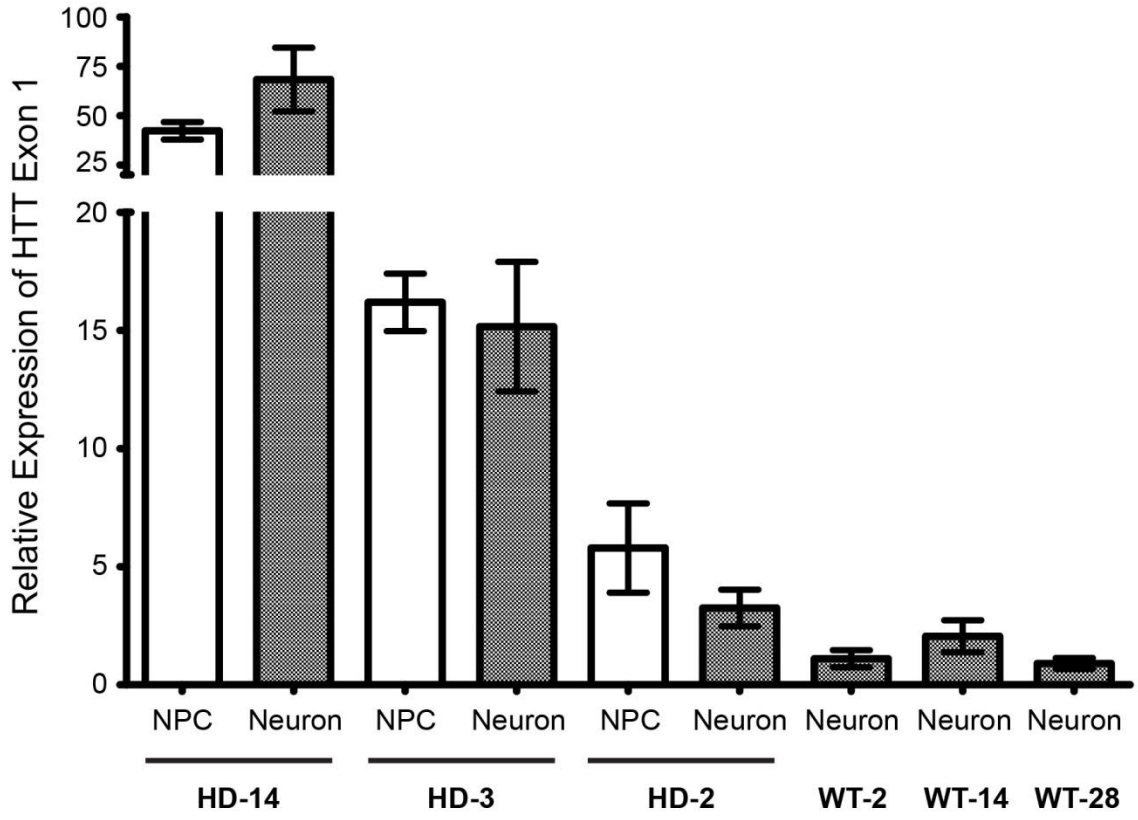


Figure 3-2. *HTT* expression is elevated in HD monkey neural cells.

HTT expression was analyzed by RT-qPCR at various stages of neural differentiation. *HTT* expression was increased in HD-NPCs and neurons compared to WT neuron expression levels. Expression values were normalized to *Ubiquitin-C* (*UBC*). All graphs are relative to expression values in WT-2 neuron and plotted as delta-delta CT method. Data are represented as mean +/- SEM.

Figure 3-3

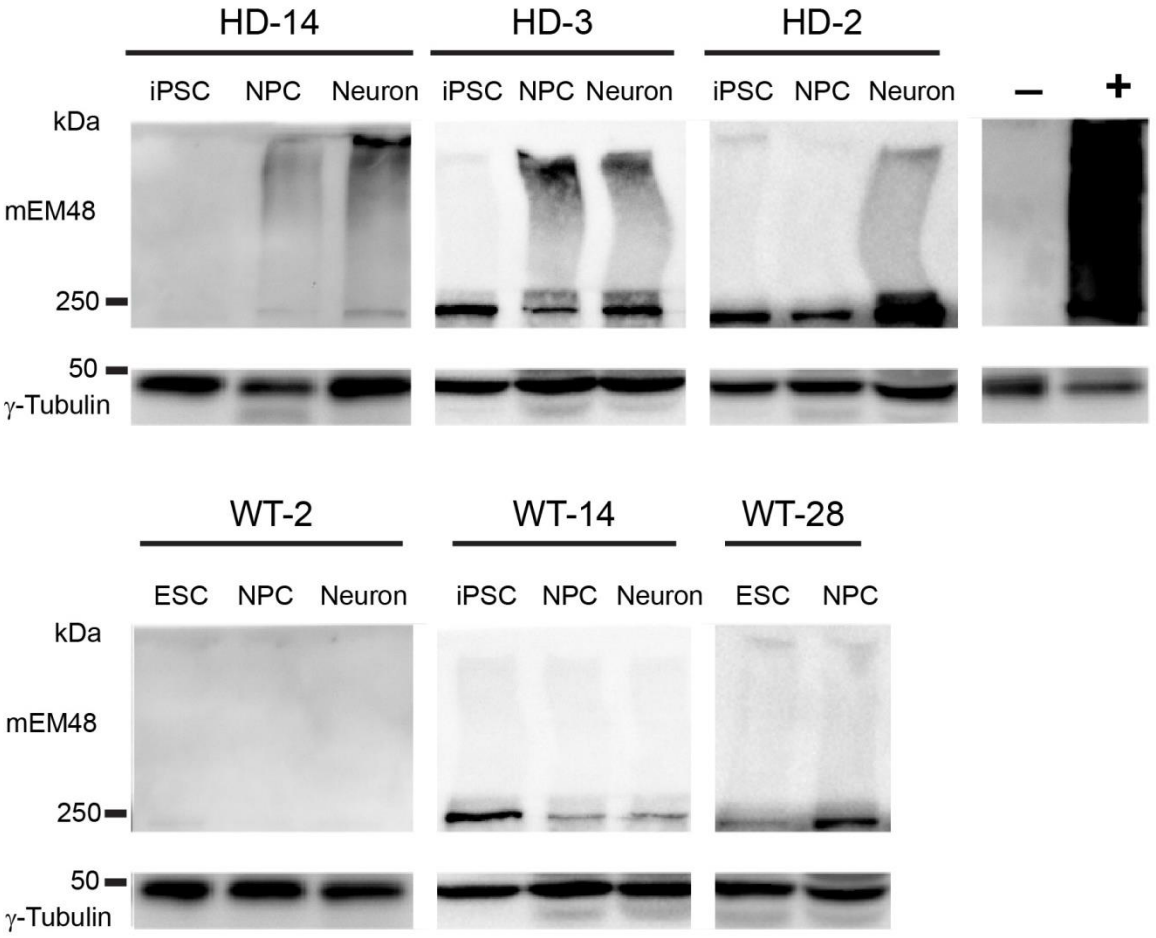


Figure 3-3. HD monkey neural cells develop oligomeric aggregation of mHTT.

Lysates from HD and WT cells at different stages of neural differentiation were analyzed for mHTT. Western blot analysis using mEM48 reveals an increase in oligomeric mHTT that correlates with neuronal differentiation stage. Notice aggregate accumulation in the stacking gel (above 250 kDa) in HD cells compared to absent accumulation in WT cell lines. Negative (-) and Positive (+) lanes are frontal cortex samples from non-transgenic and HD monkeys, respectively. γ-Tubulin was included as a loading control.

Figure 3-4

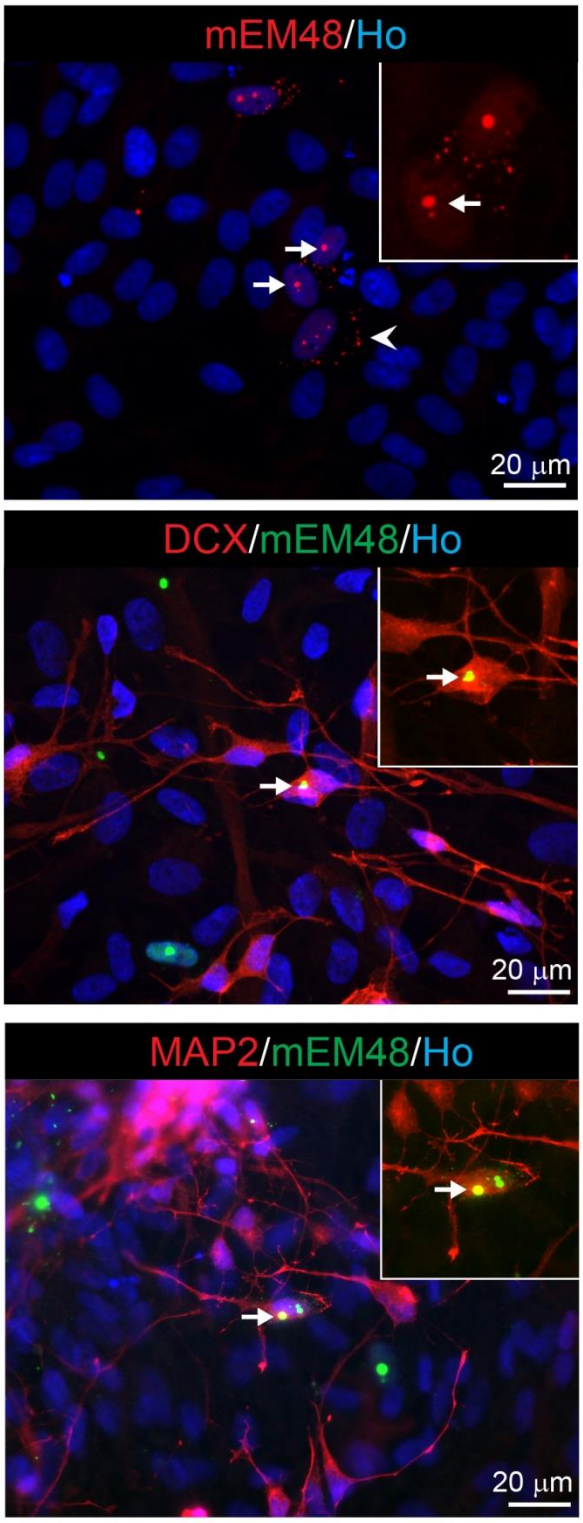


Figure 3-4. HD monkey neural cells develop mHTT nuclear inclusions.

HD monkey NPCs were differentiated for 21 days. Following neural differentiation, immunocytochemical staining revealed mHTT nuclear inclusions.

Top: Staining of HD-14 neurons with mEM48 (red) reveals distinct nuclear inclusions and cytosolic aggregates of mHTT. Arrows indicate nuclear inclusions. Arrow heads indicate cytosolic aggregates. Middle: mHTT inclusions (green) can be seen co-labeled with DCX (red). Bottom: mHTT inclusions (green) can be seen co-labeled with MAP2 (red). Nuclear staining using Hoechst (blue). Scale bars: 20μm.

Figure 3-5

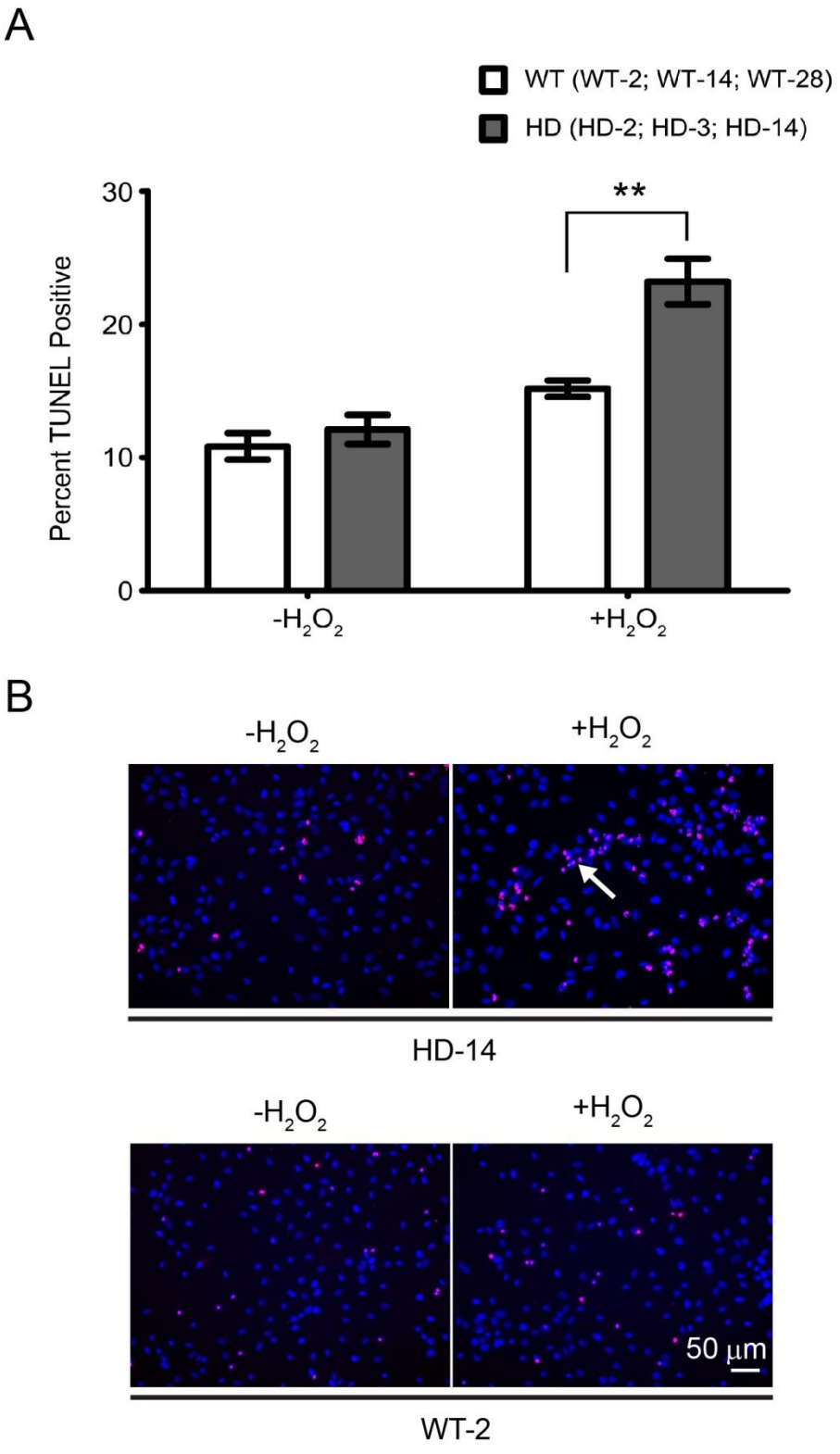

A
WT (WT-2; WT-14; WT-28)
HD (HD-2; HD-3; HD-14)
Percent TUNEL Positive
30
20
10
0
**
-H2O2
+H2O2
B
-H2O2
+H2O2
HD-14
-H2O2
+H2O2
50 μm
WT-2

Figure 3-5. HD monkey NPCs show increased vulnerability to oxidative stress.

Oxidative stress was induced in cell culture by exposure to hydrogen peroxide (H_2O_2) for 24 hours. After exposure cells were fixed and stained for cell death by TUNEL assay.

(A) Analysis of TUNEL positive cells reveals significant increase in cell death in H_2O_2 exposed HD-NPCs compared to H_2O_2 exposed WT-NPCs. TUNEL-positive cell percentage was averaged for 3 HD lines (HD-2, HD-3, HD-14) and 3 WT lines (WT-2, WT-14, WT-28). Percentage of TUNEL-positive cells were calculated from total number of cells from image fields randomly selected. Data are represented as mean±SEM. (**$p < 0.001$ ANOVA).

