Induced pluripotent HD monkey stem cells derived neural cells for drug discovery

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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 pathogenesis has been extensively studied, effective treatment of HD has yet to be developed. Therapeutic discovery research in HD has been reported using yeast, cells derived from transgenic animal models and HD patients and induced pluripotent stem cell (iPSCs) from patients. A transgenic nonhuman primate model of HD (HD monkey) shows neuropathological, behavioral and molecular changes similar to an HD patient. Additionally, neural progenitor cell (NPC) derived from HD monkeys can be maintained in culture and differentiated to neural cells with distinct HD cellular phenotypes including the formation of mHTT aggregates, intranuclear inclusions and increased susceptibility to oxidative stress. Here, we evaluated the potential application of HD monkey NPCs (HD-NPCs) and neural cells (HD-NCs) as an in vitro model for HD drug discovery research.

Introduction

Neurodegenerative diseases affect millions of patients 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. HD is a fatal neurodegenerative disorder caused by trinucleotide repeat expansion. The expansion of cytosine-adenine-guanine (CAG) repeats in the first exon of the HTT gene on chromosome 4p16.3 results in the development of HD. An individual that has more than 36 CAG repeats will develop HD. 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.¹ Drugs and small molecules are expected to elicit systemic benefit on HD phenotypes. Besides the challenge in developing

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effective therapeutics, the lack of a preclinical large animal model has limited the option for assessing efficacy of novel therapeutics with outcome measurements 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. The recent development of an HD monkey model that developed HD clinical features with progression similar to human HD patients suggested the potential of this unique model system for preclinical study. Since the HD monkey model holds great promise as a preclinical animal model of HD, the current study has evolved based on the recent development of HD monkey NPCs (HD-NPCs) and their derivative neural cells (HD-NCs) that developed HD cellular features including increased susceptibility to oxidative stress, increased apoptosis, the formation of mHTT aggregate and intranuclear inclusions. To further determine if HD-NPCs and HD-NCs derived from pluripotent stem cells (PSCs) of HD monkeys are a potential in vitro platform for HD drug discovery research, we examined 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 effects 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-NCs can be used as an in vitro platform for drug discovery research of HD.

Materials and Methods

Neural progenitor cell culture and neural differentiation

WT-2 (rZH-2: WT Embryonic Stem Cells (ESCs)), WT-14 (RiPS-14: WT iPSCs), HD-2 (RPg-2: HD ESCs) and HD-3 (RiPS-3: HD iPSCs) derived NPCs were used in this study. NPC derivation and culture were described previously. In brief, NPCs were cultured with neural proliferation medium (NPM), which was composed 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 coated with 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 an established protocol. In brief, WT-NPCs and HD-NPCs were seeded onto P/L coated tissue culture dish in 30,000 cell/cm² concentration. On the next day, neural differentiation medium, composed 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 cultured for four days. 0.2 μg/mL sonic hedgehog (SHH) (R&D) and 0.1μg/mL fibroblast growth factor-8 (FGF-8) (R&D) were then added into differentiation medium. Ascorbic acid (200mM, Sigma) was added into the differentiation medium on day eight and cultured until day 21 when differentiated neural cells were collected for analysis.

To determine drug response, NPCs and NCs were treated with 10 μM RI (Sigma), 10 μM ME (Sigma), and 0.1 μM MB (Sigma) for 24 hours prior to analysis. Except for MB treatment, 0.1 μM MB (Sigma) was supplemented in the last seven days of differentiation. All samples were collected for viability, cytotoxicity and apoptosis analysis.
**Immunocytochemistry**

NPCs and NCs cultured on P/L-coated glass slides were fixed in 4% PFA after treatment with candidate drugs. Fixed cells were permeabilized and blocked 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. Slides were washed twice in PBS followed by incubation with secondary antibody for one hour at room temperature. Images were taken by using fluorescence microscope equipped with CellSens software (Olympus). To quantify the percentage of positive cells, five pictures were captured randomly and counted in two biological replicas by examiner with no knowledge of the samples. A total of 1,000 to 2,500 cells were counted for each treatment. Primary and secondary antibodies are listed in Supplementary Table 1.

**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 SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) using CFX96 Real-Time Detection System (Bio-Rad). qPCR primer sequences are listed in Supplementary Table 2.

