Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter know, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Lindsey J. Sniffen April 6, 2018

Effect of soluble TNF signaling on blood-brain barrier permeability and neuroinflammation in a 5xFAD mouse model of Alzheimer's disease fed a high-fat high-fructose diet

by

Lindsey J. Sniffen

Dr. Malú G. Tansey, PhD
Adviser

Program of Neuroscience and Behavioral Biology

Dr. Malú G. Tansey
Adviser

Dr. Gillian Hue
Committee Member

Dr. Jennifer Zelger
Committee Member

2018

Effect of soluble TNF signaling on blood-brain barrier permeability and neuroinflammation in a 5xFAD mouse model of Alzheimer's disease fed a high-fat high-fructose diet

By

Lindsey J. Sniffen

Dr. Malú G. Tansey
Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Program of Neuroscience and Behavioral Biology

2018

Abstract

Effect of soluble TNF signaling on blood-brain barrier permeability and neuroinflammation in a 5xFAD mouse model of Alzheimer's disease fed a high-fat high-fructose diet
By Lindsey J. Sniffen

In the United States, there are over 5 million people living with Alzheimer's Disease (AD), and the prevalence is expected to increase as the population ages (Alzheimer's Association, 2016). One risk factor for AD is metabolic disorders such as type 2 diabetes which increases risk for AD by 65% (Xu, 2011). Consuming a high-fat and high-sugar diet causes weight gain and is a risk factor for metabolic disorders. Both metabolic disorders such as type 2 diabetes and AD have an increase in chronic low-level inflammation in the brain and body that can activate the immune system (de Sousa Rodrigues, 2016). A proposed mechanism for this inflammation is through soluble tumor necrosis factor (solTNF). Increased inflammation can damage the blood-brain barrier (BBB) and increase its permeability. The BBB consists of membrane proteins such as claudin 2 (CLDN2) and zona occludins-1 (ZO-1) on the surface of endothelial cells to form tight junctions (Stamatovic, 2008). Chemokine (C-C motif) ligand 2 (CCL2) attracts CCR2 expressing macrophages to the site of inflammation (Khoury, 2007). Cluster of differentiation 45 (CD45) is highly expressed on monocytes and is increased with increases of neuroinflammation (Jeong, 2013). IL-6 is a proinflammatory cytokine that is secreted by T-cells and brain immune cells. Using qPCR, we observed hippocampal mRNA expression of TNF, CCL2, ZO-1, CLDN2, CD45, and IL-6. We also looked at hippocampal protein expression of ZO-1. We analyzed 5-point familial Alzheimer's Disease (5xFAD) and wild-type mice either fed a control diet (CD) or high-fat high-fructose (HFHF) diet. Some mice from each group were also administered Xpro® 1595, which inhibits solTNF signaling (MacPherson, 2017), to observe its effects on inflammatory markers. Our study demonstrates that HFHF diet increases hippocampal inflammation as measured by TNF mRNA in WT mice, XPro reduces TNF mRNA levels in the hippocampus of TG mice, and 5xFAD mice fed a HFHF diet have increased CCL2 mRNA in the hippocampus that is ameliorated by blockade of solTNF signaling with XPro®1595.

Effect of soluble TNF signaling on blood-brain barrier permeability and neuroinflammation in a 5xFAD mouse model of Alzheimer's disease fed a high-fat high-fructose diet

By

Lindsey J. Sniffen

Dr. Malú G. Tansey
Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Program of Neuroscience and Behavioral Biology

2018

Acknowledgements

I would like to thank Dr. Malú Tansey for supporting this research opportunity, Dr. Lori Eidson for her consistent mentorship, and Dr. Elizabeth Rodrigues and Mary Herrick for their support throughout this project. I would also like to thank Sean Kelly for teaching me how to perform qPCR, and Madelyn Houser for training me in Western blotting, and Billie Jeanne Duke for preparing stocks and reagents. The privilege of working with the Tansey lab has been a rewarding experience, and I hope to continue to learn from the Tansey lab members. Finally, I would like to thank my committee members, Dr. Gillian Hue, Dr. Jennifer Felger, and Dr. Malú Tansey, for their insight and support of this research project.

Table of Contents

Introduction……………………………………………………………………………………1

Methods……………...……………………………………………………………………...6

Table 1…………………………………………………………………………………6

Table 2…………………………………………………………………………………7

Results…………………………………………………………………………………………10

Figure 1……………………………………………………………………………..11

Figure 2……………………………………………………………………………..14

Figure 3……………………………………………………………………………..18

Figure 4……………………………………………………………………………..19

Figure 5……………………………………………………………………………..20

Figure 6……………………………………………………………………………..22

Discussion……………………………………………………………………………..23

References……………………………………………………………………………..28

Introduction

Alzheimer's Disease (AD) affects more than 5 million Americans and is the sixth leading cause of death in the US (Alzheimer's Association, 2016). AD is the most common form of dementia, and the symptoms may include memory difficulties, communication issues, inability to focus, and problems with reasoning. The exact cause of this disease is still unknown. One proposed mechanism is accumulating amyloid plaques and tau tangles, which are found along with brain atrophy in post mortem AD brains. In AD tau proteins aggregate together in the cell, which leads to a collapse of the cytoskeleton and neuronal death (Hardy, 1991). Amyloid plaques form outside of the cell when one misfolded protein accumulates with other misfolded proteins, resulting from the genetic amyloid precursor protein (APP) cleaved incorrectly. The neuronal cell death from Tau and amyloid plaques cause microglia to be called to the damaged site as signaled by inflammatory proteins. Microglia act as the brain's resident immune system and function similarly to macrophages by repairing or stopping further neuronal damage, but microglia may contribute to protein misfolding with chronic inflammation. Together with the blood brain barrier (BBB), a semipermeable membrane that regulates trafficking to the brain, they are responsible for protecting the brain.

The increase in prevalence of AD in the United States may be attributed to an increase in American Western diet consumption that is high in fat and sugar (de Sousa Rodrigues, 2016). Consuming a high-fat and high-sugar diet causes weight gain and is a risk factor for obesity and diabetes. Metabolic disorders such as type 2 diabetes increase risk for AD by 65% (Xu, 2011). Metabolic disorders can cause low-level chronic inflammation that interferes with the gut-brain axis (de Sousa Rodrigues, 2017). High-fat and high-sugar diet can promote gut inflammation, which is associated with an increase in intestinal permeability. It can also increase adipose tissue,

and obese patients have increased activated macrophages in these adipose tissues which can cause metabolic stress (Surmi and Hasty, 2008). This can activate the immune system in the periphery and the brain (de Sousa Rodrigues, 2016). This process occurs, in part, through the hypothalamic-pituitary-adrenal (HPA) axis. Chronic activation of the HPA axis by metabolic stress causes a chronic increased level of cortisol (Simsek, 2016). Chronic increased cortisol production increases oxidative stress, leading to increased inflammatory processes (Simsek, 2016). This peripheral inflammation due to diet consumption can increase permeability in the BBB, leading to further increased brain inflammation.

A predicted pathway for this response is through soluble tumor necrosis factor (solTNF). SolTNF is found in the brain, peripheral nervous system plasma and cerebral spinal fluid (CSF). It is trafficked through the BBB into the brain where it is involved in inflammatory signaling (Selkoe, 2013). This pro-inflammatory state is marked by an increase in the number of monocytes, microglia, and higher levels of inflammatory markers. solTNF binds to tumor necrosis factor receptor 1 (TNFR1) to initiate a pathway that activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which signals for pro-inflammatory processes at the nuclear level (McCoy and Tansey, 2008).

The blood-brain barrier is a vital structure made up of astrocytes and endothelial cells that prevents potential hazards and peripheral immune cells from crossing into the brain (Pardridge, 2005). When the highly selective semipermeable BBB becomes damaged, it becomes less selective and more permeable leading to detrimental effects (Varatharaj, 2016). Aging and other pro-inflammatory states cause the BBB to become more permeable and susceptible to the peripheral immune system (Simsek, 2016). Tight junctions between endothelial cells create the BBB, and these tight junctions are formed by several transmembrane proteins such as occludin,

claudins, and junctional adhesion molecule (JAM). These proteins attach to the cytoskeleton of endothelial cells by the protein complex zona occludens -1 (ZO-1; Stamatovic, 2008). Overgaard et al. 2011 found that increased expression of claudin 2 (CLDN2) increases permeability of tight junctions. ZO-1 expression is decreased in mice with a more permeable BBB (Dimitrijevic, 2006). A more permeable BBB is more susceptible to peripheral immune cell trafficking into the brain. Chemokine (C-C motif) ligand 2 (CCL2) is a signal of inflammation that attracts CCR2 expressing macrophages to the site of inflammation (Khoury, 2007). Increased CCL2 in the brain due to BBB damage may cause increased trafficking of peripheral macrophages that express CCR2 in addition to resident macrophages. Additionally, CCL2 interaction with ZO-1 has been shown to decrease ZO-1 expression (Stamatovic, 2003).