(B) Images represent TUNEL staining of HD-14 and WT-2 NPCs after 24 hour exposure in normal growth media or media treated with H_2O_2. Notice increased number of TUNEL positive HD-14 NPCs after treatment. TUNEL positive cells (red). Nuclear staining using Hoechst (blue). Scale bars: 50μM.

Figure 3-6

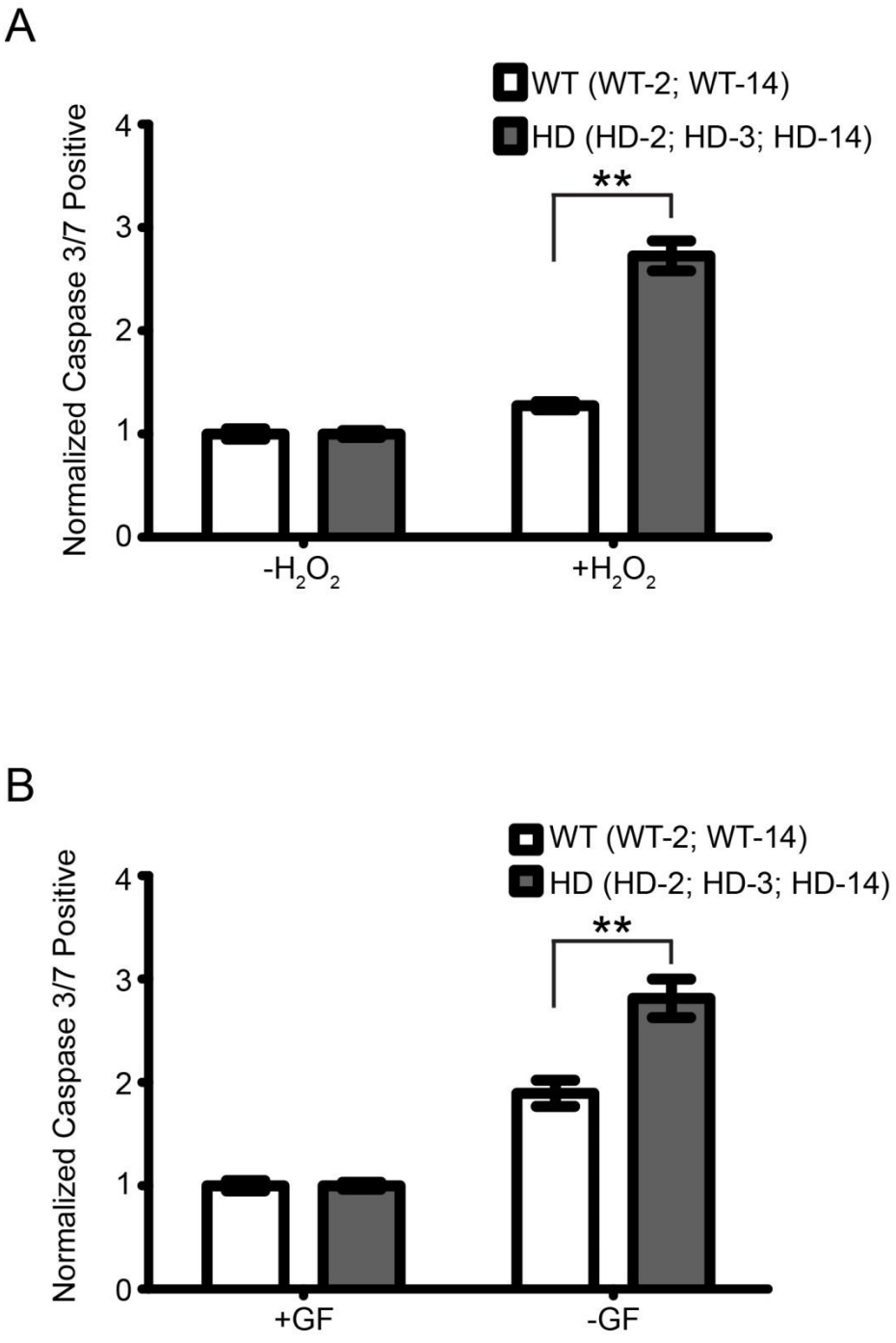

A
WT (WT-2; WT-14)
HD (HD-2; HD-3; HD-14)
Normalized Caspase 3/7 Positive
0
1
2
3
4
**
-H2O2
+H2O2
B
WT (WT-2; WT-14)
HD (HD-2; HD-3; HD-14)
Normalized Caspase 3/7 Positive
0
1
2
3
4
**
+GF
-GF

Figure 3-6. HD monkey NPCs show increased apoptosis in response to cellular stress. Oxidative stress was induced in cell culture by exposure to hydrogen peroxide (H_2O_2) for 24 hours. After exposure cells were harvested and caspase 3/7 activity was determined.

(A) Analysis of caspase 3/7 assay reveals significant increase in cell death in H_2O_2 exposed HD-NPCs compared to H_2O_2 exposed WT-NPCs. Apoptotic population percentages were averaged for 3 HD lines (HD-2, HD-3, HD-14) and 2 WT lines (WT-2, WT-14). Values represent fold change from untreated sample. Data are represented as mean±SEM (**p < 0.001 ANOVA).

(B) Cell stress was induced in cell culture by withdrawal of growth factors for 24 hours. After 24 hours, cells were harvested and assessed for caspase 3/7 activity. Analysis of caspase 3/7 assay reveals significant increase in cell death in HD-NPCs compared to WT-NPCs after growth factor withdrawal. Apoptotic population percentage was averaged for 3 HD lines (HD-2, HD-3, HD-14) and 2 WT lines (WT-2, WT-14). Values represent fold change from untreated sample. Data are represented as mean±SEM (**p < 0.001 ANOVA)

Chapter 4

Reversal of Huntington's disease associated cellular phenotypes support role of *in vitro* model for therapeutic evaluation

This chapter will be published in **Stem Cell Reports:**

Carter, R.L., Chen, Y., Kunkanjanawan, T., Xu, Y., Putkhao, K., Moran, S.P., Yang, J., Huang A.H.C., Parnpai, R., and Chan A.W. Reversal of cellular phenotypes in neural dells derived from Huntington's disease monkey induced pluripotent stem cells. *Stem Cell Reports*. 2014 (Under review)

4.1 Abstract

Pluripotent stem cell (PSC) models of Huntington's disease (HD) provide a unique opportunity to investigate therapeutic strategies in a high-throughput manner. As mechanisms of neurodegeneration are identified, specific pathways are uncovered as targets for therapeutic intervention. Genetic HD animal models are well suited to evaluate impact of a candidate therapy on behavior and movement defects, however developing corresponding *in vitro* systems is important to uncover the underlying molecular impacts of a therapeutic treatment and evaluate cytotoxicity. In this study we assess the impact of two popular therapeutic strategies for HD in induced pluripotent stem cell (iPSC) derived neural cells from HD monkeys. Using an RNA interference (RNAi) approach, expression of huntingtin (*HTT*) was reduced in HD neural progenitor cells (HD-NPCs). Knock down of *HTT* resulted in the reduction of oligomeric aggregates, and rescued apoptosis in HD-NPCs exposed to hydrogen peroxide (H_2O_2), as well as rescued apoptosis in differentiated HD neurons. We also show rescue of excitotoxicity in HD neural cells by treatment with *N*-methyl-D-aspartate receptor (NMDAR) antagonist memantine. This data demonstrates that HD monkey neural cells respond to therapeutic treatment. Furthermore, these results support the utility of our HD model as a platform to evaluate gene therapy and pharmacologic treatments *in vitro*.

4.2 Introduction

A promising direction in the search for novel therapeutic strategies in HD is the use of PSC derived neural cells as a platform for drug validation and discovery (Grskovic et al., 2011; Inoue and Yamanaka, 2011). Patients with HD have a very few disease

modifying treatments available, and the search for novel therapeutics is an active field of research. The inherent property of PSCs to differentiate to disease relevant neural subtypes allows for studies that assess the impact of candidate treatments on neuronal protection and function (Johnson and Davidson, 2010; Zuccato et al., 2010). Ideally HD stem cell models should produce robust and reproducible cellular phenotypes that mirror pathological features observed in human patients (Niclis et al., 2009; Saha and Jaenisch, 2009). The establishment of such models allow researchers to monitor phenotype progression as neurons develop, and may provide a key advantage in evaluating the timing at which therapy is most efficacious (Grskovic et al., 2011).

HD is a monogenetic disorder caused by a single, highly penetrant mutation (MacDonald et al., 1993). This makes HD an exceptional candidate for gene therapy approaches. Evidence suggest that suppression of *HTT* using non-coding small RNAs may be a viable therapeutic option (Bilsen et al., 2008; Pfister et al., 2009; Appl et al., 2012; Yu et al., 2012). Non-coding small RNAs are nucleotides capable to reduce mRNA expression via (RNAi) processing (Zhang and Friedlander, 2011). Several studies using RNAi approaches to suppress *HTT* expression in HD mice demonstrate that reduction of the mHTT protein rescues behavioral and neuropathology phenotypes in HD mice (Harper, 2005; Wang et al., 2005; Machida et al., 2006). Reports also indicate that transient reductions provide a sustained therapeutic benefit, providing further support for RNAi approaches in HD therapy (DiFiglia et al., 2007; Boudreau et al., 2009; Drouet et al., 2009; Kordasiewicz et al., 2012). Another approach using homologous recombination to correct the endogenous mutant allele of *HTT* demonstrated reversal HD associated phenotypes HD patient iPSCs (An et al., 2012). These provided proof of concept that

may lead to the exploration of other gene silencing methods to correct endogenous mutations.

Another approach in HD therapy is the development of small molecules that target specific pathways involved in pathogenesis. The few drugs that are available to the HD patient population are mainly for alleviation of symptoms, suppression of involuntary movements, improving mood, and regulation of appetite and sleep (Ross and Shoulson, 2009; Mestre and Ferreira, 2011; Morse et al., 2011). There has been very little progress in the discovery of drug treatments that slow disease progression. There is a particular interest in the discovery of small molecule compounds that inhibit the toxic effects of mHTT and reduce neurodegeneration (Fecke et al., 2009; Morse et al., 2011). iPSC derived models of HD may provide a powerful diagnostic tool in the discovery and validation of compounds that repair mitochondrial function, protect cells from toxic aggregates, or prevent excitotoxicity (Kaye and Finkbeiner, 2013). This strategy has been employed for a number of neurological disorders, leading to the discovery and pre-clinical validation of therapies (Marchetto et al., 2010; Egawa et al., 2012; Lee et al., 2012; Kondo et al., 2013). This approach depends on the identification and validation of effective targets, followed by phenotypic assays that screen for the reversal of cellular phenotypes observed in cellular based assays (Fecke et al., 2009; Johnson and Davidson, 2010; Morse et al., 2011). In addition, pre-clinical animal models, such as HD monkeys, that physiologically mirror disease progression in humans allow for novel drug candidates to be assess for safety and efficacy.

Genetically targeting *HTT* expression in iPSCs provides a unique opportunity to evaluate RNAi approaches in disease relevant cells that mirror the genetic background of

the donor (An et al., 2012). This rationale can also be applied in evaluating drug compounds in iPSC derived neural cell populations, allowing for the screening of candidates that lead to subsequent pre-clinical studies. In the context of this study, neural cells derived from HD monkey iPSCs express *mHTT* transgene and recapitulate HD associated phenotypes. Here we show that HD monkey neural cell respond to genetic and pharmacologic treatment. Aggregation and cell death phenotypes were reversed by RNAi mediated suppression of *HTT*. Furthermore neural toxicity was rescued by treatment with NMDAR antagonist memantine. The reversal of HD associated phenotypes in our model suggest that iPSC derived HD monkey neural cells provide an *in vitro* platform to evaluate therapeutic impact of new gene and drug treatments, which can be translated to *in vivo* assessment in HD monkeys.

4.3 Methods

shRNA virus preparation and infection

HD monkey NPCs were seeded at density of 5 x 10^4 cells per cm^2 in a 35-mm tissue culture dish. 2 mL concentrated lentivirus (pFH1-siHD-Ubi-Zeo) were added together with 8 μg/mL of poloybrene (Sigma, St. Louis, MO) in neural proliferation medium. After 48 hours infected cells were selected by the supplement of Zeocin (InvivoGen, 100μg/mL). NPCs were treated with Zeocin for 10 days before assessment of *HTT* knockdown. shRNA-HD target sequence:
GCGACCCTGGAAAAGCTGATGAAGGAAGGCCTTCATCAGCTTTTCCAGGGTC
GCTTTTTTGGAA

In vitro differentiation and memantine treatment

For *in vitro* differentiation of NPCs to the neuronal lineage, NHP NPCs were seeded at density of $5x10^4$ cells/cm^2 on a P/L-coated glass chamber (Fisher Scientific) in neural proliferation medium. The following day medium was replaced with differentiation medium [DMEM/F12 (Life Technologies), P/S (Invitrogen), 2 mM L-glutamine, 1x N2 (Invitrogen), and 1x B27 (Life Technologies)]. At 4 days of neural induction, SHH (R&D, 0.2 μg/mL) and FGF8 (R&D, 0.1 μg/mL) were added to medium. At day 8, 200 μM ascorbic acid (Sigma) was added in combination with SHH and FGF8. Cell were culture in differentiation medium for 21 days prior to treatment. At day 21 of neural differentiation, Memantine (Sigma, 10mM) was supplemented into culture medium and incubated for 24 hours prior to cytotoxicity assay.

Real-Time Quantitative PCR (RT-qPCR)

Total RNA from cell samples was prepared using TRIzol® (Life Technologies), and genomic DNA was removed from the RNA sample using Turbo DNA-free (Applied Biosystems) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using a RNA-to-cDNA kit (Applied Biosystems). RT-qPCR was performed using SsoAdvanced Universal SYBR Green Suppermix (Bio-Rad) and SYBR green primers on a CFX96 Real-Time Detection System (Bio-Rad). RT-qPCR primer sequences are listed in **Table 2-1.** PCR conditions followed manufacturer's instruction (Bio-Rad): Initial 95°C polymerase activation step for 30 sec followed by amplification cycles 95°C for 10 sec and 55°C for 30 sec for 40 cycles.

Western Blot Analysis

Cell samples were lysed in RIPA buffer including protease inhibitors at 4°C for 30 min followed by sonification. Homogenates were centrifuged at 14,000 g at 4°C for 15 min.

Protein supernatant was collected and protein samples were quantified using Bio-Rad DC Protein Assay (Bio-Rad). Equal amounts of protein extract (100μg) were loaded and separated by electrophoresis in 9% SDS-PAGE gel. Proteins were transferred to a PVDF membrane overnight at 4^{o}C run at 30V. Blots were probed with primary antibodies: mEM48 (1:100) and γ-tubulin (1:500, Sigma), and secondary antibody: peroxidase-conjugated AffiniPure donkey anti-mouse IgG (1:1000; Jackson ImmunoResearch). mEM48 antibody recognizes mHTT with increased signal to aggregates.

Cell Stress and Apoptosis Assays

Hydrogen peroxide (H_2O_2): NPCs were treated with 5 mM H_2O_2 in culture medium for 24 hr. Following incubation cells were harvested and stained to assess caspase 3/7 activity using Muse™ Caspase-3/7 Kit (Millipore) according to the manufacturer's instructions. Cell counts were analyzed using Muse Cell Analyzer (Millipore).

