THE REGULATION OF NUR77 IN RESPONSE TO INFLAMMATION, AMYLOID-BETA, AND EXERCISE IN AN LPS-INDUCED ALZHEIMER'S DISEASE MODEL

by

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ABSTRACT

According to the Alzheimer's Association, Alzheimer's Disease (AD) is the sixth leading cause of death in the United States, and is the only leading cause of death that cannot be prevented or slowed. Nur77 is a member of the NR4A subfamily of orphan nuclear receptors. Nur77 is down regulated in transgenic APP + PS1 murine models of AD, and the pharmacological enhancement of the nuclear receptor leads to enhanced memory consolidation in wild type animals. Taken together, this information suggests that Nur77 inhibition may be a factor contributing to the characteristic memory loss seen in AD. Additionally, inflammatory cytokines induce Nur77 expression in target cells. Previous research in our lab indicates that the intraperitoneal administration of the inflammation-inducing agent lipopolysaccharide (LPS), over seven consecutive days, significantly increases Aβ peptide levels in the hippocampus of mice leading to reduced cognitive function (Kahn et al., 2012). Our lab has shown that Nur77 increases as cytokines increase during the first 5 days of the 7 –day LPS injection protocol. However, on the 7^{th} day, when Aβ is elevated and mice exhibit endotoxin tolerance, Nur77 is reduced. Additionally, exercise has been shown to play a significant role in AD models. If mice are allowed to run for two weeks following the 7-day LPS injection protocol, the hippocampal Aβ-levels return to the amount expressed in the saline-injected control group. It is well-known that exercise enhances cognitive function. Recent studies have also shown that exercise enhances the expression of Nur77 in the skeletal muscle of mice. In this study, we demonstrate that there is a trending decrease in Nur77 across treatment groups: both of the LPS groups expressed less Nur77 than both of the Sal groups. While there was not a significant difference between condition groups, the data suggests that

there is a pattern of increased Nur77 expression in mice administered LPS that were allowed to exercise for 14 days, which may contribute to enhanced cognition.

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INTRODUCTION

Alzheimer's Disease (AD) was first introduced by Dr. Alois Alzheimer when he presented his findings on his patient, Auguste Deter, at the Southwest German Medical Conference in 1906 (Bio.com). Deter first came to Alzheimer at the age of fifty-one presenting with erratic behavior, disorientation, and severe memory loss. After her death, at the age of fifty-five, Alzheimer performed an autopsy on her brain that revealed the common pathologies of AD that we are familiar with today: shrinkage of the cerebral cortex, abnormal protein clumping, and fiber tangles. The term "Alzheimer's Disease" was coined in the Eighth Edition of the *Handbook of Psychiatry* in 1910 (National Institute on Aging 2015).

According to the 2014 Alzheimer's Disease Facts and Figures Report produced by the Alzheimer's Association, Alzheimer's Disease is the sixth leading cause of death in the United States and is the only leading cause of death that cannot be prevented or slowed. From the years 2000-2008, deaths attributed to Alzheimer's Disease has increased by sixty-six percent, while the other five leading causes of death have decreased in prevalence (Alzheimer's Disease Facts and Figures 2014). In 2010, 4.7 million people were suffering from AD and in 2050 it is projected that 14.8 million people will be diagnosed with the disease (Hebert et al. 2013). Sadly, one in every three people will die of Alzheimer's Disease or some other form of dementia (Alzheimer's Disease Facts and Figures 2014).

Not only is AD extremely prevalent and likely to increase in upcoming years, but the cost of care to treat Alzheimer's Disease is extremely daunting as well. The total annual cost associated with AD is currently between $157 billion and $215 billion dollars.

$11 billion dollars of which is paid by Medicare on a yearly basis (Hurd et al. 2013). With the rising numbers of people being diagnosed with this disease, the financial burdens associated with it are only projected to rise. Therefore, it is extremely important to understand Alzheimer's Disease in order to find a cure.