Another marker of inflammation is cluster of differentiation 45 (CD45), which is highly expressed on monocytes (Jeong, 2013). Therefore, if there is more inflammation, more monocytes will be recruited to the brain and will increase the number of cells that have high expression of CD45. Interleukin-6 (IL-6) and tumor necrosis factor (TNF) are proinflammatory cytokines and therefore markers of inflammation. IL-6 is secreted by T-cells and brain immune cells, and stimulates an immune response after trauma. TNF is not only found peripherally, but is also synthesized in the brain. The Dominant-Negative TNF inhibitor Xpro® 1595 (Xpro) inhibits solTNF signaling by forming inactive heterotrimers with native solTNF to prevent TNFR1 binding (Steed, 2003) thereby decreasing neuroinflammation (MacPherson, 2017). This selective solTNF neutralization was also found to decrease amyloid plaques in 5-point mutation Familial Alzheimer's Disease (5xFAD) mice. Therefore, it may decrease BBB permeability and affect ZO-1, CLDN2, IL-6, and CD45 expression.

This study analyzed changes in mRNA and protein expression following Western diet manipulation in wild- type C57BL/6 mice and transgenic 5xFAD mice. 5xFAD mice have 5 human familial Alzheimer's Disease (AD) gene mutations. Three amyloid precursor protein mutations from the Swedish, Florida and London families, along with 2 mutations in presenilin-1. This model demonstrates dysregulation in the production of amyloid, early Aβ42 deposition, and age-dependent cognitive decline and neurodegeneration by 4-5 months of age (Oakley, 2006) making it ideal for studying AD. The mice were fed either a control chow diet (CD) or a high-fat high-fructose diet (HFHF). Diet was started at 2 months as this is the age 5xFAD mice begin to show amyloid plaques (Oakley, 2006). This study analyzed the hippocampus, as it is susceptible to damage and increased permeability in the BBB, and is implicated in the pathogenesis of AD (Khoury, 2007). ZO-1 and CLDN2 were analyzed to investigate BBB permeability, CCL2 to investigate immune cell trafficking, and TNF, CD45, and IL-6 to investigate changes in inflammation.

We hypothesize that HFHF diet combined with a genetic predisposition for AD increases inflammation leading to increased BBB permeability and neuroinflammation. Additionally, we hypothesized that solTNF would mediate inflammation and changes in BBB permeability in the hippocampus. If HFHF diet damages the BBB and increases permeability, then we expect an upregulation of CLDN2 and a downregulation of ZO-1. Additionally, if HFHF diet increases inflammation and transport into the brain, then we expected an upregulation of TNF, CD45, CCL2, and IL-6 in both wild-type (WT) and transgenic 5xFAD (TG) mice, and to a greater extent in the transgenic genotype due to gene-diet interaction. If Xpro® 1595 decreases inflammation, then we expected to see downregulation of TNF, CD45, CCL2, and IL-6 in all conditions (region, genotype, and diet), especially TG HFHF. If Xpro® 1595 decreases

inflammation, then there should be a decrease in permeability in the BBB and we expect upregulation of ZO-1 and downregulation of CLDN2. Additionally, we expected a decrease in expression of TNF, CD45, CCL2, and IL-6 with Xpro® 1595 administration. Furthermore, we analyzed the weight changes of the mice to see if HFHF-induced obesity correlated with gene and protein expression, as mice that are more susceptible to weight gain may have increased expression of neuroinflammatory markers. Only female mice were used in this study as female 5xFAD mice show increased amyloid plaque formation and AD pathology as compared to males (Dinkins, 2015). It has been previously shown that a HFHF diet causes both male and female 3xFAD mice to gain similar amounts of weight and have similar amounts of abdominal adipose fat (Barron, 2013).

Methods

Experimental Design

Table 1. Experimental groups

Genotype	Diet	Treatment	Number of mice
Wild-type C57BL/6J	Control	Saline	6
		Xpro® 1595	6
	High-fat high-fructose	Saline	8
		Xpro® 1595	6
Transgenic 5xFAD	Control	Saline	7
		Xpro® 1595	6
	High-fat high-fructose	Saline	9
		Xpro® 1595	4

Female animals started control chow or HFHF diet treatment at 2 months of age, and diet was continued until 4 months of age. Two months of age is early adulthood and 4 months is mature adulthood. Drug administration of Xpro® 1595 (10 mg/kg, subcutaneous) or saline was started at 3 months of age and was administered once every 3 days for 4 weeks. At experiment end, animals were anesthetized with isoflurane and brains were rapidly removed. The brains were not perfused but the meninges and vasculature were stripped. The hippocampus was microdissected on dry ice and flash frozen in 2-methylbutane on dry ice. The hippocampus was stored at -80 degrees Celsius until processing.

qPCR

RNA was extracted from the hippocampus under RNase-free conditions. Tissue was lysed with TRIzol on ice. RNA was isolated and purified with QIAshredder and RNeasy kits according to manufacturer's protocol (Qiagen, 2012). Protein was saved for further analysis in sodium dodecyl sulfate (SDS) and stored at -80 degrees Celsius. Concentration and purity of RNA was analyzed with a Nano-Drop 2000 spectrophotometer. RNA samples were standardized to 4ug concentration and then reverse transcribed using Applied Biosystems RNA to cDNA

reagents. cDNA was run in triplicate using a SYBR green master mix with primers *ZO-1, CLDN2, TNF, CCL2, CD45*, and *IL-6*. (see Table 2 for primer sequences). The universal two-step RT-PCR cycling conditions used with SYBRgreen mastermix on the 7900HT Sequence Detection System were: 50 °C (2 min), 95 °C (10 min), 40 cycles of 95 °C (15 s) and 60 °C (1 min). Samples were run in triplicate, and the coefficient of variation within the triplicates was no more than 4%. TaqMan method of qPCR has a higher specificity, but with proper protocol the cost-effective use of SYBR green can produce accurate results (Tajadini, 2014).

Table 2. Primer sequences designed and purchased from Integrated DNA Technologies (IDT)

CLDN2	Forward- CAC CCA CAG ATA CTT GTA AGG Reverse- AGC CTC TAA TCC CTT ATT TCA C
ZO-1	Forward- CCT GAA GGA ATT GAG CAA GA Reverse- GCA GAG TTT CAC CTT TCT CT
CCL2	Forward- CAC CCA CAG ATA CTT GTA AGG Reverse- AGC CTC TAA TCC CTT ATT TCA C
TNF	Forward- CTG AGG TCA ATC TGC CCA AGT AC Reverse- CT CAC AGA GCA ATG ACT CCA AAG
CD45	Forward- TCA TGG TCA CAC GAT GTG AAG A Reverse- AGC CCG AGT GCC TTC CT
IL-6	Forward- GAG GAT ACC ACT CCC AAC AGA CC Reverse- AAG TGC ATC ATC GTT GTT CAT ACA

To analyze data, the triplicates of each sample's cycle threshold (Ct) were averaged. Several mouse housekeeping genes (Hprt1, TATA, Cyclo, 36B4, and GAPDH; Applied Biosystems, Foster City, CA) were run in order to determine the optimal endogenous control, but none of the housekeeping controls nor their geometric means remained invariant among all eight experimental groups. Thus, to ensure standardization in total cDNA across groups, cDNA was quantified via the PicoGreen Assay (Invitrogen, Carlsbad, CA) and then standardized so that all samples started quantitative RT-PCR with 1ug cDNA (Kelly, 2015). Then each WT sample's Ct was normalized to the control condition by subtracting the average WT CD saline Ct, and for each TG sample's Ct was normalized to TG CD saline Ct. Then each sample's Ct was converted to fold change by the formula 2^{Ct}. Fold changes were analyzed using a 2-way ANOVA using

GraphPad Prism. One 2-way ANOVA test with post hoc analysis was run with WT mice data and another with TG mice. For both genotypes, diet and Xpro-treatment were the two main effects compared, and alpha was set at 0.05.

Western Blot of ZO-1

Protein concentration was calculated using a bicinchoninic acid assay (BCA) of total protein, and western blots were done as previously described (Lee, 2012). Protein was prepared in 5X Laemmli sample buffer and loaded on pre-cast 4-20% 15-well sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (SDS-PAGE; Bio-Rad, Hercules, CA, USA), transferred onto Polyvinylidene (PDVF) membranes (Millipore), and probed for ZO-1 (1:4000, rabbit anti-mouse antibody; Life Technologies) and β-actin (1:2000, mouse anti-mouse antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse, respectively) at 1:2000 (Jackson ImmunoResearch Lab, West Grove, PA, USA). Immunoreactive bands were visualized with SuperSignal West Pico HRP substrate (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions and imaged on Azure Chemi Gel documentation station. The exposure time was 120 seconds and brightness and contrast was adjusted to be the same for each blot. Using Photoshop, images were converted to grayscale and 600 dots per inch (dpi). The lanes were plotted and the areas and percentage of total areas of peaks were found using ImageJ for both ZO-1 and beta-actin. Each percentage was normalized to a reference sample between gels. The average of the WT CD saline group and TG CD saline group was found, and each sample was normalized to the mean respectively. The normalized housekeeping protein beta-actin was subtracted from the ZO-1 values and plotted in Prism. One 2-way

ANOVA test with post hoc analysis was run with WT mice data and another with TG mice. For both, diet and Xpro-treatment were the two main effects compared, and alpha was set at 0.05.

