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Lu Qian April 13th 2015

Modeling of Huntington's Disease Astrocytes from Non-human Primate Neural Progenitor Cells

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
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Bachelor of Sciences with Honors

Biology Department

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Abstract

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Huntington's disease (HD) is an autosomal dominant disease characterized by an overexpansion of CAG repeats in the mutant huntingtin gene *(mHTT)*. Recently, increasing evidence has shown that astrocytes, a type of glial cells responsible for the mediation of neural and glial functions and the structural integrity of the blood brain barriers, can accumulate mutant huntingtin protein and compromise their neural supportive functions. Animal models have provided a useful approach to study the pathological mechanism of HD. However, deficiencies still exist in these models, causing incomplete or erroneous recapitulation of the HD phenotype. To further minimize the difference between animal models and HD patients, this study employed neural progenitor cells (NPCs) reprogrammed from rhesus monkeys' induced pluripotent cells (iPSCs) as a base-stone to derive astrocytes *in vitro*. To create a HD monkey model, monkeys were transfected with exon1 with 72 CAG repeats in *mHTT*. In comparison to iPSCs, NPCs exhibited a greater capacity to generate a homogenous neural population and mirror the HD characteristics. Results of this study implied that HD astrocytes derived from monkey NPCs could recapitulate the down-regulation of PGC1-alpha, GLT-1, and SOD2 found in HD patients, as well as the up-regulation of glutamate receptors and the existence of intranuclear inclusions, which indicated the presence of poly-glutamine aggregates and hence an essential HD phenotype. A dramatic alteration in astrocyte morphology was also perceived. Nonetheless, this HD astrocyte model revealed

phenotypes unobserved in HD patients in terms of increased BDNF expression, which could be attributed to the proteins' positive correlation with glutamate receptors.

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Acknowledgements

I am extremely grateful and indebted to my advisor Dr. Chan for his continual guidance and assistance throughout my thesis research, and also the members in Chan's lab for their constructive advice and feedbacks. I would like to thank my thesis committees, Dr. Erri Aisen and Dr. Patrick Cafferty, for devoting their time to this research.

Finally, I would like to thank my family and friends who had supported me throughout this project.

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Chapter 1

Background

1.1 Overview of Huntington's Disease (HD)

Huntington's disease (HD) is an autosomal dominant genetic disease, and is characterized by neurodegeneration in the central neural system (CNS). An overexpansion of CAG tirnucleotides in the mutant huntingtin gene causes HD manifestation (Biagioli et al., 2015). On average, 3-10 per 100,000 people are diagnosed with HD in Western Europe and North America, and HD juvenile cases account for 4.92% with an early onset age of 20 years (Gil & Rego., 2008; Quarrell et al., 2012). Studies on 831 HD patients from the National Huntington Disease Research Roster have revealed that 5.7% of HD patients' deaths are due to suicides, and at least 27.6% patients demonstrate suicidal ideation (Farrer., 1986). Therefore, the adverse outcome of HD is not confined to individuals' ravaged health, but extends to a negative societal impact.

No cures have been developed for HD, and current clinical treatments target motor and psychiatric symptoms as well as disease management. Similar to other neurodegenerative disorders, HD is characteristic of a progressive development (Zielonka et al., 2014). Adult Huntington's disease (AHD) typically occurs with a CAG expansion over 40 repeats and appears between 35 and 55 years, and has a slow progression of chorea that eventually leads to dystonia. However, for juvenile Huntington's disease (JHD) where the CAG expansion tends to exceed 60 repeats, dystonia is more prominent than chorea at an early stage, and JHD patients demonstrate faster symptomatic progression than AHD patients (Quarrell et al., 2013). JHD patients also manifest more extensive and severe pathologies evidenced from their extra neurodegeneration in the

frontal and temporal lobes (Quarrell et al., 2013). Basal ganglia, which are nuclei in the base of the forebrain, have been identified as the major target of HD (Alexander, Crutcher & Delong, 1990). The atrophy of basal ganglia partially explains the pronounced motor and cognitive symptoms in HD patients.

Though neurodegeneration in HD selectively targets the striatum at the onset of the disease, it spreads to other cerebral regions like the brainstem as the disease progresses (Rüb et al., 2014). The invasion exacerbates the aggravation and complication of HD symptoms. Cognitive decline, psychiatric abnormalities, and motor function dysfunction are involved in the symptoms of HD (Papoutsi et al., 2014). More discernible symptoms include involuntary movements and HD chorea (Papoutsi et al., 2014). Following the loss of motor control, patients demonstrate postural instability and walk imbalance (Reilmann et al., 2014).

1.2 Genetics and Pathological Mechanisms of Huntington's Disease (HD)

HD is caused by an unstable expansion of CAG (encodes for glutamine) repeats in exon1 of the huntingtin *(HTT)* gene. A CAG expansion exceeding 35 units is expected to induce HD symptoms (Biagioli et al., 2015). The length of the poly-glutamine expansion on the mutant HTT protein (mHTT) positively correlates with the disease severity: a longer expansion implies an earlier age of disease onset. It has also been suggested that the poly-glutamine length is associated with the instability of the *HTT* allele, and a higher instability of parental alleles may result in more severe HD symptoms of the affected offspring (The Huntingtin's Disease Collaborative Research Group., 1993).

Wild type huntingtin protein (HTT) is indispensable in maintaining cellular activities, including gene regulation and intracellular vesicle transport. HTT is involved in the autophagy pathway and is vital for fetal survival during gastrulation; inactivation of *HTT* at late embryonic stages can induce postnatal neurodegeneration (Ochaba et al., 2014). Autophagy is necessary for cell metabolism, because it regulates dramatic cellular changes and cell differentiation in response to the hormonal fluctuation during gastrulation. Defects in autophagy pathway may result in insufficient protein degradation and thus disrupt fetal development (Mizushima & Levine., 2010). Besides autophagy, HTT also plays roles in the endocytosis and the assembly of cellular cytoskeletons, transcription and transportation of brain derived neurotrophic factor (BDNF), and the inhibition of the cytochrome-c release to resist cell apoptosis (Gil & Rego., 2008).

Although a detailed mechanism of the molecular pathology of HD still remains unclear, our understanding about HD has been steadily improved. First of all, the

overexpansion of CAG trinucleotide in HD presents characteristics of both the gain-of-function and the loss-of-function mutation. The gain-of-function can be concluded from the selective atrophy of striatal neurons despite a somatic expression of mHTT (Leegwater-Kim & Cha., 2004). These symptoms cannot be precisely recapitulated by a simple *HTT* knockout, indicating an acquisition of *de novo* function of mHTT (Cattaneo et al., 2001). One hypothesis is that cells may exhibit varying tolerances to the mHTT toxicity, in which the striatum exhibits a faster rate of disease progression than other affected regions (Cattaneo et al., 2001). Recently, a further refined hypothesis has been proposed: the adverse impacts of mHTT on GABA-mediated neurotransmissions would preferentially localize the neural atrophy in the striatum, as the striatum is rich with GABAergic medium-sized spiny neurons (Wang & Qin., 2006). McKinstry et al have found that HTT can negatively regulate the maturation of excitatory synapses of striatal neurons. Accordingly, HTT insufficiency may result in an inadequate inhibition of neural hyperexcitaion. HTT insufficiency can also cause synaptic immaturity and pre-mature aging of neurons, eventually leading to striatal neurodegeneration (McKinstry et al., 2014).

Second, failures of ubiquitination might amplify the cytotoxicity of mHTT. Post-translational modifications of mHTT involve caspase-mediated cleavages that produce poly-glutamine residues. The cleavage product forms intranuclear inclusions and cytoplasmic aggregates, and induces neural apoptosis (Wang & Qian., 2006). Because the ubiquitin-proteasome system is the major pathway for the poly-glutamine aggregate clearance, an age-dependent decline of the system's capacity can lead to aggregate accumulation and cellular cytotoxicity (Li et al., 2010). This might explain an age-

dependent progression of HD symptoms. Apart from the defected ubiquitin-proteasome system, a recruitment-sequestration model has been proposed and offers an interesting insight (Thakur, Yang & Wetzel., 2004). This model argues that cytoplasmic poly-glutamine tracts are able to elongate themselves by recruiting fragments from local benign poly-glutamine proteins; this extraction breaks the structure of benign HTT, and leads to an aggravation of mHTT-induced phenotypes accompanied with a loss-of-function of HTT (Thakur, Yang & Wetzel., 2004).

Mitochondrial damage is also closely related to striatal neuodegeneration. Two major types of mitochondrial damages have been identified in HD: reactive oxygen species (ROS) damages fostered by an intracellular iron accumulation, and apoptosis induced by calcium ion dysregulation. The work of Mena and colleagues has revealed a detailed mechanism of an iron-triggered ROS attack. ROS are generated from the activities of the electron transport chain and are produced when oxygen receives the leaking electrons from the transport chain. An excessive amount of redox-active iron engaging in the electron transport chain can exacerbate ROS' impacts by catalyzing the production of hydroxyl radical, which is a strong oxidant. The subsequent radical production can accelerate glutathione consumption and lipid peroxidation and cause mitochondrial DNA damage. Mitochondrial impairments are explicit in HD neurons, as neurons are highly susceptible to ROS and the mitochondrial damage because of their elongated morphology and heavy reliance on ATP for energy and axonal transport (Mena et al., 2015). Interestingly, iron accumulation is not confined to HD neurons; a high concentration of intracellular irons is also found in oligodendrocytes responsible for neuronal myelination (Mena et al., 2015). Studies have proposed that these glial cells can

produce a large amount of hydroxyl radicals, thereby imposing oxidative threats on neighboring neurons (Bartzokis et al., 2007; van den Bogaard et al., 2013).

A defensive mechanism against the oxidative stress, calcium-mediated autophagy can protect neurons from excitotoxicity (Luo., 2014). In HD, a disrupted calcium homeostasis might result from impaired calcium buffering regulated by endoplasmic reticula and mitochondria. Though autophagy is a calcium-mediated process, increasing evidence has indicated that the dysregulation of the intracellular calcium concentration could interrupt the autophagy pathway, driving cells to apoptosis (Luo., 2014).

