IDENTIFYING DNMT3A INTERACTIONS WITHIN WILD-TYPE AND MUTANT HTT- EXPRESSING NEURONS

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Abstract

Huntington's disease (HD) is a progressive and fatal neurodegenerative disorder whose pathogenic mechanism is still not entirely understood. However, previous studies have indicated the prevalence of transcriptional dysregulation in the presence of mutant huntingtin (mHtt). DNA methylation of the promoter region is a mechanism that affects transcription; more specifically, it is a mechanism that represses transcription and has also been observed to be abnormal in the presence of mHtt. Furthermore, a previous study conducted in 2016 by Pan et al. showed rehabilitating effects against mHtt-induced toxicity by the inhibition and knockdown of certain DNA methyltransferase enzymes (DNMTs), DNMT1 and DNMT3A, which catalyze DNA methylation. The current study hypothesizes that specific differences within the DNMT3A multi-protein complex between wild-type (WT) and mHtt-expressing are neuroresponsible for the abnormal recruitment of DNMT3A to individual loci in the HD context. These differences cause the repression of specific genes essential to neuronal health. To validate the hypothesis, co-immunoprecipitation of DNMT3A within WT and HD models, followed by mass spectrometry, will be performed to note any differences in the identified proteins. If this study's results support the hypothesis, a greater understanding of HD's pathogenesis will be attained, and an increased potential of identifying a therapeutic solution for HD.

Keywords: Huntington's disease, neurodegenerative disorder, DNA methylation, DNMT, DNMT3A, interacting proteins

Introduction

Huntington's disease (HD) is a progressive and fatal neurodegenerative disorder characterized by involuntary movements, cognitive impairment, and mood disturbances. HD affects around 30,000 people in the United States, with another 150,000 or more people at risk of developing the condition (Hersch & Rosas, 2008). HD is caused by an abnormal expansion of a CAG repeat in the *HTT* gene, thereby encoding an abnormal expansion of polyglutamine repeats in the huntingtin (Htt) protein or mutant huntingtin (mHtt). Normally, this CAG sequence is repeated 10-35 times within a healthy gene, but in a patient that suffers from HD, this sequence is repeated 36-120 times (Lee et al., 2012). Neurons, especially those of the cortex and striatum, are particularly vulnerable to mHtt; thus, HD causes neuronal dysfunction and death (Ross et al., 2014).

Currently, the pathogenic mechanism of HD is still not clearly understood. However, increasing evidence suggests that

transcriptional dysregulation is a significant factor in the progression of HD (Lee, Hwang, Kim, Kowall, & Ryu, 2013). DNA methylation of the promoter region is a common epigenetic signaling tool used by cells to repress the expression of specific genes. This tool is believed to reduce gene expression by impairing the binding of transcriptional activators. DNA methyltransferase enzymes (DNMTs) catalyze this process; these enzymes do this by converting cytosine of eukaryotic DNA into methylated cytosine. DNMTs accomplish this by transferring a methyl group onto the C5 position of the cytosine to form methylated cytosine or 5-methylcytosine. DNMT3A, DNMT3B, and DNMT1 are members of the DNMT family; the former two are de novo methyltransferases, and the latter enzyme is a maintenance methyltransferase (Moore, Le, & Fan, 2013).

Many studies have been conducted to analyze the connection between DNA methylation and HD. One such study, published in 2016 by Pan et al., indicated that abnormal DNA methylation might play a key role in the progression of HD. This was done by showing an increase in the expression of certain genes key to neuronal health that are observed to be repressed in the HD condition . Results were obtained through pharmacologically inhibition and shRNA knockdown of DNMT1 and DNMT3A in mutant Htt- expressing cortical neurons and R6/2 HD mice (Pan, Y et al., 2016). A study published by Ng et al. in 2013 found that many of the genes associated with neurogenesis and neuronal differentiation that experience changes in expression in the HD conditions also show significant changes in DNA methylation (Ng et al., 2013). Another study, published by Mollica et al. in 2016, found that the genes associated with DNA repair machinery expression were downregulated due, at least in part, to abnormal DNA methylation (Mollica et al., 2016).

Based on these previous findings, this study hypothesizes that in the HD context, abnormal DNA methylation is causing the repression of genes essential to neuronal health through abnormal recruitment of DNMT3A to particular loci due to an aberrance in its multi-protein complex. This aberrance then results in neuronal dysfunction and death. This study will attempt to validate this, focusing on just DNMT3A for feasibility.

