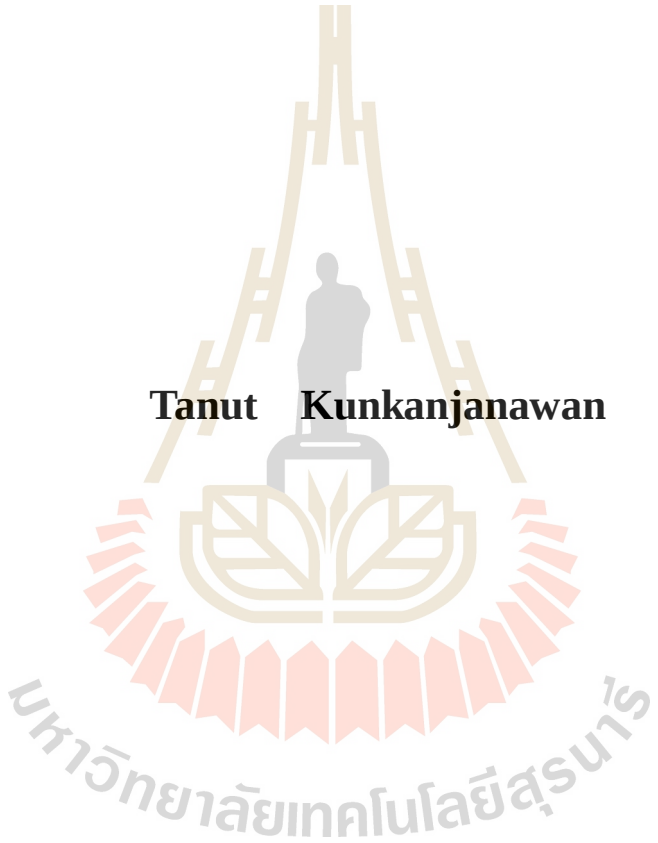
TRANSGENIC NON-HUMAN PRIMATE HUNTINGTON'S DISEASE MODEL FOR DRUG DISCOVERY RESEARCH AND MicroRNA-196a THERAPY

Tanut Kunkanjanawan



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แบบจำลองโรคฮันติงตันจากลิงดัดแปลงพันธุกรรมสำหรับการทดสอบยา และการรักษาด้วย MicroRNA-196a

นายธณัท กุลกาญจนาวรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ธณัท กุลกาญจนาวรรณ : แบบจำลองโรคฮันติงตันจากลิงดัดแปลงพันธุกรรมสำหรับการทดสอบยาและการรักษาด้วย MicroRNA-196a (TRANSGENIC NON-HUMAN PRIMATE HUNTINGTON'S DISEASE MODEL FOR DRUG DISCOVERY RESEARCH AND MicroRNA-196a THERAPY) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. รังสรรค์ พาลพ่าย, 146 หน้า.

โรคฮันติงตัน (Huntington's disease: HD) เป็นโรคความเสื่อมของระบบประสาทซึ่งเกิดจากการเพิ่มของนิวคลีโอไทด์ CAG ซึ่งถอดรหัสเป็น polyglutamine (polyQ) ในยีนฮันติงติน (Huntingtin: Htt) การเพิ่ม polyQ นำมาสู่การสร้างโปรตีนฮันติงตินกลายพันธุ์ที่เกิดการเกาะกันเป็นกลุ่มก้อน ในปัจจุบันยังไม่มีการรักษาโรคฮันติงตัน แบบจำลองโรคฮันติงตันในลิงดัดแปลงพันธุกรรมซึ่งแสดงออกถึงการดำเนินไปของโรคและลักษณะทางพยาธิสภาพเหมือนกับมนุษย์แสดงถึงศักยภาพในการใช้ลิงดัดแปลงพันธุกรรมเป็นต้นแบบของโรคฮันติงตัน ปัจจุบันพบว่าลิงดัดแปลงพันธุกรรมโรคฮันติงตันมีการเปลี่ยนแปลงทางประสาทพยาธิสภาพโครงร่างประสาทวิทยาพฤติกรรม และ สารชีวโมเลกุลที่เหมือนกับผู้ป่วยจริง นอกจากนี้เซลล์ต้นกำเนิดระบบประสาท (neural progenitor cells: NPCs) จากลิงดัดแปลงพันธุกรรมโรคฮันติงตันได้ถูกศึกษาและแสดงให้เห็นถึงความสามารถแปรสภาพไปเป็นเซลล์ประสาทซึ่งสามารถพัฒนาลักษณะจำเพาะของโรคฮันติงตันได้แก่ การเกาะกันเป็นก้อนของโปรตีนกลายพันธุ์ และ ก้อนโปรตีนในนิวเคลียส แม้กระทั่งความไวต่อสภาวะความเครียดออกซิเดชัน ในงานวิจัยนี้เซลล์ต้นกำเนิดระบบประสาทและเซลล์ประสาทของลิงฮันติงตันได้ถูกทดสอบการเป็นต้นแบบในการวิจัยเพื่อค้นคว้ายาในหลอดทดลองจึงได้ใช้ riluzole (RI) memantine (ME) และ methylene blue (MB) ทดสอบในเซลล์ต้นกำเนิดระบบประสาทและเซลล์ประสาทของลิงฮันติงตัน ซึ่งยาเหล่านี้ได้แสดงถึงความสามารถในการป้องกันผลของโรคฮันติงตันและโรคความเสื่อมของโรคระบบประสาทอื่น ๆ มาแล้ว ในการศึกษานี้พบว่ายาเหล่านี้สามารถป้องกันระบบเซลล์ประสาทจากโรคฮันติงตันได้ในการทดสอบความเป็นพิษต่อเซลล์ apoptosis และ การเกาะกันเป็นกลุ่มก้อนของโปรตีนฮันติงตินที่กลายพันธุ์

ในขณะนี้ความสนใจในพยาธิวิทยาของโรคฮันติงตันได้หันมาสู่การเปลี่ยนแปลงการแสดงออกของยีนที่เกี่ยวข้องกับโรค ซึ่งรวมไปถึงการศึกษาในอาร์เอ็นเอที่ไม่มีการถอดรหัส (non-coding RNA) ในงานวิจัยนี้ได้พบแสดงออกที่เพิ่มขึ้นของ miR-196a ทั้งในสมองลิงดัดแปลงพันธุกรรมโรคฮันติงตันและสมองของผู้ป่วย อีกทั้งเมื่อเพิ่มการแสดงออกของ miR-196a ซึ่งพบว่าความเป็นพิษต่อเซลล์จากโรค และ apoptosis ถูกทำให้บรรเทาลงในเซลล์ต้นกำเนิดระบบประสาทและเซลล์ประสาทของลิงดัดแปลงพันธุกรรมโรคฮันติงตัน และยังพบว่าการแสดงออกของยีนที่เกี่ยวข้องกับ apoptosis ลดลงเช่นกัน จากการย้อมด้วย mitotracker พบว่ารูปร่างและการทำงานของไมโตคอนเดรียถูก

ทำให้ดีขึ้นและพบการเพิ่มการแสดงออกของยีน *CBP* และ *PGC-1α* ในเซลล์ต้นกำเนิดระบบประสาทของลิงดัดแปลงพันธุกรรมโรคฮันติงตัน ดังนั้นการเพิ่มการแสดงออกของ miR-196a สามารถการแก้ไขสภาพของเซลล์ในเซลล์ต้นกำเนิดระบบประสาทและเซลล์ประสาทที่เป็นโรคฮันติงตัน จากผลการทดลองสามารถบ่งชี้ถึงการควบคุมพยาธิสภาพของโรคฮันติงตันของ miR-196a ซึ่งอาจจะนำมาสู่การเข้าใจถึงการควบคุมเชิงโมเลกุลของโรคฮันติงตันและการพัฒนาการรักษาแนวใหม่ได้

โดยสรุปงานศึกษาวิจัยนี้แสดงให้เห็นว่าแบบจำลองโรคฮันติงตันจากลิงดัดแปลงพันธุกรรมเป็นแบบจำลองที่มีศักยภาพในการเป็นตัวแทนพยาธิสภาพของโรคฮันติงตัน การค้นคว้ายา และการพัฒนาการรักษาด้วยยีน การเพิ่มการแสดงออกของ miR-196a แสดงให้เห็นถึงศักยภาพในการป้องกันพยาธิสภาพของโรคฮันติงตัน และแสดงถึงโอกาสในการรักษาซึ่งอาจจะมีผลต่อการควบคุมโรคความเสื่อมทางระบบประสาทอื่นๆด้วย

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2558 ลายมือชื่ออาจารย์ที่ปรึกษา _____

TANUT KUNKANJANAWAN : TRANSGENIC NON-HUMAN PRIMATE HUNTINGTON'S DISEASE MODEL FOR DRUG DISCOVERY RESEARCH AND MicroRNA-196a THERAPY. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 146 PP.

TRANSGENIC NON-HUMAN PRIMATE HUNTINGTON'S DISEASE MODELING/DRUG SCREENING/ MicroRNA-196a THERAPY

Huntington's disease (HD) is a neurodegenerative disease caused by an expansion of CAG trinucleotide repeat encoded for polyglutamine (polyQ) in the Huntingtin (*Htt*) gene which leads to the formation of mutant HTT (mHTT) protein aggregates. Currently, no cure for HD was found. Transgenic HD nonhuman primate (HD-NHP) animal models have been developed. These animals recapitulate a progressive development of clinical and pathological features similar to human HD patients, which supports the potential preclinical application of the HD-NHP model. To date, phenotypes observed in HD-NHP include neuropathological, neuroanatomical, behavioral and biomolecular abnormalities. Additionally, neural progenitor cells (NPCs) derived from HD monkeys have been develolped. These cells are capable of differentiating into neurons, and upon differentiation developed distinct HD cellular phenotypes. Observed phenotypes included the formation of mHTT aggregates and intranuclear inclusions, as well as increased susceptibility to oxidative stress. In this study potential application of HD monkey NPCs (HD NPCs) and neurons (HD neurons) were evaluated as an *in vitro* platform for drug discovery research. To demonstrate proof-of-concept in this system, HD NPCs and neurons were treated with

riluzole (RI), memantine (ME), and methylene blue (MB). These known compounds have been shown to have a protective effect in HD and other neurodegenerative diseases. In this context, three compounds were tested for their neuroprotective properties on HD phenotypes cytotoxicity, apoptosis, and aggregation of mHTT protein.

Recently attention to HD pathology has turned to genetic alterations associated with the disease. This includes the investigation of non-coding RNA. Elevated expression of miR-196a has been observed in both HD-NHP and human HD brains. Cytotoxicity and apoptosis were ameliorated by the overexpression of miR-196a in HD-NHP neural progenitor cells (HD-NPCs) and differentiated neurons (HD-neurons). The expression of apoptosis related genes was down regulated. Mitochondrial morphology and activity were improved as indicated by mitotracker staining and the upregulation of *CBP* and *PGC-1α* in HD-NPCs overexpressing miR-196a. Here, the amelioration of HD cellular phenotypes in HD-NPCs and HD-neurons overexpressing miR-196a were demonstrated. Our results also suggested that the regulatory role of miR-196a in HD pathogenesis may hold the key for understanding molecular regulation of HD and can be used to develop novel therapeutic.

In summary, this study demonstrated HD-NHP model is a potential model for HD pathology, drug discovery, and development of gene therapy. Moreover, overexpression of miR-196a showed protective potential on HD pathology and presented a unique therapeutic opportunity that may impacts across related neurodegenerative diseases

School of Biotechnology Student's Signature _____

Academic Year 2015 Advisor's Signature _____

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Tanut Kunkanjanawan

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

3NP	=	3-Nitropropionic acid
AAV	=	Adeno-assciated virus
ALS	=	Amyloidtrophic lateral sclerosis
ANXA1	=	*Anexin-1A*
AR	=	Androgen receptor
ATP	=	Adenosine triphosphate
Aβ	=	Amyloid-β
BCL2L1	=	B-cell lymphoma 2 like protein
Bdnf	=	*Brain-derived neurotrophic factor* gene
Bim	=	*Bcl-2 interacting mediator of cell death* gene
CaMKIV	=	*Calcium/calmodulin-dependent protein kinase IV* gene
CBP	=	CREB binding protein
CELF2	=	Elav-like family member 2
CN	=	Caudate nucleus
CREB	=	Cyclic-AMP response element binding protein
CUGBP	=	Elav-like family member 2 (CELF2)
D2R	=	*Dopamine D2 receptor* gene
DA neuron	=	dopaminergic neuron
DARPP-32	=	Dopamine- and cAMP-regulated phosphoprotein
DPSCs	=	Dental pulp derived mesenchymal stem cell

LIST OF ABBREVIATIONS (Continued)

DRP1	=	Dynamin-related protein 1
ERRs	=	Estrogen-related receptors
ESC	=	Embryonic stem cell
FMRP	=	Fragile X mental retardation protein
FOXO	=	*Forkhead box protein O* gene
FRET	=	Förster resonance energy transfer
FXS	=	Fragile X syndrome
G6PD	=	Glucose 6-phosohate dehydrogenase
GTPases	=	Guanosine triphosphatases
HAT	=	Histone acetyltransferase
HD	=	Huntington's disease
HIV-1	=	Human immunodeficiency virus type 1
HSPs	=	Heat-shock protein
Htt	=	*Huntingtin* gene
HTT	=	Huntingtin protein
ICC	=	Immunocytochemistry
iPSCs	=	Induced pluripotent stem cells
Jacob	=	*Juxtasynaptic attractor of caldendrin on dendritic boutons protein* gene
MAPKs	=	Mitogen-activated protein kinase
MB	=	Methylene blue

LIST OF ABBREVIATIONS (Continued)

ME	=	Memantine
MFN	=	Mitofusin proteins
mHtt	=	Mutant *Huntingtin* gene
mHTT	=	Mutant Huntingtin protein
miRNA	=	MicroRNAs
mLIF	=	Mouse leukemia inhibitory growth factor
MSN	=	Medium spiny GABAergic neuron
NCoR	=	Nuclear receptor co-repressor
NDM	=	Neural differentiation medium
NES	=	Nuclear transport signal
NGFR	=	Nerve growth factor receptorv
NHP	=	Non-human primate
NLS	=	Nuclear localization signal
NMDARs	=	N-methyl-D-aspartate receptor
NO	=	Nitric oxide
NPCs	=	Neural progenitor cell
NRSF	=	Neuron-restrictive silencer factor
OPA1	=	Optical atrophy 1
P bodies	=	Processing body
P/L	=	Polyornithine/Laminin
P/S	=	Penicillin/Streptomycin

LIST OF ABBREVIATIONS (Continued)

PD	=	Parkinson's disease
PGC-1α	=	*Peroxisome proliferator-activated receptor-γ coactivator 1α*
PKA	=	Protein kinase A
polyQ	=	Polyglutamine
PPARγ	=	*Peroxisome proliferator-activated receptor γ* gene
PSCs	=	Pluripotent stem cells
Puma	=	*Pro-apoptotic Bcl2 homology domain 3 (BH3)-only member* gene
qPCR	=	Real-Time Quantitative PCR
REST	=	RE1-Silencing Transcription Factor
RI	=	Riluzole
RISC	=	RNA-induced silencing complex
ROS	=	Reactive oxygen species
SBMA	=	Spinal and bulbar muscular atrophy
SDH	=	Mitochondrial complex II/succinate dehydrogenase
SHH	=	Sonic Hedgehog
siRNAs	=	Small inhibitory RNAs
Sp1	=	Specificity protein 1
TAFs	=	TBP-associated factors
Tat	=	HIV-1 trans-activator to transcription
TBP	=	TATA-binding protein

LIST OF ABBREVIATIONS (Continued)

TCA cycle	=	Tricarboxylic acid cycle
TFIID	=	A multisubunit complex consists of TBP and TAFs
TRAIL	=	*Tumor necrosis factor related apoptosis-inducing ligand* gene
UPS	=	Ubiquitin-proteosome system
VMAT2	=	Vesicular monoamine transporter 2
WT	=	Wild type
YAC	=	Yeast artificial chromosome

CHAPTER I

INTRODUCTION

1.1 Background

Huntington's disease (HD) is a neurodegenerative disease caused by the expansion of CAG trinucleotide (polyglutamine; polyQ) tract in *Htt* gene which leads to the formation of mHTT protein aggregation (Deng et al., 2004; Reiner et al., 1988; White et al., 1997). The progressive degeneration of HD characterized by movement, cognitive, and emotional disorder (Ross and Tabrizi, 2011). The expansion of CAG in the exon 1 of the *Htt* gene affects the severity and disease onset, by the presence of more than 40 CAGs causes HD within a normal lifespan, and longer CAG repeats lead to juvenile onset (Roos, 2010). In the brain, neural degeneration occurs begin in striatal medium spiny neurons (MSNs) expressing dopamine- and cAMP-regulated phosphoprotein (DARPP-32) that subsequently expanded throughout the whole brain (Sapp et al., 1997). Although the general cause of HD is known, the pathogenesis and treatment of HD had yet to be discovered (Johnson and Davidson, 2010).

Transgenic animal play an important role in understanding disease pathogenesis and advancing diagnostic tools that may lead prevention, early intervention, and cures of human diseases (Chan, 2009; Pouladi et al., 2013). Transgenic rodent models differences from humans (Chen et al., 2012). In 2008, the first NPH HD model was generated by using lentiviruses expressing exon 1 of the human *Htt* gene with 84 CAG repeats. Transgenic HD NHP developed HD phenotype similar to human (Yang et al.,

2008; Chan et al., 2014). Recently, NHP HD NPCs were reported as the ideal HD model for drugs discovery and the development of novel treatment (Carter et al., 2014).

To ameliorate HD phenotype, researchers have tried different approaches including gene therapy (Kordasiewicz et al., 2012), stem cells (Chen et al., 2014; Mu et al., 2014) and small molecule (Janero, 2014; Kulkarni and Saxena, 2014; Mason and Barker, 2009) in HD models. HD drug discovery studies has been undertaken in *in vitro* platforms such as yeast (Outeiro and Giorgini, 2006), *ex vivo* brain slice (Murphy and Messer, 2004), genetically modified 293/HEK cell (Shao et al., 2008), primary neuronal culture from transgenic rodents (Giampà et al., 2013), transgenic rodent cell line such as PC12 (Wang et al., 2005), transgenic NHP cell line (Carter et al., 2014) and MSNs derived from human iPSCs (The HD iPSC Consortium, 2012). However, an effective drug or small molecule that is able to cure HD still needs to be discovered.

Gene therapy is another attractive approach in HD research. The interaction of transcription factors with HTT and their recruitment to the mHTT aggregates which is likely to compromise the transcription factor function may lead to down regulation of the target gene which are activated by these transcriptions factor (Sugars and Rubinsztein, 2003). Because mHTT protein disrupts many parts of neuronal system such as transcriptomic (Kocerha et al., 2013), miRNA system (Lee et al., 2011), proteomic (Chen et al., 2012), apoptosis (Hackam et al., 2000; Vis et al., 2005), and mitochondrial biogenesis (Milakovic and Johnson, 2005; Song et al., 2011), removal of m*Htt* gene may be the perfect cure. However, *Htt* knockdown resulted in disturbed cell migration, reduced proliferation and increased cell death specifically to early neural development in mice model (Tong et al., 2011). Because of the WT and m*Htt* is

different in the number of CAG repeated, the selective transient repression or knockdown only m*Htt* is required (Kordasiewicz et al., 2012).

MicroRNAs (miRNA) are small noncoding RNA molecules, 19 to 24 nucleotides in the length, that contribute to the regulation of crucial processes, such as cell proliferation, apotosis, development and differentiation (Ambros, 2004). mHTT inhibits the formation of processing body (P bodies) by interacting with Argonaute 1 and 2, two proteins involved in miRNA silencing pathway which is globally inactivate level of miRNA in HD (Savas et al., 2008). Deregulation of miRNA systems were reported in postmortem brain of HD patient (Johnson et al., 2008; Martí et al., 2010). Marti and colleagues identified different expression of miRNAs in frontal cortex and striatum of HD patients. Many of the altered miRNAs were found such as, miR-128, miR-139-3p, miR-222, miR-382, miR-433, and miR-485-3p were reduced, on the other hand miR-100, miR-151-3p, miR-16, miR-219-2-3p, miR-27b, miR-451, and miR-92a were found to increase in all methods (Martí et al., 2010). Post mortem miRNA array analysis of transgenic HD NPH brain shown elevated expression of miR-196a when compared with control brain (unpublished data). Moreover, miR-196a overexpresses can ameliorate clinical forms in animal models of spinal and bulbar muscular atrophy (SBMA) (Miyazaki et al., 2012), human immunodeficiency virus type 1 (HIV-1) associated neurocognitive disorders (Bagashev et al., 2014), and Huntington's disease (Cheng et al., 2013; Hoss et al., 2014). The present studies aim to investigate the potential of NHP-HD NPCs and the differentiated neural cells for drug discovery research and the therapeutic benefit of miR-196a in NHP-HD NPCs model.

1.2 Research objectives

1.2.1 To examine the potential of drugs discovery in NHP-HD NPCs and differentiated neural cells model.

1.2.2 To discover the therapeutic effect of miR-196a overexpression in NHP-HD model NPCs and differentiated neural cells.

1.3 Research hypotheses

1.3.1 NHP-HD NPCs and differentiated neural cells could be used for *in vitro* drug screening platform in HD.

1.3.2 Overexpression of miR-196a in NHP-HD NPCs could ameliorate HD cellular phenotype, cytotoxicity and apoptosis.

1.4 Scope of the study

1.4.1 NHP WT and HD NPCs and differentiated neural cells were used as an *in vitro* model for investigating the potential of NPH NPCs and neurons for HD drugs discovery. Two WT and two HD NPCs and neural cells derived from both NHP ESCs and iPSCs were used in the study. [Rilizole (2-amino-6-trifluoro-methoxy-benzothiazole; RI), Memantine (1-amino-3,5-di-methyl-adamantane; ME), and Methylene blue (MB)] were added into culture media of NPCs and differentiated neurons. Viability and cytotoxicity were analyzed by MTT assay and Glucose 6-phosohate dehydrogenase (G6PD) cytotoxicity assay. Moreover, drugs that treated neurons were preformed apoptosis assay by measuring percentage of cleaved caspase-3 positive cell in Map2 positive cells. Furthermore, the reductions of mHTT protein aggregation in treated neural cell were determined by western blot.

1.4.2 NHP WT and HD NPCs overexpressed miR-196a were generated by using lentiviral vector followed by zeocin antibiotic selection. The levels of miR-196a and mHtt gene expression were determined in control and miR-196a overexpressed NPCs and differentiated neural cells. Moreover, NPCs and neural cell properties were confirmed by using ICC and expression of HD related genes were determined by qPCR. Furthermore, cell viability was determined by MTT assay, G6PD cytotoxicity assay and cleaved caspase-3 apoptosis assay. Mitochondrial morphology and properties were evaluated by using mitotracker staining and qPCR analysis of mitochondrial related genes.

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CHAPTER II

LITERATURE REVIEW

2.1 General introduction to Huntington's disease

Huntington's disease (HD) is a progressive neurodegenerative disorder first described in 1872 by an American physician named George Huntington. HD is an inherited disorder with age-dependent, adult- and juvenile onset. The classical symptoms of HD are progressive involuntary movements (leading to jerky movements), cognitive decline and psychiatric impairment. Additional signs include progressive weight loss, personality changes, alteration of wake-sleep cycle, and mental problems (such as showing a difficulty in reasoning and judgment) (Zuccato et al., 2010). Symptoms typically start at a mean age of 45 years (Bates et al., 2015) and the survival time is approximately 15-20 years after disease onset (Cattaneo et al., 2005). In juvenile HD, symptoms may begin before 30 years of age (Roze et al., 2011). The prevalence is estimated to be 5 per 100,000 persons worldwide (Kumar et al., 2015). According to the current diagnostic criteria, an HD patient is a person who carries a CAG-expanded allele (discussed in topic 2.2) of the HTT gene or has a family history of HD and develops movement disorders (Reilmann et al., 2014). The course of HD is divided into three phases: premanifest, prodromal and manifest (Fig 2.1). Premanifest phase, also called presymptomatic period, is a period which individuals show no subjective symptoms. Prodromal period refers to a period of increasing motor, and/or cognitive impairment, while the Manifest phase is a period that individuals show clinical motor and/or cognitive signs and symptoms that have resulted in

quality of life restrictions. A CAG-Age Product (CAP) score is a parameter that is used to normalize HD clinical data, therefore enabling the study of HD progression (Penney et al., 1997). This score is calculated based on the number of CAG repeats, the age at death, and the severity of neuropathology.

