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## Wearing Memory Thin: The Effects of High Fat Diet on Neuroinflammation and Memory

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The average American diet currently exceeds the recommended amount of fat necessary for our bodies to function. It is widely known that consuming too much fat can lead to obesity and disease, but a high fat diet (HFD) can also lead to memory impairment. Previous studies suggest that neuroinflammation may be the mechanism through which a HFD produces this memory impairment. In the present study, it was hypothesized that a HFD would increase the neuroinflammatory molecule, glial fibrillary acidic protein (GFAP) in the hippocampus, a brain region important for memory. Male Sprague-Dawley rats were fed a regular chow diet or a high fat (chow plus lard) diet for 8 weeks and then were tested for hippocampal-dependent spatial memory using the Morris water maze. Western blots were conducted to analyze GFAP levels. The HFD resulted in poor water maze performance; however, it did not increase GFAP levels in the hippocampus. Further studies will analyze the effects of the HFD on other neuroinflammatory markers in the hippocampus, such as interleukin-6 and tumour necrosis factor-alpha.

The average American diet contains too much fat. The upper percentile of total and saturated fats in the diets of U.S. citizens exceeds the highest recommended amount of fats that should be in a person's diet (Elmadfa & Kornsteiner, 2009; U.S. Department of Agriculture and U.S. Department of Health and Human Services, 2010). While too much fat can be detrimental to overall health, researchers have found that high fat diets can also impair memory. In animal studies, rats that were fed a high fat diet performed poorly on various cognitive tests (Greenwood & Winocur, 1990; Valladolid-Acebes et al., 2011). For example, one experiment utilized the Radial Arm Maze (RAM) to analyze memory. The RAM assessed memory by tracking a variety of mistakes, such as reentering a maze arm, which suggested




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memory impairment. Another cognitive test that has been used to assess the memory of animals on a high fat diet is the Morris water maze. One study found that experimental animals on the diet spent greater amounts of time searching for an escape platform than did animals in the control group (Greenwood & Winocur, 1990). Since the rats were motivated by the temperature of the water to escape onto the platform, longer search times suggested memory impairment.

There is evidence that suggests that the connection between diet and memory occurs because the high fat diet causes chronic inflammation in the brain (De Souza et al., 2005; Pistell et al., 2010). High fat diets have been shown to increase several proinflammatory cytokines in the brain of mice (Thirumangalakudi et al., 2008; Pistell et al., 2010). Neuroinflammation can even impair memory by itself. For instance, one experiment administered a series of inflammation-inducing substances in the brains of mice, which resulted in an increase in the inflammatory marker, interleukin-6 (IL-6) and impaired spatial memory (Oitzl et al., 1993). Another study administered similar injections in order to induce neuroinflammation and also found memory impairment (Lee, et al., 2008). Even though neither of these studies incorporated a specific diet, the results showed that increased levels of proinflammatory cytokines impaired cognitive functioning in mice (Lee et al., 2008; Oitzl et al., 1993). Chronic diet-induced neuroinflammation even contributes to the development of neurodegenerative diseases, such as Alzheimer's and Parkinson's (Mattson, 2003).

Taken together, past studies suggest a mechanism that begins with high fat consumption which leads to neuroinflammation and results in memory impairment. The present study focused on testing this specific mechanism between high fat diet and memory impairment. As a first step of this study, rats were fed either a control diet or a HFD. Then levels of the inflammatory marker, glial fibrillary acidic protein




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(GFAP) were measured in the rats' brains. We predicted that GFAP would be significantly increased in the brains of high-fat fed, memory-deficient animals.

The motivation for this particular experiment was a study by Pistell and colleagues (2010). In this study, researchers found that after high fat consumption, the memory of mice was impaired. It was also found that GFAP levels were significantly increased in the cortex. Thus, it was hypothesized that GFAP would also be increased in the hippocampus. The hippocampus was chosen as the focus of the study because it is essential to memory processes and might be the location of the diet-induced memory impairment.

