11-2014

Neural Stem Cells as a Model to Study Huntington's Disease

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https://doi.org/10.33015/dominican.edu/2014.bio.08

Neural Stem Cells as a Model to Study Huntington's Disease

A thesis submitted to the faculty of
Dominican University of California
in partial fulfillment of the requirements
for the degree

Master of Science
in
Biology

By
Rawan Bakhsh
San Rafael, California
November, 2014

CERTIFICATION OF APPROVAL

I certify that I have read *Neural Stem Cells as a Model to Study Huntington's Disease* by Rawan Bakhsh, and I approved this thesis to be submitted in partial fulfillment of the requirements for the degree: Master of Sciences in Biology at Dominican University of California.

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Abstract

Huntington's disease (HD) is a heritable neurodegenerative disorder that affects muscle coordination and diminishes cognitive abilities, by affecting the medium spiny neurons in the brain. In HD patients, neurons are damaged and destroyed because of the toxicity of the mutant Huntington protein (mHtt). The mechanism of how mHtt protein affects the neurons is unknown. In this study we explored the effects of mHtt expression by looking at changes in huntingtin localization, changes in the expression and co-localization of related proteins and differences in cell morphology. We examine how this expression affects the cytoskeletal structures using neural stem cells Q7 (wild type) and Q140 (mHtt) and differentiated neurons as a model for studying HD. In addition we looked at the interference of mHtt protein with RRAS, the downstream signaling components of Plexin/Semaphorin pathway of the neurons. Our work began with optimization of the growth conditions of the cell lines of our model cell system. We then focused on developing protocols for differentiation into neurons, and continued with immunocytochemistry studies and confocal microscopy for imaging the fluorescently labeled cells. We found differences in growth rate and morphology between Q7 and Q140 cell lines. We studied the effects of mHtt protein on the differentiation process of the neurons and noticed differences in the mHtt protein expression between both differentiated cell lines. There is evidence that mHtt interferes with cell adhesion, motility, and molecules related to signaling and cytoskeleton remodeling. The results of these studies leave us with a well-characterized tool for the study of HD.

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LIST OF ABBREVIATIONS

CAG	Cytosine-Adenine-Guanine
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
ES	Embryonic Stem cells
FGF2	Fibroblast Growth Factor 2
HD	Huntington Disease
Hdh	Huntington disease homozygous
Htt	Huntingtin protein
mHtt	Mutant Huntingtin protein
NGC	Normal Goat serum
NSCs	Neural Stem Cells
NSEM	Neural Stem cells Expansion Medium
PS	Pluripotent Stem cells
RT	Room Temperature
WT	Wild Type

Introduction

Huntington's disease (HD) is an inherited disorder characterized by neuronal dysfunction and degeneration in the brain that affects muscle coordination and diminishes cognitive abilities. It is caused by a variant of the Huntingtin gene that includes an extended stretch of glutamine residues. It is named after George Huntington, the physician who first described the illness in 1872. HD used to be known as Huntington's chorea, from Greek for choreography, or dance. HD affects both women and men and all ethnic groups. People from two to 80+ years of age can develop HD.

Approximately 200,000 Americans are at risk of inheriting the disease from an affected parent. Those who have a parent with HD have a 50 percent chance of inheriting the defective gene. This disease results in a gradual patient decline over a period of 10 - 25 years, typically leading to a very poor quality of life with complete dependence on others for care.

In HD patients, the presence of mutant Huntingtin protein (mHtt) is associated with neuronal damage and eventual death due to severe neurodegeneration. There are multiple theories regarding the mechanism of the mHtt toxicity, but there is no definitive evidence for the cellular process that causes this disease. Normal functioning Huntingtin protein (Htt) contains a stretch of 10-35 glutamines (the number of CAG repeats present in the Huntington gene). Patients with more than 40 CAG repeats in their Huntingtin gene are considered to have the condition. The mechanism of the cellular toxicity for this protein is not yet clearly elucidated, but many of the studies are hovering around one hypothesis,

that is mHtt toxicity is related to direct interaction with the neuronal signaling pathways (Miller, JP., et al., 2012).

Neurons are the basic building blocks of the nervous system. Neurons are specialized in receiving and transmitting the signaling information. They are the information processing units of the brain. Each part of the neuron plays a role in the communication of information throughout the body. Any malfunction in this system can lead to serious imbalance of the human body and with HD patients the malfunction goes much further causing the death of the neurons and eventually the end of human life. What has been understood about the mHtt protein so far that it is a toxic protein for the neurons and it may interact with some of downstream signaling component of Plexin/Semaphorin pathway of the neurons.

Slit /Robo and Plexin/ Semaphorin are signaling pathways controlling neural outgrowth and axonal guidance. Any interaction with mHtt may be associated with mHtt toxicity. In both of these pathways extracellular ligands bind to their cognate receptors and signal through downstream effectors to inhibit binding of integrin to extracellular matrix. This inhibition leads to growth cone collapse in the case of migrating axons. In this regard, Slits and Semaphorin are thought of as repulsive signals in that they inhibit cell migration and axonal outgrowth of neurons. According to work done by the Hughes lab at the BUCK Institute for Age Research, RRAS was identified as a downstream component of the Plexin/Semaphorin pathway and as a potent loss of function suppressor of death in cells expressing mHtt, by screening for RNAi modifiers of mHtt toxicity (Miller JP et al. 2012) (Fig1).

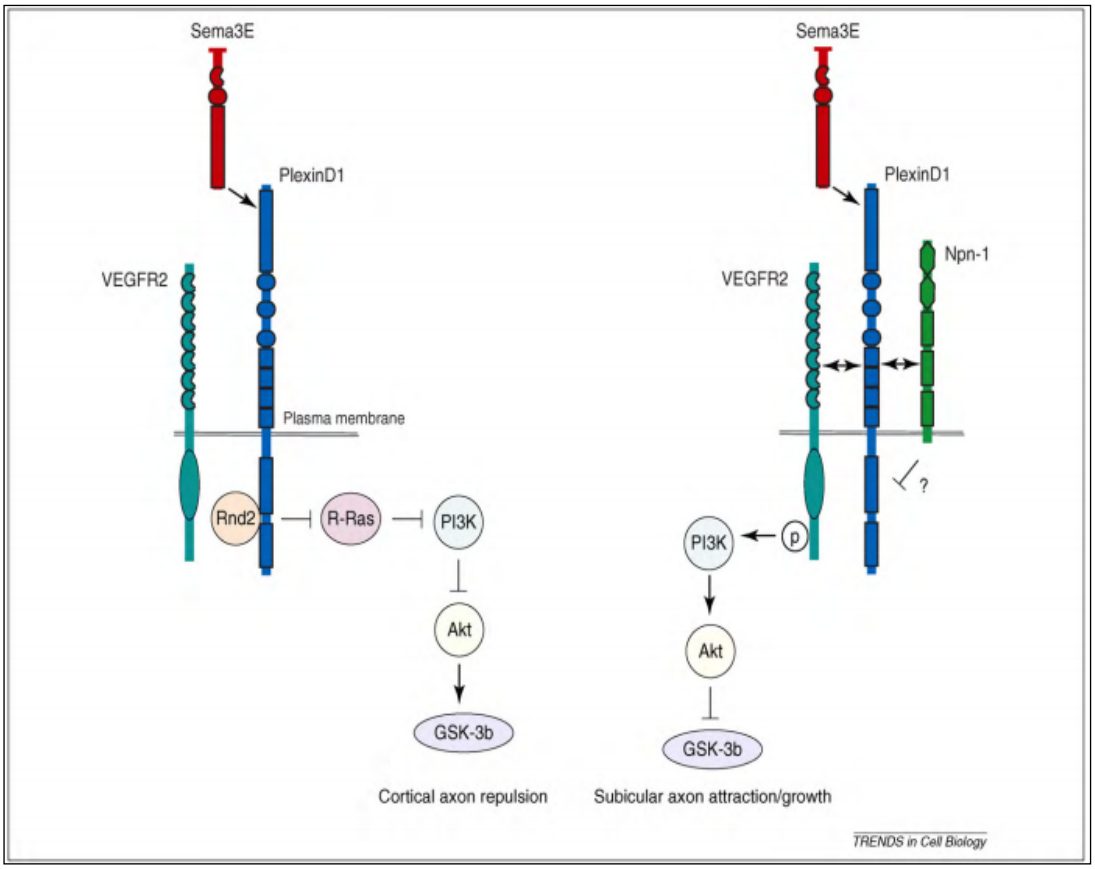

Figure 1: The Plexin-Semaphorin signaling pathway. Receptor activation inhibits RRAS activity, which leads repulsive signaling (in relation to axon guidance). RRAS inhibition alters cytoskeletal dynamics to favor retraction of filopodia and detachment from the extracellular matrix. (Derijck, A. A., 2010)

The lack of effective interventions to HD disease means that patients and the families that care for them have very few options and must suffer with the debilitating symptoms. Developing such interventions requires an understanding of the mechanism of the way

mHtt protein negatively impacts neuronal function. Mouse models of HD are important for understanding disease progression and for the development of therapeutics (Figiel, et al., 2012, Pouladi et al., 2013) Cell culture models derived from these mice are an important tool for studies of the effects of mHtt protein interactions because they can provide neurons similar to the neurons predominantly affected in Huntington's patients.. These disease models provide researchers with a replenishing supply of live cells, without the need for extracting from live animal brains. The neural stem cells are very important tool in studying the HD because they are non-immortalized self-renewing cells with widespread differentiation potential. While human cell HD model systems have the advantage of a better match to the true disease state, they are not a good for these early studies because they are more difficult to grow and the differentiation process is excessively long.

