QUANTITATIVE MEASUREMENT OF THE EXPRESSION OF TWO GENES IN THE CORETX AND CEREBELLUM OF A MOUSE MODEL OF JUVENILE ALZHEIMER'S

By

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A Thesis Submitted to The Honors College

In Partial Fulfillment of the Bachelors degree
With Honors in

BIOCHEMISTRY

THE UNIVERSITY OF ARIZONA

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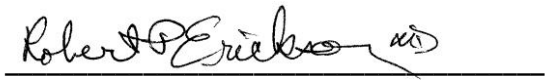

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Abstract

There is a continuing effort in order to connect the two neurodegenerative diseases: Alzheimer's Disease and Niemann-Pick Type C Disease (NPC1) "Juvenile Alzheimer's". Here in this study, we attempted to determine if there was a connection through the gene expression in inflammatory genes, *CD14* and *ETS-1*, in a NPC1 mouse model, *Npc1*^{nmf164}, using quantitative real time PCR. Hydroxypropyl-beta-cyclodextrin (HPβCD) is reported to provide a beneficial effect on NPC1, and there was interest in seeing if HPβCD treatment could return the expression levels to wildtype levels. Instead, only a significant decrease of the *CD14* and *ETS-1* in cerebellar tissue of untreated NPC1 affected mice (when compared to wildtype levels), and no significant difference for the treated, affected mice was seen. CD14 p value = 0.026, and ETS-1 p value = 0.014. There was no significant difference found in cortex tissue for either *CD14* or *ETS-1*.

Introduction

Niemann Pick Type C (NPC) is a rare autosomal recessive disorder and is characterized by impaired motor function, speech complications (dysarthria), yellow discoloration of the skin (jaundice), inflammation of the liver and spleen, and cerebellar dysfunction (Heidenreich and Erickson, 1997). This is due to a mutation within the intracellular cholesterol transport system. There are two forms, NPC1, which consists of a mutation in the NPC1 protein located within the endosome and which makes up 95% of NPC human occurrences; the other form is NPC2, which is 5% of the human cases, and is caused by a mutation in the NPC2 protein. The NPC2 phenotype is more severe than the NPC1 mutant, (Patterson, 2003).

There is currently one null mutation mouse model, *Npc1*^{nih}, and one single nucleotide polymorphism (SNP), *Npc1*^{nmf164} mouse model. The *Npc1*^{nih} mutant was created through a retrotransposon insertion that created a knock out of the NPC1 gene, effectively removing the protein (Zhang, et al, 2008). This mutant shows severe cerebellar dysfunction, and reduced survival when compared to wild-type (WT), and may represent a more severe, infantile form of the NPC1 human disease. The second mutant, *Npc1*^{nmf164}, has a base substitution that causes a missense mutation within an area that is correlated with a large portion of the human variants (Maue, et al, 2011). This mutant, is less severe and may correlate with the juvenile/late onset form of the NPC1 human disease.

Hydroxypropyl-beta-cyclodextrin (HPβCD) is known to be capable of shuttling cholesterol and other important lipids without the endosomal transport system. Research sparked anew when it was shown that the blood brain barrier of mice was still open up to up to 7 days after birth (Griffin et al. 2004) and that there was such a dramatic effect from HPβCD treatment when start at 7 days (Liu et al, 2009). Since one of the central components of NPC is neurodegeneration, it would be vital to ensure that the HPβCD could reach the brain. Previous research has shown that HPβCD cannot cross the blood brain barrier once closed (Camargo et al. 2001). We believe that treatment of HPβCD can be beneficial to the *Npc1*^{nmf164} mutant mouse model, if started at 7 days post-natal, and with continued treatment throughout life.

Recently, Gjoneska, E. et al (2015) looked into the gene expression in an inducible mouse model of Alzheimer's Disease (AD), and found a variety of gene expression differences when compared to WT. They looked into various groupings of genes that range from a variety of purposes, including genes related to inflammatory pathways, and transcription factors (Gjoneska, E. et al 2015). A previous study done by Borbon and Erickson (2011), showed that when the *Npc1*^{nih} mouse model was bred and combined with an AD mouse model (APP/PS1), there was increased amyloid plague production, giving some merit to calling NPC1 "juvenile Alzheimer's". In a review by Fiorenza et al (2013), there is noticeable effect of cholesterol metabolism on the formation of APP and Aβ, which are both vital for the manifestation of AD.