1.3 Astrocytes in Neurodegenerative Diseases

Although neurodegeneration is an ultimate phenotype of HD, neurons are not the sole unit in the CNS. Normal neural homeostasis, growth sustenance, and extracellular environment maintenance cannot be maintained without an active support of other cell types in the CNS. Glial cells account for a major population of these supportive cells, and constitute nearly 90% of the human brain (Allen & Barres., 2009). Increasing evidence has shown that damaged glial cells and their abnormal activities can accelerate atrophy in most neurodegenerative diseases, including Alzheimer's disease and HD (Sica., 2015). Since mHTT is expressed in the entire CNS, it is important to understand the susceptibility of these glial cells to mHTT, and how mHTT affects their capability to regulate the CNS environment and neural activities.

Astrocytes are a type of glial cells that are able to influence the progression and severity of neurodegenerative disorders. Astrocyte function encompasses the connections between neurons and the blood-brain barrier, balance of the extracellular ions and neurotransmitters, synthesis/release of neurotrophic factors, and the release of antioxidants (Kimelberg & Nedergaard., 2011). Distinct anatomical features of astrocytes allude to their peculiar function in maintaining the CNS. Their extensions of the outward radiating processes establish broad contacts not only among themselves, but also between neurons, oligodendrocytes and the blood-brain barrier, creating a convoluted CNS network (Sica., 2015). This network facilitates a complicated cross-talk between neural cells and their supporter cells for neurotransmission, nutrient exchange, and metabolic waste removal (Sica., 2015). In the blood-brain barrier, astrocytes control its integrity and

permeability to lymphocytes' trafficking, which is associated with neuroinflammation and microglial activation (Romero-Sandoval & Sweitzer., 2015).

Astrocyte activation, or astrogliosis, is prevalently observed in neurodegenerative disorders and embodies changes in gene expression, morphology, and function. Upon activation, astrocytes up-regulate spinal cord astrocytic S100 beta, vimentin and glial fibrillary acidic protein (GFAP) (Romero-Sandoval & Sweitzer., 2015; Kamphuis et al., 2015). Reactive astrocytes display a hypertrophic morphology with enlarged cellular volumes, thicker branches, and a blurred territoriality of the overlapping processes (Romero-Sandoval & Sweitzer., 2015). Increased cell proliferation also features astrogliosis (Hsiao et al., 2012).

Up-regulation of GFAP is considered as a protective mechanism against CNS injuries. Studies have shown that GFAP-null mice are more susceptible to spinal cord injuries, neural toxicity-induced seizures, and neurodegeneration (Nawashiro et al., 2005; Nawashiro et al., 1998). Moreover, astrocytes extracted from GFAP-null mice are also incapable of reconstructing blood-brain barrier properties with endothelial cells *in vitro* (Kamphuis et al., 2015). These demonstrate the importance of GFAP in both sustaining neuroprotection and maintaining the structure and permeability of the blood-brain barrier. Therefore, down-regulated GFAP in astrocytes might lead to a breach or a loosened structure of the blood-brain barrier and expose neurons to metabolic toxicants in the blood streams, which would further induce neural apoptosis in neurodegenerative diseases. Thus it might be interesting to determine whether mHTT expression would suppress the transcription and function of GFAP and inhibit astrocyte activation.

In addition to GFAP dysregulation, an elevation of brain-derived neurotrophic factor (BDNF) has been reported in reactive astrocytes with the presence of CNS injuries, and can potentially lead to neuropathic pains (Wang et al., 2011). BDNF is a nerve growth factor synthesized within astrocytes and prompts neural survival. Besides its neuron-supportive function, BDNF also activates astrocytes and microglial cells through stimulating the release of calcium from intracellular stores, and attenuates the excitation of GABAergic-neurons (Wang et al., 2011). Increased BDNF expression therefore induces astrocyte activation and hence GFAP overexpression (Wang et al., 2011).

However, genetic and morphological changes in reactive astrocyte are not exclusively beneficial and may disturb astrocytes' capability to support neural survival. Disrupted ionic and transmitter homeostasis in reactive astrocytes can exacerbate neural hyperexcitation and accelerate neural atrophy (Steifert & Steihäuser., 2013). Such hyperexcitation can be provoked through: 1) a reduced density instead of a total amount of Kir channels on the astrocyte processes to uptake extracellular potassium ions, 2) a reduced level of glutamine synthetase in astrocytes and hence insufficient GABA synthesis to counteract neural hyperexcitation, and 3) improper localization of glutamate transporters due to altered cytoskeletal mapping (Robel et al., 2015). The expression of glutamate transporter GLAST and K^+ transporters is also decreased in reactive astrocytes, and might therefore contribute to impaired neurotransmission and neural dysfunction (Hol & Pekny., 2015; Orre et al., 2014).

Besides these adverse impacts of reactive astrocytes, what accentuates astrocytes' role in HD pathogenesis and symptom aggravation is its involvement in neuroinflammation. Chronic neuroinflammation is a critical feature observed in the

majority of neurodegenerative diseases. Reactive astrocytes can readily respond to proinflammatory mediators through astrogliosis that is initiated by astrocytes' interaction with neurons and microglial with the presence of CNS injuries. Cytokine signaling is another route for astrogliosis and usually involves the activities of TGF-alpha, CNTF, IL-6 and LIF (Hol &Pekny., 2015). Unlike the wild type astrocytes striving to reduce the inflammatory stress, HD astrocytes show an up-regulated expression and activation of NF-κB central to the stimulation of downstream inflammatory mediators like TNF alpha and IL-1beta (Hsiao et al., 2013). Interestingly, studies have shown that mHTT can enhance the activity of IκB kinase (IKK) responsible for NF-κB activation, and therefore result in astrocyte overactivation and a prolonged inflammatory response (Hsiao et al., 2013). This indicates that astrocytes expressing mHTT might be reactive and pro-inflammatory.

Apart from neuroinflammation, astrocytes also participate in CNS cholesterol metabolism, and the dysregulation of which can exacerbate neurodegeneration. The work of Lenoi and Caccia shows a detailed mechanism of neurodegeneration induced by cholesterol insufficiency in HD astrocytes. Energy delivery to neurons is one of the essential roles of astrocytes, because most neurons stop cholesterol synthesis after brain maturation, and the blood-brain barrier is impermeable to peripheral cholesterol trafficking (Leoni & Caccia., 2015). Besides energy refueling, cholesterol also enhances synaptogenesis, maintenance and stabilization of synapses and neuritis growth (Leoni & Caccia., 2015). In HD astrocytes, a declined production and exocytosis of cholesterol was observed (Valenza et al., 2010). This down-regulation is closely relevant to aggravated neural atrophy. Meanwhile, since cholesterol also regulates the fluidity of the

plasma membrane, a possible mechanism emerges that cholesterol insufficiency might disrupt the structure of the mitochondrial membrane and affect the function of TCA cycle related enzymes embedded in the membrane (Leoni & Caccia., 2015).

In summary, because astrocytes are extensively involved in the synaptic maintenance, energy metabolism and inflammation of neurons, it is important to investigate their mHTT-induced functional deficits and roles in HD pathogenesis. Interactions between neurons and glial cells at both a cellular and a molecular level may therefore provide new insights about the disease's pathogenesis and hence the development of HD therapy.

1.4 Animal Models to Recapitulate HD Symptoms and The Pathogenic Mechanism

To date, different animal models have been generated to study the progression and pathogenesis of HD. It is believed that an ideal HD model should have: 1) an explicit manifestation of neural and behavioral abnormalities, 2) recapitulation of the disease progression and mechanism, 2) a rapid disease onset proper for laboratory studies, and 3) a low within-species variability (Wang & Qin., 2006). Though few animal models could satisfy the full criteria, the dilemma encourages the development of HD animal models that can facilitate to generate comprehensive knowledge of the disease and hence guide the development of promising therapies. The work of Pouladi et al gives an inclusive review of HD animal models and their strengths and weaknesses.

Mice models are frequently used in HD studies and classified into chemically induced models and transgenic models. For example, neurotoxins such as ibotenic acid and 3-nitropropinoic acid are usually used to generate chemically induced mice models (Pouladi et al., 2013). These mice can rapidly develop excitotoxic lesions and atrophy of GABAergic neurons. However, several factors can undermine the accuracy of this model. First, since the provocation of neurodegenerative symptoms is acute, the model cannot mirror a slow disease progression typical of HD (Pouladi et al., 2013). Second, extrastriatal lesions may mislead the interpretation of motor symptoms (Wang & Qin., 2006). Meanwhile, mice share a different cerebral structure with higher primates, and this difference may result in *de novo* motor abnormalities unobserved in HD patients (Pouladi et al., 2013).

Transgenic mouse models have been widely used to recapitulate the HD symptom and examine the underlying pathological mechanism of HD. One of the representative

transgenic mouse models is R6/2 mouse that overexpresses exon1 in *mHTT* with CAG trinucleotide expansions. Major features of the R6/2 mice include well-characterized disease progression and an early disease onset (Wang & Qin., 2006). However, R6/2 mice are resistant to excitotoxicity and have limited neural atrophy in comparison to HD patients (Wang & Qin., 2006). Moreover, R6/2 mice carrying truncated *HTT* fragments might manifest inadequate or misleading HD phenotypes, as these artificial fragments are more or less different from a full-length *Htt* mutation naturally occurring in patients (Wang & Qin., 2006).

Besides *in vivo* studies, cell cultures emerge as an alternative to investigate function alterations of a specific cell type caused by mHTT. These *in vitro* models can largely distinguish autonomous changes from intercellular interactions (Carter et al., 2014). Reprogramming of HD patients' somatic cells into induced pluripotent stem cells (iPSCs) has been developed as an alternative approach to study HD pathogenesis and the development of HD therapeutics (Juopperi et al., 2015). Advantages of this iPSC approach in HD research include the following. First, HD iPSCs reprogrammed from patients' inherit identical mutation of the mHTT (Juopperi et al., 2015). Second, iPSCs can differentiate into a wide range of cell types *in vitro* including neurons and glial cells. This enables researchers to examine interactions between neurons and glial cells with identical genetic background, and minimizes individual variations. Last but not the least, iPSCs are capable of self-renewal and fast proliferation, which makes the maintenance and expansion of the cell line relatively convenient (Carter et al., 2014). Interestingly, iPSCs implants in mice have also shown capability of grafting, survival, migration and neurogenesis (Juopperi et al., 2015).