In this study we are using the neural stem cell lines derived from the brains of homozygous fetal Hdh CAG Q7 (wild-type) and heterozygous Q140 (mutant Htt) knock-in mice. The cell lines have been developed by (Ritch, Valencia et al. 2012), and differentiated neurons as models. The Q140 mutant Htt came from a generated knock-in mouse that has rotating sequences of the polyglutamine stretch located in exon 1 of the *Hdh* mouse gene. This gene is homologous to the human *HD* gene and was replaced by a mutant polyglutamine repeat. The mutation is expressed under the *Hdh* promoter in the full-length huntingtin protein (Fig. 2A).

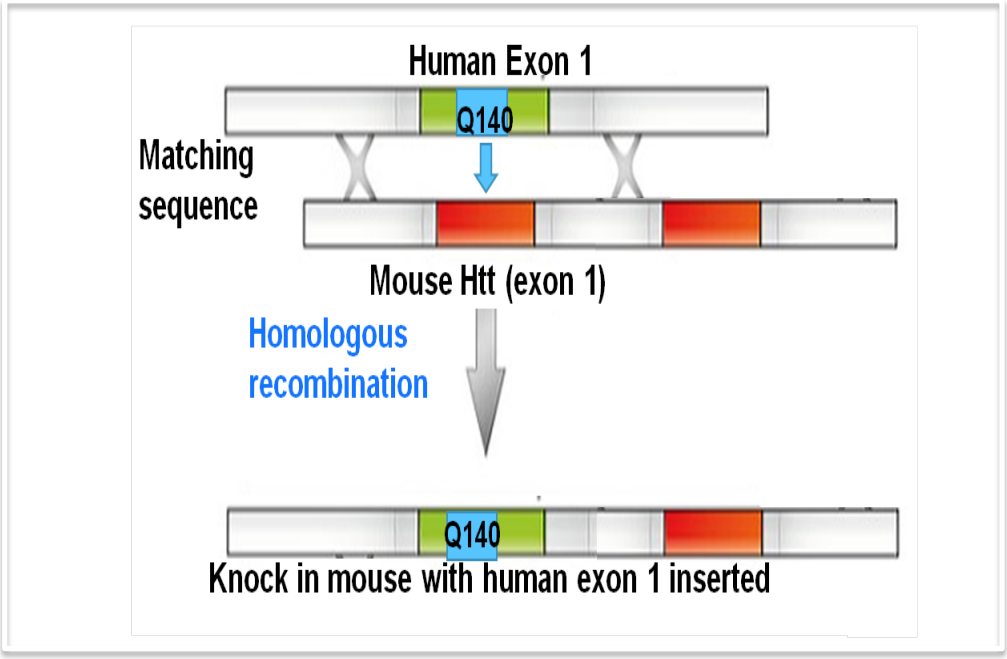

Figure 2A: HD mice created by homologous recombination of human exon 1 with 140 CAG repeat into the endogenous mouse gene for Htt.

The characterization of the specific cell lines that we are using (Q7 and Q140) has demonstrated their relevance in helping to understand HD. One benefit of these lines is; they follow previously defined phenotypes for HD patients, such as lowered levels of cholesterol increased levels of reactive oxygen species, and a reduced motility (Ritch, Valencia et al. 2012). Also these cell lines grow as adherent radial glial-like stem cells, making them relatively easy to culture *in vitro*. These cell lines can be derived from fetal and adult brains as well as from induced pluripotent stem cells (iPS) cells and Embryonic stem (ES) cells (Conti, Pollard et al. 2005, Goffredo, Conti et al. 2008, Spiliotopoulos, Goffredo et al. 2009). This allows some flexibility to utilize the type of cell line ideal for

the study. Another advantage of these NS cells is that they remain highly neurogenic even after 100 passages they can still undergo complete neuronal differentiation in 2-3 weeks. In addition, these models do not exhibit the neurochemical characteristics, receptors and antigenic features typical of mature neurons (Conti et al., 2005; Goffredo et al., 2008; Pollard et al., 2006). Finally, the cell lines are useful for the study of some therapies such as those using proteases inhibitors to modulate the toxicity of mutant huntingtin.

Despite the discovery in 1993 of the mutation responsible for Huntington's Disease, the mechanism of how the mutant protein disrupts cell function remains elusive. Neurodegenerative diseases such as HD, Alzheimer's and Parkinson's disease are complicated by the fact that they all have relatively slow progression from the first onset of symptoms.. Huntingtin protein (Htt) is found in many of the body's tissues*, with the highest levels of expression in the brain. Studies suggest that this protein may be involved in chemical signaling, transporting materials, attaching (binding) to proteins and other structures, and protecting the cell from self-destruction (apoptosis). Many studies have found that Htt protein appears to play an important role in nerve cells (neurons) in the brain and is essential for normal development before birth, but the mutation that results in HD is an increase in the copies of the amino acid glutamine (CAG) in a stretch of repeated glutamines at the amino-terminus of the Htt (Fig 2B). This "expanded polyglutamine" huntingtin acquires toxic properties, presumably through mechanisms that involve its reduced solubility and aberrant interactions with other cellular proteins that do not occur with the normal Htt protein.

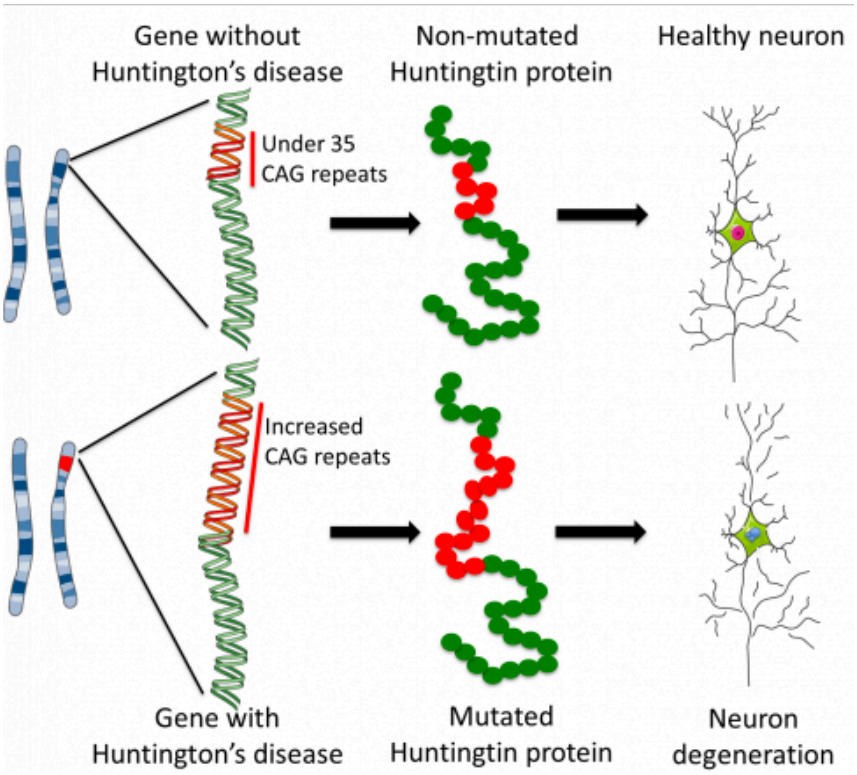

Figure 2B: Insertion of additional CAG repeats in the Huntingtin gene translates into an extended stretch of glutamine residues in the protein. The mutant Huntingtin (mHtt) protein leads to neurodegeneration through an unknown mechanism of action.

(Cambray, 2014)

There is evidence indicating that the signaling protein RRAS is involved in mutant huntingtin toxicity. (Miller, Yates et al. 2012). The authors found that mutant Htt and RRAS are co-localized in STHdh$^{Q111/Q111}$ Cells and in BACHD and R6/2 Mouse Models. They also identified multiple components of the RRAS signaling pathway as loss-of-function suppressors of mutant huntingtin toxicity in human and mouse cell models. Loss-of-function in orthologous RRAS pathway members also suppressed motor dysfunction in a Drosophila model of Huntington's disease (Miller, Yates et al. 2012).

These previous results support the the possibility of a functional link between mHtt protein and RRas. Second we are investigating this relationship using the neurons, the primary cells damaged in Huntington's disease. The goal of this study is to provide insights into the mechanism of mHTT toxicity and to identify any other molecular players such as RRas that could play a role in this process.