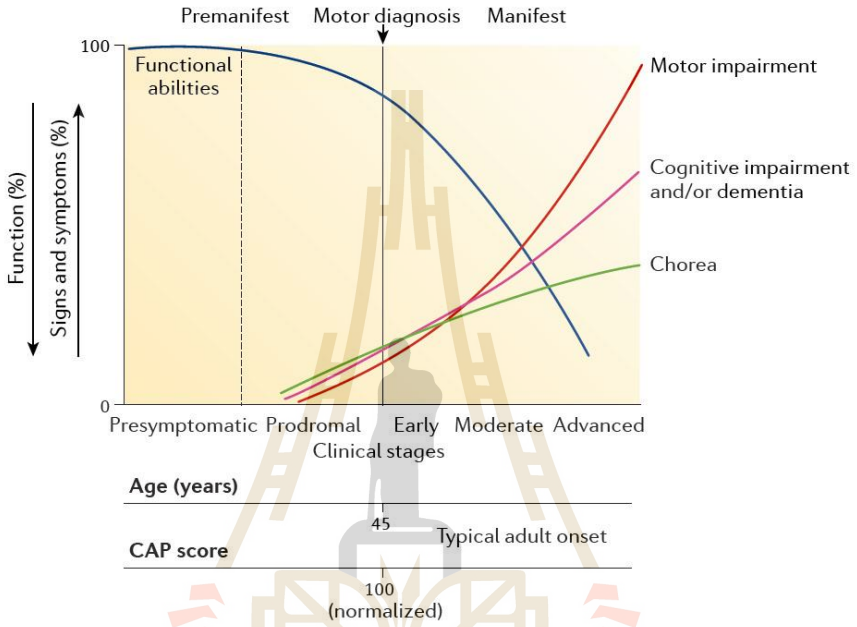

Figure 2.1. Time course of clinical HD. Mean disease onset is approximately at 100 CAP score; typically around 45 years old. Premanifest period is the period before diagnosable signs are present, also termed presymptomatic period. Prodromal period is when motor, cognitive and behavioral change gradually appears. The manifest period can be further divided into early, moderate, and advanced stages. Early stage: patients who are still active and able to take care themselves. Moderate stage: affected individuals who become unable to perform complex functions, such as driving, but still able to take care of their daily activity. Advanced or late stage: patients who are unable to take care of themselves and potentially require long-term institutional care (Bates et al., 2015).

Histological study in post-mortem brain of HD patients shows a different degree of neuronal atrophy specifically in corpus striatum (caudate nucleus; CN, putamen and globus pallidus). Based on macroscopic and microscopic change in the striatum, severity of HD is classified into five grades. The detailed classification (summarized in table 2.1) is well established by the neuropathologist, Jean Paul Vonsattel, in 1985. In brief, grade 0 refers to individuals who have clinical evidences of HD, but no detectable pathological change (both macroscopic and microscopic level) in the brain. Grade 1 refers to HD cases, where the pathological changes, 50% neuronal loss and moderate fibrillary astrocytosis in the tail of the CN, are observed only at the microscopic levels (Fig 2.2A). The CN atrophy is observed at the macroscopic level in grade 2 patients. In this grade, the convex outline of the ventricular surface of CN is still retained (Fig 2.2B). Mild neuronal loss and fibrillary astrocytosis are mainly found in the medial half of the CN head and dorsal portion of the putamen. In grade 3, the CN is moderately to severely damage. In this grade, CN is shrunk, and the medial outline of the CN head is thereby forming a straight-line border (Fig 2.2C). In grade 4, the CN is extremely shrunk and the medial outline of the CN head is medially concave (Fig 2.2D). Neuronal losses are up to 95% of total CN and fibrillary astrocytosis are scattered throughout the CN. Pathological changes have also been reported in another brain area: hypothalamus and cerebral cortex (Kassubek et al., 2004). Collectively, this massive neurodegeneration contribute greatly to HD clinical complexity.

Table 2.1 Detailed classification of five grades (0 to 4) of neuropathological severity of HD, modified from Vonsattel et al., 1985.

	Pathological changes														
	Atrophy					Neuronal depletion					Fibrillary astrocytosis				
Grade	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**
Medial CN[a]	-	-	1	2-3	4	-	1-2	2	3	4	-	1-2	2-3	3	4
Lateral CN[a]	-	-	1	2-3	4	-	0-1	1	2	4	-	0-1	1-2	2	4
Putamen[b]	-	-	1	2	3	-	-	1-2	2-3	4	-	0-1	1-2	2-3	4
Medial globus pallidus[c]	-	-	0	1	1	-	-	0	0-1	0-1	-	-	0	0-1	0-1
Lateral globus pallidus[c]	-	-	0	1	1	-	-	0	1	1	-	-	1-2	1-2	1-2

Atrophy is determined based on gross examination.
-, 0 = within normal limit, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe.

a CAP (caudate nucleus, nucleus accumbens septi, and putamen) level sample. CAP level sample were taken at the caudoputaminal jucntion, **b** CAP and GP (globus pallidus) level sample. GP level include claustrum, CN, globus pallidus, paraventricular and supraoptic nuclei with nucleus basalis. **c** GP level sample.

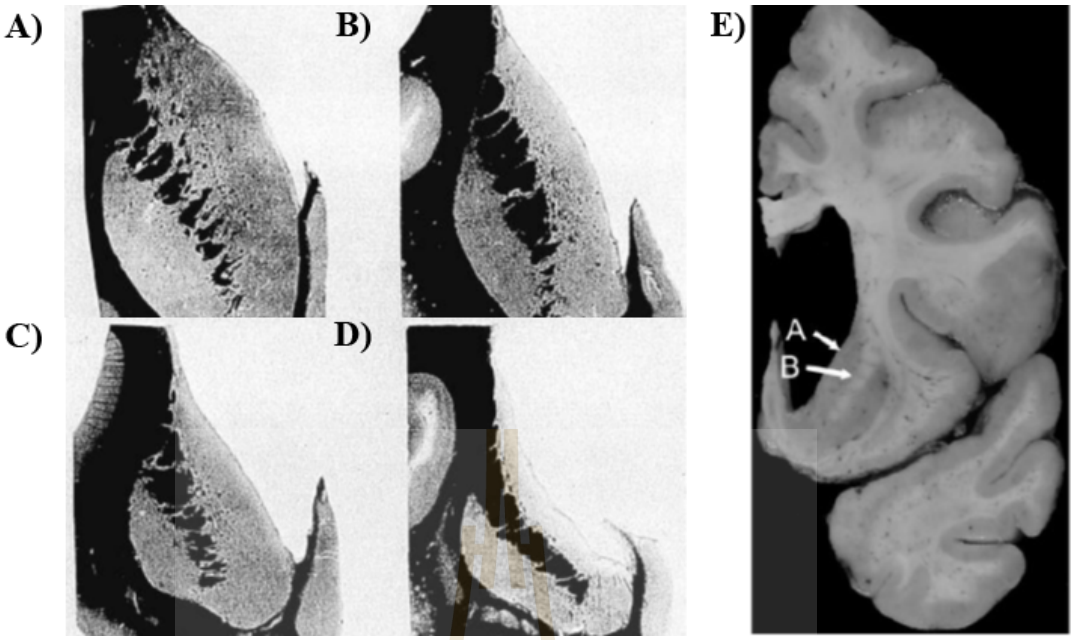

Figure 2.2. Grading criteria used to classify HD severity. A-D) Coronal section of CN showing different degrees of striatal atrophy in HD brain (Vonsattel et al., 1985). Grade 0 and 1; no macroscopic change observed in the striatum. B) Grade 2; mild CN atrophy with convex medial outline of the ventricular surface. C) Grade 3; moderate to severe CN atrophy with flattened (nearly straight) medial outline of the ventricular surface. D) Grade 4; severe CN and putamen atrophy with concave medial outline of the ventricular surface. E) Right cerebral hemisphere of grade 4 HD brain (Hadzi et al., 2012). As shown by cortical thining and medially concaveness of the medial outline of the CN head, both striatal and cortex are dramatically involved in HD pathogenesis. Arrows indicated the medial outline of the CN head and its internal capsule, light A and light B respectively.

2.2 Genetic and molecular biology of HD

HD is an autosomal dominant monogenic disorder caused by mutation within the coding region of a gene located on chromosome 4p16.3, *IT-15* (Macdonald et al., 1993). Upon proof that this mutation is responsible for HD, *IT-15* is then renamed *huntingtin* (*Htt*). HD is a group of trinucleotide $(CAG)_n$ repeat disorders. Individuals with 10-26 and 27-35 CAG repeats in the *Htt* are considered as normal and intermediate HD, respectively. Individuals who have more than 39 CAG repeats in the *Htt* will develop HD (Kumar et al., 2015). Moreover, the number of CAG repeat is highly associated with HD pathogenesis. Patients carrying longer CAG tracts develop early disease onset with more aggressive development than patient carrying shorter CAG tracts (Andrew et al., 1993). In juvenile HD, the number of CAG repeats are more than 60 (Landles and Bates, 2004).

HTT is a 348-kDa soluble protein encoded by *Htt* gene. HTT is a multidomain protein, some of which have been identified, which include polyglutamine (polyQ), HEAT repeats, caspase cleavage site, a functionally active COOH-terminal nuclear transport signal (NES), a less active nuclear localization signal (NLS), and post-translation modification sites (Fig 2.3A) (Cattaneo et al., 2005). It has been shown that polyQ stretch region exerts HTT physiological functions via the formation of a polar zipper structure that participates in the binding of HTT to its partners, such as transcription factors that contain a polyQ region (Li et al., 2003). In HD, the translation of expanded CAG repeats result in a long stretches of polyQ tracts at the N-terminal region of HTT. It was suggested that this pathogenic polyQ expansion induces conformation changes of HTT by increasing the length of the random coil that promote protein dysregulation and intracellular accumulation of mutant huntingtin

(mHTT) (Kim et al. 2009). Figure 2.3B illustrates domain flexibility effect of different polyglutamic lengths on the secondary structure of HTT using Förster resonance energy transfer (FRET) technique (Caron et al., 2013). HTT also contains a well-characterized protease cleavage site. Caspases are cysteine protease that play multiple roles, both apoptotic and non-apoptotic, in the nervous system. Several caspases (including caspase 2, 3, 6 and 7) and calpain are involved in HTT cleavage (Fryer and Zoghbi, 2006; Hermel et al., 2004). At present, the pathological relevance of mHTT fragment is, however, not completely understood.

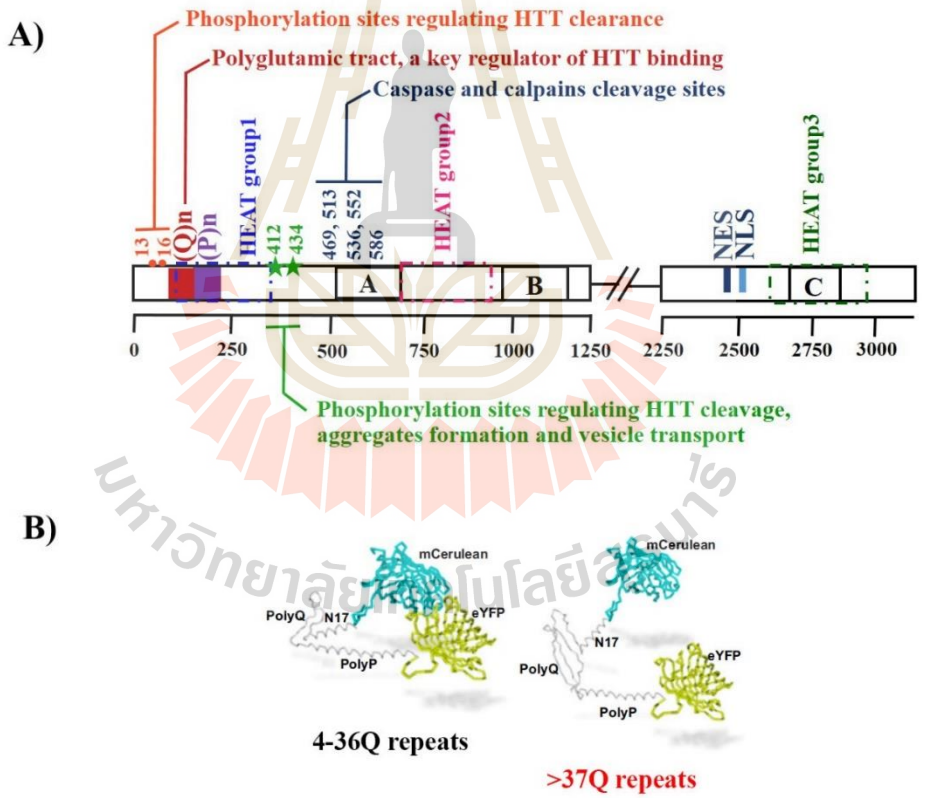

Figure 2.3. Schematic diagram and 3D conformation model of HTT. A) HTT structure with its known functional domains (modified from Zuccato et al., 2010). Polyglutamine tract, (Q)n, in human HTT begins at the 18th

amino acid and follows by the polyproline sequence, (P)n. Downstream of the polyQ, there are three main clusters of HEAT repeats: HEAT group 1, 2, 3. The caspase and calpain cleavage sites are located at the amino acid position 513, 552, 586 and 469, 536, respectively. A box is the region that cleaved in both cerebral cortex and striatum. B and C boxes are the regions that cleaved mainly in cerebral cortex and striatum, respectively. B) Models of HTT with wild-type (left) and mutant (right) polyglutamine lengths (modified from Caron et al., 2013). As a flexible domain, the polyglutamine tract allows the flanking domains (N17; the first 17 amino acids, and polyproline region) to come into close proximity.

Various approaches have been used to determine the physiological function of HTT. HTT is present throughout the body with the highest levels in the largest neurons in the brain: cortical pyramidal cells, pallidal neurons, and Purkinje cells (Gutekunst et al., 1995; Trottier et al., 1995). Insights in HTT functions are mostly derived from animal model studies. Several studies indicated that HTT plays vital roles include neurogenesis and neuroprotection throughout life. From murine study, *Htt* ablation ($Htt^{-/-}$) and *Htt* reduction ($Htt^{-/+}$) results in embryonic lethality at day 8.5 and significant reductions of subthamic nucleus neurons in the survived offsprings, respectively (Nasir et al., 1995). In zebrafish, morpholino-induced inhibition of HTT production result in developmental defects of the anterior-most region of the neural plate; the potential origins that give rise to telencephalon and pre-placodal tissue (Henshall et al., 2009). In addition to its roles in CNS formation, HTT also has anti-

apoptotic property. HTT prevents cell from dying by triggering molecular events that promoted cell survival such as inhibits pro-caspase9 processing (Rigamonti et al., 2001), and inhibits the formation of the pro-apoptotic HIPPI-HIP1 complex (Gervais et al., 2002), etc. Furthermore, HTT induces the production of BDNF, a neurotrophic factor that is involved in neurons protection. Collectively, these studies indicated that HTT is vital to maintaining a functional nervous system and mutation in *Htt* results in complex pathological events that affected the whole brain and body.

2.3 Mechanisms underlying HD pathogenesis

Compelling data from studies in human HD patients and experimental HD models show that mHTT elicit its neurodegeneration through distinct mechanisms including glutamate excitotoxicity, protein misfolding, proteolytic cleavage of mHTT, transcriptional dysregulation, mitochondrial dysfunction, and alteration of vesicle trafficking, etc.

To narrow the scope of this thesis, the main focus will be on the mechanisms possess a powerful therapeutic approach (discussed further in topic 2.5-2.7) in HD which include glutamate excitotoxicity, mitochondrial dysfunction, and gene dysregulation.

2.3.1 Protein misfolding and mHTT aggregation.

Correct structural conformation of protein is important for maintaining proper cell functions. The intracellular aggregation of HTT (Fig 2.4A and 2.4B) serve as pathological evidences leading to the hypothesis that protein misfolding is one of the mechanisms underlying HD pathogenesis (Hatters, 2008). Until now, the mechanisms by which the polyQ expansions facilitate aggregate formation are still

under debate. Recently, study supported that the first 17-amino-acid sequence of the HTT exon 1 (HTT^{NT}) enhances aggregation propensity when flanking with expanded polyQ sequence (Fig 2.4C) (Thakur et al., 2009). The same study also showed that isolated HTT^{NT} peptide itself has aggregation-resistant tendency.

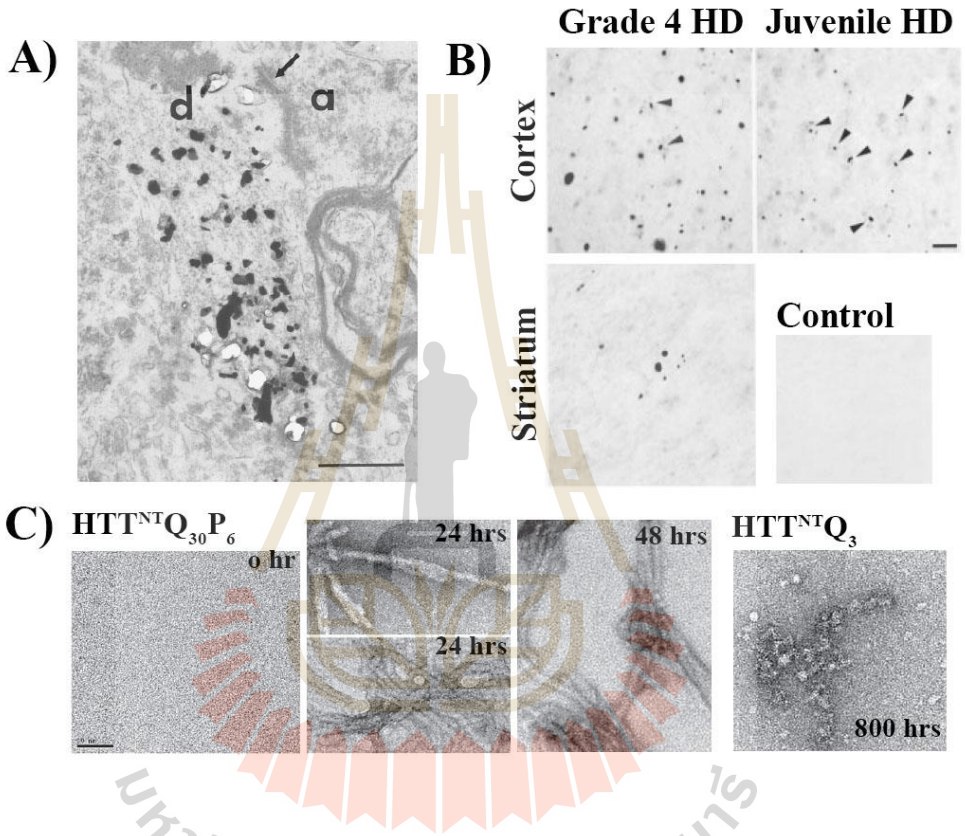

Figure 2.4. Aggregation of HTT. A and B) HTT immunoreactivity in postmortem brains from patients with HD, modified pictures from Gutekunst et al., 1999 . A) Electron micrograph of immunogold-labeled native HTT in insular cortex from an adult HD brain grade1. Labelled aggregates located in parallel with the orientation of two dendrites. Arrow, (d) and (a) indicate synaptic contact, dendrite, and an axon terminal, respectively. Scale bar, 500 nM. B) Different densities of HTT aggregates (arrowheads) are present in cortex and striatum region of grade 4 and

juvenile HD brain. In control striatum, aggregates were absent. Scale bar, 50 μm. C) Electron micrographs of HTT^{NT}-related aggregates, modified pictures from Thakur et al., 2009. Within 24 hours, *in vitro* incubation of chemically synthesized $HTT^{NT}Q_{30}P_6$ peptides formed oligomeric and protofiber aggregated structures. These structures appeared to be more fibril later on in the time course of experiments. In contrast, there were no detectable aggregation formed after 800 hours of $HTT^{NT}Q_3$; HTT^{NT} peptide containing less sequence of polyQ, incubation.

Polyglutamine inclusions display amyloidogenic-like features that consist of β-sheet-rich fibrils resembling those found in Alzheimer's disease and exhibit amyloid features such as low solubility (Zuccato et al., 2010). There are evidences demonstrated that protein clearance mechanisms are also disrupted by mHTT aggregates. The folding process and clearance of misfolded polypeptides in cells happened by the sequential actions of Heat-shock proteins (HSPs) and ubiquitin-proteosome system (UPS) (Mcclellan et al., 2005). In HTT exon 1 inducible cell lines, ubiquitinated mHTT were colocalized with Hsp70, Hsp40, and 26S proteasome subunits (Waelter et al., 2001). These findings suggest that mHTT aggregates is problematic for UPS activity and the misfolding of mHTT contributes greatly to HD pathogenesis.

2.3.2 Glutamate excitotoxicity

One of the first pathogenic signatures of HD was the preferential vulnerability of striatal projections neuron. In affected striatum, medium spiny GABAergic neurons (MSN), but not interneurons, are selectively degenerated. It has

long been postulated that overactivation of N-methyl-D-aspartate receptor (NMDARs; a specific type of ionotropic glutamate receptors) and the differential expression of glutamate receptor subtypes between striatal projection neuron and interneurons are the underlying mechanisms of this phenomenon (Ferrante et al., 1985).

Excitotoxicity is a mechanism of neuronal death driven by excessive activation of glutamate receptors leading to a numbers of deleterious consequences, including dysregulation of intraneuronal Ca^{2+} homeostasis, generation of nitric oxide (NO) and other reactive oxygen species (ROS), and alteration of mitochondrial permeability (Dong et al., 2009). Before the identification of the causative gene, striatal degeneration patterns similar to those observed in HD patients were successfully replicated by intrastriatal injection of glutamate or some of its analogs, quinolinic acid and kainic acid, into rodent models (Mcgeer and Mcgeer, 1976; Schwarcz et al., 1984). Findings from these chemical induced models suggested that glutamate excitotoxicity is one of the mechanisms underlying HD.

An insight into NMDARs signaling and its roles in pathophysiological scenarios have been extensively reviewed (Hardingham and Bading, 2010). In brief, NMDARs are glutamate-gated ion channels that favor Ca^{2+} ions. Signaling through NMDARs can contribute to both neuroprotection and pro-death responses depending on receptor location, synaptic versus extrasynaptic site (Fig 2.5).

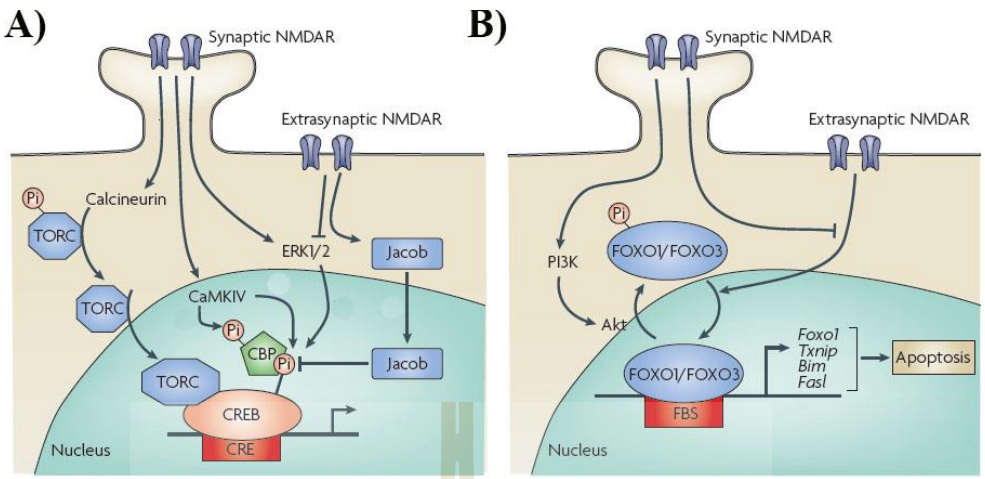

Figure 2.5. Opposing effects of synaptic and extrasynaptic NMDARs signaling, modified pictures from Hardingham and Bading, 2010. A). Synaptic NMDARs promote neuronal survival via phosphorylation of CREB signaling. Activation of CaM kinase IV accelerates phosphorylation of CREB and its coactivator CREB binding protein (CBP). Stimulation of synaptic NMDARs leading to CREB phosphorylation by promoting sustained Ras-ERK1/2 activity. B) Sustained activation of Akt-mediated prosphorylation and nuclear export of FOXO3a caused by synaptic NMDARs stimulation, contribute to the neuroprotective effects by inactivates pro-death FOXO pathway. Pro-death signaling, in contrast, is promoted through a direct antagonism of synaptic NMDARs-activated survival pathways.

