A COMPARATIVE STUDY OF AGE AND TRANSPLANTATION OF INDUCED
PLURIPOTENT STEM CELL FOR TREATING BEHAVIORAL AND PATHOLOGICAL
DEFICITS IN THE YAC 128 MOUSE MODEL OF HUNTINGTON'S DISEASE

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ABSTRACT

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by Rebecca Culver

Huntington's disease (HD) is a devastating neurodegenerative disease that results in

death at about 10-20 years after onset of motor deficits. Although no effective treatments are

available at this time, stem cell transplants are being considered for treating HD because of their

potential to replace lost neurons. Previously, our lab has generated induced pluripotent stem cells

(iPSCs) and transplanted them into 3-nitropropionic treated rats, which models HD. The iPSCs

survived, differentiated into region specific neurons, and ameliorated behavioral deficits. While

these results are encouraging, replication of this work using other HD models is needed to

provide converging evidence for these findings.

The goal of this study was to evaluate the efficacy of iPSC transplantation within the

YAC 128 transgenic mouse model of HD and to determine if the age of the mice at the time of

transplantation influences functional outcomes following transplantation. Behavioral and

histological parameters were used to evaluate the effectiveness of the transplantation at either 10-

or 12- months of age.

Results showed no improvement in motor function after iPSC transplantation in HD mice

regardless of the age at the time of transplantation. Although iPSC treatment significantly

increased MSN counts in 12-month-old HD mice compared to vehicle control HD mice, this did

not result in improved motor function. Interestingly, 12-month-old WT mice had significant

motor dysfunction following iPSC transplantation. Results from this study suggest that the

beneficial effects of transplanted iPSCs we previously reported may not generate to other rodent

models of HD.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Huntington's disease (HD) is a disabling neurodegenerative disease that is caused by an autosomal dominant genetic mutation. The predominant symptoms include abnormal involuntary movements, as well as cognitive and emotional disturbances, which typically begin prior to the onset of the motor abnormalities (Zuccato et al., 2010). Ultimately the disease results in death, which occurs about ten to twenty years after the onset of the motor abnormalities (Purdon et al., 1994). The advancing symptomatic stages of HD occur in parallel with the progressive degeneration of medium spiny neurons in the striatum (Reiner et al., 1988). Even with a causative explanation for this destructive disease, an effective treatment has yet to be elucidated.

At this time, the only treatment options available focus on alleviating the motor symptoms. However, the use of stem cell transplants may provide a new and beneficial treatment option because of their potential to replace lost neurons. In earlier studies, some HD patients showed improvement following fetal striatal transplantations but, there was a high degree of variability in the degree of symptom relief between patients (Bachoud- Lévi et al., 2000 & 2006). Although early results from this work were encouraging, many logistical, immunological, and ethical issues surrounding the use of fetal cells for treating HD remain to be addressed (Cicchetti et al., 2009 & 2014 and Keene et al., 2009).

As an alternative to transplantation of fetal cells, the use of induced pluripotent stem cells (iPSCs) to treat neurodegenerative diseases has been proposed (Fink et al., 2014). The development of iPSCs followed from the work of Takahashi and Yamanaka (2006), which demonstrated the possibility of reprogramming differentiated somatic cells back to an embryonic stem cell (ESC)-like state by forcing the expression of four transcription factors, Oct4, Klf4,

Sox2, and c-Myc. Independent studies have demonstrated that iPSCs share morphology, growth, and gene expression that is similar to ESCs (Wernig et al., 2007) and can form functional germ cells (Okita et al., 2007). The therapeutic potential of iPSCs makes these cells very attractive to study because they can be derived from human patients to model neurodegenerative diseases *in vitro*, which then can be used to better understand pathological mechanisms and to screen potential pharmaceutical interventions (Ito et al., 2012). Furthermore, since the cells can be generated from the patient, this reduces the likelihood that the cells will be rejected by the immune system when used for transplantation, although the clinical utility of this approach for HD patients may be limited unless the faulty gene in these iPSCs can be corrected before transplantation. The use of iPSCs to regenerate lost cells in neurodegenerative diseases and provide functional outcomes has been supported by previous *in vitro* and *in vivo* work. Wernig and colleagues (2008) confirmed that mouse fibroblast-derived iPSCs could be reprogrammed into neuronal lineages, *in vitro*. In addition, they found that iPSC-derived dopamine neuronal cells could improve behavioral symptoms in a rat model of Parkinson's disease (PD). While iPSCs have been extensively studied to treat PD, successful outcomes have also been found in rodent models of Alzheimer's disease, amyotrophic lateral sclerosis, spinal muscular atrophy, stroke, and spinal cord injury (Ito et al., 2012).

Our lab has generated rat-derived iPSCs and transplanted these cells into the 3-nitropropionic acid (3-NP) rat model of HD. The results from these studies included survival of iPSCs after transplantation, a significant preservation of motor function following treatment of iPSCs, and differentiation of iPSCs into the region-specific medium spiny neurons (Fink et al., 2013; Fink et al., 2014). While our previous work offers additional support that iPSCs may be a

viable treatment for HD, it is important to replicate this work within a model that carries the genetic mutation known to cause the disease.

The goals of this study were to evaluate the efficacy of iPSC transplantation within the YAC 128 transgenic mouse model of HD and determine if the age of the mice influences functional outcomes following transplantation. In 2003, Slow and colleagues created the YAC 128 transgenic mouse model which contains the entire human *HD* gene, including the promoter and 128 CAG repeats. The YAC 128 model is characterized by having age-dependent motor and cognitive deficits and a significant loss of neurons within the striatum.