Correlation Analysis of Weight Change

Mice weight gain varied; therefore, regression analyses were done to observe if this correlated with the expression of qPCR inflammation markers. Percent weight change was calculated for each mouse by the formula = ((final weight-initial weight)/final weight) *100. Values were plotted in Prism against the respective mouse's mRNA or protein fold change using correlation analysis. Groups were analyzed by genotype, diet, and Xpro treatment.

Results

Neuroinflammatory mRNA in the Hippocampus

WT mice showed an increase in hippocampal TNF mRNA expression when fed a HFHF diet compared to CD [$F(1, 22)=5.6$; $p=0.027$]. Xpro® 1595 had no effect on TNF mRNA expression in WT mice. TG mice showed a decrease in TNF expression in XPro-treated mice compared to saline-treated mice regardless of diet type [$F(1, 22)=4.64$; $p=0.042$]. Post hoc analysis was not significant in either genotype group. WT mice may show an increase in IL-6 mRNA expression when fed a HFHF diet compared to CD mice [$F(1, 22)=4.27$; $p=0.0506$]. As α was equal to 0.05, it did not reach significance, but indicates a trend. TG mice show no significant changes in IL-6 expression due to diet or drug treatment. Both WT and TG mice had no significant changes in CD45 mRNA expression due to diet or drug treatment as referenced in Figure 1.

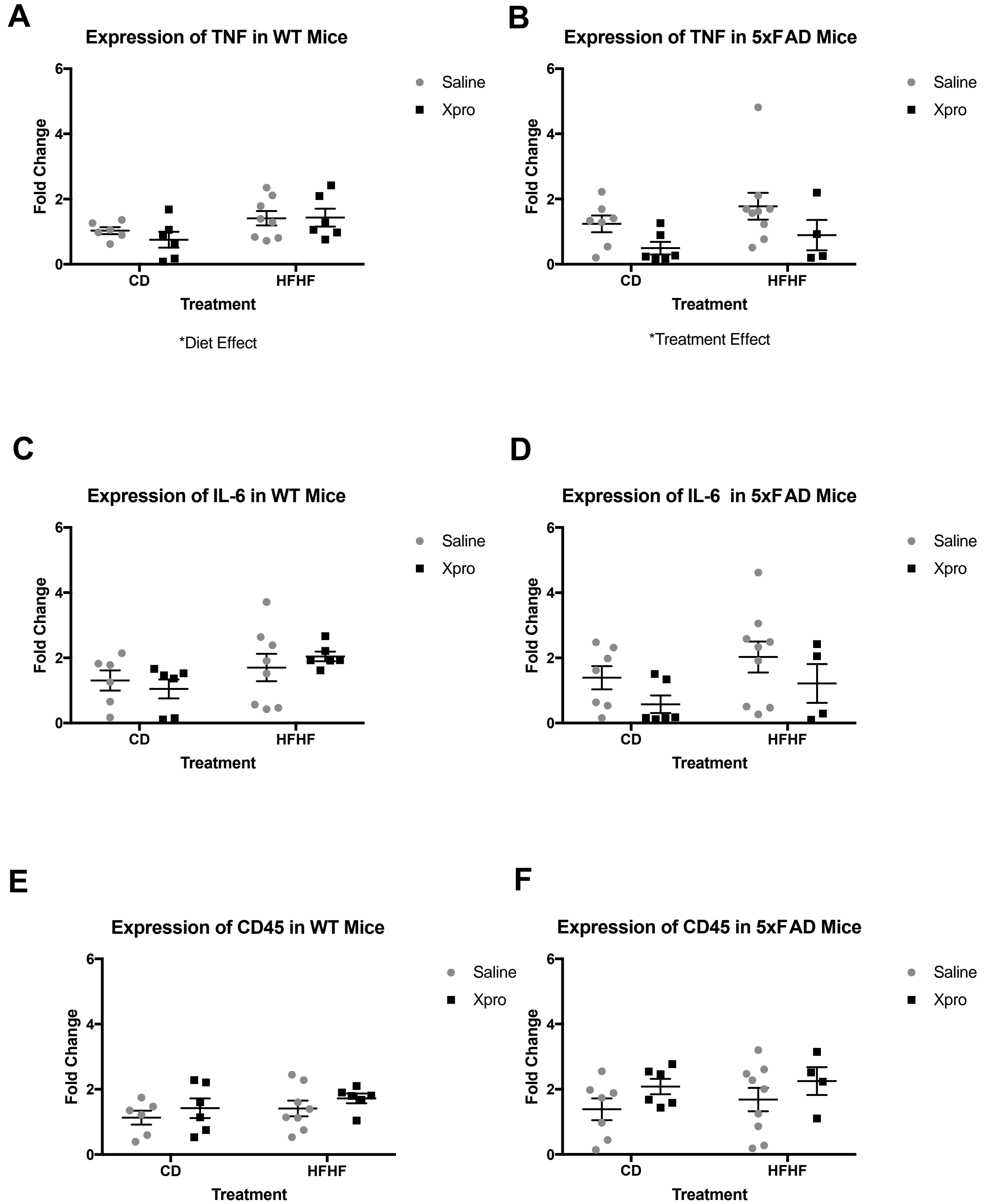


Fig. 1. A) TNF mRNA expression increased in WT mice fed HFHF diet [$F(1, 22)=5.6$; $p=0.027$].

B) TNF mRNA expression decreased in TG mice due to Xpro® 1595 administration [F(1,

22)=4.64; p=0.042]. C, D) No significant differences in IL-6 expression in WT mice or TG mice. E, F) No significant differences in CD45 expression in WT mice or TG mice.

Blood-Brain Barrier mRNA in the Hippocampus

WT mice had no significant change in CCL2 mRNA expression, however there was a trend of an increase in expression in HFHF diet compared to CD [$F(1,21)=3.62$; $p=0.071$]. TG mice had an increase in CCL2 mRNA expression in HFHF diet mice compared to CD mice [$F(1,22)=4.33$; $p=0.049$]. Post hoc analysis showed a significant increase from CD saline-treatment to HFHF saline-treatment ($p=0.03$). There were no significant differences when CD saline-treated mice were compared to CD Xpro-treated $p>0.99$, or compared to HFHF Xpro-treated $p=0.99$. Post hoc analysis was significant when CD Xpro-treated mice were compared to HFHF saline-treated mice $p=0.04$, and not significant when compared to HFHF Xpro-treated $p=0.99$. There were no significant differences when HFHF saline-treated mice were compared to HFHF Xpro-treated mice $p=0.14$. There were no significant changes in CLDN2 mRNA expression in either WT or TG mice regardless of diet or Xpro-treatment. WT mice showed an interaction of diet and drug treatment in ZO-1 mRNA expression [$F(1,22)=12.34$; $p=0.002$]. Post hoc analysis showed CD Xpro-treated mice had a significant decrease in ZO-1 mRNA expression as compared with CD saline-treated mice, HFHF saline-treated mice, and HFHF Xpro-treated mice ($p=0.008$, $p=0.0001$, $p<0.0001$, respectively). There were no significant differences between CD saline-treated mice compared to HFHF saline-treated mice ($p=0.08$). HFHF Xpro-treated mice had a significant decrease in ZO-1 mRNA expression compared to CD saline-treated mice ($p=0.007$). There was no significant difference between HFHF saline-treated

mice and HFHF Xpro-treated mice ($p=0.55$). TG mice showed no significant differences in ZO-1 mRNA expression due to diet or drug treatment as referenced in Figure 2.