Stimulation of synaptic NMDARs promote neuroprotection via three distinct mechanisms; 1) nuclear Ca^{2+} signaling mediates induction of survival genes, such as *calcium/calmodulin-dependent protein kinase IV* (*CaMKIV*), cyclic-AMP response element binding protein (CREB)-dependent gene and *brain-derived neurotrophic*

factor (*Bdnf*), 2) suppression of apoptosis gene, such as *pro-apoptotic Bcl2 homology domain 3 (BH3)-only member gene* (*Puma*), *caspase 9*, *forkhead box protein O* (*FOXO*), and *p53*, 3) enhances antioxidant defenses via modulating thioredoxin (TRX)–peroxiredoxin (PRX) system. Pro-death signaling, in contrast, is promoted through extrasynaptic NMDARs suppressed CREB activity and FOXO activation. Extrasynaptic NMDARs exert CREB shut-off signal via CREB dephosphorylating pathway: 1) inactivation of Ras-ERK1/2 signaling and 2) nuclear translocation of juxtasynaptic attractor of caldendrin on dendritic boutons protein (Jacob). Stimulation of extrasynaptic NMDARs promote cytosol to nuclear translocation of FOXO3a, causing transcriptional induction of cell death-promoting genes such as Bcl-2 interacting mediator of cell death (Bim) and tumor necrosis factor related apoptosis-inducing ligand (TRAIL), subsequently contributes to NMDAR-dependent neuronal death (Dick and Bading, 2010).

Hyperactivation of extrasynaptic NMDARs signaling and alteration of glutamate clearance may be a key factor in excitotoxic-mediated HD pathogenesis. In HD yeast artificial chromosome (YAC) mice expressing normal (18) or pathological (128) CAG repeats in HTT exhibited different NMDARs activity on MSNs. The striatum from YAC128 mice showed increase extrasynaptic NMDARs expression but no effect on synaptic NMDARs activity (Milnerwood et al., 2010). This enhanced extrasynaptic NMDARs activity was associated with reductions in nuclear CREB activation and blockage of extrasynaptic NMDARs with memantine reactivated CREB signaling and reversed motor learning in YAC128 mice. In addition, alteration of extrasynaptic NMDARs activity was found to support the expression of Rhes, a small guanine nucleotide-binding protein that is enriched in striatum. In HEK293 HD model,

Rhes binds to mHTT and overexpression of Rhes enhances mHTT sumoylation (Subramaniam et al., 2009). Modification with SUMOylation has been reported to promote mHTT toxicity by increasing the amount of toxic oligomer of fragmented mHTT and enhancing transcriptional repression (Steffan et al., 2004). Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) is a member of a transcriptional co-activators family encoded by *peroxisome proliferator-activated receptor gamma coactivator 1a* (*PPARGC1A or PPARγ coactivator 1a*) gene (Taherzadeh-Fard et al., 2011). In physiological condition, PGC-1α regulates expression of mitochondrial respiratory genes including *nuclear respiratory factor 1* (*NRF-1*) and *nuclear respiratory factor 2* (*NRF-2*) (Scarpulla, 2006). In HD patients and in several HD models, PGC-1α is a key neuroprotective CREB target that has been reported to downregulate the expression (Mcgill and Beal, 2006). Reduction of PGC-1α expression, therefore, leads to impaired energy metabolism caused by disruption of mitochondrial function (Cui et al., 2006). Taken together, mHTT promotes neurotoxicity by disrupting the balance between synaptic and extrasynaptic NMDARs activity that trigger pro-death cascades through the upregulation extrasynaptic NMDARs.

2.3.3 Mitochondrial dysfunction

Having intense energy demands, neurons are highly dependent on mitochondrial adenosine triphosphate (ATP) production and are very sensitive to disturbance of energy metabolism (Duan et al., 2014). Increase evidence suggests that mHTT could lead to abnormal cellular metabolism in HD patients. Mitochondrial dysfunctions in HD can be the result of mitochondrial Ca^{2+} overload caused by NMDARs hyperactivation, inhibition of mitochondrial complex II, excessive

mitochondrial fission and abnormal mitochondrial trafficking, oxidative stress induced mitochondrial damage, and downregulation of key factor (e.g. PGC-1α) controlling mitochondrial biogenesis, etc (Duan et al., 2014). Deficiency in mitochondrial complex II/succinate dehydrogenase (SDH) subunit has been reported in the striatum of HD patients (Browne et al., 1997). Complex II/ SDH is composed of four nuclearly encoded subunits that play an important role in energy production and cellular respiration. SDH catalyses the oxidation of succinate into fumarate in Tricarboxylic acid cycle (TCA cycle) and fuels ATP synthesis by generating proton motive force across the mitochondrial inner membrane (Rustin et al., 2002). In partnership with ubiquinone, SDH controls superoxide production and protects mitochondrial inner membrane from oxidative damage by its specific redox properties. Loss of complex II/SDH subunits results in functional alteration of mitochondrial membrane potential and respiratory chain impairment in striatal neurons expressing mHTT (Benchoua et al., 2006). Fission-fusion are essential in the proliferation and distribution of mitochondria and are required for maintaining proper mitochondrial functions by allowing the exchange of damaged mitochondrial genome DNA and its metabolite with healthy mitochondria (Knott et al., 2008). Mitochondrial fission and fusion process are mediated by large guanosine triphosphatases (GTPases) of the dynamin family. Dynamin-related protein 1 (DRP1) plays a role in mitochondrial fission while mitofusin proteins (MFN1 and MFN2) and optical atrophy 1 (OPA1) regulate mitochondrial fusion (Johri et al., 2011). In HD, it has been shown that mHTT abnormally binds to DRP1 and increase DRP1-GTPase activity, leading to excessive mitochondrial fission and neuronal death (Fig 2.6A) (Guo et al., 2013). Moreover, the same study also showed that the expression level of DRP1 is proportionally associated

with mitochondrial accumulation of p53 and the DRP1/p53 complex initiate mitochondrial damage and neuronal death in HD subsequently. Of note, p53 signaling is composed of a network of genes that is linked to mitochondrial depolarization and stress signaling. p53 initiates the upregulation of pro-apoptotic downstream targets, *Bax* and *Puma* (Bae et al., 2005).

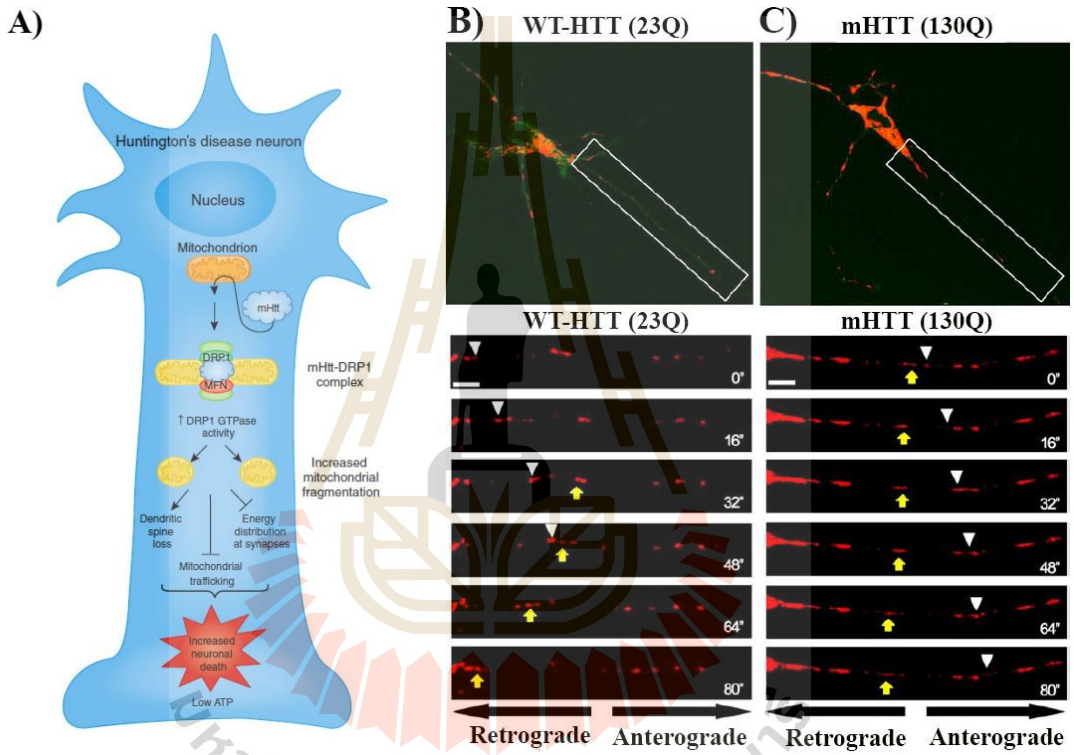

Figure 2.6. mHTT and mitochondrial dysfunction. A) Mitochondrial fission-fusion imbalance caused by mHTT, pictures from Johri et al., 2011. mHTT directly bind to DRP1 to promote DRP1-GTPase activity. Excessive mitochondrial fission triggers mitochondrial fragmentation. This results in defective transport of mitochondria to synapses, synaptic spine loss and consequent neuronal death. Effects of mHTT on mitochondrial transportation rate, modified pictures from Orr et al., 2008. B) and C) are

representative images of striatal neurons transfected with GFP-wild-type HTT (23 polyQ repeats) and GFP-mHTT (130 polyQ repeats), respectively. Below are the time-series pictures of the region high-lighted in white framed rectangles. Both anterograde (marked by white triangles) and retrograde (yellow arrow) moving mitochondria tended to travel shorter distances in neurons expressing GFP-mHTT compared with those neurons expressing GFP-wild-type HTT. Scale bar, 5 μM.

In addition, blockage of mitochondrial trafficking may serve as supporting evidence that mHTT causes mitochondrial dysfunction. Proteolytic cleavage of mHTT gives rise to N-terminal polyQ-containing fragments that binds directly to mitochondria and that block mitochondrial transport in cultured striatal neurons (Orr et al., 2008). Figure 2.6B and 2.6C are representative time-lapse confocal images of mitochondria in striatal neurons overexpressed wild-type (23) or pathological (130) polyQ repeats in HTT fused to GFP. In 130Q-transfected neurons, the distance of mitochondria movement, both anterograde (marked by white triangles) and retrograde (yellow arrow) direction, are less than those observed in wild-type (23Q) neurons. Moreover, the same study also reported that the synaptosomal fraction isolated from Q150 knock-in mouse brain are reduced in ATP level. Collectively, these findings demonstrate that mHTT disrupts mitochondrial bioenergetics through various mechanisms.

2.3.4 Transcriptional dysregulation

Microarray studies suggested transcriptional dysregulation is an early event in neuropathological process of HD (Zuccato et al., 2010). HTT interacting partners include specificity protein 1 (Sp1), TAFII130 (Dunah et al., 2002), p53 and CREB signaling

(Steffan et al., 2000), RE1-Silencing Transcription Factor (REST) (Johnson and Buckley, 2009), and nuclear receptor co-repressor (NCoR) (Boutell et al., 1999).

Initiation of transcription by RNA polymerase II machinery requires the assembly of transcription factors to form a preinitiation complex at the promoter region of the target gene. Sp1 is a transcriptional activator that binds to promoters with GC-rich sequence and is required for the recruitment of transcription factor II D (TFIID; a multisubunit complex consists of TATA-binding protein (TBP) and TBP-associated factors (TAFs)) (Dunah et al., 2002). HTT interacts directly with Sp1 and coactivator TAFII 130 in human CN lysate of grade 1 HD brain (Dunah et al., 2002). In the same studies, the reversion of transcriptional inhibition of the *dopamine D2 receptor (D2R) gene* in cultured striatal cells derived from HD mice were achieved by co-expression of Sp1 and TAFII130. Additionally, mHTT inhibits the interaction between nuclear Sp1 and nerve growth factor receptor (NGFR) promoter region by tightly bind to the C-terminal region of Sp1 (Li et al., 2002). mHTT, therefore, suppresses the expression of Sp1-related genes (Fig 2.7).

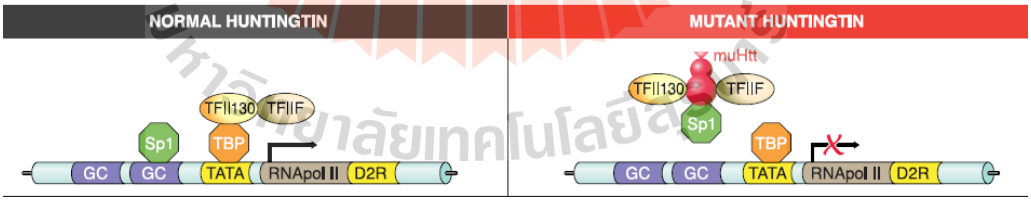

Figure 2.7. Critical pathway implicated in HD pathogenesis, modified pictures from Zuccato et al., 2010. In HD, mHTT interacts with transcriptional activator and basal transcription machinery: Sp1, TAFII130, and other TAFs. These lead to the reduction of *D2R* expression, since the binding between Sp1 and D2R promoter is required for the initiation of the transcription processes.

One of the key regulators of striatal degeneration in HD is CREB/cAMP response element (CRE) transcriptional pathway (Choi et al., 2009). In response to an array of stimuli, several types of protein kinase, such as protein kinase A (PKA), mitogen-activated protein kinase (MAPKs), and CaMKIV, trigger CREB-mediated transcriptional cascade via phophorylate CREB at serine 133 (Shaywitz and Greenberg, 1999). In the nervous system, more than 100 of putative CREB-dependent genes (table 2.2) have been identified (Lonze and Ginty, 2002). In HD, a shift from synaptic to extrasynaptic NMDARs signaling results in dephosphorylation of CREB that lead to transcriptomic dysregulation of CREB-dependent genes. For instance, mHTT represses PGC-1α expression by occupying the PGC-1α promoter and interfering with CREB/ TAF4 signaling pathway (Cui et al., 2006). As a consequence, downstream events of PGC-1α are likely impaired in HD. These include mitochondrial biogenesis (*NRFs*), glucose utilization (*estrogen-related receptors; ERRs*), fatty acid oxidation (*PPARα/δ*), and antioxidants (*PPARβ*) (Ventura-Clapier et al., 2008).

In vertebrate, REST serves as a transcriptional repressor that orchestrates neuronal gene expression (Ballas et al., 2005). It has been shown that mHTT inhibits the transcription of BDNF by promoting nuclear translocation of REST (Zuccato et al., 2003). Downregulation of BDNF, therefore, worsens HD pathology (Strand et al., 2007).

Table 2.2 An abbreviated list of CREB target genes, modified table from Lonze and Ginty, 2002; Mayr and Montminy, 2001

Function	Gene symbol	Gene name
Neuropeptides/ Neurotransmitters		
	DβH	Dopamine β-hydroxylase
	Syn1	Synapsin I
	Ache	Acetylcholinesterase
Neurotrophic factors/ Growth factors		
	BDNF	Brain derived neurotrophic factor
	FGF-6	Fibroblast growth factor-6
	IGF-I	Insulin-like growth factor I
	TGF-β2	Transforming growth factor- β2
Structural		
	CDH1	E-cadherin
	FN1	Fibronectin
	ICAM-1	Intracellular adhesion molecule 1
Channels/ Transporters		
	GLUT2	Glucose transporter 2
	ATP1α1	Na^+/K^+-ATPaseα
Cellular metabolism/ Transcription		
	Bcl-2	B-cell lymphoma 2
	GS	Glutamine synthetase
	PPARGC1A or PPARγ coactivator 1a	Peroxisome proliferator-activated receptor-γ coactivator 1a
Signal Transduction		
	iNOS	Inducible nitric oxide synthase
	nNOS	Neuronal nitric oxide synthase

2.4 The disease modeling of HD

To study disease pathogenesis and develop novel therapeutics, nonhuman disease models that replicate human disease are created for biomedical research (Chan, 2009). Disease models play an important role in the understanding of disease pathogenesis and advancing diagnosis tools that may lead to the development of early intervention and cure of human disease (Chan, 2009; Pouladi et al., 2013). HD was discovered in 1872, but effective treatment has not been developed. Close to 10,000

publications have been reported on HD and one of the major scopes of these articles is to create HD models.

Yeast and mammalian cell line model have been used to understand the cellular dysfunction in HD (Bard et al., 2014). Yeast models have the advantage of being less sensitive to polyQ mediated toxicity, while at the same time having a complete genetic analysis to rule out the participation of other cellular factor in the aggregation process (Krobitsch and Lindquist, 2000). Transgenic N-terminal fragment of HTT with multiple polyQ repeat and fused with GFP protein yeast model are suitable for proteomic studies (Krobitsch and Lindquist, 2000) and screening the HTT protein aggregate reducing compounds (Outeiro and Giorgini, 2006; Sontag et al., 2012). Although HD mammalian stable cell lines models, such as HEK 293 FT or PC12, overexpressing *mHtt* gene (Cheng et al., 2013; Shao et al., 2008; Wang et al., 2005) shows homogenous pathology and cytotoxicity effect of HD, they could not demonstrate the neuronal environment and neural biological impact of HD (Bard et al., 2014). Alternatively, primary neural cell derived from transgenic HD animal model (Lee et al., 2006; Roos et al., 1993; Snyder et al., 2011; Sontag et al., 2012; Wu et al., 2006) or HD patient (Kaye and Finkbeiner, 2013; The HD iPSC Consortium, 2012) could provide an insight into neuropathological development and demonstrate neuronal synaptic pathology (Carter and Chan, 2012; Carter et al., 2014).

Insight into the pathogenesis of HD were derived from various models, such as worms (*Caenorhabditis elegans*), fruitflies (*Drosophila melangaster*), mice, rats, sheep, pigs and monkeys (Pouladi et al., 2013). For instant, intrastriatal injection of glutamate receptor agonist has resulted in selective loss of the GABAergic projection neurons. The first neurotoxin models used ibotenic acid (Guldin and Markowitsch,

1981) and kainic acid (Braszko et al., 1981). In addition, mitochondrial toxin such as 3-Nitropropionic acid (3NP) was used to generate lesion models (Lee et al., 2006). A number of genetic approaches have been used to generate animal model of HD. Knock in *mHtt* fragmentation in *C. elegans* (Parker et al., 2001) and *D. melanagaster* (Jackson et al., 1998) model enhanced modeling of mechanosensory defect, neuronal dysfunction and neurodegeneration. Transgenic rodent models have been widely used in biomedical researches. R6/1, R6/2, and N171-Q82 mice model of HD express truncated N-terminal fragment of mHTT, which typically exhibit a rapid onset of symptoms, including motor, cognitive and behavioral abnormalities, weight loss and reduction in lifespan (Ferrante, 2009; Menalled and Chesselet, 2002). Full length *mHtt* expression such as YAC 128 and BAC HD mice demonstrate behavioral and neurodegenerative phenotype but more aggressive progression was observed in transgenic animal models expressing human *mHtt* than in the knock in lines, even with the exact bases of difference (Ferrante, 2009; Menalled and Chesselet, 2002; Pouladi et al., 2013). However, there are limitations in rodent models systems such as life span, metabolism, genetic constitution and physiological differences from human (Chen et al., 2012). Transgenic HD sheep model has been made by microinjection of full length human *Htt* cDNA containing 73 CAG repeats under the control of human *Htt* promoter. HD sheep exhibit reduce expression of DARPP32 which is consistent with HD patients, even no obvious clinical (Reid et al., 2013). Lentiviral with N-terminal fragment of mHTT 124 repeat using human *Htt* promoter was microinjected into performed in Tibetan miniature pigs embryo. However, no aggregation formation in brain tissue is detected up to 16 months of age, and no developmental or gross motor abnormalities are observed. The 16 months old showed reduced intensity of

DARPP32 immunoreactivity in neostriatal TgHD neuron compared to WT control (Baxa et al., 2013).

In 2008, the first NPH HD model was reported by using lentiviruses expressing exon 1 of the human *mHtt* gene with 84 CAG repeats (Yang et al., 2008). Transgenic HD NHP showed HD phenotypes similar to humans (Yang et al., 2008). Transgenic monkey HD modeling were in dental pulp derived mesenchymal stem cell (DPSCs) (Snyder et al., 2011), induced pluripotent stem cells (iPSCs) (Chan et al., 2010), embryonic stem cell (ESC) derived from *in vitro* fertilization using HD NHP sperm (Putkhao et al., 2013) and neural progenitor cells (NPCs) (Carter et al., 2014). Moreover, NHP HD NPCs were reported as the ideal HD model *in vitro* for drugs discovery and phenotype reversal by *mHtt* knock out (Carter et al., 2014).

2.5 HD and drugs discovery

Currently, there is no treatment that can stop, postpone, or reverse the progression of HD (Kaplan and Stockwell, 2012). Scientists are trying many methods to ameliorate HD by using, drug and small molecule (Fecke et al., 2009; Huang et al., 2011), gene therapy (An et al., 2012; Cheng et al., 2013; Kordasiewicz et al., 2012), and stem cells (Eckmann et al., 2014). However, gene and stem cell therapy will not be possible for the global scale HD patient. In 2008, tetrabenazine, which decreases the uptake of dopamine by inhibiting vesicular monoamine transporter 2 (VMAT2), was approved by U.S. FDA to control the involuntary sporadic movement of the extremities and face associated with chorea (Hayden et al., 2009; Wang et al., 2010). Furthermore, antipsychotic medications, such as haloperidol, clozapine, chlorpromizine, and olanzapine, which function by decreasing dopamine levels, are also sometimes

prescribed to reduce chorea (Jankovic, 2009; Phillips et al., 2008). Drugs such as lithium, valproate, and carbamazeprine are used to ameliorate mania and mood swings in HD patients (Pidgeon and Rickards, 2013). Hence, the current HD medication were prescribed for symptomatic relief but not to directly remit the HD pathology (Pidgeon and Rickards, 2013). To develop effective treatment to cure HD, therapeutic discovery research in HD has been reported using yeast model (Krobitsch and Lindquist, 2000), brain slice model (Murphy and Messer, 2004), transgenic 293/HEK cell (Shao et al., 2008), transgenic animal cell line (Carter et al., 2014; Cifra et al., 2011; Wu et al., 2006), and human differentiated MSN (Kaye and Finkbeiner, 2013).

Riluzole (RI) or 6-(trifluoromethoxy) benzothiazol-2-amine have been reported to inhibit multiple ion channels such as glutamate-gated channels, voltage-gated channels, and volume-sensitive chloride channels (Hebert et al., 1994). RI interferes glutamate-mediated excitotoxicity by blocking voltage-gated sodium channel and inhibits the release of glutamate at the presynaptic terminus (Cifra et al., 2011). In amyloidtrophic lateral sclerosis (ALS), RI is the only drug licensed for symptomatic treatment to delay disease progression and has improved survival rates (Georgoulopoulou et al., 2013; Lee et al., 2013). RI suppressed the bursting of motor neurons elicited by inhibition of glutamate uptake in an ALS model (Cifra et al., 2011). In PD model, RI attenuated dopaminergic neuron (DA neuron) degeneration and suppressed reactive astrocytosis in the striatum (Carbone et al., 2012). RI has the protective effects on DA neurons *in vitro* that against neuronal injuries by reduction of energy demand via the blocking of multiple ion channels, attenuate oxidative injury by reducing lipid peroxidation and inhibition of cytosolic phospholipase A_2, an enzyme that is linked to oxidative stress (Storch et al., 2000). In HD rodent model, RI showed

significant reduction in glutamate induced apoptosis in YAC128 MSNs (Wu et al., 2006). Furthermore, RI reduced mHTT aggregation, improved behavioral function, and increase the life span of R6/2 transgenic mice (Schiefer et al., 2002). In HD patients, RI enhances neurite formation in damaged motorneuron, decreased loss of grey matter and increase serum BDNF (Squitieri et al., 2009).

Memantine (ME) or 1-amino-3, 5-di-methyl-adamantane is a specific, moderate-affinity, uncompetitive, open-channel NMDAR antagonist (Möbius, 2003; Wu et al., 2006). In rat model of HD striatal degeneration by using 3NP induction, ME treatment could reduce the lesion volume and apoptosis by reducing calpain activation (Lee et al., 2006). 3NP inhibits mitochondrial SDH which reduces the capacity of mitochondrial to regulated cytosolic Ca^{2+} concentration. ME regulates Ca^{2+} entering through NMDAR which is tightly regulated by mitochondrial Ca^{2+} reuptake system in neurons and modulate 3NP effects (Lee et al., 2006). Increased function of NMDAR receptor in HD could contribute to selective striatal excitotoxicity and could be prevented by inhibitor of NMDAR and mGluR1/5 receptor in transgenic YAC128 mouse model (Zhang et al., 2008). The elevation of NMDAR activity interfered with intracellular Ca^{2+} signaling pathways that coupled with survival or death is deregulated in HD. A shift in the balance of synaptic Ex-NMDAR blocker attenuates mHTT-induced striatal atrophy and motor learning deficits in YAC128 model (Dau et al., 2014; Milnerwood et al., 2012). A pilot study examined ME as a treatment for HD patients. A daily dose of ME significantly improved motor symptoms such as chorea but failed to improve patient's cognitive and behavioral function (Ondo et al., 2007). Thus, clinical benefits of RI and ME have led to an open label clinical trial (Mason and Barker, 2009) that demonstrated neuroprotective effect was not only in HD (Ondo

et al., 2007; Squitieri et al., 2009) but also in Parkinson's disease (PD) (Cifra et al., 2011; Wesnes et al., 2015)

Methylene blue (MB) belongs to the phenothaizinium family. In ALS, MB inhibits the effect of nitric oxide, which mediates microglial responses to injury in ALS-linked transgenic $SOD1^{G93A}$ mice model that received local transfusion into lateral spinal cord with MB (Dibaj et al., 2012). $SOD1^{G93A}$ mice treated with MB had delayed ALS disease onset and motor dysfunction in early preclinical stage (Dibaj et al., 2012). However, systemic treatment in $SOD1^{G93A}$ mice had no effect on disease onset or survivals (Lougheed and Turnbull, 2011). In AD, MB reduced soluble amyloid-β (Aβ) and rescued early cognitive deficit in AD transgenic mice (Medina et al., 2011; Mori et al., 2014; Paban et al., 2014). MB also preserve cognitive performance, reduce Tau aggregation, Tau phosphorylation and pathologic Tau formation in $Tau^{\Delta K}$ mice (Hochgrafe et al., 2015). MB modulates AD pathology via increased macroautophgy, elevated HSP70 ATPase activity and facilitates chaperone mediated clearance of Tau (Hochgrafe et al., 2015; Jinwal et al., 2009). Moreover, MB has been used in Phase IIb clinical trial for treatment of mild to moderate AD with an improvement in cognitive function and slowing the progression of AD (Gura, 2008). MB also has the potential to inhibit Aβ oligomerization, primary pathogenic species, by promoting less toxic fibrillization (Necula et al., 2007). In HD, MB slow down the aggregation of mHTT protein even aggregation has been initiated and aggregated species are presented *in vitro*. In primary neurons expressing mHTT, MB reduced the formation of both oligomeric and insoluble HTT and increased survival of MB treated primary neurons of *Drosophila* and improved behavioral phenotype in R/2 mice

(Sontag et al., 2012). In R/2 treated mice, reduced striatal HTT aggregation and elevated BDNF level was in observed (Sontag et al., 2012).