This study compared the efficacy of iPSC transplantation between 10-and 12-month-old YAC 128 mice and aged-matched wild-type (WT) mice. The specific ages were chosen according to results from the literature (Slow et al., 2003) and from previous work within our lab, which indicated that YAC 128 mice have progressive neuronal loss and by 12 months of age, as well as a significant decrease in striatal neuronal count compared to control mice. In addition, a significant correlation was found between neuronal counts and rotarod performance at 12 months of age for YAC 128 mice, indication that a greater loss of neurons in the striatum was associated with a reduced ability to perform the rotarod task. Furthermore, by 12 months of age, YAC 128 mice show a significant reduction in the volume of the striatum, cortex, and globus pallidus (Van Raamsdonk et al., 2005).

However, these deficits have not been observed in 10-month-old mice. Slow and colleagues (2003) also studied 9-month-old mice and found only trends in the parameters that were significant in 12-month-old mice. Ten-month-old YAC 128 mice have not been extensively studied in the past. However, previous work within our lab has shown that there is no significant

difference in accelerod performance between YAC 128 mice and control littermates when testing began at 10-months of age (data not published).

Given this background, this study focused on analyzing the effectiveness of the iPSC transplantation in YAC 128 mice at 10- and 12-months of age, along with age-matched WT littermates. Behavioral and histological parameters were used to evaluate the effectiveness of the transplantation in mice at these two different ages. This study was designed to address the question of whether or not iPSC transplantation can be effective in ameliorating behavioral and anatomical deficits that are present before and/or at the onset of the disease symptoms.

CHAPTER II

MATERIALS AND METHODS

Animals

Male and female wild-type and HD YAC 128 mice (Jackson Laboratory, Bar Harbor, ME) were used in the study. There were eight groups in the study, which included 10-month-old and 12-month-old YAC 128 and aged-matched wild-type (WT) mice transplanted with vehicle control or iPSCs, n= 6-7. All animal tests and procedures were performed in strict adherence to the Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Central Michigan University. All animals were housed under a reverse day-light cycle and were kept at a constant room temperature of 22 °C. All mice were kept in a polycarbonate cage with 1-3 animals per cage were given access to food and water *ad libitum*.

Induced Pluripotent Stem Cell Culture

Cultures of iPSCs followed a modified version of a previously published protocol generated within our lab (Fink et al., 2013). Briefly, adenoviral-generated iPSCs, cryopreserved in a 10% solution of DMSO (Sigma-Aldrich, St. Louis, MO), were thawed and plated on a layer of Poly-L-Lysine (Sigma) in iPSC culture medium, [Dulbecco's Modified Eagles Media (DMEM; Thermo Fisher Scientific, Waltham, MA), supplemented with 10% knockout serum (KOS; Thermo Fisher), 1X non-essential amino acids (NEAA; Thermo Fisher), 1x β-mercaptoethanol (Thermo Fisher), 20 ng/mL basic fibroblast growth factor (bFGF; Thermo Fisher), 10 ng/mL mouse leukemia inhibitory factor (LIF; Thermo Fisher) and 5 mg/mL

streptomycin and 5 UI/mL penicillin]. The cell culture media was changed daily and the cells were passaged once a week.

Transplantation

Once the mice reached 10- or 12-months of age, they were randomly assigned to receive intrastriatal injections of either iPSC or Hanks Balanced Salt Solution (HBSS; Thermo Fisher). The animals were anesthetized with a combination of isoflurane and oxygen, their heads were shaved and fixed into stereotaxic ear-bars with lambda and bregma kept on a horizontal plane. A mid-sagittal incision was made to expose bregma and burr holes were drilled at 0.50 mm AP and ±1.75 mm ML from bregma. A 5 µL Hamiltan syringe was then lowered into each of the burr holes to a depth of -2.5 mm from dura. Approximately 400,000 iPSCs, pre-labeled with Hoechst 33342 (Sigma) and suspended in 2 µL of HBSS, were injected into the striatum of each hemisphere at a rate of 1 µL/min. Non-transplanted control mice received equivalent injections of HBSS. Once the injections were complete, the needle was left in place for 3 minutes and the needle was withdrawn, the skin was sutured, and the mice were placed into a recovery cage until they were mobile. Mice were monitored for 5 days and their sutures were removed by 10 days, post-transplantation.

Accelerod

The accelerod task was used to evaluate motor coordination by measuring the latency of the mice to fall from a rotating rod (San Diego Instruments, San Diego, CA) as it progressively accelerated from 5 to over 15-rpm. A week prior to surgery, mice were trained for 5 consecutive days, with 5 testing sessions occurring each day. A day prior to surgical transplantation, baseline measurements were recorded. Testing resumed at 4-5 days post-transplantation and continued

once per week for 10 consecutive weeks. For each testing day, the latency to fall off of the accelerating rod was recorded for 5 trials. Each trial was followed by a 45-second rest period. The measurements for the 5 trials were averaged to obtain one measurement for each testing day.

Open Field

The open field behavioral apparatus was used to assess spontaneous motor activity of the mice for 1 hour test periods at baseline, one day prior to surgery, and weekly for 10 weeks post-transplantation. Mice were individually placed into Plexiglas monitor boxes, which measured 41cm x 41cm x 30 cm high and was connected to a computer containing Monitor Software (Hamilton-Kinder, Poway, CA) three parameters of motor activity were recorded: (1) total distance traveled; (2) total time resting; and (3) time spent in the periphery (next to the walls of the open-field apparatus).

Clasping

Clasping is an abnormal response observed in transgenic mice, which is characterized by the withdrawal of fore and/or hind limbs into the torso when the mouse is suspended by the tail. In this study, baseline clasping was recorded one day prior to surgery and once a week for 10 consecutive weeks post-transplantation. In order to assess clasping behavior, mice were suspended by their tails at a height of 30 centimeters for three, 30-second trials. Within this study, clasping was operationally defined as the withdrawal of a limb into the torso for at least 1 second. For each trial, a clasping score ranged from 0 to 4, where 0 represents the absence of limbs clasped, 1 represents the clasping of any one limb, 2 represents the clasping of any two limbs, 3 represents the clasping of any 3 limbs, and 4 represents the clasping of all limbs. The clasping scores were averaged to obtain one score for each animal per testing week.