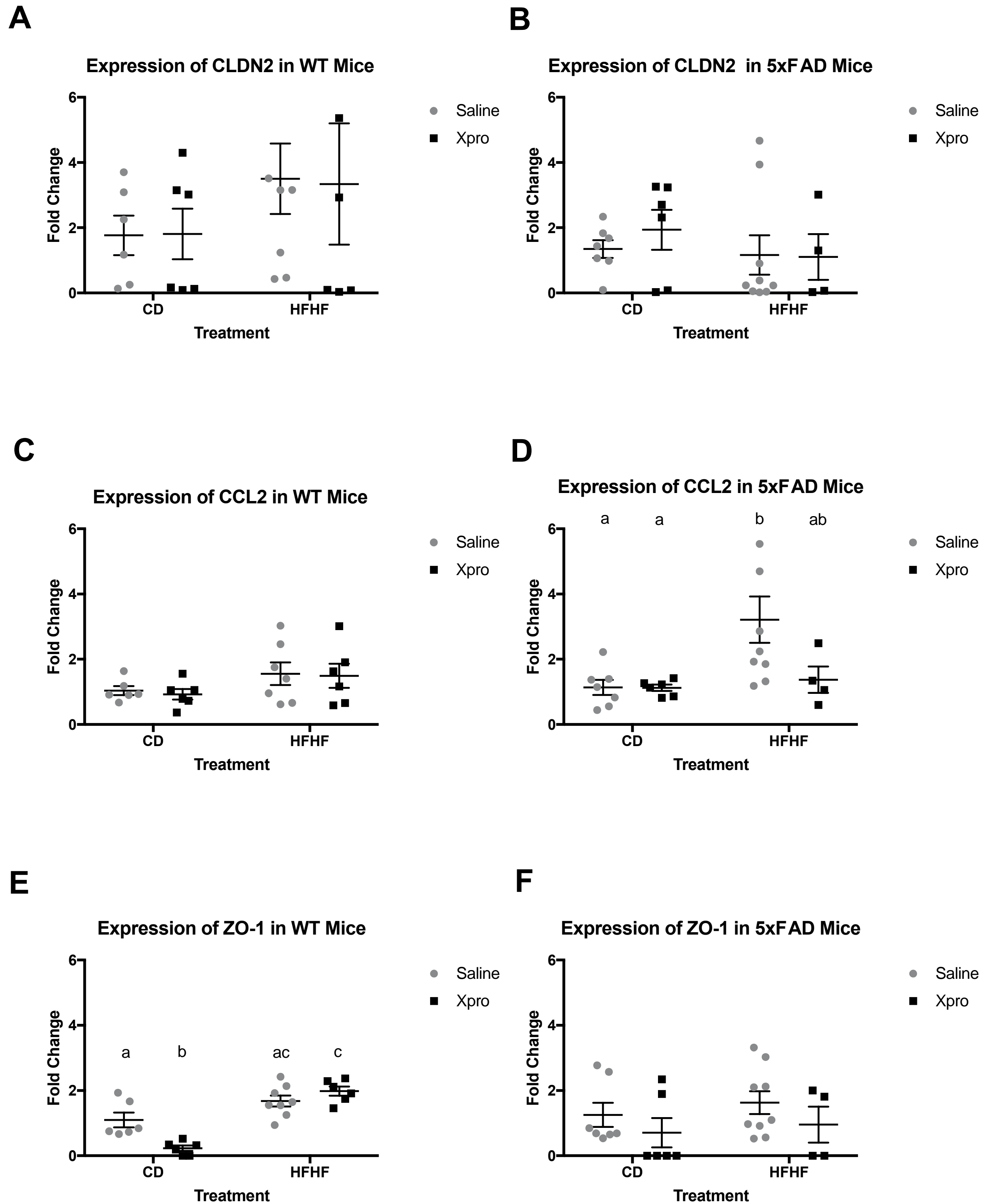


Fig. 2. A, B) Diet, genotype, and Xpro® 1595 administration have no significant effects on CLDN2 mRNA expression C) Diet and Xpro® 1595 administration have no significant effects

on CCL2 mRNA expression in WT mice. D) TG mice had a significant increase in CCL2 mRNA expression in HFHF diet mice compared to CD [$F(1,22)=4.3$; $p=0.049$]. Post hoc analysis showed a significant increase in CCL2 in HFHF saline-treated mice compared to CD saline-treated mice $p=0.03$ and CD Xpro-treated mice $p=0.04$. E) ZO-1 mRNA expression main diet effect and interaction in WT mice [$F(1,22)=48.9$; $p<0.0001$]; and [$F(1,22)=12.34$; $p=0.002$], respectively. Post hoc analysis showed CD saline-treated mice compared to CD Xpro-treated mice ($p=0.008$), compared to HFHF saline-treated ($p=0.08$), and compared to HFHF Xpro-treated mice ($p=0.007$). CD Xpro-treated mice compared to HFHF saline-treated mice ($p=<0.0001$) and HFHF Xpro-treated mice ($p<0.0001$). F) ZO-1 mRNA expression of TG mice is not significant due to diet or Xpro-treatment.

Correlation Analysis of Weight Changes

A 2-way ANOVA comparing the average weight of each group overtime for WT mice and a second 2-way ANOVA comparing TG mice was performed. WT mice showed an interaction of time and diet and treatment [$F(24,184)=3.455$; $p<0.0001$], a main time effect [$F(8,184)=51.36$; $p<0.0001$], no main diet-treatment effect [$F(3,23)=2.5$; $p=0.085$], and matching of subjects [$F(23,184)=30.92$; $p<0.0001$]. Post hoc analysis showed no significant differences. TG mice showed an interaction of time and diet and treatment [$F(24,160)=6.84$; $p<0.0001$], a main time effect [$F(8,160)=60$; $p<0.0001$], a main diet-treatment effect [$F(3,20)=5.77$; $p=0.005$], and matching of subjects [$F(20,160)=34.06$; $p<0.0001$]. Post hoc analysis showed a significant increase in weight in HF saline and HF Xpro mice compared to CD saline mice ($p=0.041$, $p=0.023$; respectively). There were no significant differences between CD saline compared to CD Xpro ($p=0.99$) or HF saline compared to HF Xpro ($p=0.74$). There were no significant

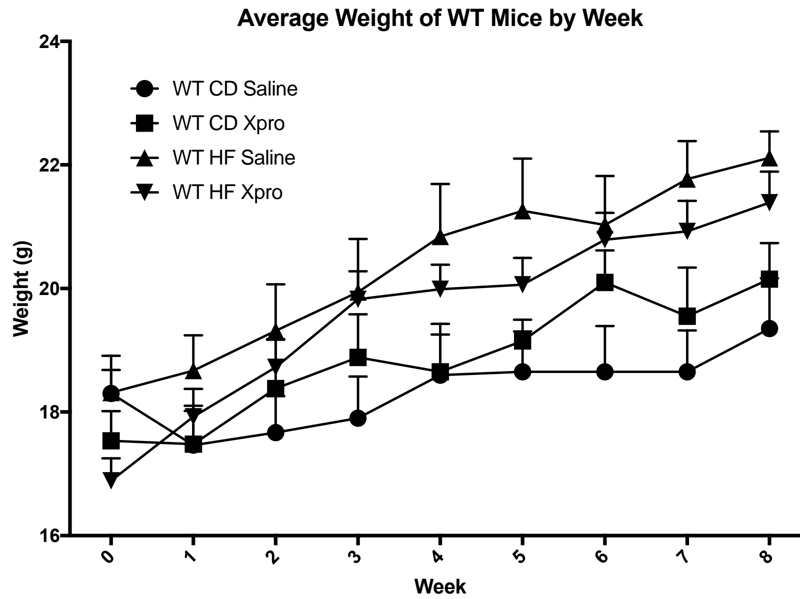
differences between CD Xpro compared to HF saline ($p=0.068$). There was a significant increase in average weight in HF Xpro mice compared to CD Xpro mice ($p=0.033$) as referenced in Figure 3.

WT mice: There were no significant correlations between weight gain and mRNA levels of hippocampal TNF mRNA in CD saline-treatment group [$p=0.24$, $R^2=0.32$], CD Xpro-treatment group [$p=0.84$, $R^2=0.012$], HFHF saline-treatment group [$p=0.21$, $R^2=0.25$], and HFHF Xpro-treatment group [$p=0.26$, $R^2=0.302$] or for CLDN2 mRNA in CD saline-treatment group [$p=0.35$, $R^2=0.22$], CD Xpro-treatment group [$p=0.74$, $R^2=0.029$], HFHF saline-treatment group [$p=0.63$, $R^2=0.041$], and HFHF Xpro-treatment group [$p=0.26$, $R^2=0.39$]. CCL2 mRNA did not correlate with weight gain in CD saline-treatment group [$p=0.76$, $R^2=0.025$], CD Xpro-treatment group [$p=0.82$, $R^2=0.014$], HFHF saline-treatment group [$p=0.33$, $R^2=0.19$], and HFHF Xpro-treatment group [$p=0.14$, $R^2=0.46$]. IL-6 mRNA did not correlate with weight gain CD saline-treatment group [$p=0.46$, $R^2=0.14$], CD Xpro-treatment group [$p=0.76$, $R^2=0.025$], HFHF saline-treatment group [$p=0.28$, $R^2=0.19$], and HFHF Xpro-treatment group [$p=0.19$, $R^2=0.38$]. CD45 mRNA did not correlate with weight gain CD saline-treatment group [$p=0.77$, $R^2=0.02$], CD Xpro-treatment group [$p=0.36$, $R^2=0.21$], HFHF saline-treatment group [$p=0.39$, $R^2=0.12$], and HFHF Xpro-treatment group [$p=0.95$, $R^2=0.001$]. ZO-1 mRNA did not correlate with weight gain CD saline-treatment group [$p=0.25$, $R^2=0.31$], CD Xpro-treatment group [$p=0.83$, $R^2=0.012$], HFHF saline-treatment group [$p=0.25$, $R^2=0.21$], and HFHF Xpro-treatment group [$p=0.82$, $R^2=0.01$] as referenced in Figure 4.