High fat diets impair cognitive functions, especially memory (Pistell, et al. 2010; Valladolid-Acebes, et al. 2011). What scientists have not yet understood is the mechanism by which a high fat diet impairs memory. High fat diets can also produce neuroinflammation, which includes an excess of specialized protein molecules called cytokines. There is some evidence which indicates that cytokines play a role in memory depletion (Tapia-González et al., 2011). Understanding more about the role of neuroinflammation can be important in reducing the risk of neurodegenerative diseases, such as Alzheimer's disease (Luchsinger, Tang, Shea, & Mayeux, 2002).

Winocur and Greenwood (1990) are two experts on the subject of high fat diet and memory. In one of their earlier studies, these two scientists found that a high saturated fat diet significantly impairs many different types of learning and memory. In this particular study, the researchers took one-month old rats and separated them into three diet groups: high saturated fat, high polyunsaturated fat, and standard rat chow. The rats were placed on this diet for three months and underwent multiple types of behavioral memory testing (Greenwood & Winocur, 1990).




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The first test, a radial arm maze (RAM), was used to test spatial memory. During this test, the rat is deposited in the middle of an octagonal maze with eight arms and must find the food at the end of some of the maze's arms. When a rat reenters previously searched arms, this indicates a sign of spatial memory impairment. In this test, lard-fed rats, whose diet was forty percent saturated fatty acids, performed the greatest number of errors, compared with the soybean-oil fed animals. The second test, the Hebb-Williams maze, involved twelve unique maze problems that the rats had to solve in order to reach a goal box. The goal of the Hebb-Williams maze is to not search the same corridor twice and to complete the maze as quickly as possible. Again, the saturated fat, or lard, group performed the worst out of the three diet groups, showing a decline in learning and memory skills. In the final test of memory, variable-interval delayed alternation (VIDA), rats were required to press a lever in order to receive food. The results of this test followed the patterns of the previous tests. The lard-fed group performed in the bottom percentile; the soybean-oil group represented the median percentile; and the chow-fed animals performed in the apical percentile (Greenwood & Winocur, 1990).

Although the lard-fed rats were impaired in all of these tasks, they were least impaired on the RAM, which uses memory that is controlled by the hippocampus. Therefore, Greenwood and Winocur (1990) suggested that perhaps hippocampal function was not affected by a high fat diet, since the RAM is hippocampal dependent. However, the authors provided multiple explanations for this result, including short diet exposure (Greenwood & Winocur, 1990).

Though specific hippocampal impairment was not expressed in this study, it has been found in many other projects. For example, a very recent study found that after only three days of saturated fat intake, spatial memory in mice was impaired (Kanoski & Davidson, 2010). This study supports the fact that a high fat diet can impair memory and emphasizes the fact that one should not only be aware of the

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amount of fat that he consumes, but also of the type of fat that he consumes. In the present research project, the goal was to support the idea that the hippocampus and other areas of the brain are affected equally by an HFD.

Another study by Valladolid-Acebes and colleagues (2011), which also used the radial arm maze, found similar results to Greenwood and Winocur (1990). In the more recent study, the researchers found that rats that were fed a high fat diet made more errors and took more time to find the food pellets. Valladolid-Acebes also found that even though the experimental rats were only on the diet for two months, the high fat diet rats gained a significant amount of weight when compared to a control group. This led the researchers to believe that obesity and memory deficits, especially those that require the use of the hippocampus, may be linked (Valladolid-Acebes et al., 2011).

One experiment by Granholm et al. (2008) fed rats ten percent saturated fat diets for two months and tested their memory using a water version of the RAM. In this behavioral test, rats had to find a hidden underwater platform, similar to what is used in the water maze, at the end of various maze arms. The results of the study showed that animals that were fed high levels of saturated fats made more working memory errors, such as reentering the same arm twice, than control animals fed a low-fat diet (Granholm et al., 2008).

