

April 2011

Optimization of Procedures for Establishing Mouse Primary Cortical Cultures and siRNA Knockdowns for Studying Huntington's Disease Proteins

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Optimization of Procedures for Establishing Mouse Primary Cortical Cultures and siRNA Knockdowns for Studying Huntington's Disease Proteins

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biochemistry (TM)

and

Biology and Biotechnology (KW)

by

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April 28, 2011

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ABSTRACT

Huntington's disease (HD) is one of the most destructive progressive neurodegenerative disorders, and is caused by a genetic mutation in the *HTT* gene encoding Huntingtin. To assist Neil Aronin's ongoing HD research at UMass Medical Center, we developed a more efficient protocol for mouse cortical dissection and plating. In addition, we optimized a procedure for transducing cortical cells with lentiviruses containing various siRNAs and GFP, and assayed the percent knockdown by qRT-PCR. In the future, these procedures will help assess the role of previously identified protein partners of mutant Huntingtin that contribute to HD pathogenesis.

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ACKNOWLEDGEMENTS

We would like to thank Neil Aronin, UMass Memorial Medical Center (Worcester) for allowing us to work in his lab and for sponsoring this project. We would also like to thank David Adams for serving as WPI Project Advisor. Special thanks to Katherine Chase or the Aronin lab for her assistance in all laboratory procedures, guidance and working with us to complete the project. In addition, we would like to thank Wanzhao Liu, Edith, and Miguel Esteves for their assistance in qRT-PCR and lentivirus construction.

BACKGROUND

Millions of people worldwide are afflicted with hundreds of genetic disorders. A genetic disorder is a type of disease that results from a mutation or abnormality in one's genetic makeup. These can be caused by a multitude of reasons; some are caused by a single mutation in a gene, while others involve many deletions or insertions of various sizes (University of Utah, 2011). Some of the best known genetic diseases are Down syndrome, Alzheimer's disease, Cystic Fibrosis, and Huntington's disease.

Huntington's Disease Description

Huntington's disease (HD) is one of the most destructive genetic brain disorders (Huntington's Disease Society of America, 2008). Scientists typically refer to it as a 'progressive neurodegenerative disorder' (Gusella et al., 1983) due to the prevalence of genes that are programmed to deteriorate neurons located in various regions of the brain. Progressive deterioration leads to severe loss of bodily functions, such as coordinated movement, thought and behavioral functions. The onset of the disease more commonly occurs later in an individual's life, and is usually detected between the ages of 30 and 50; however, rare cases of juvenile HD have been seen in children as young as two (Huntington's Disease Society of America, 2008). Since HD is a genetic condition, the mutation is passed through generations (National Institute of Neurological Disorders and Stroke, 2010) and is inherited through autosomal dominance (Gusella et al., 1983). For this reason it has a greater risk of affecting the offspring of individuals that have HD. For example, if one parent has HD they have a 50 percent chance of passing it to their offspring – causing them to be afflicted with the disease (**Figure 1**). Likewise, if that

child does not receive the gene for HD, they cannot pass the gene to their children. As a result, if both parents have Huntington's, there is a 100 percent chance that their offspring will have the gene for the disease. Upon inheritance, the individual will eventually get the disease; it is hard to predict the onset as patients develop symptoms at varying rates and severity. Considering the fact that Huntington's is a highly inheritable disease, 97 to 99 percent of people that have Huntington's had a known family member with the disease (National Institute of Neurological Disorders and Stroke, 2010).

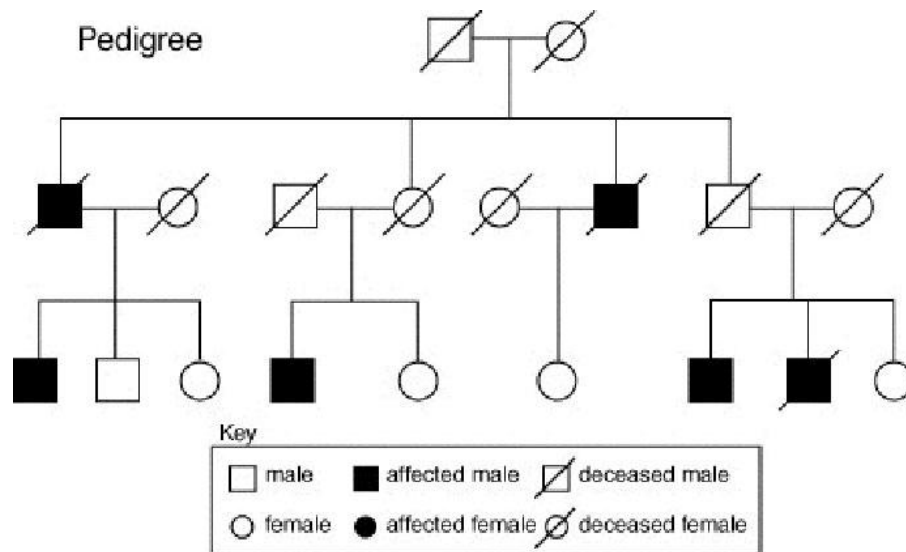


Figure 1: Example Pedigree Chart of Huntington's Disease. (Neuroethics, 2011)

People Affected by HD

Huntington's disease affects about 250,000 Americans; whether they have it or are at risk of getting it (Huntington's Disease Society of America, 2008). It is estimated that approximately five to ten people out of 100,000 people have HD. Due to late-onset of this disease, this number is not necessarily accurate as most individuals have had children before realizing they have HD, unknowingly affecting more of the population (Gusella et al., 1983). HD is not a sex-linked genetic disorder, so it affects both men and

women equally. Interestingly, it appears to be more prevalent in people of European decent – as opposed to African, Japanese, and Chinese descendants (Genetics Home Reference, 2008).

Causes of Huntington's

Huntington's disease is caused by a genetic mutation in a gene *HTT*, used to make the protein Huntingtin. Its exact role remains unknown, but it has been shown to play a crucial part in brain neurons. The Huntington's disease *HTT* mutation (*mHTT*) has a specific DNA segment interrupted by a CAG trinucleotide repeat (cytosine, followed by an adenine, and then a guanine), which is repeated many times continuously (Figure 2). Individuals with normal, wild-type *HTT* have a CAG sequence that is generally repeated between 10-35 times, while people with *mHTT* have a repeat from 36 to over 120 times.

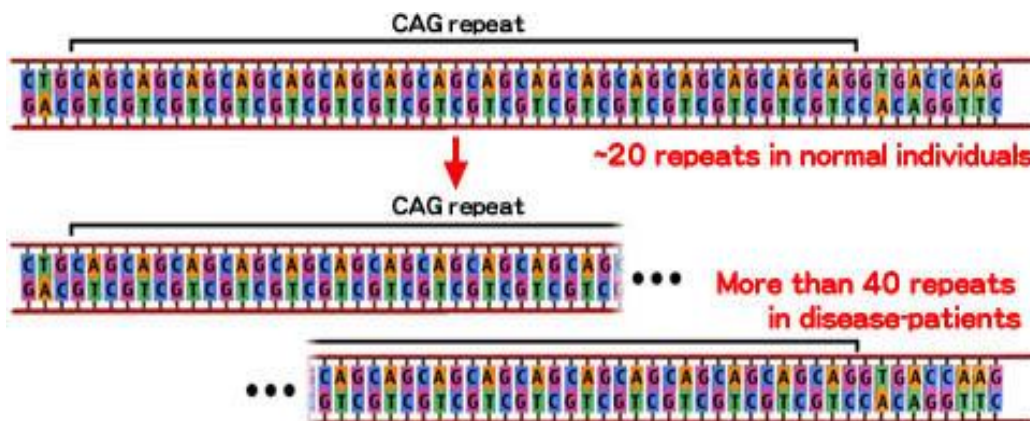


Figure 2: Example of CAG Repeat Sequences in *HTT*. Shown are example CAG trinucleotide repeat sequences in normal individuals (top row) and HD patients (bottom row) (“What are Polyglutamine Diseases?”, 2002)

People who have over 40 CAG repeats almost always have the disease, while people that have 36-40 will not necessarily have HD (Genetics Home Reference, 2008).

If a person has between 27 and 35 repeats, their children are at risk for HD because as the gene is inherited, it has an increased chance of lengthening.