Methods

Antibodies

Four DNMT3A antibodies were used for western blotting (WB): rabbit polyclonal anti-DNMT3A (PA5-77945, Thermo Scientific), mouse monoclonal anti- DNMT3A (MA5-16171, Thermo Scientific), rabbit polyclonal anti-DNMT3A (ab2850, Abcam), and rabbit polyclonal anti-DNMT3A (sc-20703, Santa Cruz). Mouse monoclonal anti-histone H3 (3538, Cell Signaling Technology), mouse monoclonal anti-lamin A/C (4777, Cell Signaling Technology), mouse monoclonal anti-alpha tubulin (T5168, Sigma), and rabbit polyclonal anti-DNMT1(5032S, Cell Signaling Technology) antibodies were also used for western blotting.

Fractionation Techniques

Two fractionation techniques were used in this study. The details of each are listed below.

Protocol One

For the first protocol, a certain section of the C57BL/6 mice brain (the forebrain) was isolated and lysed in NP-40 lysis buffer (10 mM HEPES, pH 7.9, 3 mM MgCl2, 10 mM KCl, 10 mM NaF, 1 mM Na3VO4, 0.5 mM DTT, 0.5% NP-40, 1x complete EDTA-free protease inhibitor cocktail), dounced 15 times with a tight pestle, and pelleted at 1,000x g for 15 minutes. Lysates were diluted 1:1 with benzonase buffer (10 mM HEPES, pH 7.9, 3 mM MgCl2, 280 mM NaCl, 0.2 mM EDTA, 10 mM NaF, 1 mM Na3VO4, 0.5 mM DTT, 0.5% NP-40, and 1x complete EDTA-free protease inhibitor cocktail) and digested with the permissive nuclease benzonase (Novagen) at a final concentration of 50 U/mL for 1 h rotating at 4 ℃. The digested lysates were then pelleted at 17,000x g for 20 minutes at 4 ℃.

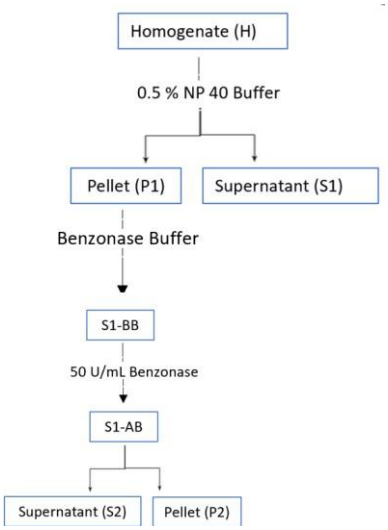

Figure 1. This diagram shows the steps of fractionation protocol one, as well as, the names and abbreviations of the different pellets and supernatants produced.

Protocol Two

For the second protocol, certain sections of the C57BL/6 mice brains (the forebrain and cortex) were isolated and lysed in cytoskeleton buffer (10 mM Pipes, pH 6.8, 100 mM NaCI, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA, 0.5%(vol/vol) Triton X-100, 10 mM NaF, 1 mM Na3VO4, 1x complete EDTA-free protease inhibitor cocktail), then pelleted at 600x g for 3 minutes after 3 min at 4°C. Pellets were then digested with RNase-free DNase I at a final concentration of 40 U/mL at 37 ℃ for 30 min in digestion buffer (10 mM Pipes, pH 6.8, 50 mM NaCI, 300 mM sucrose, 3 mM MgCI2, 1 mM EGTA, 10 mM NaF, 1 mM Na3VO4, 1x complete EDTA-free protease inhibitor cocktail, and 0.5% [vol/vol] Triton X-100). Ammonium sulfate was then added to produce a final concentration of 0.25 M. The pellet was left to rotate at 37 ℃ for ten minutes and then was pelleted once again at 21130x g for twenty minutes. NaCl was then added to the pellet to produce a final concentration of 2 M and was left to rotate for 30 minutes at 4 ℃. It was then pelleted once again at 21130x g for twenty minutes, and urea was added to produce a final concentration of 8M. Finally, the pellet was incubated for 15 minutes at 37 ℃.