Mielke et al. (2006) conducted a longitudinal study on mice that were fed a forty-five percent fat diet for a year. The researchers used two behavioral tests, the traditional Morris water maze and an operant bar-pressing task, to test memory. In the bar-pressing task, mice were placed in a cage with a lever. When pressed, this lever would release food pellets on the other side of the cage. In order to reach the reward, the mice had to find an alternate route around an obstacle in the middle of the test cage (Mielke et al., 2006). Mielke et al. (2006) found that high fat fed mice were impaired in the operant-




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learning task, because they could not successfully learn the task. However, no impairment was found in the experimental mice when it came to the water maze task, which is dependent on the hippocampus. Researchers explained this phenomenon by mentioning a supposed genetic inheritance for mice to do well on the water maze that was seen in past studies (Mielke et al., 2006). This reason supports the use of the rat model in the present experiment instead of the mouse model.

It is hypothesized that an excess of proinflammatory cytokines in the brain could be the key to the diet-memory relationship (De Souza et al., 2005; Tapia-González et al., 2011). The primary goal of these specialized proteins is to signal to the immune system when repair is needed in the body. The consequence of high fat consumption is that it significantly increases the amount of these proteins, resulting in a dangerously excessive amount that can cause memory impairment (De Souza et al., 2005; Tapia- González et al., 2011).

A study by Pistell and colleagues (2009) examined the multiple steps of the diet-induced cognitive decline. Pistell et al. (2009) implemented three types of diets: a 41% percent fat diet, a 60% fat diet, and a control diet for a total of four months. Using the Western Blot and densitometry processes, the researchers quantified the amount of inflammatory proteins in the cerebral cortices of the mice (Pistell et. al, 2009). They found considerably higher levels of certain cytokines in HFD-fed mice: tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-6 (IL-6), and glial fibrillary acidic protein (GFAP), which are the three biomarkers that will be examined in the present study.

An experiment by Thirumangalakudi et al. (2008) found increases of various proinflammatory molecules after consumption of certain diets. Mice were fed either a high fat or high cholesterol diet for two months. After diet consumption, the brain samples of all of the rats displayed an



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increase in three proinflammatory molecules, including one that was incorporated in my study, GFAP (Thirumangalakudi et al., 2008).

These same biochemical processes were used in the present study to analyze brain samples in rats that were fed a high fat lard diet and compare them to the brain samples of the control diet rats. The main difference between the Pistell study and the present one is that Pistell et al. examined biomarkers in the cerebral cortex, rather than in the hippocampus. It has also been shown that cytokines increase in numerous parts of the brain, such as the hypothalamus. For example, one study fed mice a high fat diet and found an increase of cytokines in the hypothalamus (De Souza et al., 2005). . However, in the present study, it was hypothesized that cytokine levels would significantly increase in the cerebral cortex and in the hippocampus. The cerebral cortex was chosen, since Pistell and colleagues found increased GFAP levels in this area; and, the hippocampus was chosen because it is an important memory region.

One experiment placed mice on one of the three diets: 41% fat, 60% fat, or low fat. After consuming their respective diets for a total of four months, the mice were euthanized, and their brains were extracted. The brain samples of the high fat mice were immunoblotted for three proinflammatory cytokines, including TNF- $\alpha$  and IL-6. Results revealed that all three of the inflammatory markers were increased in the hypothalamus. (De Souza et al., 2005)

Research in this field has already been used to better understand neurodegenerative diseases. One of these diseases, Alzheimer's, involves the breaking down of connections in the brain and can destroy a person's capacity to remember things. One study analyzed the risk of Alzheimer's disease in ninety-eight elderly persons in New York State (Luchsinger et al., 2002). Though many factors were recorded and considered, the researchers found that individuals that consumed the highest amount of calories and saturated fat had a higher risk of developing Alzheimer's disease. Also, individuals that were diagnosed





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with the disease maintained a higher caloric diet (Luchsinger et al., 2002). The researchers' hypothesis that calories affect an individual's risk of developing a neurodegenerative disease is further supported by a literature review by Mattson (2003). Mattson found consistent results that showed more calories consumed lead to higher risks for diseases such as Alzheimer's and Parkinson's. Results also showed a correlation between limiting calories and a lowered risk of neurodegeneration in the hippocampus (Mattson, 2003).