The pathologies first observed by Alois Alzheimer in 1906 are what separate AD from other forms of dementia. The abnormal protein clumping is now identified as Amyloid-Beta (Aβ) senile plaques (Dubac 2013). Aβ is a 42 amino acid protein that is cleaved from a larger protein called Amyloid Precursor Protein (APP) (Yonkin 1998). Our lab has shown that the majority of Aβ is created outside of the brain. β-secretase and γ-secretase, in response to a stimulus such as inflammation, cleaves APP into Aβ. This Aβ peptide is then able to cross the blood-brain barrier (BBB), enter the brain, and aggregate into senile plaques. These plaques are neuro-toxic and themselves act as inflammatory agents, leading to neuronal cell death (Choi et al. 2013). The fiber tangles refers to neurofibrillary tangles (NFTs), which are microtubule-associated hyper-phosphorylated tau (Selkoe, 2001). These tangles occur within the neurons and interfere with cell signaling. Alzheimer also noticed a decrease in brain volume in Auguste Deter's brain. This shrinkage of the cerebral cortex is due to the significant reduction of neurons and synapses that are a result of the Aβ plaques and the NFTs (Mattson, 2004).

The senile plaques and NFTs are thought to be the cause of the cognitive defects that are displayed in AD. These pathologies initially begin in the hippocampi of individuals, the area of the brain that is associated with learning new information. The disease first begins with mild impairments, such as minor short-term memory loss (Facts and Figures 2014). Patients with AD progressively get worse as the pathologies spread to

other parts of the brain, leading to severe cognitive decline. Alzheimer's Disease eventually leads to death when the parts of the brain that control involuntary functions, such as breathing and heart rate, are affected resulting in loss of function (Heneka & O'Banion, 2007).

Inflammation, both in the periphery and the CNS, has been shown to play a large role in Alzheimer's Disease. It is well-known that chronic inflammatory diseases, such as Type II diabetes, cardiovascular disease, and hypertension, increases one's risk of developing AD (Schmidt et al., 2002). This is explained by studies that have shown that neuroinflammation can be stimulated by repeated bouts of peripheral inflammation (Cunningham et al., 2013). Kahn et al. has demonstrated that systemic inflammation in non-transgenic mice, resulting from 7 days of LPS injections, leads to memory deficits, a common symptom of AD (2012). This inflammation also leads to an increase in Aβ in the CNS (Kahn et al., 2012). Researchers Tuppo and Arias looked into the use of non-steroidal anti-inflammatory drugs as a possible treatment option for Alzheimer's Disease (2005). Unfortunately, by the time people start showing symptoms associated with AD, the disease has progressed to a point that these anti-inflammatory drugs are unable to reverse the memory impairment (Tuppo & Arias, 2005).

My research focuses on Nur77, a member of the NR4A nuclear orphan receptor family. A nuclear orphan receptor protein is a protein that does not have a known ligand that binds to it. Nur77 is a transcription modulator that responds to oxidative stress and other neurological stressors, inducing a CREB-dependent neuroprotective pathway (Acton 2011). Another function of Nur77 is to reduce the expression of pro-inflammatory cytokines in response to stress (Shao et al. 2010). Nur77 is also necessary

for long-term potentiation and memory consolidation in hippocampal-dependent tasks as it is elevated in the hippocampus of mice immediately after learning (Bridi & Abel 2013). Interestingly, inflammation generally leads to an increase in the expression of Nur77 in target cells, so one would think that the inflammation associated with AD would increase the production of Nur77 (Pei et al. 2005). However, this is not the case. In a study performed by Dickey et al., (2004), Aβ actually suppresses Nur77 in mice which may exacerbate neuronal loss and memory impairment in AD-related pathologies. Exercise has been shown to increase the expression of Nur77 in muscle cells and could possibly have an effect in the brain as well (Kanzleiter et al. 2009).