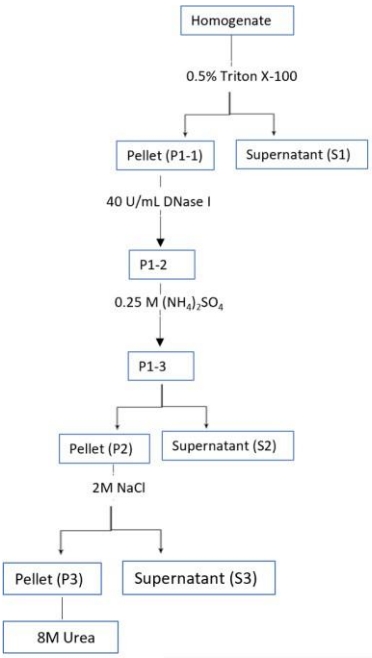

Figure 2. This diagram shows the steps of fractionation protocol two, as well as, the names and abbreviations of the different pellets and supernatants produced.

Lysates, Primary Neuron Cultures, and Transfection

The primary cortical culture was created from the cerebral cortex tissues of 16-day embryos from pregnant Swiss Webster mice. Cells were plated onto six Poly-l-lysine coated 10 cm plates, with 6.5 million cells per plate. Neurons were transfected with Htt-expressing lentivirus (three plates for WT Htt and 3 plates for mHtt) after 5 days in vitro. All plates were cultured for a total of 10 days in vitro.

Lysates were created from forebrains of 78-day old, WT, male C57BL/6 mice, and from cortices of 44-day old, WT, and DNMT3A homozygous knockout, male, C57BL/6 mice. The DNMT3A knockout was conditional; only neurons of the mice were affected.

Subcellular Fractionation and Western Blot Analyses

Fractionation and western blotting were employed to determine the most prominent location of DNMT3A within WT neurons using the lysates and neuronal cultures. Cytoplasmic and nuclear fractions were prepared using two different protocols to determine which could separate the cells' soluble and insoluble components best. This step was performed as a sufficient amount of DNMT3A needs to be extracted to perform co-IP. The main areas within the neurons that were analyzed were the cytoplasm and the nucleus. More specifically, within the nucleus, DNMT3A was analyzed to determine if it was mostly floating around in the free space of the nucleus, bound to the nuclear matrix, or bound to the chromatin located within.

Results

DNMT3A (PA5-77945) Antibody Showed Greater Performance Than DNMT3A (MA5- 16171) Antibody

WB analyses were done for DNMT3A in subcellular fractions obtained from WT mouse forebrain lysates and prepared using fractionation protocol with one of two different DNMT3A antibodies. Two different antibodies were tested as DNMT3A has two identified isoforms, DNMT3A1 and DNMT3A2; the former contains the N-terminal while the latter does not. Both contain the C-terminal, however.

This result is shown in Figure 3. It was desired to measure both forms of DNMT3A, so the

DNMT3A (MA5-16171) antibody, which binds to the C-terminal region of DNMT3A, was tested alongside the DNMT3A (PA5- 77945) antibody, which binds to the N- terminal region of DNMT3A.

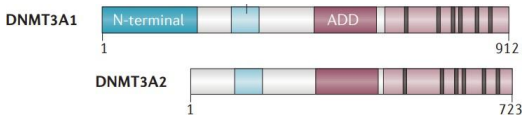

Figure 3. This shows the two isoforms of DNMT3A: DNMT3A1 and DNMT3A2. DNMT3A1 contains an N-terminal region, while DNMT3A2 does not. Adapted from "DNMT3A in haematological malignancies" by L. Yang, R. Rau, and M.A. Goodell, 2015, Nature reviews, Cancer, 15(3), p. 152–165. (Yang, Rau, & Goodell, 2015).

As shown in Figure 4, the results of this WB suggested that the DNMT3A (PA5-7794) antibody performed well, but the strength of the DNMT3A (MA5-16171) antibody's performance was difficult to determine. As can be seen in Figure 4-A, the WB using the DNMT3A (PA5-77945) antibody indicates a clear line of bands around 130 kDa, the size of DNMT3A1, while the results of the WB using the DNMT3A (MA5-16171) antibody are not as precise, as shown in Figure 4-B.

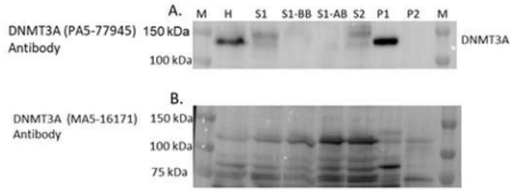

Figure 4. This shows the results of WB analyses done on subcellular fractions from WT mouse forebrain lysates prepared using fractionation protocol one and with A.) DNMT3A (PA5-77945) antibody, B.) DNMT3A (MA5-16171) antibody.