Histology

At the conclusion of the behavioral testing, all mice were sacrificed for histological analysis. Briefly, mice were given an overdose of sodium pentobarbital (Fatal Plus; Vortech Pharmaceuticals, Dearborn, MI) and transcardially perfused with 0.1M phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in PBS. The brains were extracted and suspended in 4% PFA for 24 hours, and then transferred to a 30% sucrose solution for 48 hours. Finally, the brains were flash-frozen using methylbutane (Sigma, St. Louis, MO) and stored at -80 °C until they were processed. The frozen brains dedicated for IHC were sectioned at 40 μm on a cryostat (Vibrotome, St. Louis, MO) and serially placed into six wells of a 12-well plate containing PBS. The sections were stored at 4°C for histological analysis. Two sets of brain sections were analyzed.

For one set of brain tissue from 3-4 mice in each group, the sections were processed with 3,3'-diaminobenzidine (DAB) and were labeled for microglia to determine the endogenous immune response following iPSC transplantation. The procedure began by placing brain sections into a 0.3% hydrogen peroxide solution for 20 minutes then the tissue was blocked for non-specific binding in 10% normal goat serum (NGS; Sigma) solution for one hour. Sections were incubated for four hours at room temperature with the primary antibody (Iba-1, 1: 4,000, Wako) then the tissue was placed in 4 °C for overnight incubation. The following day, tissue sections were rinsed three times with 0.1% TBS-Tween (Sigma) and incubated with the appropriate conjugated secondary antibody (biotin, 1:250, Vector Laboratories, Burlingame, CA) for one hour at room temperature. Tissue was rinsed three times with 0.1% TBS-Tween before and after being placed in avidin-biotin peroxidase solution (Vector) for one hour. Finally, tissue was incubated in the DAB solution (Vector) for 5 minutes. After the DAB incubation period, the

8

tissue was transferred into deionized H_2O. The tissue was mounted onto positively charged glass slides and was allowed to dry overnight. Finally, the slides were rinsed with xylene (Sigma, St. Louis, MO) for two minutes and were coverslipped with Eukitt® quick-hardening mounting medium (Sigma, St. Louis, MO).

The other set of tissue from the brains of 3-4 animals in each group was designated for fluorescent immunohistochemistry to determine differences in neuronal counts between study groups. The procedure began by placing brain sections into a 10% NGS solution to block for non-specific binding. Next, tissue was incubated with the primary antibodies (NeuN, 1:500, Millipore, Billerica, MA; DARPP-32, 1:500, Abcam, Cambridge, UK) overnight at 4 °C. The next day, the sections were rinsed and incubated with the secondary antibody (Alexa Fluro 488, 1:1000, Thermo Fisher) for one hour at room temperature. After the incubation in the secondary antibody, the tissue was rinsed, mounted on glass microscope slides, and coverslipped with Fluromount mounting medium (Sigma, St. Louis, MO).

Imaging and Analysis

Once the tissue was processed for histological markers, cells labeled positive for NeuN, DARPP-32, and Iba-1 were counted within the striatum for all experimental groups. The landmarks used to define the borders of the striatum were the corpus callosum, lateral ventricles, and anterior commissure. The cell counts were recorded from both striatum in three random chosen sections on microscope slides. NeuN and DARPP-32 images were captured using fluorescent microscopy and consistent exposure times were maintained for each slide. Iba-1 slides were captured using bright-field microscopy. Once images were captured, estimation of total neuronal profiles were performed using MBF Stereo Investigator Software (MBF

9

Bioscience, Williston, VT). DARPP-32 and NeuN cell counts were performed using 200 x 200 counting frames evenly distributed in the striatum. Iba-1 cell counts were performed using 600 x 600 count frames. Estimation of total cell counts was obtained and averaged for three brain sections.

Statistics

All statistical analysis was performed using SPSS v24. Accelerod, open-field, and clasping data was analyzed using a repeated measures analysis of variance (ANOVA), which measured the difference between groups and treatments across the eleven weeks of behavioral testing. Histological data was analyzed for group differences using a one-way ANOVAs. The statistical significance was set at an alpha level of 0.05 and, when appropriate, a Fisher's Protected Least Significant Difference (PLSD) test was performed for *post-hoc* analysis.

CHAPTER III

RESULTS

Behavioral Assessments

Accelerod

A repeated measures ANOVA indicated significant differences in latency to fall off the accelerod task among the four groups of 10-month-old mice, (F[3, 23] = 7.26, p = .001) (Figure 1). LSD *post hoc* analysis revealed that there was a significant genotype effect however, there was no significant difference between vehicle control and iPSC transplanted mice. WT mice had significantly higher latency to fall times than did HD mice.