**Western Blot Analysis**

After treatment for 24 hours (RI and ME) or seven days (MB), cells were harvested and total protein was extracted by using RIPA buffer with protease inhibitor. Total protein concentration was quantified by Bio-Rad DC Protein Assay (Bio-Rad). Equal amount of protein extract was loaded and separated by electrophoresis in 9% SDS-PAGE gel. Proteins were transferred onto a PVDF membrane and probed with primary antibodies mEM48 and γ-tubulin (Millipore) at 4°C overnight followed by thorough washes and incubation with secondary antibody. Positive band was visualized by using SuperSignal West FemtoChemiluminescent Substrate (Thermo Scientific) and imaged by ChemiDoc Imaging System (Bio-Rad).

**Cell viability and G6PD cytotoxicity assay**

For cell viability, WT-NPCs or HD-NPCs were plated in a P/L coated 96 wells plate. Next day, NPCs were treated with drugs in culture medium for 24 hours. Cell viability assay was performed by MTT assay (ATCC) following manufacturer’s instructions.

For cell cytotoxicity, WT-NPCs or HD-NPCs (or WT-NCs or HD-NCs) were treated with drugs in culture medium for 24 hours. Vybrant G6PD Cytotoxicity Assay Kit (Life Technologies) was used to determine cytotoxicity according to manufacturer’s instructions.

For NCs, WT-NPCs or HD-NPCs were differentiated 21 days in 24 wells plate coated with P/L. On day 21 fresh media with drugs was replaced followed by 24 hours incubation. Media was then collected and spun down to collect all suspended cells. The cell pellets were then re-suspended in G6PD reagent with lysis buffer and added into culture well. The
percentage of G6PD released in media was normalized with total neural cell lysate from corresponding well.\(^4\)

**Statistical analysis**

All experiments were composed of three biological replicas. Statistical analysis was performed by using SPSS 12.0 (SPSS, Inc., USA). Data was represented as mean ± standard error. Statistical differences were calculated using Analysis of variance (ANOVA). Differences were considered significant at *\(^p< 0.05\) and statistically significant at **\(^p< 0.01\).

**Results**

**Characterization of NPCs and NCs**

WT-NPCs and HD-NPCs derived from WT and HD monkey PSCs were used to determine if they could be used 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-NCs. WT-NPCs and HD-NPCs expressed progenitor cell markers, which include Sox-2, Pax-6, Musashi-1, and Nestin (Supplementary Fig. 1A). The expression of mHTT was significantly up regulated in the two HD-NPC lines and their differentiated NCs (HD-2 and HD-3) when compared to the two WT-NPC lines and their differentiated NCs after normalized to endogenous HTT levels (WT-2 and WT-14) (Fig. 1A). Moreover, HD-3 has a much higher expression level of mHTT than HD-2, which was consistent to prior report (Fig. 1A).\(^4\) Western blot analysis of WT-14 and HD-3 NPC and NCs revealed the accumulation of mHTT aggregate in HD-NCs, but not in WT-NCs and NPCs (Fig. 1B). G6PD Cytotoxicity assay was used to determine cytotoxicity in NPCs and NCs. No difference was found on cytotoxicity in WT and HD NPCs (Fig. 1C). However, a significant increase in cytotoxicity was observed in HD-NCs when compared to WT-NCs (Fig. 1D). These results suggested the impact of mHTT in cytotoxicity and mHTT protein aggregation in HD cells. Interestingly, both cytotoxicity and mHTT aggregation were significantly increased in HD-NCs when compared to corresponding HD-NPCs. This finding suggested a differential susceptibility to mHTT in NPCs and NCs, thus variations in response to treatments are also expected in HD-NPCs and HD-NCs.

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

RI,\(^9\) ME\(^10\) and MB\(^8\) 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-NPCs and HD-NPCs (Fig. 2A-H). This result is consistent with cytotoxicity observed in NPCs in that no significant difference in cytotoxicity was observed when compared between WT-NPCs and HD-NPCs.

**Assessing drugs response in WT and HD differentiated NCs by G6PD cytotoxicity assay**

WT-NPCs and HD-NPCs were differentiated into NCs followed by treatment with RI, ME and MB. All differentiated NCs expressed neural specific markers including Tuj-1, doublecortin (DCX), microtubule-associated protein-2 (Map-2), and tyrosine hydroxylase...
(TH) (Supplementary Fig. 1B). The formation of intranuclear inclusions and mHTT aggregates was observed in HD-NCs with a significant increase in cleaved caspase-3 positive cells (Supplementary Fig. 1B). RI, and ME were administered into culture media on the last day of neural differentiation for 24 hours while MB was added into culture media during the last seven days of differentiation. RI, ME and MB did not have an effect on NCs derived from the two WT-NPC lines (Fig. 3A and 3B). However, significant reduction in cytotoxicity in HD-2 and HD-3 NCs was observed (Fig. 3C and 3D, respectively). These results suggested that cytotoxicity in HD-NCs can be ameliorated by RI, ME and MB.