In order to continue to build upon the connection of AD and NPC1, we examined the gene expression of two genes *CD14*, which is correlated with the inflammatory pathway, and *ETS-1*, which belongs to a family of transcription factors, and is related to microglia activation and proliferation (Jantaratnotail, et al, 2013). We looked at the expression of these two genes within the cortex and cerebellum of WT mice, affected mice, and affected mice treated with HPβCD. We hypothesized that the *CD14* and *ETS-1* gene expressions would be elevated in the affected mice when compared to WT mice; this would be similar to the results found from Gjoneska, E. et al (2015). There is also the question as to whether or not HPβCD can have an

effect on the expression of this two genes by providing an artificial fix to the failed cholesterol transport system, and thereby lowering the expression of these two genes.

Materials and Methods

Mice

Mice used in this study were on the *Npc1*^{nmf164} mutant on the BALB/cJ background inbred strain from Jackson Laboratory. Through backcrossing, the mice used are descended from a N5 backcross generation that are 98.5% BALB/cJ. The *Npc1*^{nmf164} mutant is characterized by a high similarity to the human juvenile form (Maue, et al, 2011). The work done by Maue, et al (2011) showed similar levels of mRNA, but reduced levels of functionally NPC1 protein, as well as a slower progression of the phenotype when compared to previous null mutations (*Npc1*^{nih}). All mice used were kept in temperature and humidity controlled rooms, with NIH31 chow and water within reach *ad libitum.*

Genotyping

At the time of weaning, 3 weeks old, mice were tail-tipped. Tails were then lysed using protease K digestion. PCR was then done on the DNA obtained, with the settings and primers from Maue, et al (2011). The PCR product was then digested using *BSTEII*, and the product run on a 1.5% agarose gel to see the digestion pattern. See Fig. 1 for an example.

Hydroxypropyl-beta-cyclodextrin Treatment

HPβCD (Sigma Aldrich, average Ms ~1,460) treatment began at 7 days old on mice litters. The entire litter was injected subcutaneously at 4g/kg of 20% HPβCD in phosphate buffered solution (PBS). At 14 days, the mice were injected again, then tail-tipped and genotyped. Starting at 21 days, only homozygous *Npc1*^{nmf164} mutant mice (GG) were continued for weekly HPβCD treatments for the remainder of their lives.

RNA Extraction

RNA was lysed from cortex and cerebellar tissues (~25mg) and then purified using columns and protocol from Fisher Scientific SurePrep RNA/DNA/protein purification kit.

cDNA Synthesis

cDNA was created in order to measure the relative expression levels of mRNA in the qRT-PCR. One PCR tube is used per tissue sample per mouse, for an end volume of 20 µl. Each tube received 1µl Oligo $(dT)_{12\text{-}18}$ primers, 1µl of dNTP mix (1:6 dilution of 100µM solution in water), 10 µl of ddH_2O, and 1µl of the corresponding purified mRNA. The mixture was then heated to 65°C for 5 mins, then chilled on ice. 4µl of 5x 1st Strand Buffer, 2 µl of 0.1M DTT, and 1 µl of RNase Out was then added to each tube, and then heated at 42° for 2 mins. 1 µl of SuperScript II Reverse Transcriptase was then added to each tube, and heated at 42°C for 50 mins, followed by 15 mins at 70°C for deactivation. The Nanodrop spectrometer was used to determine the ng/µl of double stranded DNA in the cDNA reaction solution after completion. RNA elution buffer was used as the blank, and 1 µl was used.

Quantitative Real Time- PCR

cDNA created from the mRNA of cortex and cerebellar tissues were used to determine the relative expression levels of *CD14* and *ETS-1* between wildtype, affected, and treated affected mice. This was done using a final volume of 20 µl of reaction mix: 10 µl of iQ SYBR Green Supermix (Bio-Rad), 0.4 µl PerfeCTa Universal PCR primer (Quanta), 0.4 µlof the CD14 or the ETS-1 primer, 7.2 µl of Nuclease Free Water, and 1 µl of the corresponding cDNA per well. A pool of all of the components listed was created, and then dispensed into each well according to final desired final concentrations, minus the 1 µl of cDNA which was individual put into each well. The Roche Lightcycler® 96 was used with custom protocols of: 95°C for 2 mins, then 40 cycles of 95°C for 5s, 52°C for 15s, and 70°C for 15s. Samples were done in triplicate. Cyclic threshold was determined using Lightcycler® 96 software, Version 1.1.

Results

Figure 1. 1.5% agarose gel with ethidium bromide staining showing genotyping of *Npc1^{nmf164}* mutant mice after BSTEII digestion. Genotype from left to right: AG, GG, AA, AG, AG, AG, GG, AA. Picture taken in UV light.