In terms of the four groups of 12-month-old mice, the analysis of accelerod performance indicated that there was a significant difference between the groups (F[3, 22] = 9.185, p = .000) (Figure 2). *Post hoc* analysis revealed that vehicle-treated WT mice were significantly different from iPSC-treated WT mice, iPSC-treated HD mice, and vehicle-treated HD mice, indicating that vehicle-treated WT mice remained on the accelerod significantly longer than iPSC-treated WT mice and both HD treatment groups.
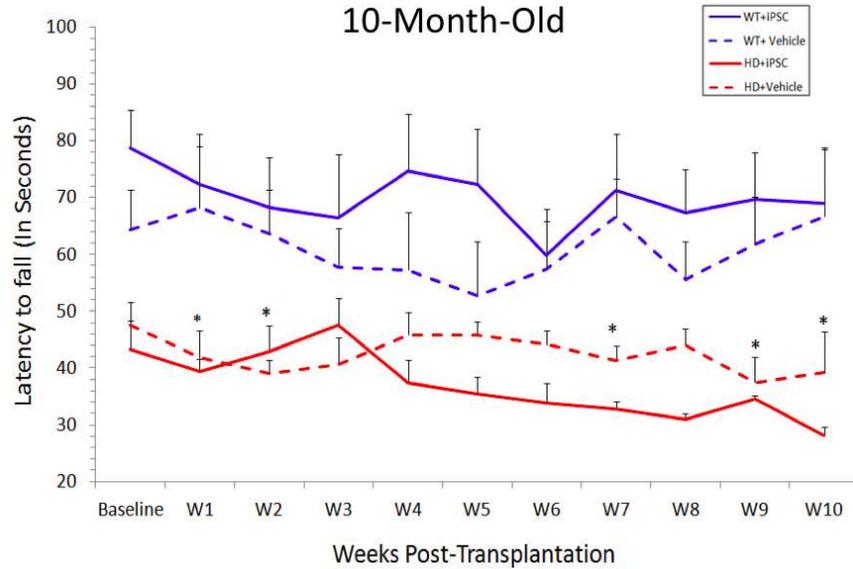
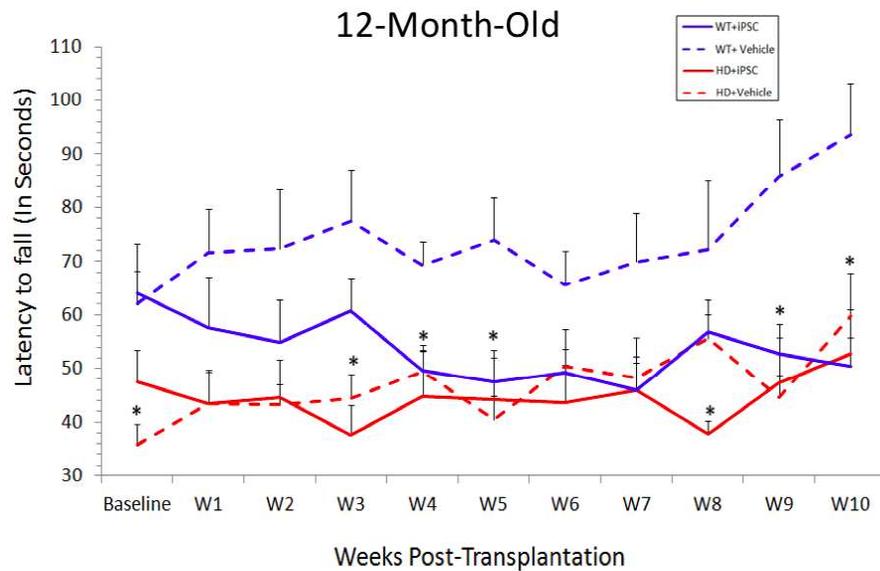Figure 1. Analysis of Motor Coordination for 10-Month-Old Mice

Figure 2. Analysis of Motor Coordination for 12-Month-Old Mice

Figure 1 and 2 Legend. Analysis of motor coordination for 10- and 12-month-old mice. Change in motor coordination following iPSC transplantation was analyzed by measuring how long the mice could remain on an accelerating rod. Average latency to fall, in seconds, was measured at baseline, one week before transplantation, and for 10 consecutive weeks post-transplantation. The 10-month-old vehicle and iPSC-treated WT mice remain on the accelerod longer than vehicle and iPSC-treated HD mice (Figure 1). In the 12-month-old groups of mice, the vehicle-treated WT mice had significantly longer latency to fall compared to iPSC-treated WT mice, iPSC-treated HD mice, and vehicle-treated HD mice (Figure 2). Line graphs represent mean values prior to surgery and for 10 consecutive weeks post-transplantation. Error bars represent SEM. Significantly different from WT+Vehicle (*$p < 0.05$).

12

Open Field

The repeated measures ANOVA statistical test was performed to determine differences in total distance traveled, total time spent resting, and time spent in the periphery. Among the four groups of 10-month-old and 12-month-old mice, there was no significant difference in the total distance traveled, total time spent resting, and time spent in the periphery between the groups, respectively, $((F[3, 23] = 0.125, p = 0.944; F[3, 22] = 0.602, p = 0.621), (F[3, 23] = 0.460, p = 0.713; F[3, 22] = 1.491, p = 0.244); (F[3, 23] = 1.04, p = 0.392; F[3, 22] = 0.729, p = 0.545))$ (Data not shown).

Clasping

A repeated measures ANOVA showed that among the four groups of 10-month-old and 12-month-old mice, there was no significant difference in the number of limbs clasped over time and between the groups $(F[3, 23] = 0.805, p = 0.892; F[3, 22] = 0.614, p = 0.613)$ (Data not shown).

Histological Results

Region Specific Neuronal Cell Counts

A one-way ANOVA indicated that there was no significant difference in the total number of DARPP-32 positive cells in the four groups of 10-month-old mice, $(F[3,11] = 1.87, p = 0.213)$ (Figure 4). However, a one-way ANOVA revealed significant differences in the average of DARPP-32 positive cells among the four groups of 12-month-old mice, $(F[3,11] = 6.508, p = .015)$ (Figure 4). LSD post hoc analysis indicated that HD+ mice, which received vehicle treatment, had significantly less DARPP-32 positive cells compared to iPSC-treated HD+ mice

and both vehicle and iPSC-treated WT mice. Furthermore, iPSC-treated HD+ mice were not

significantly different from vehicle and iPSC-treated WT mice. A one-way ANOVA was

conducted to determine group differences between average number of NeuN-positive cells. This

analysis indicated that there were no significant differences between the four groups of 10-

month-old mice, (F[3,11] = 3.332, p = 0.077) (Figure 3) however, there was a significant

difference among the four groups of 12-month-old mice, (F[3,11] = 4.62, p = 0.037) (Figure 3).