TG mice: Hippocampal TNF mRNA did not correlate with weight gain in CD saline-treatment group [$p=0.33$, $R^2=0.187$], CD Xpro-treatment group [$p=0.37$, $R^2=0.201$], HFHF saline-treatment group [$p=0.68$, $R^2=0.026$], and HFHF Xpro-treatment group [$p=0.88$,

R^2=0.013]. CLDN2 mRNA did not correlate with weight gain in CD saline-treatment group [p=0.25, R^2=0.249], CD Xpro-treatment group [p=0.54, R^2=0.102], HFHF saline-treatment group [p=0.39, R^2=0.106], and HFHF Xpro-treatment group [p=0.169, R^2=0.691]. Hippocampal CCL2 mRNA did not correlate with weight gain in CD saline-treatment group [p=0.42, R^2=0.17], CD Xpro-treatment group [p=0.42, R^2=0.168], HFHF saline-treatment group [p=0.89, R^2=0.004], and HFHF Xpro-treatment group [p=0.92, R^2=0.003]. Hippocampal IL-6 mRNA did not correlate with weight gain in CD saline-treatment group [p=0.11, R^2=0.42], CD Xpro-treatment group [p=0.81, R^2=0.017], HFHF saline-treatment group [p=0.25, R^2=0.18], and HFHF Xpro-treatment group [p=0.72, R^2=0.081]. For hippocampal CD45 mRNA, CD saline-treatment group [p=0.2, R^2=0.3], CD Xpro-treatment group [p=0.64, R^2=0.06], HFHF saline-treatment group [p=0.08, R^2=0.37], and HFHF Xpro-treatment group [p=0.88, R^2=0.014]. Hippocampal ZO-1 mRNA did not correlate with weight gain in CD saline-treatment group [p=052, R^2=0.09], CD Xpro-treatment group [p=0.87, R^2=0.007], HFHF saline-treatment group [p=0.26, R^2=0.18], and HFHF Xpro-treatment group [p=0.73, R^2=0.07] as referenced in Figure 5.

A



B

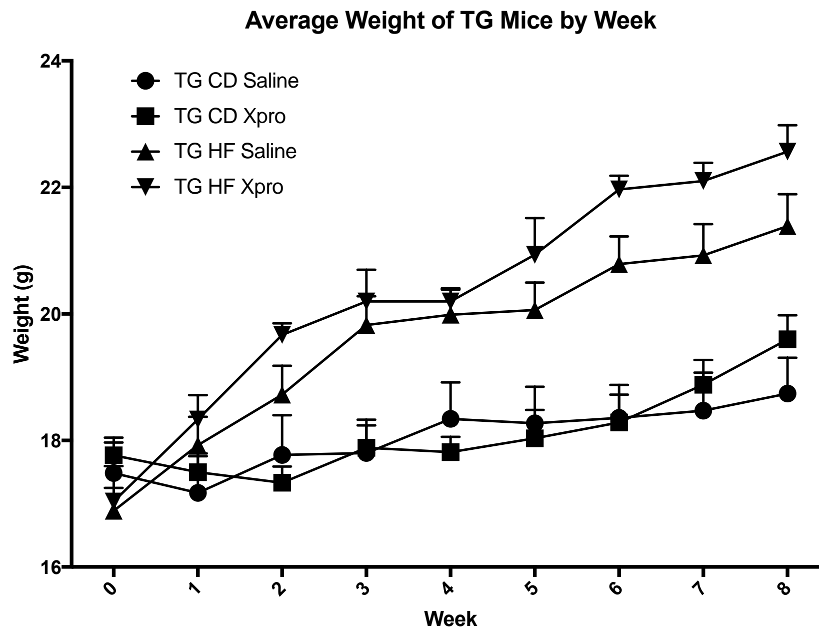


Fig. 3. A) Average weight of the WT groups increases over time, however there are no significant differences in post hoc analysis. B) There is an signficiant increase in average weight gain over time in TG HF saline mice compared to TG CD saline mice (p=0.041) and in TG HF Xpro mice compared to TG CD Xpro mice (p=0.033).

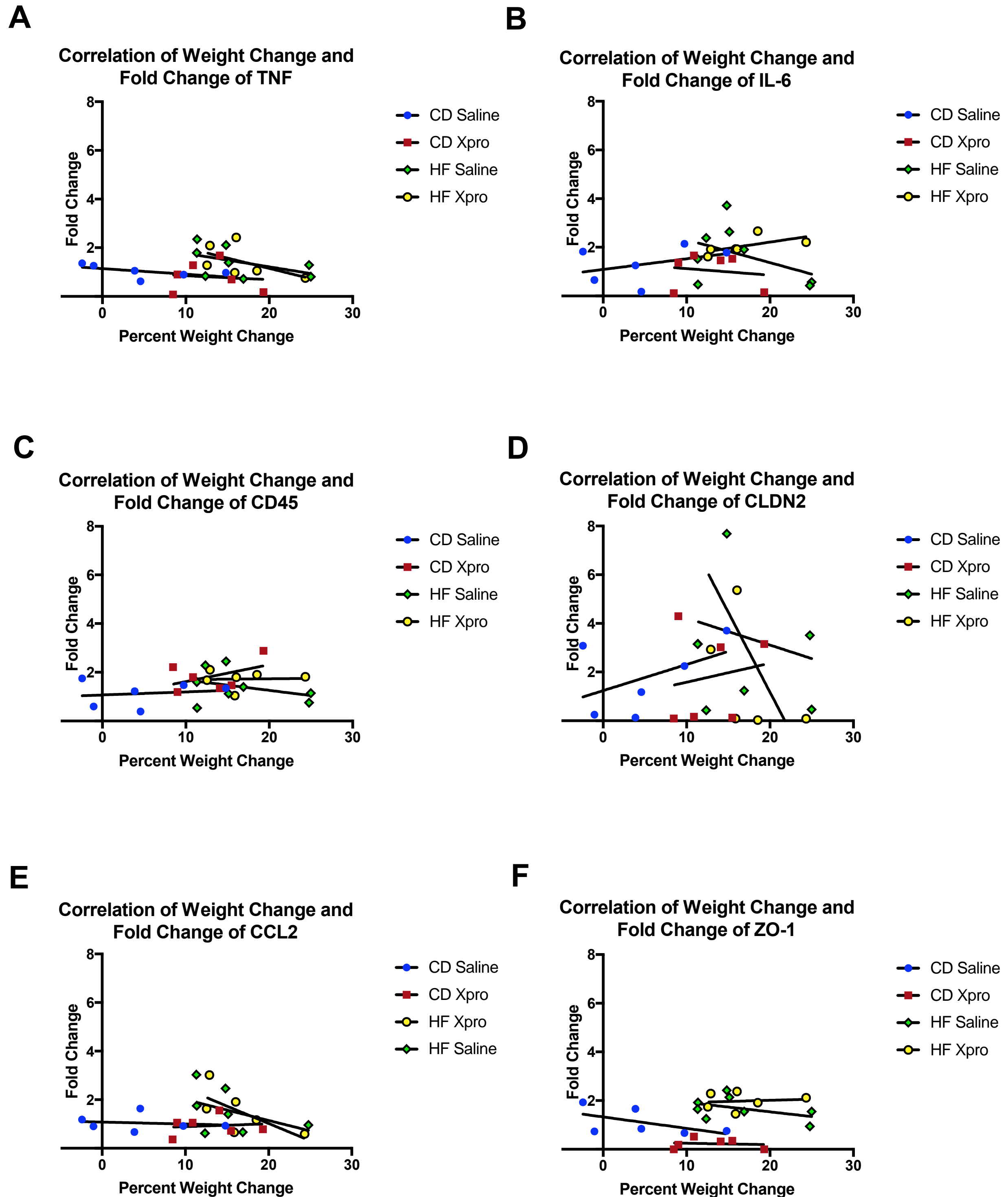


Fig. 4. A, B, C, D, E, F) There were no significant correlations between percent weight change and fold change of hippocampal mRNA in WT mice regardless of genotype or diet.