Identifying Genotypes in order to determine control groups.

The SNP in the *Npc1^{nmf164}* is a change from an A (Adenine) nucleotide to a G (Guanine) nucleotide in one or both of the alleles within the mice. The mouse (*Mus musculus)* is a diploid mammal, therefore, each animal will have two copies of the NPC1 gene. The possible genotypes are: AA, AG, and GG. Each letter denotes a copy; AA means the genotype of the mouse has both of the NPC1 copies with an A. This SNP is vital to the mouse model, as a homozygous recessive (GG) will have the phenotype similar to the human NPC1 disease; the heterozygous genotype (AG) will not, because the copy with the A SNP is dominant and the mouse will be phenotypically normal.

BSTEII is a digestion enzyme that will cut the DNA when it reads a specific sequence within the DNA. For the *Npc1^{nmf164}* mutant, when the G allele is present, the correct sequence appears, and the BSTEII will cut the DNA. This can be seen in lanes 1,2,4,5, and 6 of the 1.5% agarose gel in Fig. 1 above. Lane 1 shows an AG, which only has partial digestion (cutting), which creates two bands on top of one another. There is only partial cutting as only one copy of the DNA has the G base substitution that allows the *BSTEII* to recognize and cut the DNA. Lane 2 shows a complete cutting of the DNA from having two copies of the G allele; this is determined by the strong, lower band. Lane 3 shows no digestion at all, so this would be indicative of the WT (AA) genotype. The lowest, faint band present in all of the lanes at the same location is presumed to be primer dimer, and does not have an effect on the genotyping of the mouse.

Selected Mice, their ages, and the cDNA created

For the purpose of this study, 4 AA mice were used as the WT control, and 5 untreated GG were the affected control. There were then 3 HPβCD treated GG (GG, Tx). Table 1 below shows the mouse number with the number of days the corresponding mouse lived before death or sacrifice for tissue extraction. The WT control is split evenly with 2 long lived, 235 days old, and 2 short lived (85 days old). The GG control is split again, but between medium aged (131,149, and 114 days old) and long lived (415 and 313 days old). Finally, the GG Tx group has 2 longer lived mice (338 and 246 days old). , and one medium aged (114 days old). There is no current knowledge as to whether or not the age of the mice will have a significant impact on the gene expression.

Table 2 shows the total cDNA found within the cDNA synthesis final volume (in ng/µl) for all of the brain cortex tissues of each mouse; the cell within the table corresponds to the mouse seen in Table 1. For example, the total cDNA from the cortex of the AA mouse 4509 is 1293.4 ng/µl. Table 3 shows the total cDNA for the cerebellar tissues. All of the values are within relatively the same range (>1000 ng/µl). Except for the cerebellar total cDNA for 4511. This low value (363 ng/µl) could indicate an error with the procedure. This cDNA value will not be used for calculations, so for the cerebellar tissue analysis, there will only be 3 AA controls.

Genotype: AA	Genotype: GG	Genotype: GG, Tx
4509, 235	4502, 131	4497, 338
4580, 85	4587, 149	4493, 246
4511, 235	4507, 415	4466, 114
4581, 85	4525, 313	
	4622, 114	

Table 1. Mouse number and age of mouse on death and/or tissue extraction (Mouse number, Age (days old)). Corresponding locations in relation to row within the columns will remain the same throughout all future tables.

AA	GG	GG, Tx
1293.4	1401.1	1496.1
1540	1119.8	1093.7
1052.5	1194.2	1148.5
1101.6	1209.2	
	1212.6	

Table 2. Total cDNA (ng/µl) from brain cortex tissue. Obtained from using the Nanodrop spectrometer, with RNA elution buffer as blank.

AA	GG	GG, Tx
1065.2	1415	1874.2
363	1077.9	1049.1
1020	1208.5	1230.4
1111.4	1315.4	
	1109.9	

Table 3. Total cDNA (ng/µl) from cerebellar tissue. Obtained from using the Nanodrop spectrometer, with RNA elution buffer as blank.

Quantitative Real Time PCR results and calculations

C_t values were obtained from the Lightcycler® 96 software, Version 1.1 based on the the qRT-PCR runs. These values are not listed, but are included into the data calculations in Table 4 and Table 5 below. Each sample was done in triplicate in order to account for error, and these 3 C_t values were then averaged to get 1 C_t value for one mouse, in either the cortex or cerebellar tissue, and for either the expression of *CD14* or *ETS-1*. The averaged C_t values were then divided by the corresponding total cDNA values for that specific mouse and specific tissue. This was done to account for the total cDNA that was present to show the ratio of expression of the gene (*CD14* or *ETS-1*) in relation to all the mRNA that was extracted and used for cDNA synthesis. The cDNA synthesis process is not specific to one mRNA, and therfore the expression of a gene must be in relation to all the genes expressed via mRNA. Finally, all the C_t : total cDNA ratios were averaged in order to have a ratio for each group seen in Table 4 and Table 5 (Average ± standard error). The grey divider indicates a separation as the gene expression being measured changes, and each gene has its own set of averages and standard errors.