LSD post hoc analysis revealed that vehicle-treated WT mice had significantly more cells than

vehicle-treated HD mice and both iPSC-treated WT and HD mice. Images displaying NeuN and

DARPP-32 positive labeling for 10-month-old and 12-month-old mice are represented in Figure
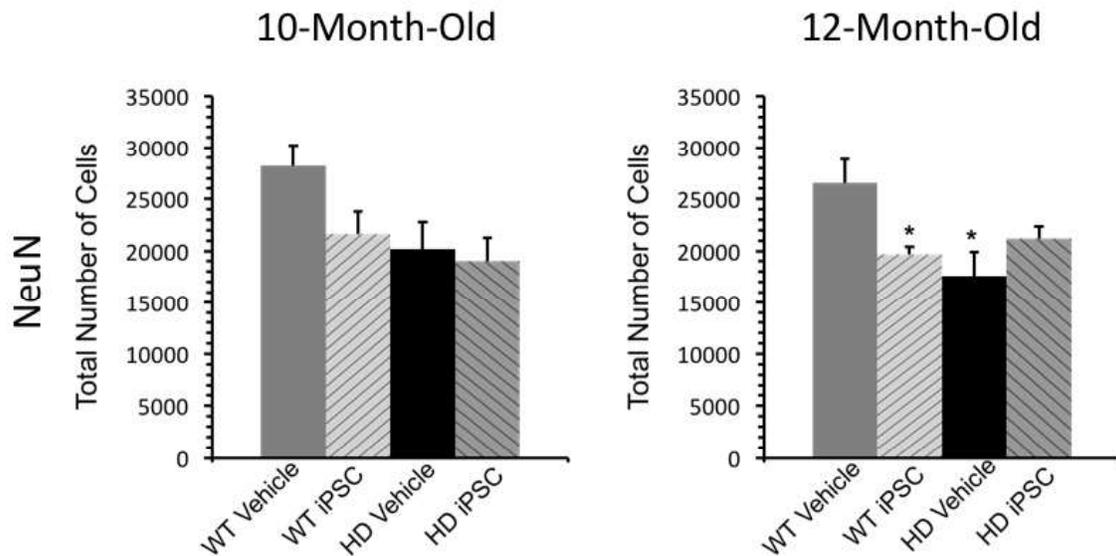
5.

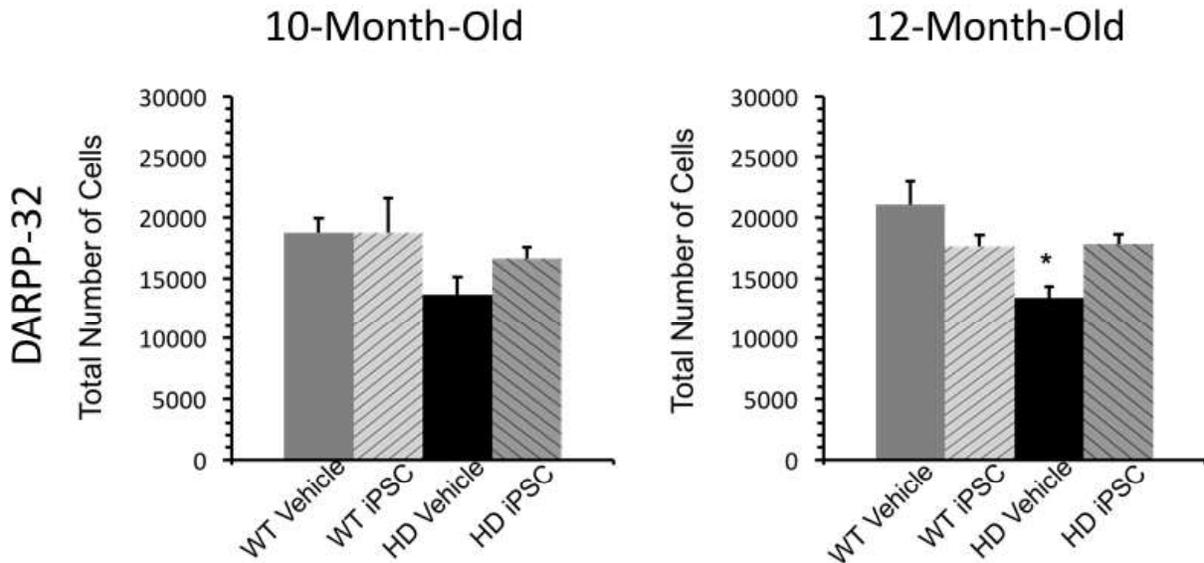Figure 3. Counts of Mature Neurons in the 10- and 12-Month-Old Mice