In this study we are exploring the effects of mHtt expression by looking at changes in huntingtin localization, changes in the expression and localization of related proteins as well as effects on differences in cell morphology. Our plan is to culture the cells, differentiate them to neurons, and then continue with Immunocytochemistry studies including use of confocal microscopy for imaging the fluorescently labeled cells. In addition we are focusing on some characteristics of the mHtt protein in the signaling pathways of neurons, by investigating the interaction of mHtt protein with some of the downstream signaling components of Plexin/Semaphorin pathways (i.e. RRAS).

One of the most important aspects of our studies is the differentiation of the neural progenitor cells into mature neurons. Previous studies that investigated pathways affected

by mHtt protein used non-neuronal cell lines and so the conditions are not as relevant to the true HD disease state. In this study we are analyzing the toxicity of mHtt protein in the neurons by using the embryonic stem cells from the brains of homozygous fetal HD CAG Q7 (wild-type) and heterozygous CAG Q140 (mutant Htt) knock-in mice and differentiated neurons as model to study the toxic effects of mutant Htt protein in those cells . We study how the expression of mHtt protein alters the growth pattern, cell morphology, and process of neuronal differentiation. Also we investigate the interaction of the mHtt protein with RRAS (downstream signaling component of Plexin/Semaphorin pathway), and we are doing this part in collaboration with Robert Hughes and Brad Gibson at the Buck institute for Research on Aging. In this study we aim to:

1- Investigate the phenotypic effects of mutant Huntington (mHtt) expression in neuronal stem cells (NSCs), by watching and comparing the WT Q7 with HD Q140 during the culturing and the differentiation process for both cell lines.

2- Explore the role of Htt in the process of neuronal differentiation in both of the wild type (Q7-WT) and the mutatant (Q140-HD) cell lines, by using the immunochemistry to label Htt and other potentially relevant proteins.

3- Study the changes in the cytoskeleton structure and neurite growth in both of Q7 and Q140 cell line.

4- Look at the co-location of mHtt protein and the RRAS at the Q140 differentiated neurons and compare them with the Q7 differentiated neurons using the immunochemistry technique. Also investigate the relationship between the Htt protein and RRAS protein in both Q7 and Q140, using the florescent microscopy images.

By comparing the cell growth and morphology, the co-localization of proteins, and even the differentiation process itself in neuronal cell populations, to the wild type, we expect our results to be particularly applicable to the human disease state. Comparisons between differentiated neurons with either the Q7 or the Q140 at different stages through the differentiation period will also be particularly useful. For example, one study looks at the co-localization of Htt with RRAS to help determine the relevance of RRAS signaling in the HD disease mechanism. Clearly the more that is understood regarding the effects of mutant huntingtin on neuron function the closer we are to identifying targets that can lead to new treatments for HD.

Research Design and Methods

1 -Culturing protocol:

Because we began our project with just one aliquot of each cell line, our first efforts were directed towards expanding these lines to have sufficient back up stocks and also share our stocks with our collaborators**.** The two cell lines are neural stem cell lines HD (Q170, mHtt) and the WT (Q7 Htt). The second goal was to characterize each cell line and optimize the methods and conditions required to propagate each line. Our studies progressed to include examination of the phenotypic effects of mutant Huntington (mHtt) expression in neuronal stem cells (NSCs).

Protocols used in this study were adapted from the neural stem cell culturing (NSC) protocol from J.J.Ritch et al. /Molecular and Cellular Neuroscience 50(2012)70-81.

1.1-Thawing the cells:

First, coat a T25 with 2.5ml of gelatin for 15 min in the hood (note: we do not coat overnight). Second, make the Neural Stem Cell Expansion medium (NSEM). Use 5 ml of pre-warmed NSEM on 15ml conical tube. After that, thaw the cryotube in water bath (but not completely thaw).Third, add 1ml of NSEM to the cryotube and transfer all what in cryotube to 15ml conical tube and spin down for

3 minutes at 1200 rpm. Meanwhile, aspirate off gelatin from T25 and add 3ml of NSEM to T25, then re-suspend pellet in 2ml of NSEM minimizing titrations. Forth, gently rock the flask side to side. When the cells are nearly confluent, split up into T75 flask using all cells.

1.2-Splitting the cells:

Before splitting the cells, coat T75 with gelatin, for T75, add 4ml of gelatin. Gently aspirate off the NSEM, and washed once with HBBS. After that, added 5ml of pre-warmed Accutase and left it in the incubator for 1 minute. Then gently collected all the cells and moved them to 15ml conical tube and spun it down at 1200RPM for 3 minutes, meanwhile aspirated off gelatin from the T75 flask and added 14ml of the pre-warmed NSEM.

The following step is aspirate off the Accutase and add 1ml of NSEM to the cells in the 15ml conical tube, then by using P1000 break up the cells gently in order to have a single cells solution (no clumps), before transferring the cell solution to the T75 flask, counted the cells using the Hemocytometer. The final volume should be ~15ml in T75.

1.3-Feeding the cells:

Media changed every other day and checked out each flask before aspirating off medium. If the cells are attached, aspirate off half of medium from T75 (~7.5ml) and added 7.5 of the pre-warmed NSEM.

If the cells floating aggregates, transfer 7.5ml of the medium from T75 to 15ml conical tube and spin it down for 3 min at 1200RPM, after that lightly snap pellet and add 7.5ml of pre-warmed NSEM to conical tube (if it necessary titrate with P1000 to get pellet into suspension) transfer all to T75.

Figure 3: Example of cell culturing steps that we did at the lap.

1.4-Freezing the cells:

Split the cells as normal but this time, re-suspend pellet in 11ml of 10% glycerol in DMEM/F12 and count them, after that wrap cryotube in Kim-wipe and place in 50ml conical holder. The following step is wrapped Styrofoam holder with under-pad and tape, then placed in -80ºC overnight (do not invert or tilt), then placed them in LN2 next day.

2 - Differentiation protocol:

To look at the impact of Htt on the process of neuronal differentiation we differentiated the cells according to the protocol of (Spiliotopoulos, Goffredo et al. 2009), this protocol takes 21days to progress, 85% of the cells will differentiate into molecularly and electrophysiological mature neurons belonging to the GABAergic lineage.

Cells were split normally using the Accutase and spin down at 1200RPM for 3 minutes, aspirate off the Accutase and add 2ml of media D1, by using P1000, gently breakdown the cells to single cells solution. Then we count the cells and plate them on to un-coating T75 flask (density: 1.0×10^5–1.5×10^5 cells/cm^2) in media D1 for 3 days (from day 0-3). The cells incubate at 37ºC and on day 3, change half of the medium D1 with fresh pre-warmed D1 media.

Media D1 (50ml) recipe**:** 47.7ml Advance DMEM/F12, 0.5ml Pen/Step, 0.5ml GlutaMAX, 0.5ml Gentamicin, 0.25ml N2, 1ml B27, 5µl FGF2 of 100ug.

The second step was On day 4, we split the cells using the Accutase and spin down at 1200RPM for 3 minutes and re-suspended gently in 2ml of media A by pipetting the cells. After that, count the cells and plate them in 12 well plastic coverslip plate that have been coated with laminin and has 2ml of pre-warmed A media in each well (density: 5×10^4 –7.5× 104 cells/cm2), and incubate at 37ºC. On day 6, we change half of medium A with pre-warmed media A and incubate at the same conditions.

Media A (50ml) recipe: 12ml DMEM/F12, 36ml Neurobasal media, 0.5ml Pen/Strep, 0.5ml GlutaMAX, 50 µl Gentamicin, 0.25ml N2, 1ml B27, 5µl FGF2 of 100 ug/ml, 10 µl BDNF of 100 ug/ml.

On day 7, we replace half media A with pre-warmed media B, and did that every other day until day 21. Replacing the media by using P1000, aspirate off 1ml of the old media and add 1ml of the fresh media.

Media D (5oml) recipe: 12ml DMEM/F12, 36ml Neurobasal, 0.5ml Pen/Strep, 0.5ml GlutaMAX, 50µl Gentamicin, 0.25ml N2, 1ml B27, 2.5µl FGF2 of100ug / ml, 15µl BDNF of 100ug / ml.

Fixing the Neurons (Day 7, 13 and 21):

Aspirate media (leave behind a small amount (~200 µl)) to facilitate gentle rinsing and rinsed each well with 2mL of PBS, then aspirate PBS. add 1mL of 10% Formalin (4% paraformaldehyde) to each well and let it sit for ~ 30 minutes at room temperature. After that, remove ~90% of the formalin with the vacuum and added 2mL of PBS to each well. Seal with Parafilm and stored the plates at 4^{o}C.