Despite of all these advantages, reprogrammed HD iPSCs still retain certain demerits. Juopperi and colleagues have reported that HD iPSCs-derived neurons lack distinct neural abnormalities and phenotypes observed in patients, and this could be attributable to the lack of microenvironmental modifications, including intercellular interactions, growth factors and appropriate stressors. More importantly, neurons derived from iPSCs tend to be mosaic with multiple cell lineages including undifferentiated iPSCs (Fong et al., 2010). This introduces difficulty in maintaining a high homogeneity of the derived cell population.

To resolve the limitations of iPSCs, another type of multi-potent cells is employed in HD studies. Neural Progenitor Cells (NPCs) are multipotent cells found in the subventricular zone in the CNS and able to self-renew and differentiate into all neural lineages including neurons and glial cells (Reetz et al., 2015). This suggests that NPCs may be able to replenish neurons and hence reverse the neurodegenerative phenotype of HD (Carter et al., 2014; Chen et al., 2013). Neurons differentiated from NPCs also exhibit a higher homogeneity of population than from iPSCs *in vitro* (Carter et al., 2014). Since NPCs are less likely to form tumors, they could become a better candidate for cell replacement therapy (Carter et al., 2014).

In conclusion, to establish an astrocyte model that can recapitulate the pathological phenotypes of HD while having the minimum variability, and that is easier to maintain and can best serve as a platform for cell replacement therapy studies, NPCs were chosen in this study as a base-stone to derive HD astrocytes *in vitro*.

Chapter 2

HD Astrocyte Modeling

2.1 Introduction

The purpose of this study is to investigate functional and genetic alterations in HD astrocytes derived from transgenic HD monkey iPSCs - generated NPCs, and how these changes could contribute to HD pathogenesis. To achieve this goal, we engendered HD astrocytes as an *in vitro* platform for HD modeling to recapitulate HD cellular phenotypes.

Astrocytes engage in key mechanisms to maintain the homeostasis of the CNS and buttress the activities and survival of neurons. Astrocyte disturbance can therefore contribute to HD pathogenesis. First, astrocyte can secrete neurotrophic factors to maintain the remodeling and growth of neural synapses. Particularly, BDNF is a nerve growth factor synthesized within astrocytes. Recently, BDNF has been found to positively correlate with Seladin-1, an anti-apoptotic factor (Sarchielli et al., 2014). BDNF not only increases the intracellular concentration of Seladin-1, but also promotes Seladin-1-induced synthesis of cholesterol, which is crucial in maintaining energy metabolism, synaptogenesis, neurotransmitter release, and myelination of neurons (Sarchielli et al., 2014; Valenza et al., 2010). Therefore, neural atrophy might be exacerbated with the presence of BDNF down-regulation.

Similar to BDNF, glutamate transporter (GLT-1) and ionotropic glutamate receptor (iGLUR) regulate important astrocyte functions. GLT-1 facilitates the uptake of extracellular glutamate and protects neurons from glutamate-induced excitotoxicity (Kawahara et al., 2005). Because glutamate-induced excitotoxicity is one of the major causes of neurodegeneration, HD astrocytes might manifest GLT-1 down-regulation that

could result in an insufficient clearance of extracellular glutamate and neural hyperexcitation. Concurrently, iGLUR over-stimulation in astrocytes can induce membrane depolarization and hence the release of neurotransmitters and growth factors to promote self-repair of neighboring neurons (Gottlieb & Matute., 1997). Yet, enhanced activities of iGLUR have been perceived in HD neurons, and are correlated with a prolonged glutamate-exposure and excitotoxicity of neurons (Fernandes & Raymond., 2009). Therefore, it is possible that HD astrocytes might embody similar iGLUR up-regulation, resulting in their elevated excitatory stress and reduced viability.

In addition, GFAP loss of astrocytes might also contribute to HD pathogenesis. GFAP is a chief intermediate filament in astrocytes and regulates cell migration and glutamate transporter localization in the cell membrane (Mol & Pekny., 2015). Consequently, a reduced GFAP level could lead to astrocyte de-differentiation and a lower density of glutamate transporters. An overall result might be accumulation of extracellular glutamate, malformation of astrocyte morphology, and alteration of the glial-neuron network in the CNS.

In line with the hypothesis that HD astrocytes would exhibit lower oxidative resilience, fewer antioxidants and mitochondrial enzymes would be expected in HD astrocytes. Since these enzymes/molecules can convert oxidative radicles to less harmful products, impaired synthesis of these enzymes might heighten the vulnerability of astrocytes to oxidative stress. Specifically, superoxide dismutase 2 (SOD2), a mitochondrial enzyme that catalyzes superoxide radicals into hydrogen peroxide, might be reduced in HD astrocytes (Lee et al., 2012). Undermined oxidative tolerance has also been perceived in HD neurons (Carter et al., 2014). Evidence has shown that impaired

antioxidant production by astrocytes that resulted from a mitochondrial dysfunction might be a primary reason for an increased oxidative stress of neurons in HD (Boussicault et al., 2014). Since the peroxisome proliferator-activated receptor-gamma coactivator (PGC1-alpha) regulates mitochondrial biogenesis and activities, its dysfunction might be involved in the pathogenic mechanism of neural atrophy (Chen et al., 2012). Consequently, it might be possible that astrocytes and neurons might share some common pathological pathways in HD, which lead to their alike cellular phenotypes.

2.2 Hypotheses

According to the reasoning above, we hypothesized that mHTT would affect astrocytes' neuroprotective function by inducing down-regulation of brain-derived neurotrophic factor (BDNF), glutamate transporter (GLT-1), superoxide dismutase 2 (SDO2), the peroxisome proliferator-activated receptor-gamma coactivator (PGC1-alpha), vimentin, and GFAP. On the other hand, ionotrphic glutamate receptors would be up-regulated, corresponding to an enhanced expression of their transmembrane domain (M1) embedded in the plasma membrane. A different morphology of HD astrocytes would also be anticipated. It is possible that structural malformations could worsen astrocytes' deficiency in maintaining the integrity of the blood-brain barrier and the CNS network.

Our lab has established a HD monkey NPC cell line that was generated by using iPSCs derived from HD monkeys. HD-NPCs can recapitulate HD cellular phenotypes including intranuclear inclusions and cells' increased susceptibility to the oxidative stress (Carter et al., 2014). The fact that HD NPCs can differentiate into neurons and mirror HD neural phenotypes indicates their potential role in modeling HD and as a platform for discovery research (Carter et al., 2014).

2.3 Material and Methods

2.3.1 Preparation of a NPC Monolayer *in vitro*

Neural progenitor cells (NPCs) are maintained and expanded according to the protocol invented by Dhara et al. After passaging, NPCs were suspended in the complete neural proliferation media (cNPM) supplemented with Neural Basal-A medium, 2mM L-glutamine, 1x penicillin/ streptomycin, 1x B27, 20ng/ml bFGF, and 10ng/ml LIF. NPCs were seeded into a 4-well plate with cover glass and a 35mm culture dish at a cell density of $7x10^4$ cells/cm^2. The working volume of the media was 0.5ml per well in a 4-well plate, and 2ml in a 35mm dish. Both the 4-well plate and the 35mm culture dish were coated with 1ug/cm^2 laminin and 20ug/ml poly-ornithine. Cells were evenly distributed and incubated at 37°c and 5% CO_2.

2.2.2 Astrocyte Differentiation from NPCs

NPCs were differentiated into astrocytes as instructed by Majumder et al, and the differentiation course was divided into two parts: day0-day2, and day3-day30. A monolayer of NPCs with approximately 90% of cell confluence was prepared for astrocyte differentiation. To start the astrocyte differentiation, cNPM was removed from the culturing system, and NPCs were provided with astrocyte differentiation media. To formulate the astrocyte differentiation media for da0 to day2, bFGF and LIF were removed from cNPM; 500nM Azacytidine (Aza-C), 20nM Trichostatin (TSA) and 20ng/ml BMP2 were added to the remaining media. From day3 to day30, cells were cultured with the astrocyte differentiation media free of Aza-C and TSA. Media were changed every 2 days throughout the 30 day-differentiation procedure.

To maximize the efficacy of astrocyte differentiation, Majumder et al incorporated both epigenetic modification and molecular signaling. Aza-C is a DNA methyltransferase inhibitor, and TSA is a histone deacetylase inhibitor. Both Aza-C and TSA can activate the expression of astrocyte markers by alleviating the hypermethylation of these genes. In addition, Majumder et al also suggest that BMP2 can suppress the potentiality of NPCs to differentiate into neurons, and therefore maintain a relatively high homogeneity of the astrocyte population (Majumder et al., 2013).

2.2.3 Immunocytochemistry

Cells were fixed by quickly adding 100ul of 4% paraformaldehyde/ 1x PBS. Solution was aspirated. Another 200ul of the same solution was added to cells and incubated for 7-15 min at room temperature. The fixation medium was aspirated, and cells were gently washed with 1x PBS 3 times each for 5 minutes. After wash, 200ul of blocking buffer (3% BSA/1x PBS/ 0.2% Triton X-100) was added to cells for 30-60 minutes at room temperature. Blocking buffer was then removed. Primary antibody (in 3% BSA/ 1x PBS) was diluted in the blocking buffer according to its best working concentration and added to cells. Cells were then incubated at 4°c overnight. Primary antibody solution was aspirated, and cells were washed with 1x PBS 3 times each for 15 minutes. Secondary antibody was diluted in the blocking buffer according to its best working concentration, and was added to cells for 30-45 minutes at room temperature. Following the incubation, cells were gently washed with 1x PBS 3 times each for 5 minutes. Hoechst staining was then performed, in which cells were incubated with 0.2ul Hoechst in 200ul 1x PBS for 5 minutes. After one last wash with 1x PBS, samples were

examined under an epifluorescent scope (Olympus BX51). Five random images were taken. See Table.1A-B for interested markers and their primary antibodies/secondary antibodies and dilutions.