### I. Diet

Twenty-four male Sprague-Dawley rats were used for this experiment. After one week of habituation, 10 rats were placed on a control diet, consisting of Purina rodent chow and tap water; and 14 rats were placed on a high fat "choice" diet (HFD), consisting of Purina rodent chow, tap water, and lard (Armour; Chicago, IL). More rats were placed in the experimental or high fat, group because it was divided into smaller groups during analysis. The rats were fed their respective diets for 8 weeks. All of the animals received fresh food every three days and weekly cage changes. The experimental animals received fresh lard every 2 days.

### II. Western Blot

After euthanization, the hippocampi and cortices were harvested from the control and HFD animals, and the tissue was homogenized lysis buffer using a mortar and pestle. The samples were then centrifuged and the supernatants were aliquoted. A protein assay was conducted in order to determine the concentration of proteins in each sample, and concentration values were obtained using a spectrophotometer. Then the brain samples were diluted in a 1:1 ratio with sample buffer and  $\beta$ -mercaptoethanol and boiled for five minutes to denature the proteins.




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Samples were loaded into a 10% Tris gel for SDS PAGE. An electrophoresis was run in order to separate the proteins by size. The proteins were then transferred from the gel to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked for one hour (5% nonfat milk and tris-buffered saline with Tween-20) before being immunoblotted. The membranes were incubated in a goat-anti-mouse primary antibody specific to GFAP (Cell Signaling; Boston, MA) at a 1:10,000 ratio in 5% nonfat milk blocker overnight at 4° Celsius (Cell Signaling; Boston, MA). The membranes were then blotted in a 1:40,000 ratio secondary goat-anti-mouse antibody (Santa Cruz Biotechnology; Santa Cruz, CA). Afterward, the membranes were incubated in a chemiluminescent HRP substrate (Millipore; Bilenica, MA) and the image was captured using X-Ray film. The films were then analyzed by densitometry using Alpha-Innotech Flourchem Imager (R & D Systems; Minneapolis, MN). The density values (arbitrary units) were analyzed using a Two-Way Mixed-Design ANOVA, which used the control diet, HFD, cerebral cortex, and hippocampus as comparing factors.

The mean density of GFAP protein bands was analyzed in the hippocampus and in the cortex of both diet groups, in order to compare the amount of GFAP expressed in control and HFD brains. The high fat diet did not increase GFAP in the hippocampus. GFAP was also analyzed in the cortex, because a previous study found that a HFD increased GFAP in the cortices of mice (Pistell, 2009); however, there was still a lack of effect.

To analyze those rats that were most affected by the diet, the rats in the HFD group were divided into three tertiles according to the amount of weight gained during the first 5 days of diet consumption. The amount of GFAP in the control group and in the top tertile of the HFD group was compared. Although there appeared to be more of a difference than when the entire HFD group was analyzed, this difference



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still was not significantly different. The hippocampus contained more GFAP than the cortex, and this difference was statistically significant ( $F(1,22)= 75.545, p < .001$ ).

It was hypothesized that a HFD would increase GFAP in the hippocampi of rats. Since the HFD did not produce an excess of GFAP in the hippocampus, the hypothesis for this study was not supported.

This lack of effect seemed to occur for a number of reasons. One reason could be the small population size of this study. Using more than 24 animals in a future study might make it easier to see an increase, especially when considering the top tertile of the HFD group. The sample size decreases when only the top tertile is analyzed; therefore, if an increase in GFAP levels does exist in the top percentage of HFD rats, this increase may become statistically significant if more rats are analyzed. Also, the duration of the diet may play a large role in this study. The fact that the rats ate the diet for only two months may suggest that the diet did not have time to produce a significant effect. Some studies kept the experimental animals on the HFD for longer durations of time, even up to twelve months (Pistell et al., 2009); therefore, longer diet duration will be considered for future studies.