Figure 4. Counts of Medium Spiny Neurons in the 10- and 12-Month-Old Mice

Figure 3 and 4 Legend. Counts of mature neuron and medium spiny neurons in the 10- and 12-month-old mice. Average number of NeuN-positive and DARPP-32-positive cells were counted to assess whether the transplanted iPSCs could improve pathological deficits in HD mice and to determine if the outcome was age-dependent. Positively labeled cells were counted within the striatum in three coronal sections and averaged to obtain one value for all mice. Although no differences were observed in the number of positively labeled DARPP-32 and NeuN cells in the 12-month groups (A & C), the vehicle-treated HD mice had significantly less DARPP-32 and NeuN-labeled cells than all other 12-month groups except for the number of NeuN-positive cells in the WT-iPSC mice (B). Bar graphs represent mean values and error bars represent SEM. Significantly different from WT+Vehicle (*$p < 0.05$).
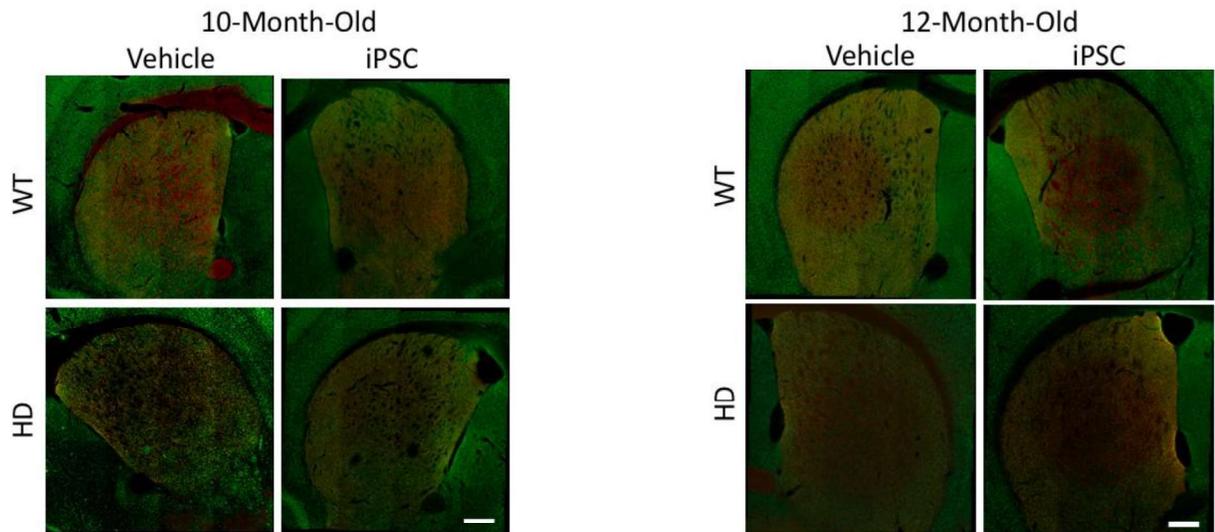
Figure 5. Images Displaying NeuN and DARPP-32 Positive Labeling for 10-Month-Old and 12-Month-Old Mice

Figure 5 Legend. Images representing NeuN (green) and DARPP-32 (red) positive labeling for 10-month-old mice and 12-month-old mice. A 20x objective was utilized for imaging and scale bars are 400 μm in length.

Microglia Cell Counts

Two separate one-way ANOVA tests indicated that there were no significant differences in total number of positively labeled Iba-1 cells among the four groups of 10-month-old (F[3,11] = 0.500, p = .692) and 12-month-old mice (F[3,11] = 1.301, p = 0.339), respectively (Figure 6). Images displaying Iba-1 positive cells among the four groups of 10- and 12-month-old mice, shown in Figure 7.
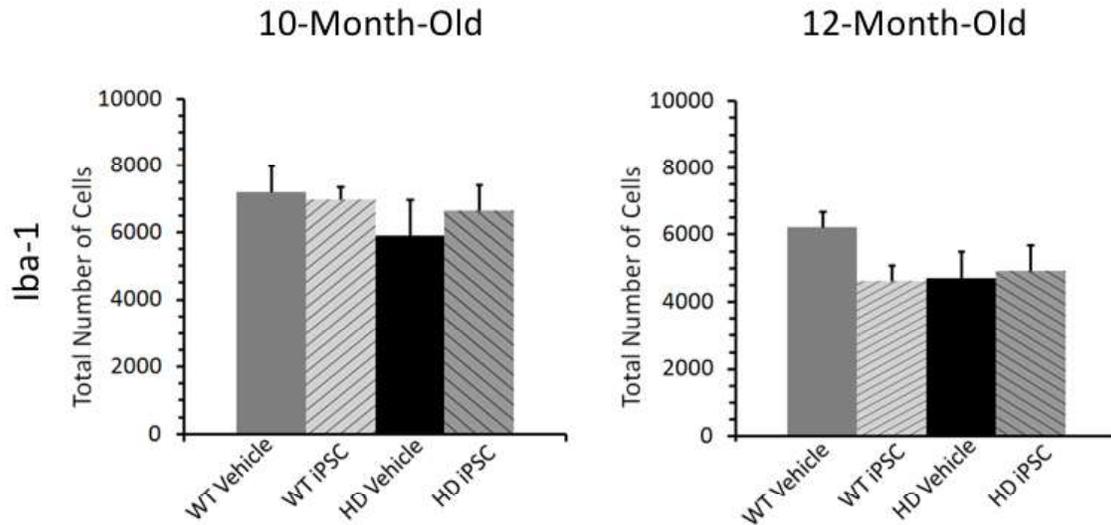
10-Month-Old

12-Month-Old

Figure 6. Microglia Cell Counts in the 10- and 12-Month-Old Mice
Figure 6 Legend. Average number of Iba-1 positive cells were counted to determine if the
endogenous immune response changed following transplantation. Positively labeled cells were
counted within the striatum for three coronal sections and averaged to obtain one value for all
mice. No between-group differences were found for the average number of Iba-1 positive cells
between the four groups of 10- and 12-month-old mice. Bar graphs represent mean values and
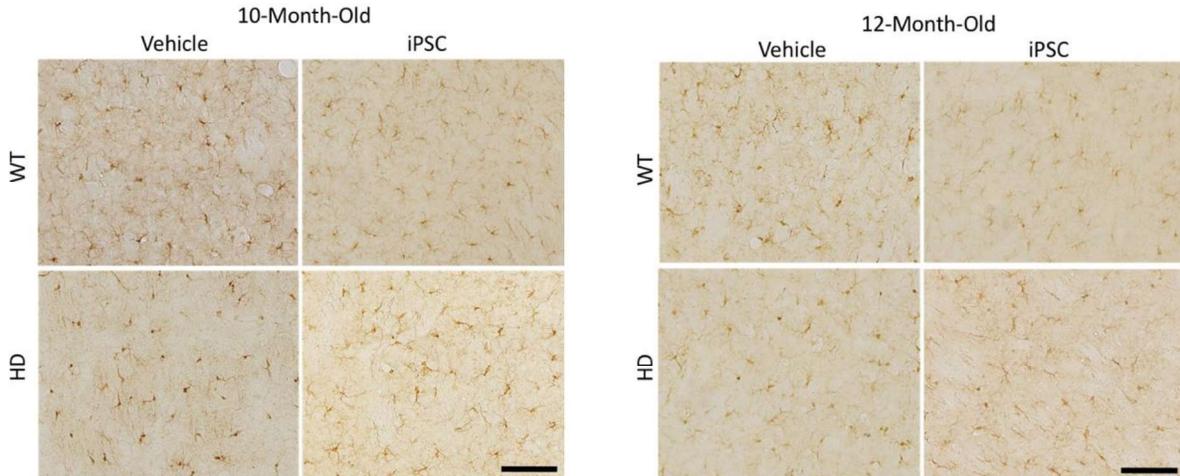error bars represent SEM. Significantly different from WT+Vehicle (*$p < 0.05$).