With this literature in mind, my project was broken up into two parts. The first part was to see how Nur77 expression was affected in our inflammation-induced Alzheimer's Disease model. In this model, we use lipopolysaccharide, or LPS, which is an element found in the cell wall of gram-negative bacteria. This endotoxin is administered to the mouse, via intraperitoneal (ip) injections, for 7 days. The mice receiving the LPS injections exhibit an immune response shortly following the injection, mimicking a bacterial infection. These mice ultimately show elevated Aβ-deposition in their hippocampi following the injection series. Furthermore, those with elevated Aβ also display a significant reduction in cognitive function compared to saline controls (Kahn et al., 2012). As mentioned earlier, Nur77 is stimulated by inflammation but it is also suppressed by Aβ. So my first step was to see how Nur77 responded to an inflammation-induced AD model.

The second portion of my research was to see if exercise increased the expression of Nur77 in the hippocampi of mice within our AD model. If Nur77 is down-regulated in

our model, then exercise may be a way to increase its expression and reduce some of the cognitive effects that are associated with Alzheimer's Disease. Previous studies from our lab have shown that two weeks of voluntary exercise (Figure 1), following the LPS-injection series, was found to significantly reduce the hippocampal-Aβ loads to levels that were no longer significantly different from saline controls (Figure 2). This discovery is promising because it suggests that the decrease in Aβ could lead to an increase in Nur77, which may be a factor contributing to recovery of cognitive function. For the first half of my study, we hypothesized that Nur77 expression should increase for the first 5 days of the injection series as a response to the release of pro-inflammatory cytokines. However, Nur77 expression should be decreased by Day 7 when Aβ-deposition occurs if there is enough Aβ present in this model to suppress Nur77 expression. For the second portion, we hypothesized that Nur77 expression would increase in response to exercise since Nur77 has been shown to be stimulated by exercise (Kanzleiter et al., 2009). The reduction in Aβ-deposition following two weeks of voluntary exercise should only contribute to this phenomenon.

Figure 1: Visual representation of a mouse exercising on the running wheels utilized during this study.