Table1.A. Summary of characteristics and functions of attempted markers

	Characteristics
mEM48	An antibody interacts with mHTT to exemplify aggregate inclusions (Carter et al., 2014)
GFAP	An intermediate filament found predominantly in astrocytes and provides nutritional and structural support of neurons (Nawashiro et al., 2006)
Vimentin	An intermediate filament more common in a subpopulation of immature astrocytes and wound systems (Pixley & Vellis., 1984)
SOD2	An antioxidant detoxifies superoxide radicles within the mitochondrial matrix (Lee et al., 2012)
beta III-Tubulin	A tubulin isoform expressed before or during the terminal mitosis of neuroblasts and indicates early neurogenesis (Roskams et al., 1997)
Nestin	An intermediate filament marks for the multi-potentiality of progenitor cells (Wiese et al., 2004)

Table1.B. Attempted primary antibodies and secondary antibodies

	Primary Antibody Dilution	Secondary Antibody Dilution
mEM48	(1:50)	Alexa Flour 594 (1:1000, A-21205, Life Technologies)
GFAP	1:500 (G9269, Sigma)	Cy-5® (1:350, Life Technologies)
Vimentin	1:300 (V4630, Sigma)	Alexa Flour 594 (1:1000, A-21205, Life Technologies
SOD2	1:150(ab1353, Abcam)	Alexa Flour 594 (1:1000, A-21205, Life Technologies
beta III-Tubulin	1:300 (MAB 1637, Chemicon)	Alexa Flour 594 (1:1000, A-21205, Life Technologies
Nestin	1:1000 (ab11306, Abcam)	Cy-5® (1:350, Life Technologies)

2.3.4 Real-time PCR to Measure Astrocytic Gene Expression

RNA samples of cells were prepared by using TRIzol®(Life Technologies). Samples were then extracted and separated from genomic DNA by using Turbo DNA-free (Applied Biosystems) as described by manufacture's protocol. To start cDNA reverse transcription, 1ug of RNA samples was used. Procedures were done with a RNA-to-cDNA kit (Applied Biosystems). Transcribed cDNA was then stored at -20°c. Soo Advanced Universal Sybr Green (Applied Biosystems) was used to formulate a master mix for real-time PCR as instructed by the manufacture. Real-time PCR was conducted on CFX96 Real-time Detection System (Bio-rad). A thermal cycle was adopted as the following: 95°c activation step for 10 minutes, 95°c amplification step for 15s, and 60°c for 60s for 40 cycles (Carter et al., 2014). See Table.2 for used primers and their sequences. All real-time PCR values were normalized to ubiquitin-C (UBC), an internal control.

Table2. Attempted primers and their sequences

	SYBR Forward Primer Sequence
GFAP	5'-TCA AGA GGA ACA TCG TGG TG-3'
PGC1-alpha	5'- CTC AAA GAC CCC AAA GGA TGC -3'
GLT-1	5'- TCA TCA TCC ACG GGG GCA TC -3'
GLUR (M1)	5'- TTG AAG GTT ATG AGG TGG AAG C -3'
BDNF	5'- GCC CAA TGA AGA AAA CAA TAA GG -3'
MAP2	5'- ATC TTT CTC CTC TGG CTT CCG -3'
Nestin	5'- TGC GGA TGA GGA AGA AAG TGG -3'
SOX2	5'- GCA GGT TGA CAT CGT TGG TAA T -3'

2.3. 5 Oxidative Stress Challenge with H_2O_2

On day30 of the differentiation, astrocytes were treated with 5mM H_2O_2 diluted in the differentiation media free of Aza-C/ TSA for 24 hours at 37°c and 4% CO_2. Effects of the oxidative challenge were measured by TUNEL analysis. After fixation with 4% paraformaldehyde/ 1x PBS, cells were stained with In Situ Cell Death Detection Kit (Roche) and with Hoechst. Protocols suggested by the manufacture were followed. Cells were then examined under an epifluorescent scope (Olympus BX51) to quantify the percentage of positive cell deaths. For quantification, 6 random images with magnification of 20x were taken, and cell counts were performed.

2.4 Results

2.4.1 Morphological Differences between HD and Wild Type Differentiated Cells

On day 2 of astrocyte differentiation, both the wild type NPCs and HD NPCs developed explicit processes accompanied with massive cell deaths (Figure.1). Extensive cell death at this stage might be largely attributed to the cytotoxicity of TSA/Aza-C. Particularly, TSA has been reported as a potent anti-breast cancer drug that could arrest cells in the G1 and G2 phases of the cell cycle and thereby inhibit cell proliferation (Vigushin et al., 2001). From day 2 to day 30, the wild type cells showed down-slowed apoptosis under microscope upon the removal of TSA/Aza-C (Figure.1). However, a similar increase in survival was not observed in HD cells (Figure.1). Instead, a continuing cell loss was observed in HD cells throughout the differentiation process (Figure.1). Regarding the astrocyte morphology, the wild type cells developed radiated and long processes as early as day 2 and continued to branch at later stages (Figure1). Branching and elongation of processes were most active at the mid-stage of the differentiation; slightly thicker branches were observed at day 30 (Figure.1) Interestingly, HD cells showed a retraction of processes after day 2, and such retraction persisted over the differentiation process until day 30 (Figure.1). The majority of the HD cells exhibited a flat morphology with few branching and radiated processes (Figure.1).

Figure. 1

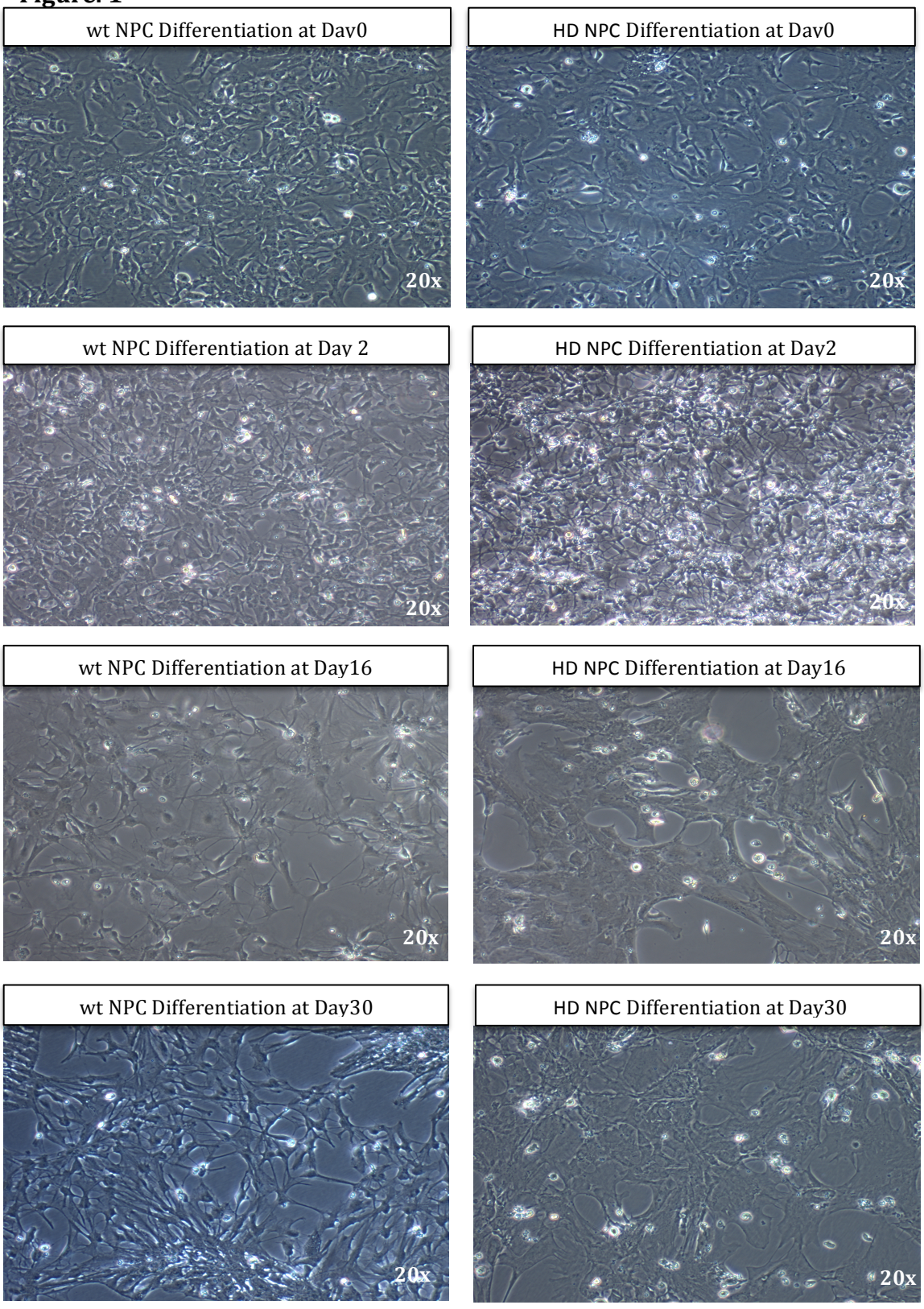

Fig.1. Astrocyte differentiation of HD NPCs and wild type NPCs on Day0, Day16, Day 30
NPCs were treated with the differentiation media containing Aza-C and TSA. Morphology changes from Day0 to Day 2 were recorded. The starting morphology was similar in HD NPCs and wild type NPCs on Day0. On Day2, both HD NPCs and wild type NPCs developed explicit processes. HD NPCs showed more cell deaths than the wild type. Upon the removal of TSA/Aza-C, wild type astrocytes developed persistent branching of processes, but HD astrocytes showed retraction of processes, which was not reversed on Day 30. Scale bar: 50 µm

2.4.2 Expression of Astrocytic Markers

Fixed astrocytes were stained with mEM48 for poly-glutamine aggregates, GFAP/vimentin for astrocyte maturation, and SOD2 for cellular antioxidative capability. beta III-tubulin (neurons) and nestin (NPCs) were used for examining the homogeneity of differentiated cells. Positive mEM48 staining was observed in the nuclei and the cytoplasm of HD astrocytes suggesting an accumulation of mHTT aggregates (Figure.2). This agreed with the pathological characteristic of HD where the mHTT would condense into insoluble aggregates inside the nucleus and form intranuclear inclusions (Rüb et al., 2013). Staining for beta III tubulin and nestin was not perceptible in HD nor wild type differentiated cells, indicating a much lower expression level compared to other markers.