TUNEL: Cell death was assessed by TUNEL analysis. Samples were prepared on P/L-coated coverslips. Samples were fixed in 4% PFA and stained using an In Situ Cell Death Detection Kit (Roche) to quantify the percentage of TUNEL-positive cells. For each replicate, 6 images were taken randomly and quantified using CellSens software (Olympus).

Cell Cytotoxicity Assay

Monkey NPCs were seeded onto P/L-coated dishes and differentiated for 21 days in neural differentiation medium. At the end of 21 days, neural cells were treated with 10 mM memantine (Sigma) for 24 hr. Vybrant Cytotoxicity Assay Kit (Life Technologies), which monitors the release of the cytosolic enzyme glucose 6-phosphate dehydrogenase

(G6PD) from damaged cells into the medium, was used according to the manufacturer's instructions to assess cytotoxicity.

Statistical Analysis

One-way ANOVA with Tukey's posttest was used for the comparison of multiple groups. All experimental data using cell cultures were represent three biological replicates, and samples were run in duplicate or triplicate. Statistical significance was defined as $P < 0.05$, and all graphs are expressed as mean ± SEM.

4.4 Results

Following the characterization of HD phenotypes in neural cells derived from HD monkeys, we wanted to test whether these phenotypes could be rescued by therapeutic treatment. RNA interference (RNAi)-mediated reduction of *HTT* in HD models has shown great promise in a number of studies (Boudreau et al., 2009; McBride et al., 2011; Yu et al., 2012). We postulated that suppression of *HTT* expression in HD-NPCs would deliver a therapeutic benefit. HD-NPCs (HD-3 and HD-14) were infected with lentivirus expressing small hairpin RNA (shRNA) targeting the *HTT* transcript (shHD) **(Figure 4-1A)**. Cells carrying shHD were selected by resistance to Zeocin to establish stable transformed NPC lines. RT-qPCR analysis confirmed that shHD resulted in approximately 50% reduction of *HTT* in HD-3 and HD-14 NPCs **(Figure 4-1B)**.

After demonstrating successful knock down in shHD expressing NPCs (sh-HDNPCs), we asked if oligomeric mHTT aggregation was also reduced in knocked down HD cells. Western blot using mEM48 specific for mHTT, revealed that oligomeric aggregates were reduced in HD-3 and HD-14 neural cells. **(Figure 4-1C)**. Together these

results demonstrate the successful suppression of *HTT* expression in HD-3 and HD-14 neural cells and show the impact of shRNA treatment on oligomeric mHTT aggregation.

We next questioned whether suppression of *HTT* could reverse the cell death phenotypes and vulnerability to oxidative stress that was observed in previous experiments. HD-3 NPCs with and without expression of shHD were challenged with H_2O_2 to induce oxidative stress. As demonstrated by caspase 3/7 assay, after 24 hour exposure to H_2O_2 the percentage of caspase positive cells was significantly reduced in sh-HDNPCs in comparison to HD-3 NPCs **(Figure 4-2)**. Having demonstrated rescue of apoptosis in NPCs, we then asked if HTT suppression could rescue cell death in HD cells after neural differentiation. Quantification of TUNEL positive cells showed that cell death was significantly reduced in HD-3 and HD-14 neural cells expressing shHD when compared to HD neural cells **(Figure 4-3A-B)**. This data suggest that shRNA suppression of *HTT* rescued cell death in sh-HDNPCs and in differentiated neural cells.

In addition to *mHTT* expression, neurodegeneration has been attributed to NMDAR mediated cytotoxicity in HD (The Hd iPSC Consortium, 2012; Daggett and Yang, 2013; Marco et al., 2013). Reports of using memantine, a well-characterized NMDAR antagonist, have shown some promise as a potential therapy in HD (Okamoto et al., 2009; Milnerwood et al., 2010). Memantine is an un-competitive, open channel, NMDAR antagonist (Parsons et al., 1999; Lipton, 2006) **(Figure 4-4A)**. Memantine bound to NMDARs inhibits glutamatergic activation of the receptor, consequently blocking influx of calcium into the synaptic terminal (Lipton, 2006; Raymond et al., 2011). Excessive influx of calcium leads to neuronal cell injury, and activation of apoptotic cascades (Arundine and Tymianski, 2003; Milakovic et al., 2006; Raymond et

al., 2011). Memantine has been approved for moderate to severe Alzheimer's disease (Reisberg et al., 2003), and a small open label trial reports memantine may provide some clinical benefit in HD (Ondo et al., 2007).

To test whether treatment with memantine could rescue cell death observed in HD neurons, 10 mM memantine was supplemented into neural differentiation medium 24 hours prior to assessing cytotoxicity. Cytotoxicity was measuring by release of G6PD, a well-known cytotoxicity assay (Batchelor and Zhou, 2004). Cytotoxicity of HD neurons treated with memantine was significantly reduced compared to untreated HD neurons, while cytotoxicity in WT neurons was unchanged (**Figure 4-4B)**. This results further confirm cell death phenotypes in monkey HD neural cells are show this cells respond to therapeutic treatment of NMDAR mediated excitotoxicity.

In summary, both the suppression of *HTT* in HD neural cells and the use of memantine to ameliorate NMDAR mediated excitotoxicity led to the rescue of cell death phenotypes. Furthermore, *HTT* suppression therapy also resulted in the reduction of oligomeric mHTT aggregation. Collectively, these results demonstrate that monkey HD neural cells respond to therapy and suggest our model will be useful as a platform for drug assessment.

4.5 Discussion

The capability of rhesus monkey neural cells to respond to *in vitro* drug and gene therapy provides proof-of-principle that neural cells derived from HD monkey PSCs may provide a resource in the assessment and development of candidate therapeutic strategies. Suppression of *HTT* expression in HD neural cells resulted in reduced oligomeric aggregation in addition to significant reduction in H_2O_2 vulnerability and cell death

following neural differentiation. In this study *HTT* was suppressed in a non-allele specific manner, reducing both the endogenous and mutant transgene copies of *HTT*. Concerns have been raised regarding the long term clinical consequences of suppressing normal HTT functions (Sah and Aronin, 2011; Lu and Yang, 2012; Matsui and Corey, 2012). Although HTT is known to play a role in neurogenesis, non-allele specific *HTT* silencing has been shown to be tolerable in a number of studies involving HD mice models (DiFiglia et al., 2007; Boudreau et al., 2009; Drouet et al., 2009).

Towards developing strategies to specifically target the *mHTT* allele for suppression, investigators have explored the use of antisense oligonucleotides (ASOs). ASOs are single-stranded oligodeoxynucleotides that suppress the synthesis of the targeted protein (Bennett and Swayze, 2010). ASOs enter the cell and reduce gene expression by the RNase-H–mediated degradation of the complementary mRNA (Bennett and Swayze, 2010; Matsui and Corey, 2012). ASOs also function to bind complementary mRNA and physically block translation of the target mRNA (Bennett and Swayze, 2010; Matsui and Corey, 2012). A number of studies have taken advantage of *mHTT* specific SNPs to suppress *mHTT* expression, suggesting that this could prove to be an effective strategy in HD cell therapy (Bilsen et al., 2008; Pfister et al., 2009). Considering these findings, specific suppression of the *mHTT* transgene in our HD monkey cells would be more a widely accepted approach for gene therapy.

In addition to allele specific targeting, the method of non-coding small RNA incorporation into neural cell can be optimized. In this study we used lentiviral mediated infection, but perhaps using adeno-associated virus (AAV), which is more widely used for clinical delivery in humans (Zhang and Friedlander, 2011; Lentz et al., 2012; Gray,

2013), would allow for a study more similar to human viral-vector delivery methods. Although both lentiviral and AAV infection approaches have demonstrated potential for clinical gene therapy applications in the central nervous system, AAV vectors provide lower immunogenicity and have the ability to deliver genes as a stable episome (Zhang and Friedlander, 2011). These features overcome safety concerns associated with insertional mutagenesis in lentiviral methods due to incorporation of DNA into the host genome. Reports of AAV delivered RNAi therapy in HD mice (DiFiglia et al., 2007; Boudreau et al., 2009; Dufour et al., 2014), as well as safety assessment in NHPs (Hadaczek et al., 2009; McBride et al., 2011) support the adoption of AAV infection for gene therapy in HD.

Excitotoxicity is considered a high priority target because it is hypothesized to play a role in many neurodegenerative diseases (Parsons et al., 1999; Reisberg et al., 2003; Lipton, 2006). Therefore validating successful molecules that mediate this neurotoxic mechanism is of great interest. Memantine was chosen for this study because it has been approved by the FDA for treatments in Alzheimer's disease and has shown to improve behavior phenotypes in HD mice (Reisberg et al., 2003; Okamoto et al., 2009; Dau et al., 2014). Our results show a reduction in cytotoxicity during neural differentiation. Cytotoxicity measurements are easily quantifiable and can be scaled up for high-throughput screening purposes. As Ca^{2+} handling and mitochondrial alterations are also part of the excitotoxicity pathway, measuring Ca^{2+} influx and mitochondrial function in neural cells would also provide quantifiable measures in evaluating compounds that act on the NMDA pathway (Lipton, 2006; Raymond et al., 2011). There are also other targets beyond the synaptic receptor that are involved in the glutamate

excitotoxicity pathway. One such target is PSD95, a postsynaptic scaffolding protein that acts on the stability and trafficking of NMDARs (Zuccato et al., 2010). It has been postulated that modulating NMDAR activity in combination with postsynaptic proteins downstream would protect HD neurons and provide improvements at clinically relevant levels (Raymond et al., 2011). As novel classes of compounds are discovered that target other players in excitotoxicity, our system demonstrates the potential of PSC derived neural cells as a platform to investigate other experimental compounds that mediate NMDAR activity.

In summary, we demonstrate the reversal of HD associated phenotypes in HD monkey neural cells derived from iPSC. Of the human HD iPSC studies that have been reported, few show reversal of robust HD phenotypes *in vitro*. Human HD patient derived iPSC lines develop mild phenotypes, presenting a challenge in building screening platforms for evaluating therapeutics strategies (Perrier and Peschanski, 2012). Our finding suggest that HD monkey neural cells tolerate and benefit from non-allele specific RNAi suppression of *HTT*, and respond to memantine drug treatment, providing proof-of-concept as a platform to evaluate the impact of candidate therapies in a disease relevant, *in vitro* system. Moreover this system is derived from HD monkeys, and presents a valuable advantage that findings can directly translate to pre-clinical studies in our HD monkeys.

Figure 4-1

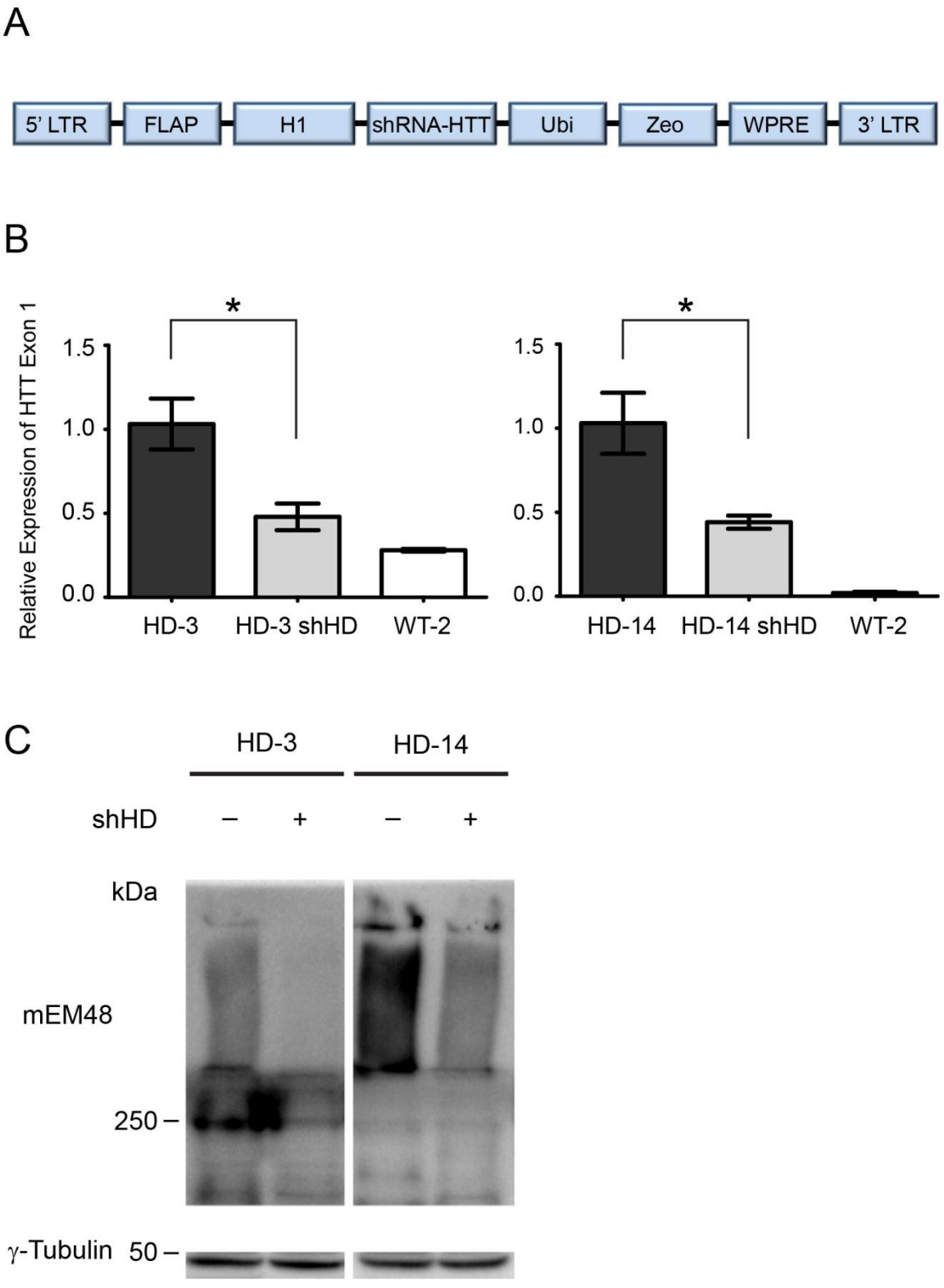

A
5' LTR
FLAP
H1
shRNA-HTT
Ubi
Zeo
WPRE
3' LTR
B
Relative Expression of HTT Exon 1
1.5
1.0
0.5
0.0
*
HD-3
HD-3 shHD
WT-2
HD-14
HD-14 shHD
C
HD-3
HD-14
shHD
kDa
mEM48
250
γ-Tubulin
50

Figure 4-1. *HTT* suppression reverses aggregation phenotype in HD monkey neural cells.

HTT expression was suppressed by introducing a small-hairpin RNA vector targeting *HTT* (shHD) into HD-NPCs. shHD was incorporated by lentiviral infection following selection for Zeocin resistance. (A) This schematic illustrates the shHD vector targeting *HTT*. Expression is under control of the H1 promoter. LTR, long terminal repeats; Flap, HIV-flap sequence; H1, human H1 promoter; shRNA-HTT, shRNA targeting *HTT*; Zeo, Zeocin resistance gene; WPRE, woodchuck post-transcriptional regulatory element.

(B) *HTT* expression was analyzed by RT-qPCR following shHD knockdown in HD-NPCs. *HTT* suppresion was confirmed by reduced *HTT* expression in HD-3 and HD-14 NPCs infected with shHD compared to non-infected HD-NPCs expression levels. Expression values were normalized to *Ubiquitin-C* (*UBC*). Data was analyzed using delta-delta CT method. Data are represented as mean +/- SEM. (* $p < 0.05$ ANOVA).