Figure 7. Images Displaying Iba-1 Positive Labeling for 10-Month-Old and 12-Month-Old Mice
Figure 7 Legend. Corresponding Images Displaying Iba-1 Positive Cells Within The Striatum Of
The 10- And 12-Month Groups. A 20x Objective was utilized for Imaging and Scale Bars Are
100 μm In Length.

CHAPTER IV

DISCUSSION

The objective of this study was to determine if the disease state or age at the time of transplantation influenced how effective iPSC transplantation could be at ameliorating behavioral and pathological deficits within the YAC 128 mouse model. The accelerod behavioral task enabled us to compare motor coordination between WT and HD mice and determine if iPSC treatment could improve motor coordination among the HD mice. Our data revealed that iPSC treatment did not improve the motor coordination of 10- and 12-month-old HD mice. The motor coordination of iPSC-treated 10- and 12-month-old WT mice was not significantly different from the aged-matched vehicle-treated HD mice. Interestingly, the iPSC-treated WT 12-month-old mice had significantly lower latency to fall than aged-matched vehicle-treated WT mice, indicating that transplanting iPSCs can adversely affect motor performance in the older WT mice.

In addition to the accelerod behavioral task, open-field testing was conducted to compare locomotion and anxiety-like behavior between the treatment groups. Previous studies have shown that as early as 6-months-of age, YAC 128 mice travel significantly less distance and spend significantly more time resting compared to aged-matched WT mice (Chiu et al., 2014; Slow et al., 2003). Furthermore, spending more time in the periphery of the open field apparatus is an indicator of anxiety-like behavior in several mouse models of HD (Pla et al., 2014). Our data indicated that there were no significant differences in total distance traveled, total time resting, and time spent in the periphery between all treatment groups, regardless of age, genotype, and treatment group.

Immunohistochemistry was performed to analyze pathological changes in all groups of the YAC mice. Previous characterization of the YAC 128 model has shown that the number of mature neurons and medium spiny neurons, within the striatum, progressively decreases as the HD mice age (Slow et al., 2003; Van Raamdonk et al., 2005). Within this study, the total number of NeuN positive cells (mature neurons) and DARPP-32 positive cells (medium spiny neurons) were calculated within the striatum. Results showed that as both WT and HD mice aged, the total number of NeuN positive cells decreased, indicating that age plays a role in the survival of neuronal cells. Our data revealed that there were no between-group differences among the four groups of 10-month-old mice in total number of NeuN and DARPP-32 positive cells. In regard to the four groups of 12-month-old mice, vehicle-treated WT mice had more NeuN-positive cells than iPSC-treated WT mice and vehicle-treated HD mice. In terms of DARPP-32 labeling, the vehicle-treated HD mice had significantly less DARPP-32 cells compared to all other groups. These findings indicate that iPSCs can reduce age-related neuronal and MSN loss, but that transplanting them into WT 12-month-old mice can accelerate neuronal loss.

A limitation of stem cell transplantation can be related to rejection by the immune system of the host. The average number of Iba-1 positive cells, which label for microglia, were counted and compared among the four groups of 10- and 12-month-old mice. Our results suggested that there was no significant difference in the average number of Iba-1 positive cells, regardless of age, genotype, or treatment group. The results were surprising given the finding of the adverse effects that the iPSCs had on accelerod performance and neuronal survival in the 12-month-old mice.

The results from the current study suggest that the previous findings with the 3-NP and QA models of HD might not generalize to other models. In the present study, we found that even

though the average number of DARPP-32 positive cells increased in 12-month-old iPSC-treated HD mice, this did not translate to an improvement in motor function. Previous research investigating iPSC transplantation into the 3-NP and the quinolinic-acid (QA) rat models of HD showed behavioral recovery and neuronal sparing following transplantation (Fink et al., 2014; Mu et al., 2014) In the previous work in our lab, iPSC transplanted 3-NP rats showed significant recovery of motor function compared to 3-NP and vehicle-treated control rats on the accelerod task, and the histological results revealed that iPSCs differentiated into region specific neurons (Fink et al., 2014). However, when iPSCs were transplanted at a late time point in the 3-NP model, the rats displayed the same neuropathological deficits as the control 3-NP rats, despite an improvement in motor function (Fink et al., 2014). A study using the QA model of HD, showed that iPSC transplantation reduced learning and memory deficits on the Morris water maze task and histological analysis revealed that transplanted iPSCs reduced striatal atrophy and differentiated into medium spiny neurons and glial cells (Mu et al., 2014). The results from the current study suggest that the previous findings with the 3-NP and QA models of HD might not generalize to other models. In the present study, we found that even though the average number of DARPP-32 positive cells increased in 12-month-old iPSC-treated HD mice, this did not translate to an improvement in motor function.