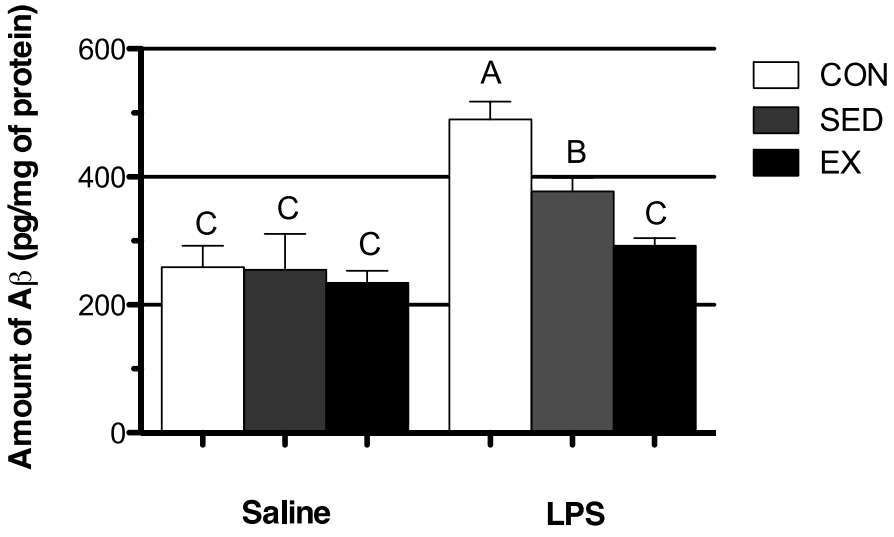

Figure 2: Two Weeks of Voluntary Exercise or Sedentary Recovery. There were no significant differences in running revolutions following administration of LPS or saline. As expected, there was a significant increase in the level of hippocampal-AB in LPS-injected CON mice, mice that received only 7 injections followed by immediate tissue removal, as compared to saline-injected CON mice. Two weeks of voluntary exercise, following LPS-administration, significantly decreased the level of hippocampal-AB as compared to matched SED mice. Interestingly, there were also no significant differences in hippocampal-AB between LPS-administered EX mice and saline administered EX and SED mice. Bars represent mean ±SEM. Letters that are different (a,b,c) represents significant differences ($p<0.05$). CON= control mice, EX= exercise mice, SED= sedentary recovery. (Weintraub, M. K., Unpublished data)

MATERIALS AND METHODS

Subjects

Twenty-four young C57BL/6J mice, 3-4 month old females, were utilized in the first portion of this study that focused on Nur77 expression in our LPS-induced AD model, while 4-6 month old males were utilized in the exercise-portion of the study. These mice were bred in the Texas Christian University (TCU) vivarium from a breeding stock acquired from Jackson Laboratory. All mice were housed in groups of three or four in polycarbonate mouse cages. They were housed in the same room that maintained a constant 12-hour light/dark cycle. All mice had access to food and water. They were cared for in accordance with the procedures put forth by the Institutional Animal Care and Use Committee of TCU.

Treatment Conditions

Mice were randomly separated into groups. Twelve mice received intraperitoneal (i.p.) injections of 250 μg/kg of LPS (Escherichia coli serotype: 055:B5; Sigma-Aldrich, St Louis, MO). These mice were further separated into four groups of 3 mice: day 1, day 3, day 5, and day 7, corresponding to the number of consecutive days of LPS received. The mice were euthanized and their hippocampi were removed four hours post-LPS injections, depending on their group. Twelve mice were used as controls and followed the same procedure, receiving i.p. injections of saline rather than LPS. The exercise study involved mice that were given 7 consecutive days of 250 μg/kg LPS or saline i.p. injections. These mice were then separated into two groups: mice that recovered for 14 days but were sedentary and mice that were allowed to exercise for 14 days. The mice in the exercise groups voluntarily ran at night, using a running wheel that detected the

number of revolutions. After two weeks of exercise or sedentary recovery, the mice hippocampi were extracted immediately following euthanization by CO_2 inhalation.

Western Blot

Hippocampus tissue was isolated post-euthanasia and proteins were extracted using Pro-Prep lysis buffer (Bio-Rad Laboratories, Hercules, CA) The proteins were then analyzed by western blot procedure. DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) was utilized to measure lysate protein concentrations and those numbers were used to calculate an aliquot concentration of 2µg/µl. 15µl of each aliquot solution was loaded into the wells of a 10% SDS polyacrylamide gel and run for 40 minutes in an SDS-Page buffer solution. The proteins were then transferred to a PVDF membrane for western blotting. The membrane was blocked in 5% milk for two hours. Primary incubations were either with a 1:200 dilution of Rabbit-Nurr77 antibody with PBST or 1:1000 dilution of Mouse-β-actin antibody with PBST (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Washes were done using PBST as well. Chemiluminescence was detected using SuperSignal West Dura (ThermoFisher Scientific, Waltham, MA) applied to the membrane and Syngene G:Box (Syngene, Frederick, MD).

Statistical Analysis

GeneTools software (Syngene) was utilized to perform densitometry analysis of banding in order to determine relative protein concentrations of Nur77 and β-Actin. Nur77 levels were normalized by dividing by β-Actin levels. In both experiments, the band intensities (DU) were analyzed using a two-way analysis of variance (ANOVA) procedure (Statview 5.0, SAS, Cary, NC), in which the interaction effects, treatment (LPS or saline) and Day (1,3,5,7) were interpreted for the first study, and treatment (LPS

or saline) and condition (exercise or sedentary) were interpreted for the second study.. Significant interactions were further analyzed using SPSS pair-wise comparisons.