The increase in CAG repeats results in a much longer Huntingtin protein. This, larger than average, piece of protein is then cut into smaller pieces which become detrimental to the body as they join together and collect in the neurons of the brain. Aggregation of these fragments causes the neurons in the brain to deteriorate, resulting in a loss of crucial elements that control everyday thoughts, movements, and actions



Figure 3: Brain Morphology of Huntington's Disease (upper) vs. Unaffected (lower). Folding@Home, 2010)

(Genetics Home Reference, 2008). The further progression of neuronal decay increases the severity of the disease, eroding the folds and separations found in the brains of non-afflicted individuals which cause the patients to suffer more (Figure 3). Some people lose up to 25 percent of their brain with HD

(University of Utah, 2011).

HD Symptoms

There are a variety of different symptoms associated with Huntington's disease, which can be broken down into the follow categories: juvenile symptoms, early symptoms, and late onset symptoms. Although juvenile and adult Huntington's disease have the same molecular basis for disease (increased CAG repeats in *HTT*), there are distinct differences in the progression and physiological depiction of the symptoms.

Juvenile Onset

Some of the first signs of juvenile Huntington's are slow movements, frequent falls, clumsy, impaired or slurred speech, and drooling. As the disease progresses, the quality of their schoolwork slowly drop because their thinking and logic skills are decreasing. Children that have Huntington's disease often have seizures; this occurs in almost 50 percent of childhood cases (Genetics Home Reference, 2008). Advancement of the disease results in children having increased movement issues, accompanied by emotional and mental alterations. Another key difference is that juvenile HD is generally faster progressing, shortening the child's lifespan to about 10 years after onset, as opposed to the 10-25 years for adults.

Adult Onset

As for adult-onset individuals, there are early signs and then symptoms that are slower to progress. The early signs include mostly cognitive and movement issues, which can be as simple as becoming clumsy, having mood swings, or experiencing depression and memory loss. As the brain continues to deteriorate these symptoms become worse, short and long term memory loss can occur, and uncontrolled movement

of the head and other body parts can also be present. The affected person can also start to have difficulty walking and talking (Huntington's Disease Society of America, 2008).

Through the progression of the disease, these symptoms become worse. Individuals become very irritable with forceful and sometimes violent outbursts. They eventually have issues making their own decisions and remembering occurrences, while their motor skills slowly depreciate. Loss of motor skills causes a HD patient to fall more often, which leads to an increased risk of injury. As this loss continues, they become incapable of swallowing and eating, and are unable to move without assistance. They slowly become dependent on others to take care of them (Huntington's Disease Society of America, 2008).

HD Treatments After Diagnosis

One of the most devastating aspects of Huntington's disease is there is no cure. Individuals who get HD will die suffering from this disease; however HD is not usually the direct cause of death. Fatality is usually caused by major complications of the disease, such as, choking, infections, or heart problems (Huntington's Disease Society of America, 2008). Although there is no way to halt the progression of Huntington's disease or reverse its effects, medications can help relieve some of the symptoms. These medications help control the involuntary muscle movements or to help treat depression. In 2008, a new medicine called tetrabenazine (**Figure 4**) was approved by the U.S Food and Drug Administration to help treat Huntington's disease (National Institute of Neurological Disorders and Stroke, 2010). Since the disease affects mobility, it is crucial for people with HD to keep active. Also, due to its effect on cognitive skills, HD patients

should try to keep their minds sharp by doing puzzles, word games, and intellectual activities (National Institute of Neurological Disorders and Stroke, 2010).

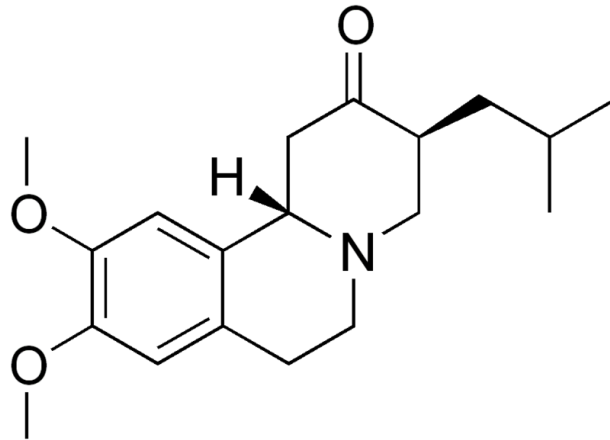


Figure 4: Chemical Structure of Tetrabenazine for Treating HD Patients. (Chemical Register, 2011)

Current HD Research

Although there is currently no cure for HD, extensive research is being done in attempts to find one. While neurobiologists study the fundamental aspects of the disease, researchers are also performing animal testing, functional and structural neuroimaging, fetal tissue work, and human clinical research (NINDS, 2010). These approaches aid our understanding of HD and may lead to a cure.

Neurobiology

Neurobiology, or the study of neurons, is one of the most fundamental forms of HD. Considering HD's deteriorating effects on the brain, neurobiology plays a crucial role in finding a cure and is a major component in ongoing research. Since the discovery of the *HTT* Huntingtin gene, neurobiologists have been trying to understand how it

contributes to HD pathogenicity (NINDS, 2010). Through previous investigations, specific parts of the brain have been labeled and their functions identified. For example, the basal ganglion area has been shown to help control movements. Knowing this type of information coupled with which areas of the brain are affected by HD will help make correlations to symptoms (Tan, 2001).

Clinical Research

Various professionals, including neurologists and psychiatrists, are working together with patients to control symptoms. For instance, clinical trials are currently being performed to study the effects of various medications including tetrabenazine on symptoms (NINDS, 2010). One way to help track the effects of various drugs is through neuroimaging.

Neuroimaging

Neuroimaging provides a significant source for data in HD research, allowing researchers to visualize what is going on in a patient's brain. One of the major types of imaging is called positron emission tomography (PET). PET is performed following the administration of a radioactive substrate (such as glucose) to yield a three dimensional, colored image of functional processes within the human body (Nordqvist, 2009). Performing routine PET scans on HD patients allows researchers to see the structures in the brain to determine how they change throughout the disease's progression, or to monitor potential improvements following therapies (NINDS, 2010).

Animal Testing

Following the identification of the HD gene (*HTT*), its effects on the brain are becoming clearer through animal experiments. For example, transgenic mice have been created that mimic the extensive *HTT* trinucleotide repeats. Researchers use such mice to perform tests and to test drugs to study the progression of the disease (NINDS, 2010).

Fetal Tissue

Most HD fetal tissue research is performed using rodents and small, non-human primates. Researchers graft fetal tissue into animals to test whether it can replace damaged areas and reverse the neuron loss caused by HD (NINDS, 2010). Fetal brains are also being analyzed to investigate the earliest stages of the disease. Early analysis may reveal aspects about HD that other research cannot.

University of Massachusetts Medical Center's HD Research

The University of Massachusetts Medical Center (UMass) is performing research which incorporates several aspects of the aforementioned methods. The goal is to “catalyze our world-class basic research into scientific discoveries with high impact clinical applications and overcome the barriers in translating knowledge into clinical practice” (UMass Medical School, 2011). In pursuing this, they have become an internationally acknowledged research institution, with several award winning and life changing discoveries, including the Nobel Prize work on RNA interference (RNAi) by Craig Mello, PhD.

RNAi is a pathway that cells use to silence gene activity. By understanding this process, researchers can manipulate specific gene activity to study the effects of decreasing the expression of a specific gene. Knocking down a gene's activity aids in identifying that gene's significance to the development and maintenance of life. In order to do so, synthesized dsRNA (or the gene encoding it) containing a sequence complementary to the gene to be knocked down is introduced inside a cell using microinjection or viral delivery. This dsRNA will be recognized as exogenous genetic material and will activate the RNAi pathway, leading to the degradation of the mRNA for that specific gene. RNAi is being used to investigate treatments for HIV and HD, and other disorders (NIGMS, 2011). Neil Aronin, MD and professor of medicine, cell biology, and physiology at UMass, has incorporated RNAi technology into his ongoing HD research (see below).

Dr. Aronin's Research

Over the last few decades, Dr. Aronin has been an influential role in the advancement of both the understanding and development of potential treatments and cures for HD. He has collaborated internationally and his work has appeared in highly reputable journals including *Science*, *Journal of Neurophysiology*, *Journal of Neuroscience*, *Proceedings of the National Academy of Sciences*, and *Cell Press*. To briefly outline his influential career:

- In 1995, he contributed to proving that there is a correlation between the presence of additional glutamine residues and the size of mutant huntingtin, as well as providing evidence that the translation of multiple mutant huntingtin protein products results from somatic heterogeneity (Aronin et al., 1995). These results

led to studies that determine the origin of the mutant huntingtin proteins and their potential contribution to pathological and functional changes in HD.