The YAC 128 mouse model is able to replicate genetic hallmarks of the disease unlike the 3-NP and QA lesion models. As early as 2 months of age, YAC 128 brains can be positively labeled for EM48, which selectively labels nuclear localized mutant huntingtin protein (mHTT). The highest concentration of EM48 labeling is found in the lateral striatum, where the most down-regulation of DARPP-32 expression appears, and the EM48 expression increases with age and expands into other brain regions (Van Raamsdonk et al., 2005). In addition, previous

research suggests that mHTT accumulates within microglia, leading to neuronal degeneration through cell-autonomous and non-cell-autonomous mechanisms (Yang et al., 2017). The chronic exposure of the mHTT may have contributed to creating an environment that does not support the successful integration of the transplanted iPSCs.

Although improvements in motor function have been reported following stem cell transplantation within the YAC 128 model, often critical variables may have contributed to those improvements. Type, age, number of passages, and other manipulations of the cells can influence outcomes. In other work from our lab, (Dey et al., 2010), we found significant behavioral and neuronal sparing following transplantation of mesenchymal stem cells (MSCs) that overexpressed brain derived neurotrophic factor (BDNF). It is well documented that MSCs provide therapeutic benefit by releasing neuroprotective factors and this benefit is enhanced by overexpressing BDNF, a neurotrophic factor reduced in HD (Kerkis et al., 2015). Recovery of motor function occurred when human iPSC-derived neuronal precursor cells were transplanted within 12-month-old YAC 128 mice (Jeon et al., 2013). Within this study, all mice received injections of cyclosporine A prior to and daily for 12 weeks post-transplantation. The administration of the immunosuppressant may have contributed to the survival and success of the transplanted cells. Taken together, the environment within the brain can play a major role in how successful the graft will be.

The degree that the immune response was responsible for the lack of an iPSC-induced behavioral benefit in the present study is questionable. Our results showed that as the mice aged, the average number of Iba-1positive cells decreased. However, there were no significant differences between genotype or treatment were found. Previous characterizations of the YAC 128 model showed that even though the total number of Iba-1 positive cells was not significantly

different between genotypes, the morphology of these cells was different (Franciosi et al., 2012 & Johnson et al., 2015). Briefly, microglia morphology is governed by signals in the environment. Within a healthy environment, microglia are typically in a patrolling state that is characterized by a small cell body and many thin ramified processes extending from the cell body. During trauma or infection, microglia become reactive and respond by increasing the size of their cell body and retracting their processes towards the cell body (Hanisch et al., 2007). HD patients and YAC 128 mice have been shown to have higher levels of reactive microglia compared to healthy controls (Crotti & Glass, 2015; Franciosi et al., 2012; Johnson et al., 2015). It is still debated as to whether these reactive microglia are neuroprotective by removing mutant huntingtin and debris from degenerated neurons, or if are they neurotoxic by releasing pro-inflammatory cytokines. Therefore, in order to understand how microglia responded to the iPSC transplantation, future investigations should study morphological changes or use specific markers to differentiate between microglia states, such as quantifying the unique cytokines released from patrolling and reactive microglia.

The outcome of the open-field testing may have been negatively influenced by the experimental procedure. Open-field testing was performed one week before surgical transplantation and once a week for 10 consecutive weeks post-transplantation. Each open-field session was conducted for 60 minutes. The combination of weekly testing and the length of time of each session may have decreased the novelty of the task in order to collect valid data. Previous experiments, utilizing the YAC 128 mouse model, have found genotype differences in total distance traveled and time spent in the center of the open field apparatus, but each of those sessions were conducted for only 10-30 minutes. (Chiu et al., 2011; Pollock et al., 2016; Slow et al., 2003). Therefore, the experimental procedure of the current study may have reduced the

desire to explore the open field apparatus and the familiarity of the environment may have reduced anxiety-like behavior in the HD mice.

Overall, our results suggest that age significantly influences the outcome of the grafted cells. The total number of medium spiny neurons appeared to be greater within 12-month-old HD mice post-transplantation compared to age-matched vehicle-treated HD mice However, this neuronal preservation did not lead to an improvement in motor function. Interestingly, 12-month-old iPSC-treated WT mice had significantly more medium spiny neurons than aged-matched vehicle-treated HD mice but, these WT mice experienced a decrease in motor function, post-transplantation. The decreased motor function may be explained by a significant decrease in the number of mature neurons that help to support motor function. As the mice aged and regardless of genotype or treatment, there were significantly less mature neurons, medium spiny neurons, and microglia. Based on these results and findings from the literature, environmental changes may be progressively changing with age that may be detrimental to the survival of transplanted iPSCs. The results of this study suggest that iPSCs did not provide a therapeutic benefit for treating HD.

Cell-replacement therapy is still a viable strategy for treating HD. However, the type of cell as well as other factors, including donor age, number of passages, and method of preparing the cells seem to be important. Priming iPSCs prior to transplantation may increase the likelihood that the transplanted cells will provide therapeutic benefit. Recent data from our lab has shown that induced neural stem cells (iNSCs), derived from iPSCs, were able to significantly improve motor function, differentiate into region-specific neurons, and increase the levels of BDNF and TrkB post-transplantation in 10-month-old HD YAC 128 mice. Similar results were

found when neural precursor cells, derived from patient iPSCs, were transplanted within 12-month-old YAC 128 mice (Jeon et al., 2014).

Although our results suggest that use of iPSCs as a therapy for HD may be more limited than we originally thought, the use of pre-differentiated iPSCs might be a more viable option. However, iNSCs, derived from patient specific iPSCs and corrected with new gene editing techniques, may be a more effective strategy for treating behavioral and pathological deficits of HD. Clearly, more work is needed to discern what the optimal cell type and preparation procedures should be to maximize the efficacy of stem cell therapies for HD.

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