2.6 MicroRNAs (miRNAs) and HD

MircroRNAs (miRNAs) are noncoding small RNAs that regulate the expression of complementary messenger RNAs. These functions of miRNAs are important for the control of animal development and physiology (Smirnova et al., 2005). The first discovered miRNAs is *lin-4*, which involves in gene regulatory network controlling the timing of *C. elegans* larval development (Lee et al., 1993; Wightman et al., 1993). In plants, miRNAs base paired with messenger RNA target by precise or nearly complementary manner that lead to cleavage and destruction of the target mRNA through RNA interference mechanism (Rhoades et al., 2002). Although miRNAs in animals are not precisely complementary to their mRNA targets and inhibit protein synthesis through an unknown mechanism (Ambros, 2004). Mammalian miRNAs play an important role in development and other processes with tissue specific or developmental specific expression pattern, additionally miRNAs are evolutionary conserved (Lewis et al., 2003). After transcription in nucleus, long RNAs with a small hairpin structure (primary miRNAs) are processed by a series of RNase III enzyme (Drosha or DGCR8 in mammals). The initial cleavage is followed by exportin-5-mediated transport to cytoplasm of 65-75 nucleotides which is further processed by the cytoplasmic RNase III endonuclease Dicer complex (Wienholds et al., 2003). Final processing by Dicer appears coupled to the assembly of miRNA into the RNA-induced silencing complex (RISC), which is the effectors of RNA interference (Kim, 2005; Squitieri et al., 2011). The key component of the RISC complex is an Argonaute

protein which is consistently found in RISC complex of a variety of organisms. The Argonaute protein family is conflicting, diverse with PAZ domain, which is involved in the miRNA binding, and PIWI domain, which is related to RNaseH endonucleases and functions in slicer activity (Song et al., 2004). miRNAs interfere target gene expression by binding to the 3' UTRs of mRNAs targets and alter translation. It has been estimated that miRNAs could regulate as many as 30% of human protein-coding genes, with each mRNA targeted by the multiple miRNAs (Filipowicz et al., 2008).

A large number of miRNAs are expressed in the brain and there is growing evidence indicating the essential role of miRNAs in brain development and neural function (Petri et al., 2014). For instance, deletion of Dicer gene results in early embryonic lethality and defects in embryonic brain development (Bernstein et al., 2003; De pietri tonelli et al., 2008). Moreover, loss of Dicer in mature DA neurons lead to a progressive midbrain DA neural cell loss and reduced locomotion similar to Parkinson's disease patients (Kim et al., 2007). Furthermore, inactivated Dicer in forebrain and hippocampal neuron changes dendrite morphology, spine length, apoptosis, microencephaly, ataxia, and lethality by three weeks after birth (Davis et al., 2008). A neuron-specific miR-124 promotes neuronal differentiation by repressing Polypyrimidine tract binding protein 1 (PTBP1) expression, which is a global repressor of alternative pre-mRNA splicing in non-neural cells (Makeyev et al., 2007). In addition, reduced expression of miR-124 sustain PTB expression and severely attenuate neural differentiation (Makeyev et al., 2007). Moreover, miR-9 and miR-124 inhibit NPCs proliferation and induce neuronal differentiation by targeting specific BAF53a and BAF45a, respectively (Yoo et al., 2009). Furthermore, overexpression of

miR-9 and miR-124 in human fibroblast is sufficient for neural conversion (Yoo et al., 2011).

In HD, miRNAs dysregulation was reported in transgenic HD rodent model (YAC128 and R6/2) and in the 3NP model that impair mitochondrial SDH complex II and Ca^{2+} homeostasis and induce striatal neurodegeneration (Brouillet et al., 2005). Nine miRNAs (miR-22, miR-29, miR-128, miR-132, miR-138, miR-218, miR-222, miR-344, and miR- 674) are common downregulated and four miRNAs (miR-34b-3p, miR-207, miR-448, and miR-669c) are upregulated in transgenic HD rodent models (Lee et al., 2011). The expression levels of Drosha and DGCR8 was higher in YAC128 mice than WT mice and Dicer1 was lower in YAC128 than WT mice (Lee et al., 2011). In transgenic HD monkey, miR-128a, miR-181c, and miR-133 downregulation and miR-194 upregulation has been reported in 11 transgenic HD monkey brains. Interestingly, miR-128a was also downregulated in the brain of pre-symptomatic and post symptomatic HD patients (Kocerha et al., 2014). miR-128a targets HD related genes including, *HIP1*, *SP1*, and *Htt* (Kocerha et al., 2014). The dysregulation of miRNAs in frontal cortex and striatum of HD patients have also been reported. Recent studies have shown miRNAs transcriptome dysregulation of HD is also reported by affecting miR-9/9*, miR-124, miR-132, miR-29b, miR-29a, miR-330, miR-17, miR-196a, miR-222, miR-485, and miR-486 (Johnson et al., 2008; Martí et al., 2010; Packer et al., 2008). Furthermore, there is strong evidence suggesting that the abnormal function of transcriptional repressor REST play a major role in gene deregulation in HD (Zuccato et al., 2007). BDNF is a REST-modulated neuronal gene that is significantly reduced in the striatum of HD patients with MSNs loss and clinical manifestations of HD (Zuccato et al., 2007; Zuccato et al., 2001). It is interesting that

miR-30a, which has been shown to target BDNF (Mellios et al., 2008), is significantly upregulated in both HD frontal cortex and striatum (Martí et al., 2010). The dysregulation of REST-miRNA generally alters the miRNAs transcriptome in HD brain, which may influence the expression of downstream target genes (Martí et al., 2010).

2.7 miRNA and HD therapeutic application

HD is a neurodegenerative disorder caused by abnormal polyQ expansion in HTT protein that disrupts the neural system. *Htt* knockdown resulted in disturbed cell migration, reduced proliferation and increased cell death specifically in early neural development in mice (Tong et al., 2011). Because of the WT and *mHtt*, there is a difference in only the number of CAGs repeated in *Htt*, selectively knockdown only *mHtt* is required (Kordasiewicz et al., 2012). However, partial reduction of *Htt* in rhesus monkey putamen have no impact on motor function, circadian behavior, neuropathology or an immune response (Mcbride et al., 2011). Although, the safety of RNAi-based therapy can be hampered by the ability of small inhibitory RNAs (siRNAs) to bind to unintended mRNAs and reduced their expression. Low off targeting potential siRNAs were screened to exhibit higher specificity as low toxicity and fewer false positives in phenotypic screens (Boudreau et al., 2011).

miR-196a was reported to play a critical role in normal development and pathogenesis of cancer, such as gastric- (Tsai et al., 2014), pancreatic- (Slater et al., 2014), esophageal- (Wang et al., 2012), breast-cancer (Lee et al., 2014) and glioblastoma multiform (Yang et al., 2014). Because miR-196a is highly expressed during early cancer development, it is considered as potential candidate of early cancer

biomarker (Lee et al., 2014; Tsai et al., 2014). MiR-196a targets Anexin-1A, a mediator of apoptosis and inhibitor of cell proliferation (Luthra et al., 2008; Pin et al., 2012), and Hox gene cluster which may be involved in neuroprotective response in HD (Severino et al., 2013). The therapeutic potential of miR-196a in neurodegenerative diseases was first described in spinal and bulbar muscular atrophy (SBMA) that was caused by polyQ expansion at the androgen receptor (AR-polyQ). By silencing *CUGBP*, Elav-like family member 2 (CELF2), miR-196a enhanced the decay of AR mRNA. Using adeno-associated virus (AAV) vector mediated delivery of miR-196a, SBMA phenotype in mouse model was ameliorated (Miyazaki et al., 2012). miR-196a was also related in fragile X syndrome (FXS) that is caused by the loss of expression of fragile X mental retardation protein (FMRP). The phosphorylation of FMRP promotes miR-196a-mediated repression of *HOXB8* without affecting the interaction between FMRP and mRNA (Li et al., 2014). Recently, miR-196a showed protective benefit in human immunodeficiency virus type 1 (HIV-1) associated neurodegenerative disorder. HIV-1 trans-activator to transcription (Tat) protein in neuron deregulation that lost its ability to promote accumulation and phosphorylation of p73 in the presence of miR-196a mimic. Interestingly, the accumulation of p73 did not lead to neural cell death but prevented neuronal deregulation and death by neutralizing HIV-1 Tat protein function (Bagashev et al., 2014).

In HD monkey, miR-196a was significantly upregulated in HD monkey's brain compared to WT brain (unpublished microarray data). The upregulation of miR-196a in HD patient brain sample was identified by using qPCR based miRNA array (Packer et al., 2008). Furthermore, miR-196a overexpression ameliorates phenotypes of HD in animal model and human HD iPSCs model by suppressed the expression of *mHtt*

predominantly through the suppression of protein synthesis and partly through protein degradation. miR-196a do not alleviate HD phenotype by targeting *mHtt* directly,but it was speculated that may reduce HD pathology through UPS system, gliosis, and the CREB pathway (Cheng et al., 2013). Moreover, a recent article reported the upregulation of HOX-related miRNAs including, miR-196a-5p, miR-196b-5p, and miR-615-3p in 12 HD patients prefrontal cortex. Interestingly, they found the size of CAG repeat and age of onset was independently inversely related to miR-196a-5p. These results suggested that Hox-related miRNAs may be involved in neuroprotective response in HD and suggested as the promising therapeutics and HD biomarkers (Hoss et al., 2014).

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CHAPTER III

Transgenic Huntington's Disease Non-Human Primate *in vitro* Model For Drug Screening

3.1 Abstract

Huntington's disease (HD) is a neurodegenerative disease caused by an expansion of CAG trinucleotide repeat (polyglutamine; polyQ) in the huntingtin (*Htt*) gene which leads to the formation of mutant HTT (mHTT) protein aggregates. In the nervous system, an accumulation of mHTT protein results in glutamate-mediated excitotoxicity, proteosome instability, and apoptosis. Although HD pathologenesis has been extensively investigated, effective treatment of HD has yet to be developed. Therapeutic discovery research in HD has been reported in using transgenic yeast, mammalian neural cell line, primary culture of medium spiny neuron (MSNs) derived from transgenic animal models, HD patient *ex vivo* MSNs, and MSNs derived from patient induced pluripotent stem cell (iPSCs). Transgenic nonhuman primate (NHP; monkey) model of HD (HD monkey) showed neuropathological, neuroanatomical, behavioral and molecular changes similar to patient. Additionally, neural progenitor cell (NPC) derived from HD monkeys can maintain in culture and are capable to differentiate to neurons that developed distinct HD cellular phenotypes including the formation of mHTT aggregates and intranuclear inclusions as well as increase susceptibility to oxidative stress. Here, we evaluated the potential application of HD monkey NPCs (HD NPCs) and neurons (HD neurons) as an *in vitro* platform

for drug discovery research in HD. To demonstrate if HD NPCs and neurons can be used for such purpose, we used riluzole (RI), memantine (ME), and methylene blue (MB) which have been shown to have a protective effect on HD and other neurodegenerative diseases, to determine if similar protective effects on HD cellular phenotype scan be replicated based on cytotoxicity, apoptosis, and aggregation of mHTT protein.

3.2 Introduction

Neurodegenerative diseases affect millions of patient and their families. Because of the complexity of disease pathogenesis, the development of treatments that could slow down the progression of these diseases has been a challenging task (Xu and Zhong, 2013). HD is a fatal neurodegenerative disorder caused by trinucleotide repeat expansion (Nasir et al., 1995; Sapp et al., 1997; Schilling et al., 1999). The expansion of cytosine-adenine-guanine (CAG) repeat in the first exon of the *Htt* gene on chromosome 4p16.3 results in the development of HD. Individual has more than 36 CAG repeats will develop HD (White et al., 1997). The age of onset is inversely correlated with the size of CAG tract while the severity of disease is directly correlated with the CAG repeat length (Langbehn et al., 2010). Different methods have been developed to ameliorate HD by using drugs and small molecules (Fecke et al., 2009; Huang et al., 2011), gene therapy (An et al., 2012; Cheng et al., 2013; Kordasiewicz et al., 2012), and stem cells (Mu et al., 2014). Among these approaches, gene and stem cell therapy have been focused on restoring neural cell functions in the affected brain regions with limited systemic effect. On the other hand, drugs and small molecules are expected to elicit systemic effect on HD phenotypes. Besides the challenge in

developing effective therapeutics, the lack of a preclinical large animal model has limited the option for assessing efficacy of novel therapeutics which outcome measurement similar to those used in humans. In fact, most of the HD rodent models only capture a fragment of HD phenotypes and are difficult to use for long-term assessment because of their short lifespan (Pouladi et al., 2013; Yang and Chan, 2011). Recent development of HD monkey model develop HD clinical features with progression similar to human HD patients suggested the potential of this unique model system for preclinical study (Chan et al., 2014). Although HD monkey model holds great promise as preclinical animal model of HD, the current study was evolved based on recent development of HD monkey NPCs (HD NPCs) and their derivative HD neurons that developed HD cellular features including increased susceptibility to oxidative stress, increased apoptosis and the formation of mHTT aggregate and intranuclear inclusions (Carter et al., 2014). To further determine if HD NPCs and HD neurons derived from iPSCs of HD monkeys are potential *in vitro* platform for HD drug discovery research, we determined the effect of three known drugs [Rilizole (2-amino-6-trifluoro-methoxy-benzothiazole; RI), Memantine (1-amino-3,5-di-methyl-adamantane; ME), and Methylene blue (MB)] that had shown to have beneficial effect on HD. Cytotoxicity, apoptosis and mHTT aggregation were used as outcome measurements to determine if HD cellular pathologies were improved. Here we hypothesize HD NPCs and HD neurons can be used as an *in vitro* platform for drug discovery research of HD.

3.3 Materials and Methods

3.3.1 Neural progenitor cell culture and neural differentiation

WT-2 (rZH-2: WT Embryonic Stem Cells (ESCs) (Putkhao et al., 2013), WT-14 (RiPS-14: WT Induced Pluripotent Stem Cells (iPSCs) (Carter et al., 2014)), HD-2 (RPg-2: 29 mutant CAG repeat HD ESCs (Putkhao et al., 2013)), and HD-3 (RiPS-3: 72 mutant CAG repeat HD iPSCs (Chan et al., 2010)) derived NPCs were produced and provided from Chan's lab. NPCs derivation and culture was described in our recent article (Carter et al., 2014). NPCs were cultured with NPM medium which was comprised of Neurobasal medium (Life Technologies), 1x B27 (Life Technologies), 2 mM L-glutamine, basic fibroblast growth factor (bFGF) (R&D, 20 ng/ml), and mouse leukemia inhibitory growth factor (mLIF) (Chemicon, 10 ng/ml) on tissue culture dish which coated by polyornithine/ laminin (P/L)(Sigma). NPCs were passaged by using stem Pro® Accutase® (Life Technologies).

WT-2, WT-14, HD-2, and HD-3 NPCs were differentiated *in vitro* by using Carter, 2014 protocol. In brief, WT and HD NPC were seeded into P/L coated tissue culture dish in 30,000 cell/cm^2 concentration. On the next day, neural differentiation medium, which was comprised of DMEM/F12 (Life Technologies), 1x P/S (Invitrogen), 2 mM L-glutamine, 1x N2 (Life Technologies), 1x B27 (Life Technologies), and 0.1 mM 2-Mercaptoethanol (Sigma) were added for four days. After that, 0.2 ug/mL Sonic Hedgehog (SHH) (R&D) and 0.1ug/mL Fibroblast growth factor-8 (FGF-8) (R&D) were added into differentiation medium for four days. At day 8, 200 mM ascorbic acid (Sigma) was supplemented into the medium together with SHH and FGF8 and culture until 21 days of differentiation had occurred. Differentiated neuronal cells were collected for further analysis.

For drugs screening, NPCs were treated with 10 uM RI (Sigma), 10 uM ME (Sigma), and 0.1 uM MB (Sigma) for 24 hrs prior to collecting sample. In neural cell, 10 uM RI (Sigma), 10 uM ME (Sigma) were treated 24 hrs before sample collection (Wu et al., 2006). But in 0.1 uM MB (Sigma) treated groups, MB was supplemented in differentiation media in last seven days of differentiation prior to collecting sample (Sontag et al., 2012). All samples were collected for further viability, cytotoxicity, or apoptosis analysis.

3.3.2 Immunocytochemistry

NPCs and neuronal cells cultured on P/L-coated glass slide were fixed in 4% PFA after 24 hrs treated with candidate drugs. Fixed cells were permeabilization and blocking with 0.2% Triton-X-100 (Sigma) and 3% Bovine Serum Albumin (BSA) (Sigma) in PBS. 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. Immunocytochemisty images were taken using fluorescence microscope (Olympus) with CellSens software (Olympus). To quantify the percentage of positive cells, five pictures were counted with two biologically replicated by taken randomly and quantified using blind investigators. Primary and secondary antibodies are listed in table 3.1

Table 3.1 Primary and secondary antibodies list

Antibody	Concentration	Vender
Nestin	1:1000	Millipore
Sox2	1:500	Stem Cell Technology
Musashi 1	1:500	Millipore
Pax6	1:300	Covance, Atlanta, GA
β-III tubulin	1:300	Millipore
Tyrosine Hydroxylase	1:100	Millipore
Map-2	1:500	Millipore
Double cortin	1:500	Millipore
mEM48	1:50	
Alexa Flour 594	1:1000	Life Technology
Cy-5	1:750	Life Technology

3.3.3 Real-Time Quantitative PCR (qPCR)

Total RNA from cell samples was prepared by using TRIzol® (Life Technologies). Genomic DNA was removed by using Turbo DNA-free Kit (Life Technologies) according to the manufacturer's instructions. Total RNA (1000 µg) was reverse transcribed using a RNA-to-cDNA kit (Applied Biosystems). qPCR was performed using SsoAdvaned Universal SYBR Green Supermix (Bio-Rad) using CFX96 Real-Time Detection System (Bio-Rad). qPCR primer sequences are listed in table 3.2.

Table 3.2: qPCR primer sequences

SYBR Primers		
Gene Symbol	**Forward Primer**	**Reverse Primer**
Ubiquitin C	CCACTCTGCACTTGGTCCTG	CCAGTTGGGAATGCAACAACTTTA
HTT Exon 1	GCGACCCTGGAAAAGCTGAT	CTGCTGCTGCTGGAAGGACT

3.3.4 Western Blot Analysis

After treating neural cell with candidate drugs for 24 hrs or seven days and harvesting the treated cell, protein was extracted from cell pellet by using RIPA buffer with protease inhibitor. Total protein concentration was quantified by Bio-Rad DC Protein Assay (Bio-Rad). Equal concentration of protein extract was loaded and separated by electrophoresis in 9% SDS-PAGE gel. Proteins were transferred to a PVDF membrane and probed with primary antibodies EM48, and γ-tubulin (Millipore) 4°C overnight. After that, secondary antibody was probed and visualizing band by using SuperSignal West FemtoChemiluminescent Substrate (Thermo Scientific). Band image was taken by Bio-Rad ChemiDoc (Bio-Rad).

3.3.5 Cell viability and G6PD cytotoxicity assay

For cell viability test, WT or HD NPCs were plated in a P/L coated 96 wells plate. Next day, NPCs were treated with candidate drugs in culture medium for 24 hrs. Cell viability assay was performed by MTT assay (ATCC) by following manufacturer's instructions. For cell cytotoxicity, WT or HD NPCs or neurons were treated with candidate drugs in culture medium for 24 hrs. Following incubation, cells were analyzed cytotoxicity by Vybrant G6PD Cytotoxicity Assay Kit (Life Technologies) according to the manufacturer's instructions. For neurons, WT or HD NPCs were differentiated 21 days in 24 wells plate, which were pre-coated with P/L. At day 21 media were changed to new culturing media, which contain drugs and incubated for 24 hrs. Then, media were collect and spinned down to collect the floating death cell. The pellets were resuspended with the G6PD reagent with the lysis buffer and added into the neural cell in culture plate. The percentage of G6PD released in media was calculated with total neural cell lysis from their individual well.

3.3.6 Statistical analysis

All experiments were repeated three times. Statistical analysis was performed using SPSS 12.0 (SPSS, Inc., USA). Data are represented as mean ± standard error. Statistical differences were calculated using Analysis of variance (ANOVA). Difference were considered significant at *p< 0.05 and statistically significant at **p< 0.01.

3.4 Results

3.4.1 Characterization of WT and HD NPCs and neurons

WT and HD NPCs derived from WT and HD monkey PSCs were used for determining if they are a suitable cell model for drug discovery research. HD cellular phenotypes including cytotoxicity, apoptosis and the formation of mHTT aggregates were used as outcome measures to determine if selected drugs can ameliorate and reverse cellular changes in HD NPCs and HD neurons. WT and HD NPCs expressed progenitor cell markers such as Sox-2, Pax-6, Musashi-1, and Nestin (Fig 3.1A). The expression of mHTT was significantly upregulated in two HD NPC lines and in their differentiated neurons (HD-2 and HD-3) when compared to the two WT NPC lines and their differentiated neurons (WT-2 and WT-14) (Fig 3.2A). Moreover, HD-3 showed a much higher expression level of mHTT than HD-2 which was consistent to prior report (Fig 3.2A) (Carter et al., 2014). Western blot analysis of WT-14 and HD-3 NPC and neurons revealed the accumulation of mHTT aggregate in HD neurons, but not in WT neurons as well as NPCs (Fig 3.2B).

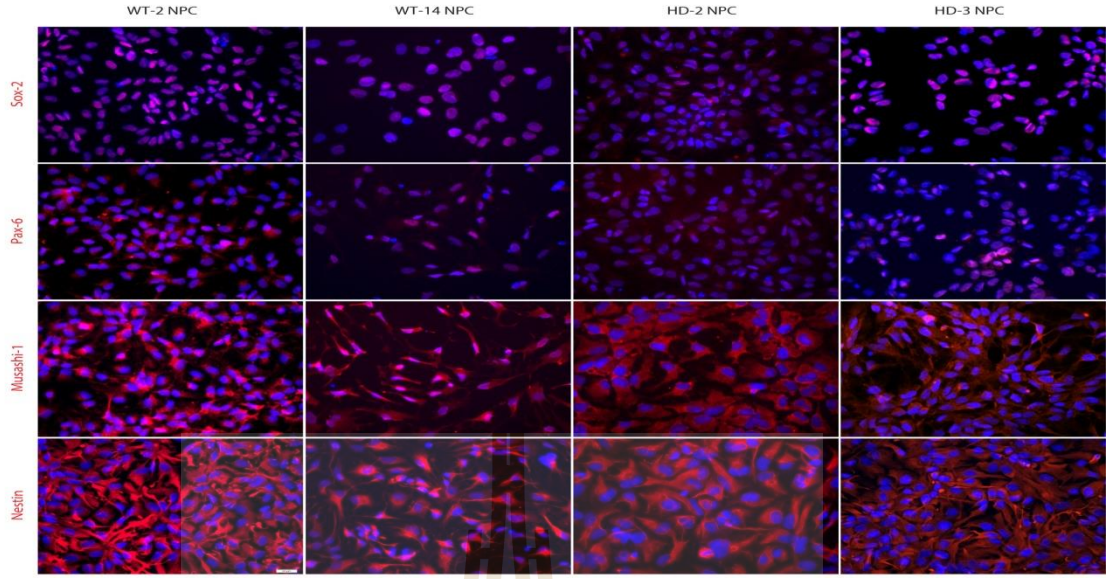

Figure 3.1 Immunocytochemistry staining of WT-2, WT-14, HD-2 and HD-3 NPCs with NPCs markers; Sox-2, Pax-6, Musachi-1, and Nestin. The pictures were taken using 40 x magnifications.