- In 1997, Dr. Aronin assisted in an experiment that showed that regions in the brain afflicted with HD accumulate and aggregate *mHTT* in the neuronal intranuclear inclusions and dystrophic neurites. Based on these findings, researchers proposed that neuronal dysfunction could result from huntingtin aggregation (DiFiglia et al., 1997)
- In 2000, Dr. Aronin worked with a team who studied the HD neuropathology. Their findings showed that autophagy and membrane tubulation of the endosomal--lysosomal system is induced by cytoplasmic aggregation of *mHTT*. This is important because they also showed that the endosomal--lysosomal system is the main pathway for removing excess huntingtin (Kegel et al., 2000).
- In 2007, Dr. Aronin silenced *mHTT* in transgenic HD mice using siRNA, showing that RNAi may be a promising therapy for HD. Transducing cc-siRNA-*HTT* effectively reduced *mHTT* protein, and blocked both neuropathology and motor deficits (DiFiglia et al., 2007).

Dr. Aronin's Current Research

Currently, Dr. Aronin and his lab are developing siRNA therapies to silence only *mHTT* mRNA, while increasing the efficiency and durability of the treatment.

Specifically, they are trying to identify the role of previously verified protein partners of mHTT that contribute to HD pathogenesis by knocking down expression of the potential partner. Various siRNAs delivered by lentiviral vectors are being tested on embryonic mouse cortical neurons (**Figure 5**). Positives identified by the primary culture screen will then be used to treat transgenic HD mice using adeno-associated viruses.

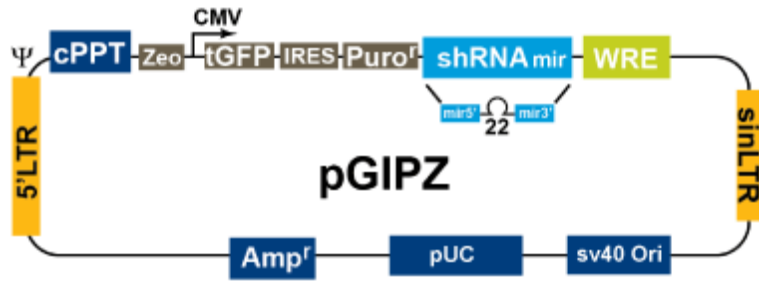


Figure 5: Diagram of a Lentivirus Vector Encoding an shRNA to Knockdown Expression of a Specific Gene of Interest (Aronin, 2010).

PROJECT PURPOSE

To assist Dr. Aronin and his team with their goal of using siRNAs to knock down expression of candidate genes that may interact with mHTT protein, the goal of this project is to optimize procedures for more efficiently deriving mouse embryonic cortical cultures, including tissue dissection, plating, and maintenance. In addition, the project will help optimize procedures for treating the primary cultures with lentiviruses encoding various siRNAs (and GFP), by assaying the percent knockdown as a measure of effectiveness. RNA will be isolated from transduced cells, then qRT-PCR will be used to assay percent knockdown. The development of these procedures will aid the Aronin lab's goal of verifying the role of previously identified protein partners of mHTT protein in HD pathogenesis.

METHODS

Materials

| Materials (Supplier) | Components | Usage |
|--|--|---|
| DMEM/F12 (Gibco) | <ul style="list-style-type: none"> • L-Glutamine • 2.438 g/L Sodium Bicarbonate | Media used for fetal mice and brain removal “pouring media” |
| Δ FBS | | Added to NB4 Active to make “plating media” |
| 5’FdU | | Antimitotic added to “feeding media” |
| HypernatE (BrainBits) | <ul style="list-style-type: none"> • Phenol Red | Media used for brain dissection and storage during dissection |
| NB4 Active (BrainBits) | <ul style="list-style-type: none"> • Neurobasal • B27 • Dipeptide L-Alanyl-L-Glutamine • Creatine • Estrogen • Cholesterol | Media used for both “plating media” and “feeding media” |
| Papain (Worthington Biochemical Corporation) | | Protease used for cell dissociation |
| Poly-L-Lysine P4707 (Sigma) | | Residue used to coat 6 well plates |
| TripLE Express (Gibco) | <ul style="list-style-type: none"> • Phenol Red | Protease used for cell dissociation |
| 5’ UtP | | Antimitotic added to “feeding media” |

Dissection Protocol

The first and most fundamental part of the project was to develop a more efficient mouse cortical dissection and plating protocol. Throughout the project, many changes were made in the original procedure to improve efficiency and viability. The dissection protocol can be divided into set-up, which remained fairly constant in this project, and fetal mouse brain dissection, which changed significantly in this project.

Prior to dissection, two sterile areas were prepared for pup and brain removal, respectively. Both areas were covered by a sterile pad and equipped with sterilized forceps and scissors. The area for pup removal, from the uterus, had a beaker of approximately 300 mL of 70% ethanol for sterilizing the pregnant mouse, a beaker of approximately 50 mL of pouring medium to rinse the uterus after removal, and a large culture dish containing 20 mL of pouring medium for the uterus. On the dissecting bench were three small culture dishes, one containing 1 mL of HypernatE (for the removal of cortical tissue), and two with 2 mL of HypernatE (one for whole brain storage, the other for cortical tissue storage), as well as a dissection microscope for cortical dissection, and ice packs for brain removal, cortical dissection, and brain storage.

In order to dissect the embryonic cortical tissue, the pregnant mouse (16 days pregnant) was killed by cervical dislocation, then rinsed with ethanol to prevent contamination. Her abdomen was cut open, and the uterus was removed. Each embryonic pup was contained inside his/her own uterine sac within the uterus, but were linked together through a large placenta (**Figure 6**). The uterus was rinsed with pouring medium and placed in the large dish for pup removal. Once the uterus had been removed, each pup was removed from their sacs using a pair of forceps and scissors. These surgical steps remained constant throughout the project.

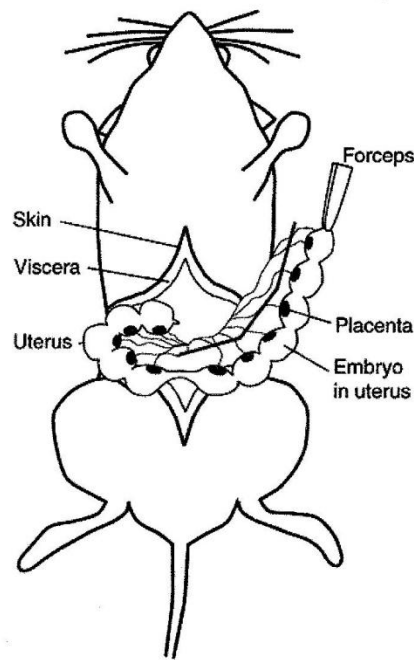


Figure 6: Diagram of the Mouse Uterus and Placenta for Removal of Pups (Nature Publishing Group, 2011).

Once all the pups had been removed, the brains could be taken out. Initially, the brain was removed by decapitating the pup and placing the head in a small culture dish with NB4 active medium. The plate was then placed under the dissection microscope for dissecting: the head was anchored by placing a set of forceps through the eyes; another set of forceps was then used to separate the skull down the sagittal suture and to pull it open to each side revealing the brain; the brain was then removed with the forceps by scooping from underneath, severing any remaining membranous and spinal connective tissue, and placed in a separate culture dish for cortical dissection. This procedure was modified to maximize the time efficiency and simplicity, and to increase the survival rate of the cortical cells. The enhanced protocol did not require decapitation of the pups, instead, the pup was anchored by its neck with a pair of forceps, then similar to before,

another set of forceps was used to separate the skull down the sagittal suture, from here there was no need to pull the skull to the sides, instead the brain could easily be removed by gently squeezing slightly below the top of the brain with the forceps and slowly lifting it up and out, pinching underneath to sever any remaining membranous and spinal connective tissue. The brain was then placed in a culture dish of HibernateE medium for cortical dissection. These modifications are shown in **Figures 7-9** (Nature Publishing Group, 2011). Each step was performed over ice to assist in preservation of the cortical cells.

