EFFECTS OF ACUTE AND CHRONIC INFLAMMATION ON VASOPRESSIN EXPRESSION IN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS

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EFFECTS OF ACUTE AND CHRONIC INFLAMMATION ON VASOPRESSIN EXPRESSION IN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS

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ABSTRACT

Inflammation can lead to a suite of behavioral changes known as sickness behaviors, which have been implicated in the etiology of chronic inflammatory disease. Here, we examine the role of acute and chronic induction of inflammation on vasopressin (AVP) expression in the paraventricular nucleus of the hypothalamus (PVN) of female mice. To test, adult female C57B6/J mice were injected with lipopolysaccharide (LPS) at two different dosages and schedules to induce acute (1.0mg/kg, once) and chronic (0.25mg/kg LPS, eight times) inflammatory responses. The acute dose has previously been demonstrated to induce sickness behaviors, as measured by the open field test and a social interaction test; however, these measures were unaffected by the chronic administration of LPS. AVP mRNA expression in the PVN was measured using fluorescent in situ hybridization (FISH) and fluorescent microscopy. Both the acute and chronic administration of LPS resulted in an increase in PVN AVP expression without a change in the area of this expression. This increase in AVP in the PVN occurred despite the lack of sickness behavior, suggesting that further work is necessary to characterize the role of AVP in the PVN in these chronic and acute inflammatory responses.

INTRODUCTION

Inflammation is defined by the series of cellular and molecular events that occur during an acute immune response to infection (Chen et al., 2018). Emerging evidence has indicated that inflammation plays a central role in many physical and mental diseases and disorders. An acute infection induces behavioral changes known as sickness behaviors, such as lethargy, depression, and anxiety (Hart, 1988). These sickness behaviors can aid in the recovery from an infection (Adelman & Martin, 2010; Maes et al., 2012). For example, sickness behaviors prevent the spreading of an infection by limiting the rate of bacterial reproduction due to a lack of iron in the gut, which resulted from a suppressed appetite (Kluger & Rothenburg, 1979). In addition, this inflammation also shifts motivations such that there is a social disinterest, which may reduce infection rates of non-infected relatives by limiting contamination of the environment (Shakhar & Shakhar, 2015).

An inappropriate prolonged immune response can result in chronic sickness behaviors, such as psychiatric disorders like major depressive disorder (Fischer et al., 2015). Indeed, several mental illnesses, such as major depressive disorder and anxiety disorders, have inflammatory components (Felger, 2018; Kayser & Dalmau, 2011). In the case of persistent infection or inflammation, the continuous production of pro-inflammatory cytokines sustains sickness behaviors (Dantzer et al., 2008). Pro-inflammatory cytokines and other inflammatory processes are correlated to sickness behaviors, in both human and animal models (Myers, 2008). As these inflammatory mediators affect normal neuronal and glial functioning, it is likely that altered neuronal and/or glial signaling may mediate the suite of behavioral changes associated with chronic sickness (Sankowski, Mader, & Valdes-Ferrer, 2015). Hypothalamic alterations of

cytokine and immune-related enzyme expression have been found in a mouse model of autoimmune disorders (Ajmone-Cat et al., 2019).

One candidate factor for mediating these changes in behaviors associated with chronic sickness may be arginine vasopressin (AVP). AVP is a neuropeptide released from the paraventricular nucleus of the hypothalamus (PVN) to regulate blood pressure, water balance, and anxiety/social behaviors (Caldwell et. al., 2008; Dantzer & Bluthe, 1992). Neurosecretory cells in the PVN co-express AVP and corticotrophin-releasing hormone (CRH) (Sawchenko, Swanson, & Vale, 1984), which then regulate the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Aguilera & Rabadan-Diehl, 2000; Wotjak et al., 1996). Therefore, AVP neurons within the PVN form a link between the limbic forebrain and the HPA axis within the PVN (Groeneweg et. al., 2011). For example, rats bred for high anxiety, display not only greater anxiety-like behavior but also depressive-like behavior, regardless of the presence of stress (Keck et al., 2002; Landgraf et.al., 1999; Wigger et al., 2004). Taken together, these data suggest that AVP from the PVN may mediate sickness behaviors (Dantzer & Bluthe, 1992). However, it is not known how the inflammatory mediators such as cytokines effect AVP expression to cause physiological and behavioral changes.

The goal of this study is to experimentally determine the effect of induced inflammation on AVP expression in the forebrain, specifically the PVN, as a measure of the neuronal manifestation of sickness in mice. Lipopolysaccharide (LPS), a major component of the cell wall of gram-negative bacteria, was used to stimulate an immune response, in two different inflammatory models. Acute and chronic inflammatory models were created based on two different LPS dosages and varied number of administrations. Acute inflammation was induced with 1 administration of 1.0 mg/kg LPS (Ming, Sawicki, & Bekar, 2015), while chronic

inflammation was induced with 8 administrations (bi-weekly for 4 weeks) of 0.25mg/kg LPS (Krishna, Dodd, & Filipov, 2016).