(C) Lysates from HD and WT cells after neural differentiation were analyzed for mHTT. Western blot analysis using mEM48 reveals reduction in oligomeric mHTT in HD-neurons after suppression by shHD. Notice aggregate accumulation in the stacking gel (above 250 kDa) is reduced in samples knocked down by shHD. γ-Tubulin was included as a loading control.

Figure 4-2

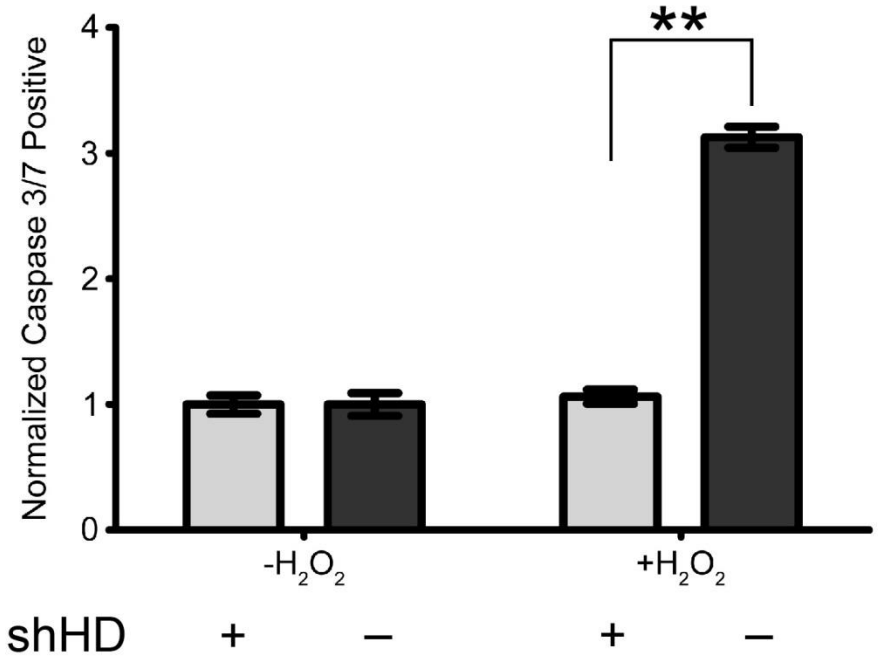


Figure 4-2. *HTT* suppression reverses vulnerability to oxidative stress in HD monkey NPCs.

Oxidative stress was induced in cell culture by exposure to hydrogen peroxide (H_2O_2) for 24 hours. After exposure HD-3 NPCs were harvested and stained for caspase 3/7 activity. Analysis of caspase 3/7 assay reveals significant decrease in the percentage of apoptotic NPCs expressing shHD compared to HD-NPCs without shHD. Values represent fold change from no H_2O_2 exposure. Data are represented as mean±SEM (**$p < 0.001$ ANOVA).

Figure 4-3

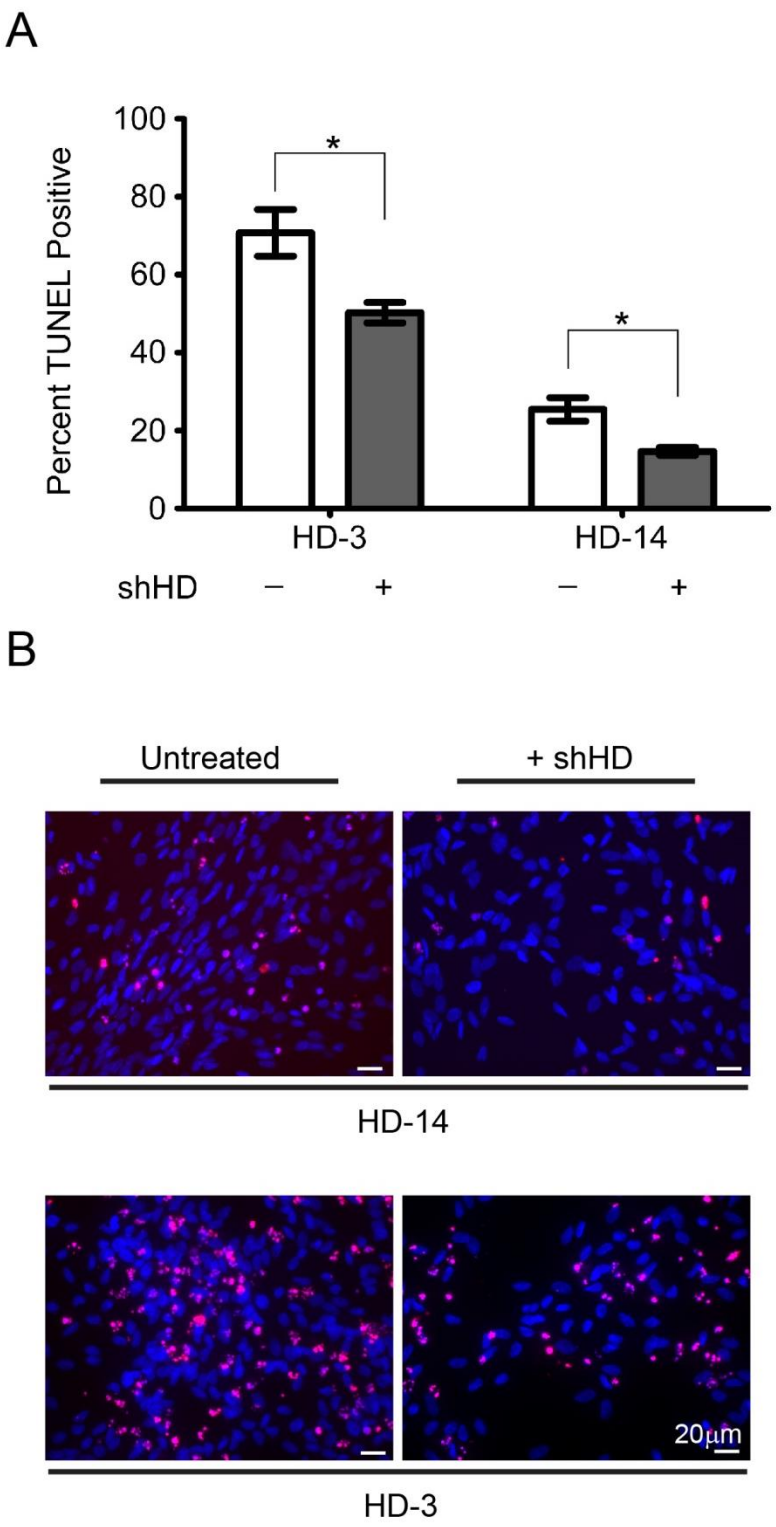

A
Percent TUNEL Positive
100
80
60
40
20
0
*
*
HD-3
HD-14
shHD
–
+
–
+
B
Untreated
+ shHD
HD-14
20μm
HD-3

Figure 4-3. *HTT* suppression reverses cell death in HD monkey neurons.

HD-3 and HD-14 NPCs were differentiated for 21 days following infection with shHD. After differentiation, cells were assessed for cell death by TUNEL assay.

(A) Analysis of TUNEL positive cells reveals that cell death was significantly reduced in HD-neurons expressing shHD compared to HD-neurons that did not express shHD. Percentage of TUNEL-positive cells was calculated from total number of cells from image fields randomly selected. Data are represented as mean±SEM. (*$p < 0.05$ ANOVA).

(B) Images represent TUNEL staining of HD-14 and HD-3 cell after neural differentiation. Notice reduced number of TUNEL positive HD-3 and HD-14 neurons expression shHD compared to HD-neurons without shHD. TUNEL positive cells (red). Nuclear staining using Hoechst (blue). Scale bars: 20μM.

Figure 4-4

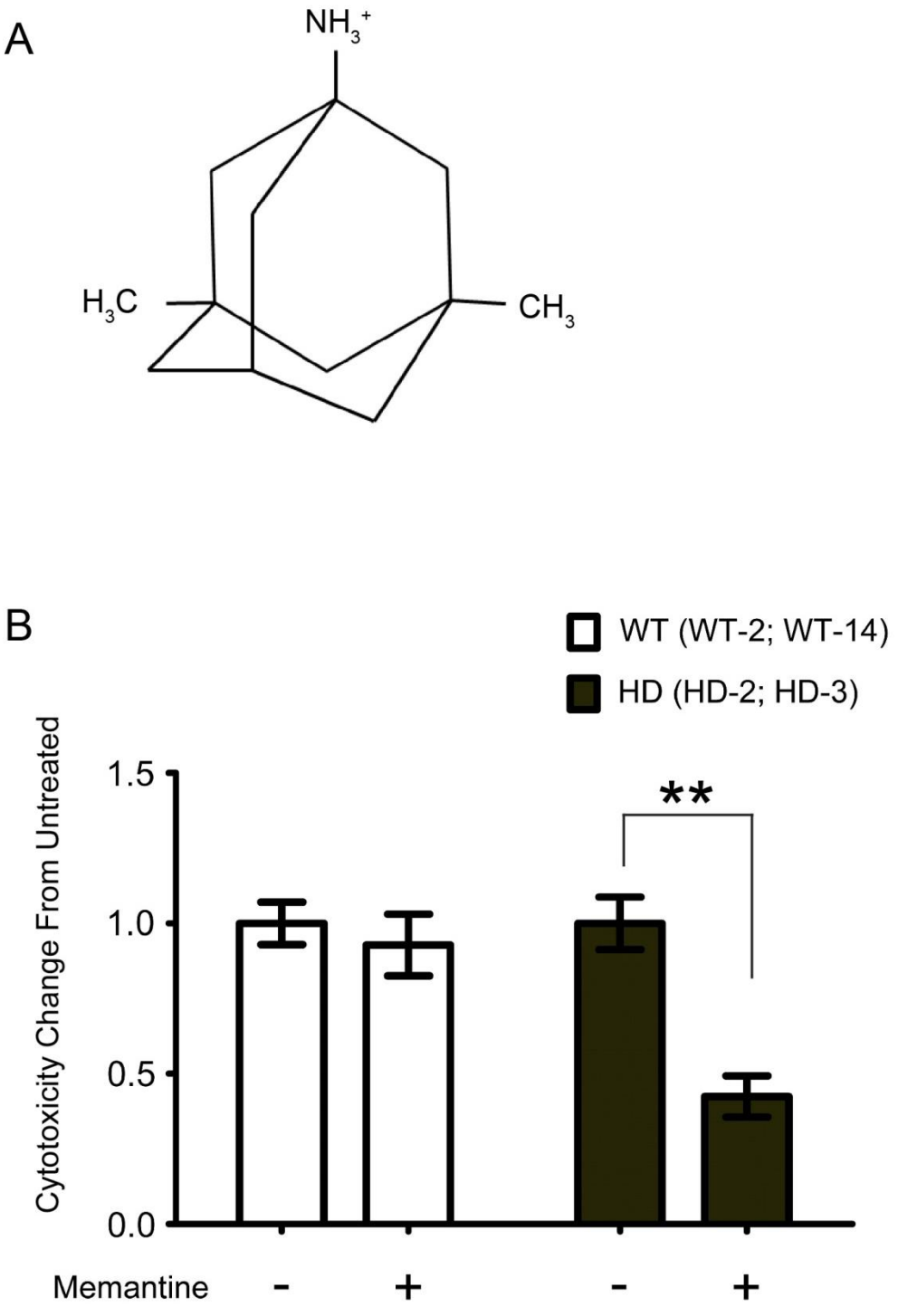

A
NH3+
H3C
CH3
B
WT (WT-2; WT-14)
HD (HD-2; HD-3)
Cytotoxicity Change From Untreated
1.5
1.0
0.5
0.0
**
Memantine
-
+
-
+

Figure 4-4. Memantine treatment rescues excitotoxicity in HD monkey neurons.

Memantine is an NMDA receptor antagonist that binds to NMDRs. (A) This schematic illustrates the chemical structure of memantine. Memantine has a molecular weight of 215.76 daltons and is classified as a small molecule drug. (B) Cells were treated with 10mM memantine after 21 days neural differentiation for 24 hr. Following treatment cytotoxicity was measured in HD (HD-2, HD-3) and WT (WT-2, WT-14) neurons. Cytotoxicity profile was measured by G6PD assay. Analysis showed that cytotoxicity is significantly reduced in HD neurons treated with memantine. Values represent mean fold change in cytotoxicity from untreated cells. Cytotoxicity values averaged from 2 HD lines (HD-2, HD-3) and 2 WT lines (WT-2, WT-28) from three repeated experiments. Data are represented as mean±SEM. (** $p < 0.01$ ANOVA).

Chapter 5

Conclusions and future directions

5.1 Summary

Developing cellular models of Huntington's disease (HD) are of great importance in understanding the cellular mechanisms of the disease, and for developing *in vitro* platforms for the validation and evaluation of therapeutic strategies. In addition to efforts to develop induced pluripotent stem cells from (iPSCs) from HD patients, generating pluripotent stem cells (PSCs) derived from HD non-human primates (NHP) provides a unique opportunity to study the development of HD associated phenotypes in an animal model that shares more similarity to humans compared to other HD models. Moreover, HD monkey neural cells may potentially be used to evaluate therapies before being investigated *in vivo* for pre-clinical studies.

We demonstrated that PSCs derived from HD monkeys differentiate to NPCs capable of generating γ-aminobutyric acid (GABA) neurons both *in vitro* and *in vivo*. We adapted methods for neural differentiation from human iPSC studies to develop a protocol for the culture and differentiation of rhesus monkey embryonic stem cells (ESCs) and iPSCs toward forebrain neurons. After demonstrating pluripotency, we derived stable NPC populations. Establishing NPC cell lines was an important step in developing an *in vitro* model for HD as it was necessary to maintain a progenitor population committed to the neural lineage. Direct differentiation from ESCs or iPSCs presents technical hurdles that result in inefficient generation of neurons and contamination from mutilineage cell types. To further characterize NPC populations, we performed immunocytochemistry and FACS analysis confirming that NPC populations were homogenous and expressed NPC markers.

As a model of HD, it was important that NPCs were capable to derived neurons. We derived neurons using *in vitro* methods toward GABA neural cell type. While other neural cell types are shown to be affected in late stages of HD. GABAergic medium spiny neurons (MSNs) are the most severely affected, and generating GABA neurons *in vitro* was an important goal. Neurons showed expression of mature neural markers MAP2 and βIII-Tubulin, and a subset of neurons were positive for GABA and tyrosine hydroxylase.

We also show that NPCs are capable to differentiate to neurons following xenograft transplantation in severe combined immunodeficiency (SCID) mice striatum. This experiment served dual purposes. These results demonstrate the survival and neural differentiation potential of transplanted monkey NPCs, and also lay the foundation for future cell replacement studies in HD mice and ultimately autologous transplantation to HD monkeys. Although at 12 and 15 weeks we were unable to observe either spontaneous or induced action potentials in recorded neurons, we did however observe excitatory postsynaptic currents that were blocked by the addiction of CNQX, suggesting that glutamatergic receptors and signaling was functional in transplanted neurons.