The data from this study produced several interesting findings. For instance, this research contradicted a study by Pistell, which found that a HFD increased GFAP in the cortex of mice (Pistell, 2009). The reason for this difference may have been the different rodent sub-species. Perhaps mice are more susceptible to a HFD than rats are.

The present finding that the hippocampus contained more GFAP than the cortex is consistent with a study that found more GFAP molecules in the hippocampus than in the cerebral cortex (Pistell et al., 2009). This study used the Western Blot process to measure GFAP in both areas and found that there were higher levels of GFAP in the hippocampus. This suggests that there were more GFAP molecules in the hippocampus than in the cerebral cortex (Martin & Callaghan, 1995). However, GFAP is an



intermediate filament protein and is present inside astrocytes at all times. Thus, another study used staining to view the amount of astrocytes containing GFAP in multiple brain areas and found more astrocytes in the hippocampus than in the cortex (Pistell et al., 2009). Therefore, it is more likely that there are simply more GFAP immunoreactive astrocytes present in the hippocampus, rather than a greater expression of the GFAP molecule (Amenta, Bronzetti, Sabbantini, & Vega, 1998).

Overall, a HFD may still produce neuroinflammation in the hippocampus. It may just be expressed by a different proinflammatory molecule. This research will be continued by observing different proinflammatory markers in the future, such as, IL-6 and TNF- $\alpha$ .

The clinical implications of this study include informing the general public about diet-induced memory impairment. This research could warn the general public about the neurological dangers posed by excess saturated fat intake, such as the risk of neurodegenerative diseases. It could also assist with understanding these diseases, including Alzheimer disease.

Ultimately, this research could help target dietary risk factors that could lead to such diseases. Eventually, it could be used to support correlations found in studies like Luchsinger (2002) and help healthcare professionals develop low-fat diets for individuals that are genetically predisposed for neurodegenerative diseases.