Ameliorating apoptosis and mHTT aggregation in HD-NCs

Activated caspase induced apoptosis has been reported as one of the pathological hallmarks in HD. Similar observation was also described in NCs derived from HD-NPCs. To examine if RI, ME and MB ameliorate apoptotic responses, they were administered into culture media of differentiated WT-NCs and HD-NCs for 24 hours followed by fixation and immunostaining using specific antibody that recognized cleaved caspase-3 and microtubule-associated protein-2 (Map2). Total cell count of Map-2 (green) and cleaved caspase-3 (red) positive cells was used to determine the ratio of apoptotic NC population (Fig. 4 A–C). In WT control NCs, the addition of RI, ME and MB did not affect the number of caspase-3 positive cells or apoptotic NCs (Fig. 4D). Whereas, significant reduction in caspase-3 positive cells was observed in HD-NCs (HD-2 and HD-3) treated with RI, ME and MB (Fig. 4B–D, E–F). Additionally, WT-14 and HD-3 NCs treated with RI, ME and MB were double immunostained using doublecortin (DCX) and mEM48 antibodies to determine if mHTT aggregation was affected in NCs. As expected, no aggregate was observed in WT-NCs with or without treatment (Fig. 5A). In contrast, mEM48 positive NCs were reduced in HD-NCs in all treatment groups (Fig. 5B), which was further confirmed by western blot analysis using mEM48 antibody (Fig. 5C–D). Between the three drugs, MB has a more robust impact on the reduction of mHTT aggregates as well as soluble form mHTT protein when compared to RI and ME (Fig. 5C–D). These results suggested that RI, ME and MB can reduce caspase-3 induced apoptosis in HD-NCs with RI being the most prominent, and MB having the least effect. In the case of reducing mHTT aggregation, MB was the most effective while ME had no significant impact on both aggregated and soluble forms of mHTT protein. These data were consistent between cytotoxicity, reduced apoptosis and the formation of mHTT aggregates in HD-NCs.

Discussion

In this study, three drugs that have shown beneficial effects in several neurodegenerative diseases including HD, PD and AD were used to determine if HD monkey iPSCs derived NPCs and NCs can be used as an 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 HD-NCs are highly susceptible to oxidative stress with HD cellular phenotypes including mHTT aggregates that can be reversed by genetic and biochemical approaches. HD-NCs express glutamate receptors, which are the target of RI and ME, and a positive impact was observed similar to prior studies. HD-NPCs and HD-
NCs are unique platforms for drug discovery research \textit{in vitro} while HD monkeys can be used as preclinical animal model for assessing therapeutic efficacy.\textsuperscript{3}

NPCs and NCs of HD were extremely sensitive to oxidative stress induced by hydrogen peroxide or by the withdrawn of growth factors in culture that resulted in a significant increase in apoptosis.\textsuperscript{4, 11} Compared to HD-NCs, HD-NPCs have lower mHTT expression level and mild HD cellular phenotypes that may limit its application in drug discovery research because of the lack of robust outcome measurements used to determine the beneficial effect of treatments. Many \textit{in vitro} platforms have been developed for drug discovery research which include HD yeast model\textsuperscript{12}, brain slice model\textsuperscript{13} genetically modified 293/HEK cell\textsuperscript{14}, primary neuronal culture from transgenic rodents\textsuperscript{15, 16}, transgenic rodent cell line such as PC12\textsuperscript{16}, transgenic NHP cell line\textsuperscript{4} and NCs derived from human iPSCs.\textsuperscript{17, 18} However, similar to HD-NPCs, robust HD cellular phenotypes are very limited in these model systems with a narrow margin for determining therapeutic effect. To the contrary, HD-NCs 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 withdrawn of growth factors. Most importantly HD-NCs can be generated by \textit{in vitro} differentiation of HD-NPCs.\textsuperscript{4}