The missing data in Table 5 corresponds to the total cDNA that was far too low (363 ng/µl). Because of this, the ratio of C_t : total cDNA cannot not be accurately determined without it being a significant outlier. The C_t : total cDNA was not included in the average and standard error calculation for either gene on cerebellar tissue for that specific mouse (4580).

C_t : total cDNA ratios in cortex tissue

	AA	**GG**	**GG, Tx**
CD14	0.02526	0.02125	0.02029
	0.02077	0.02592	0.02608
	0.02793	0.02741	0.02437
	0.0288	0.02338	
		0.02377	
Average	0.02569 ± 0.0018	0.02435 ± 0.00107	0.02358 ± 0.00172
ETS-1	0.0252	0.02211	0.02152
	0.02402	0.02579	0.02581
	0.02762	0.02902	0.02607
	0.02807	0.02328	
		0.02365	
Average	0.02623 ± 0.00097	0.02477 ± 0.00122	0.02447 ± 0.00256

Table 4. C_t : total cDNA ratios for brain cortex tissue. Values obtained by: C_t / total cDNA, for corresponding values. C_t values were obtained from average of triplicates. Average values also includes the standard error.

C_t : total cDNA ratios in cerebellar tissue

	AA	GG	GG,Tx
CD14	0.02667	0.02002	0.0165
		0.02762	0.02786
	0.02883	0.02477	0.02294
	0.03071	0.02414	
		0.02595	
Average	0.02874 ± 0.00117	0.0245 ± 0.00127	0.02243 ± 0.00329
ETS-1	0.03196	0.02076	0.01798
		0.0302	0.02893
	0.02868	0.02408	0.02308
	0.02838	0.02155	
		0.0227	
Average	0.02967 ± 0.00115	0.02386 ± 0.00168	0.02333 ± 0.0316

Table 5. C_t : total cDNA ratios for cerebellar tissue. Values obtained by: C_t / total cDNA, for corresponding values. C_t values were obtained from average of triplicates. Average values also includes the standard error. Missing value in AA column was omitted due to total cDNA corresponding to it was eliminated.

Cortex tissue had no significant change in gene expression

The averages and the standard errors were normalized and graphed as percent's using the AA control average and standard error as the comparison point. Figure 2 shows this normalization for the expression of *CD14* on the brain cortex tissues for each group of mice. It should be noted that the graph starts as 0.8. Using a 1 sided t-test, the statistical significance was calculated between each group. Between the AA group and GG, there is no statistical significance (p value = 0.27). There was no statistical significance between the AA and the GG Tx group (p value = 0.22). There was also no statistical significance between the GG and the GG Tx (p value = 0.36).

Figure 3 shows the averages and the standard errors for the *ETS-1* gene in the cortex tissue. The graph has the y-axis start at 0.88. The 1-sided t-test was also done for the ETS-1 levels in cortex tissues. Between AA and GG, there was no statistical significance (p value = 0.19). For AA and GG Tx, there was no statistical significance as well (p value = 0.19). GG and GG Tx are not statistical different (p value = 0.44).