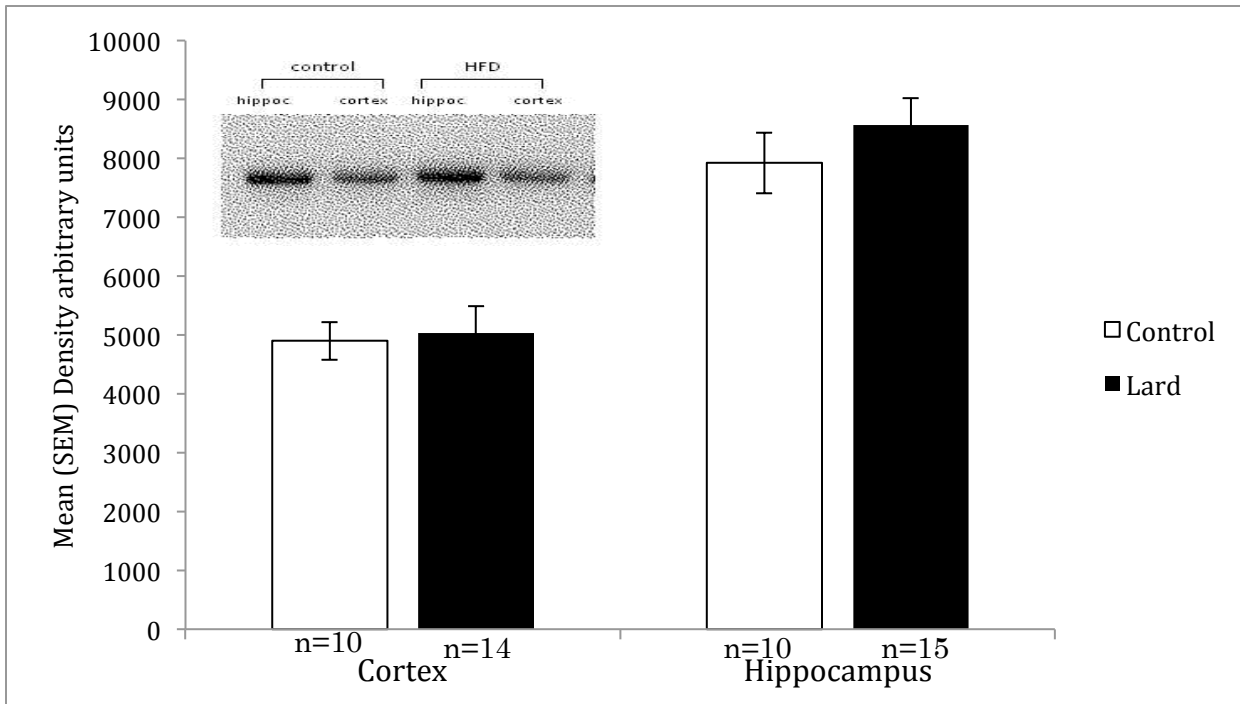


Fig 1. Comparison of the control and HFD rats in the cerebral cortex and in the hippocampus. Inset: Example of control and HFD protein bands on x-ray film.

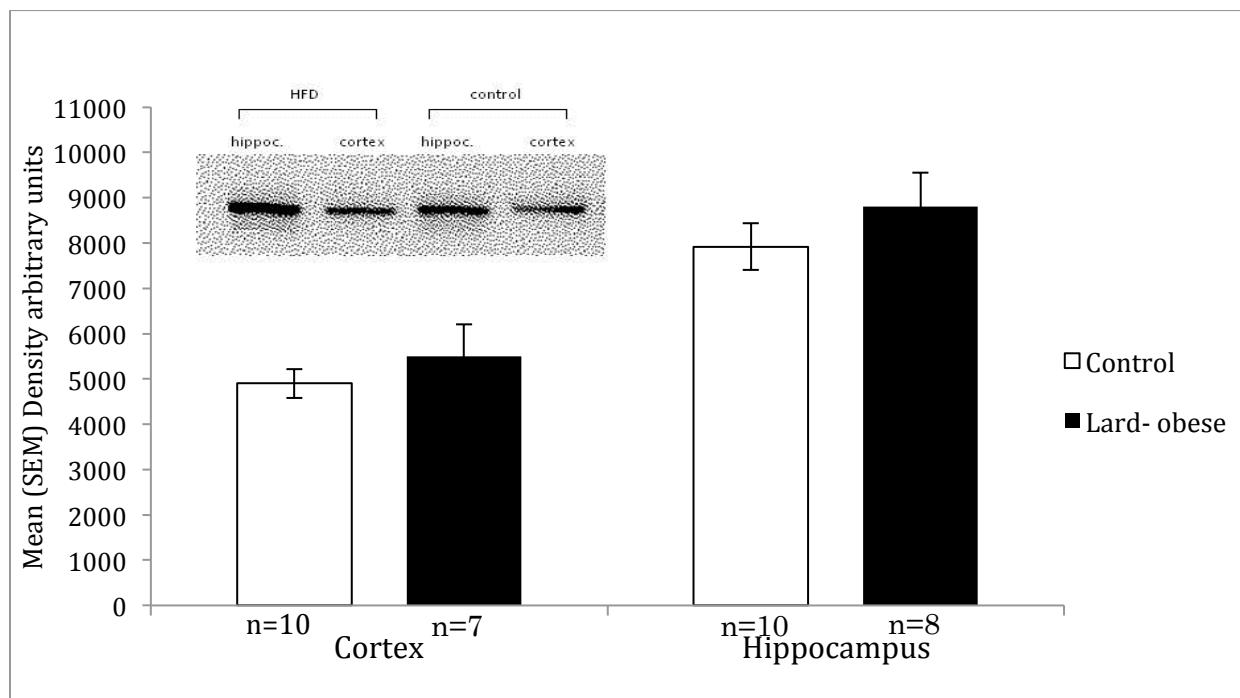


Fig 2. Comparison of the control and HFD-obese rats in the cerebral cortex and in the hippocampus. Inset: Example of control and HFD protein bands on x-ray film.

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