Astrocyte differentiation of HD and wild type NPCs was assessed by immunocytochemistry using antibodies for GFAP and vimentin. GFAP is a marker for mature astrocytes, and vimentin is highly expressed in immature astrocytes (Pixley &Vellis., 1984). As expected, the wild type differentiated cells demonstrated a robust expression of both GFAP and vimentin, suggesting a transition state between maturing astrocytes and mature astrocytes (Figure.3-4). Since the co-expression of vimentin and GFAP is a hallmark of reactive astrocytes, it was possible that wild type differentiated cells might contain a subpopulation of reactive astrocytes (Janeczko., 1993). In comparison with the wild type, HD differentiated cells showed a much weaker staining of GFAP but a stronger expression of vimentin (Figure.3-4), revealing their immaturity and incompleteness of differentiation.

Moreover, wild type differentiated cells also exhibited a higher expression level of SOD2 than HD cells (Figure.5). A subcellular distribution of SOD2 in wild type cells

extended from the cell body to the processes (Figure.5). In comparison, SOD2 expression was much lower in HD cells and mostly located in perinuclear regions (Figure.5). No well-defined processes with positive SOD2 staining were displayed in HD cells (Figure.5). Interestingly, HD differentiated cells also revealed flattened and elongated nuclei as well as membrane indentions in comparison to the wild type (Figure 6). This finding was consistent with previous studies on neurodegenerative diseases caused by poly-glutamine aggregates (Nagaoka et al., 2003).