RESULTS

We first sought to determine if Nur77 expression would increase following peripheral inflammation and if there was enough Aβ-deposition in this model to suppress Nur77. In order to answer this question, we performed multiple Western Blots, a technique which uses fluorescence to detect protein concentration, from hippocampus tissue removed from mice that had received 1, 3, 5, or 7 days of LPS injections. (Figure 3A). A 2(Trx: SAL, LPS) x 2(Day: 1,3,5,7) ANOVA showed a significant interaction for day and treatment on Nur77 expression. $F(3,15) = 6.70$, $p=0.004$. SPSS pair-wise comparisons indicated that there is no significant differences in Nur77 expression between SAL(M=0.14) and LPS(M=0.09; p= ns) after 1 Day of injections. After 3 injections there is a significant increase in Nur77 expression in the SAL group (M=0.64) compared to the LPS group(M=0.51; p=0.023). After 5 injections there is a significant decrease in Nur77 expression in the SAL group (M= 0.37) compared to the LPS group (M=0.53; p=0.004). After 7 days of injections there is no significant difference between the SAL group (M=0.12) and the LPS group(M=0.06; p=ns). Figure 3B shows visual representations of the western blot images utilized to create Figure 3A.

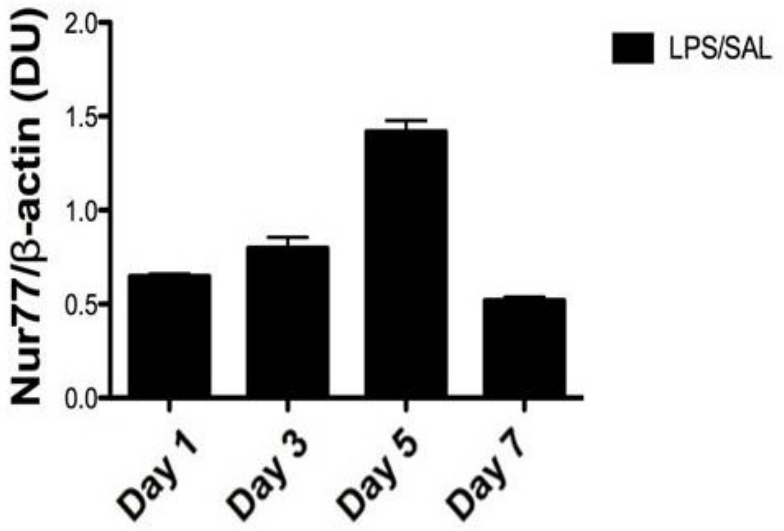

Figure 3A: **Densitometry Analysis**. Tissue samples were analyzed from LPS and SAL groups for each time point. Nur77 expression was normalized by dividing Nur77 by β-actin. The LPS average was then divided by the SAL average at each time point to indicating the trend in Nur77 expression. N= 3.

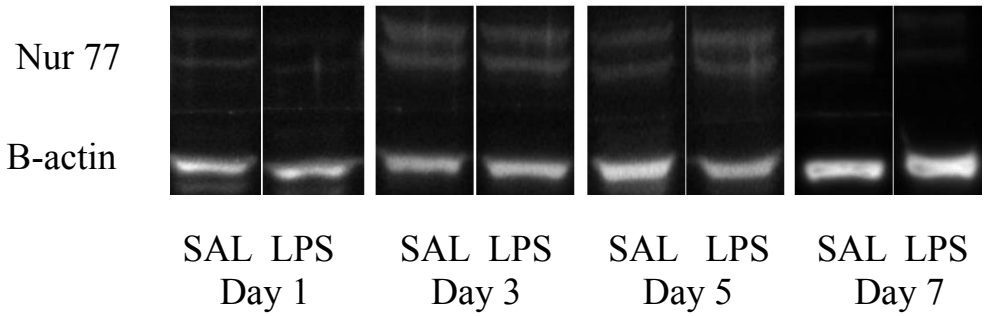

Figure 3B: Western Blot. Images comparing Nur77 levels in LPS and saline injected mice days 1, 3, 5, 7. Images provided by Tanner Robertson and myself.