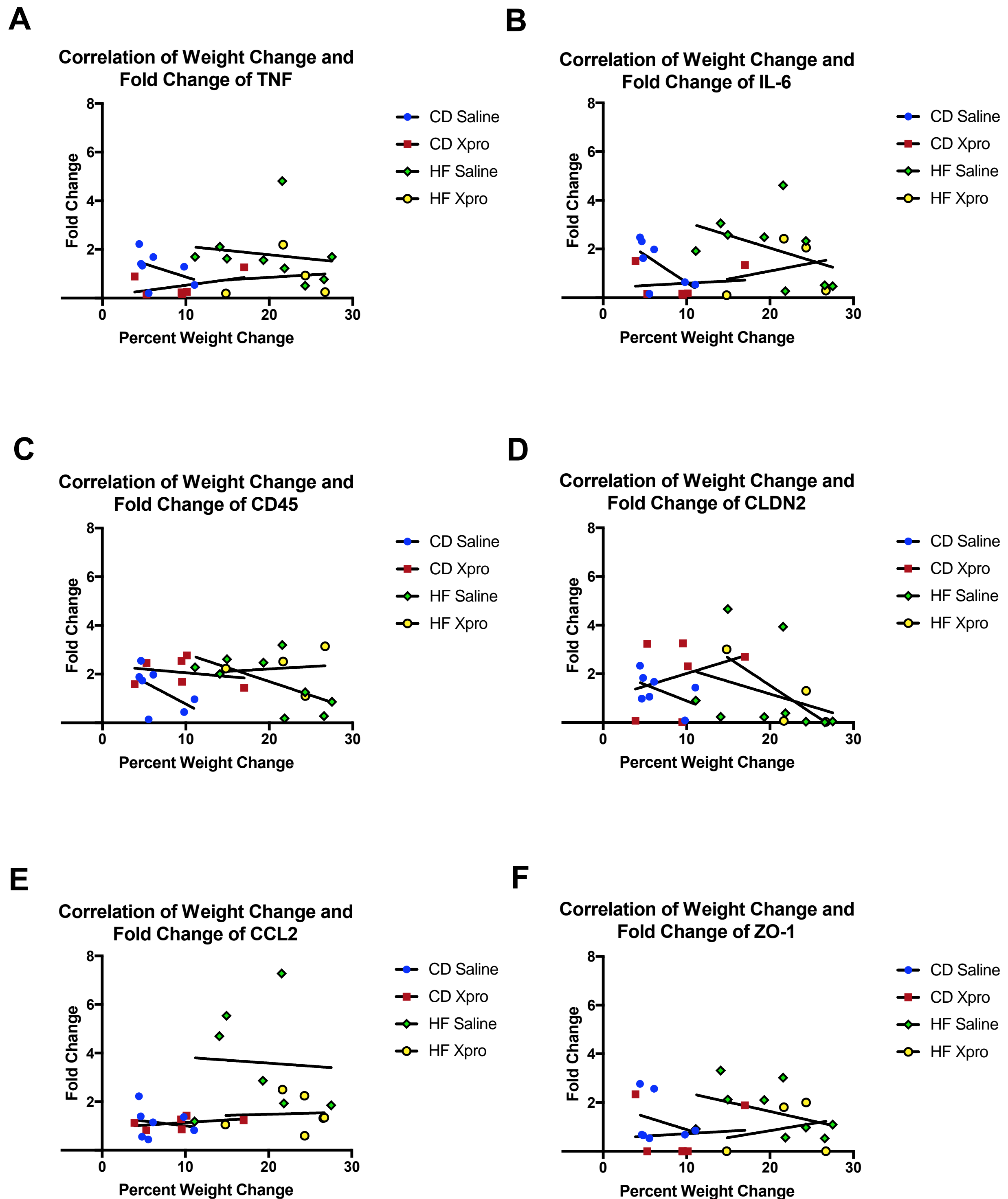


Fig. 5. A, B, C, D, E, F) There were no significant correlations between percent weight change and fold change of hippocampal mRNA in TG mice regardless of genotype or diet.

ZO-1 Hippocampal Protein Expression and Correlation Analysis with Percent Weight Change

ZO-1 protein expression was not significant due to diet or drug treatment effects in either WT [$F(1,15)=0.72$, $p=0.41$; $F(1,15)=0.002$, $p=0.97$, respectively] or TG mice [$F(1,11)=0.056$, $p=0.82$; $F(1,11)=1.78$, $p=0.21$, respectively]. Correlation analysis in WT mice showed that protein expression was not significantly correlated with weight change in CD saline-treated mice [$p=0.79$, $R^2=0.103$], CD Xpro-treated mice [$p=0.75$, $R^2=0.06$], or HFHF saline-treated mice [$p=0.132$, $R^2=0.47$]. An increase in weight was significantly correlated with an increase in ZO-1 protein expression in HFHF Xpro-treated mice [$p=0.025$, $R^2=0.85$]. Correlation analysis in TG mice showed that protein expression was not significantly correlated with weight change in CD saline-treatment group [$p=0.898$, $R^2=0.025$], CD Xpro-treatment group [$p=0.87$, $R^2=0.007$], or HFHF saline-treatment group [$p=0.44$, $R^2=0.31$]. No correlation was performed for HFHF Xpro-treatment group because n=2 as referenced in Figure 6.

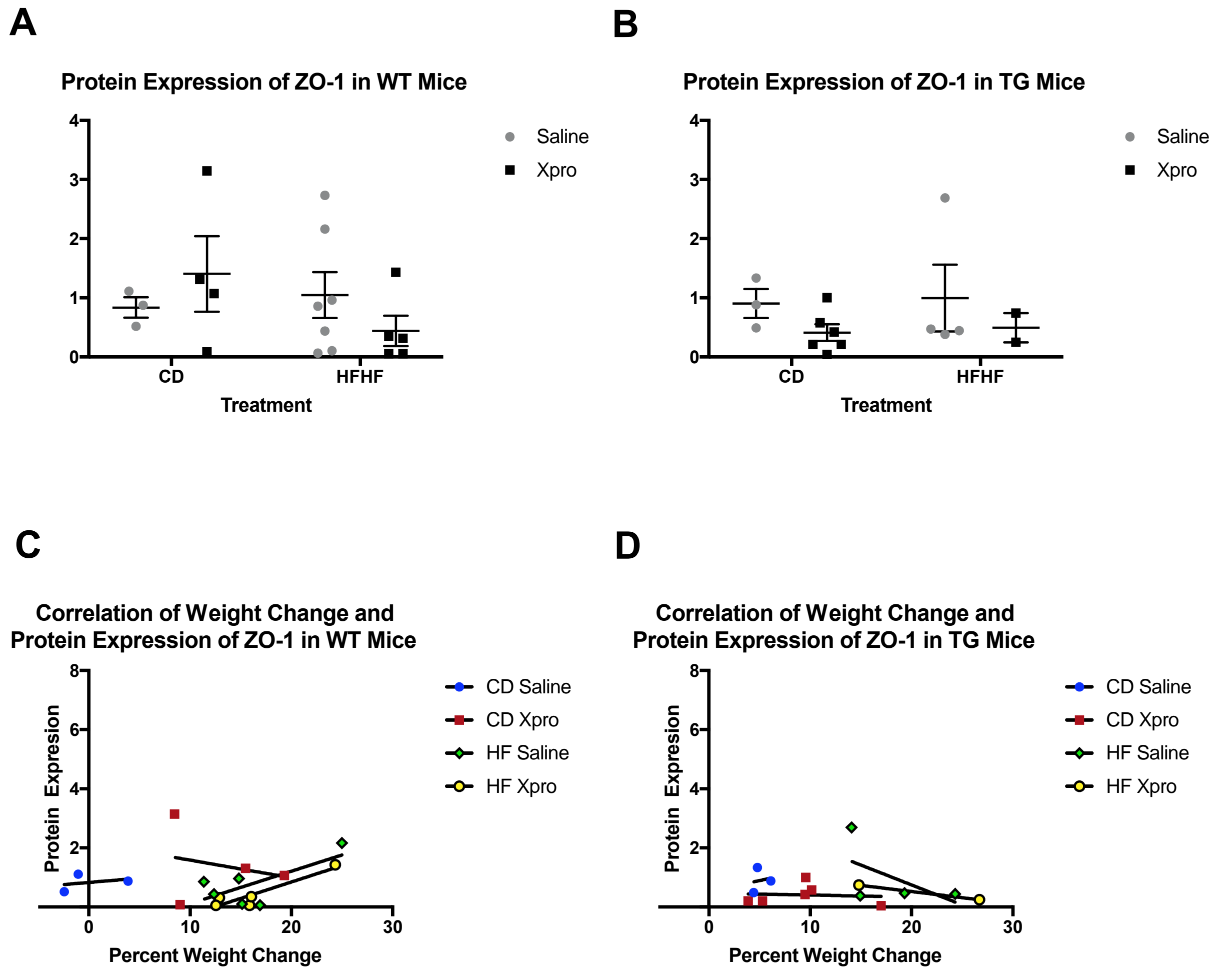


Fig. 6. A, B) There were no significant diet or drug treatment main effects in ZO-1 protein expression in either WT or TG mice. C) There was a significant correlation between weight change and ZO-1 protein expression in HFHF Xpro-treated WT mice [$F(1,3)=17.16$, $p=0.025$, $R^2=0.85$]. D) ZO-1 protein expression in TG mice was not correlated with percent weight change regardless of drug treatment or diet.

Discussion

High-fat high-fructose (HFHF) diet causes an increase in chronic low-level inflammation, which may increase BBB permeability, and increase neuroinflammatory processes (Varatharaj, 2016) that contribute to neurodegenerative diseases including Alzheimer's Disease (AD). TNF is a cytokine that signals for increased inflammation in the periphery and the brain, and Xpro® 1595 is a soluble TNF signaling inhibitor, which reduces inflammation (MacPherson, 2017). We found a significant increase in TNF mRNA expression in WT mice that were fed a HFHF diet. This is aligned with previous literature that TNF is increased in the brain when there is an increase in peripheral stress and metabolic disease (Mu, 2017), and supports our hypothesis that chronic metabolic challenge increases neuroinflammation. Xpro® 1595 had no significant effects on WT mice, but, despite no increase in TNF in mice fed HFHF diet, XPro®1595 reduced expression of hippocampal TNF mRNA in TG mice fed either CD or HFHF diet. Increasing concentration or frequency of the dose may reduce TNF more effectively in WT mice.