To ensure that the line of bands seen in the WB of the DNMT3A (PA5-7794) antibody is indeed DNMT3A and to determine how well the DNMT3A (MA5-16171) antibody performs, additional WB analyses were performed. As shown in Figure 5, these four WB analyses were performed with four different DNMT3A antibodies: PA5-77945, MA5-16171, ab2850, and sc-20703. Ab2850 is another DNMT3A antibody on hand and was thus included in this experiment. Sc-20703 served as the control due to previous validation of its competence.

Supernatant one (S1) cellular fractions prepared from WT and homozygous DNMT3A knockout mice cortex lysates were used for each of these WB analyses; these fractions were prepared using fractionation protocol two. The meaning of S1 is shown in Figure 2, as are the meanings of other abbreviations discussed later. As shown in Figure 5-D, only the DNMT3A (PA5-77945) antibody produced similar results to that of the control, validating its capability. WB analyses using a tubulin antibody were also performed to ensure that these results were not biased due to technical inconsistencies. The results of this did just that; this is shown in Figure 5 as well.

However, this fractionation protocol did not seem to extract DNMT3A from the pellets very well, specifically pellet one (P1), so fractionation protocol two was used for subsequent fractionation.

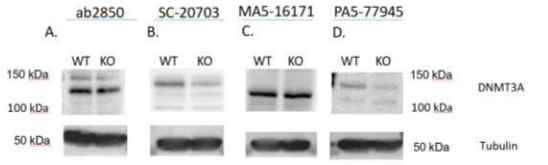

Figure 5. This shows the results of four WB analyses performed using four different DNMT3A antibodies on fractions prepared using S1 from fractionation protocol two, prepared from WT and homozygous DNMT3A knockout mice cortex lysates. The following indicates which antibodies were used for which WB analyses: A.) ab2850, B.) sc-20703, C.) MA5-16171, and D.) PA5-7794.

Western Blot Analysis for Histone

Then, WB analyses for histone H3 in WT forebrain fractions obtained from protocol one and WT forebrain and cortex fractions obtained from fractionation protocol two were done. This protein served as a marker to indicate the part of the cell each fraction tested pertained to, which would give insight into the primary location of DNMT3A. Figure 6, which shows the results of the WB on the WT forebrain fractions obtained from protocol two, indicates histone H3 to be in the homogenate (H), supernatant 3 (S3), pellet 1-2 (P1-2), and pellet 1-3 (P1-3). The results of the WB on the cortex fractions obtained from protocol two show a clearly botched WB, as shown in figure 7-E. This seems to be due to the migration of the homogenates.

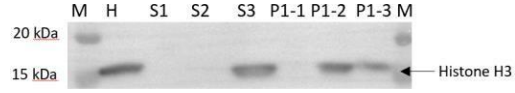

Figure 6. This shows the results of the western blot done on subcellular fractions from mouse forebrain lysates prepared using fractionation protocol two and with histone H3 antibody.

Western Blot Analysis for DNMT3A, DNMT1, and Lamin on Cortex Lysate Fractions

Many WB analyses were performed for an array of proteins: DNMT3A, DNMT1, and lamin A/C. These WB analyses were performed on WT and DNMT3A homozygous knockout cortex lysate fractions obtained from fractionation protocol two.

The WB analysis of DNMT3A using DNMT3A (PA5-77945) antibody on WT cortex lysate fractions indicates the greatest amount of DNMT3A to be in the fractions of homogenate (H), supernatant two (S2), the pellet before the addition of DNase I (P1-BD), and the pellet after the addition of DNase I (P1-AD). This is shown in Figure 7-C. This analysis is reasonable, as supernatant two is obtained from pellet one, as shown in Figure 2, so if P1 contains a significant amount of DNMT3A, then S2 should as well, and vice versa. S2 is believed to contain DNMT3A that was bound to chromatin. The chromatin binding is due to the fact that this sample was obtained after using DNase I on pellet one. The results of this were as expected, as the role of DNMT3A is mostly believed to be involved with gene expression regulation.

Figure 7-C also shows the results of this WB done on the DNMT3A homozygous knock- out homogenate (H) and supernatant one (S1) fractions. The bands shown for these columns are much weaker than those present for the WT samples columns, confirming this line of bands to be DNMT3A truly.