The next step was to see if exercise could increase the expression of Nur77 in the brains of our inflammatory-induced AD mice. We ran western blots using lysates that were retrieved from the hippocampi of mice two weeks post-sedentary or two weeks post-exercise recovery (Figure 4A). Once again we used a two-way ANOVA to analyze our data. We found there to be a trending main effect between the LPS and the

saline (SAL) treatment groups, in which the saline mice with 14 days of exercise recovery (SAL/Ex) and the saline mice with 14 days of sedentary recovery (SAL/Sed) expressed more Nur77 than the LPS/Ex and LPS/Sed animals. The statistical power was too low, due to such a small sample size, that we were unable to determine significant differences across conditions, exercise versus sedentary. Figure 4B shows the visual representations of the Western Blots that were run.

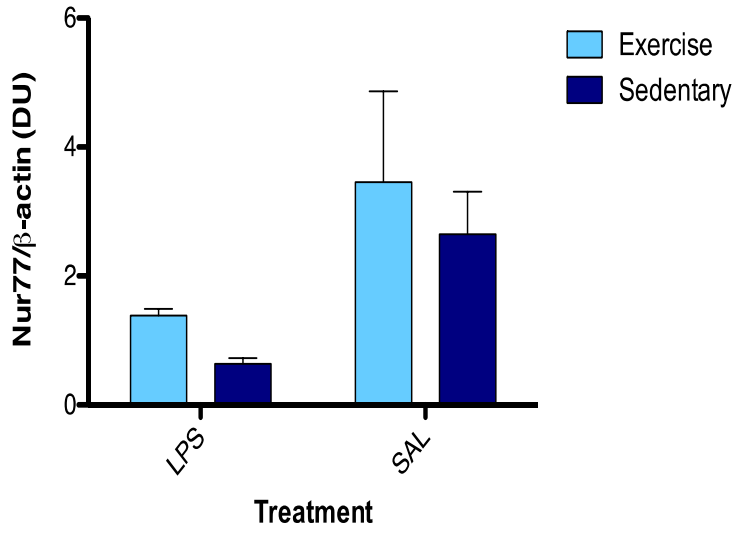

Figure 4A:
Densitometry analysis. Lysates isolated from hippocampal tissue 2 weeks following ex or sed recovery. N=3. Nur77 expression was normalized by dividing Nur77 by β-actin.

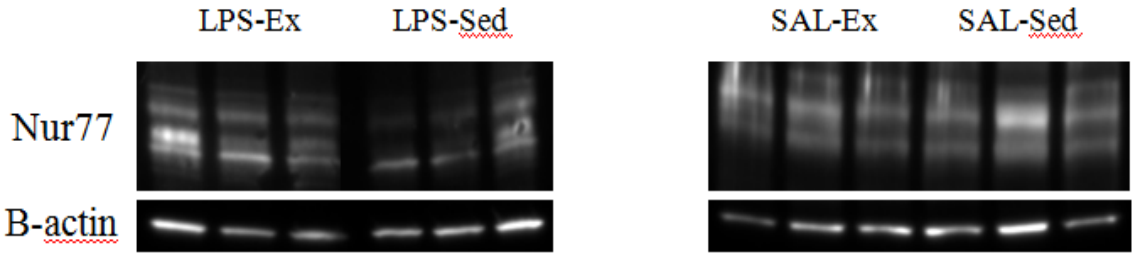

Figure 4B: Western Blot. Images comparing Nur77 levels in LPS-Ex, LPS-Sed, SAL-Ex, and SAL-Sed mice.