Figure. 2

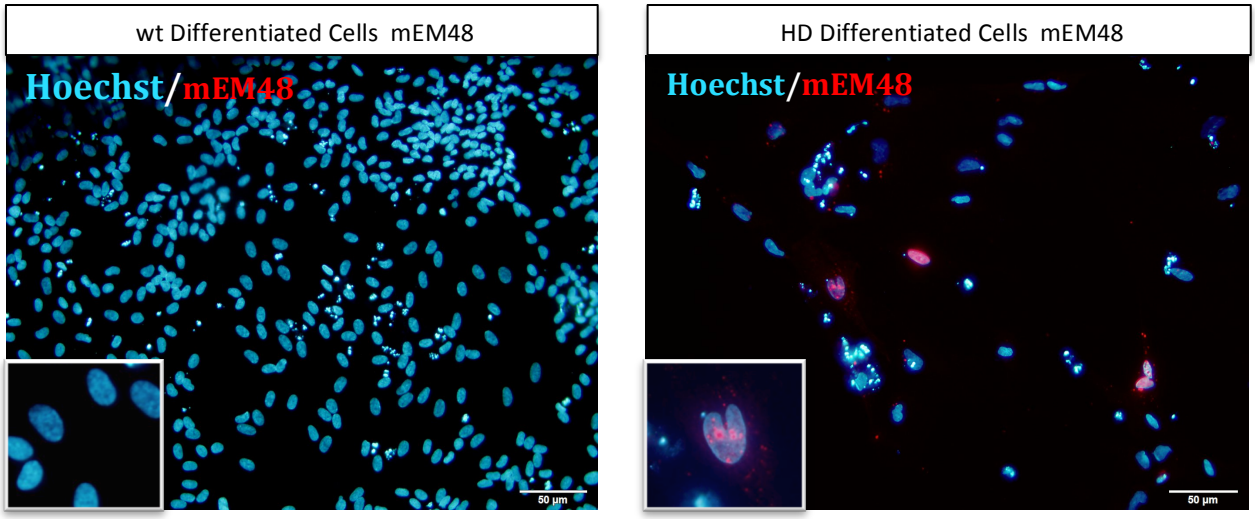

Figure. 3

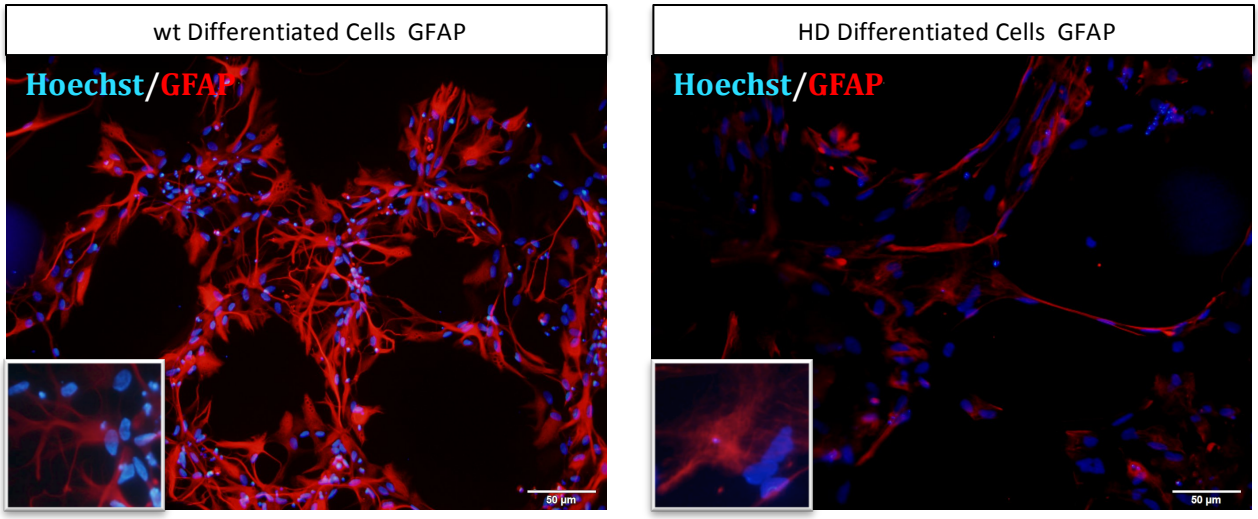

Figure. 4

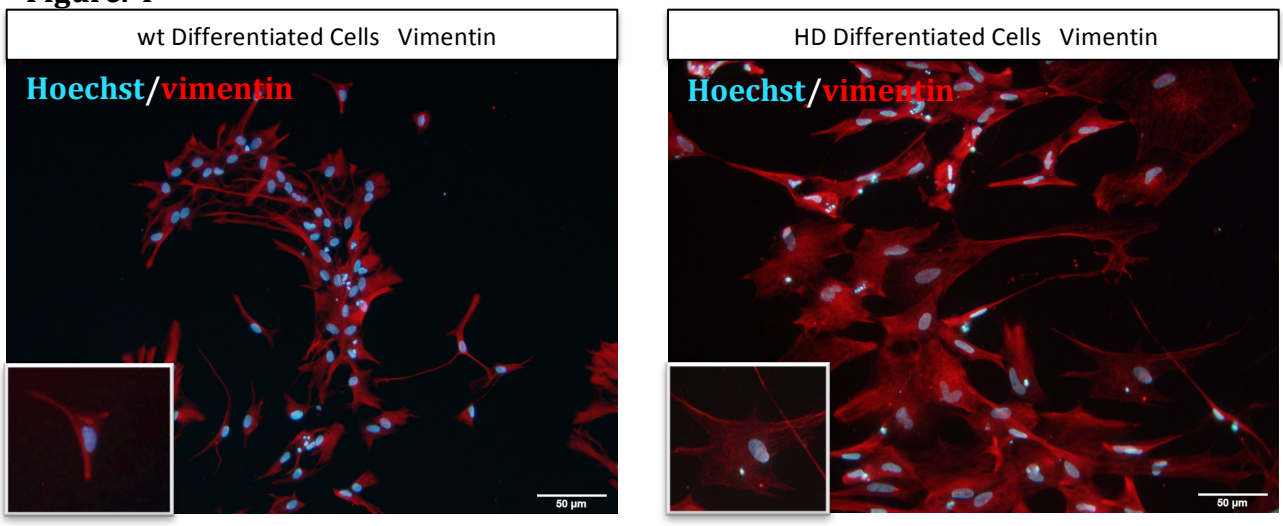

Figure. 5

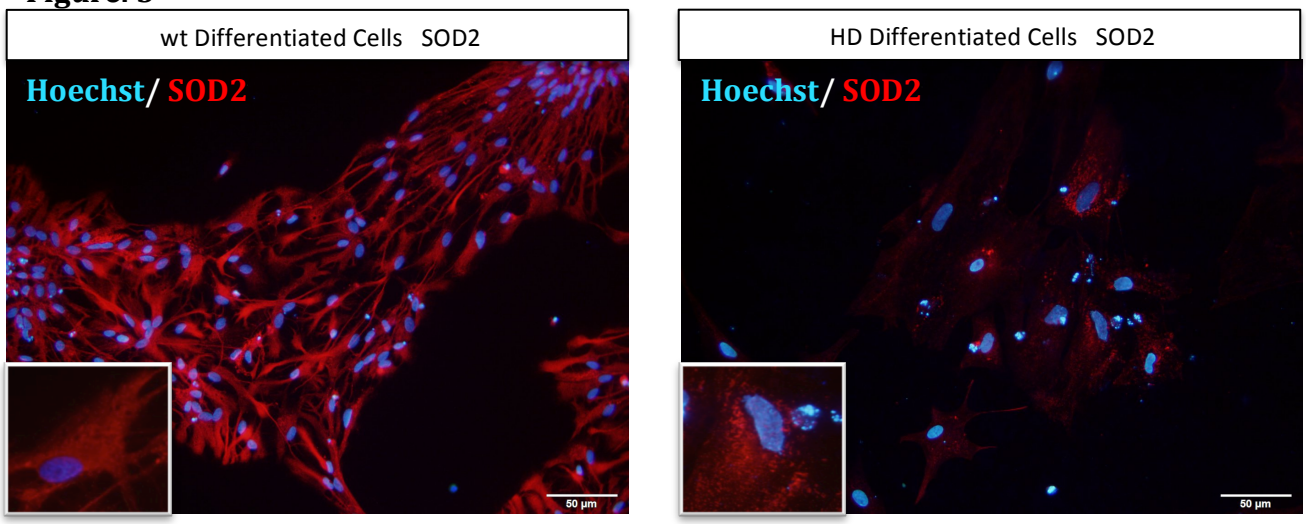

Figure. 6

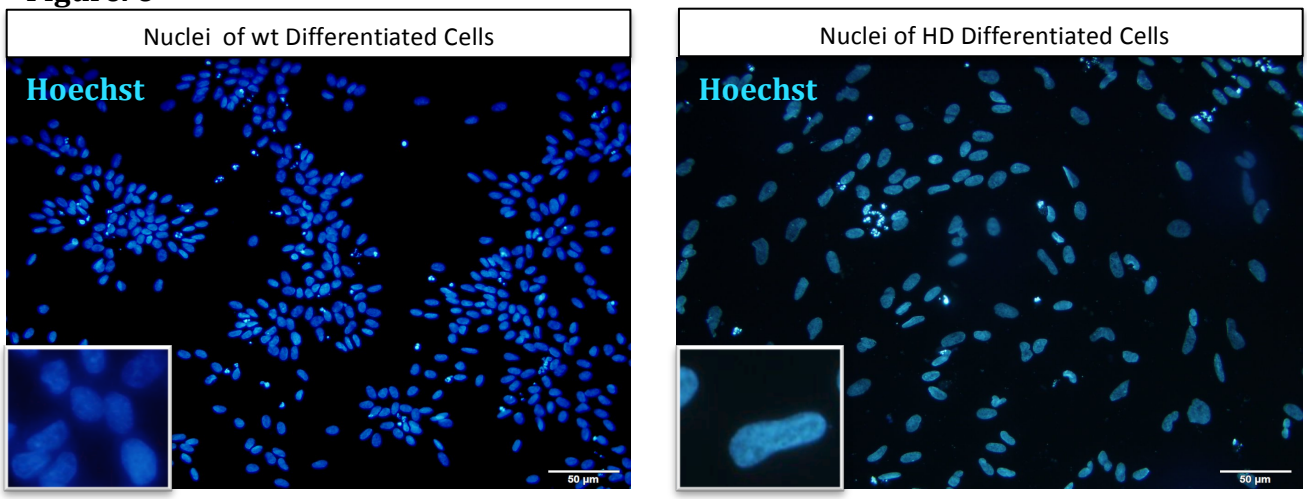

Fig.2. Immunocytochemistry with mEM48
Both HD astrocytes and wild type astrocytes were stained for mEM48 for poly-glutamine aggregates. Only HD astrocytes exhibited positive mEM48 staining (red) inside the nuclei and the cytoplasm, indicating the presence of poly-glutamine aggregates. Scale bar: 50 µm

Fig.3. Immunocytochemistry with GFAP
HD astrocytes and the wild type astrocytes were stained for GFAP (red). Both the HD astrocytes and the wild type astrocytes revealed positive GFAP staining. However, the wild type astrocytes demonstrated much more potent GFAP expression and larger GFAP-positive population than HD astrocytes. Scale bar: 50 µm

Fig.4. Immunocytochemistry with Vimentin
Both HD astrocytes and wild type astrocytes showed positive Vimentin staining (red). HD astrocyte exhibited a flat morphology with a greater size than the wild type. Scale bar: 50 µm

Fig.5. Immunocytochemistry with SOD2
HD astrocytes and wild type astrocytes were stained for SOD2 (red). The wild type astrocytes showed much more potent expression of SOD2 distributed in the entire cell. HD astrocytes showed a weak signal of SOD2, which was majorly localized within the mitochondria surrounding the nucleus. Scale bar: 50 µm

Fig.6. Nuclear sizes of HD astrocytes and wild type astrocytes at Day30
On Day30, HD astrocytes and wild type astrocytes were harvested and stained with Hoechst for nuclei visualization. Compared with the wild type astrocytes, HD astrocytes exhibited a larger nuclear size as well as nuclear membrane indentations. Scale bar: 50 µm

2.4.3 Real-time PCR on Classic Markers for Astrocyte Function

Significant down-regulation of GFAP, GLT-1 and PGC1-alpha was observed in HD astrocytes when compared with the wild type astrocytes (Figure.7). GFAP was the primary astrocyte intermediate filament. Its reduction could result in the instability of cytoskeletons composed of intermediate filaments, and thus lead to deformed astrocyte morphologies. An undermined GLT-1 expression was also observed in two out of three replicates of HD astrocytes (Figure.7). This observation coincided with previous studies where EAAT2 (or GLT-1) expression was decreased in both the striatum and the cortex of HD patients and was accompanied with a significantly impaired striatal glutamate uptake (Rebec., 2013). The down-regulation of PGC1-alpha in HD astrocytes implied impairment in the mitochondrial biogenesis (Figure.7). The loss-of-function of PGC1-alpha has been reported in HD, Alzheimer's disease and other neurodegenerative disorders (Chen et al., 2012). As a transcriptional co-activator, PGC1-alpha controls the activity of mitochondrial regulators such as succinate dehydrogenase and the electron transport chain complexes, and its disturbance might therefore contribute to the mitochondrial inactivity (Chen et al., 2012).

The suppression of GFAP, GLT-1 and PGC1-alpha suggested that HD astrocytes derived from our HD NPC model were able to recapitulate certain pathological features of HD astrocytes. However, these HD astrocytes also expressed higher levels of nestin and SOX_2 than the wild type astrocytes (Figure.7). Interestingly, the expression of BDNF and TM1 was up-regulated in HD astrocytes (Figure.7). Increased iGLUR(TM1) expression has been suspected as a trigger of neural excitotoxicity in HD (Fernandes & Raymond., 2009). However, BDNF enhancement has not been perceived in HD patients.

Figure.7

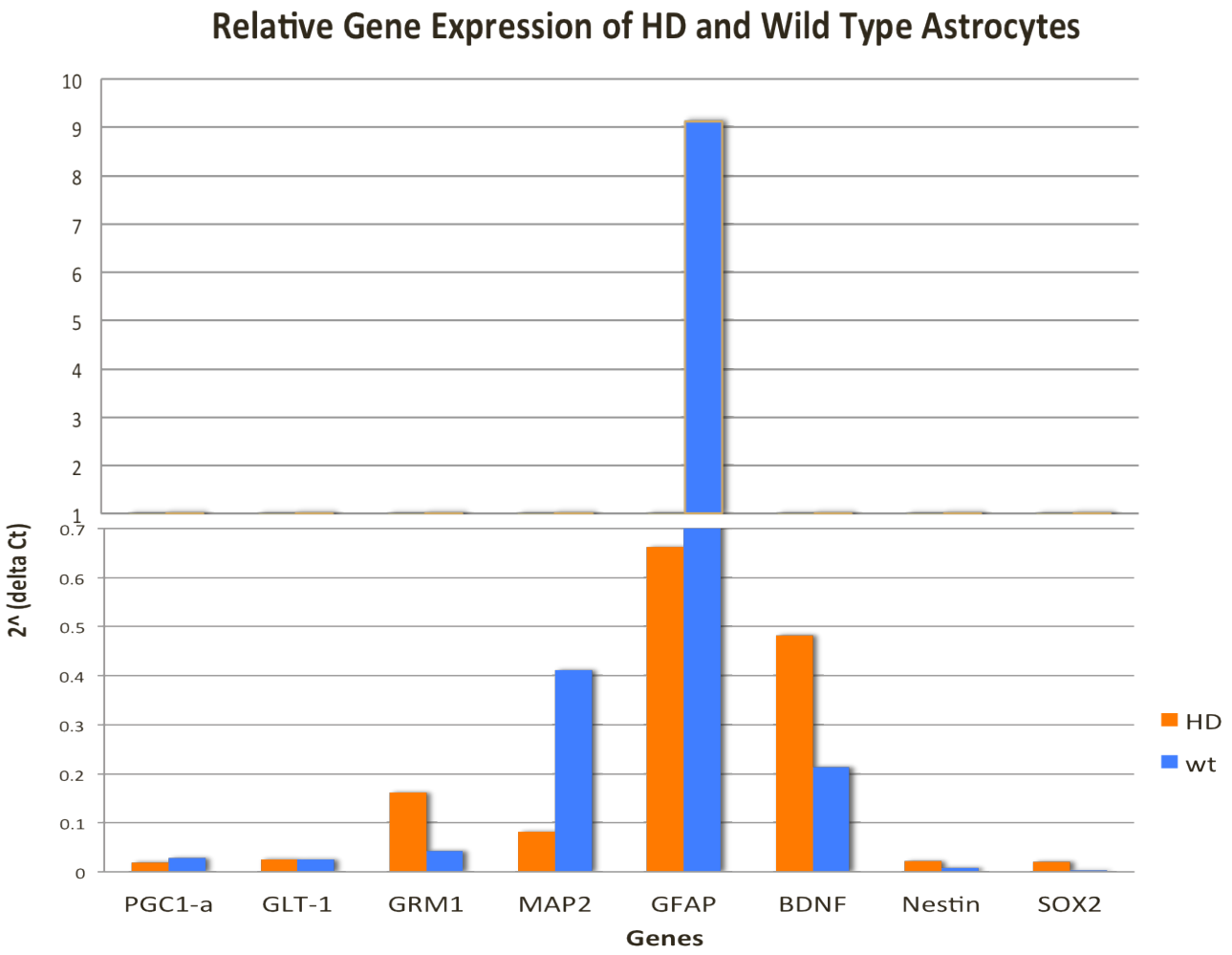

Fig.7. Relative gene expression of functional and identity markers in HD and wild type astrocytes.
In comparison with the wild type, HD astrocytes demonstrated down-regulation of GFAP and PGC1-alpha. Two replicated out three in real-time PCR also revealed a decrease in GLT-1 expression in HD astrocytes. However, BDNF and GRM1 (or iGLUR(TM1)), and nestin and SOX_2 were up-regulated in HD astrocytes.