3- Labeling protocol:

Wash the cells 2x with 1ml PBS at room temperature (RT), then permeabilize the cells by adding 1ml PBS-Triton (0.5%) for 5-15 min at RT. After that wash 2x with PBS at RT. The next step is block with 1ml 5% of normal goat serum (5%NGC in PBS) for 30 min at RT. During the blocking step prepare Petri dishes for incubation: fold and then cut out circles of paper towels and placed 4 layers into the bottom of Petri dish, one dish for every 4 cover slips. Then moistened the paper and placed a slightly smaller circle of Parafilm on top. After that, incubate using 100µl primary antibody solution diluted in 5% NGC. Then pipet 100µl of antibody solution onto the Parafilm, use forceps to carefully take the cover slip over so that side with attached cells is the one facing down in contact with the antibody solution. We incubated over night at 4ºC. Move the cover slips back to the 12-well plates. Then we wash 2x with PBS at RT. In dark room incubate the cells with 500µl of the secondary antibodies diluted in PBS (1:500) for 1-2 hours at RT. then washed 2x with PBS at RT, then Count stained nucleus with 1ml of DAPI for 1-2 hours at RT, then wash 2x with PBS at RT. Add distilled H2O then for the final step remove the cover slip from the wells to be placed on a microscope slide containing 1 drop close to 50µl of ProLong Gold mounting media and let them dry for 24 hours.

4- The Examination Using Confocal Microscopy:

The four available laser channels will be used to capture images consecutively from each of our fluorescently labeled proteins. The removal of light from other planes of focus will more easily resolve the relevant details of neuron morphology such as neurite branching and even smaller structures such as filopodia.

Results

1- Bright-Field Microscopy:

1.1 Differences in growth rate between WT (Q7) and HD (Q140).

We found that the growth rate of the Q7 cell line was consistently greater than that of the Q140 cell line. This was determined through watching the growth process and recording the progress of cell line expansion with a camera attached to an inverted microscope (Fig4). We also counted the cells using the Haemocytometer on different days through the culturing period (Fig5). During the initial period of optimization, a variety of growth conditions were tested on both Q7 and Q140 cell lines. Variables that were investigated included media components, growth fractors, passaging protocol and seeding density. Several conditions were identified as particularly important for the successful growth of both NSC lines. The addition of freshly thawed fibroblast growth factor 2 (FGF2) a minimum of every other days was needed to maintain a healthy cell population. Also important was the settling out of cell clusters that formed in the Q140 plates. For this investigation we were looking for conditions that could be used for both cell lines so that growth conditions were varied as little as possible. For experiements, both lines were subjected to the same conditions except the seeding density of Q140 was sometimes doubled to accout for slower growth rates.

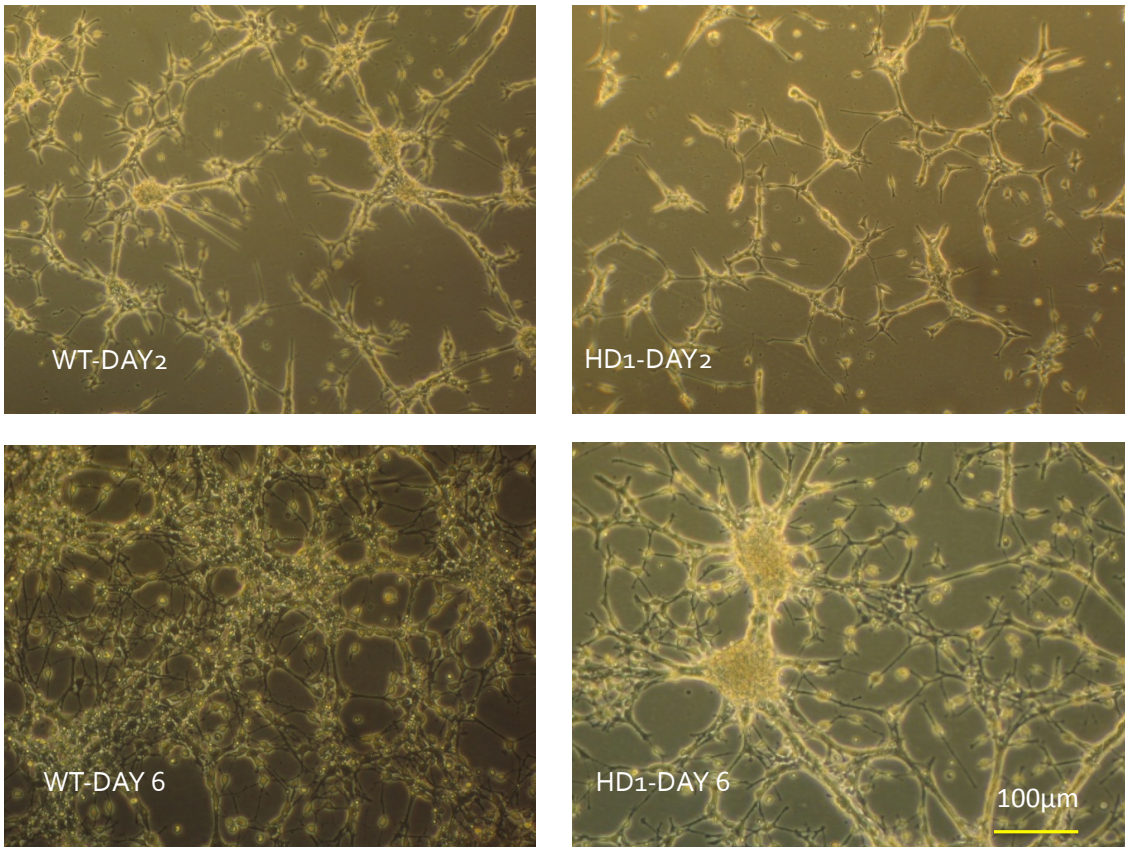

Figure 4: Q7 WT cells (left) grow and divide more rapidly compared to Q140 HD (right)

Pictures were taken on day 2 and day 6 after passaging. Initial seeding with 1 x 10^6 cells on a T-75 culture dish.

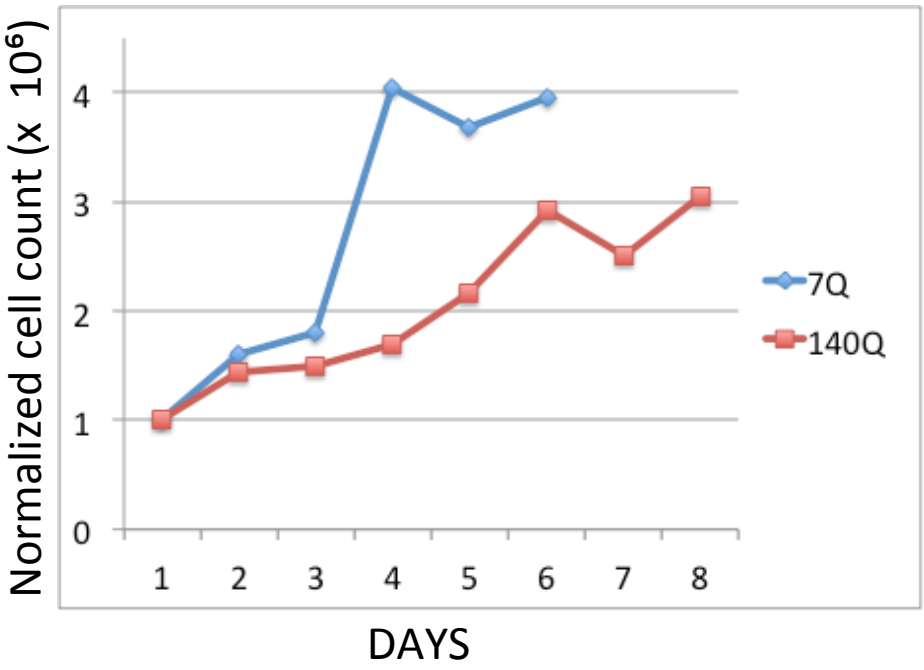

Figure 5: Normalized growth curves for Q7WT and Q140HD cell lines, show the differences on the growth rate between Q7 and Q140.

Cells were counted each time before passaging.

1.2 Morphology Differences between WT and HD

To study the morphology changes in both Q7 and Q140 cell lines we watched and recorded any differences between the WT Q7 and HD Q140. By analyzing the bright-field microscopy images, we notice that the Q140 cells behavior is different compared to Q7 cells. Q140 cells tend to grow as a hybrid mixture of monolayer cells, which are the same cells attached to neurospheres, on the other hand Q7 cells grow as a flat layer of single cells (Fig6).