DISCUSSION

Our first goal was to see how Nur77 responded to our LPS-stimulated AD model. Nur77 was first stimulated by inflammation, but by day 7, Nur77 expression had dropped in the presence of Aβ. Figure 3 shows the ratio (LPS/SAL) of Nur77 expression. The statistics indicate that there is no significant difference in Nur77 expression between SAL and LPS groups on Day 1. On Day 3, however, there seems to be a stark increase in Nur77 expression in both the SAL group and the LPS group. Even more strange is the fact that the SAL mice expressed more Nur77 than the LPS mice. This is unusual because one would expect that the SAL control group should not be portraying large changes in Nur77 expression. This phenomenon could be due to the fact that the there was only an N of 2 for the LPS pair at this time point, while there was an N of 3 for the SAL group. The SAL mice may have also been experiencing sickness or stress which could be affecting Nur77 expression. The repeated injections may also be causing enough inflammation at the injection site in the SAL mice to cause an increase in Nur77 expression. A larger sample size would be required to determine whether this increase in Nur77 on Day 3 for the SAL control group can be trusted. On Day 5 there is a significant increase in Nur77 expression compared to SAL controls. This increase is likely the result of an increase in the production of pro-inflammatory cytokines due to LPS-induced macrophage activation. This makes sense because, as mentioned earlier, Nur77 expression is upregulated in response to inflammation (Pei et al. 2005). There is no significant difference in Nur77 expression between LPS and SAL groups on Day 7. This corresponds with our hypothesis. On the 7^{th} day, when Aβ is present, there is a decrease in Nur77 expression, mirroring the means produced by both treatment groups on Day 1. Dickey et al. found

that the presence of Aβ suppresses Nur77 expression in transgenic mice (2004). Therefore, it makes sense that the Aβ in our model would produce the same results. However, there is a problem with this conclusion that must be addressed. The mice in our model exhibit a phenomenon known as endotoxin tolerance. This just means that after 4 or 5 days of receiving daily injections, the mice immune systems begin to recognize that they have "seen" LPS before and they stop secreting cytokines in order to avoid going into septic shock (Kahn et al. 2012). Figure 5 graphically depicts this endotoxin tolerance by measuring Il-1β, a pro-inflammatory cytokine, after 1 injection, 4 injections, and 7 injections of LPS. This graph, provided by Kahn et al., closely resembles Figure 3A, which shows that Nur77 expression gradually increases until Day 5, but drops on Day 7. Taking endotoxin tolerance into account, the reduction in Nur77 expression on Day 7 could be a result of the decreased secretion of pro-inflammatory cytokines, it could be suppression from the deposited Aβ, or it could be a mixture of the two.

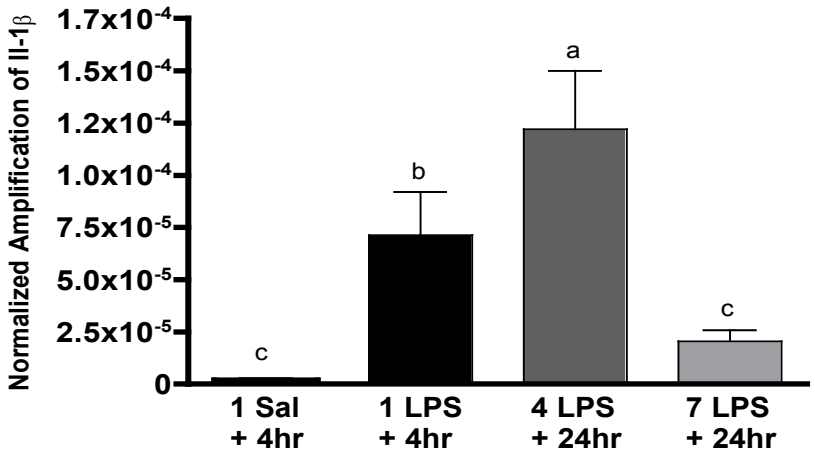

Figure 5: Central cytokine levels. The decrease of central IL-1B from day 4 to day 7 indicates the development of endotoxin tolerance in this murine model. Graph provided by Kahn et al., 2012.