2.4.4 Oxidative Challenge with H_2O_2

After a 24 hr-H_2O_2 treatment, TUNEL staining was used to identify apoptotic cells, and the Hoechst staining to determine the total cell population. To minimize false-positive signals caused by the cell/dye debris in the background, only TUNEL signals co-localizing with the nuclei stained with Hoechst were counted as positive apoptotic cells (Figure.8). H_2O_2-treated HD astrocytes demonstrated an elevated rate of apoptosis that was approximately 10% higher than the untreated HD astrocytes, and 14% higher than both the treated and untreated wild type astrocytes (Figure.9). In comparison with the HD astrocytes, the wild type astrocytes did not exhibit much difference in the oxidative challenge, and their rate of apoptosis held relatively constant at 4% (Figure.9). This hinted at a higher susceptibility of HD astrocytes to the oxidative stress. This observation coincided with the reduced expression of PGC1-alpha in HD astrocytes, since PGC1-alpha was a transcription cofactor involved in astrocytes' anti-oxidative defense and the mitochondrial biogenesis (Mena et al., 2015). Therefore, it was possible that the down-regulation of PGC1-alpha in HD astrocytes would suppress the expression of antioxidants and mitochondrial biogenesis, eventually leading to their increased vulnerability to ROS.

Figure. 8

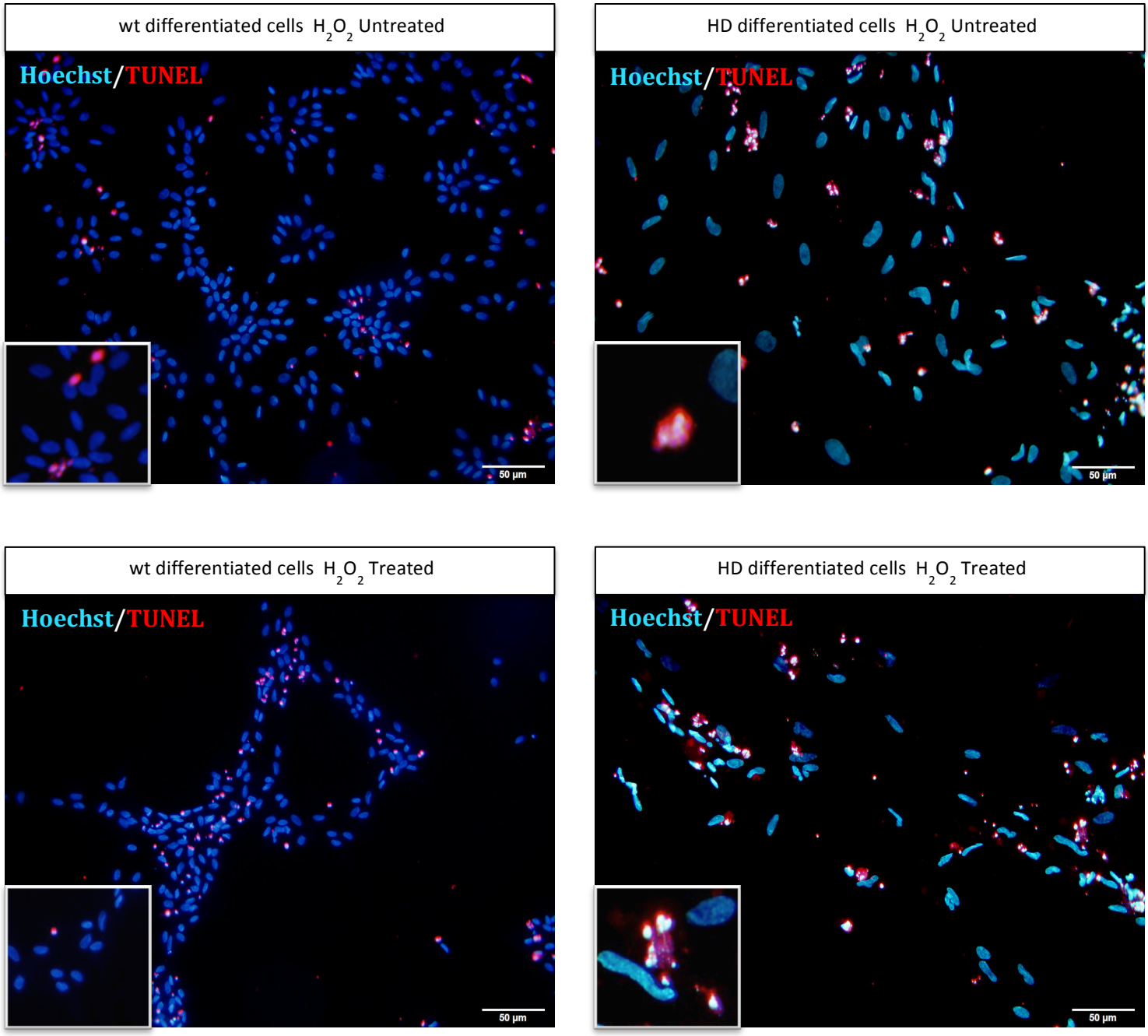

Fig.8. TUNEL analysis on cells treated/untreated H_2O_2.
TUNEL analysis (red) was applied to quantify the cell apoptosis before and after the oxidative challenge. In comparison with the wild type astrocytes, HD astrocytes showed a higher rate of apoptosis both before and after the treatment. Scale bar: 50 µm

Figure. 9

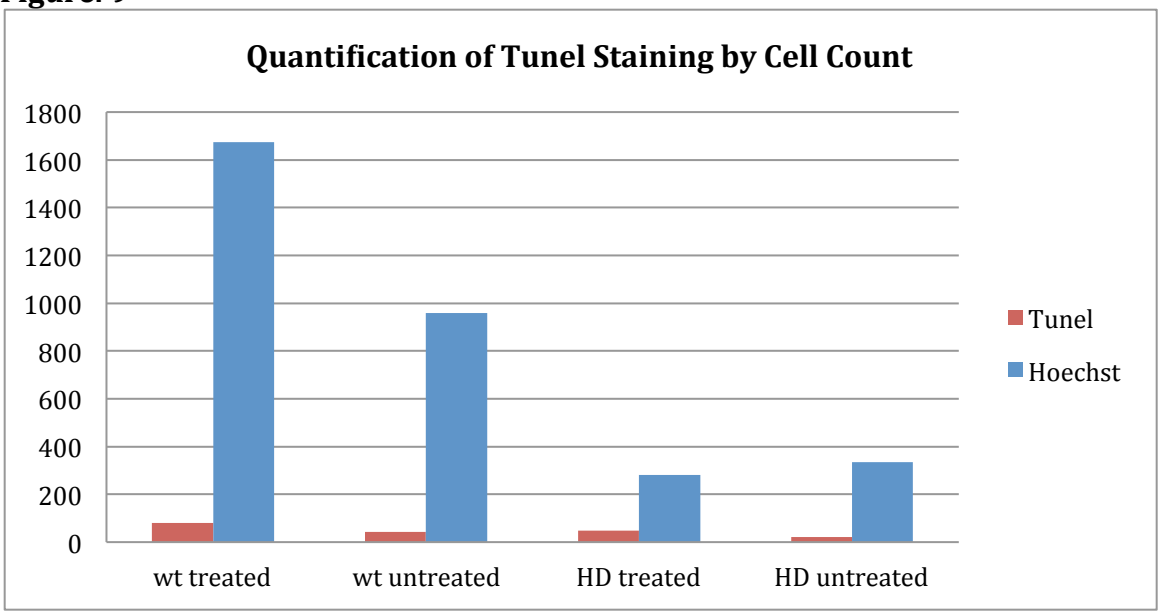

Figure. 10

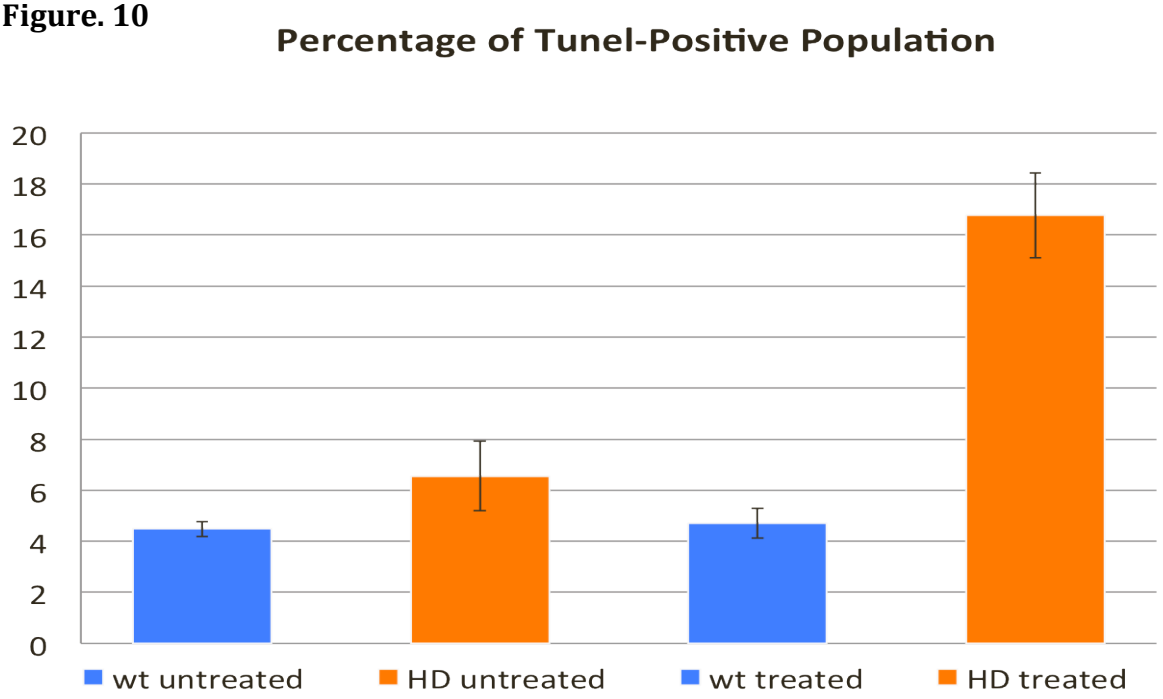

Fig.9. Quantification of TUNEL snalysis by cell counts
Six random images were taken to conduct cell counts. Cells with positive Hoechst staining were included to count for a total population. Cells with positive TUNEL staining were regarded as apoptotic.