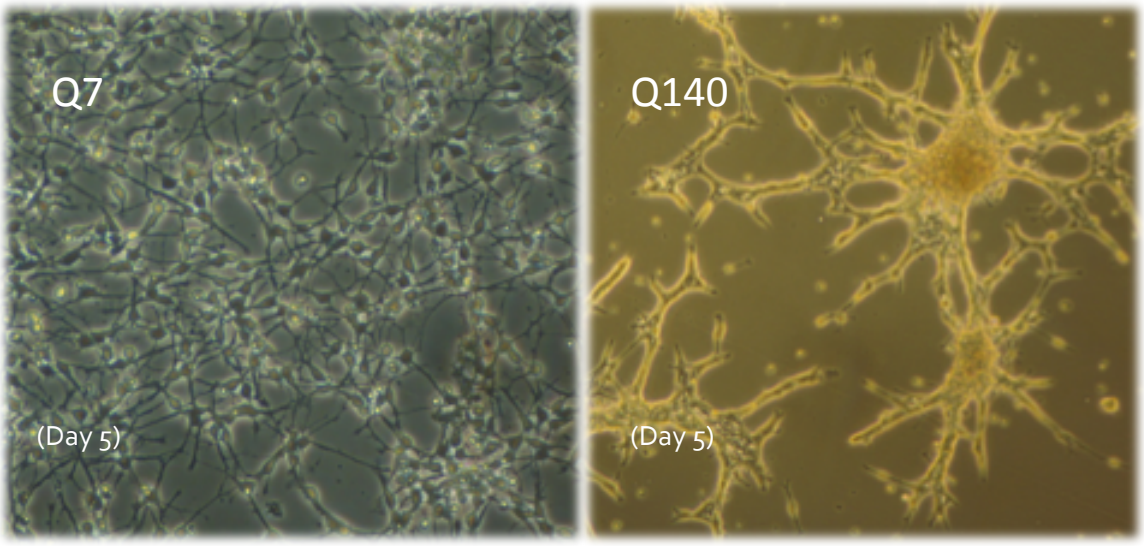

Figure 6: Q7 and Q140 NSCs imaged on day 5 after passaging. Q7 cells are growing as a single layer while Q140 cells are growing as a layer of clustered cells.

Another morphological difference in the Q140 cell line that was noteworthy was a greater tendency of neurites to grow in curved paths. This can sometimes be observed as the neurite tending to circle in one direction towards the cell body. (Fig7a). Finally, in some preparations, the neurons

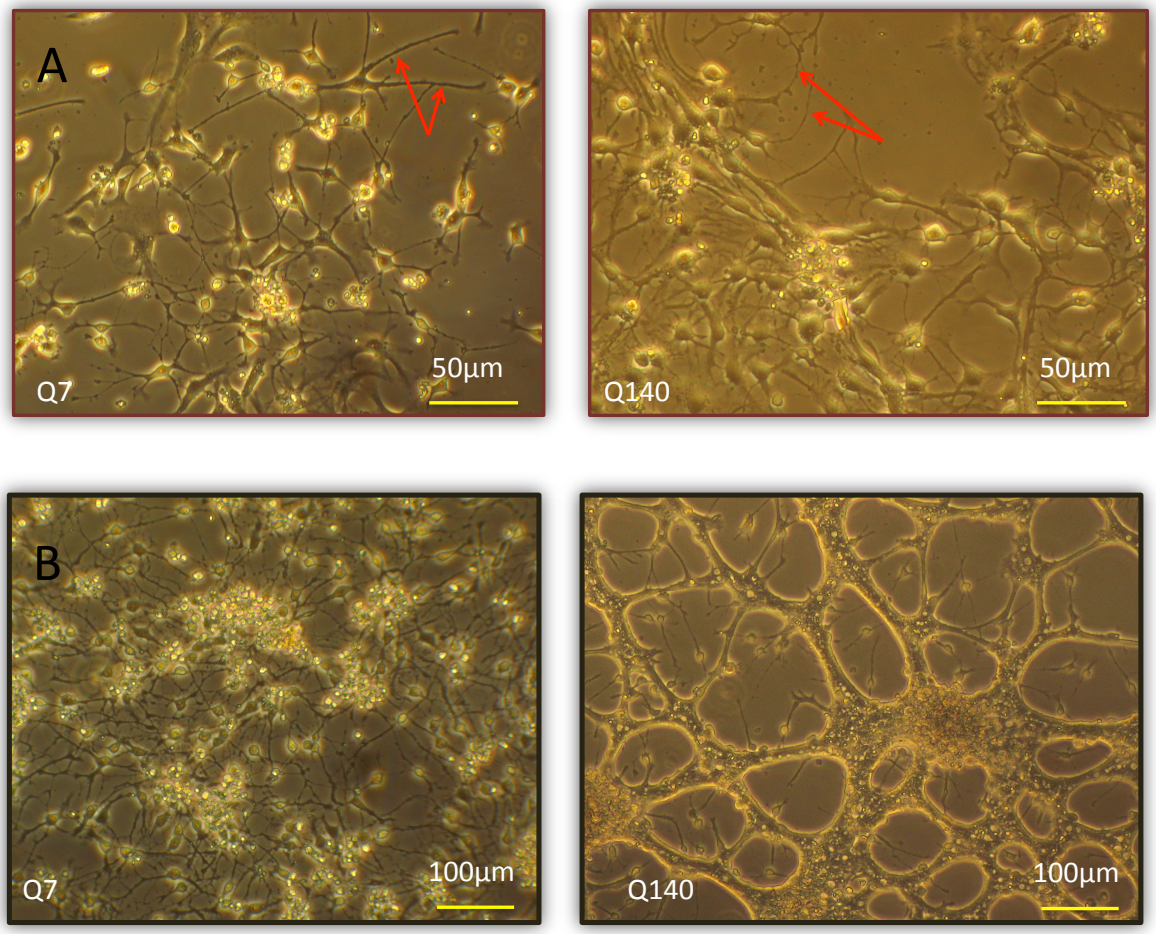

Figure 7: Morphological differences in live differentiated neurons.

A) Q140 (right) showing circular growth patterns as compared to the more straight and outwardly projecting Q7 neurons . B) When crowded, neurons with the Q140 mutation can form clusters with less distinct cell projections than observed in Q7

2- Confocal Microscopy results:

2.1 - mHtt affects the differentiation process (proportion of MAP2 positive cells).

We used the same differentiation protocol to differentiate both Q7 and Q140 cells. We labeled for both GABA and MAP2 protein at the final stage of the differentiation process (day 21) in order to view the impact of Htt protein on the process of the neuronal differentiation. From the result we observed that all of the Q7 differentiated neurons are expressing GABA and MAP2, but not all of the Q140 differentiated neurons are expressing GABA (fig8.A&B).

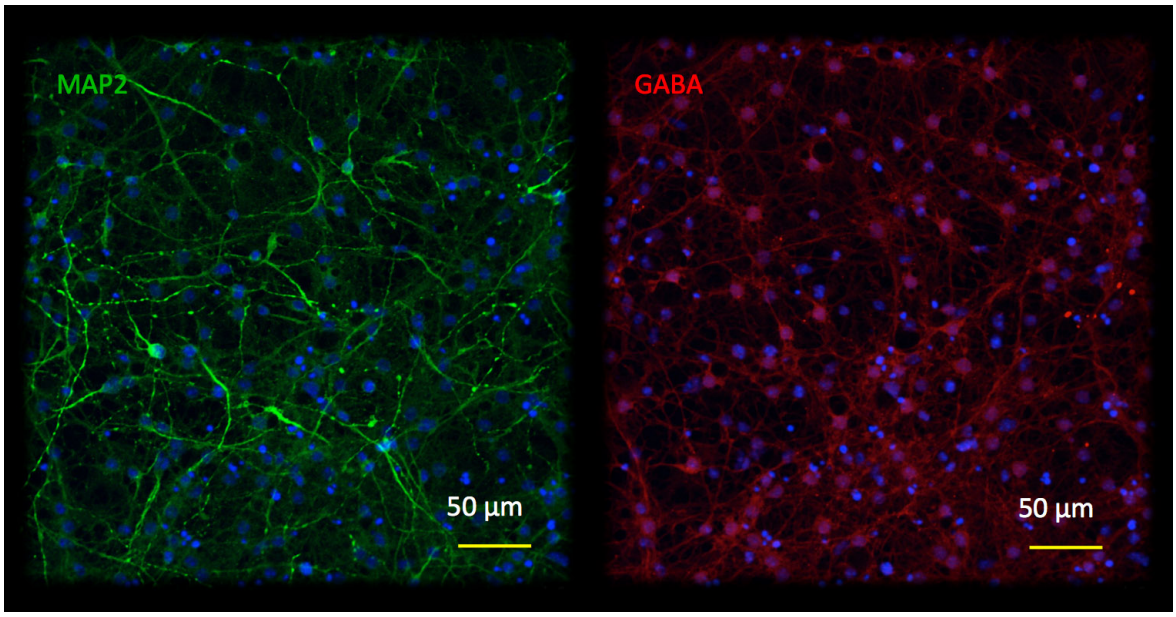

Figure 8.A: Imaging of MAP2 and GABA expression reveals effect that Q140 has on the differentiation process. Here, all differentiated neurons show some expression of MAP2 but not all of the cells express GABA.