G6PD Cytotoxicity assay was used to determine cytotoxicity in NPCs and neurons. No different was found on cytotoxicity in WT and HD NPCs (Fig 3.2C), but, a significant increase in cytotoxicity was observed in HD neurons when compared to WT neurons (Fig 3.2D). These results suggested the differences in cytotoxicity and mHTT protein aggregation between WT and HD cells. Interestingly, both cytotoxicity and mHTT aggregation were significantly increased in HD neurons when compared to corresponding HD NPCs. This finding suggested a differential susceptibility to mHTT in NPCs and neurons, thus variations in response to treatments are also expected in HD NPCs and neurons.

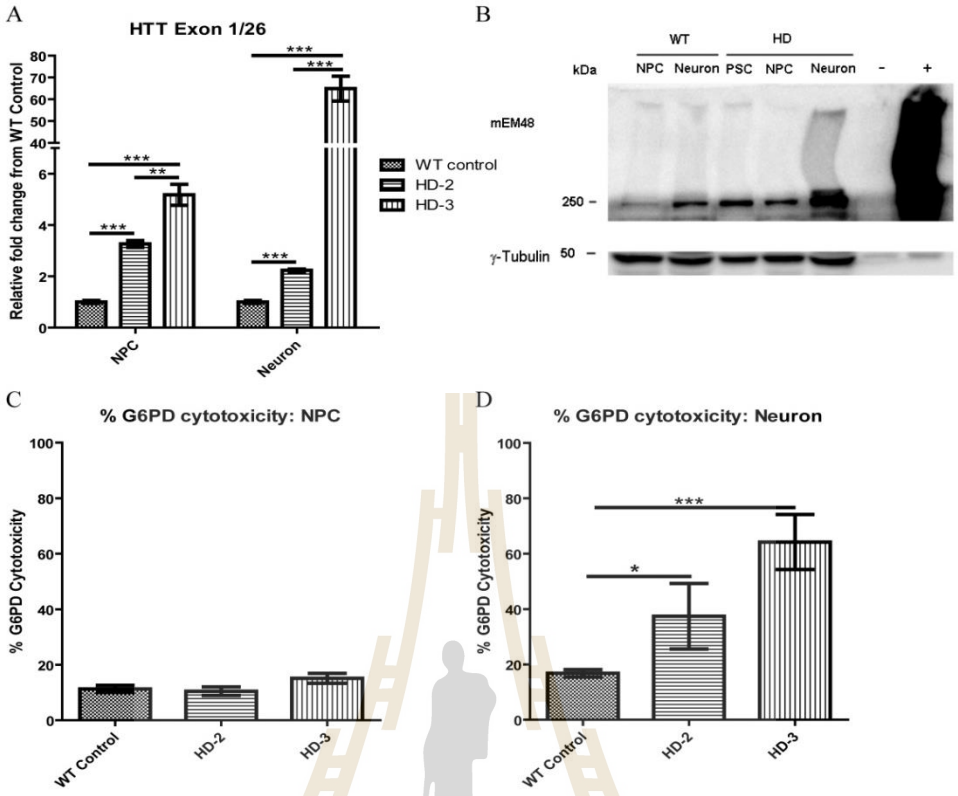

Figure 3.2 Characterization of WT and HD NPCs and differentiated neural cell. SYBR green RT-qPCR analysis of m*Htt* gene exon 1 normalized with WT *Htt* gene exon 26 (*Htt* exon 1/*Htt* exon26) in WT controls, HD-2 and HD-3 NPCs and differentiated neural cells **(A),** Western Blot analysis of mutant HTT protein aggregation of WT and HD NPCs and differentiated neuron detected by EM48 antibody and normalized with γ-Tubulin. Negative control using WT brain and positive control using HD brain protein **(B),** NPCs and neural cells cytotoxicity screening by using G6PD cytotoxicity assay **(C-D).** Data are represented as mean±SEM of fold change from average WT gene expression. (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$: ANOVA

3.4.2 Assessing drug responses in WT and HD NPCs using MTT assay and G6PD cytotoxicity assay

RI (Landwehrmeyer et al., 2007; Squitieri et al., 2008; Squitieri et al., 2009), ME (Anitha et al., 2010; Dau et al., 2014; Hjermind et al., 2011; Ondo et al., 2007; Wu et al., 2006), and MB (Mori et al., 2014; Sontag et al., 2012) were used to treat HD NPCs and determine if viability and cytotoxicity can be improved. After 24 hours treatment, no significant improvement in viability and cytotoxicity were observed in WT and HD NPCs (Fig 3.3A-H). This result is consistent with cytotoxicity observed in NPCs that no significant different in cytotoxicity was observed when compared between WT and HD NPCs.

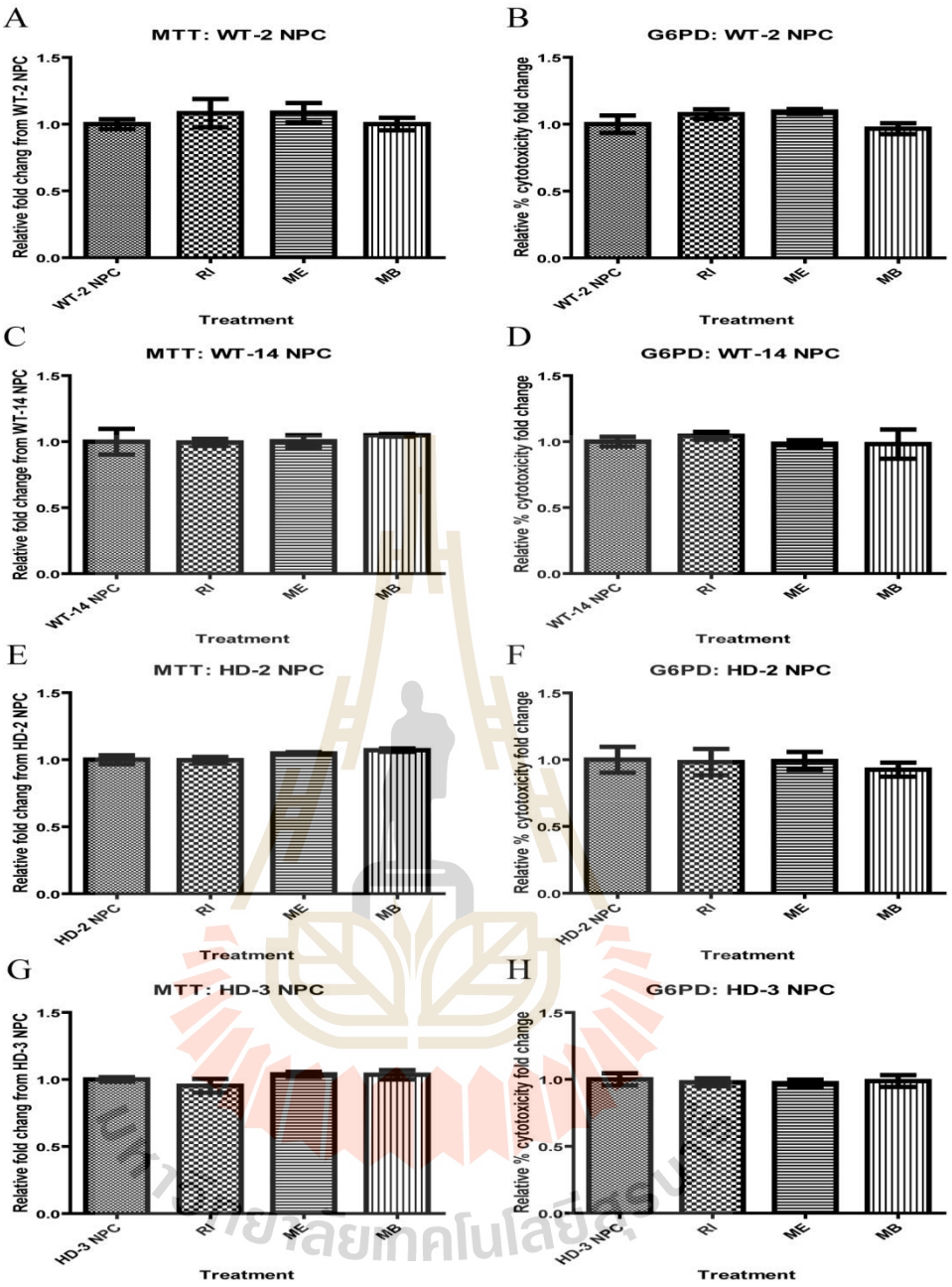

Figure 3.3 Drugs screenings in WT and HD NPCs using MTT assay and G6PD cytotoxicity assay. NPCs viability screening by using MTT assay after 24 hrs treated with candidate drugs, 10 uM RI, 10 uM ME, 0.1 uM MB **(A, C, E, G).** NPCs cytotoxicity screening by using G6PD cytotoxicity assay after 24 hrs treated with candidate drugs, 10 uM RI, 10 uM ME,

0.1 uM MB **(B, D, F, H)**. Data are represented as mean±SEM of fold change comparing with untreated control. (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$: ANOVA)

3.4.3 Assessing drugs response in WT and HD differentiated neurons by G6PD cytotoxicity assay

WT and HD NPCs were differentiated into neurons followed by treatment with RI, ME, and MB. All differentiated neurons expressed neural specific markers including Tuj-1, DCX, Map-2, and TH (Fig 3.4). The formation of intranuclear inclusions and mHTT aggregates was observed in HD neurons and significant increase in cleaved caspase-3 positive cells (Fig 3.4). RI, and ME were administered into culture media on the last day of neural differentiation for 24 hrs while MB was added into the culture media for 7 days until complete differentiation. RI, ME and MB did not alleviate cytotoxicity on neurons derived from two different WT NPC lines (Figs 3.5A and 3.5B). However, significant reduction in cytotoxicity in HD-2 and HD-3 neurons was observed (Figs 3.5C and 3.5D, respectively). These results suggested that cytotoxicity in HD neurons can be ameliorated by RI, ME and MB.

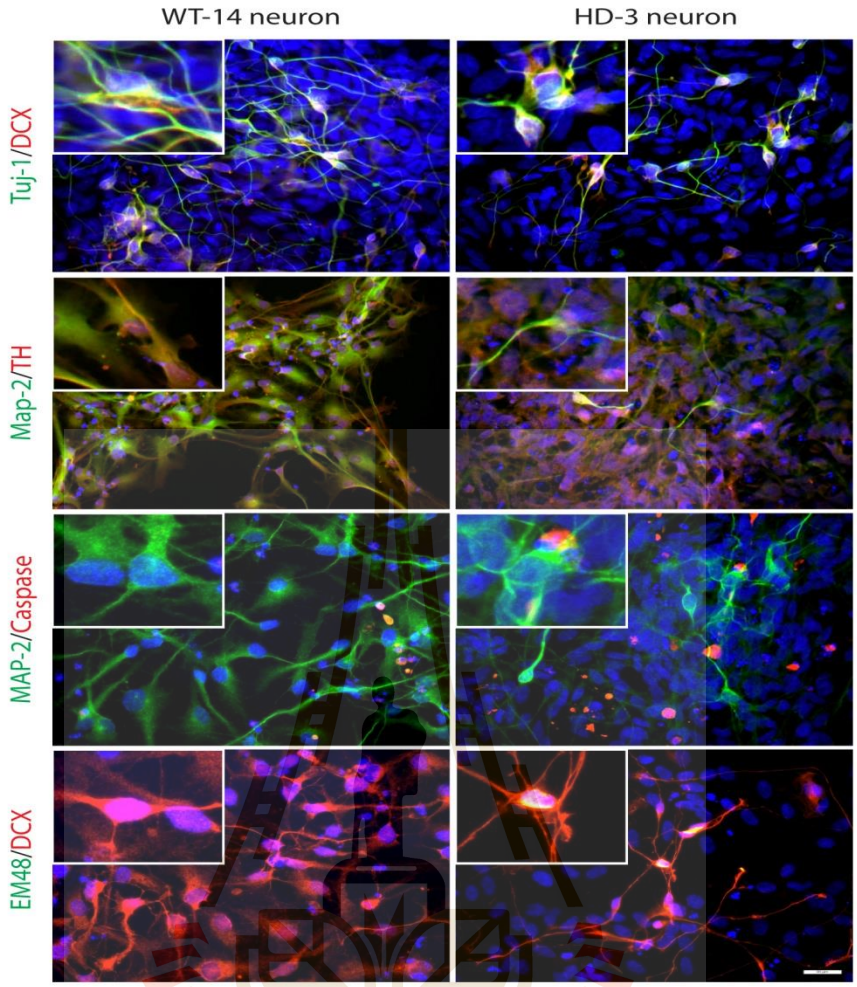

Figure 3.4 Immunocytochemistry staining of WT-14 and HD-3 differentiated neuron with Neural markers; βIII tubulin (Tuj-1), Doublecortin (DCX), Tyrosine hydroxylase (TH), Microtubule associated protein 2 (Map-2). For apoptosis and mHTT protein aggregation detection both neurons were stained with cleaved caspase-3and EM48 respectively. The pictures were taken using 40 x magnifications.

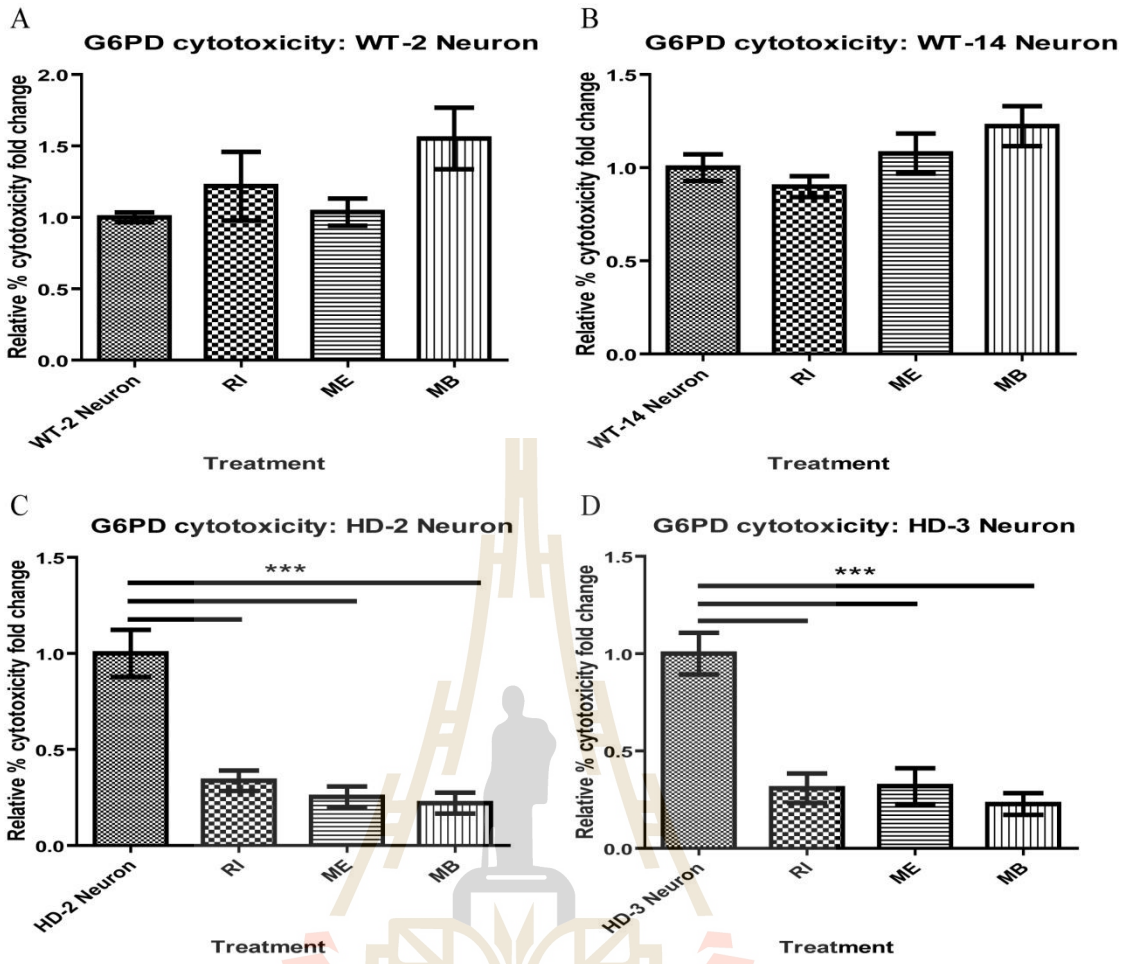

Figure 3.5 Drug screenings in WT and HD differentiated neural cells using G6PD cytotoxicity assay. WT-2 **(A)**,WT-14 **(B)**, HD-2 **(C)** and HD-3 **(D)** differentiated neuron cytotoxicity screening using G6PD cytotoxicity assay after 24 hrs treated with 10 uM RI, 10 uM ME, and treated for 7 days for 0.1 uM MB**.** Data are represented as mean±SEM of fold change comparing with untreated control. (*** $p < 0.001$: ANOVA)

3.4.4 Ameliorating apoptosis and mHTT aggregation in HD neurons

Activated caspase induced apoptosis has been reported as one of the pathological hallmarks in HD (Squitieri et al., 2011). Similar observation was also described in neurons derived from HD NPCs (Carter et al., 2014). To examine if RI, ME, and MB ameliorate apoptotic responses, they were administered into neural culture media of differentiated WT and HD neurons for 24 hours followed by fixation and immunostaining using specific antibody that recognized cleaved caspase-3 and Map2 (microtubule-associated protein-2). Total cell count of Map-2 (green) and cleaved caspase-3 (red) positive cells was used to determine the ratio of apoptotic neurons in neural cell population (Figs 3.6A and 3.6B). In WT control neurons, the addition of RI, ME and MB did not result in the increase of caspase-3 positive cells or apoptotic neurons (Fig 3.6C). Whereas, significant reduction in caspase-3 positive cells was observed in HD neurons (HD-2 and HD-3) treated with RI, ME, and MB (Figs 3.6B and 3.6D-E). Moreover, WT-14 and HD-3 neurons treated with RI, ME and MB were double immunostained using doublecortin (DCX), neural specific antibody, and EM-48 to determine if mHTT aggregation was affected in neural cells. As expected, no aggregate was observed in WT control neurons with or without treatment (Fig 3.7A). In contrary, EM-48 positive neurons were dramatically reduced in HD neuron in all treatment groups (Fig 3.7B), which was further confirmed by western blot analysis using mEM48 antibody (Fig 3.7C). Between the three drugs, MB has the most significant impact on the reduction of mHTT aggregates as well as soluble form mHTT protein when compared to RI and ME (Fig 3.7C). These results suggested that RI, ME, and MB can reduce caspase-3 induced apoptosis in HD neurons with RI as the most prominent and MB as the least. In case of suppressing mHTT aggregation, MB

was the most effective while ME has no significant impact on both aggregated and soluble form of mHTT protein. These data were consistent between cytotoxicity, reduced apoptosis and the formation of mHTT aggregates in HD neurons.

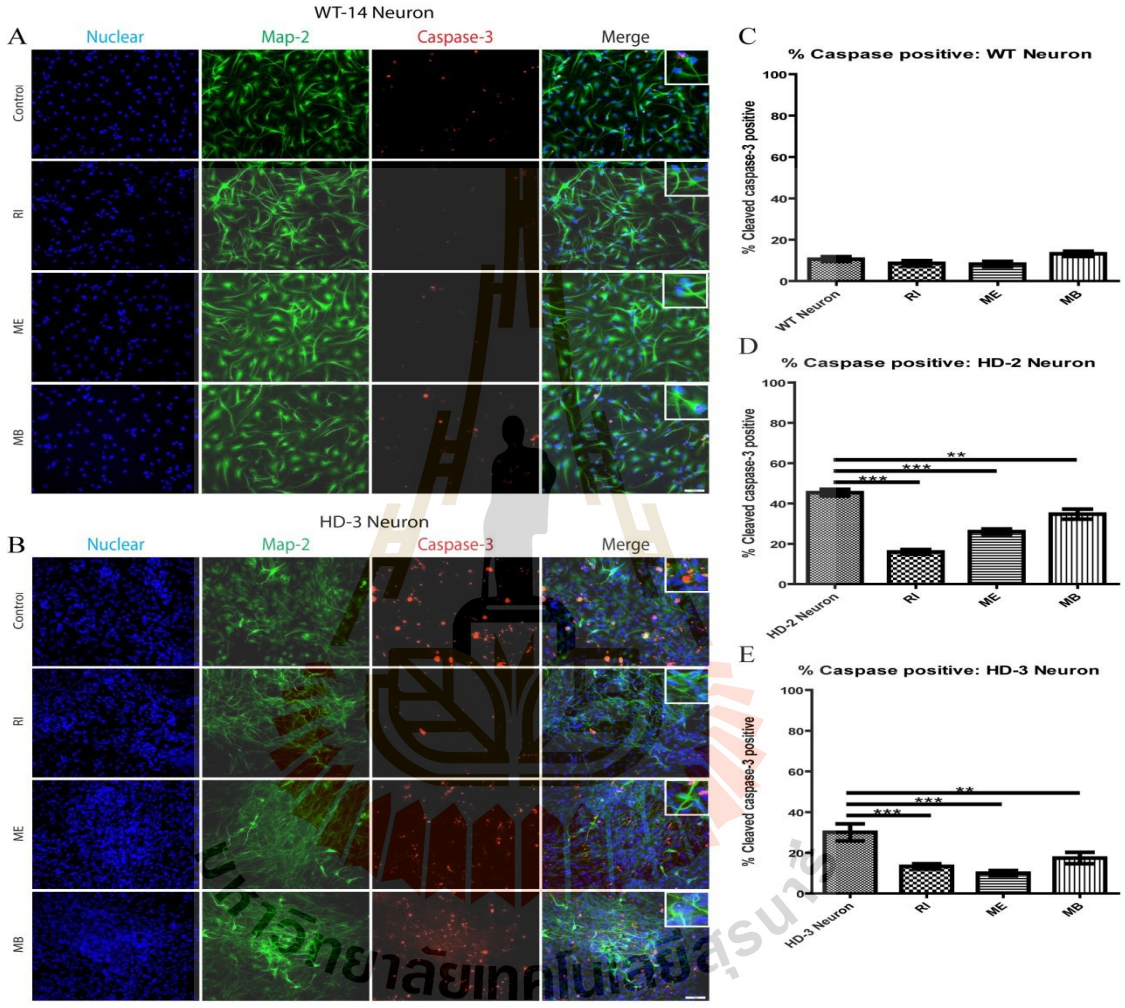

Figure 3.6 Drugs reduced differentiated HD neuron apoptosis via cleaved caspase-3. Immunocytochemistry staining of WT-14 **(A)**, and HD-3 **(B)** differentiated neural cells after 24 hrs treated with 10 uM RI, 10 uM ME, and 7 days treated for 0.1 uM MB before stained with cleaved caspase-3 and Map-2 antibody. The pictures were taken using 20 x magnifications. The graph shows % of cleaved caspase -3 positive WT

controls **(C)**, HD-2 **(D)**, HD-3 **(E)** differentiated neural cells 24 hrs treated with 10 uM RI, 10 uM ME, and 7 days treated for 0.1 uM MB from five pictures, in which two were biologically replicated, whose average cells counted were 500-700 cells per picture**.**

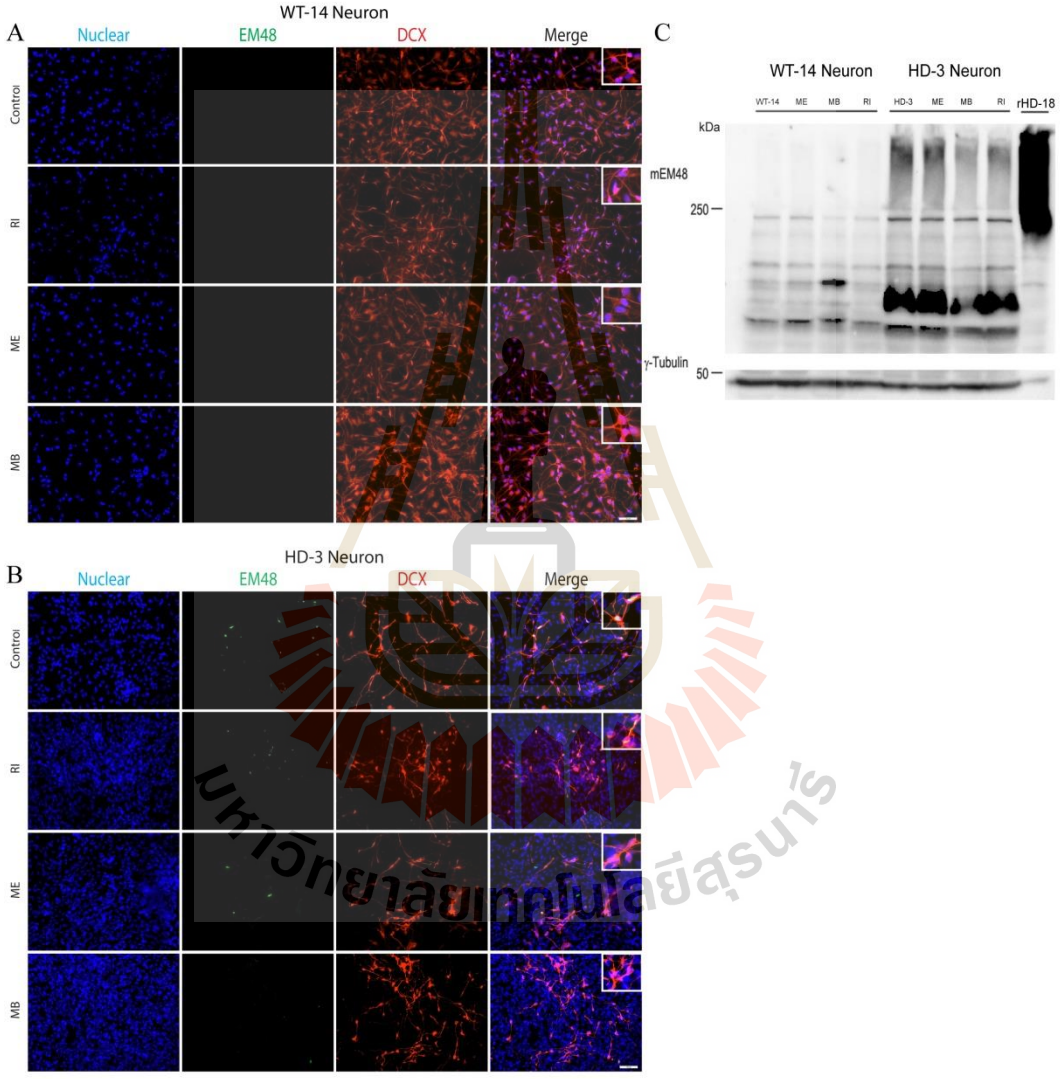

Figure 3.7 HTT protein aggregation reduction effect of candidate drugs in HD neural cells. Immunocytochemistry staining of WT-14 **(A)**, and HD-3 **(B)** differentiated neuron after 24 hrs treated with 10 uM RI, 10 uM ME, and 7 days treated for 0.1 uM MB before stained with EM-48 and

DCX antibody. The pictures were taken using 20 x magnifications. Western Blot analysis of mHTT protein aggregation of W-14 and HD-3 neuron after 24 hrs treated with 10 uM RI, 10 uM ME, and 7 days treated for 0.1 uM MB using EM48 antibody and normalized with γ-Tubulin. Positive control used rHD-18 brain protein **(C)**.