Improvement in cytotoxicity, caspase-3 induced apoptosis and the reduction of mHTT protein aggregates were observed in HD-NCs 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.\textsuperscript{19} RI ameliorates glutamate-mediated excitotoxicity by blocking the voltage-gated sodium channel and inhibits the release of glutamate at the presynaptic terminus.\textsuperscript{20} 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).\textsuperscript{9} In YAC128 MSN model, RI significantly reduced glutamate induced apoptosis.\textsuperscript{7} Besides HD, the neuroprotective effect of RI has also been reported in PD model by attenuating dopaminergic neuron degeneration and suppressed reactive astrocytosis in the striatum.\textsuperscript{21} Furthermore, in amyloidotic lateral sclerosis (ALS), RI improves the survival rate of ALS patients.\textsuperscript{22, 23} ME is a specific, moderate-affinity, uncompetitive, open-channel N-methyl-D-aspartate glutamate receptor (NMDAR) antagonist.\textsuperscript{7} 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.\textsuperscript{24} The elevation of NMDAR activity alters intracellular Ca\textsuperscript{2+} signaling pathways that lead to cell death, and a synaptic Ex-NMDAR blocker can attenuate mHTT-induced striatal atrophy and motor learning deficits in YAC128 model.\textsuperscript{25} Thus, glutamate receptor antagonist such as RI and ME have been considered for reducing cytotoxicity in HD, and two open label clinical trials on these two drugs have shown neuroprotective effect not only in HD,\textsuperscript{9} but also in PD\textsuperscript{26} and AD.\textsuperscript{27}

MB belongs to the phenothiazinium family, which has been used in Phase IIb clinical trial for the treatment of mild to moderate AD. Significant improvement in cognitive function and slowing down of AD progression has been reported.\textsuperscript{28} MB also reduced soluble A\textsubscript{\textbeta} and rescued early cognitive deficit in AD transgenic mice.\textsuperscript{29} 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.\textsuperscript{8} In primary neurons expressing mHTT, MB reduced the formation of both oligomeric and insoluble mHTT and increased the survival of neurons.\textsuperscript{8}

Our studies on RI, ME, and MB in HD-NPCS and NCs demonstrated similar beneficial effects in ameliorating HD cellular phenotypes, which were consistent with prior studies in different model systems.\textsuperscript{18} Reduction in cytotoxicity and caspase-3 induced apoptosis via glutamate receptor inhibitors such as RI and ME suggested our HD-NPCS and NCs response to drugs in a similar manner to the other model systems. Similar effect on the reduction of mHTT protein aggregate and soluble form mHTT protein was also observed in HD-NCs treated with MB and RI.\textsuperscript{8, 20} In 3-nitropropionic induced HD model, ME has shown to reduce HTT levels that further suggested its potential in reducing proteolytic HTT fragment\textsuperscript{30}. However, a similar suppression effect in mHTT by ME has not been reported in other model systems which is consistent to our findings (Figure 5C). To demonstrate the effect on mHTT aggregation, we chose to use HD-3 because more robust mHTT aggregation has been observed.\textsuperscript{4} HD-3 has 72 CAG repeats while HD-2 has only 29 CAG repeats with much lower mHTT aggregation that limited it from assessing impact on mHTT aggregation. While mechanism and therapeutic effect of RI, ME and MB in HD have yet to be determined, our HD-NPCS and NCs offered a unique alternative platform not only for understanding the underlying mechanism of these effective drugs, but also a novel platform with robust phenotypic outcome measures for drug discovery research in HD.

In conclusion, this study is not aimed to discover novel drugs for HD, but for validating the potential application of HD-NPCs and NCs 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. Although our prior study has suggested the potential of HD-NPCS and NCs in drug discovery research based on the positive effect of ME in ameliorating cytotoxicity, in-depth validation using different assessment tools and methods was not performed.\textsuperscript{4} Here we perform direct comparison on protective effect of three known drugs using multiple assays to confirm if HD-NPCS and NCs can effectively response to drugs that have shown benefits on neurodegenerative diseases. While HD-NPCS and HD-NCs can serve as an in vitro drug discovery platform, HD monkeys with similar genetic constitution as the HD-NPCS and HD-NCs can be used for preclinical assessment prior to HD monkeys preclinical study followed by human clinical trial to further ensure the safety and potential benefit of the new treatment.\textsuperscript{2, 3}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