TNF is an activator of CCL2 release, and CCL2 recruits macrophages to a site of inflammation (Hasan, 2005). CCL2 is associated with increased trafficking of peripheral macrophages into the brain due to BBB damage (Khoury, 2007). TG mice demonstrated significant increases in CCL2 mRNA expression due to HFHF diet. Acute inflammation increased CCL2 expression, which causes increased infiltration of CCR2 macrophages in the hippocampus (Howe, 2017), indicating that in TG mice, there may be increased trafficking of macrophages into the brain due to HFHF diet-induced chronic inflammation. Xpro® 1595 administration seems to decrease CCL2 mRNA in HFHF diet-fed mice; however, the decrease is not significant ($p=0.14$). With significance, this would also complement the TNF mRNA results. We expected an increase in CCL2 mRNA expression due to diet because there was an increase in

TNF expression due to diet in WT mice and CCL2 and TNF levels correlate according to the literature (Hasan, 2015). Our results indicated no significant effects of CCL2 mRNA expression in the WT mice, however, with increased N's there may be a significant increase in CCL2 expression as our p value was close to 0.05 ($p=0.071$).

ZO-1 or tight junction protein 1 (TJP-1) is a scaffolding protein that links tight junction fibril proteins such as claudins and occludins to the cell cytoskeleton (Itoh, 1997). Previous literature demonstrates that ZO-1 expression decreases when there is an increase in BBB permeability (Dimitrijevic, 2006). We predicted a decrease in ZO-1 expression in HFHF diet mice compared to CD mice due to the increase in TNF inflammation from metabolic disease. We expected an increase in ZO-1 expression in Xpro-treated mice compared to saline-treated mice, as inhibiting TNF decreases inflammation. Our results indicated an interaction between diet and drug treatment in WT mice. When WT mice fed CD were administered Xpro® 1595 they had a significant decrease in ZO-1 expression as compared with WT mice fed a CD and given saline. These results are contrary to our predictions that XPro®1595 would increase ZO-1 expression to restore BBB function.

While excessive TNF decreases ZO-1, little is known about the constitutive regulation of the BBB by TNF (Rochfort, 2015). As TNF can be beneficial to normal neuron and glia functioning (Tanaka 2000, Hattori et al. 1993), and regulate junctional protein turnover (Rochfort, 2015), inhibition of TNF in the absence of chronic inflammation may disrupt normal TNF-mediated tight junction protein formation. WT mice did not have a significant change in ZO-1 mRNA expression when fed a HFHF diet and given saline treatment when compared to CD saline-treatment ($p=0.08$). While these results do not support our hypothesis that HFHF-diet induce inflammation would disrupt the BBB, we may need to investigate other BBB proteins.

One study found that diabetic rats had no change in hippocampal ZO-1 expression compared to control rats, but reductions in occludins and claudin-5, which were attributed to the increased BBB permeability, were noted (Yoo, 2016). Our results may indicate that inflammation caused by HFHF does not increase BBB permeability through ZO-1 expression.

TG mice had no significant changes in ZO-1 mRNA expression due to diet or drug treatment which may be due to the variability of the data. Additionally, ZO-1 mRNA expression may be altered due to the 5xFAD genotype and may be more resistant to changes due to diet and drug treatment. Our TG mice results do not support our hypothesis that genetic risk for AD and Western Diet compound to disrupt the BBB. Further investigation of other BBB proteins is warranted. CLDN2 is a pore forming BBB protein that is linked to ZO-1 and demonstrates an increase in expression when there is increased BBB permeability (Overgaard, 2011). Our results showed no significant differences in CLDN2 expression. This may mean TNF mediated inflammation does not affect CLDN2 mRNA expression. Additionally, there are other claudins that respond to inflammation such as CLDN5 which decrease due to inflammation (Gunzel and Yu, 2013) which may respond to TNF mediated inflammation.

Hippocampal IL-6 and CD45 mRNA expression did not significantly increase due to the chronic inflammation of a HFHF diet, and Xpro administration did not have an effect in either WT or TG mice. We predicted an increase in IL-6 expression, as it was previously found that TNF induces IL-6 signaling in astrocytes (Benveniste, 1990). CD45 is also a marker of inflammation and was expected to increase due to HFHF diet (Jeong, 2013). CD45 is expressed on peripheral macrophages and we expected an increase in infiltration due to HFHF diet weakening the BBB (MacPherson, 2017). Additionally, previous studies have indicated that mice with metabolic syndrome have a decrease in memory tasks associated with increases in IL-6 and

TNF expression in the hippocampus (Dinel, 2011). However, our results did not support the previous literature, which may be due to the variability of mRNA expression we found. A proposed factor for the variability found in some of the inflammatory markers mRNA expression was the variability in mouse weight change. Some mice gained less weight than others within the same group. However, we found no significant correlations between percent weight change and mRNA expression.

Due to the interaction found in the ZO-1 mRNA expression results, a western blot was done to clarify these results by looking at protein expression. Post-transcriptional changes can sometimes occur to the mRNA and alter the resulting protein expression. Our findings indicated that the increased inflammation from HFHF diet did not significantly alter ZO-1 protein expression in WT or TG mice. Additionally, Xpro® 1595 administration had no significant effects on WT or TG hippocampal ZO-1 protein. However, the number of mice analyzed on the western blot was reduced compared to qPCR because many mice had low protein concentration. This is partly because we analyzed the hippocampus, which is a small part of the brain that yields low levels of protein. Additionally, there may be differences in mRNA and protein expression in different regions of the hippocampus. Previous studies show more BBB breakdown in the dentate gyrus and cornu ammonius 1 (CA1) regions of the hippocampus (Montagne, 2015). These regional effects would have been lost in our studies as the entire hippocampus was processed for protein levels.

We also performed correlation analyses on ZO-1 protein expression and percent weight change. Our findings showed a significant correlation in HFHF diet WT mice administered Xpro® 1595. Mice that gained more weight had increased protein expression of ZO-1. This may mean that with more samples, there may be a significant increase in ZO-1 protein expression due

to increased inflammation in the HFHF diet WT mice. This supports our ZO-1 mRNA expression results that there is an increase in ZO-1 expression due to HFHF diet and Xpro-treatment. This may mean that the diet and drug treatment interaction is still present in WT mice, and HFHF diet increases ZO-1 expression only when administered Xpro® 1595.

In the future, we would like to assess TNF, ZO-1, CLDN2, and CCL2 mRNA expression and protein levels in the hypothalamus and cortex of the brain. The hypothalamus is involved with the regulation of hunger and insulin secretion and may an important factor in the cause of obesity and diabetes (Osundiji, 2012). Additionally, we would look at protein expression in these areas to determine correlation with mRNA levels. We may also perform immunohistochemistry to gain a better understanding of how the BBB is disrupted due to a HFHF diet and how Xpro® 1595 may affect it. Furthermore, we can at the effects of HFHF diet over longer period in 5xFAD mice to learn about the effects of HFHF diet on amyloid plaque formation and Alzheimer's pathology.

In conclusion, our study demonstrates that HFHF diet increases hippocampal inflammation as measured by TNF mRNA in WT mice, XPro reduces TNF mRNA levels in the hippocampus of TG mice, and 5xFAD mice fed a HFHF diet have increased CCL2 mRNA in the hippocampus that is ameliorated by blockade of solTNF signaling with XPro®1595. Work is ongoing to increase the number of animals in each group and further elucidate the combinatorial effect of genetic susceptibility for AD and metabolic challenge.

References

Alzheimer’s Association. (2016). 2016 Alzheimer’s disease facts and figures. Retrieved from http://www.alz.org/facts/

Barron, A. M., Rosario, E. R., Elteriefti, R., & Pike, C. J. (2013). Sex-specific effects of high fat diet on indices of metabolic syndrome in 3xTg-AD mice: implications for Alzheimer's disease. *PLoS One,* 8(10): e78554.

Benveniste, E. N., Sparacio, S. M., Norris, J. G., Grenett, H. E., & Fuller, G. M. (1990). Induction and regulation of interleukin-6 gene expression in rat astrocytes. *Journal of Neuroimmunology,* 30(2): 201-212.

De Sousa Rodrigues, M. E., Bekhbat, M., Houser, M., Chang, J., Walker, D., Jones, D. P., Oller do Nascimento, C., Barnum, C. J. & Tansey, M. (2016). Chronic psychological stress and high-fat high-fructose diet disrupt metabolic and inflammatory gene networks in the brain, liver, and gut and promote behavioral deficits in mice. *Brain, Behavior, and Immunity,* 59: 158-172.