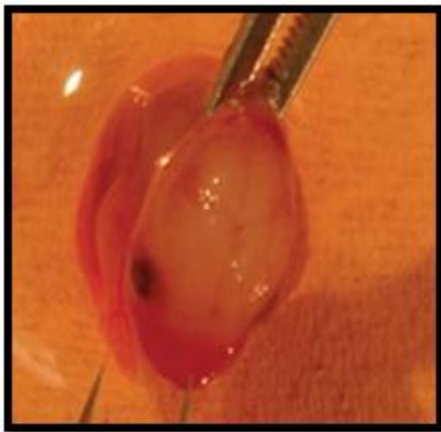


Figure 7: Brain removal, pup anchoring.

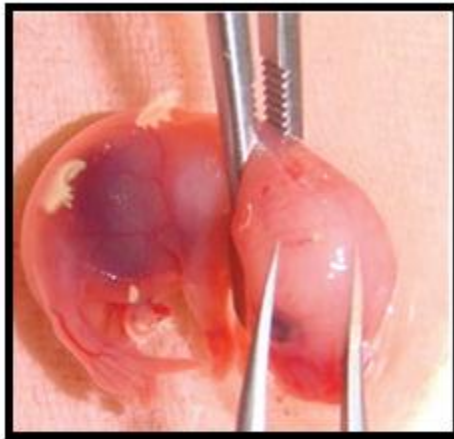


Figure 8: Brain removal, sagittal incision.



Figure 9: Final Brain Removal.

After the brains were removed, another dissection was performed to remove the cortical tissue. Due to the small size of the brain, and especially the cortex (**Figures 10 – 12**) (Nature Publishing Group, 2011), this procedure was assisted through use of a dissection microscope. The cortical tissue was accessed by placing the brain face up and using a pair of forceps to gently push from the sagittal suture out until the top layer unfolded, forming a crescent moon shape (**Figure 11**). The outermost lighter arc of this shape is the cortex, which was removed using forceps, and placed in a separate small culture dish of HypernateE medium. This procedure was then repeated for the opposite side of the brain.

Eventually this procedure was changed to a more efficient means of removing the cortex, containing less non-cortical tissue contamination. The improved method follows the same sequence of events with a slight alteration to the first step. Before pushing open the top layer to reveal the cortex, the membrane enclosing the brain was removed; to do so the brain was placed upside down to easier view the membranous tissue (blood vessels used as guidelines) and forceps were used to gently unwrap the brain. The procedure then continued as aforementioned. The removal of this membrane allowed for more efficient separation of the cortical tissue into individual cells for non-clustered plating.

These changes in the procedure led to a more efficient protocol, which allowed the cells to survive for up to two weeks, enabling lentiviral treatments. Details of each of the procedures performed are shown in Appendices, A-D.

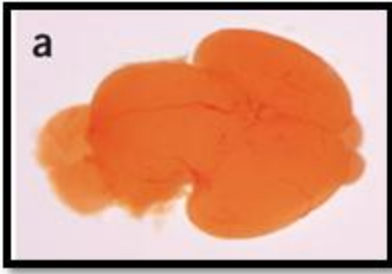


Figure 10: Total Fetal Brain.

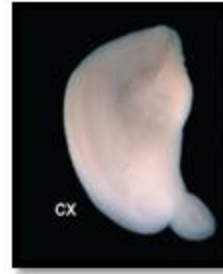


Figure 11: Crescent Moon of the Brain Frontal Lobe.



Figure 12: Cerebral Cortex Tissue.

Plating Procedure

Prior to dissection, typically six to ten 6-well plates were prepared at least one day in advance: 1 mL of poly-L-lysine was pipetted into each well, then the plates were placed inside the 37°C incubator overnight. After approximately 24 hours, the plates were removed from the incubator and placed in the hood, then the poly-L-lysine was collected for reuse. The wells were rinsed twice with sterile water, and left to dry in the sterile hood. If plates were prepared in excess of one day prior to dissection, once dried they were labeled and stored in a plastic ziploc bag inside the 8°C refrigerator for later use. Also prepared was the NB4 active plating medium containing 5% Δ FBS. This medium was placed in the 37°C water bath during the dissection.

After dissection, the removed cortices were taken off ice and placed in the sterile hood. Using forceps, the cortical tissue was removed from the small culture dish and placed in a 1.5mL centrifuge tube containing protease solution to separate the cells. In the beginning stages of the project, the cells were placed in trypsin, however later it was determined that papain (shown in Appendix B) was a more efficient protease for this process. Upon addition of protease, the cells were then placed in a 37°C incubator for approximately 45 minutes, with agitation every five to ten minutes. Post incubation, the cells were placed in the hood and slowly triturated ten times with a fire polished pipette. The cells were then observed under a microscope to ensure cell separation. If still clustered, the cells were triturated an additional five times and allowed to settle. Once settled, the cell suspension was collected and placed in a 15mL conical tube for a cell count.

In order to perform a cell count, 90 μL of trypan blue and 10 μL of the suspension was mixed in a single well of a microassay plate. After thoroughly mixing, the solution was put in a hemocytometer and placed under the microscope. The cells in each of the four primary quadrants were then individually counted, averaged and then multiplied by 1×10^5 . Using this number, the final volume containing 0.5×10^6 cells/mL was calculated to dilute the remaining cell solution with the plating medium. Upon dilution, 2 mL of the appropriate solution was placed in each well. The plates were then labeled with the cell type, date, and initials. A final observation under the microscope was done to ensure they were single, nonclustered cells. The plates were then placed in the 37°C incubator.

Cell Feeding

In preparation for media change, about 250 mL of feeding medium was made by adding antimetabolites to the NB4 active media. The basic proportions for 50 mL of medium were as follows: 0.484 $\mu\text{L}/\text{mL}$ of 5'UtP (24.2 $\mu\text{L}/50\text{mL}$ NB4 active medium), 0.2402 $\mu\text{L}/\text{mL}$ of 5'FdU (12.0 $\mu\text{L}/50\text{ mL}$ NB4 active medium). Prior to each media change, the feeding medium was placed in the 37°C water bath in order to prevent the growing cells from going into shock.

Initially, the first media change occurred 24 hours after plating, and again every other day for approximately two weeks, cell viability permitting. Due to significant cell death, it was determined that the cells were remaining in the plating medium for too long. As a result, the first media change was performed six hours after plating. This was done by removing 1 mL of the medium to be replaced with 1 mL of fresh medium. In the beginning of the project, this was done by aspiration, but was changed to pipetting for more accurate and gentle removal. As mentioned above, this method was then repeated every 48 hours contingent on cell survival.

Lentivirus Addition

After approximately five days of cell growth, lentivirus was added to the cells with fresh medium and placed back in the incubator. Twelve hours later, the medium was changed again; after this initial change the medium change resumed the 48 hour schedule with only one difference: for biosafety reasons the tips and medium were treated with bleach after use. Initially, the lentivirus was added to the medium at a multiplicity

of infection (MOI) (number of viral particles per cell) of 0.5, but upon evaluation of the initial knockdown results, the MOI was increased to 1.0 and the tests are being repeated. High titer replication-deficient lentiviral stocks were prepared by Dr. Miguel Esteves, using a lentivirus based system that carries a GFP expression cassette with the shRNA in the 3'UTR in the context of miR-30 sequences (shown previously in **Figure 5**). To calculate the volume of lentivirus stock needed per well with a cell concentration of 1×10^6 cells/well, a simple calculation was performed using the following (a complete list of data found in Appendix E):

$$\frac{\left(\frac{50 \times 10^6 \text{ viral particles}}{[\text{Titer}] \text{ tu/mL}} \right)}{1000}$$

| Gene | GIPZ Clone | Lenti Stock | Titer (tu/mL) | MOI 50 (Volume/well (μL)) | Growth Medium Volume (μL) |
|-------|-------------|-------------|---------------|---------------------------|---------------------------|
| CCR10 | V2LMM_12449 | L647 | 7.55E+08 | 66.2 | 933.8 |

After determining the volume of lentiviral stock needed for each well, all but 1 mL of medium was removed from the wells, the lentivirus was added, and a calculated volume of feeding medium was added, bringing the final volume of the wells to 2 mL. The plates were then gently rocked to spread the lentivirus evenly over the cells, and returned to the 37°C incubator. All pipettes and tips used in this process, and any used after transduction, were treated with bleach before disposal. After three days, the GFP became visible in the cells, and after six days the cells were photographed and counted under the microscope (green versus clear) to calculate the percent transduced. On the seventh day, the cells were harvested and prepared for quantitative real-time PCR by Wanzhao, an associate of Aronin's laboratory.

qRT-PCR

To test the knock down efficiency of the siRNAs, once the cells were harvested RNA was extracted and the expression of specific genes was assayed by quantitative real-time PCR with SYBR green. RNA was extracted and reverse transcribed to create cDNA. This cDNA was then PCR-amplified through a series of 40 cycles, recording the fluorescence at the end of each as data for analysis. The protocol followed was as per QIAGEN included with their qRT-PCR materials (*QuantiTect® SYBR® Green PCR Handbook*).