3.5 Discussion

In this study, three drugs that have shown beneficial effect in several neurodegenerative diseases including HD, PD and AD were used to determine if HD monkey iPSCs derived NPCs and neurons can be a reliable *in vitro* platform for drug discovery research because of their unique HD cellular phenotypes such as cytotoxicity, apoptosis and protein aggregation. We have shown that HD NPCs and neurons are highly susceptible to oxidative stress and HD cellular phenotypes including mHTT aggregates can be reversed by genetic and biochemical approaches. HD neurons expressed glutamate receptor which are the target of RI ad ME (Carter et al., 2014). HD NPCs and neurons are unique platform to evaluate if drug screening can be achieved *in vitro* while HD monkeys can also be used preclinical animal model for assessing therapeutic efficacy.

NPCs and neural cells of HD were extremely sensitive to oxidative stress induced by hydrogen peroxide or by the removal of growth factors in culture with a significant increase in apoptosis (Carter et al., 2014; Zhang et al., 2010). Compared to HD neurons, HD NPCs have lower mHTT expression level and much milder HD cellular phenotypes that may limit its application in drug discovery research because of the lack of robust outcome measurement to determine beneficial effect of treatments.

Many *in vitro* platforms have been developed for drug discovery research witch include HD yeast model (Outeiro and Giorgini, 2006), brain slice model (Murphy and Messer, 2004), genetically modified 293/HEK cell (Shao et al., 2008), primary neuronal culture from transgenic rodents (Chen et al., 2000; Giampà et al., 2013), transgenic rodent cell line such as PC12 (Wang et al., 2005), transgenic NHP cell line (Carter et al., 2014) and medium spiny neurons (MSNs) derived from human iPSCs (The HD iPSC Consortium, 2012). However, similar to HD NPCs, robust HD cellular phenotypes are very limited in these model systems with a narrow margin for determinations effect. HD neurons developed robust cellular phenotypes including the increase of mHTT aggregates, the formation of intranuclear inclusions, increase in caspase-3 induced apoptosis, and cytotoxicity caused by oxidative stress and the removal of growth factors.

The significant improvement in cytotoxicity, caspase-3 induced apoptosis, and the reduction of mHTT protein aggregates was observed in HD neurons treated with RI, ME, and MB. RI has been reported to inhibit ion channels such as glutamate-gated channels, voltage-gated channels, and volume-sensitive chloride channels (Hebert et al., 1994). RI ameliorates glutamate-mediated excitotoxicity by blocking voltage-gated sodium channel and inhibits the release of glutamate at the presynaptic terminus (Cifra et al., 2011). In HD patients, RI enhances neurite formation and growth in damaged motor neurons, reduces the loss of gray matter and increases serum brain-derived neurotrophic factor (BDNF) (Squitieri et al., 2009). In YAC128 MSN model, RI significantly reduced glutamate induced apoptosis (Wu et al., 2006). Besides HD, neuroprotective effect of RI has also been reported in PD model by attenuating dopaminergic neuron degeneration and suppressed reactive astrocytosis in the striatum

(Carbone et al., 2012). Furthermore, in amyloidtrophic lateral sclerosis (ALS), RI improves the survival rate of ALS patients (Georgoulopoulou et al., 2013; Lee et al., 2013). ME is a specific, moderate-affinity, uncompetitive, open-channel N-methyl-D-aspartate glutamate receptor (NMDAR) antagonist (Möbius, 2003; Wu et al., 2006). Increased function of NMDAR was observed in YAC128 mouse model that was caused by selective striatal excitotoxicity and can be prevented by the inhibition of NMDA and mGluR1/5 receptor activity (Zhang et al., 2008).The elevation of NMDAR activity interfers intracellular Ca^{2+} signaling pathways that lead to cell death in HD. A synaptic Ex-NMDAR blocker can attenuate mHTT-induced striatal atrophy and motor learning deficits in YAC128 model (Milnerwood et al., 2012). Thus, glutamate receptor antagonist such as RI and ME have been considered for reducing cytotoxicity in HD and open label clinical trials on these two drugs (Mason & Barker, 2009) have shown neuroprotective effect not only in HD (Ondo et al., 2007; Squitieri et al., 2009) but also in PD (Cifra et al., 2011; Wesnes et al., 2015) and AD (Sun et al., 2014).

MB belongs to the phenothaizinium family which has been used in Phase IIb clinical trial for the treatment of mild to moderate AD (Gura, 2008). Significant improvement in cognitive function and slowing down of AD progression has been reported (Gura, 2008). Additionally, MB may inhibit Aβ oligomerization, primary pathogenic species in AD, by promoting less toxic fibrillization which is less toxic, (Necula et al., 2007). MB also reduced soluble Aβ and rescued early cognitive deficit in AD transgenic mice (Medina et al., 2011; Mori et al., 2014; Paban et al., 2014). In HD, MB can slow down the aggregation of mHTT protein even when aggregation has already been initiated, and aggregated species were presented *in vitro* (Sontag et al., 2012). In primary neurons expressing mHTT, MB reduced the formation of both

oligomeric and insoluble mHTT and increased the survival of neurons (Sontag et al., 2012).

Our studies on RI, ME, and MB in HD NPCS and neurons demonstrated similar beneficial effects in ameliorating HD cellular phenotypes which were consistent with prior studies in different model systems (Carter et al., 2014; The HD iPSC Consortium, 2012). Reduction in cytotoxicity and caspase-3 induced apoptosis via glutamate receptor inhibitors such as RI and ME suggested our HD NPCs and neurons response to drugs in a similar manner to the other model systems. These results suggested HD NPCs and neurons can be used for assessing therapeutic benefits of novel drugs or small molecules. Similar effect on the reduction of mHTT protein aggregate and soluble form mHTT protein was also observed in HD neurons treated with MB and RI (Cifra et al., 2011; Lee et al., 2006; Mcfarland et al., 2013; Niatsetskaya et al., 2009; Sontag et al., 2012; Tauffenberger et al., 2013; Wu et al., 2006; Yu et al., 2013). While mechanism and therapeutic effect of RI, ME and MB in HD have yet to be determined, our HD NPCs and neurons offered a unique platform not only for understanding the underlying mechanism of these effective drugs but also a novel platform with robust outcome phenotypic measure for drug discovery research in HD.

In conclusion, this study is not aimed to discover new drugs for HD but for validating the potential application of HD NPCs and neurons in drug discovery research by using drugs that have been known for their therapeutic benefits in neurodegenerative diseases such as HD, PD and AD as a proof of principle. While HD NPCs and neurons can serve as an *in vitro* drug discovery platform, HD monkeys with similar genetic constitution as the HD NPCs and neurons can be used for preclinical

ssessment prior to human clinical trial to further ensure the safety and potential benefit of the new treatments (Chan 2014).

3.6 References

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CHAPTER IV

miR-196a Ameliorates Cytotoxicity and Cellular Phenotype in Transgenic Huntington's disease Monkey Neural Cells

4.1 Abstract

Huntington's disease (HD) is an inherited neurodegenerative disorder caused by the expansion of polyglutamine (polyQ) tract that leads to motor, cognitive and psychiatric impairment. Currently, there is no cure for HD. A transgenic HD nonhuman primate (HD-NHP) model was developed with progressive development of clinical and pathological features similar to human HD which suggested the potential preclinical application of the HD-NHP model. Elevated expression of miR-196a was observed in both HD-NHP and human HD brains. Cytotoxicity and apoptosis was ameliorated by the overexpression of miR-196a in HD-NHP neural progenitor cells (HD-NPCs) and differentiated neurons (HD-neurons). The expression of apoptosis related genes was also down regulated. Mitochondrial morphology and activity were improved as indicated by mitotracker staining and the upregulation of *CBP* and *PGC-1α* in HD-NPCs overexpressing miR-196a. Here we demonstrated the amelioration of HD cellular phenotypes in HD-NPCs and HD-neurons overexpressing miR-196a. Our results also suggested the regulatory role ofmiR-196a in HD pathogenesis that may hold the key for understanding molecular regulation in HD and developing novel therapeutics.

4.2 Introduction

HD is an autosomal dominant neurodegenerative disorder caused by the expansion of CAG trinucleotide repeats located at the first exon of the *HTT* gene (Berrios et al., 2001; Galkina et al., 2014; Macdonald et al., 1993; White et al., 1997). Clinical features of HD include cognitive, psychological, and motor deficits (Roos, 2010; Spires et al., 2004; Yang et al., 2008; Yoon et al., 2006; Zizak et al., 2005). Molecular instability, a core component in disease pathogenesis and progression, has been investigated by transcriptomic and small RNA profiling approaches (Asli and Kessel, 2010;Bernstein et al., 2003; Bernstein et al., 2003; Cao et al., 2006; Chan and Kocerha, 2012; Kocerha et al., 2014). Dysregulation of genes and non-coding RNA such as micro RNAs (miRNAs) in the brain are highly correlated with neuropathological changes in diseases such as HD (Chan and Kocerha, 2012; Cheng et al., 2013; Hebert et al., 2008; Hoss et al., 2014; Kocerha et al., 2009a; Kocerha et al., 2009b; Lee et al., 2011; Sehm et al., 2009; Severino et al., 2013; Yu et al., 2012). Dysregulated expression of peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (*PGC-1α*), a regulator of mitochondrial biogenesis and oxidative stress (Chiang et al., 2010; Tsunemi et al., 2012), CREB binding protein (*CBP*), a histone acetyltransferase (HAT) transcriptional co-activator (Choi et al., 2009; Jiang et al., 2006) and brain derived neurotrophic factor (*BDNF*) are all important for the maintenance and survival of neurons (Gambazzi et al., 2010; Giampà et al., 2013), and are all dysregulated in HD. In addition to transcriptomic dysregulation, alteration of miRNA expression level has also been reported in neurological disorders, including psychiatric disorders, autism spectrum disorder, Alzheimer's disease, Parkinson's disease and HD (Chan and Kocerha, 2012; Hebert et al., 2008; Kocerha et al., 2009;

Kocerha et al., 2013; Soldati et al., 2013). Recent miRNA profiling study on human HD prefrontal cortex identified five miRNAs that are located in the *Hox* gene cluster were upregulated in HD when compared to control (Hoss et al., 2014). Three of the five miRNAs (miR-196a-5p, miR-196b-5p and miR-615-3p) have near zero levels in the control which suggested their potential as biomarker for HD (Hoss et al., 2014). Among these three candidates, over expression of miR-196a ameliorates spinal and bulbar muscular atrophy (SBMA) (Miyazaki et al., 2012) and HD (Cheng et al., 2013) cellular and clinical phenotypes that demonstrate the therapeutic potential of miR-196a in neurodegenerative diseases. Moreover, miR-196a was also highly expressed during early cancer development and is a candidate for an early cancer biomarker (Lee et al., 2014; Tsai et al., 2014). Although the *HTT* gene is not a putative target of miR-196a, miR-196a targets genes that are involved in neuronal differentiation, neurite outgrowth, cell death and survival further suggested its role in HD pathogenesis (Bagashev et al., 2014; Cheng et al., 2013; Li et al., 2014; Miyazaki et al., 2012). Among these mRNA targets, *Anexin-1A* (*ANXA1*) is a mediator of apoptosis and inhibitor of cell proliferation (Luthra et al., 2008; Pin et al., 2012). The relation between miR-196a and *Hox* gene cluster further suggested its involvement in neuroprotective response in HD (Hoss et al., 2014; Severino et al., 2013).

This study evolved based on the recent development of the HD-NHP model (Chan et al., 2014; Chen et al., 2014; Kocerha et al., 2014; Putkhao et al., 2013; Yang et al., 2008) and the development of HD-neurons from iPSCs derived HD-NPCs (Carter et al., 2014; Chan et al., 2010; Chen et al., 2014). HD-NHPs develop progressive decline in cognitive, behavioral and motor functions as they aged (Chan et al., 2014; Chan et al., 2015; Moran et al., 2015; Yang et al., 2008). Transcriptional

dysregulation of mRNA and miRNA in peripheral blood and brain tissues were also observed in HD-NHP (Kocerha et al., 2013; Kocerha et al., 2014). HD-NHP brains revealed the formation of mutant HTT aggregates and nuclear inclusion which further suggested the potential of HD-NHP as a large animal model for studying HD pathogenesis (Carter et al., 2014; Chen et al., 2014). Additionally, HD-NPCs and their derivative neurons develop HD cellular phenotypes including the accumulation of mutant HTT aggregates, nuclear inclusion, mitochondrial dysfunction and increase susceptibility to oxidative stress (Carter et al., 2014). Most importantly, HD cellular phenotypes in HD-NPCs and neurons can be reversed by genetic and small molecule approaches, which suggested the potential of HD-NPCs as an *in vitro* platform for studying HD pathogenesis and drug discovery research (Carter et al., 2014). Here we use HD-NPCs and HD-neurons to investigate if the over expression of miR-196s can rescue or ameliorate HD cellular phenotypes including cell viability, apoptosis, mitochondrial functions and dysregulated gene expression.

4.3 Materials and Methods

4.3.1 Information of monkey and human brain tissues used

All transgenic HD monkey were housed under the guideline of the IACUC approved procedure and the support of the devision of Animal Resources at the Yerkes National Primate Research Center (YNPRC). All procedures were approved by YNPRC/Emory Animal Care and Biosafty Committees. Transgenic HD monkey, HD1 (29Q) and HD7 (70Q) was created by transfection of mature oocyte by using lentiviarus carrying a *mHTT* gene compose of Exon 1 of the *HTT* gene with extended CAG tract under the regulation of polyubiquitin C promoter (HD1) and

human huntingtin promoter (HD7) (Yang et al., 2008). Brain sample of WT and HD monkeys were recovered from euthanized HD1 and HD7 monkey and preserved in -80 ^{o}C. Human HD patients and normal brain tissues were provided by Emory Alzheimer's Disease Research Center and the Emory Neuroscience NINDS Core Facilities (ENNCF) Neuropathology Core Service at Emory University. The use of human tissues was followed and compiled with NIH guideline.

4.3.2 Establishment of HD-NHP NPCs over expressing miR-196a

A lentiviral vector carried miR-196a under the control of Tet-On inducible system (Tet-hsa-miR-196a) with zeocin resistant gene regulated by human polyubiquitin promoter (Ubi-zeo) placed downstream of the Tet-hsa-miR-196a (pLV-miR-196a) was used in this study. High titer LV-miR-196a was prepared by the co-transfection of 0.68 ug of pVSVG, 1.014 ug of Δ8.9, and 1.35 ug pLV-miR-196a into 293FT cell (Chan 2014; Yang et al., 2008). Two days after transfection, LV-miR-196a was collected, concentrated by ultracentrifugation and followed by transfection of wild-type NPCs (WT-NPCs or WT-2 NPCs) and HD-NPCs (or HD-3 NPCs). NPCs were seeded at 20,000 cells/cm^2 the day before infection. On the day of viral infection, NPCs culture was replaced with fresh neural proliferation medium (NPM; Neurobasal-A medium (Life Technologies) supplemented with B27 (Life Technologies), penicillin/streptomycin (P/S), 2 mM L-glutamine, basic fibroblast growth factor (bFGF) (R&D, 20 ng/ml), and mouse leukemia inhibitory growth factor (mLIF) (Chemicon, 10 ng/ml) with concentrated virus supplemented with 8 ug/mL polybrene (Sigma) for two day and then replaced with NPM supplemented with 100 ug/mL zeocin for selection of NPCs overexpressing miR-196a (196a-NPC). Overexpression

of miR-196a was determined by qRT-PCR using has-miR-196aTaqMan probe (Applied Biosystems).

4.3.3 Neural progenitor cell culture and neural differentiation

WT-2 NPCs (WT-NPCs) and HD-3 NPCs (HD-NPCs) were derived by NHP pluripotent stem cell (Carter et al., 2014). Maintenance and neural differentiation of NPCs was performed as described by Carter and colleagues (Carter et al., 2014). In brief, NPCs were cultured on polyornithine/laminin (P/L) coated tissue culture dish with NPM medium. NPCs were dissociated by using Accutase® (Life Technologies).

To *in vitro* differentiate NPCs to neuron cells, 30,000 NPCs/cm^2 were seeded onto P/L coated tissue culture dish. Neural proliferation medium was replaced with neural differentiation medium (NDM; DMEM/F12 (Life Technologies) supplemented with P/S (Invitrogen), 2 mM L-glutamine, 1x N2 (Invitrogen), 1x B27 (Life Technologies), and 0.1 mM 2-Mercaptoethanol (Sigma)) for four days. On day 5, 0.2 µg/mL Sonic Hedgehog (SHH; R&D) and 0.1µg/mL Fibroblast growth factor-8 (FGF-8; R&D) were supplemented into NDM for four days. On day 8, 200 mM ascorbic acid (Sigma) was also added into the medium until the end of neural differentiation on day 21 (Carter et al., 2014).

4.3.4 Immunocytochemistry

The immunocytochemistry protocol was performed as described in chapter III 3.3.2

4.3.5 Real-Time Quantitative PCR (qPCR)

Total RNA from tissue and cell samples was prepared by using TRIzol® (Life Technologies). Genomic DNA was removed by using Turbo DNA-free Kit (Life Technologies) according to the manufacturer's instructions. Total RNA (1000 µg) was

reverse transcribed using a RNA-to-cDNA kit (Applied Biosystems). For miR-196a, 300ug of total RNA was reverse transcribed using TaqmanMiroRNA Reverse Transcription Kit (Applied Biosystems) with hsa-miR-196a and RNU6B RT primer. qPCR was performed using SsoAdvaned Universal SYBR Green Supermix (Bio-Rad). For miRNA expression, qPCR was performed using TaqMan® gene microRNA expression primersusing CFX96 Real-Time Detection System (Bio-Rad). qPCR primer sequences are listed in table 4.1.

Table 4.1 qPCR primer sequences

Taqman ® Primer		
Gene Symbol	**Taqman ® Primer Context Sequence**	
RNU6B	CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT	
hsa-miR-196a	UAGGUAGUUUCAUGUUGUUGG	
SYBR Primers		
Gene Symbol	**Forward Primer**	**Reverse Primer**
Ubiquitin C	CCACTCTGCACTTGGTCCTG	CCAGTTGGGAATGCAACAACTTTA
HTT Exon 1	GCGACCCTGGAAAAGCTGAT	CTGCTGCTGCTGGAAGGACT
Caspase-3	TCGCTTTGTGCCATGCTGAAAC	TGTTGCCACCTTTCGGTTAACC
BCL2L1	CGGGATGGGGTAAACTGG	AGGTGGTCATTCAGGTAAGTGG
ANXA1	ATGAAAGGTGCTGGAACTCG	TCTCCCTTGGTTTCATCCAG
BDNF	GCCCAATGAAGAAAACAATAAGG	AGCAGAAAGAGAAGAGG GGC
CBP	AGCGAAACCAACAAACCATCC	TGGGGTCTATGGGATTTGGGT
PGC1α	CTAAAGACCCCAAAGGATGC	GCGGTGTCTGTAGTGGCTTG

4.3.6 Western Blot Analysis

Proteins were extracted from cell pellet by using RIPA buffer with protease inhibitor. Total protein concentration was quantified by Bio-Rad DC Protein Assay (Bio-Rad). Equal concentration of protein extract were loaded and separated by electrophoresis in 9% SDS-PAGE gel. Proteins were transferred onto a PVDF membrane and probed with primary antibodies cleaved caspase-3 (Milipore) and α-tubulin (Milipore) at 4°C overnight. The next day, secondary antibody was probed and visualizing band by using SuperSignal West FemtoChemiluminescent Substrate (Thermo Scientific). Image was taken by using Bio-Rad ChemiDoc (Bio-Rad).

4.3.7 Cell Stress and Apoptosis Assays

For cell viability, NPCs were seeded in P/L coated 96 wells plate the day before treatment. Withdrawal of growth factor or supplement with 10 uM MG-132 (Selleckchem) in culture for 24 hrs were used to induce cell stress followed by viability and cytotoxicity assays. Cell viability assay was performed by MTT assay (ATCC) according to manufacturer's instructions. Cell cytotoxicity was measured by using Vybrant Cytotoxicity Assay Kit (Life Technologies) according to the manufacturer's instructions. For cleaved caspase-3 protein expression, cells were fixed and stained with cleaved caspase-3 antibody (Millipore).

4.3.8 Mitochondria Morphology

Mitochondria with active mitochondrial membrane potential were determined by staining with Mitotracker® Green FM (Life Technologies). Cells were then fixed and nuclei were labeled by using Hoechst 33342. Images were acquired using a Zeiss LSM 510 NLO META confocal microscope (Oberkochen, Germany).

4.3.9 Statistical analysis

All experiments were repeated three times. Statistical analysis was performed using SPSS 12.0 (SPSS, Inc., USA). Data are represented as mean ± standard error. Statistical differences were calculated using t test. Difference were considered significant at *p< 0.05 and statistically significant at **p< 0.01.

4.4 Results

4.4.1 Upregulation of miR-196a in HD-NHP brain and NPCs

Expression of miR-196a and mutant *HTT* (*mHTT*) transgene was measured by qPCR in different regions of the HD-NHP brain (Fig 4.1A-C). *mHTT* transgene was highly expressed in the caudate and putamen of HD-NHP's brain compared to the wildtype non-transgenic rhesus macaque (Fig 4.1A). In addition to caudate and putamen, miR-196a was also highly expressed in thalamus, cerebellum, motor cortex and frontal cortex of HD-NHP (Fig 4.1B). These findings were consistent with miR-196a expression pattern in human HD striatum where miR-196a was significantly increased in Stage 1 and 3 (n=4) HD patients when compared to control (n=4) (Fig 4.1C). Expression of miR-196a was also measured in undifferentiated pluripotent stem cells (PSCs), NPCs, and differentiated neural cells (Fig 4.1D). The expression of miR-196a was significantly higher in HD cells compared to the control of each corresponding stage (Fig 4.1D).