Green: MAP2, Red: GABA, Blue: DAPI (nuclear stain)

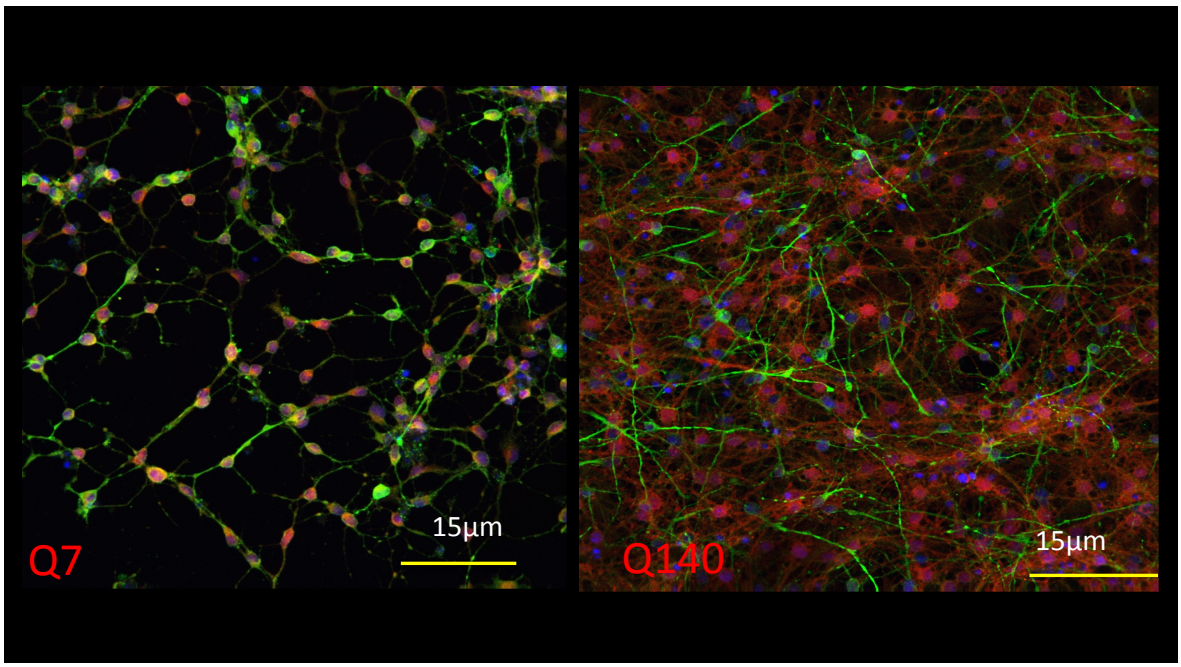

Figure 8B: 20X confocal microscopy image for Q7 differentiated cells labeled for both GABA red and MAP2 green (on the left side of picture) compared to the Q140 cells (on the right side of picture) where GABA red and MAP2 green. Both were under the same differentiation conditions.

2.2 - Difference in the Htt expression in differentiated neurons.

The Q140 mature neurons (expressing GABA and MAP2) were inconsistent regarding their relative level of Htt expression. Sometimes they showed a higher level of Htt protein expression compared to the Q7 mature neurons when we labeled for the Htt protein and MAP2 protein (Fig9A) to study the effects of the mHtt protein expression in the neuronal stem cells. However usually there is little difference in the Htt protein expression in neurons that label positively for GABA and this is true for both Q7 and Q140 (Fig9B).

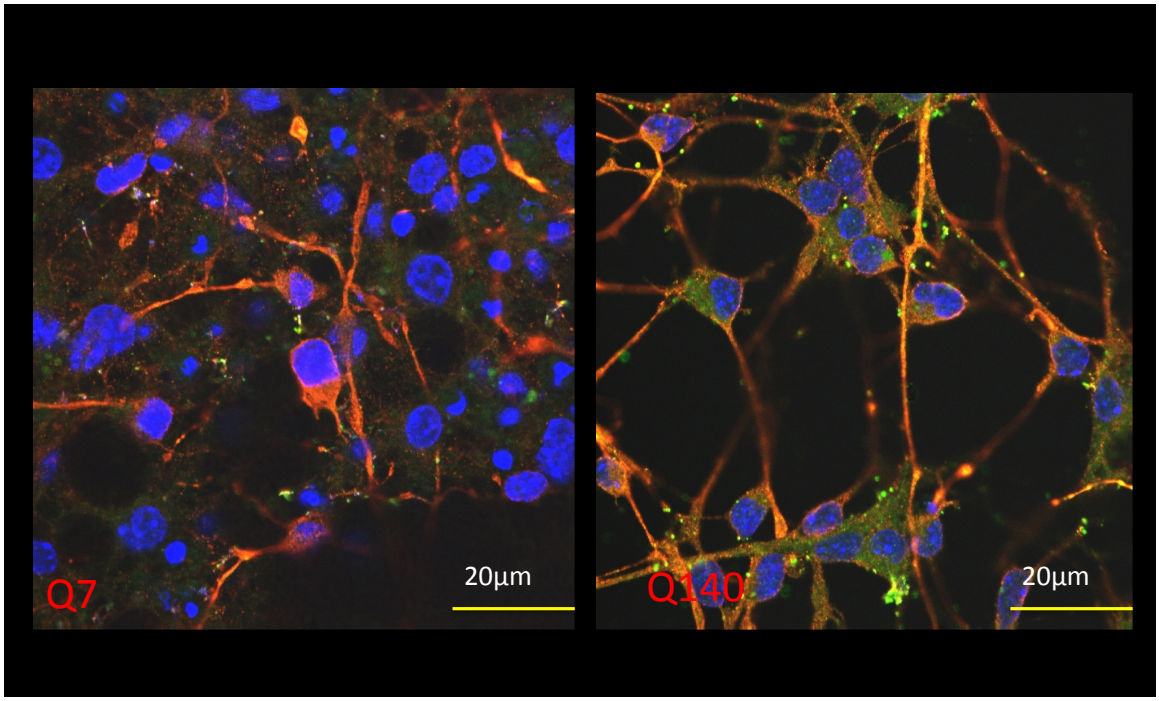

Figure 9A: Confocal microscopy images suggest differences in the Htt protein expression between Q7 differentiated cells and Q140 differentiated cells. Laser intensities used to collect images are the same for Q7 and Q140.

Green: Huntingtin (Htt), Red: MAP2

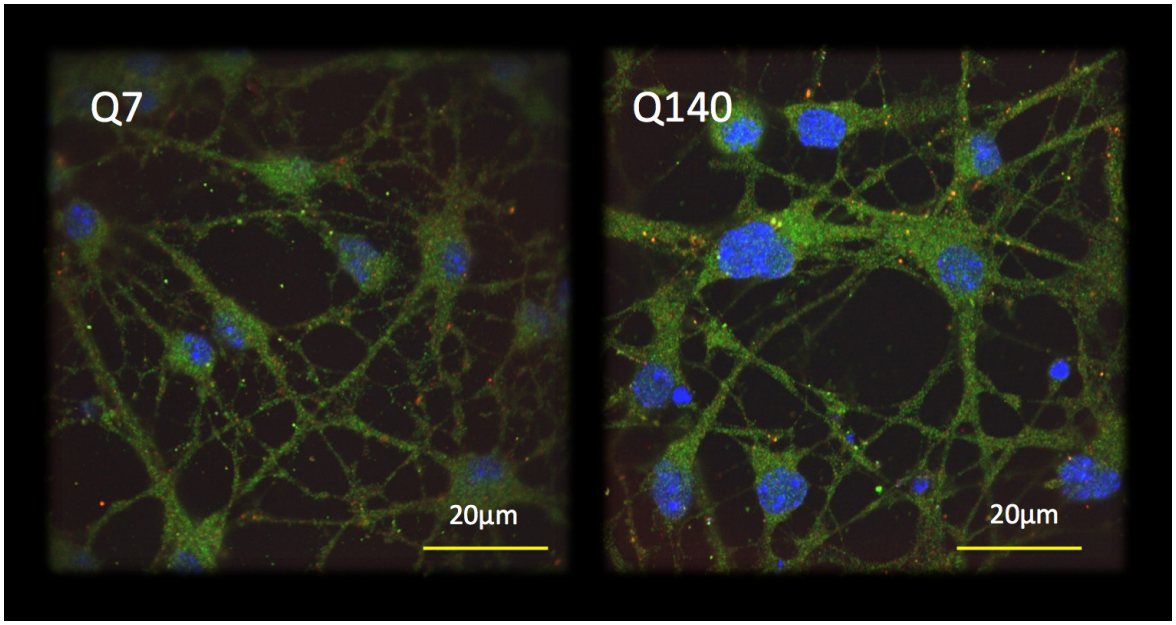

Figure 9B: Differentiated neurons showing similar expression of the Huntingtin protein levels in Q7 and Q140 mature neurons. Samples are labeled using an antibody that recognizes both Htt and mHtt.

Note there are differences between the size of nuclei relative to the cell body.

Green: Huntingtin Protein, Blue: DAPI nuclear stain.