We also set out to characterize HD associated cellular phenotypes in PSC derived HD monkey neural cells. Our research group's report on the development of neuropathologic and clinical HD phenotypes in HD monkeys led us to investigate cellular phenotypes in HD monkey neural derivatives. HD cells showed increased expression of huntingtin (*HTT)* and developed mHTT aggregates along neural induction. HD neural cells were more vulnerable to cellular stress and exhibited cell death phenotypes. The development of classic features of HD, including nuclear inclusion and oligomeric

aggregates, is not seen in human HD-iPSC studies. This may possibly be due to the genetic differences of *HTT* between animal models and humans, however EM48 positive inclusions have been shown to develop in HD mice with full-length human *mHTT* constructs (Gutekunst et al., 1999; Van Raamsdonk, 2005). Like many other animal models, our transgenic HD monkeys carry a *HTT* exon 1 fragment with CAG repeats (Yang et al., 2008) and expression of N-terminal HTT fragment has been reported to produce a more severe phenotype than the full-length *HTT* mutation in HD mice models (Pouladi et al., 2013). Carrying an N-terminal fragment construct and having *HTT* expression driven by the human poly-ubiquitin-C promoter may lead to exacerbated pathology. This can be considered a strength for our model compared to more phenotypically mild human HD-iPSC studies. For purposes in drug screening and uncovering disease mechanisms, strong robust phenotypes are necessary. This is evident in reports of human HD-PSCs studies that produce only mild phenotypes in their cell populations (Niclis et al., 2009; Chae et al., 2012; Feyeux et al., 2012; Zhang et al., 2012), or require chemical induction to enhance phenotypes (Jeon et al., 2012; Cheng et al., 2013).

In this study we demonstrate the potential of HD monkey cells as a platform to evaluate gene therapy and drug treatment for HD. HD monkey neural cells respond to genetic suppression of *HTT*. Reduction in nuclear and oligomeric aggregation in HD neural cells correlated with knock down of *HTT* expression. *HTT* reduction reversed vulnerability to oxidative stress, and cell death phenotypes. HD neurons also respond to memantine treatment, decreasing cytotoxicity measurements after treatment. These results provide a framework for developing this model into a platform used to assess the

efficacy of novel therapeutic candidates. Furthermore these results have implications on cell replacement applications. Reports describing successful transplantation and integration of monkey neural progenitors into targeted brain regions of NHPs (Hashimoto et al., 2012; Emborg et al., 2013) point to the feasibility of cell replacement studies in NHP models of neurodegenerative disease. These reports, combined with rescue of neurodegeneration using RNAi suppression of HTT in HD-NPCs provide the foundation for a long-term test to assess the safety and efficacy of grafting genetically corrected pluripotent cells that could be accomplished in our HD monkey models. (Carter and Chan, 2012; Chen et al., 2014)

5.2 Future Directions

In this study we describe methods to efficiently derive NPC lines from PSCs and generate neurons as an *in vitro* model for HD. Monkey NPCs were characterized using a panel of pan-neural progenitor markers, however to further define regional fate of our progenitor population we would need to determine the expression of neural sub-type specific markers. NKX2.1 is a transcription factor that is specifically expressed in early basal forebrain progenitors (Liu et al., 2013). Additional region specific neural markers including FOXG1, MEIS2, and LHX8 would aid in defining progenitor region identity (Liu et al., 2013). Including these additional markers to the panel used to characterize forebrain NPCs would provide a more complete status of neural differentiation and provide information as to what neuronal sub-type monkey NPCs were fated towards.

An additional method for deriving neuronal cultures from somatic tissues utilizes direct reprogramming strategies to induce neural differentiation directly from somatic cells, without requiring pluripotency (Lu et al., 2013; Qiang et al., 2013). This method

involves forced expression of neurogenic morphogens and transcription factors that transform somatic cells to neural stem cells. This methods has been applied in human and non-human primates (Lu et al., 2013; Qiang et al., 2013). On one hand this approach overcomes the technical hurdles associated with managing ESC and iPSC culture, however the reagent cost and reproducibility of this approach may limit its use as a standard operating protocol until further optimizations are made.

Further towards the goal of generating a more pure neuronal culture and reducing heterogeneity, is employing cell sorting strategies. A number of surface markers exist for identifying neuronal populations. Reports using these markers in tandem with FACS support this strategy as an efficient means of generating a more narrowly defined neural culture (Yuan et al., 2011). This approach shows promise, however the lasting effect on neuron health and physiological function after sorting remains to be determined. Another lingering question in using sorting strategies is whether purified cells maintain regional identity after continued culture, and continue to yield expected neural sub-types. Pursuing these strategies to better define neural cell populations and increase homogeneity would benefit the downstream utility of *in vitro* assays. These improvements may lead to stronger, more informed conclusions, as comparisons on the impact of mHTT among specific neural sub-types could be investigated.

In this study we provide data demonstrating that our model recapitulates HD associated phenotypes including, cell death, vulnerability to oxidative stress, and aggregation phenotypes. We suspect that further investigation would identify additional cellular pathways that are impaired in our HD model. Reports of alterations in mitochondrial structure and energy metabolism have been described in rodent and human

HD cellular models (An et al., 2012; The Hd iPSC Consortium, 2012; Jonson et al., 2013). In addition, human iPSC studies have reported transcriptional changes in HD cells when compared to controls (An et al., 2012; Feyeux et al., 2012;
The Hd iPSC Consortium, 2012). Our lab reports transcriptional dysregulation was observed in peripheral blood samples accompanied by cognitive and neuroanatomical changes in our HD rhesus monkey cohort over a longitudinal study (Kocerha et al., 2013; Chan et al., 2014). Together this evidence suggest that upon further characterization we may uncover additional phenotypes in HD monkey neural cells.

Neural function is also an interesting feature that could potentially be investigated using our model. Receptor activity and synaptic firing is impaired in HD and would further validate our system as a model for HD phenotypes. In this study, we found measuring electrophysiological properties of HD neurons challenging. Our finding are consistent with other reports that suggest that extensive neurodegeneration in differentiated HD neurons hamper action potential firing and synaptic integration into neural networks (The Hd iPSC Consortium, 2012). Our findings demonstrating improvement of neuron survival by suppressing *HTT* and ameliorating excitotoxicity, may provide a strategy in overcoming this hurdle. A recovery in neuronal function in neurons after therapeutic treatment would further support the utility of HD neural cells to model and evaluate therapies *in vitro.*

In this study our primary focus centered on neural pathology and replication of HD associated phenotypes in neurons. This strategy could be further expanded to include an investigation on glial cell pathology. Although glial cells are not the primary cell type implicated in HD, there is evidence that glial cell defects may play a role in a non-cell

autonomous manner (Juopperi et al., 2012; Williams et al., 2014). Future studies in this direction would provide novel insights, as this area of HD research has not been well investigated.

An addition goal of this work is to develop a model that replicates cellular features of HD that can serve as phenotypic measurements to evaluate drug and gene therapies. These results demonstrate the therapeutic potential of RNAi mediated reduction of HTT as well as show the therapeutic benefit of treating excitotoxicity using memantine. The next steps for utilizing this system involve targeting other pathways in involved in HD pathogenesis for therapeutic treatment. There is evidence that improvement in mitochondrial performance and management of reactive oxidative species in the cell using coenzyme Q_{10} may lead to improved cell health in HD (Schilling et al., 2001; Ferrante et al., 2002). Furthermore, strategies that improve aggregate clearance and increase the activity of protein folding complexes may also be interesting therapeutic targets (Fecke et al., 2009; Zuccato et al., 2010). HTT has also been shown to reduce expression of important neurotrophic factors, mainly BDFN (Zuccato and Cattaneo, 2009; Xie et al., 2010; Arregui et al., 2011; Rosenblum et al., 2014). Employing strategies to upregulate BDNF expression would provide a major therapeutic benefit to neuronal health and viability. Lastly, microRNAs (miRNAs) have been explored in their relationship to HD (Packer et al., 2008; Cheng et al., 2013). Unpublished microarray data from brain tissues from our HD monkeys identified novel candidates that may be involved in HD pathogenesis. In a recent report, modulation of miR-196a was shown to ameliorate HD phenotypes in both HD mice and human HD-iPSCs (Cheng et al., 2013). This report in combination with results from this study

highlight the potential of identifying new therapeutic targets in HD monkeys, that can be developed towards treatments that translate to human studies.

5.3 Conclusion

In this study, we developed an *in vitro* model of HD from PSC's generated from transgenic HD rhesus monkeys. HD Monkeys PSCs generated NPC and GABA neuron populations that recapitulated key HD associated cellular phenotypes. Phenotypes were reversed using gene therapy and pharmacological approaches, supporting the potential for our system as a pre-clinical model from which insights into HD pathology and therapeutic approaches may be further investigated *in vivo* in our HD monkey cohort. Furthermore, this works contributes to the methodology of investigating neurological disorders using iPSCs, and provides a foundation for exploring cell replacement therapy in non-human primates.

References

An, Mahru C., Zhang, N., Scott, G., Montoro, D., Wittkop, T., Mooney, S., Melov, S.Ellerby, Lisa M., 2012. Genetic Correction of Huntington's Disease Phenotypes in Induced Pluripotent Stem Cells. Cell Stem Cell. 11, 253-263.

Appl, T., Kaltenbach, L., Lo, D. C.Terstappen, G. C., 2012. Targeting mutant huntingtin for the development of disease-modifying therapy. Drug Discovery Today. 17, 1217-1223.

Arregui, L., Benítez, J. A., Razgado, L. F., Vergara, P.Segovia, J., 2011. Adenoviral Astrocyte-Specific Expression of BDNF in the Striata of Mice Transgenic for Huntington's Disease Delays the Onset of the Motor Phenotype. Cellular and Molecular Neurobiology. 31, 1229-1243.

Arundine, M.Tymianski, M., 2003. Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell Calcium. 34, 325-337.

Aubry, L., Bugi, A., Lefort, N., Rousseau, F., Peschanski, M.Perrier, A. L., 2008. Striatal progenitors derived from human ES cells mature into DARPP32 neurons in vitro and in quinolinic acid-lesioned rats. Proceedings of the National Academy of Sciences. 105, 16707-16712.

Bachevalier, J., Machado, C. J.Kazama, A., 2011. Behavioral outcomes of late-onset or early-onset orbital frontal cortex (areas 11/13) lesions in rhesus monkeys. Annals of the New York Academy of Sciences. 1239, 71-86.

Bachevalier, J.Nemanic, S., 2008. Memory for spatial location and object-place associations are differently processed by the hippocampal formation, parahippocampal areas TH/TF and perirhinal cortex. Hippocampus. 18, 64-80.

Bae, B.-I., Xu, H., Igarashi, S., Fujimuro, M., Agrawal, N., Taya, Y., Hayward, S. D., Moran, T. H., Montell, C., Ross, C. A., Snyder, S. H.Sawa, A., 2005. p53 Mediates Cellular Dysfunction and Behavioral Abnormalities in Huntington's Disease. Neuron. 47, 29-41.

Batchelor, R. H.Zhou, M., 2004. Use of cellular glucose-6-phosphate dehydrogenase for cell quantitation: applications in cytotoxicity and apoptosis assays. Analytical Biochemistry. 329, 35-42.

Beal, M. F., Brouillet, E., Jenkins, B. G., Ferrante, R. J., Kowall, N. W., Miller, J. M., Storey, E., Srivastava, R., Rosen, B. R.Hyman, B. T., 1993. Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. J Neurosci. 13, 4181-4192.

Bellin, M., Marchetto, M. C., Gage, F. H.Mummery, C. L., 2012. Induced pluripotent stem cells: the new patient? Nature Reviews Molecular Cell Biology. 13, 713-726.

Bennett, C. F.Swayze, E. E., 2010. RNA Targeting Therapeutics: Molecular Mechanisms of Antisense Oligonucleotides as a Therapeutic Platform. Annual Review of Pharmacology and Toxicology. 50, 259-293.

Bilsen, P. H. J. v., Jaspers, L., Lombardi, M. S., Odekerken, J. C. E., Burright, E. N.Kaemmerer, W. F., 2008. Identification and Allele-Specific Silencing of the Mutant Huntingtin Allele in Huntington's Disease Patient-Derived Fibroblasts. Human Gene Therapy. 19, 710-718.

Bogdanov, M. B., Andreassen, O. A., Dedeoglu, A., Ferrante, R. J.Beal, M. F., 2001. Increased oxidative damage to DNA in a transgenic mouse model of Huntington's disease. J Neurochem. 79, 1246-1249.

Boudreau, R. L., McBride, J. L., Martins, I., Shen, S., Xing, Y., Carter, B. J.Davidson, B. L., 2009. Nonallele-specific Silencing of Mutant and Wild-type Huntingtin Demonstrates Therapeutic Efficacy in Huntington's Disease Mice. Molecular Therapy. 17, 1053-1063.

Bradley, C. K., Scott, H. A., Chami, O., Peura, T. T., Dumevska, B., Schmidt, U.Stojanov, T., 2011. Derivation of Huntington's disease-affected human embryonic stem cell lines. Stem Cells Dev. 20, 495-502.

Browne, S. E., Bowling, A. C., MacGarvey, U., Baik, M. J., Berger, S. C., Muqit, M. M., Bird, E. D.Beal, M. F., 1997. Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. Ann Neurol. 41, 646-653.

Butterfield, D. A., Howard, B. J.LaFontaine, M. A., 2001. Brain oxidative stress in animal models of accelerated aging and the age-related neurodegenerative disorders, Alzheimer's disease and Huntington's disease. Curr Med Chem. 8, 815-828.

Camnasio, S., Carri, A. D., Lombardo, A., Grad, I., Mariotti, C., Castucci, A., Rozell, B., Riso, P. L., Castiglioni, V., Zuccato, C., Rochon, C., Takashima, Y., Diaferia, G., Biunno, I., Gellera, C., Jaconi, M., Smith, A., Hovatta, O., Naldini, L., Di Donato, S., Feki, A.Cattaneo, E., 2012. The first reported generation of several induced pluripotent stem cell lines from homozygous and heterozygous Huntington's

disease patients demonstrates mutation related enhanced lysosomal activity. Neurobiology of Disease. 46, 41-51.

Carroll, J. B., Warby, S. C., Southwell, A. L., Doty, C. N., Greenlee, S., Skotte, N., Hung, G., Bennett, C. F., Freier, S. M.Hayden, M. R., 2011. Potent and Selective Antisense Oligonucleotides Targeting Single-Nucleotide Polymorphisms in the Huntington Disease Gene / Allele-Specific Silencing of Mutant Huntingtin. Molecular Therapy. 19, 2178-2185.

Carter, R. L.Chan, A. W., 2012. Pluripotent Stem Cells Models for Huntington's Disease: Prospects and Challenges. J Genet Genomics. 39, 253-259.

Castiglioni, V., Onorati, M., Rochon, C.Cattaneo, E., 2012. Induced pluripotent stem cell lines from Huntington's disease mice undergo neuronal differentiation while showing alterations in the lysosomal pathway. Neurobiology of Disease. 46, 30-40.

Chae, J. I., Kim, D. W., Lee, N., Jeon, Y. J., Jeon, I., Kwon, J., Kim, J., Soh, Y., Lee, D. S., Seo, Kang S., Choi, N. J., Park, Byoung C., Kang, Sung H., Ryu, J., Oh, S. H., Shin, Dong A., Lee, Dong R., Do, Jeong T., Park, I. H., Daley, George Q.Song, J., 2012. Quantitative proteomic analysis of induced pluripotent stem cells derived from a human Huntington's disease patient. Biochemical Journal. 446, 359-371.

Chan, A. W., 2009. Transgenic primate research paves the path to a better animal model: are we a step closer to curing inherited human genetic disorders? J Mol Cell Biol. 1, 13-14.

Chan, A. W., Xu, Y., Jiang, J., Rahim, T., Zhao, D., Kocerha, J., Chi, T., Moran, S., Engelhardt, H., Larkin, K., Neumann, A., Cheng, H., Li, C., Nelson, K., Banta,

H., Zola, S. M., Villinger, F., Yang, J., Testa, C. M., Mao, H., Zhang, X.Bachevalier, J., 2014. A two years longitudinal study of a transgenic Huntington disease monkey. BMC Neurosci. 15, 36.