The WB analysis for DNMT1 indicates the greatest amount to be in S1, P1-2, and pellet three (P3). This is shown in Figure 7-A and 7-B. This WB was done to determine the location of DNMT1, which could then be compared to the location of DNMT3A. It seems that these two locations are not the same.

Figure 7-D shows the results of the WB analysis for lamin A/C on the WT and DNMT3A homozygous knockout cortex lysate fractions. This again served as a marker indicating the part of the cell each fraction tested pertained to. This figure shows lamin A to be in both WT and DNMT3A KO homogenate fractions and the pellet before DNase I fraction. On the other hand, Lamin C is shown in the same fractions as lamin A and additionally in the pellet after DNase I fraction. It is reasonable for the homogenates to contain lamin A/C, but not for only P1-AD and/or P1-BD to contain lamin A/C. If lamin A/C remained in the pellet rather than being released into the supernatant, then it must be present in the fractions of pellet two and three as well, which is not the case here. This indicates some technical error, such as the proteins degrading, especially as the samples were incubated at 37 ℃ for quite a bit of time, as called for by fractionation protocol two.

Lastly, these WB analyses fractionation protocol two was able to extract proteins from the pellets quite well, or at least better than fractionation protocol one.

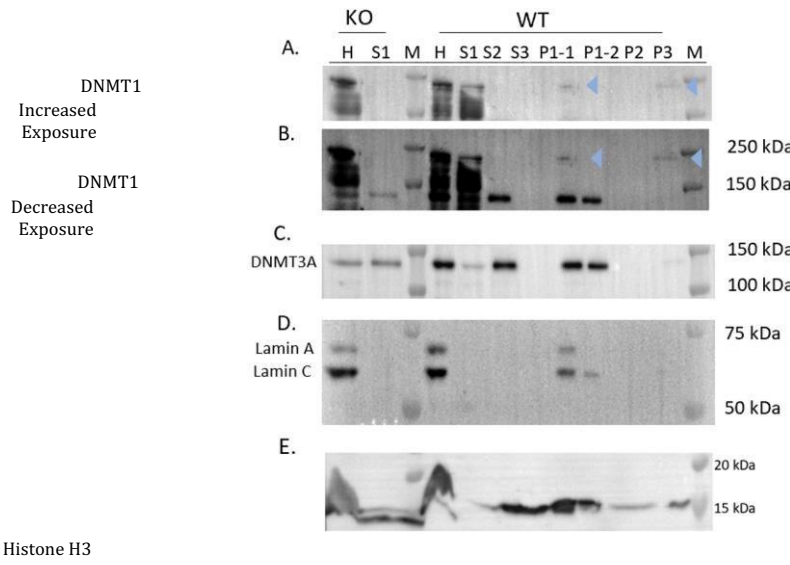

Figure 7. This shows the results of the WB analyses done on WT and DNMT3A homozygous knockout cortex lysate subcellular fractions prepared using fractionation protocol two and with A.) DNMT1 antibody (image shown with increased exposure), B.) DNMT1 antibody (image shown with decreased exposure), C. DNMT3A (PA5-77945) antibody, D.) Lamin A/C antibody and E.) histone H3 antibody. The arrows indicate DNMT1.

Western Blot Analysis for DNMT3A in WT and mHtt-Expressing Cortical Neurons Finally, a WB analysis for DNMT3A in WT and mHtt-expressing cortical neuron fractions obtained from protocol two with the DNMT3A (PA5-77945) antibody was done. The purpose of this was to analyze the location of DNMT3A within these two models and note any differences. As shown in Figure 8, there seems to be a greater amount of DNMT3A in the S3 and P3 of the mHtt-expressing neurons than that of the WT neurons. Both, though, seem to contain a relatively similar amount of DNMT3A within the S2 and P1 fractions.

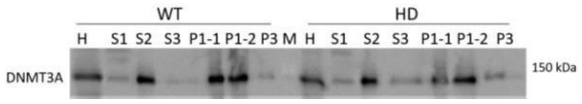

Figure 8. This shows the results of the WB performed for DNMT3A in WT and mHtt-expressing cortical neuron fractions obtained from protocol two with the DNMT3A (PA5-77945) antibody.

Discussion/Conclusion

The purpose of this study was to determine any differences in the multi-protein complex of DNMT3A within wild-type and mHtt-expressing neurons. This was done in hopes of gaining a greater understanding of the mechanism that guides DNMT3A to a specific gene to catalyze the methylation of it to then allow for a greater understanding of normal and abnormal DNA methylation, the mechanism of Huntington's disease progression, and become closer to developing a solution for this disease. This work has not been done in an HD context; most of the work related to identifying DNMT3A interactors has been done in cancer cells or proliferating cells, which dramatically enhances this study's significance.