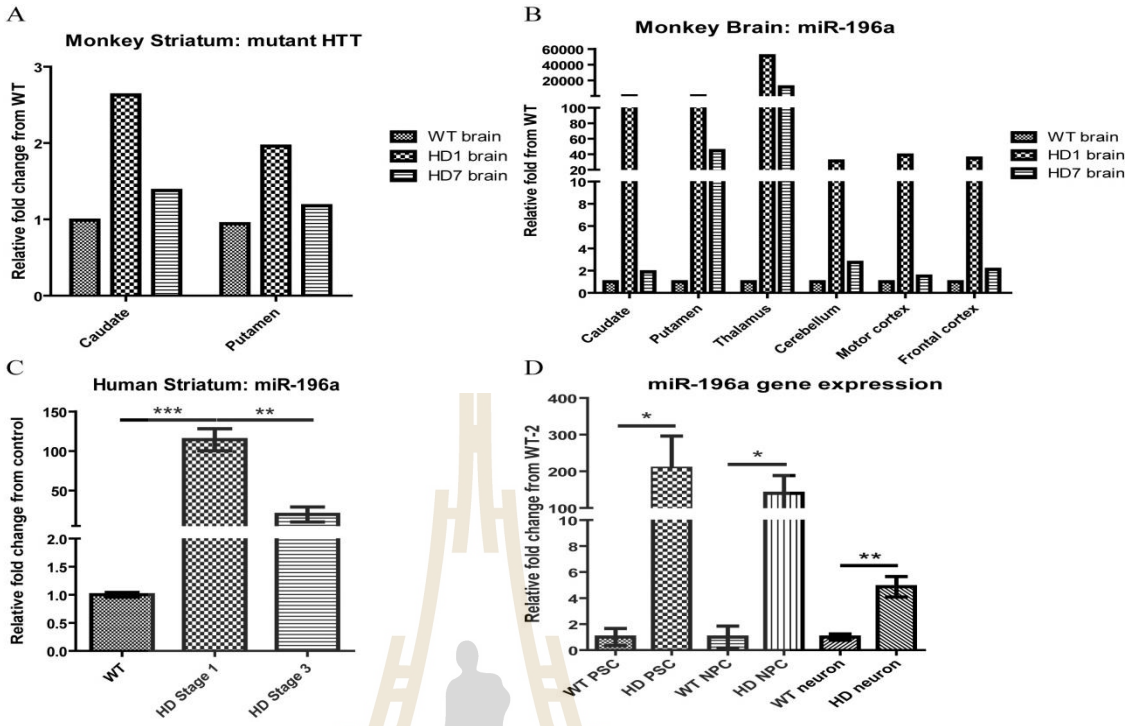

Figure 4.1 Relation between miR-196a and HD. *mHTT* gene expression analysis (*HTT* exon1/*HTT* exon26) of WT, HD1, and HD7 NHP brain part striatum (Caudate and Putamen) by SYBR green RT-qPCR. The expression level was calculated to relative fold change to WT brain (A). miR-196a gene expression analysis of WT, HD1, and HD7 monkey brain by microRNA Taqman RT-qPCR. The expression level was calculated to relative fold change to WT brain (B). miR-196a gene expression analysis of human WT, HD patient stage1, and stage 3 striatum by microRNA Taqman RT-qPCR. The expression level was calculated to relative fold change to WT striatum (D). miR-196a gene expression analysis of NHP WT and HD cell line (PSCs, NPCs and neuron) by microRNA Taqman RT-qPCR. The expression level was calculated to relative fold change to WT cell line within same stage (E). Data are represented as mean±SEM. (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$: T-test)

4.4.2 Impact of over expression of miR-196a in NPCs and neural cells

Over expression of miR-196a in WT-NPCs and HD-NPCs was confirmed by quantitative measurement in WT-NPCs and HD-NPCs with (WT-196a NPCs and HD-196a NPCs) or without (WT-NPCs and HD-NPCs) being introduced with miR-196a transgene by lentiviruses. miR-196a expression was significantly increased in NPCs of WT-196a NPCs and HD-196a NPCs when compared to WT-NPCs and HD-NPCs, respectively (Fig 4.3A). Elevated expression of miR-196a in WT-NPCs and HD-NPCs did not affect NPC property based on the expression of NPC markers including Sox-2, Pax-6, Musashi-1, and Nestin (Fig 4.2A). Additionally, the expression of *mHTT* was not affected by the overexpression of miR-196a as expected that *HTT* is not the putative target of miR-196a (Fig 4.3B). To further determine the morphology with extended neurite outgrowth compared to the stump shape morphology in HD neurons without relationship between *HTT* and miR-196a, we analyzed miR-196a expression in *HTT* knocked down HD NPCs (shHD-3) that was previously described (Carter et al., 2014) and compared with WT and HD NPCs. There was no difference in the expression of miR-196a in shHD-3 and HD NPCs, and were both significantly higher than WT NPCs (Fig 4.2B). Our next step was to determine if overexpressing miR-196a affect neural differentiation. All NPCs with or without overexpressing miR-196a were *in vitro* differentiated into neural cells (Carter et al., 2014). We confirmed the overexpression of miR-196a in neural cells derived from both WT-196a NPCs and HD-196a NPCs when compared to those derived from WT-NPCs and HD-NPCs (Fig 4.3C). However, the overexpression of miR-196a has no effect on the expression of *mHTT* (Fig 4.3D). Immunostaining using neural specific antibodies demonstrated the expression of neuronal markers in all groups mHTT protein inclusions bodies in HD-miR-196a neuron were still detected (Fig 4.3E). Additionally, HD-miR-

196a derived neurons showed improved neural the overexpression of miR-196a (Fig 4.3E).Our results further suggested the overexpression of miR-196a did not affect NPC property and neural differentiation.

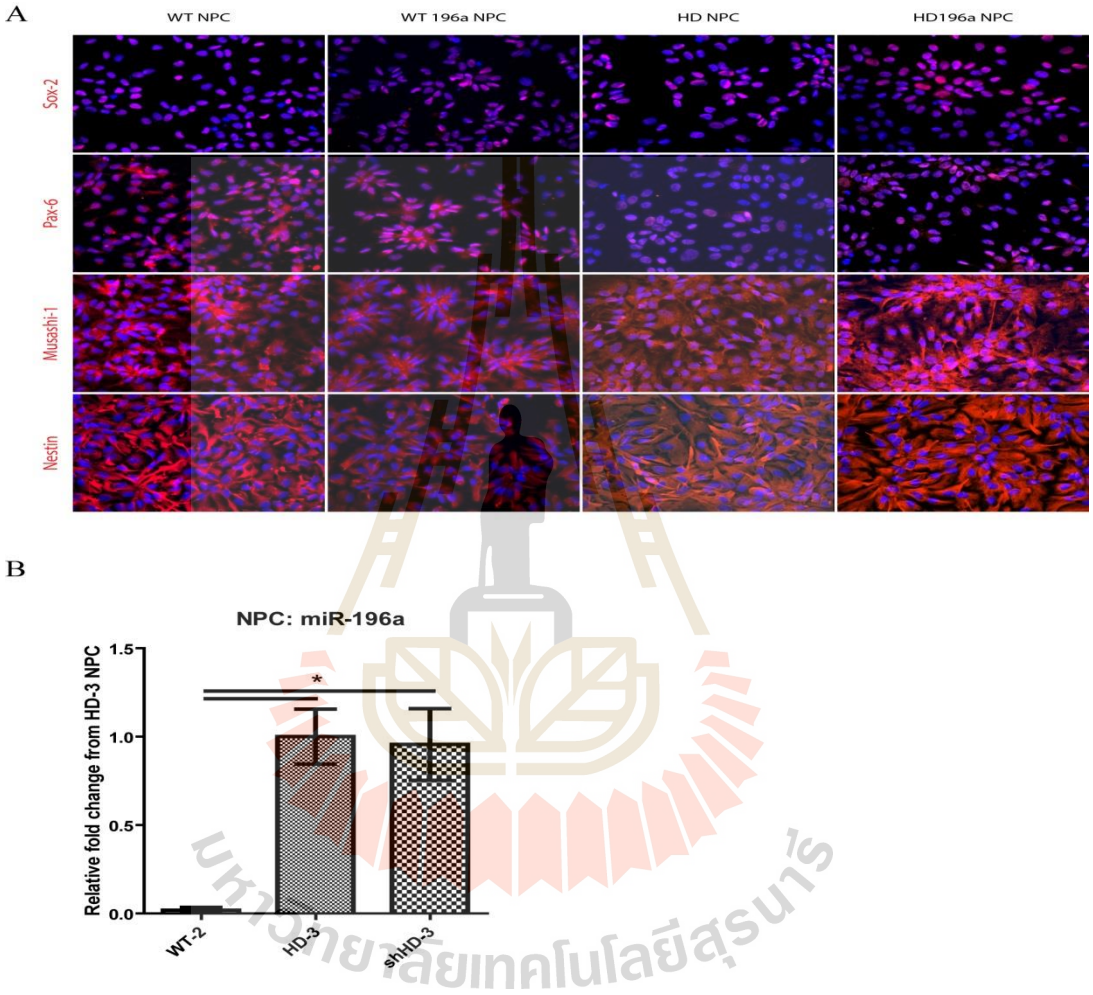

Figure 4.2 The effect of miR-196a on NPCs characteristic and m*Htt* expression. Images represent immunocytochemistry staining of WT, WT-196a, HD and HD-196a NPCs. All NPCs of each line were stained by NPCs markers (Sox2, Pax6, Musashi-1, and Nestin) (A). miR-196a gene expression analysis of WT, HD, and shHD-3 NPCs by microRNA Taqman RT-qPCR. The expression level was calculated to relative fold change to HD NPCs (B). Data are represented as mean±SEM. (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$: One way ANOWA)

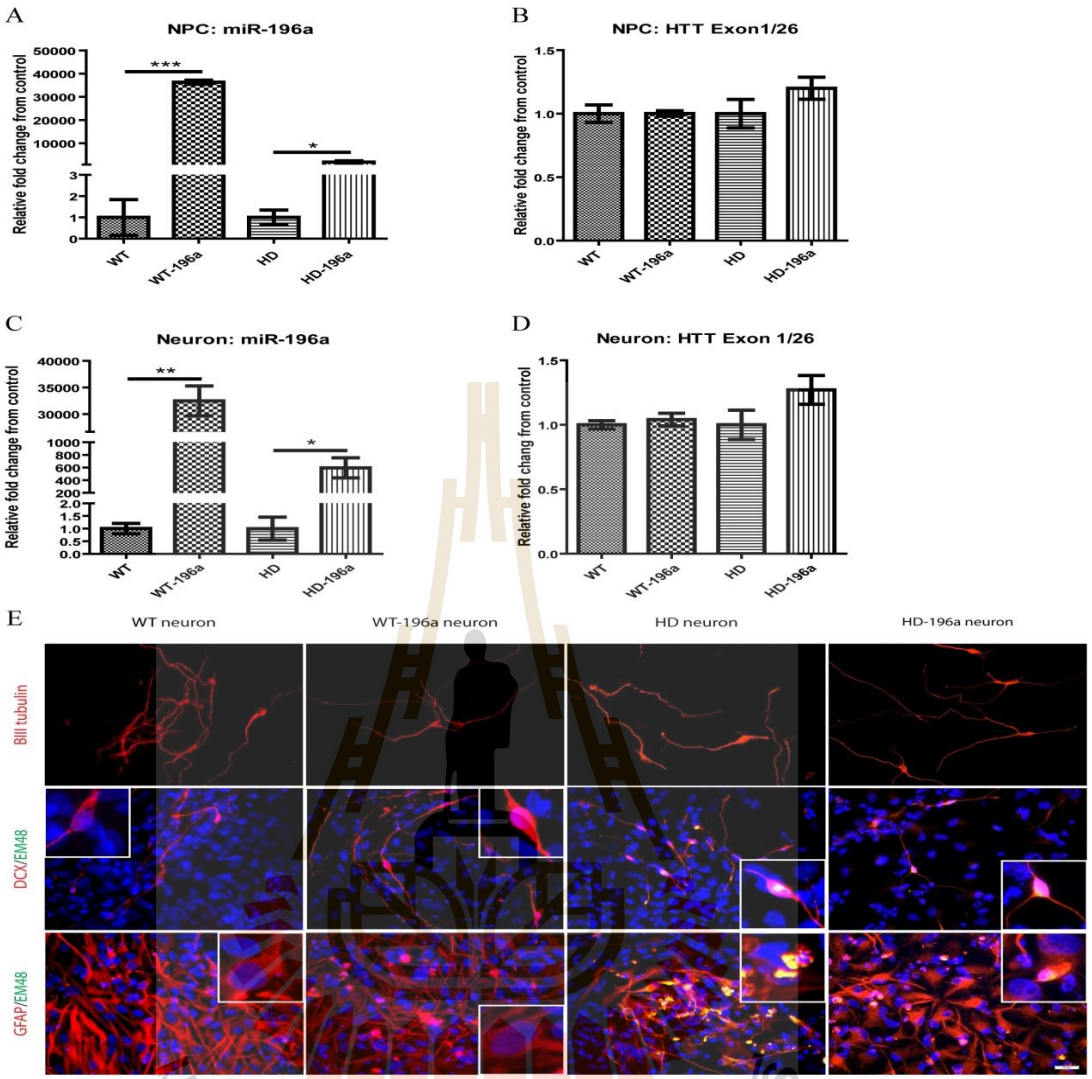

Figure 4.3 miR-196a over expressed NPCs and differentiated neural cells characterization. miR-196a gene expression analysis of miR-196a over expressed NPCs at 7 days after zeocin selection by microRNA Taqman RT-qPCR. The expression level was calculated to relative fold change to individual WT or HD NPCs (A). Mutant *HTT* gene expression analysis (*HTT* exon1/*HTT* exon26) of miR-196a overexpressed NPCs by SYBR green RT-qPCR. The expression level was calculated to relative fold change to non miR-196a overexpressed WT or HD NPCs (B). miR-196a

gene expression analysis of miR-196a over expressed neuron at 21 days of differentiation by microRNA Taqman RT-qPCR. The expression level was calculated to relative fold change to individual WT or HD neuron (C). Mutant *HTT* gene expression analysis (*HTT* exon 1/*HTT* exon26) of miR-196a overexpressed neuron at 21 days of differentiation by SYBR green RT-qPCR. The expression level was calculated to relative fold change to non miR-196a overexpressed WT or HD neuron (D). Images represent immunocytochemistry staining of WT, WT-196a, HD and HD-196a differentiated neural cells. All differentiated neural of each line were stained by neuronal markers (βIII tubulin, Doublecortin, and GFAP) and nuclear inclusion and cytosolic aggregates of mutant HTT (mEM48) (E). Data are represented as mean±SEM. (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$: T-test)

4.4.3 Effect of miR-196a on gene expression in HD NPCs and neural cells

To determine the impact and the role of miR-196a in HD pathogenesis, a panel of genes related to HD (*HTT*, Huntingtin-interacting protein 1:*HIP-1*), apoptosis (B-cell lymphoma 2 like protein 1: *BCL2L1*, Annexin1A: *ANX1A*) and neural cell growth (*BDNF*) were quantitatively measured by qPCR in NPCs and differentiated neural cells. Comparative gene expression between HD and WT NPCs and differentiated neurons were shown in figure 4.4A-P. The expression of *mHTT* (Fig 4.3B) was not different in HD-NPCs and HD neurons (Fig 4.3D) with or without the overexpression of miR-196a (Fig 4.5A). *HIP-1* is a pro-apoptotic protein (Hackam et al., 2000) that interact with HTT protein and its expression was also not affected by the

overexpression of miR-196a in both NPCs and neural cells (Figs 4.5B-C). *BCL2L1* is a member of the BCL-2 protein family that acts as anti- or pro-apoptosis regulators and is expressed at the outer mitochondrial membrane. BCl-2 was expressed at higher levels in HD patients than control postmortem brains and are primarily found in degenerating or apoptotic neurons (Vis et al., 2005). In NPCs, the expression of *BCL2L1* was significantly reduced when miR-196a was overexpressed (Fig 4.5D). Similarly, the expression of *BCL2L1* was reduced significantly in HD neural cells overexpressing miR-196a, however, upregulation was observed in WT-196a neural cells (Fig 4.5E). *ANX1A* is a mediator of apoptosis and inhibitor of cell proliferation which has been reported in various cancer types (Pin et al., 2012; Queiroz et al., 2014; Zhu et al., 2013) and is a target of miR-196a (Luthra et al., 2008). Significant reduction in *ANX1A* was found in WT and HD-NPCs and neurons overexpressing miR-196a (Figs 4.5F and 4.5G) that suggested the neuroprotective effect of miR-196a. Finally, *BDNF* is one of the neural growth factors that is highly dysregulated in HD and resulted in synaptic dysfunction in the brain (Gambazzi et al., 2010).The expression level of BDNF was not different in HD and WT NPC (Fig 4.4G), though in HD neuron the expression was significant lower comparing to WT neuron (Fig 4.4H). When miR-196a was overexpressed in HD NPC and neurons, BDNF was not significantly upregulated in NPCs but was significant increased in HD neurons (Figs 4.5H and 4.5J).

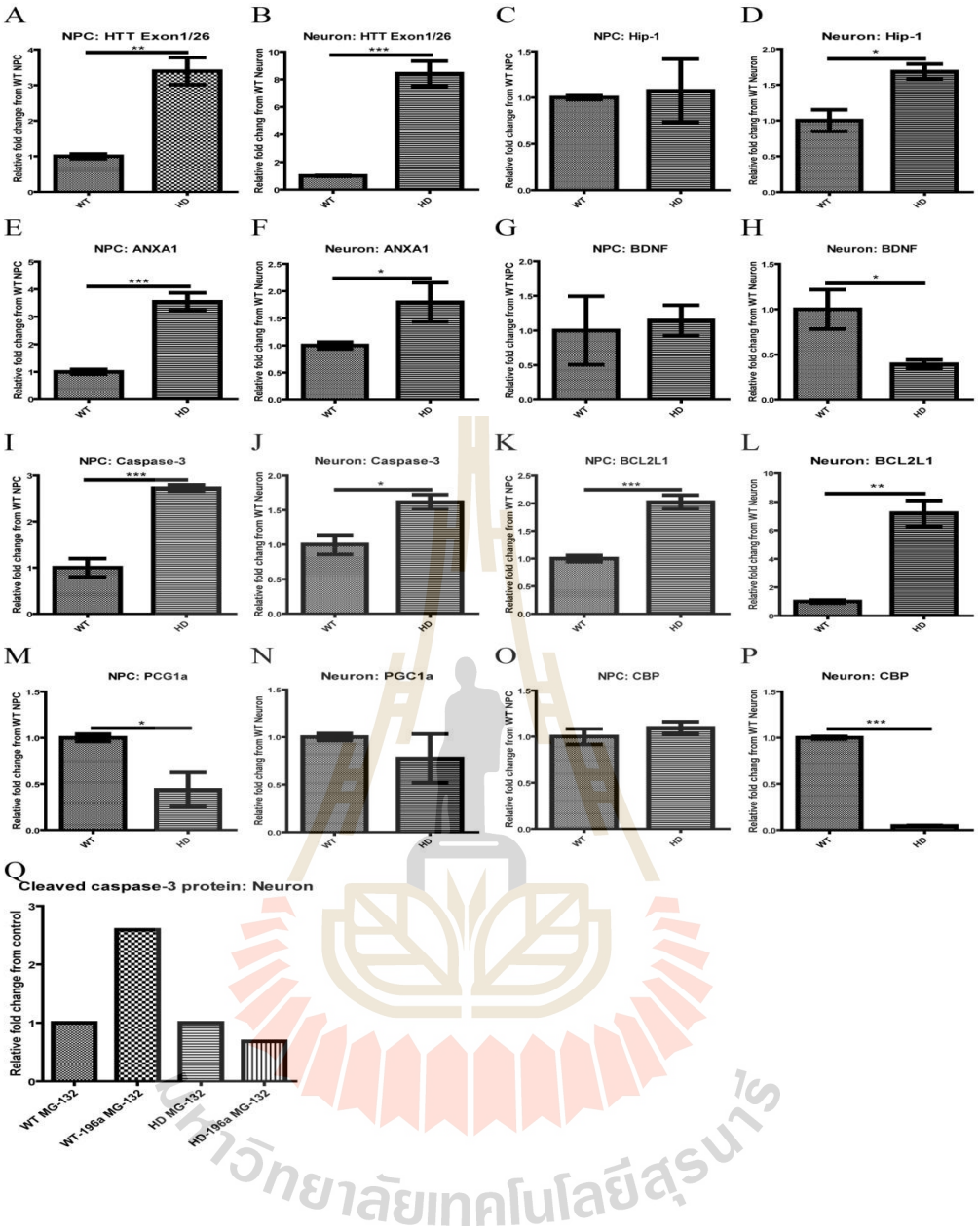

Figure 4.4 HD related related genes expression of NPCs and differentiated neural cells. SYBR green RT-qPCR analysis of mutant *HTT* gene exon 1 normalized with WT *HTT* gene exon 26 (*HTT* exon1/*HTT* exon26) (A, B), *Hip-1* (C, D), *ANXA1* (E, F), *BDNF* (G, H), *Caspase-3* (I, J), *BCL2L* (K, L), *PCG1α* (M, N), *CBP* (O, P), in WT and HD NPCs and differentiated neuron. And cleaved caspase-3 protein analyzed using western blotting. The graph showed western blot band intensity analysis of WT, WT miR-196a, HD, and HD miR-196a neuron after treated with MG-132 for 24 hrs (Q).

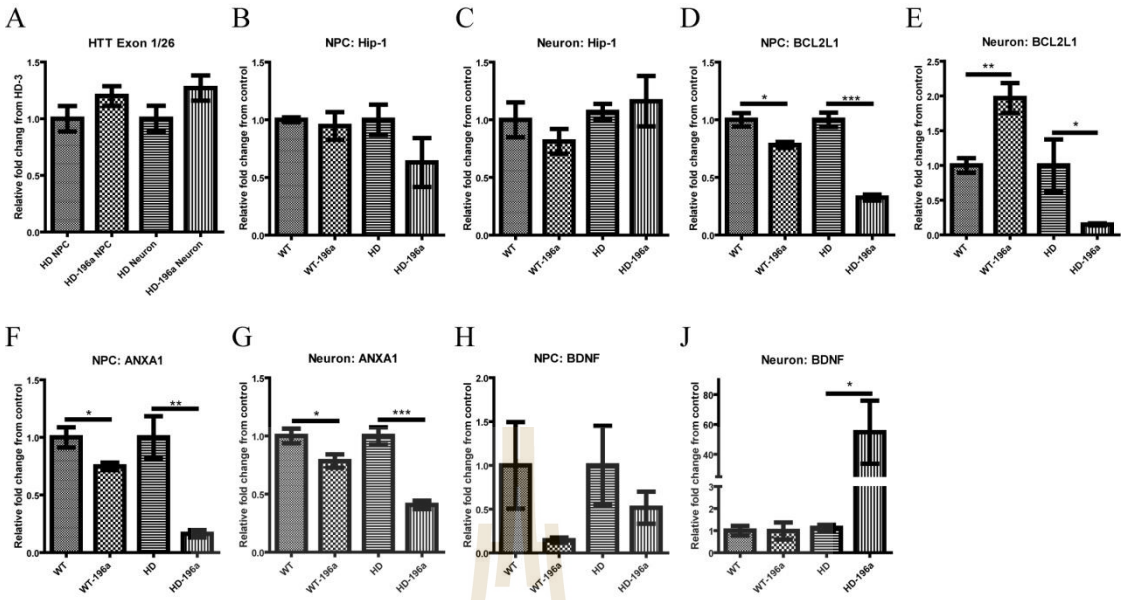

Figure 4.5 HD related and miR-196a related genes expression of over expressed NPCs and differentiated neural cells. SYBR green RT-qPCR analysis of mutant *HTT* gene exon 1 normalized with WT *HTT* gene exon 26 (*HTT* exon1/*HTT* exon26) in HD and HD-196a NPCs and differentiated neuron (A). SYBR green RT-qPCR analysis of *Hip-1* (B-C), *BCL-2L1* (D), *ANXA1* (F-G), and *BDNF* (H-J) -gene in WT, WT-196a, HD, and HD-196a NPCs and differentiated neuron. The expression level was calculated to relative fold change to individual WT or HD NPCs or neuron. Data are represented as mean±SEM. (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$: T-test)

4.4.4 Ameliorate cytotoxicity and apoptosis in NPCs and neural cells by miR-196a.

To investigate if the overexpression of miR-196a can ameliorate cytotoxicity and apoptosis in HD NPCs and neural cells, MG-132, a proteasome

inhibitor was used to induce cell stress followed by MTT assay and G6PD cytotoxicity analysis. HD cells have shown to be highly sensitive to cell stress induced by MG-132 in animal model and HD patient fibroblasts and neurons (Cheng et al., 2013; Seo et al., 2007; Snider et al., 2003). Overexpression of miR-196a in both WT-NPCs and HD-NPCs significantly improve viability (Fig 4.6A). In HD-NPCs overexpressing miR-196a also reached similar levels as WT-NPCs which indicated improvement of cell survival and enhanced NADH production (Fig 4.6A). Neuroprotective effect of miR-196a was determined by the withdrawal of growth factors (bFGF and mLIF) or the supplement of 10 uM of MG-132 for 24 hours followed by G6PD cytotoxicity assay. The withdrawal of growth factors or the supplement of MG-132 has no significant effect on cytotoxicity in WT-NPCs with or without the overexpression of miR-196a (Fig 4.6B). However, the overexpression of miR-196a in HD-NPCs showed beneficial effect on cell cytotoxicity when growth factors were withdrawn or treated with MG-132 (Fig 4.6C). To further confirm if miR-196a improves apoptosis in NPCs, WT- and HD-NPCs were challenged with MG-132 followed by immunostaining using antibody that specifically recognized cleaved caspase-3 followed by cell count analysis. MG-132 treatment induced apoptosis in both WT and HD-NPCs (Figs 4.6D and 4.6E). In WT-NPCs, overexpression of miR-196a significantly increased apoptosis, while there was no significant impact on apoptotic rate between WT-NPCs with or without overexpressing miR-196a when challenged with MG-132 (Figs 4.6D and 4.6I). A significant reduction in cleaved caspase3 positive cells was observed in HD-NPCs overexpressing miR-196a with or without MG-132 challenge (Figs 4.6E and 4.6I). Since neuroprotective effect of miR-196a was clearly shown in HD-NPCs, our next step was to determine if a similar effect was found in neurons. Cytotoxicity in HD

neurons was significantly higher compared to WT neural cells (Fig 4.6F) and the overexpression of miR-196a ameliorated cytotoxicity in both WT and HD neural cells (Figs 4.6G and 4.69H). Overall, we demonstrated the overexpression of miR-196a could ameliorate cytotoxicity and apoptosis in HD NPCs and neurons.