Chan, A. W. S., 2013. Progress and Prospects for Genetic Modification of Nonhuman Primate Models in Biomedical Research. ILAR Journal. 54, 211-223.

Chan, A. W. S., Cheng, P.-H., Neumann, A.Yang, J.-J., 2010. Reprogramming Huntington Monkey Skin Cells into Pluripotent Stem Cells. Cellular Reprogramming (Formerly "Cloning and Stem Cells"). 12, 509-517.

Chen, Y., Carter, R. L., Cho, I. K.Chan, A. W. S., 2014. Cell-based therapies for Huntington's disease. Drug Discovery Today.

Chen, Y., Niu, Y.Ji, W., 2012. Transgenic Nonhuman Primate Models for Human Diseases: Approaches and Contributing Factors. Journal of Genetics and Genomics. 39, 247-251.

Cheng, P.-H., Li, C.-L., Chang, Y.-F., Tsai, S.-J., Lai, Y.-Y., Chan, Anthony W. S., Chen, C.-M.Yang, S.-H., 2013. miR-196a Ameliorates Phenotypes of Huntington Disease in Cell, Transgenic Mouse, and Induced Pluripotent Stem Cell Models. The American Journal of Human Genetics. 93, 306-312.

Choo, Y. S., 2004. Mutant huntingtin directly increases susceptibility of mitochondria to the calcium-induced permeability transition and cytochrome c release. Human Molecular Genetics. 13, 1407-1420.

Conti, L.Cattaneo, E., 2010. Neural stem cell systems: physiological players or in vitro entities? Nature Reviews Neuroscience.

Costa, V.Scorrano, L., 2012. Shaping the role of mitochondria in the pathogenesis of Huntington's disease. The EMBO Journal.

Coyle, J. T.Puttfarcken, P., 1993. Oxidative stress, glutamate, and neurodegenerative disorders. Science. 262, 689-695.

Coyle, J. T.Schwarcz, R., 1976. Lesion of striatal neurones with kainic acid provides a model for Huntington's chorea. Nature. 263, 244-246.

Daggett, A.Yang, X. W., 2013. Huntington's disease: easing the NMDAR traffic jam. Nature Medicine. 19, 971-973.

Dau, A., Gladding, C. M., Sepers, M. D.Raymond, L. A., 2014. Chronic blockade of extrasynaptic NMDA receptors ameliorates synaptic dysfunction and pro-death signaling in Huntington disease transgenic mice. Neurobiology of Disease. 62, 533-542.

Davies, S.Ramsden, D. B., 2001. Huntington's disease. Mol Pathol. 54, 409-413.

Davies, S. W., Turmaine, M., Cozens, B. A., DiFiglia, M., Sharp, A. H., Ross, C. A., Scherzinger, E., Wanker, E. E., Mangiarini, L.Bates, G. P., 1997. Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell. 90, 537-548.

Deng, Y. P., Albin, R. L., Penney, J. B., Young, A. B., Anderson, K. D.Reiner, A., 2004. Differential loss of striatal projection systems in Huntington's disease: a quantitative immunohistochemical study. J Chem Neuroanat. 27, 143-164.

Dhara, S. K., Hasneen, K., Machacek, D. W., Boyd, N. L., Rao, R. R.Stice, S. L., 2008. Human neural progenitor cells derived from embryonic stem cells in feeder-free cultures. Differentiation. 76, 454-464.

Dhara, S. K.Stice, S. L., 2008. Neural differentiation of human embryonic stem cells. Journal of Cellular Biochemistry. 105, 633-640.

DiFiglia, M., 1997. Aggregation of Huntingtin in Neuronal Intranuclear Inclusions and Dystrophic Neurites in Brain. Science. 277, 1990-1993.

DiFiglia, M., Sapp, E., Chase, K. O., Davies, S. W., Bates, G. P., Vonsattel, J. P.Aronin, N., 1997. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science. 277, 1990-1993.

DiFiglia, M., Sena-Esteves, M., Chase, K., Sapp, E., Pfister, E., Sass, M., Yoder, J., Reeves, P., Pandey, R. K., Rajeev, K. G., Manoharan, M., Sah, D. W. Y., Zamore, P. D.Aronin, N., 2007. Therapeutic silencing of mutant huntingtin with siRNA attenuates striatal and cortical neuropathology and behavioral deficits. Proceedings of the National Academy of Sciences. 104, 17204-17209.

Dong, G., Ferguson, J. M., Duling, A. J., Nicholas, R. G., Zhang, D., Rezvani, K., Fang, S., Monteiro, M. J., Li, S., Li, X. J.Wang, H., 2011. Modeling Pathogenesis of Huntington's Disease with Inducible Neuroprogenitor Cells. Cellular and Molecular Neurobiology. 31, 737-747.

Dragatsis, I., Levine, M. S.Zeitlin, S., 2000. Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. Nat Genet. 26, 300-306.

Drouet, V., Perrin, V., Hassig, R., Dufour, N., Auregan, G., Alves, S., Bonvento, G., Brouillet, E., Luthi-Carter, R., Hantraye, P.Déglon, N., 2009. Sustained effects of nonallele-specificHuntingtinsilencing. Annals of Neurology. 65, 276-285.

Dufour, B. D., Smith, C. A., Clark, R. L., Walker, T. R.McBride, J. L., 2014. Intrajugular Vein Delivery of AAV9-RNAi Prevents Neuropathological Changes and Weight Loss in Huntington's Disease Mice. Molecular Therapy.

Egawa, N., Kitaoka, S., Tsukita, K., Naitoh, M., Takahashi, K., Yamamoto, T., Adachi, F., Kondo, T., Okita, K., Asaka, I., Aoi, T., Watanabe, A., Yamada, Y., Morizane, A., Takahashi, J., Ayaki, T., Ito, H., Yoshikawa, K., Yamawaki, S., Suzuki, S., Watanabe, D., Hioki, H., Kaneko, T., Makioka, K., Okamoto, K., Takuma, H., Tamaoka, A., Hasegawa, K., Nonaka, T., Hasegawa, M., Kawata, A., Yoshida, M., Nakahata, T., Takahashi, R., Marchetto, M. C. N., Gage, F. H., Yamanaka, S.Inoue, H., 2012. Drug Screening for ALS Using Patient-Specific Induced Pluripotent Stem Cells. Science Translational Medicine. 4, 145ra104-145ra104.

Emborg, Marina E., Liu, Y., Xi, J., Zhang, X., Yin, Y., Lu, J., Joers, V., Swanson, C., Holden, James E.Zhang, S.-C., 2013. Induced Pluripotent Stem Cell-Derived Neural Cells Survive and Mature in the Nonhuman Primate Brain. Cell Reports.

Erceg, S., Ronaghi, M.Stojković, M., 2009. Human Embryonic Stem Cell Differentiation Toward Regional Specific Neural Precursors. Stem Cells. 27, 78-87.

Faber, P. W., Alter, J. R., MacDonald, M. E.Hart, A. C., 1999. Polyglutamine-mediated dysfunction and apoptotic death of a Caenorhabditis elegans sensory neuron. Proc Natl Acad Sci U S A. 96, 179-184.

Fecke, W., Gianfriddo, M., Gaviraghi, G., Terstappen, G. C.Heitz, F., 2009. Small molecule drug discovery for Huntington's Disease. Drug Discovery Today. 14, 453-464.

Ferrante, R. J., Andreassen, O. A., Dedeoglu, A., Ferrante, K. L., Jenkins, B. G., Hersch, S. M.Beal, M. F., 2002. Therapeutic effects of coenzyme Q10 and remacemide in transgenic mouse models of Huntington's disease. J Neurosci. 22, 1592-1599.

Feyeux, M., Bourgois-Rocha, F., Redfern, A., Giles, P., Lefort, N., Aubert, S., Bonnefond, C., Bugi, A., Ruiz, M., Deglon, N., Jones, L., Peschanski, M., Allen, N. D.Perrier, A. L., 2012. Early transcriptional changes linked to naturally occurring Huntington's disease mutations in neural derivatives of human embryonic stem cells. Human Molecular Genetics. 21, 3883-3895.

Fong, C.-Y., Gauthaman, K.Bongso, A., 2010. Teratomas from pluripotent stem cells: A clinical hurdle. Journal of Cellular Biochemistry. 111, 769-781.

Gage, Fred H.Temple, S., 2013. Neural Stem Cells: Generating and Regenerating the Brain. Neuron. 80, 588-601.

Gaj, T., Gersbach, C. A.Barbas, C. F., 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends in Biotechnology. 31, 397-405.

Garcia, M., Vanhoutte, P., Pages, C., Besson, M. J., Brouillet, E.Caboche, J., 2002. The mitochondrial toxin 3-nitropropionic acid induces striatal neurodegeneration via a c-Jun N-terminal kinase/c-Jun module. J Neurosci. 22, 2174-2184.

Gerrard, L., Rodgers, L.Cui, W., 2005. Differentiation of Human Embryonic Stem Cells to Neural Lineages in Adherent Culture by Blocking Bone Morphogenetic Protein Signaling. Stem Cells. 23, 1234-1241.

Gil, J. M.Rego, A. C., 2008. Mechanisms of neurodegeneration in Huntington's disease. Eur J Neurosci. 27, 2803-2820.

Godin, J. D., Colombo, K., Molina-Calavita, M., Keryer, G., Zala, D., Charrin, B. C., Dietrich, P., Volvert, M.-L., Guillemot, F., Dragatsis, I., Bellaiche, Y., Saudou, F., Nguyen, L.Humbert, S., 2010. Huntingtin Is Required for Mitotic Spindle Orientation and Mammalian Neurogenesis. Neuron. 67, 392-406.

Goldberg, Y. P., Telenius, H.Hayden, M. R., 1994. The molecular genetics of Huntington's disease. Curr Opin Neurol. 7, 325-332.

Götz, M.Huttner, W. B., 2005. The cell biology of neurogenesis. Nature Reviews Molecular Cell Biology. 6, 777-788.

Gray, S. J., 2013. Gene therapy and neurodevelopmental disorders. Neuropharmacology. 68, 136-142.

Gunawardena, S., Her, L. S., Brusch, R. G., Laymon, R. A., Niesman, I. R., Gordesky-Gold, B., Sintasath, L., Bonini, N. M.Goldstein, L. S., 2003. Disruption of axonal transport by loss of huntingtin or expression of pathogenic polyQ proteins in Drosophila. Neuron. 40, 25-40.

Gutekunst, C. A., Li, S. H., Yi, H., Mulroy, J. S., Kuemmerle, S., Jones, R., Rye, D., Ferrante, R. J., Hersch, S. M.Li, X. J., 1999. Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. J Neurosci. 19, 2522-2534.

Hadaczek, P., Forsayeth, J., Mirek, H., Munson, K., Bringas, J., Pivirotto, P., McBride, J. L., Davidson, B. L.Bankiewicz, K. S., 2009. Transduction of nonhuman primate brain with adeno-associated virus serotype 1: vector trafficking and immune response. Hum Gene Ther. 20, 225-237.

Hardingham, G. E.Bading, H., 2010. Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. Nature Reviews Neuroscience. 11, 682-696.

Harper, S. Q., 2005. From the Cover: RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. Proceedings of the National Academy of Sciences. 102, 5820-5825.

Hashimoto, K., Shimada, H., Okada, Y., Ibata, K., Ebise, H., Ota, S.-i., Tomioka, I., Nomura, T., Maeda, T., Kohda, K., Yuzaki, M., Sasaki, E., Nakamura, M.Okano, H., 2012. Efficient Derivation of Multipotent Neural Stem/Progenitor Cells from Non-Human Primate Embryonic Stem Cells. PLoS One. 7, e49469.

Hedreen, J. C.Folstein, S. E., 1995. Early loss of neostriatal striosome neurons in Huntington's disease. J Neuropathol Exp Neurol. 54, 105-120.

Hu, J., Liu, J., Yu, D., Chu, Y.Corey, D. R., 2012. Mechanism of allele-selective inhibition of huntingtin expression by duplex RNAs that target CAG repeats: function through the RNAi pathway. Nucleic Acids Research. 40, 11270-11280.

Imarisio, S., Carmichael, J., Korolchuk, V., Chen, C.-W., Saiki, S., Rose, C., Krishna, G., Davies, Janet E., Ttofi, E., Underwood, Benjamin R.Rubinsztein, David C., 2008. Huntington's disease: from pathology and genetics to potential therapies. Biochemical Journal. 412, 191.

Inoue, H.Yamanaka, S., 2011. The Use of Induced Pluripotent Stem Cells in Drug Development. Clinical Pharmacology & Therapeutics.

Itsykson, P., Ilouz, N., Turetsky, T., Goldstein, R. S., Pera, M. F., Fishbein, I., Segal, M.Reubinoff, B. E., 2005. Derivation of neural precursors from human embryonic

stem cells in the presence of noggin. Molecular and Cellular Neuroscience. 30, 24-36.

Jacobsen, J. C., Bawden, C. S., Rudiger, S. R., McLaughlan, C. J., Reid, S. J., Waldvogel, H. J., MacDonald, M. E., Gusella, J. F., Walker, S. K., Kelly, J. M., Webb, G. C., Faull, R. L. M., Rees, M. I.Snell, R. G., 2010. An ovine transgenic Huntington's disease model. Human Molecular Genetics. 19, 1873-1882.

Jeon, I., Lee, N., Li, J.-Y., Park, I.-H., Park, K. S., Moon, J., Shim, S. H., Choi, C., Chang, D.-J., Kwon, J., Oh, S.-H., Shin, D. A., Kim, H. S., Do, J. T., Lee, D. R., Kim, M., Kang, K.-S., Daley, G. Q., Brundin, P.Song, J., 2012. Neuronal Properties, In Vivo Effects and Pathology of a Huntington's Disease Patient-Derived Induced Pluripotent Stem Cells. Stem Cells. N/A-N/A.

Johnson, C. D.Davidson, B. L., 2010. Huntington's disease: progress toward effective disease-modifying treatments and a cure. Hum Mol Genet. 19, R98-R102.

Jonson, I., Ougland, R., Klungland, A.Larsen, E., 2013. Oxidative stress causes DNA triplet expansion in Huntington's disease mouse embryonic stem cells. Stem Cell Research. 11, 1264-1271.

Joung, J. K.Sander, J. D., 2012. TALENs: a widely applicable technology for targeted genome editing. Nature Reviews Molecular Cell Biology. 14, 49-55.

Juopperi, T. A., Kim, W. R., Chiang, C.-H., Yu, H., Margolis, R. L., Ross, C. A., Ming, G.-l.Song, H., 2012. Astrocytes generated from patient induced pluripotent stem cells recapitulate features of Huntington's disease patient cells. Molecular Brain. 5, 17.

Kaye, J. A.Finkbeiner, S., 2013. Modeling Huntington's disease with induced pluripotent stem cells. Molecular and Cellular Neuroscience. 56, 50-64.

Kim, M., Lee, S.-T., Chu, K.Kim, S. U., 2008. Stem cell-based cell therapy for Huntington disease: A review. Neuropathology. 28, 1-9.

Kirkeby, A., Grealish, S., Wolf, Daniel A., Nelander, J., Wood, J., Lundblad, M., Lindvall, O.Parmar, M., 2012. Generation of Regionally Specified Neural Progenitors and Functional Neurons from Human Embryonic Stem Cells under Defined Conditions. Cell Reports. 1, 703-714.

Kocerha, J., Liu, Y., Willoughby, D., Chidamparam, K., Benito, J., Nelson, K., Xu, Y., Chi, T., Engelhardt, H., Moran, S., Yang, S. H., Li, S. H., Li, X. J., Larkin, K., Neumann, A., Banta, H., Yang, J. J.Chan, A. W., 2013. Longitudinal transcriptomic dysregulation in the peripheral blood of transgenic Huntington's disease monkeys. BMC Neurosci. 14, 88.