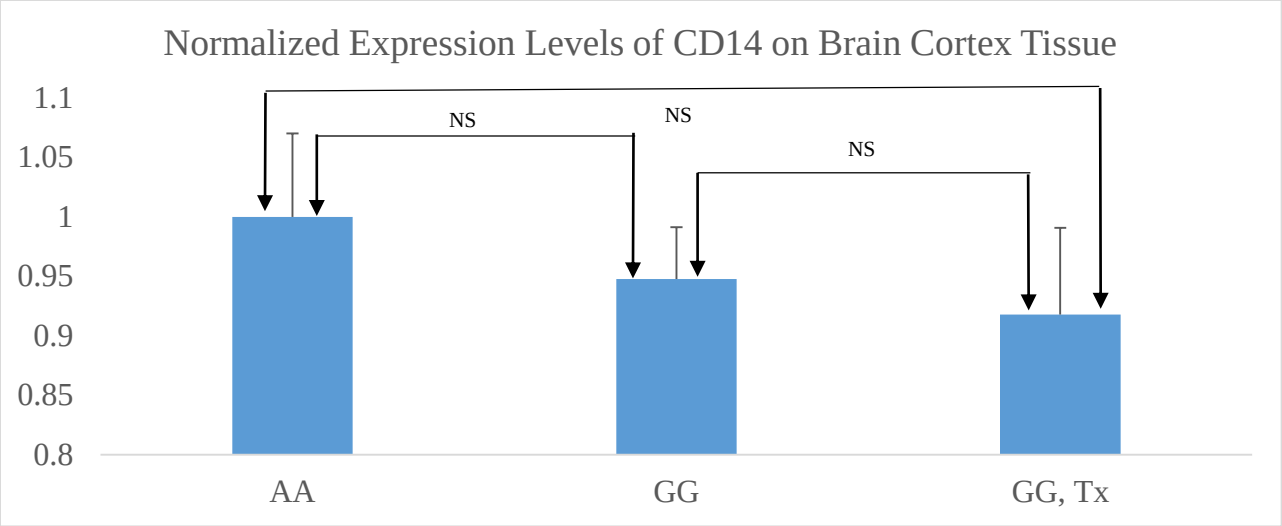

Figure 2. Normalized expression level for *CD14* on Brain Cortex tissue based on the C_t/total cDNA (ng/µl). Means and standard errors are graphed as a percentage based on the expression level of AA (WT). y-axis starts at 0.8. There is no significant difference between any of the groups (AA vs GG, AA vs GG, Tx, and GG vs GG, Tx). AA n=4; GG n=5; GG, Tx n= 3. "NS" denotes not significant.

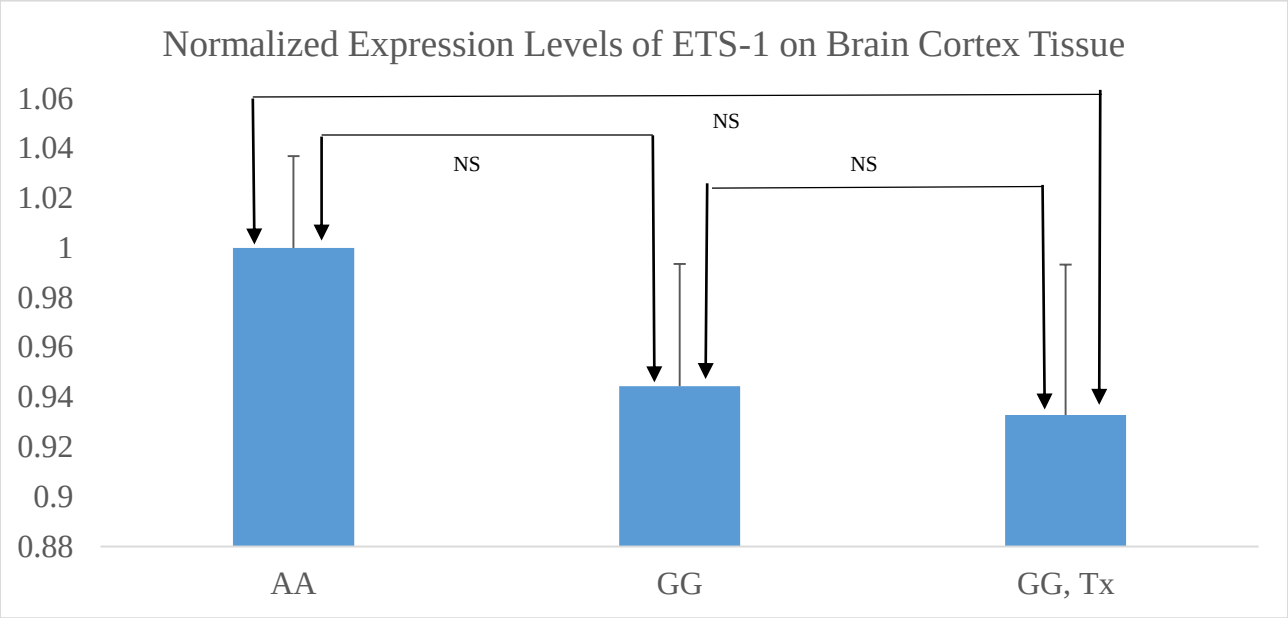

Figure 3. Normalized expression level for *ETS-1* on Brain Cortex tissue based on the C_t/total cDNA (ng/µl). Means and standard errors are graphed as a percentage based on the expression level of AA (WT). There is no significant difference between any of the groups (AA vs GG, AA vs GG, Tx, and GG vs GG, Tx). AA n=4; GG n=5; GG, Tx n= 3. "NS" denotes not significant.