2.3 - RRAS expression differences.

In the Q140 differentiated neurons we observe from the labeled result for RRAS and the Htt protein, that RRAS expression level is higher compared to the Q7 differentiated neurons (Fig10A). Also combining the labeling for RRAS antibody and Htt antibody we observe a co-localization of RRAS with mHtt in Q140 differentiated

cells (fig10B). The expression pattern of RRAS was more punctate in 140Q compared to 7Q.

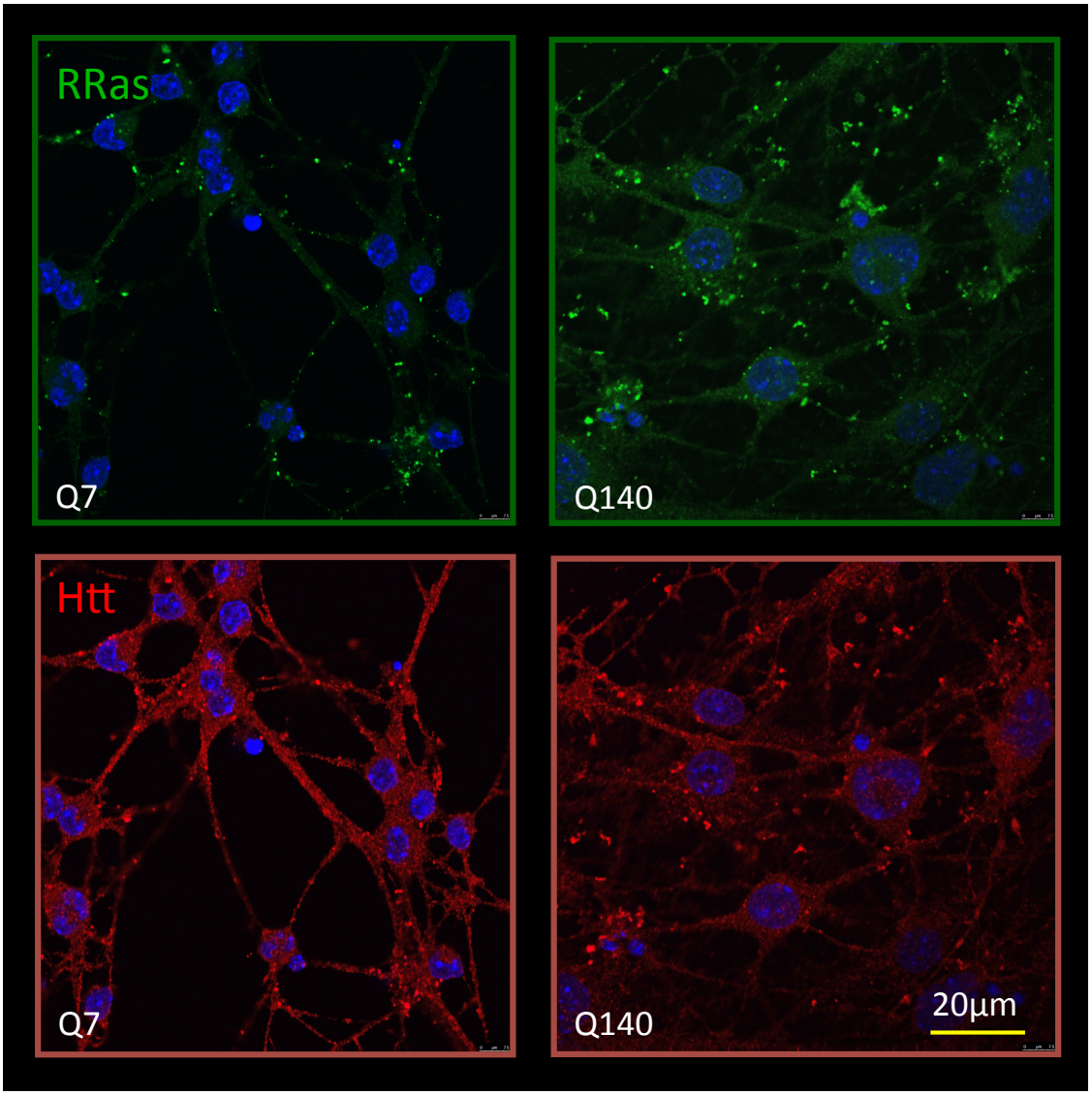

Figure10A: The relative expression level of RRAS is higher in Q140 cells. Comparison of Q7 and Q140 indicates a difference in expression levels. Huntingtin expression is about equal in both cell lines, while in Q7, there is relatively less RRAS than Huntingtin.

Green: RRAS, Red: Huntingtin (Htt or mHtt)

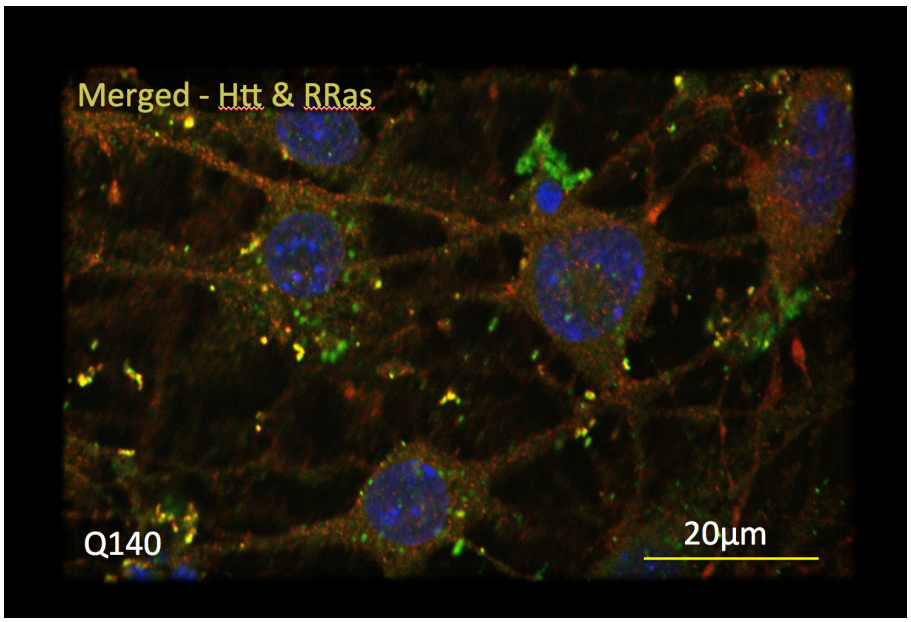

Figure 10B: - Merging red and green channels shows Htt and RRAS are co-localized in the cytoplasm.

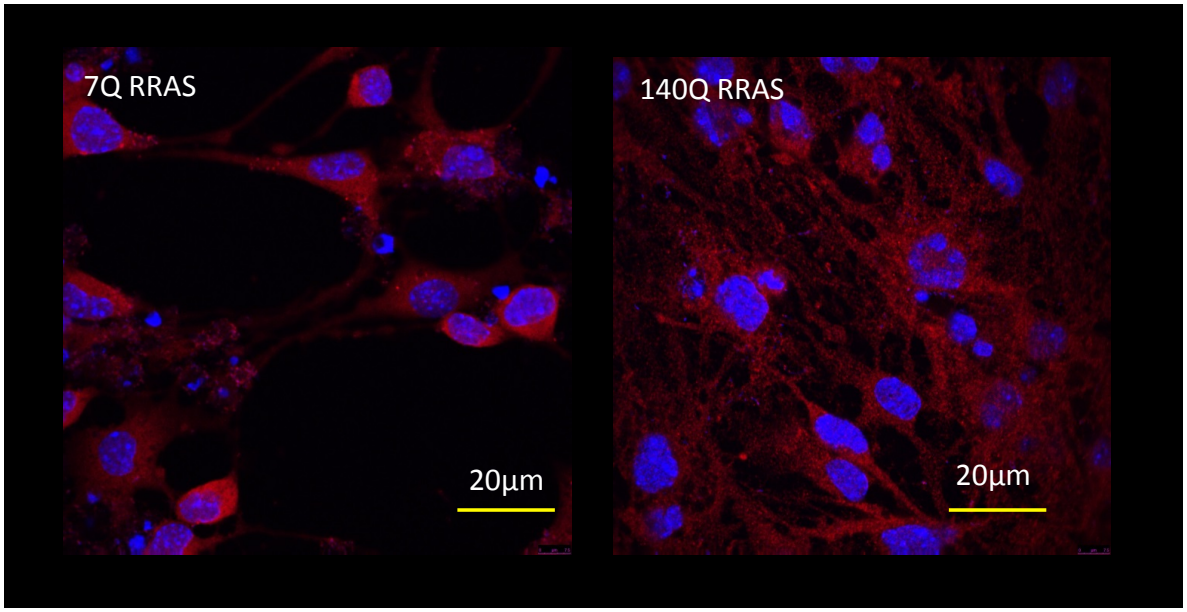

Figure 10C: The pattern of RRAS expression was observed as more punctate in the mutant Q140 and smooth in Q7. Morphological differences between these cell lines is clear in the more definable cell bodies of the Q7 cells.