All experimental procedures described in this study were approved by Emory Environmental Health and Biosafety committee. We thank Ms. Leslee Sinclair for her assistance in proofing and editing. Tanut Kunkanjanawan and Rangsan Pranpai are supported by the Royal Golden Jubilee PhD program of Thailand Research Fund. YNPRC is supported by the Office of Research and Infrastructure Program (ORIP)/OD P51OD11132. This study is supported in part by grant awarded by the NINDS (NS084163) and the ORIP/NIH (OD010930; Transgenic Huntington’s Disease Monkey Resource) to AWSC.
References


Figure 1. Characterization of WT and HD NPCs and differentiated neural cell
(A) RT-qPCR analysis of exon 1 of HTT (mHTT and endogenous HTT) and normalized with
exon 26 of HTT gene (HTTexon 1/HTT exon26) expression of WT-2, WT-14, HD-2 and
HD-3 NPCs and differentiated neural cells.
(B) Western Blot analysis of mHTT protein aggregation of WT-14 NPCs, HD-3 NPCs and
their differentiated NCs was detected by mEM48 antibody and normalized with γ-Tubulin.
Negative control: WT brain. Positive control: HD monkey brain protein
(C-D) NPCs and neural cells cytotoxicity was determined by using G6PD cytotoxicity assay.
Data are represented as mean±SEM of relative fold change to the average of WT control
gene expression (1A) and cytotoxicity (%; 1C-D). (* p < 0.05, ** p < 0.01, and *** p <
0.001: ANOVA)
Figure 2. Effect of drugs on WT and HD NPCs was determined by using MTT assay and G6PD cytotoxicity assay

(A, C, E, G) NPCs viability was determined by using MTT assay after 24 hrs treatment with drugs, 10 uM RI, 10 uM ME, 0.1 uM MB
(B, D, F, H) NPCs cytotoxicity was examined by using G6PD cytotoxicity assay after 24 hrs treatment with drugs, 10 uM RI, 10 uM ME, 0.1 uM MB, NT-No Treatment

Data are represented as mean±SEM of fold change comparing with untreated control. (* p < 0.05, ** p < 0.01, and *** p < 0.001: ANOVA)
Figure 3. Effect of drugs on WT and HD differentiated NCs was determined by using G6PD cytotoxicity assay.
Cytotoxicity of WT-2 NC (A), WT-14 NC (B), HD-2 NC (C) and HD-3 NC (D) differentiated NC was examined by using G6PD cytotoxicity assay after 24 hrs treatment with 10 μM RI, 10 μM ME, and 7 days treatment for 0.1 μM MB. NT-No Treatment
Data are represented as mean±SEM of fold change comparing with untreated control. (*** p < 0.001: ANOVA)
Figure 4. Effect on apoptosis in differentiated HD-NCs

(A–C) Immunostaining of WT-14 (A), HD-2 (B), and HD-3 (C) differentiated NCs after 24 hrs treatment with 10 uM RI, 10 uM ME, and 7 days treatment for 0.1 uM MB by using cleaved caspase-3 and Map-2 antibody. Scale bar = 20μm

(D–F) Percentage of cleaved caspase-3 positive WT controls (D), HD-2 (E), HD-3 (F) differentiated NCs after 24 hrs treatment with 10 uM RI, 10 uM ME, or 7 days treatment of 0.1 uM MB. A total of 1,000 to 2,500 cells were counted for each treatment.

Data are represented as mean±SEM comparing with untreated control. NT-No Treatment.
(* p < 0.05, ** p < 0.01, and *** p < 0.001: ANOVA)
Figure 5. Effect of drugs on mHTT protein aggregation in HD-NCs

(A, B) Immunostaining of WT-14 (A), and HD-3 (B) differentiated NCs after 24 hrs treatment with 10 uM RI, 10 uM ME, or 7 days treatment of 0.1 uM MB before stained with mEM48 and DCX antibodies. Scale bar = 20μm.

(C) Western Blot analysis of mHTT protein aggregation of WT-14 and HD-3 NCs after 24 hrs treatment with 10 uM RI, 10 uM ME, or 7 days treatment of 0.1 uM MB using mEM48 antibody and normalized with γ-Tubulin. Positive control: HD monkey brain. NT-No Treatment.
(D) Quantitative measurement of mHTT aggregate by measuring optical intensity of immunoblot of WT-14 and HD-3 NCs after 24 hrs treatment with 10 uM RI, 10 uM ME, or 7 days treatment of 0.1 uM MB using mEM48 antibody and normalized with $\gamma$-Tubulin as shown in (Figure 5C).