Koch, P., Opitz, T., Steinbeck, J. A., Ladewig, J.Brustle, O., 2009. A rosette-type, self-renewing human ES cell-derived neural stem cell with potential for in vitro instruction and synaptic integration. Proceedings of the National Academy of Sciences. 106, 3225-3230.

Kondo, T., Asai, M., Tsukita, K., Kutoku, Y., Ohsawa, Y., Sunada, Y., Imamura, K., Egawa, N., Yahata, N., Okita, K., Takahashi, K., Asaka, I., Aoi, T., Watanabe, A., Watanabe, K., Kadoya, C., Nakano, R., Watanabe, D., Maruyama, K., Hori, O., Hibino, S., Choshi, T., Nakahata, T., Hioki, H., Kaneko, T., Naitoh, M., Yoshikawa, K., Yamawaki, S., Suzuki, S., Hata, R., Ueno, S.-i., Seki, T., Kobayashi, K., Toda, T., Murakami, K., Irie, K., Klein, William L., Mori, H.,

Asada, T., Takahashi, R., Iwata, N., Yamanaka, S.Inoue, H., 2013. Modeling Alzheimer's Disease with iPSCs Reveals Stress Phenotypes Associated with Intracellular Aβ and Differential Drug Responsiveness. Cell Stem Cell. 12, 487-496.

Kordasiewicz, Holly B., Stanek, Lisa M., Wancewicz, Edward V., Mazur, C., McAlonis, Melissa M., Pytel, Kimberly A., Artates, Jonathan W., Weiss, A., Cheng, Seng H., Shihabuddin, Lamya S., Hung, G., Bennett, C. F.Cleveland, Don W., 2012. Sustained Therapeutic Reversal of Huntington's Disease by Transient Repression of Huntingtin Synthesis. Neuron. 74, 1031-1044.

Labbadia, J.Morimoto, R. I., 2013. Huntington's disease: underlying molecular mechanisms and emerging concepts. Trends in Biochemical Sciences. 38, 378-385.

Laforet, G. A., Sapp, E., Chase, K., McIntyre, C., Boyce, F. M., Campbell, M., Cadigan, B. A., Warzecki, L., Tagle, D. A., Reddy, P. H., Cepeda, C., Calvert, C. R., Jokel, E. S., Klapstein, G. J., Ariano, M. A., Levine, M. S., DiFiglia, M.Aronin, N., 2001. Changes in cortical and striatal neurons predict behavioral and electrophysiological abnormalities in a transgenic murine model of Huntington's disease. J Neurosci. 21, 9112-9123.

Laowtammathron, C., Cheng, E. C. H., Cheng, P.-H., Snyder, B. R., Yang, S.-H., Johnson, Z., Lorthongpanich, C., Kuo, H.-C., Parnpai, R.Chan, A. W. S., 2010. Monkey hybrid stem cells develop cellular features of Huntington's disease. BMC Cell Biology. 11, 12.

Lee, G., Ramirez, C. N., Kim, H., Zeltner, N., Liu, B., Radu, C., Bhinder, B., Kim, Y. J., Choi, I. Y., Mukherjee-Clavin, B., Djaballah, H.Studer, L., 2012. Large-scale screening using familial dysautonomia induced pluripotent stem cells identifies compounds that rescue IKBKAP expression. Nature Biotechnology. 30, 1244-1248.

Lentz, T. B., Gray, S. J.Samulski, R. J., 2012. Viral vectors for gene delivery to the central nervous system. Neurobiology of Disease. 48, 179-188.

Li, J. Y., Popovic, N.Brundin, P., 2005. The use of the R6 transgenic mouse models of Huntington's disease in attempts to develop novel therapeutic strategies. NeuroRx. 2, 447-464.

Li, S.Li, X. J., 2006. Multiple pathways contribute to the pathogenesis of Huntington disease. Mol Neurodegener. 1, 19.

Li, S. H., Lam, S., Cheng, A. L.Li, X. J., 2000. Intranuclear huntingtin increases the expression of caspase-1 and induces apoptosis. Hum Mol Genet. 9, 2859-2867.

Li, S. H., Schilling, G., Young, W. S., 3rd, Li, X. J., Margolis, R. L., Stine, O. C., Wagster, M. V., Abbott, M. H., Franz, M. L., Ranen, N. G.et al., 1993. Huntington's disease gene (IT15) is widely expressed in human and rat tissues. Neuron. 11, 985-993.

Li, X., Wang, C. E., Huang, S., Xu, X., Li, X. J., Li, H.Li, S., 2010. Inhibiting the ubiquitin-proteasome system leads to preferential accumulation of toxic N-terminal mutant huntingtin fragments. Human Molecular Genetics. 19, 2445-2455.

Lipton, S. A., 2006. Paradigm shift in neuroprotection by NMDA receptor blockade: Memantine and beyond. Nature Reviews Drug Discovery. 5, 160-170.

Liu, Y., Weick, J. P., Liu, H., Krencik, R., Zhang, X., Ma, L., Zhou, G.-m., Ayala, M.Zhang, S.-C., 2013. Medial ganglionic eminence–like cells derived from human embryonic stem cells correct learning and memory deficits. Nature Biotechnology. 31, 440-447.

Liu, Y., Weick, J. P., Liu, H., Krencik, R., Zhang, X., Ma, L., Zhou, G. M., Ayala, M.Zhang, S. C., 2013. Medial ganglionic eminence-like cells derived from human embryonic stem cells correct learning and memory deficits. Nat Biotechnol. 31, 440-447.

Lu, J., Bradley, Robert A.Zhang, S.-C., 2014. Turning Reactive Glia into Functional Neurons in the Brain. Cell Stem Cell. 14, 133-134.

Lu, J., Liu, H., Huang, Cindy T.-L., Chen, H., Du, Z., Liu, Y., Sherafat, Mohammad A.Zhang, S.-C., 2013. Generation of Integration-free and Region-Specific Neural Progenitors from Primate Fibroblasts. Cell Reports. 3, 1580-1591.

Lu, X.-H.Yang, X. W., 2012. “Huntingtin Holiday”: Progress toward an Antisense Therapy for Huntington's Disease. Neuron. 74, 964-966.

Lunn, J. S., Sakowski, S. A., Hur, J.Feldman, E. L., 2011. Stem cell technology for neurodegenerative diseases. Annals of Neurology. 70, 353-361.

Ma, L., Hu, B., Liu, Y., Vermilyea, S. C., Liu, H., Gao, L., Sun, Y., Zhang, X.Zhang, S. C., 2012. Human Embryonic Stem Cell-Derived GABA Neurons Correct Locomotion Deficits in Quinolinic Acid-Lesioned Mice. Cell Stem Cell.

MacDonald, M. E., Ambrose, C. M., Duyao, M. P., Myers, R. H., Lin, C., Srinidhi, L., Barnes, G., Taylor, S. A., James, M., Groot, N., MacFarlane, H., Jenkins, B., Anderson, M. A., Wexler, N. S., Gusella, J. F., Bates, G. P., Baxendale, S., Hummerich, H., Kirby, S., North, M., Youngman, S., Mott, R., Zehetner, G., Sedlacek, Z., Poustka, A., Frischauf, A.-M., Lehrach, H., Buckler, A. J., Church, D., Doucette-Stamm, L., O'Donovan, M. C., Riba-Ramirez, L., Shah, M., Stanton, V. P., Strobel, S. A., Draths, K. M., Wales, J. L., Dervan, P., Housman, D. E., Altherr, M., Shiang, R., Thompson, L., Fielder, T., Wasmuth, J. J., Tagle, D., Valdes, J., Elmer, L., Allard, M., Castilla, L., Swaroop, M., Blanchard, K., Collins, F. S., Snell, R., Holloway, T., Gillespie, K., Datson, N., Shaw, D.Harper, P. S., 1993. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell. 72, 971-983.

Machida, Y., Okada, T., Kurosawa, M., Oyama, F., Ozawa, K.Nukina, N., 2006. rAAV-mediated shRNA ameliorated neuropathology in Huntington disease model mouse. Biochemical and Biophysical Research Communications. 343, 190-197.

Mali, P., Esvelt, K. M.Church, G. M., 2013. Cas9 as a versatile tool for engineering biology. Nature Methods. 10, 957-963.

Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S. W.Bates, G. P., 1996. Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. Cell. 87, 493-506.

Marchetto, M. C., Brennand, K. J., Boyer, L. F.Gage, F. H., 2011. Induced pluripotent stem cells (iPSCs) and neurological disease modeling: progress and promises. Human Molecular Genetics. 20, R109-R115.

Marchetto, M. C. N., Carromeu, C., Acab, A., Yu, D., Yeo, G. W., Mu, Y., Chen, G., Gage, F. H.Muotri, A. R., 2010. A Model for Neural Development and Treatment of Rett Syndrome Using Human Induced Pluripotent Stem Cells. Cell. 143, 527-539.

Marco, S., Giralt, A., Petrovic, M. M., Pouladi, M. A., Martinez-Turrillas, R., Martinez-Hernandez, J., Kaltenbach, L. S., Torres-Peraza, J., Graham, R. K., Watanabe, M., Lujan, R., Nakanishi, N., Lipton, S. A., Lo, D. C., Hayden, M. R., Alberch, J., Wesseling, J. F.Perez-Otano, I., 2013. Suppressing aberrant GluN3A expression rescues synaptic and behavioral impairments in Huntington's disease models. Nat Med. 19, 1030-1038.

Mateizel, I., 2005. Derivation of human embryonic stem cell lines from embryos obtained after IVF and after PGD for monogenic disorders. Human Reproduction. 21, 503-511.

Matsui, M.Corey, D. R., 2012. Allele-selective inhibition of trinucleotide repeat genes. Drug Discovery Today. 17, 443-450.

Maucksch, C., Vazey, E. M., Gordon, R. J.Connor, B., 2013. Stem cell-based therapy for Huntington's disease. Journal of Cellular Biochemistry. 114, 754-763.

McBride, J. L., Boudreau, R. L., Harper, S. Q., Staber, P. D., Monteys, A. M., Martins, I., Gilmore, B. L., Burstein, H., Peluso, R. W., Polisky, B., Carter, B. J.Davidson, B. L., 2008. Artificial miRNAs mitigate shRNA-mediated toxicity in the brain:

Implications for the therapeutic development of RNAi. Proceedings of the National Academy of Sciences. 105, 5868-5873.

McBride, J. L., Pitzer, M. R., Boudreau, R. L., Dufour, B., Hobbs, T., Ojeda, S. R.Davidson, B. L., 2011. Preclinical Safety of RNAi-Mediated HTT Suppression in the Rhesus Macaque as a Potential Therapy for Huntington's Disease. Molecular Therapy. 19, 2152-2162.

Mestre, T. A.Ferreira, J. J., 2011. An evidence-based approach in the treatment of Huntington’s disease. Parkinsonism & Related Disorders.

Milakovic, T., Quintanilla, R. A.Johnson, G. V. W., 2006. Mutant Huntingtin Expression Induces Mitochondrial Calcium Handling Defects in Clonal Striatal Cells: FUNCTIONAL CONSEQUENCES. Journal of Biological Chemistry. 281, 34785-34795.

Miller, B. R., Walker, A. G., Shah, A. S., Barton, S. J.Rebec, G. V., 2008. Dysregulated Information Processing by Medium Spiny Neurons in Striatum of Freely Behaving Mouse Models of Huntington's Disease. Journal of Neurophysiology. 100, 2205-2216.

Miller, V. M., 2005. CHIP Suppresses Polyglutamine Aggregation and Toxicity In Vitro and In Vivo. Journal of Neuroscience. 25, 9152-9161.

Milnerwood, A. J., Gladding, C. M., Pouladi, M. A., Kaufman, A. M., Hines, R. M., Boyd, J. D., Ko, R. W. Y., Vasuta, O. C., Graham, R. K., Hayden, M. R., Murphy, T. H.Raymond, L. A., 2010. Early Increase in Extrasynaptic NMDA Receptor Signaling and Expression Contributes to Phenotype Onset in Huntington's Disease Mice. Neuron. 65, 178-190.

Minami, M., Li, C., Dabrowska, J., Hazra, R.Rainnie, D. G., 2011. Synergistic Activation of Dopamine D1 and TrkB Receptors Mediate Gain Control of Synaptic Plasticity in the Basolateral Amygdala. PLoS One. 6, e26065.

Morse, R. J., Leeds, J. M., Macdonald, D., Park, L., Toledo-Sherman, L.Pacifici, R., 2011. Pharmaceutical Development for Huntington's Disease.

Mussolino, C.Cathomen, T., 2012. TALE nucleases: tailored genome engineering made easy. Current Opinion in Biotechnology. 23, 644-650.

Nemanic, S., Alvarado, M. C.Bachevalier, J., 2004. The hippocampal/parahippocampal regions and recognition memory: insights from visual paired comparison versus object-delayed nonmatching in monkeys. J Neurosci. 24, 2013-2026.

Nguyen, H. N., Byers, B., Cord, B., Shcheglovitov, A., Byrne, J., Gujar, P., Kee, K., Schule, B., Dolmetsch, R. E., Langston, W., Palmer, T. D.Pera, R. R., 2011. LRRK2 mutant iPSC-derived DA neurons demonstrate increased susceptibility to oxidative stress. Cell Stem Cell. 8, 267-280.

Nicholas, Cory R., Chen, J., Tang, Y., Southwell, Derek G., Chalmers, N., Vogt, D., Arnold, Christine M., Chen, Y.-Jiun J., Stanley, Edouard G., Elefanty, Andrew G., Sasai, Y., Alvarez-Buylla, A., Rubenstein, John L. R.Kriegstein, Arnold R., 2013. Functional Maturation of hPSC-Derived Forebrain Interneurons Requires an Extended Timeline and Mimics Human Neural Development. Cell Stem Cell. 12, 573-586.

Niclis, J. C., Trounson, A. O., Dottori, M., Ellisdon, A. M., Bottomley, S. P., Verlinsky, Y.Cram, D. S., 2009. Human embryonic stem cell models of Huntington disease. Reprod Biomed Online. 19, 106-113.

Nori, S., Okada, Y., Yasuda, A., Tsuji, O., Takahashi, Y., Kobayashi, Y., Fujiyoshi, K., Koike, M., Uchiyama, Y., Ikeda, E., Toyama, Y., Yamanaka, S., Nakamura, M.Okano, H., 2011. Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. Proceedings of the National Academy of Sciences. 108, 16825-16830.

Okamoto, S.-i., Pouladi, M. A., Talantova, M., Yao, D., Xia, P., Ehrnhoefer, D. E., Zaidi, R., Clemente, A., Kaul, M., Graham, R. K., Zhang, D., Vincent Chen, H. S., Tong, G., Hayden, M. R.Lipton, S. A., 2009. Balance between synaptic versus extrasynaptic NMDA receptor activity influences inclusions and neurotoxicity of mutant huntingtin. Nature Medicine. 15, 1407-1413.

Ondo, W. G., Mejia, N. I.Hunter, C. B., 2007. A pilot study of the clinical efficacy and safety of memantine for Huntington's disease. Parkinsonism & Related Disorders. 13, 453-454.

Orr, H. T.Zoghbi, H. Y., 2007. Trinucleotide Repeat Disorders. Annual Review of Neuroscience. 30, 575-621.

Packer, A. N., Xing, Y., Harper, S. Q., Jones, L.Davidson, B. L., 2008. The Bifunctional microRNA miR-9/miR-9* Regulates REST and CoREST and Is Downregulated in Huntington's Disease. Journal of Neuroscience. 28, 14341-14346.