RESULTS

The goal of this project was to help develop a more efficient protocol for embryonic mouse cortical tissue dissection, plating, and maintenance, and to optimize their transduction with lentiviruses encoding siRNAs against candidate genes thought to encode proteins that interact with *mHTT* protein. As described in Methods, the initial protocol for embryonic cortical tissue dissection, plating, and maintenance was significantly enhanced to eventually produce healthy primary neurons with a viable lifespan of about 2 weeks for further use in Dr. Aronin's investigations. **Figures 13 and 14** show healthy viable neurons. Further evidence supporting the viability of these cells is the successful transduction of GFP-lentivirus into the cultured primary neurons (green fluorescing cells in **Figure 14**).

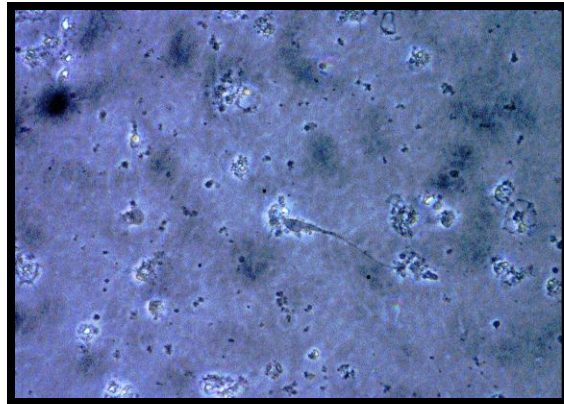


Figure 7: Photomicrograph of Healthy Mouse Early Stage Primary Neurons.

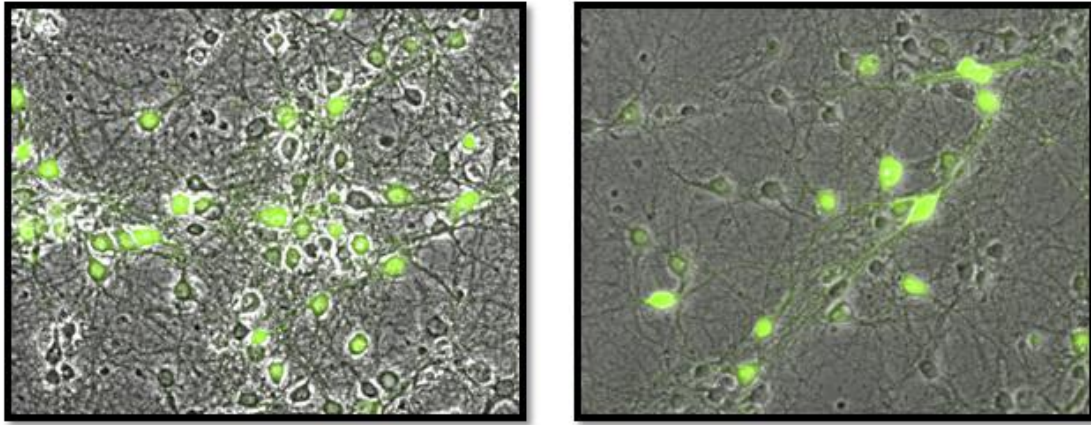


Figure 14: Fluorescence Micrographs of Mouse Cortical Cells Transduced with GFP-Lentivirus. Green cells denote successfully transduced cells.

Six days after transduction with 20 different siRNAs or scrambled siRNAs (and lentivirus not encoding any siRNA or GFP as negative controls), the number of fluorescing and clear cells per well were recorded (**Figure 15**).

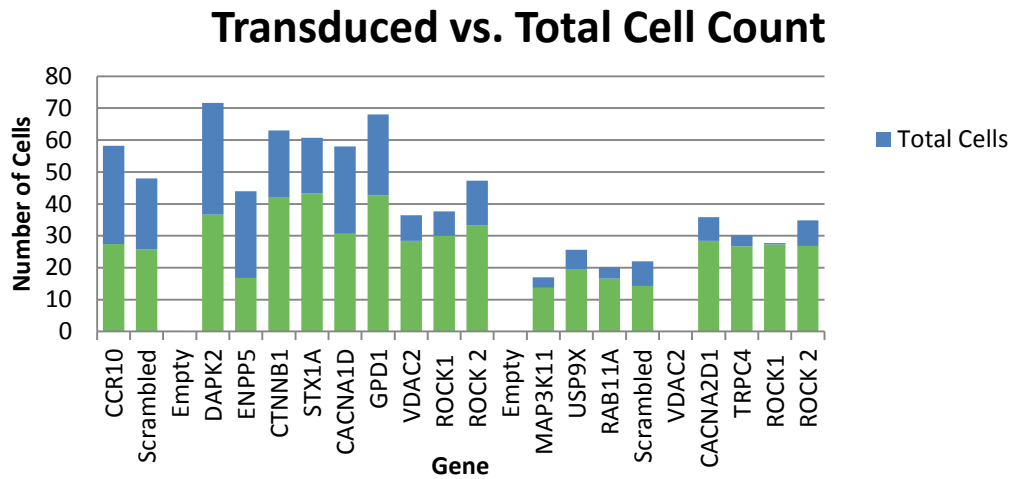


Figure 8: Number of Cells Transduced (Green) Versus Total Cell Count (Blue) for various siRNA Lentiviruses.

From this GFP data, the percent of cells transduced was calculated (**Figure 16**). Although this data does not reflect siRNA efficiency, it helps show the transduction efficiency and reproducibility of the transduction protocol. The data shows that the protocol is efficient, and it reproducibly transduces the mouse primary neurons.

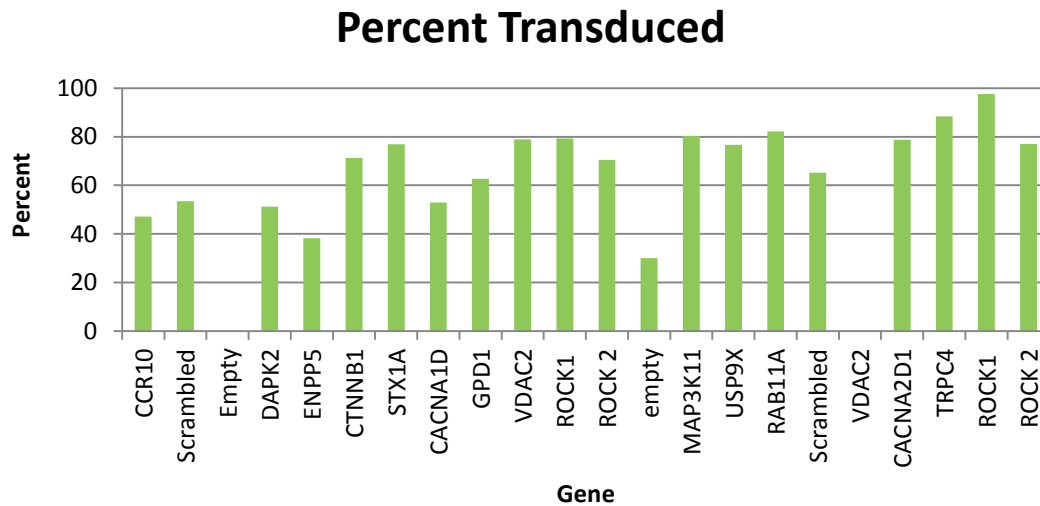


Figure 9: Percent of Cells Transduced with Various siRNA Lentiviruses.

Seven days after transduction, the cells were harvested and prepared for qRT-PCR with the goal of achieving at least 70% knockdown for each candidate mRNA. RNA was extracted from each well and reverse transcribed to create cDNA. The cDNA was then subjected to PCR to quantitate the cellular levels of specific candidate mRNAs. **Figure 17** shows the mRNA knockdowns for the first trial at a viral MOI of 0.5. Only 5 out of the 18 genes had $\geq 70\%$ knockdown (ENPP5, STX1A, TRPC4, ROCK1 and ROCK 2). Figure 17 also shows the data collected thus far on the repeat experiments at MOI of 1.0. So far, 2 of the 5 tested mRNAs achieved the $\geq 70\%$ knockdown target. Using the greater MOI of virus, each gene showed at least a 9.7 increase in the percent knockdown.