Antibody and Fractionation Protocol Choice

Based upon the results of this study previously discussed, protocol fractionation two and DNMT3A (PA5- 77945) antibody seemed to work best due to the clear and specific results they provided. Therefore, these two were used for the rest of the experiments mentioned in this paper, with success, and will be continued to be used in future experiments related to this study.

The DNMT3A (PA5-77945) antibody is the antibody that binds to the N-terminal region of DNMT3A, the region that both isoforms of DNMT3A do not contain. Using this antibody does come with the disadvantage of not being able to measure and capture DNMT3A2, the isoform that does not contain the N-terminal region. Although, DNMT3A1, the isoform that does contain that region, has been observed to be much more abundant in the brain compared to DNMT3A2, so for our purposes, capturing and analyzing DNMT3A1 should be sufficient (Chen, Ueda, Xie, & Li, 2002).

WB Analyses for Histone H3, DNMT3A, DNMT1, and Lamin

Regarding the WB analyses of DNMT1 and DNMT3A in the mouse cortex lysates, it seems that these two proteins do not have the same primary subcellular location. If this is true, this could also provide some insight into the mechanism of DNA methylation, as the location of molecules is associated with the role they perform. Thus, this would certainly be interesting and valuable to confirm. At this point, the WB of DNMT3A has matched what was expected; its primary location is bound to chromatin, due to its gene related purpose. Therefore, this fraction, or supernatant two, will be used further in this project as part of co-IP.

Western Blot Analysis for DNMT3A in WT and mHtt-Expressing Cortical Neurons

Lastly, the results of the WB for DNMT3A in WT and mHtt-expressing cortical neurons were quite interesting. These results suggest that the amount of DNMT3A at specific subcellular locations may not be the same, as less DNMT3A seems to be in the S3 and P3 fractions of the WT neurons when

compared with that of the mHtt-expressing neurons. If this were true, it could provide insight into the reason for the progression of HD. A possibility for the different locations could be due to a particular protein within the DNMT3A multi-protein complex present in the mHtt-expressing neurons, but not in the WT neurons, or vice versa, as is hypothesized to be in the case by this study.

Future Directions

No definitive conclusion can be drawn from the WB analyses results for the various proteins (DNMT1, DNMT3A, lamin A/C, and histone H3) done within this study thus far. At this point, what has been observed can only be deemed assumptions. They cannot be confirmed without further validation through repeated trials using in vivo and in vitro samples.

Repeated trials using vivo and in vitro samples would be one of the next steps for this project, along with correcting specific technical errors such as those previously mentioned and using other marker proteins besides histone and lamin A/C to indicate what part of the cell each fraction obtained from the fractionation technique is from. After further trials, the location of DNMT3A can be more conclusive. Then, this process can be repeated for HD samples obtained in vivo and in vitro. Once the definite location of DNMT3A within the mHtt-expressing neuron is determined, the locations of DNMT3A within these two models (WT vs. HD) can be accurately compared.

Another aspect that needs to be completed is co-IP using the S2 fraction prepared from fractionation protocol one and from WT and mHtt-expressing cortical neurons. Then, specific WB analyses would need to be done: a WB for DNMT3A would need to be done first to analyze this antibody's effectiveness in this technique. Then, a WB for MECP2 would need to be done; this is a protein that has been shown to interact with DNMT3A in neurons (M., Bröhm, et al., 2018). By analyzing if this interaction is still present after fractionation protocol two and with the DNMT3A (PA5- 77945) antibody, then it is assumed that these two did not affect the interactions of DNMT3A to a considerable degree. Of course, this may not be the case, but this is the most feasible method to determine this.

If this proves successful, a much greater magnitude of primary cortical culture will be created and then transfected with either WT or mHtt-expressing lentivirus. Then, fractionation will be done on these neurons with fractionation protocol two, and co-IP will be performed with DNMT3A (PA5-77945) antibody and the S2 fractions made from these cortical neurons. Mass spectrometry will then be performed on the samples obtained from the co-IP to identify the proteins present within these samples and determine any differences between the WT and HD samples. Finally, any notable differences will be validated through repeated trials using in vivo and in vitro samples.

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