Dimitrijevic, O. B., Stamatovic, S. M., Keep, R. F., &Andjelkovic, A. V. (2006). Effects of the chemokine CCL2 on blood-brain barrier permeability during ischemia-reperfusion injury. *J Cereb Blood Flow Metab,* 26(6): 797-810.

Dinel, A. L., André, C., Aubert, A., Ferreira, G., Layé, S., & Castanon, N. (2011). Cognitive and emotional alterations are related to hippocampal inflammation in a mouse model of metabolic syndrome. *PloS One,* 6(9): e24325.

Dinkins, M. B., Dasgupta, S., Wang, G., Zhu, G., He, Q., Kong, J. N. & Bieberich, E. (2015).

The 5XFAD mouse model of Alzheimer's disease exhibits an age-dependent increase in anti-ceramide IgG and exogenous administration of ceramide further increases anti-ceramide titers and amyloid plaque burden. *J Alzheimers Dis,* 46(1): 55-61.

Gunzel, D. & Yu, A. S. L. (2013). Claudins and the modulation of tight junction permeability. *Physiol Rev,* 93(2): 525-569.

Hattori, A., Tanaka, E., Murase, K., Ishida, N., Chatani, Y., Tsujimoto, M., Hayashi, K., Kohno, M. (1993). Tumor necrosis factor stimulates the synthesis and secretion of biologically active nerve growth factor in non-neuronal cells. *J Biol Chem* 268: 2577-2582.

Howe, C. L., LaFrance-Corey, R. G., Goddery, E. N., Johnson, R. K., & Mirchia, K. (2017). Neuronal CCL2 expression drives inflammatory monocyte infiltration into the brain during acute virus infection. *J Neuroinflammation,* 14(1): 238.

Jeong, H. K., Ji, K., Min, K., & Joe, E. H. (2013). Brain inflammation and microglia: facts and misconceptions. *Exp Neurobiol,* 22(2): 59-67.

Kelly, S. D., Harrell, S. D., & Neigh, G. N. (2015). Chronic stress modulates regional cerebral glucose transporter expression in an age-specific and sexually-dimorphic manner. *Physiol Behav,* 126: 39-49.

Khoury, J. E., Toft, M., Hickman, S. E., Means, T. K., Terada, K., Geula, G., & Luster, A. D. (2007). Ccr2 deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease. *Nature Medicine*. 13, 432-438.

Itoh, M., Nagafuchi, A., Moroi, S., Tsukita, S. (1997). Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments. *Journal of Cell Biology*. 138(1): 181–92.

Lee, J. K., Chung, J., Druey, K. M., & Tansey, M. (2012). RGS10 exerts a neuroprotective role

through the PKA/c-AMP response-element (CREB) pathway in dopaminergic neuron-like cells. *Journal of Neurochemistry*, 122(2): 333-343.

Martorana, A., Esposito, Z., & Koch, G. (2010). Beyond the cholinergic hypothesis: do current drugs work in Alzheimer's disease?. *CNS Neuroscience & Therapeutics,* 16(4): 235–245.

MacPherson, K. P., Sompol, P., Kannarkat, G. T., Chang, J., Sniffen, L., Wildner, M. E., Norris, C. M., Tansey, M. G. (2017). Peripheral administration of the soluble TNF inhibitor XPro1595 modifies brain immune cell profiles, decreases beta-amyloid plaque load, and rescues impaired long-term potentiation in 5xFAD mice. *Neurobiol Dis,* 102: 81-95.

McCoy M. & Tansey M. (2008). TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. *Journal of Neuroinflammation,* 5: 45.

Montagne, A., Barnes, S. R., Sweeney, M. D., Halliday, M. R., Sagare, A. P., Zhao, Z., Toga, A. W., Jacobs, R. E., Liu, C. Y., Amezcua, L., Harrington, M. G., Chui, H. C., Law, M., & Zlokovic, B. V. (2015). Blood-brain barrier breakdown in the aging human hippocampus. *Neuron,* 85(2): 296-302.

Mu, Z. P., Wang, Y. G., Li, C. Q., Lv, W. S., Wang, B., Jing, Z. H., Song, X. J., Lun, Y., Qiu, M. Y., & Ma, X. L. (2017). Association between tumor necrosis factor-alpha and diabetic peripheral neuropathy in patients with type 2 diabetes: a meta-analysis. *Molecular Neurobiology,* 54: 983-996.

Oakley, H., Cole, S. L., Logan, S., Maus, E., Shao, P., Craft, J., Guillozet-Bongaarts, A., Ohno, M., Disterhoft, J., Van Eldik, L., Berry, R., & Vassar, R. (2006). Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *J Neurosci*. 26(40): 10129-10140.

Overgaard, C. E., Daugherty, B. L., Mitchell, L. A., & Koval, M. (2011). Claudins: control of barrier function and regulation in response to oxidant stress. *Antioxid Redox Signal,* 15(5): 1179-1193.

Pardridge, W. M. (2005). The blood-brain barrier: bottleneck in brain drug development. *NeuroRx,* 2(1): 3-14.

Osundiji, M. A., Lam, D. D., Shaw, J., Yueh, C. Y., Markkula, S. P., Hurst, P., Colliva, C., Roda, A., Heisler, L. K., & Evans, M. L. (2012). Brain glucose sensors play a significant role in the regulation of pancreatic glucose-stimulated insulin secretion. *Diabetes,* 61(2): 321-328.

Qiagen. (2018). PCR protocols and applications. Retrieved from https://www.qiagen.com/au/resources/molecular-biology-methods/pcr/

Rochfort, K. D. & Cummins, P. M. (2015). The blood-brain barrier endothelium: a target for pro-inflammatory cytokines. *Biochem Soc Trans,* 43(4): 702-706.

Selkoe, D. J. (2013). The therapeutics of Alzheimer's disease: where we stand and where we are heading. *Annals of Neurology, 74(3)*. 328-336.

Sigma-Aldrich. (2018). Universal SYBR green quantitative pcr protocol. Retrieved from http://www.sigmaaldrich.com/technical-documents/protocols/biology/sybr-green-qpcr.html

Simsek, S., Yüksel, T., Kaplan, I., Uysal, C., & Aktas, H. (2016). The levels of cortisol and oxidative stress and DNA damage in child and adolescent victims of sexual abuse with or without post-traumatic stress disorder. *Psychiatry Investig,* 13(6): 616-621.

Stamatovic, S. M., Keep, R. F., & Andjelkovic, A.V. (2008). Brain endothelial cell-cell junctions: how to 'open' the blood brain barrier. *Current*

Neuropharmacology, 6(3): 179–92.

Stamatovic, S. M., Keep, R. F., Kunkel, S. L., & Andjelkovic, A. V. (2003). Potential role of MCP-1 in endothelial cell tight junction 'opening': signaling via Rho and Rho kinase. *J Cell Sci,* 116: 4615–4628.

Steed, P. M., Tansey, M. G., Zalevsky, J., Zhukovsky, E. A., Desjarlais, J. R., Szymkowski, D. E., Abbott, C., Carmichael, D., Chan, C., Cherry, L., Cheung, P., Chirino, A.J., Chung, H. H., Doberstein, S. K., Eivazi, A., Filikov, A. V., Gao, S. X., Hubert, R. S., Hwang, M., Hyun, L., Kashi, S., Kim, A., Kim, E., Kung, J., Martinez, S. P., Muchhal, U. S., Nguyen, D. H., O'Brien, C., O'Keefe, D., Singer, K., Vafa, O., Vielmetter, J., Yoder, S. C., & Dahiyat, B. I. (2003). Inactivation of TNF signaling by rationally designed dominant-negative TNF variants. *Science,* 301(5641): 1895-1898.

Surmi, B. K. and Hasty, A. H. (2008). Macrophage infiltration into adipose tissue. *Future Lipidol,* 3(5): 545-556.

Tajadini, M., Panjehpour, M., & Javanmard, S. H. (2014). Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. *Adv Biomed Res,* 3: 85.

Tanaka, M., Ito, S., & Kiuchi, K. (2000). Novel alternative promoters of mouse glial cell line-derived neurotrophic factor gene. *Biochem Biophys Acta,* 1494: 63-74.

Varatharaj, A. & Galea, I. (2016). The blood-brain barrier in systemic inflammation. *Brain, Behavior, and Immunity*, 60: 1-12.

Xu, Q., Park, Y., Huang, X., Hollenbeck, A., Blair, A., Schatzkin, A., & Chen, H. (2011). Diabetes and risk of Parkinson's disease. *Diabetes Care,* 34: 910-915.

Yoo, D. Y., Yim, H. S., Jung, H. Y., Nam, S. M., Kim, J. W., Choi, J. H., Seong, J. K., Yoon, Y.

S., Kim, D. W., & Hwang, I. K. (2016). Chronic type 2 diabetes reduces the integrity of the blood-brain barrier by reducing tight junction proteins in the hippocampus. *J Vet Med Sci,* 78(6): 957-962.