Gene CACNA1D showed the largest increase of an additional 25.4% knockdown. Out of the two genes that had $\geq 70\%$ knock down at MOI 1.0 (ENPP5 and GPD1), one had not already met this qualification at MOI 0.5 (GPD1). This data shows that the protocols optimized in this project efficiently transduce mouse cortical neurons to silence several selected genes of choice.

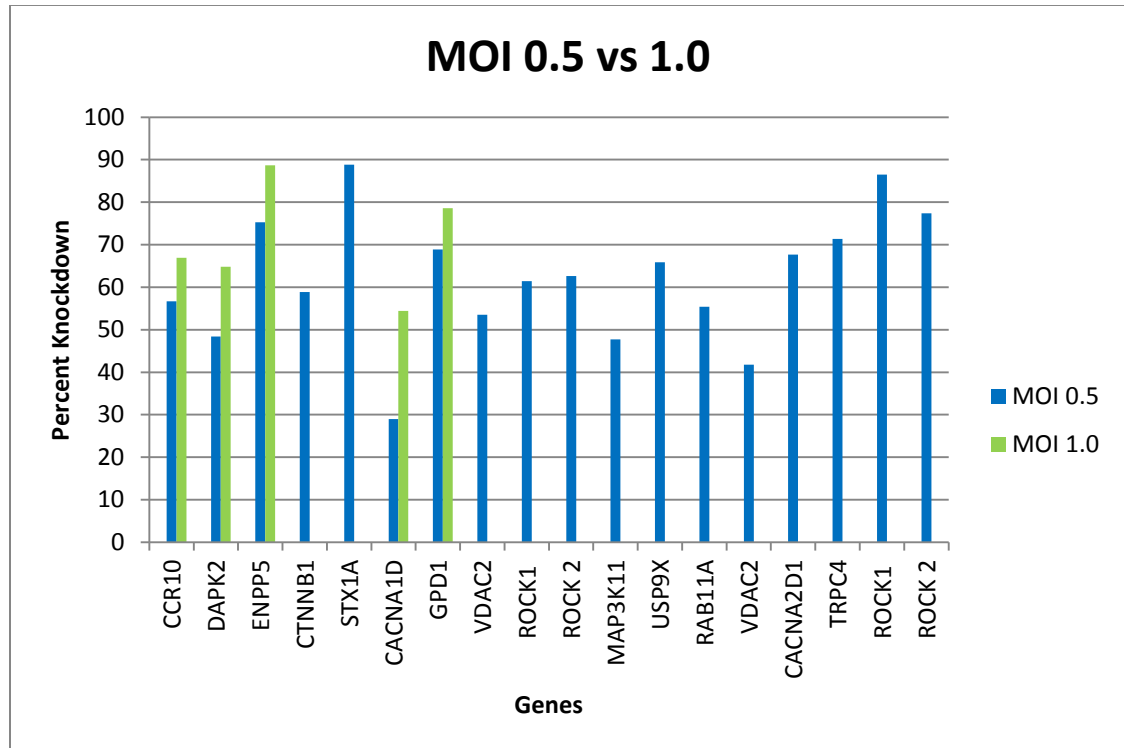


Figure 10: Comparison of Percent Knockdown for Several Target Genes at MOI 0.5 (Blue) vs. 1.0 (Green).

DISCUSSION

This project used previous advances related to the siRNA silencing of specific genes to establish protocols for applying their use to Huntington's disease, especially to investigate candidate genes previously thought to encode proteins that interact with *mHTT* protein. Since it has been established that mouse and human genomes contain many similar genes, the findings in mice may eventually apply to human HD patients.

A few problems were encountered throughout the project. Most were minor and easily resolved; others were more difficult and called for specialized attention. One of the initial problems encountered was the mice were not getting pregnant; this was speculated to be due to issues with the light timers and sounds from the construction blasts interrupting the mice's internal clocks and mating habits. As a result, the pace of the project varied, and it was difficult to progress. This was eventually resolved by ordering the pregnant mice and having them delivered to UMass directly, ensuring pregnancy. Another issue was lack of neuronal cell growth. Upon further investigation, it was found that the major issue was that the cells were being over triturated, causing them to die prior to plating. Although this was easy to fix, there was another situation in which the cells became contaminated, which impeded progression for approximately two weeks. This required outside assistance to ensure that the incubator was sterile and working properly. Despite these issues, the project progressed at a relatively smooth and consistent pace.

The data collected throughout this project showed that the enhanced protocol for mouse cortical dissection and plating enabled cultured primary neurons to sustain a

healthy viable lifespan for about 2 weeks, which should facilitate the lab's ongoing HD research. Using the lentiviral protocol, several different siRNA's were successfully transduced into the cultured primary neurons. qRT-PCR showed how efficiently the siRNAs were able to knock down the mRNAs; the goal was to achieve $\geq 70\%$ knockdown. Upon evaluation of the initial results at viral MOI of 0.5, the virus was increased to MOI 1.0 to attain at least 70% knockdown for each protein, and the experiments were repeated (at this point only a few genes have been analyzed at the increased MOI). Most labs consider 50 – 90% acceptable for successful knockdown. The MOI can be increased to 2.0 if needed in the future.

In continuation, these siRNAs (shRNA genes encoding them) will be inserted into Adeno-associated viruses (AAV) which will be unilaterally injected into mouse striatum to assess the role of these proteins in HD pathogenesis. These injections will contain either scAAV9-shRNAmir, or control vectors, injected into HD and WT four month old littermates. Eighty days post-injection, the brains will be collected for qRT-PCR analysis of the knockdown efficiency, as well as the histological and biochemical status of the disease. By analyzing this data, it is expected that the roles of these proteins will be better understood in correlation to HD pathogenicity. If successful, Dr. Aronin's lab will continue to add to the foundations of our knowledge of HD, allowing researchers to refine their investigations to focus on the beginning stages of HD to help find a cure.

If the knockdown of the genes is confirmed *in vivo*, accompanied by a decrease in the pathophysiology of the disease, a group of WT and *mHTT* mice can be re-injected with that specific AAV siRNA and observed. These observations, in correlation with task and behavioral assays, as well as neuroimaging, will allow researchers to compare

the progression of Huntington's disease between non-afflicted mice, afflicted non-treated mice, and siRNA treated afflicted mice. In doing so, they will be able to accurately define the effects of each protein on the disease, and gauge whether the siRNA can be used for human therapy. If siRNA therapy works in humans, this could provide a major breakthrough for treating this debilitating disease, extending the lifespan of millions of people affected by Huntington's disease.

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APPENDICES

Appendix A

Project _____
Continued from Page _____

Notebook No. _____

5

Things to do before the day of Dissection:

Coating Plates for Primary neurons

Coat plates or wells with poly-L-Lysine for cortical cells and Poly Ornithine for Striatal cells.

Place 1ml of coating in each well (6 well plates). Let them rock for 3 hours at room temperature. Remove coating from one corner and save until it is used three times. Rinse gently with water and remove from one corner. Let plates dry and store in plastic bag in cold room or fridge. Label with date they were coated and the reagent.

These can be made in batches and used for weeks.

Make at least one day ahead.

Check to see if the reagent needs to be reordered.

Prepare instruments:

Be sure you have all instruments clean and sterile on the day before dissection.

Place cleaned instruments in racks and cover with foil. Label with "Aronin 270D" and place in autoclave or if it is busy, leave for the glassware tech to do after the autoclave is empty. She will leave them on the bench in 270D when she brings the glassware to the lab.

Media can be prepared one day ahead.

It will be good for two weeks.

Read and Understood By _____

Signed _____

Date _____

Signed _____

Date _____

Procedure for Dissections

Bring mouse to lab.

Lay out bench pad with bed of paper towels for mouse.

Lay out two forceps and two sissors.

Prepare one beaker of 70% alcohol for dipping mouse.

Prepare one beaker of DMEM F12 for pouring.

Set out two large cell culture plates containing DMEM-F12 for uterus and heads.

Set out two small plates with Neurobasal medium for brains and cortex.

Kill mouse and immediately dip in alcohol.

Place on towel and cut through skin and muscle layers using one pair of scissors and one forcep.