Panov, A. V., Burke, J. R., Strittmatter, W. J.Greenamyre, J. T., 2003. In vitro effects of polyglutamine tracts on Ca2+-dependent depolarization of rat and human mitochondria: relevance to Huntington's disease. Arch Biochem Biophys. 410, 1-6.

Panov, A. V., Gutekunst, C.-A., Leavitt, B. R., Hayden, M. R., Burke, J. R., Strittmatter, W. J.Greenamyre, J. T., 2002. Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. Nature Neuroscience.

Park, I.-H., Arora, N., Huo, H., Maherali, N., Ahfeldt, T., Shimamura, A., Lensch, M. W., Cowan, C., Hochedlinger, K.Daley, G. Q., 2008. Disease-Specific Induced Pluripotent Stem Cells. Cell. 134, 877-886.

Parmar, M.Björklund, A., 2012. Generation of Transplantable Striatal Projection Neurons from Human ESCs. Cell Stem Cell. 10, 349-350.

Parsons, C. G., Danysz, W.Quack, G., 1999. Memantine is a clinically well tolerated N-methyl-D-aspartate (NMDA) receptor antagonist--a review of preclinical data. Neuropharmacology. 38, 735-767.

Perrier, A.Peschanski, M., 2012. How Can Human Pluripotent Stem Cells Help Decipher and Cure Huntington’s Disease? Cell Stem Cell. 11, 153-161.

Pfister, E. L., Kennington, L., Straubhaar, J., Wagh, S., Liu, W., DiFiglia, M., Landwehrmeyer, B., Vonsattel, J.-P., Zamore, P. D.Aronin, N., 2009. Five siRNAs Targeting Three SNPs May Provide Therapy for Three-Quarters of Huntington's Disease Patients. Current Biology. 19, 774-778.

Pouladi, M. A., Morton, A. J.Hayden, M. R., 2013. Choosing an animal model for the study of Huntington's disease. Nat Rev Neurosci. 14, 708-721.

Pruszak, J., Sonntag, K. C., Aung, M. H., Sanchez-Pernaute, R.Isacson, O., 2007. Markers and methods for cell sorting of human embryonic stem cell-derived neural cell populations. Stem Cells. 25, 2257-2268.

Putkhao, K., Kocerha, J., Cho, I.-K., Yang, J., Parnpai, R.Chan, A. W. S., 2012. Pathogenic cellular phenotypes are germline transmissible in a transgenic primate model of Huntington's Disease. Stem Cells and Development. 121129024937001.

Qiang, L., Fujita, R.Abeliovich, A., 2013. Remodeling Neurodegeneration: Somatic Cell Reprogramming-Based Models of Adult Neurological Disorders. Neuron. 78, 957-969.

Qiang, L., Inoue, K.Abeliovich, A., 2013. Instant Neurons: Directed Somatic Cell Reprogramming Models of Central Nervous System Disorders. Biological Psychiatry.

Raymond, L. A., André, V. M., Cepeda, C., Gladding, C. M., Milnerwood, A. J.Levine, M. S., 2011. Pathophysiology of Huntington's disease: time-dependent alterations in synaptic and receptor function. Neuroscience. 198, 252-273.

Reiner, A., Albin, R. L., Anderson, K. D., D'Amato, C. J., Penney, J. B.Young, A. B., 1988. Differential loss of striatal projection neurons in Huntington disease. Proc Natl Acad Sci U S A. 85, 5733-5737.

Reisberg, B., Doody, R., Stoffler, A., Schmitt, F., Ferris, S.Mobius, H. J., 2003. Memantine in moderate-to-severe Alzheimer's disease. N Engl J Med. 348, 1333-1341.

Reubinoff, B. E., Itsykson, P., Turetsky, T., Pera, M. F., Reinhartz, E., Itzik, A.Ben-Hur, T., 2001. Neural progenitors from human embryonic stem cells. Nat Biotechnol. 19, 1134-1140.

Robinton, D. A.Daley, G. Q., 2012. The promise of induced pluripotent stem cells in research and therapy. Nature. 481, 295-305.

Rosenblum, S., Smith, T. N., Wang, N., Chua, J. Y., Westbroek, E., Wang, K.Guzman, R., 2014. BDNF Pre-treatment of Human Embryonic-Derived Neural Stem Cells Improves Cell Survival and Functional Recovery after Transplantation in Hypoxic-Ischemic Stroke. Cell Transplant.

Ross, C. A.Poirier, M. A., 2004. Protein aggregation and neurodegenerative disease. Nature Medicine. 10, S10-S17.

Ross, C. A.Shoulson, I., 2009. Huntington disease: pathogenesis, biomarkers, and approaches to experimental therapeutics. Parkinsonism Relat Disord. 15 Suppl 3, S135-138.

Rotblat, B., Southwell, A. L., Ehrnhoefer, D. E., Skotte, N. H., Metzler, M., Franciosi, S., Leprivier, G., Somasekharan, S. P., Barokas, A., Deng, Y., Tang, T., Mathers, J., Cetinbas, N., Daugaard, M., Kwok, B., Li, L., Carnie, C. J., Fink, D., Nitsch, R., Galpin, J. D., Ahern, C. A., Melino, G., Penninger, J. M., Hayden, M. R.Sorensen, P. H., 2014. HACE1 reduces oxidative stress and mutant Huntingtin toxicity by promoting the NRF2 response. Proc Natl Acad Sci U S A. 111, 3032-3037.

Ruggieri, M., Riboldi, G., Brajkovic, S., Bucchia, M., Bresolin, N., Comi, G. P.Corti, S., 2014. Induced neural stem cells: Methods of reprogramming and potential therapeutic applications. Progress in Neurobiology. 114, 15-24.

Sah, D. W. Y.Aronin, N., 2011. Oligonucleotide therapeutic approaches for Huntington disease. Journal of Clinical Investigation. 121, 500-507.

Saha, K.Jaenisch, R., 2009. Technical Challenges in Using Human Induced Pluripotent Stem Cells to Model Disease. Cell Stem Cell. 5, 584-595.

Sanberg, P. R., Calderon, S. F., Giordano, M., Tew, J. M.Norman, A. B., 1989. The quinolinic acid model of Huntington's disease: locomotor abnormalities. Exp Neurol. 105, 45-53.

Sapp, E., Schwarz, C., Chase, K., Bhide, P. G., Young, A. B., Penney, J., Vonsattel, J. P., Aronin, N.DiFiglia, M., 1997. Huntingtin localization in brains of normal and Huntington's disease patients. Ann Neurol. 42, 604-612.

Sasaki, E., Suemizu, H., Shimada, A., Hanazawa, K., Oiwa, R., Kamioka, M., Tomioka, I., Sotomaru, Y., Hirakawa, R., Eto, T., Shiozawa, S., Maeda, T., Ito, M., Ito, R., Kito, C., Yagihashi, C., Kawai, K., Miyoshi, H., Tanioka, Y., Tamaoki, N., Habu, S., Okano, H.Nomura, T., 2009. Generation of transgenic non-human primates with germline transmission. Nature. 459, 523-527.

Sassone, J., Colciago, C., Cislaghi, G., Silani, V.Ciammola, A., 2009. Huntington's disease: The current state of research with peripheral tissues. Experimental Neurology. 219, 385-397.

Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G. P., Davies, S. W., Lehrach, H.Wanker, E. E., 1997. Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. Cell. 90, 549-558.

Schilling, G., Coonfield, M. L., Ross, C. A.Borchelt, D. R., 2001. Coenzyme Q10 and remacemide hydrochloride ameliorate motor deficits in a Huntington's disease transgenic mouse model. Neurosci Lett. 315, 149-153.

Schwarcz, R.Coyle, J. T., 1977. Striatal lesions with kainic acid: neurochemical characteristics. Brain Res. 127, 235-249.

Schwarcz, R., Whetsell, W. O., Jr.Mangano, R. M., 1983. Quinolinic acid: an endogenous metabolite that produces axon-sparing lesions in rat brain. Science. 219, 316-318.

Snyder, B. R., Cheng, P.-H., Yang, J., Yang, S.-H., Huang, A. H. C.Chan, A. W. S., 2011. Characterization of dental pulp stem/stromal cells of Huntington monkey tooth germs. BMC Cell Biology. 12, 39.

Sonntag, K.-C., Pruszak, J., Yoshizaki, T., van Arensbergen, J., Sanchez-Pernaute, R.Isacson, O., 2007. Enhanced Yield of Neuroepithelial Precursors and Midbrain-Like Dopaminergic Neurons from Human Embryonic Stem Cells Using the Bone Morphogenic Protein Antagonist Noggin. Stem Cells. 25, 411-418.

Tabar, V.Studer, L., 2014. Pluripotent stem cells in regenerative medicine: challenges and recent progress. Nature Reviews Genetics. 15, 82-92.

Takahashi, K.Yamanaka, S., 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell. 126, 663-676.

The Hd iPSC Consortium, 2012. Induced Pluripotent Stem Cells from Patients with Huntington's Disease Show CAG-Repeat-Expansion-Associated Phenotypes. Cell Stem Cell. 11, 264-278.

Tibbitts, D., Rao, R. R., Shin, S., West, F. D.Stice, S. L., 2006. Uniform adherent neural progenitor populations from rhesus embryonic stem cells. Stem Cells Dev. 15, 200-208.

Tiscornia, G., Vivas, E. L.Belmonte, J. C. I., 2011. Diseases in a dish: modeling human genetic disorders using induced pluripotent cells. Nature Medicine. 1570-1576.

van den Bogaard, S. J. A., Dumas, E. M., Milles, J., Reilmann, R., Stout, J. C., Craufurd, D., van Buchem, M. A., van der Grond, J.Roos, R. A. C., 2012. Magnetization Transfer Imaging in Premanifest and Manifest Huntington Disease. American Journal of Neuroradiology. 33, 884-889.

van der Bom, I. M. J., Moser, R. P., Gao, G., Sena-Esteves, M., Aronin, N.Gounis, M. J., 2011. Frameless multimodal image guidance of localized convection-enhanced delivery of therapeutics in the brain. Journal of NeuroInterventional Surgery. 5, 69-72.

Van Raamsdonk, J. M., 2005. Selective degeneration and nuclear localization of mutant huntingtin in the YAC128 mouse model of Huntington disease. Human Molecular Genetics. 14, 3823-3835.

Verlinsky, Y., Strelchenko, N., Kukharenko, V., Rechitsky, S., Verlinsky, O., Galat, V.Kuliev, A., 2005. Human embryonic stem cell lines with genetic disorders. Reprod Biomed Online. 10, 105-110.

von Horsten, S., Schmitt, I., Nguyen, H. P., Holzmann, C., Schmidt, T., Walther, T., Bader, M., Pabst, R., Kobbe, P., Krotova, J., Stiller, D., Kask, A., Vaarmann, A., Rathke-Hartlieb, S., Schulz, J. B., Grasshoff, U., Bauer, I., Vieira-Saecker, A. M., Paul, M., Jones, L., Lindenberg, K. S., Landwehrmeyer, B., Bauer, A., Li, X. J.Riess, O., 2003. Transgenic rat model of Huntington's disease. Hum Mol Genet. 12, 617-624.

Walker, A. G., Miller, B. R., Fritsch, J. N., Barton, S. J.Rebec, G. V., 2008. Altered Information Processing in the Prefrontal Cortex of Huntington's Disease Mouse Models. Journal of Neuroscience. 28, 8973-8982.

Wang, C. E., Tydlacka, S., Orr, A. L., Yang, S. H., Graham, R. K., Hayden, M. R., Li, S., Chan, A. W. S.Li, X. J., 2008. Accumulation of N-terminal mutant huntingtin in mouse and monkey models implicated as a pathogenic mechanism in Huntington's disease. Human Molecular Genetics. 17, 2738-2751.

Wang, Y.-L., Liu, W., Wada, E., Murata, M., Wada, K.Kanazawa, I., 2005. Clinico-pathological rescue of a model mouse of Huntington's disease by siRNA. Neuroscience Research. 53, 241-249.

Williams, E. C., Zhong, X., Mohamed, A., Li, R., Liu, Y., Dong, Q., Ananiev, G. E., Mok, J. C. C., Lin, B. R., Lu, J., Chiao, C., Cherney, R., Li, H., Zhang, S. C.Chang, Q., 2014. Mutant astrocytes differentiated from Rett syndrome patients-specific iPSCs have adverse effects on wild-type neurons. Human Molecular Genetics.

Xie, Y., Hayden, M. R.Xu, B., 2010. BDNF Overexpression in the Forebrain Rescues Huntington's Disease Phenotypes in YAC128 Mice. Journal of Neuroscience. 30, 14708-14718.

Yamanaka, S., 2012. Induced Pluripotent Stem Cells: Past, Present, and Future. Cell Stem Cell. 10, 678-684.

Yang, D., Wang, C. E., Zhao, B., Li, W., Ouyang, Z., Liu, Z., Yang, H., Fan, P., O'Neill, A., Gu, W., Yi, H., Li, S., Lai, L.Li, X. J., 2010. Expression of Huntington's disease protein results in apoptotic neurons in the brains of cloned transgenic pigs. Human Molecular Genetics. 19, 3983-3994.

Yang, S.-H., Cheng, P.-H., Banta, H., Piotrowska-Nitsche, K., Yang, J.-J., Cheng, E. C. H., Snyder, B., Larkin, K., Liu, J., Orkin, J., Fang, Z.-H., Smith, Y., Bachevalier,

J., Zola, S. M., Li, S.-H., Li, X.-J.Chan, A. W. S., 2008. Towards a transgenic model of Huntington's disease in a non-human primate. Nature. 453, 921-924.

Yang, W., Dunlap, J. R., Andrews, R. B.Wetzel, R., 2002. Aggregated polyglutamine peptides delivered to nuclei are toxic to mammalian cells. Hum Mol Genet. 11, 2905-2917.

Yu, D., Pendergraff, H., Liu, J., Kordasiewicz, H. B., Cleveland, Don W., Swayze, Eric E., Lima, Walt F., Crooke, Stanley T., Prakash, Thazha P.Corey, David R., 2012. Single-Stranded RNAs Use RNAi to Potently and Allele-Selectively Inhibit Mutant Huntingtin Expression. Cell. 150, 895-908.

Yuan, S. H., Martin, J., Elia, J., Flippin, J., Paramban, R. I., Hefferan, M. P., Vidal, J. G., Mu, Y., Killian, R. L., Israel, M. A., Emre, N., Marsala, S., Marsala, M., Gage, F. H., Goldstein, L. S.Carson, C. T., 2011. Cell-surface marker signatures for the isolation of neural stem cells, glia and neurons derived from human pluripotent stem cells. PLoS One. 6, e17540.

Zhang, K., Yi, F., Liu, G.-H.Belmonte, J. C. I., 2012. Huntington's disease: Dancing in a dish. Cell Research.

Zhang, N., An, M. C., Montoro, D.Ellerby, L. M., 2010. Characterization of Human Huntington's Disease Cell Model from Induced Pluripotent Stem Cells. PLoS Curr. 2, RRN1193.

Zhang, Y.Friedlander, R. M., 2011. Using non-coding small RNAs to develop therapies for Huntington's disease. Gene Therapy. 18, 1139-1149.

Zhou, Y.-y.Zeng, F., 2013. Integration-free Methods for Generating Induced Pluripotent Stem Cells. Genomics, Proteomics & Bioinformatics. 11, 284-287.

Zuccato, C.Cattaneo, E., 2009. Brain-derived neurotrophic factor in neurodegenerative diseases. Nature Reviews Neurology. 5, 311-322.

Zuccato, C., Valenza, M.Cattaneo, E., 2010. Molecular mechanisms and potential therapeutical targets in Huntington's disease. Physiol Rev. 90, 905-981.