Cerebellar tissue shows significant differences between wildtype and affected mice

The averages and standard errors are graphed as percentages based on the expression levels of the AA group in Figure 4 and Figure 5 for the cerebellar tissue. Figure 4 shows the expression level of *CD14* between the 3 groups. Using a 1-sided t-test, there was found to be a statistical significance between AA and GG (p value = 0.026). There was no statistical significance between AA and GG Tx (p value = 0.09) or GG and GG Tx (p value = 0.30). While the difference between the AA and GG Tx is not significant, there is still a notable decrease in expression level of *CD14* in GG Tx. There was a greater range of C_t values within the GG Tx group, and combined with one of the AA values being excluded due to low total cDNA, the ranges overlapped enough to make these two groups not statistically different (p value >0.05)(see Fig4).

Figure 5 is similar to Figure 4. The averages and standard errors again were graphed as percentages using the AA group values as the basis, but for *ETS-1* expression values in cerebellar tissues. A 1 sided t-test was used. Only between the AA and GG groups was there a statistical significance found (p value = 0.014). AA and GG Tx showed difference but it was not significant (p value = 0.087). Between GG and GG Tx, there was no significant difference found (p value = 0.44).

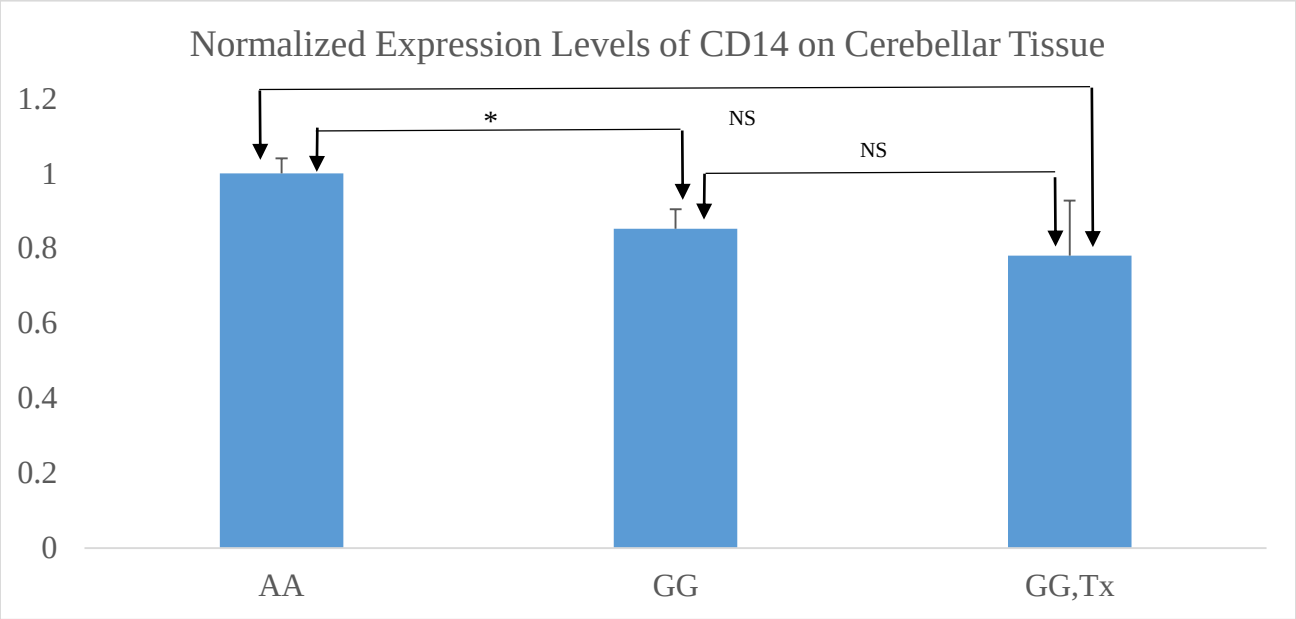

Figure 4. Normalized expression level for *CD14* on Brain Cortex tissue based on the C_t/total cDNA (ng/µl). Means and standard errors are graphed as a percentage based on the expression level of AA (WT). There was no significant difference between GG vs GG, Tx. There was difference, but not significant, AA vs GG, Tx (p = 0.093). There was a significant difference between AA vs GG (p = 0.026) AA n=4; GG n=5; GG, Tx n= 3. "NS" denotes not significant. "*" denotes statistical significance.