Fig.10. Percentage of cells displaying positive TUNEL staining
A rate of apoptosis was obtained by dividing the TUNEL-positive population by the total population in each group. Wild type astrocytes held a relatively constant apoptosis rate at 4% regardless the H_2O_2 treatment. HD astrocytes showed a higher rate of apoptosis, which was further elevated upon the oxidative challenge.

2.5 Discussion

Huntington's disease (HD) is a lethal neurodegenerative disorder with an early onset at 30 years of age and progresses with motor, cognitive and psychiatric disturbances. Typical motor abnormalities include chorea and immobility that compromise HD patients' independence (Zielonka et al., 2014).

As cell therapy becomes a promising candidate for HD treatments, it is necessary to realize that neurons are not the sole therapeutic target. Increasing evidence has highlighted a vital role of astrocyte dysfunction in the aggravation of HD symptoms and the importance to correct awry astrocytes as a potential HD therapy (Chan & Surmeier., 2014). To explore the pathological characteristics of HD astrocytes, a non-human primate model was established in this study by differentiating HD NPCs into astrocytes. The goal of this study was to determine whether this HD astrocyte model could recapitulate cellular phenotypes of HD patients and thus serve as a model to study HD pathological mechanisms and cell therapies.

The real-time PCR results demonstrated that the derived HD astrocytes were able to mirror the down-regulation of GLT-1 and PGC1-alpha widely observed in HD patients (Figure.7). Intranuclear inclusions were identified exclusively in HD astrocytes and implied the accumulation of mHTT aggregates (Figure.2). Studies have introduced multiple mechanisms regarding the disruption of GLT-1 localization. The work of Rebec illustrates an association between perturbed GLT-1 insertion in cell membrane and mHTT expression. Specifically, mHTT can inhibitorily interact with HIP14, a palmitoyl-acyl transferase, and result in the inhibition of GLT-1 palmitoylation. Since GLT-1insertion requires palmitoylation, mHTT indirectly blocks the localization of GLT-1 in

the membrane (Rebec., 2013). The transcriptional abnormalities of GLT-1 and PGC1-alpha further suggested an impaired capability of HD astrocytes to remove the extracellular glutamate and to maintain mitochondrial homeostasis for energy metabolism and antioxidant production. These perturbed astrocyte functions partially provoked the pathogenesis of HD by not only inducing neural hyperexcitation, but also undermining the astrocytes' resistance to ROS, hence impairing their neuroprotective capacity. Increased apoptosis of HD astrocytes in the H_2O_2 challenge suggested their worsened vulnerability to ROS as well. Mitochondrial dysfunctions have been determined as one of the critical attributes to the neurodegeneration in HD (Boussicault et al., 2014). Mena and colleagues have unfolded a persistently high concentration of reactive redox-iron in the affected regions of HD patients, especially in oligodendrocytes and microglia cells (Mena et al., 2015). A high-iron accumulation is detrimental, since the redox iron in the mitochondria could catalyze the formation of hydroxyl radicals and reactive aldehydes, and therefore it imposes a high oxidative stress on the neighboring neurons (Mena et al., 2015). Therefore, we suspected a high iron level in HD astrocytes as well. Given a convoluted neural-glial network and an intimate astrocyte-neuron contact in the CNS, it was possible that an excessive amount of ROS derived from HD astrocytes could exacerbate neural atrophy.

On the other hand, a higher expression level of BDNF and TM1 was observed in HD astrocytes (Figure.7). TM1 up-regulation might signify an increased level of iGLUR because it is a transmembrane domain of iGLUR. Both BDNF and iGLUR epitomize essential astrocyte functions in buttressing neural survival. Particularly, BDNF plays a critical role in modeling the synaptic plasticity of neurons (Sarchielli et al., 2014). We

supposed that changes in BDNF expression went hand-in-hand with the changes in TM1. This speculation was confirmed with clues from previous studies. The work of Adachi et al and Seifer & Steinhäuser reveals an underlying mechanism that illustrates a positive correlation between BDNF and iGLUR (Adachi et al., 2014; Serifert & Steinhäuser., 2013). In their studies, they found that the activation of iGLUR could lead to calcium influx and therefore increase intracellular calcium concentration. The elevated calcium concentration would further stimulate the glutamate release from the astrocytes and lead to neural excitation. More importantly, the elevated calcium level could also facilitate the calcium-dependent phosphorylation of the cyclic AMP-responsive element binding protein (CREB) by activating a series of kinases including PKA and MAPK. Phosphorylated CREB would then bind to Calcium-response elements (CaRE) on the BDNF promoter, stimulating the BDNF expression (Adachi et al., 2014; Seifer &Steinhäuser., 2001). In short, iGLUR activation could stimulate BDNF promoter through a calcium cascade and elevate BDNF expression.

However, it is important to note that the enhanced BDNF expression may not be parallel to a strengthened neuroprotective function of astrocytes. BDNF activation requires a furin-mediated cleavage as a posttranslational modification, and only activated BDNF is able to promote neural survival and growth; pro-BDNF not cleaved by furin has exactly the opposite activity and can induce cell apoptosis (Chen et al., 2015). It would be therefore interesting and necessary to investigate mHTT's impact on the activity of furin and interaction with other molecular machineries for post-translational modifications.

Reasons for an augmented expression of TM1 in astrocytes still remains esoteric. However, such augmentation seems to agree with the iGLUR-induced apoptosis in HD

neurons. NMDAR is a type of iGLUR. An increased NMDAR peak current has been observed in HD medium-sized spiny neurons (Fernandes & Raymond., 2009). Reinforced glutamate stimulation can elevate the concentration of the cytosolic calcium, leading to perturbed enzyme activation and mitochondrial dysfunction (Fernandes & Raymond., 2009). Particularly, increased cytosolic calcium ions can dissipate the mitochondrial membrane potential and thereby prevent ATP synthesis, and activate mitochondrial permeability transition prior to cell apoptosis (Fernandes & Raymond., 2009). This would consequently raise the susceptibility of HD astrocytes to calcium-mediated apoptosis. To explain TM1 up-regulation, a compensatory mechanism was suspected. Increased synthesis and distribution of iGLUR have been perceived in the dendrites of neurons to facilitate the long-term potentiality (LTP) and modulate the responsiveness of local synapses (Kacharmina et al., 2000). Therefore, it might be possible that process retraction and malformation of the derived HD astrocytes due to impaired intermediate filament can activate a compensatory pathway to retrieve a normal synaptic function of astrocyte processes.

Consistent with previous studies, these HD astrocytes exhibited a persistently larger nuclear size than the wild type (Nagaoka et al., 2003) (Figure.6). Membrane indentation and nuclear malformation were also noticed (Nagaoka et al., 2003). Nagaoka and colleagues revealed a positive correlation between intranuclear inclusions and enlarged nuclei. They noticed that the nuclei of HD neurons with CAG intranuclear inclusions were larger than those without inclusions, and nuclear deformities tended to occur with nuclear membrane indentation. This phenomenon however was opposite to nuclear shrinkage that usually preceded cell apoptosis and neural atrophy in HD. As an

explanation, it was proposed that intranuclear inclusions could trigger certain defensive mechanisms to resist apoptosis, and thus correlate with a nuclear size larger than expected (Nagaoka et al., 2003). Apart from intranuclear inclusions, nucleus enlargement might also result from iGLUR-mediated cell swelling (Fernandes & Raymond., 2009).

Furthermore, the derived HD astrocytes also showed a retraction of processes and a flat morphology during differentiation (Figure.1). This striking morphological change implied potential cytoskeletal instability caused by mHTT, preventing astrocytes from maintaining their structural integrity and forming a solid blood-brain barrier with endothelium cells *in vivo* (Sica., 2015). Disappearance of these processes indicated a loss of astrocyte end-feet, which could jeopardize astrocytes' vascular contact and thus their exchange of nutrients and removal of metabolic wastes in the CNS (Nawashiro et al., 2006). Besides, lowered expression of GFAP and up-regulation of nestin and SOX2 suggested the immaturity of HD astrocytes (Figure.3, Figure.7). Overall, we concluded that these HD astrocytes were underdeveloped and compromised in function and morphology and were prone to pre-mature deaths.

Finally, limitations in this study should be specified. First, in comparison to the wild type, HD astrocytes exhibited variations in gene expression hence a relatively high degree of estimated uncertainty. This could be attributed to either technical errors or dwindled cell population after an extensive cell loss. It was possible that a dramatic reduction of cell population might alter intercellular signaling and thus either lead cells to apoptosis, or trigger certain defensive mechanisms to improve cell survival. Second, despite their capacity of fast self-renewal and proliferation, reprogrammed NPCs still retain some instability because of their multi-potency and compromised growth

conditions due to aging and multiple times of passages. Finally, the fact that these derived HD astrocytes were prone to pre-mature deaths also caused difficulty in maintaining an expandable astrocyte cell line for further studies.

2.6 Conclusion

In conclusion, this thesis aimed to establish a HD astrocyte model from non-human primate NPCs, and utilize it as a platform to study HD pathogenesis and cell therapies. Derived HD astrocytes recapitulated down-regulated expression of functional markers including GLT-1, PGC1-alpha and GFAP, and up-regulation of iGLUR (TM1). These alterations might contribute to mitochondrial dysfunction, structural instability, excitotoxicity, and oxidative intolerance of astrocytes in neurodegenerative diseases. Meanwhile, HD astrocytes showed perturbed process development, suggesting their impaired capacity to maintain the CNS homeostasis. HD astrocytes in this study also manifested phenotypes unobserved in HD patients, which might be attributed to certain compensatory or self-defense mechanisms. In summary, the generated HD astrocytes demonstrated compromised function and morphology in comparison to the wild type cells and were prone to pre-mature deaths. This observation might help explore how mHTT-affected astrocytes could exacerbate neurodegeneration in HD and provide a clue for the development of astrocyte-centered HD therapeutics. Future investigation might include a co-culture system incorporating both neurons and other glial cells to better approximate an authentic CNS signaling environment *in vivo*. Experiments on the alteration of functional markers throughout the astrocyte differentiation might be covered as well to investigate HD astrocyte pathology.

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