Discussion and Conclusion

Through our investigation of the phenotypic effects of mHtt expression neuronal stem cells, we noted several significant differences between the WT-Q7 and HD-Q140. Observing growth characteristics, we found out that Q140 cells multiply more slowly than Q7. Even when the dishes of Q140 were fed and cared for additional time, the total yield was never reached greater than 50% that of a comparable dish of Q7 cells. (Fig5) It is challenging to determine the direct cause of this phenotypic difference, but the effect is consistent and reproducible, and was also observed in a second Q140 cell line that we tested. It is clear that mHtt interferes sufficiently with the cellular processes so as to measurably inhibit proliferation and this result is consistent with the loss of neurons observed during the progression of Huntington's disease.

Most analysis of differentiated neurons was done using fluorescent labeling techniques, but phase-contrast imaging of live cells revealed a few differences worth noting. Consistent with observations by our collaborator Brandon Tavshanjian, we found that some of the neurites of Q140 cells grow along a curved path, tending to circle back towards the cell body (Fig7). This unusual growth pattern has not been yet been noted in the literature and is of interest because it may be an indication of altered axon guidance signaling caused by the presence of mHtt. Because these cell lines have not yet been well characterized, any documented differences in their growth or morphology offer valuable information that contributes meaningfully to our understanding of the cellular mechanism of Huntington's disease.

One of the most clear phenotypic differences we found in the NSCs was a very clear clustering of Q140 cells. This is in contrast with the consistent monolayer pattern of growth observed in the Q7 cells (Fig4). To confirm it was not an unrelated issue with the cell line, we cultured a second Q140 cell line side by side and found the clustering phenotype again. This was an important observation for two reasons. First, it was helpful that the Q140 cell clusters are immediately recognizable under phase contrast imaging, which allows for an unambiguous distinction between the cell lines. It is also relevant because it directly supports our hypothesis. The tendency of the Q140 cells to cluster is likely to be a consequence of the mHtt interfering with the signaling pathways that are involved in cell migration and cell adhesion.

There were additional consequences of the clustering that directly affected culturing and care of these cells. The cells contained within the clusters are protected from Accutase treatment and are also resistant to physical disruption and disruption by mechanical means. One clear example of the practical implications of this is when treating with the enzyme solution Accutase used to detach cells during passaging protocols. Clustered cells are so resistant to the enzymes that identical treatments of Q7 and Q140 can lead to ten-fold differences in survival rates. Optimization of these steps was essential in obtaining similar seeding densities for differentiation. Clustered cells have a greater capacity to survive in general, for example in media even that is limited in the nutrition sources. On the other side Q7 cell line grows fast as layer of single cells, and is more sensitive external effects such as Accutase or old media. Q7 cells did not survive well

more than 4 days in the same media. During the time we spent in culturing, extending and watching both cell lines behavior it was demonstrated that the expression of mHtt in the Q140 cells results in the cells to behaving differently compared to Q7 cells, even when both are placed under the same conditions.

In our differentiation studies we treat both Q7 and Q140 cell lines to a set of conditions that induce the neural stem cells to differentiate into mature neurons. Cell lines are grown side by side under identical conditions so the only variable during the culturing phase of these studies is the presence of the mutant huntingtin protein in the cell. Our protocol takes 21 days to differentiate the cells to mature neurons, most of which we show are GABAergic.

The results from the immunochemistry labeling showed expression of GABA and MAP2 in close to 100% of the differentiated neurons, but this number was not always observed from Q140 cells. This reduced number of mature GABA neurons was an unexpected result. We found that all of the differentiated neurons were expressing MAP2 marker but not all of them show expression of GABA marker (Fig8B). We applied the same differentiation protocol and labeling process to both cell lines repeatedly, and each time we had the same result. Because GABA is a marker of GABAergic neurons this result shows that the proportion of cell types is different in cells that have been differentiated in the presence of mHtt. MAP2 and GABA do not provide clues regarding what caused the changes in differentiation, but the clear difference we observe in the two populations of differentiated cells indicates that mHtt can influence the process of differentiation into neurons.

An important part of our study included labeling of the Huntington protein (Htt) within both cell lines. The antibody we used binds to both Htt and mHtt so we can use it to label both the Q7 and Q140 lines for Huntingtin expression. We also label for GABA or MAP2 to help identify the stage of differentiation the cells are in. Labeling of Htt varies from sample to sample somewhat, but there was no clear trend of different expression levels of Htt when comparing Q7 and Q140 (Fig9A). It is important to point out that just the capturing the pattern of expression Htt in these cells in an important result that facilitates a better understanding of this model HD system. This is illustrated in Figure 11, which clearly show the pattern of Htt expression in NSCs.

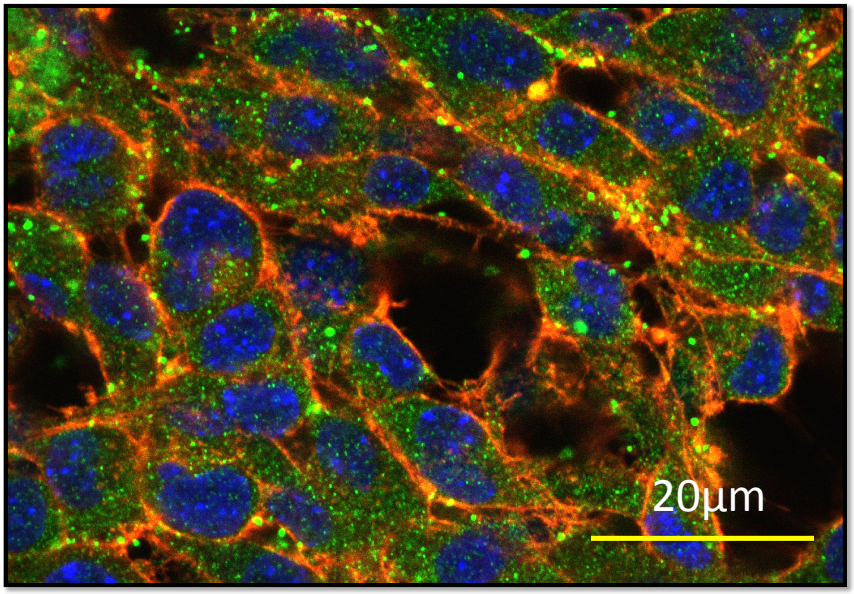

Figure 11: Neural stem cells showing cytoplasmic expression of Huntingtin (green). Cell structures are visible as a result of actin (red) and nuclear (blue) stains.

Another interesting area that we were able to investigate is the relationship between the mHtt protein and the RRAS protein. Our collaborators suggested that the expression of mHtt is able to activate RRAS, and this activation leads to altered cell association with

the extracellular matrix and altered cell motility. In our lab and as a part of our immunochemistry studies, we labeled both Htt protein and RRAS protein in Q7 and Q140 differentiated neurons.

The first observation is that in Q140 neurons there is a higher level of RRAS expression. The expression is not only high but labeling intensity was similar to that of mHtt (Fig10A). For Q7 cells we did not find that the expression of RRAS was equal to the level to Htt protein expression (Fig10B). The second observation is the co-localization of mHtt protein and RRAS in the Q140 compared to Q7, where we only find RRAS close to the nucleus and not as co-localized with Htt protein (Fig10B). These observations support the hypothesis suggested by the Hughes lab, and indicate there may be a direct relationship between RRAS and mHtt protein. RRAS is a downstream singling component of the Plexin/Semaphorin pathway. Plexins function as receptors for the repulsive axonal guidance molecules Semaphorins, Intracellular domains of Plexins are responsible for initiating cellular signal transduction inducing axon repulsion. Intracellular domains of Plexins are responsible for initiating cellular signal transduction inducing axon repulsion. Plexin-B1 possesses an intrinsic GTPase-activating protein activity for RRAS and induces growth cone collapse through RRAS inactivation. Also recent advances have revealed molecular mechanisms for Plexin mediated cytoskeletal leading to repulsive responses. Further studies of this pathway will help confirm whether mHtt is interacting with RRAS in such a way that this interference is a causative factor in the mechanism of mHtt toxicity in the disease state.

As a conclusion we have found multiple lines of evidence that mHtt interferes with cell adhesion, motility, and molecules related to the cytoskeleton signaling and remodeling. The cell lines that we are working with are almost completely uncharacterized and our studies represent a very useful set of information regarding their propagation and differentiation. Development of this functional model of HD was an important step in the elucidation of cellular mechanisms of this debilitating disease.. Our studies all point towards a role of mHtt in affecting cytoskeleton dynamics. Though some observations are preliminary and will be the subject of continued investigations, most gave meaningful support to our hypothesis. Finally, the results of theses studies leave us with a well-characterized tool for the study of HD.

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