In order to know whether it is the reduction in cytokines or the Aβ leading to the drop in Nur77 expression, a new study must be conducted in which the mice are administered poly(I:C), a viral mimetic. Mice do not exhibit endotoxin tolerance when they are given poly(I:C) (Weintraub et al. 2014). The mouse immune system never stops producing cytokines in response to this mimetic, so if there is still a drop in Nur77 expression on Day 7, we would be able to attribute it to Aβ deposition.

No matter the cause, Nur77 is in fact down-regulated by day 7 in our Alzheimer's disease model. The next part of my research looked into whether or not exercise could be used as a mechanism to increase the expression of Nur77, and thus contribute to enhanced cognition. Figure 4A displays the results from the western blots of tissue isolated from the mice that received the regular injection series followed by two weeks of sedentary or exercise recovery. While there were no significant differences between groups, there is a definite trend indicating that both exercise (ex) and sedentary mice (sed) receiving LPS had reduced expression of Nur77 compared to the ex and sed mice receiving SAL. This supports our previous claim that Aβ may be contributing to reduced Nur77 expression. Exercise does seem to play a role in increasing the expression of Nur77, however, because our statistical power is so low, we were not able to determine significance. If we were to increase our sample size to more than three subjects per group, there is a high chance that we would find a significant difference between both the treatment groups (LPS vs. SAL) as well as the condition groups (Ex vs. Sed) in Nur77 expression. If we do find that Nur77 is upregulated with exercise, this would provide a mechanism to increase one of the transcription factors needed to support neuronal health.

The next step in this project would be to use drugs to increase Nur77 to see if that helps maintain cognitive function within our AD model. Nur77 has been a major focus in cancer research, and certain anti-cancer drugs have been shown to upregulate Nur77 expression (Ashton Acton 2013). Cytosporone B is an agonist for Nur77 (Yanyan et al. 2008). Along with our normal 7-day LPS injection series, we would also administer Cytosporone B to force the expression of Nur77. We would then run behavioral tests on these mice to see if cognitive deficits, associated with Aβ deposition in our model, can be avoided with this increase in Nur77.

Interestingly, Nur77 has been a hot-research topic, as it has been shown to polarize macrophages from one phenotype to another. Macrophages can be broken down into two different types: M1 macrophages and M2 macrophages. M1 macrophages produce a pro-inflammatory immune response, which often leads to tissue damage. M2 macrophages, on the other-hand, promote cell growth and tissue repair (Mills 2012). When Nur77 was knocked-out in an atherosclerotic murine model, monocytes were polarized toward an M1 phenotype, which led to exacerbated atherosclerotic plaques (Hanna et al. 2012). Microglia are the macrophages of the brain, and previous research in our lab has suggested that the microglia are likely responsible for the clearing of Aβ after two weeks of sedentary or exercise recovery. The exercise mice are able to dispose of the Aβ more efficiently than the sedentary mice, and our lab has hypothesized that exercise may be leading to a change in the phenotype of the M1 (pro-inflammatory) microglia, to the M2 (anti-inflammatory) microglia. It would be interesting to see if the increased expression of Nur77 could be contributing to this shift in microglia phenotype. We are now introduced to a chicken or the egg conundrum. If Nur77 does play a role in

microglia phenotype polarization, then the increase in Nur77 could be leading to a change in the microglia, which could be leading to Aβ clearance, which could lead to an even greater increase in Nur77 expression. Or it could be that Nur77 does not influence the microglia, but that some other factor related to exercise is causing a change in the microglia. This would lead to Aβ clearance and an increase in Nur77. More research needs to be done to better understand this mechanism. Although there has been little research connecting Nur77 and other members of the NR4A nuclear orphan receptors to Alzheimer's Disease, it is a promising avenue that should be further explored.

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