Quickly remove uterus by holding one end with clean forceps and snipping with clean scissors.

Pour DMEM-F12 over uterus and place it in large plate.

Cut open with clean scissors and remove pups.

Snip heads off pups and place in the other large plate.

Using sterile forceps remove brain from each head and place in one small plate.

Use smallest forceps to open brain and remove cortical tissue.

Place tissue in the last small dish. You need to work fairly quickly and keep tissue moist at all times.

Take tissue to hood and place in 50 ml tube. The total amount of volume should be 2 ml.

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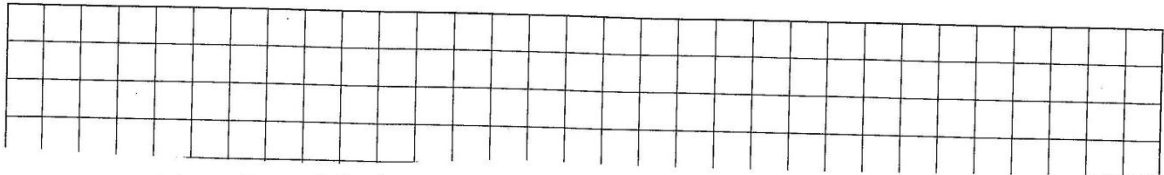
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Using a fire polished pipette, separate the cells by trituration (20X).

Place a cell strainer in the top of a 50 ml tube and wash the cells through the strainer.

Wash sides of tube with media to get all cells down to the bottom.

Place 10 ul of cell suspension in small tube and add 90 ul of tripan blue. (Find these in drawer next to hood.)

Place 10ul in cytometer and count four outside quadrants.

Average quadrants and multiply by ten. Example:

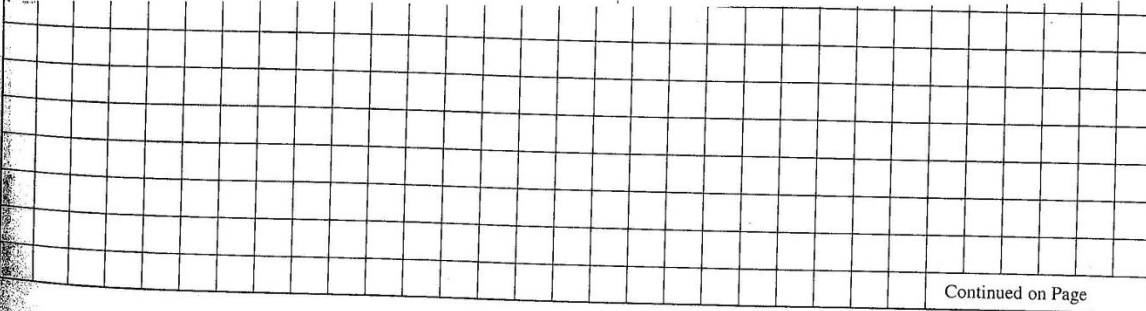
$$20 + 21 + 25 + 26 = 92 \quad 92 / 4 = 23 \quad 23 \times 10 = 230$$

This number multiplied by 10^4 gives you the number of cells per milliliter. So, $230 \times 10^4 = 2.3 \times 10^6$ cells per ml.

Next calculate the volume you need to plate cells in your 6 well plate.

After your cells are plated you need to change the media to Neurobasal plus antimitotics (warmed). This can be done 4-6 hours after plating or the following day.

Feed the cells every three to four days by replacing most of the media with fresh, warmed media.



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Date _____

Appendix B

Papain Dissociation System Protocol Summary

Sterile procedures should be used throughout:

1. Add 32ml of EBSS (Vial 1) to the albumin-ovomucoid inhibitor mixture (Vial 4) and allow the contents to dissolve while preparing the other components. Mix before using and equilibrate with O₂:CO₂. Reconstitute for the first use, then store at 2-8°C and reuse.
2. Add 5ml of EBSS (Vial 1) to papain vial (Vial 2). Place Vial 2 in 37°C water bath for ~10 minutes or until the papain is completely dissolved and the solution appears clear. If solution appears alkaline (red or purple) equilibrate the solution with 95%O₂:5%CO₂. The solution should be used promptly but can be held at room temperature during the dissection. A separate papain vial is provided for each dissociation. (If desired the papain can be transferred to a centrifuge tube or other container before proceeding.)
3. Add 500ul of EBSS to a DNase vial (Vial 3). Mix gently—DNase is sensitive to shear denaturation. Add 250ul of this solution to the vial containing the papain. This preparation contains a final concentration of approximately 20 units/ml papain and 0.005% DNase. Save the balance of the DNase vial to use in Step #7. A separate DNase vial is provided for each dissociation.
4. Place tissue in the papain solution. Tissue should be slightly minced or cut into small pieces (this can be done separately or on the side of the tube containing the papain.) Displace the air in vial with sterile O₂:CO₂. Do not bubble gas through the solution. Immediately cap the vial.
5. Incubate the vial containing the tissue at 37°C with constant agitation (a rocker platform is ideal) for 30 min to 1 ½ hrs. The amount of time must be determined empirically; however, embryonic tissue generally requires less time than postnatal tissue.
6. Triturate the mixture with 10ml pipette. Allow any pieces of undissociated tissue remaining after trituration to settle to the bottom of the tube. Vigorous trituration of neuronal tissue results in a high yield of cells, most of which are spherical and devoid of proximal processes. Gentle trituration results in more undissociated tissue fragments and a lower yield of cells although many of these now retain their proximal processes.
7. Carefully remove the cloudy cell suspension, place in sterile screw capped tube and centrifuge at 300 x g for 5 minutes at room temperature. Be careful to avoid including any pieces of undissociated tissue. During this time prepare medium to resuspend the pelleted cells. Mix 2.7ml EBSS (Vial 1) with 300ul reconstituted albumin-ovomucoid inhibitor solution (Vial 4) in a sterile tube. Add 150ul of DNase solution (Vial 3) saved at Step #3. $2.7 \times \frac{150}{300} = 3.15 \text{ ml}$
8. Discard the supernatant and immediately resuspend the cell pellet in the diluted DNase/albumin-inhibitor solution prepared in Step #7. $1000 \mu\text{l}$
9. Prepare discontinuous density gradient: Add 5ml of albumin-inhibitor solution (Vial 4) to a centrifuge tube, carefully layer the cell suspension on top, then centrifuge at 70 x g for 6 minutes at room temperature. The interface between the two layers of the gradient should be clearly visible although minimal mixing at this boundary does not affect the result. Dissociated cells pellet at the bottom of the tube, membrane fragments remain at the interface. 2.5 ml ai sol. $1000 \mu\text{l}$
10. Discard the supernatant and immediately resuspend the pelleted cells in medium for cell culture or for flow cytometric analysis.

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Appendix C

Dissection Protocol: Cortical Cells – version one

Prior to Dissection Day

- Coat 6 well plates with Poly-L-Lysine
 - Place 1mL of Poly-L-Lysine in each well and leave in incubator overnight
 - Aspirate coating and rinse with 1mL of water twice
 - Label and date plates, store in plastic bag in refrigerator
- Sterilize instruments to be used during dissection

Dissection Day

Turn on sterilizer to heat

Prepare for Dissection and Plating:

- Remove coated plates from refrigerator and place in hood to warm
- Prepare NB4 active with 5% Δ FBS and place in warm water bath (2.5mL in 50 mL)
- Remove Trypsin from refrigerator and place in hood
- Place 1.5mL centrifuge tubes in hood

On Lab Bench:

- Prepare lab bench: two draped areas
 - Removal of fetal mice: forceps and scissors, one beaker with about 300 mL of 70% ethanol, one beaker with about 50mL of pouring media (DMEM-F12), one large cell culture plate with 5mL of cold DMEM-F12 for uterus
 - Brain dissection: forceps and scissors, one large cell culture plate with 5mL cold DMEM-F12 for heads, three small cell culture plates with 2mL of cold HybernatE for brains and cortex, microscope, ice to place brains on
- Kill mouse and immediately dip in 70% ethanol
- Place on drape and cut through skin and muscle layers using a pair of sterile scissors and forceps
- Remove uterus by holding one end with a new pair of sterile forceps and snipping with a new pair of sterile scissors
- Pour DMEM-F12 over uterus and place in large cell culture plate
- Move to other drape, cut uterus open with a new pair of sterile scissors to remove pups
- Snip head off the pups and place in second large cell culture plate and put on ice
- Using sterile forceps and a microscope, remove brain from each head and place in small plate on ice