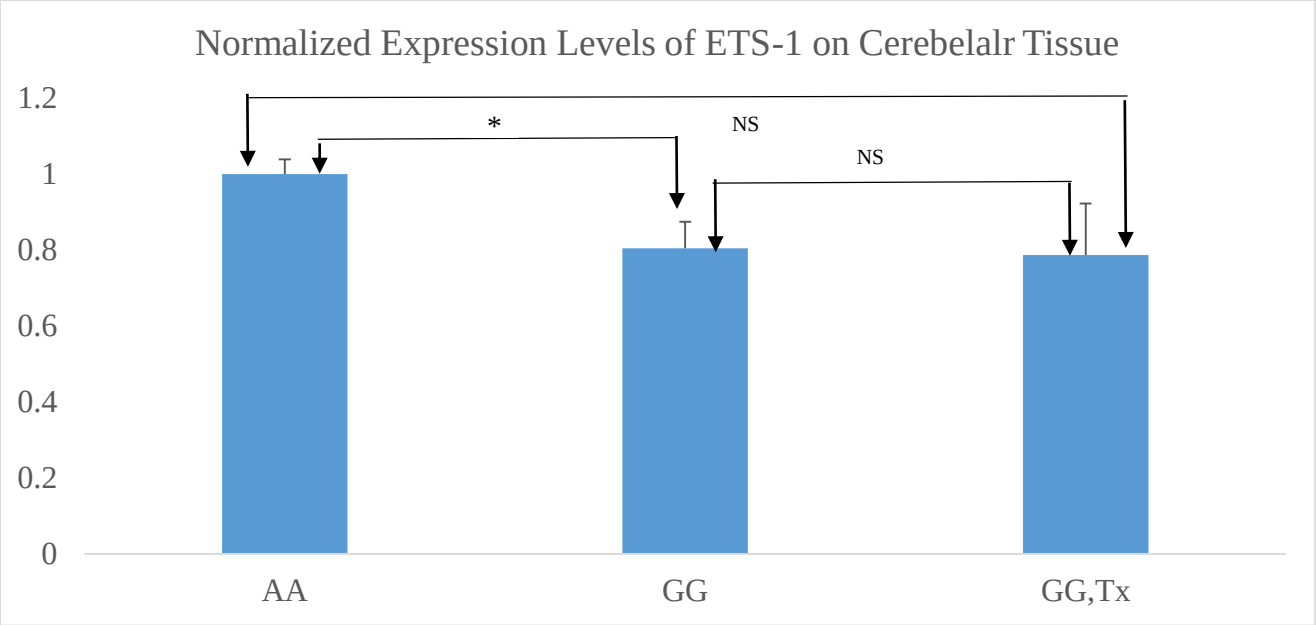

Figure 5. Normalized expression level for *ETS-1* on Brain Cortex tissue based on the C_t/total cDNA (ng/µl). Means and standard errors are graphed as a percentage based on the expression level of AA (WT). There was no significant difference between GG vs GG, Tx. There was difference, but not significant, AA vs GG, Tx ($p = 0.087$). There was a significant difference between AA vs GG ($p = 0.014$) AA n=4; GG n=5; GG, Tx n= 3. "NS" denotes not significant. "*" denotes statistical significance.

Discussion

Genes and Primers

Originally, there were four genes that were going to be looked into: *SPI1*, which is correlated with an increased immune response; *ABCA1*, which is a paralogue of the cholesterol transport, ABCA7; *CD14* and *ETS-1 (*Gjoneska, E. et al, 2015). The primers necessary to test for the expression of *SPI1* and *ABCA1* were designed and ordered, but failed during the optimization process. The optimization process looked into what was the optimal temperature for the PCR reaction to occur to get the best results. A range of temperatures was used for all of the primer sets; forward and reverse primer for each gene. The *SPI1* and *ABCA1* primers did not work under any temperature conditions. Sequences were checked, and there was no issue seen. However, the optimization was only done on cerebellar tissue for testing. The *SPI1* and *ABCA1* genes may not be active at significant levels for PCR to occur in cortex and cerebellar tisue, which could explain the failure of the optimization. NPC1, and the *Npc1*^{nmf164}, are reported to have significant effects on the liver and spleen (Heidenreich RA, Erickson RE,1997). *SPI1* and *ABCA1* might be more prevalent in these two tissues that were not tested in this study. Future experiments could look into determining the gene expression of all four of the genes within liver and spleen tissues of the three groups (AA, GG, and GG Tx).