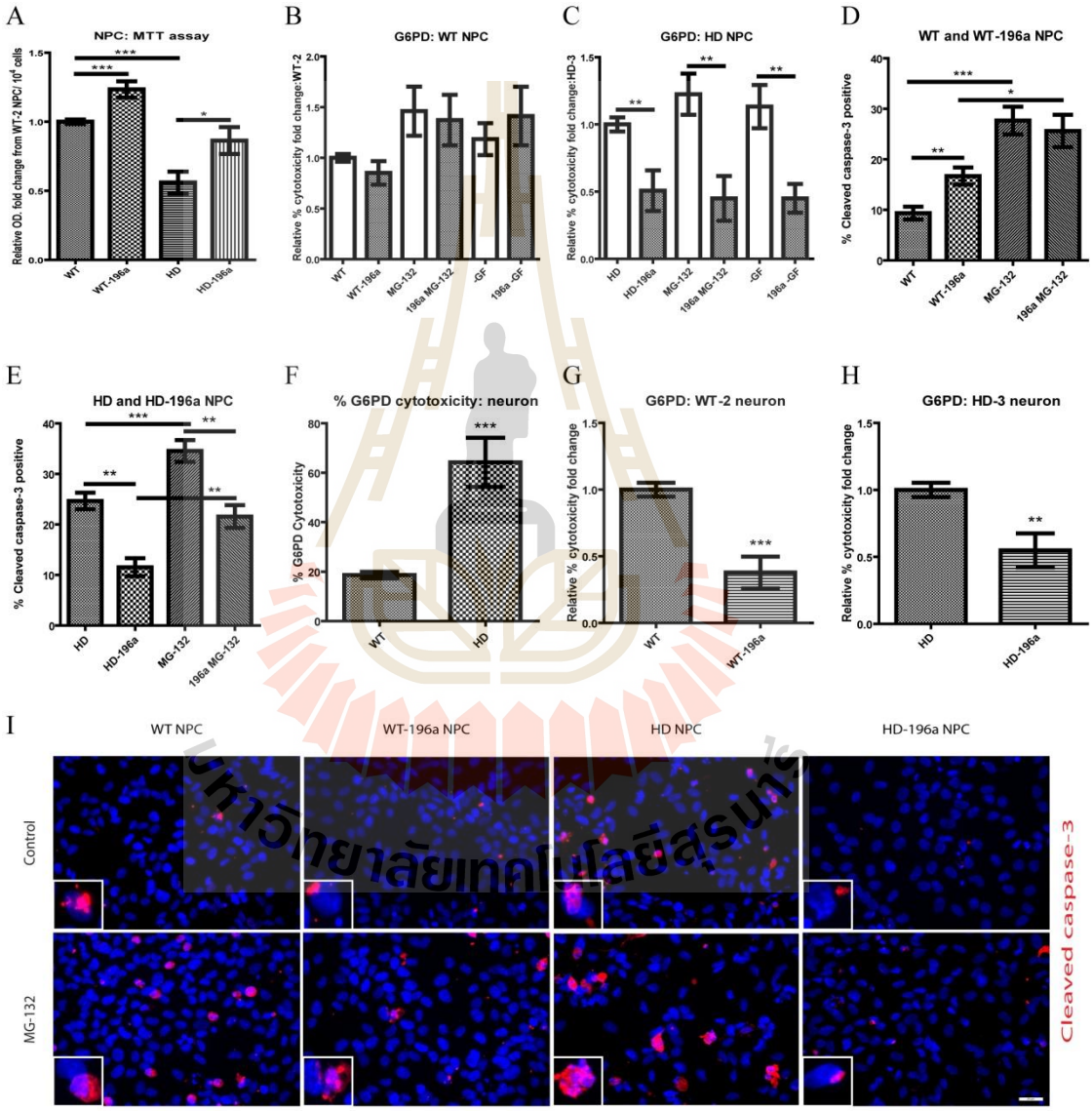

Figure 4.6 miR-196a reduced cytotoxicity and apoptosis in both WT-2 and HD-3 NPCs and differentiated neurons. MTT analysis showed cell viability of WT, WT-196a, HD and HD -196a NPCs (A). G6PD cytotoxicity

analysis of WT (B) and HD NPCs (C) with or without miR-196a over expression. NPCs were induced stress or apoptosis by growth factors withdrawn or MG-132 treated for 24 hrs. The % G6PD cytotoxicity level was calculated to relative fold change to individual WT or HD untreated control NPCs. WT, WT-196a, HD and HD-196a NPCs were induced apoptosis by MG-132 for 24 hrs before fixed and stained with cleaved caspase-3 antibody. The graph show % of cleaved caspase -3 positive NPCs cell from 5 pictures counting with 2 biological replicated which average cell counted were 2000-1200 cells (D, E). Immunocytochemistry staining images of WT, WT-196a, HD and HD-196a NPCs. NPCs were induced apoptosis by MG-132 for 24 hrs before fixed and stained with cleaved caspase-3 antibody (I).G6PD cytotoxicity analysis of differentiated neuron. WT compared with HD neuron (F). Differentiated neuron of WT (G) or HD (H) with or without miR-196a over expression were analyzed G6PD cytotoxicity at days 21 for differentiation. The % G6PD cytotoxicity level was calculated to relative fold change to individual WT or HD untreated control neuron. Data are represented as mean±SEM. (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$: T-test)

4.4.5 Effect of miR-196a on mitochondrial morphology and functions

Mitochondrial dysfunction has been demonstrated in HD animal models and patients that include the alteration of calcium buffering capacity (Panov et al., 2002), impaired bioenergetics (Acuña et al., 2013; Milakovic and Johnson, 2005),

increase oxidative stress and fission and fusion homeostasis (Jin et al., 2013). A recent report by Song and colleagues (Song et al., 2011) demonstrated mitochondrial fragmentation in HD neurons and fibroblasts. Impaired mitochondrion were fragmented with shorter and round morphology which was resulted from the increase mitochondrial fission rates over mitochondrial fusion in HD neurons (Song et al., 2011). NPC mitochondrial morphology was revealed by staining with Mitotracker (Molecular Probe) and imaged by using confocal microscopy. In HD-NPCs, short and fragmented mitochondrion surrounding the nucleus suggested extensive mitochondrial fission and was morphologically different from the elongated thread of mitochondrion in WT-NPCs which suggested a balance ratio of mitochondrial fusion (Fig. 4.7A). When miR-196a was overexpressed in HD-NPCs, reduction of mitochondrial fragmentation was observed with a more elongated thread of mitochondrion suggesting increase of mitochondrial fusion (Fig. 4.7A). To further confirm if mitochondrial function was improved by miR-196a, the expression of genes that were known to be involved in mitochondrial functions and were dysregulated in HD was quantitatively measured. Down regulation of *PGC-1α*, a regulator of mitochondrial biogenesis and oxidative stress (Chiang et al., 2010; Tsunemi et al., 2012) and CREB binding protein (*CBP*), a histone acetyltransferase (HAT) transcriptional co-activator that affect *PGC-1α* (Choi et al., 2009; Jiang et al., 2006; Martin et al., 2011) have been reported in HD. Up regulation of *CBP* was observed in HD-NPC expressing miR-196a (Fig. 4.7B) while the expression level of *PGC-1α* was not different with an increase expression of miR-196a (Fig. 4.7C). However, up regulation of both *CBP* and *PGC-1α* was observed in HD neurons overexpressing miR-196a (Figs. 4.7D and 5E). Similar expression pattern for *CBP* and *PGC-1α* was also observed in WT neurons except that *PGC-1α*

was not significantly increased in WT neurons expressing miR-196a (Fig. 4.7E). Our results suggested that miR-196a may improve mitochondrial function by the upregulation of *CBP* and *PGC-1α* to promote oxidation phosphorylation and reduce oxidative stress, which was consistent with the amelioration of cytotoxicity by miR-196

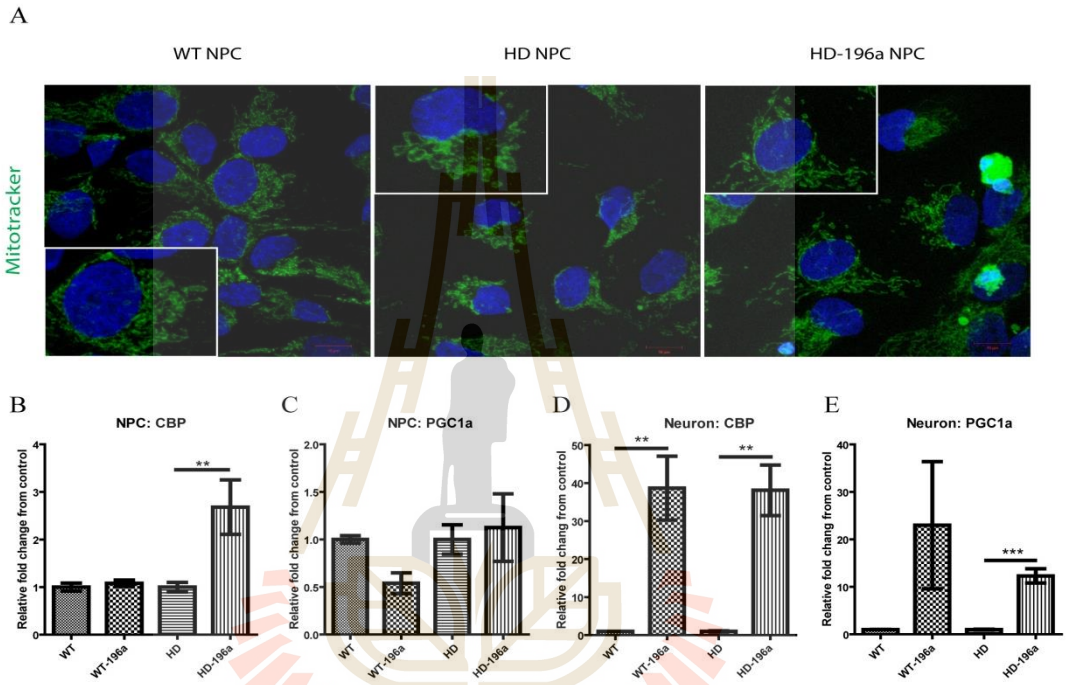

Figure 4.7 miR-196a improved mitochondrial morphology, genes expression. WT, HD and HD-196a NPCs staining of active mitochondrial membrane potential using Mitotracker® Green FM and nuclei labeled using Hoechst 33342 (100x magnification) (A). SYBR green RT-qPCR analysis of WT, WT-196a, HD and HD-196a (B,C) NPCs and (D,E) differentiated neuron. The expression level of (B or D) *CBP* and (C or E) *PGC1a* was calculated to relative fold change to individual WT or HD control. Data are represented as mean±SEM. (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$: T-test)

4.5 Discussion

HD is a neurodegenerative disorder caused by polyglutamine expansion in the HTT protein. The expansion of polyQ tract in HTT protein elicits systemic impact on the central nervous system and peripheral tissues (Her et al., 2014; Tong et al., 2011; Wade et al., 2014) which include dysregulation in global gene and miRNA expression (Kocerha et al., 2014; Lee et al., 2011), proteome (Chen et al., 2012), apoptosis (Hackam et al., 2000; Vis et al., 2005), and mitochondrial biogenesis (Milakovic and Johnson, 2005; Song et al., 2011). Based on miRNA array study on WT and HD monkey brains, miR-196a was one of the dysregulated miRNAs that was also found to be dysregulated in human HD brains (Hoss et al., 2014). Clinical benefit of miR-196a has been reported in neurodegenerative disorder such as SBMA via targeted silencing of CUGBP, Elav-like family member 2 (CELF2) (Miyazaki et al., 2012), and human immunodeficiency virus type 1 (HIV-1) associated neurodegeneration by interfered HIV-1 trans-activator of transcription (Tat) protein (Bagashev et al., 2014). Amelioration of HD phenotypes was also observed in HD mouse and stem cell models (Cheng et al., 2013; Hoss et al., 2014). However, the role of miR-196a in HD pathogenesis has not yet fully understand while HTT is not the direct target of miR-196a. We have shown that HD NPCs and differentiated neurons exhibited transcriptomic dysregulation, increase apoptosis and cytotoxicity that were similar to HD cellular phenotypes (Carter et al., 2014; Chen et al., 2014; Kocerha et al., 2009; Putkhao et al., 2013). Most importantly, these HD specific cellular phenotypes can be reversed by genetic and chemical treatment which suggested the potential of this progenitor cell model for studying pathogenic role of miR-196a in HD (Carter et al., 2014). Here we used the HD NPC model to investigate the impact of miR-196a on HD

cellular phenotypes which include susceptibility to oxidative stress, gene dysregulation and mitochondrial abnormality.

The overexpression of miR-196a did not affect progenitor properties and neural differentiation capability in both WT- and HD- NPCs (Fig 4.2). As expected, the expression of *mHTT* and *Hip-1* was not altered by the overexpression of miR-196a since *HTT* is not the putative target of miR-196a based on targetscan database (http://www.targetscan.org). One may argue that the *mHTT* transgene that was expressed in HD NPCs only carries the exon1 of the *HTT* gene driven by human polyubiquitin C promoter (Carter et al., 2014; Chan et al., 2014; Yang et al., 2008). Although *mHTT* might not be the target of miR-196a, the expression of endogenous *HTT* gene was also unaltered which suggested both endogenous *HTT* and *mHTT* genes were not affected by the overexpression of miR-196a (Fig 4.3B).

Transcriptomic dysregulation in HD brain, fibroblast ,and peripheral blood cells have been reported (Chang et al., 2012; Johnson and Buckley, 2009; Kocerha et al., 2013; Kocerha et al., 2014; Marchina et al., 2013; Mastrokolias et al., 2015). Among these dysregulated genes, we selected three candidate genes (BCL-2, ANXA1 and BDNF) that were dysregulated in the brain of HD with distinct cellular functions. BCL-2 protein was cleaved by caspase protease which is activated by *mHTT* (Cheng et al., 1997). The caspase-dependent cleavage inactivates BCL-2 antiapoptotic function and converts BCL-2 into BCL-2 associated X-protein-like protein that enhances cell death (Cheng et al., 1997; Macdonald et al., 1993). Upregulation of *BCL-2* may persist in HD patient brain as a compensatory response to suppress apoptosis and slow down degeneration in neurons. Our results in HD NPC and neurons indicated a significant increase in *BCL-2* expression compared to the control cells (Figs 4.4K and 4.4L.)

while the expression of miR-196a can suppress the expression of *BCL-2* in both NPC and neurons of the HD and WT cells (Figs. 4.5D and 4.5E). Our result in progenitor cell model was consistent with human HD brain in mimicking HD pathogenesis while the down regulation of *BCL-2* in miR-196a expressing cells suggested the possible pathogenic role of miR-196a in HD. *ANX1A* is targeted by miR-196a and suppressed apoptosis in cancer cells overexpressing miR-196a (Luthra et al., 2008). In HD patient blood, *ANXA1* was upregulated in presymtomatic and symptomatic patients compared to the controls (Borovecki et al., 2005). Consistently, we also observed the upregulation of *ANXA1*in HD NPCs and differentiated neurons (Figs 4.4E and 4.4F). The overexpression of miR-196a in HD NPCs and neurons can downregulate the expression of *ANX1A* to promote cell survival and reduced apoptosis. Finally, BDNF is one the most important growth factors that promote neural cell growth and survival, and has shown to be dysregulated in cell and animal models as well as in HD patients (Gambazzi et al., 2010; Giampà et al., 2013; Muller, 2014; The HD iPSC Consortium, 2012). The overexpression of miR-196a in HD- neurons enhanced *BDNF* expression which benefit neural cell survival (Fig. 4.5H) and improve neuronal morphology (Fig. 4.3E). The improvement of NPCs viability demonstrated by MTT assay and reduced cytotoxicity as shown by G6PD assay in miR-196s expressing NPCs and neurons further suggested the beneficial effect of miR-196a in ameliorating HD cellular defects. MTT assayis a commonly used method to examine cell proliferation based on the production of NADH and NADPH (Huet et al., 1992). Positive result in MTT test suggested improvement in mitochondrial functions based on the upregulation of *CBP* and *PGC1α* (Figs. 4.7B–E), and favorable changes in mitochondrial homeostasis (Fig. 5A). On the other hand, G6PD cytotoxicity assay was used for detecting cell death by

quantitative measurement of G6PD enzyme activity on enzyme leaked from cell with compromised plasma membrane (Batchelor and Zhou, 2004). Reduction of G6PD cytotoxicity was observed in miR-196a expressing HD NPCs and neurons with or without treatment with MG-132 (a proteosome inhibitor and apoptosis inducer), or induce cell stress by the withdrawal of growth factors. The resistant to MG-132 challenge in NPCs and neurons overexpressing miR-196a suggested the enhancement of proteosome function which has been demonstrated in mouse, and human stem cell model (Cheng et al., 2013; Seo et al., 2007). Caspases, cysteine protease characterized by their high specificity for substrates with an aspartic acid at the site of cleavage in the P1 position, play a prominent role in apoptosis (Pop and Salvesen, 2009). Additionally, caspase-3 inhibition by small molecule demonstrated neuroprotective effect in transgenic HD rat and mouse model (Chen et al., 2000; Toulmond et al., 2004). Overexpression of miR-196a reduce cleaved caspase-3 production in HD-196a NPCs and neural cells suggested the anti-apoptotic effect of miR-196a (Figs. 4.6D, 4.6I and 4.4Q).

Mitochondrial dysregulation in HD has been reported in various HD models (Eckmann et al., 2014; Jin et al., 2013; Kim et al., 2010; Milakovic and Johnson, 2005; Panov et al., 2002). Mitochondrial fragmentation in HD was due to the disturbance of mitochondria fusion and fission homeostasis (Kim et al., 2010). mHTT has a stronger binding affinity with dynamin-reated protein 1 (Drp-1) than WT HTT which resulted in the increase of mitochondrial fission rate in HD patient lymphoblast (Panov et al., 2002). Moreover, loss of Drp-1 function by shRNA silencing reduced mitochondrial fragmentation and neuronal cell death (Song et al., 2011). In HD NPCs overexpressing miR-196a, mitochondrial staining demonstrated the reduction of fragmentation in HD

NPCs. The expression of *CBP* and *PGC1α*, genes related to mitochondrial biogenesis function were significantly reduced in HD (Jiang et al., 2006; Martin et al., 2011). *PGC1α* is a transcriptional coactivator involved in energy homeostasis, adaptive thermogenesis, α-oxiadtion of fatty acid and glucose metabolism (Puigserver and Spiegelman, 2003). The expression of *PGC1α* is transcriptional controlled by CBP which is depleted in HD (Jiang et al., 2006). The overexpression of miR-196a resulted in the upregulation of both *CBP* and *PGC1α* genes, and improvement in mitochondrial functions and resistance to oxidative stress in HD NPC and neurons (Figs. 4.7B-4.7E).

Our findings demonstrated the upregulation of miR-196a could be a compensatory response in HD to defend against cell cytotoxicity, apoptosis, transcriptional dysregulation, proteosome and mitochondrial dysfunctions that lead to neuronal cell death. Although targets of miR-196a such as *ANXA1* and *BCL-2* have strongly suggested the role of miR-196a in regulating neural cell response to stress and adverse cellular conditions such as the accumulation of mHTT aggregates, continue effort in identifying gene targets of miR-196a that were dynamically changes during HD progression will lead to insight on the role of miR-196a in HD pathogenesis. Here, we showed that NHP HD NPCs and differentiated neurons replicate cellular changes and responses similar to those observed in other cell and animal models as well as in HD patient's brains.Thus HD NPCs and neurons could be used as an *in vitro* platform for studying HD pathogenesis, drug discovery research, and the development of biomarkers and novel therapeutics.

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CHAPTER V

OVERALL CONCLUSION

Huntington's disease (HD) is an inherited neurodegenerative disease caused by an expansion of CAG trinucleotide repeat (polyglutamine; polyQ) in the huntingtin (*Htt*) gene which leads to the formation of mutant HTT (mHTT) protein aggregates. An accumulation of mHTT protein results in glutamate-mediated excitotoxicity, proteosome instability, and apoptosis in nervous system that leads to motor, cognitive and psychiatric impairment. Currently, there is no cure for HD. A transgenic HD nonhuman primate (HD-NHP) model was developed with progressive development of clinical and pathological features similar to human HD which suggested the potential preclinical application of the HD-NHP model.

Our studies on RI, ME, and MB in HD NPCS and neurons demonstrated beneficial effects in ameliorating HD cellular phenotypes. Reduction in cytotoxicity and caspase-3 induced apoptosis via glutamate receptor inhibitors such as RI, ME and MB suggested our HD NPCs and neurons response to drugs in a similar manner to the other model systems. Moreover, the reduction of mHTT protein aggregate and soluble form mHTT protein was also observed in HD neurons treated with MB and RI. These results suggested HD NPCs and neurons can be used for assessing therapeutic benefits of novel drugs or small molecules.

Elevated expression of miR-196a was observed in both HD-NHP and human HD brains. Furthermore, clinical benefit of miR-196a has been reported in

neurodegenerative disorder such as SBMA, and HIV-1 associated neurodegeneration. Amelioration of HD phenotypes was also observed in HD mouse and stem cell models. Moreover, the overexpression of miR-196a did not affect progenitor properties and neural differentiation capability in both WT- and HD- NPCs. Our findings demonstrated the upregulation of miR-196a could be a compensatory response in HD to defend against cell cytotoxicity, apoptosis, transcriptional dysregulation, proteosome and mitochondrial dysfunctions that lead to neuronal cell death. Although targets of miR-196a such as *ANXA1* and *BCL-2* have strongly suggested the role of miR-196a in regulating neural cell response to stress and adverse cellular conditions such as the accumulation of mHTT aggregates, continue effort in identifying gene targets of miR-196a that were dynamically changes during HD progression will lead to insight on the role of miR-196a in HD pathogenesis.

Here, we showed that NHP HD NPCs and differentiated neurons replicate cellular changes and responses similar to those observed in other cell and animal models as well as in HD patient's brains. Thus HD NPCs and neurons could be used as an *in vitro* platform for studying HD pathogenesis, drug discovery research, and the development of biomarkers and novel therapeutics.

BIOGRAPHY

Tanut Kunkanjanawan was born in Bangkok, Thailand on July 8th, 1985. He finished his high school from Mahidolwitayanusorn School in Nakorn Pathom. In 2009, he received his Doctor of Veterinary Medicine degree (DVM) from the faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand. Following graduation, he began his career as a Veterinarian at, Kalaprapruk Animal Hospital Thailand. At that time, his cousin began to open the Bangkok Stem Cell, the cryogenic stem cell bank of cord blood and Mesenchymal stem cell banking for new born babies and Adipose Mesenchymal Stem Cell for adult. He starts to get interested in the field of stem cell-based therapy to help his cousin run the company. Five years later, he applied for a PhD. degree program at the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology with Assoc. Prof. Dr. Rangsun Parnpai. During his study, he received a scholarship from the Royal Gloden Jubilee PhD. Program. During five years of his education, he preformed his experiment in Assoc. Prof. Dr. Rangsun Parnpai's laboratory and Assoc. Prof. Dr. Anthony W.S. Chan, DVM, 's laboratory at Yerkes National Primate Research Center, Emory University, USA.