- Use forceps to open the brain and remove cortical tissue and place in second small plate on ice

In Hood:

- Using forceps place all cortical tissue pieces from small cell culture plate in 15mL conical tube containing 1ml Triple E Express (trypsin)
- Place tissue/enzyme into 37°C incubator for 20-30 minutes
- After 30 minute digestion remove the supernatant and re-suspend pieces in 1mL of warm 5%ΔFBS in NB4 active media
- Allow tissue to settle and remove most of the media and replace with new media
- Transfer re-suspended cells to 15mL conical tube (or triturate in the small tube if desired)
- Triturate 15-20 times slowly using a wet glass fire-polished pipette
- Allow 2 minutes for large pieces to settle out and put supernatant into a new 15ml conical tube. If there are many pups, use a 50 ml conical tube.
- Mix 90ul of trypan blue with 10ul of cells using a sterile pipette tip. Place 10ul in hemocytometer and count four quadrants. Average the four numbers and multiply by 1.0×10^5 . Calculate volume needed to get 0.5×10^6 cells/mL.
- Make dilution using 5%FBS in NB4 active media
- Plate diluted cells by adding 2mL into each well of the coated 6 well plates
- Check cells using microscope to be sure they are single cells
- Place in 37°C incubator
- Make feeding media (NB4 active + antimetabolites) for days to come:
 - Calculate media based on number of plated wells (2mL/well):
6 ml per plate every other day for 14 days = 42 mL, so make 50 mL for each 6 well plate
 - 0.484μL/mL of 5'UtP (24.2μL / 50 mL NB4 active)
 - 0.2402 μL/mL of 5'FdU (12.0μL / 50 mL NB4 active)

Feeding

- After 6 -12 hours: change media by removing 1mL of media using a pipette (making sure not to disturb cells) and adding 1mL of feeding media
 - Store in 37°C incubator and repeat every 48 hours

Appendix D

Dissection Protocol: Cortical Cells – version two

Prior to Dissection Day

- Coat 6 well plates with Poly-L-Lysine
 - Place 1mL of Poly-L-Lysine in each well and leave in incubator overnight
 - Aspirate coating and rinse with 1mL of water twice
 - Label and date plates, store in plastic bag in refrigerator
- Sterilize instruments to be used during dissection

Dissection Day

Turn on sterilizer to heat

Prepare for Dissection and Plating:

- Remove coated plates from refrigerator and place in hood to warm
- Prepare NB4 active with 5% Δ FBS and place in warm water bath (2.5mL in 50 mL)
- Prepare the papain solutions and place in hood
- Place 1.5mL centrifuge tubes in hood

On Lab Bench:

- Prepare lab bench: two draped areas
 - Removal of fetal mice: forceps and scissors, one beaker with about 300 mL of 70% ethanol, one beaker with about 50mL of pouring media (DMEM-F12), one large cell culture plate with 5mL of cold DMEM-F12 for uterus
 - Brain dissection: forceps and scissors, one large cell culture plate with 5mL cold DMEM-F12 for heads, three small cell culture plates with 2mL of cold HybernatE for brains and cortex, microscope, ice to place brains on
- Kill mouse and immediately dip in 70% ethanol
- Place on drape and cut through skin and muscle layers using a pair of sterile scissors and forceps
- Remove uterus by holding one end with a new pair of sterile forceps and snipping with a new pair of sterile scissors
- Pour DMEM-F12 over uterus and place in large cell culture plate
- Move to other drape, cut uterus open with a new pair of sterile scissors to remove pups
- Anchor pups using a forcep and clasp the neck, using a second forcep make an incision down the sagittal suture line

- Gently squeeze from just beneath the brain while pushing up; separate the brain from the body by pinching the membranous and connective tissue and scooping the brain from beneath
- Place each brain in the small dish of HyphenateE on ice
- Use forceps to open the brain and remove cortical tissue and place in second small plate on ice

In Hood:

- Using forceps place all cortical tissue pieces from small cell culture plate in 1.5mL centrifuge tube containing Papain solution
- Place tissue/enzyme into 37°C incubator for 40-50 minutes, mixing every 10 minutes
- After 40 - 50 minute digestion remove the supernatant and re-suspend pieces in 1mL of warm 5%ΔFBS in NB4 active media
- Allow tissue to settle and remove most of the media and replace with new media
- Transfer re-suspended cells to 15mL conical tube (or triturate in the small tube if desired)
- Triturate 10 times slowly using a wet glass fire-polished pipette
- Allow 2 minutes for large pieces to settle out and put supernatant into a new 15ml conical tube. If there are many pups, use a 50 ml conical tube.
- Mix 90ul of trypan blue with 10ul of cells using a sterile pipette tip. Place 10ul in hemocytometer and count four quadrants. Average the four numbers and multiply by 1.0×10^5 . Calculate volume needed to get 0.5×10^6 cells/mL.
- Make dilution using 5% FBS in NB4 active media
- Plate diluted cells by adding 2mL into each well of the coated 6 well plates
- Check cells using microscope to be sure they are single cells
- Place in 37°C incubator
- Make feeding media (NB4 active + antimetabolites) for days to come:
 - Calculate media based on number of plated wells (2mL/well):
6 ml per plate every other day for 14 days = 42 mL, so make 50 mL for each 6 well plate
 - 0.484μL/mL of 5'UtP (24.2μL / 50 mL NB4 active)
 - 0.2402 μL/mL of 5'FdU (12.0μL / 50 mL NB4 active)

Feeding

- After 6 -12 hours: change media by removing 1mL of media using a pipette (making sure not to disturb cells) and adding 1mL of feeding media
 - Store in 37°C incubator and repeat every 48 hours

Appendix E

| Gene | GIPZ Clone | Lenti Stock | Titer (tu/mL) | MOI 50:100 (Volume per well in μL) | Growth Medium Volume (μL) |
|-------------|-------------------|--------------------|----------------------|--|---|
| CCR10 | V2LMM_12449 | L647 | 7.55E+08 | 66.2 | 933.8 |
| Scrambled | - | L648 | 2.18E+09 | 22.9 | 977.1 |
| Empty | - | L649 | 4.23E+08 | 118.2 | 881.8 |
| DAPK2 | v2LMM_188057 | L650 | 2.71E+09 | 18.5 | 981.5 |
| ENPP5 | v2LMM_30736 | L651 | 5.33E+08 | 93.8 | 906.2 |
| CTNNB1 | V2LMM_16708 | L653 | 5.92E+09 | 8.4 | 991.6 |
| STX1A | V3LMM_415458 | L654 | 9.44E+09 | 5.3 | 994.7 |
| CACNA1D | V2LHS_112214 | L655 | 3.53E+09 | 14.2 | 985.8 |
| GPD1 | V3LHS_640972 | L656 | 6.51E+09 | 7.7 | 992.3 |
| VDAC2 | v2lmm20438 | l665 | 4.20E+08 | 119.0 | 881.0 |
| ROCK1 | v3lhs_338550 | L666 | 2.60E+09 | 19.2 | 980.8 |
| ROCK 2 | v2lmm_10488 | L667 | 3.20E+09 | 15.6 | 984.4 |
| empty | N/A | L668 | 2.50E+08 | 200.0 | 800.0 |
| MAP3K11 | V3LMM_501715 | L673 | 2.17E+10 | 2.3 | 997.7 |
| USP9X | V3LMM_477651 | L674 | 4.15E+09 | 12.0 | 988.0 |
| RAB11A | V2LMM_48972 | L675 | 2.05E+09 | 24.4 | 975.6 |
| Scrambled | - | L648 | 2.18E+09 | 22.9 | 977.1 |
| MAP3K11 | V3LMM_501715 | L673 | 2.17E+10 | 2.3 | 997.7 |
| USP9X | V3LMM_477651 | L674 | 4.15E+09 | 12.0 | 988.0 |
| RAB11A | V2LMM_48972 | L675 | 2.05E+09 | 24.4 | 975.6 |
| VDAC2 | v2lmm20438 | L686 | 1.70E+09 | 29.4 | 970.6 |
| CACNA2D1 | V2LMM_75500 | L688 | 7.30E+09 | 6.8 | 993.2 |
| TRPC4 | V2LMM_62022 | L689 | 7.30E+09 | 6.8 | 993.2 |
| ENPP5 | v2LMM_30736 | L695 | 6.80E+09 | 7.4 | 992.6 |