Inflammation, CD14, and ETS-1and HPβCD

Inflammation of the liver, spleen, and some parts of the brain are characteristic of NPC1 (Heidenreich RA, Erickson RE,1997). The *Npc1*^{nmf164} also shows significant inflammation (Maue, et al, 2011). Because of this, it would be expected that genes related to the immune

response, and therefore inflammation, would be increased. The expectation was that *CD14* and *ETS-1* would be elevated, and that the HPβCD would be able to return these values to WT levels. However, the results indicate that there was hardly any inflammation occurring due to the expression levels of the two genes. This raises the interesting question as to whether or not there is an inflammatory (immune) response to the neurodegeneration in the *Npc1^{nmf164}* mouse model of NPC1.

Not only did the two genes not have increased expression levels, but the HPβCD did not increase the expression levels of these two genes to return to WT. In all of the cases, cortex and cerebellum, *CD14* and *ETS-1*, HPβCD lowered the expression level even further, but not significantly. This is the opposite of what was expected, as HPβCD has been reported to be effective in treating the symptoms of NPC1 in another mice model, *Npc1^{NIH}* (Griffin et al. 2004). Even one treatment at 7 days old has been shown to be significant in mobilizing the cholesterol with organs (Liu et al. 2009). The goal of the HPβCD treatment is to aid in the mobilization of cholesterol within the body, in order to counteract the failed endosomal cholesterol transport associated with NPC1. By doing this, one would expect the body to then return to normal conditions as the problem of cholesterol transport has been resolved. However, the results showed that HPβCD treatment did not affect the expression levels *CD14* and *ETS-1* in terms of making them similar to WT levels.

Comparison to Alzheimer's disease mouse model and cholesterol components of both diseases

Gjoneska, E. et al (2015) observed a nearly 2 fold increase, but no statistically significant, of *CD14* expression the within the inducible AD mice model, CK-p25. The expression of *ETS-1* was not directly measured, but it was implied with the 3 fold increase of *SPI1*, which encodes the regulator for *ETS-1*, and other ETS like proteins. If *SPI1* is significantly increased, then the ETS proteins could be significantly increased. Those findings are in contrast to the no difference found for *CD14* expression in the cerebellar or cortex tissue; and small decrease in *ETS-1* found in both cortex and cerebellar tissue in the Fig.4 and Fig.5.

While the results show no connection between AD and NPC1 in the scope of *CD14* and *ETS-1* gene expression in two mouse models, there still are other similarities that could suggest a connection. Both disease have a dementia component that is related to the presence of nuerofibrillary tangles (Borbon et al, 2012), and this dementia coined the term "Juvenile Alzheimer's" for NPC1. AD and NPC1 are distinctly different in their root causes and manifestation; AD deals protein plagues damaging brain cells, while NPC1 is a failure in lysosomal cholesterol mobilization. However, in a recent review by Fiorenza, M.T. et al (2013), there was found to be abnormal cholesterol handling in the formation amyloid precursor protein (APP) and amyloid β (Aβ) formation. This is due to the pathway that gets cholesterol from the astrocyte that does the modifications and movement of cholesterol using NPC1 like proteins, and getting them to neurons for use (Fiorenza et al, 2013). The death of the cells in AD, and the production of APP and Aβ could be in part due to a failed cholesterol transport system, or a failed cholesterol system could exacerbate the symptoms of the APP and Aβ formation. In the end, there is still connections being made between AD and NPC1, despite the results above showing there is no connection in the expression if *CD14* and *ETS-1*.

Issues with Npc1^{nmf164} mouse model could indicate other beneficial factors

A concurrent study was being doing using some of the same mice, specifically the GG and GG Tx mice. HPβCD was tested if the survival and motor function of the *Npc1^{nmf164}* mouse

model of NPC1 could be restored. The GG controls used in this study lived far longer than expected, based on past average life spans; 112 ± 4 days (Maue et al, 2012). The GG controls were now living as long, if not longer than the HPβCD treated mice. Most of these long-lived GG were traced back to one breeding pair. This could indicate a background mutation may have occurred. A background mutation is a mutation in another part of the genome of the model organism that does not directly affect the protein/gene of interest. This could affect how the cholesterol moves in the body, how the body handles HPβCD, or many other cases that could be beneficial for the survival of the mouse. There is currently work being done to understand how the background mutation affected the GG controls of the concurrent study, and possibly this study on *CD14* and *ETS-1* gene expression. If the background mutation was so significant in increasing lifespan, it could have had a large effect on inflammation, or gene expression (*CD14* or *ETS-1*). The mice affected by this background mutation have not produced any offspring so the mutation has been isolated. But, there is an interest in discovering what this background mutation and what beneficial factor did it provide to the *Npc1*^{nmf164} mouse model.

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