Since this project serves to draw relationships between an acute model of sickness and a chronic model of sickness, behavioral testing is necessary to confirm the presence of sickness. Prior research has indicated a correlation between acute inflammation and sickness behaviors such as increased anxiety-related behavior and reduced motor behavior (Whylings et. al., 2019). Thus, behavioral signs of chronic sickness will be tested using an open field test and a same-sex social interaction test to measure mobility and social interaction, respectively, in mice (Ikeda, Sato, & Mizuguchi, 2013; Seibenhener & Wooten, 2015). The sickness behaviors associated with inflammation are characterized as anxiety and depression, which are both clinically are more prevalent in women than in men (Albert, 2015; McLean et. al., 2011).

Furthermore, this project aims to explore the effect of LPS-induced systemic inflammation on PVN AVP expression, in female mice, under both acute and chronic models of sickness. LPS is administered at different times in the light cycle due to possible influence of circadian rhythms in the suprachiasmatic nucleus (SCN) on the PVN (Wu et al., 2018). Preliminary data showed that mice administered LPS during the dark phase had a greater difference in AVP mRNA when compared to the control. As LPS can stimulate the release of proinflammatory cytokines (Ngkelo et. al., 2012), it is predicted that AVP mRNA expression will be enhanced in the presence of LPS. This characterization of PVN AVP will allow better understanding of the mechanisms underlying the relationship between acute and chronic inflammation, AVP, and sickness behavior.

METHODOLOGY

I. Animals

Sixteen adult (60-90 days) female C57B6/J mice (Jackson Labs) were group housed in ventilated transparent OptiMouse plastic cages with Bed-O-Cobs bedding (Animal Care Systems), a nestlet square, and *ad libitum* food and water. Animals were housed in a 12:12 light/dark cycle, with lights on designated as ZT 0. All animal procedures were approved by the Georgia State University Institutional Animal Care and Use Committee and were in accordance with the regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

II. LPS

LPS (*e. coli*, 0111:B4, Sigma), a bacterial coat protein, was used to stimulate an immune response. LPS was diluted and then stored in 0.05 mg/ml aliquots at −20 °C. It was thawed to room temperature before administration.

III. Experiment 1 (Acute Inflammation)

8 Subjects were given an intraperitoneal injection once during the dark phase, five hours after lights off (ZT 17) with 1.0 mg/kg LPS (n = 4) or equivalent volume of 0.9% sterile saline (n = 4). Animals were euthanized 3 hours after injection.

IV. Experiment 2 (Chronic Inflammation)

11 Subjects were given intraperitoneal injections bi-weekly during the light phase, one hour before lights off (ZT 11), for four weeks with 0.25mg/kg LPS (n = 5) or an equivalent volume of 0.9% sterile saline (n = 6). Behavioral tests (open field and social interaction) were run the day after the last injection was administered. Testing occurred during the dark phase, one hour after lights off (ZT 13), after one hour of acclimation of subjects to the testing rooms. Animals were then euthanized during either day (ZT 8-9; n = 6) or night (ZT 20-21; n = 5), 2 days after the last injection (8 total injections).

a. Open Field

Subjects were placed in the center of an 43x43x43 cm open field chamber and allowed to freely investigate for 10 minutes. Arenas were cleaned with 70% ethanol between trials. The total distance traveled was automatically tracked and scored by Activity Monitor (Med Associates, Inc.,) on a computer connected to the open field arenas. The center of the open field was defined as half of the total area of the chamber. Total distance and proportion of distance traveled in the center of the open field to the total distance traveled were measured as a quantification of mobility, which is an indicator of sickness behavior.

b. Social Interaction

Subjects were placed with a same sex stimulus animal for 10 minutes in a 19.1x14.0x8.6 (WxLxH) open cage containing AlphaDri bedding. A video was recorded and analyzed (Noldus Observer) to evaluate total interaction time and number of interactions between the test and novel mice. Interactions by the test

mice are defined as any active social contact or investigation within approximately 2cm, including following, sniffing, and climbing on or under the novel mice.

V. Brain Collection and Sectioning

All subjects were euthanized via CO_2 asphyxiation, during the dark phase (ZT 20-21). Brains were flash-frozen in 2-methylbutane, and stored at -80℃ until sectioning. Frozen neural tissue was sectioned coronally at 20μm with a cryostat (Leica CM3050 S) into three series, and subsequently stored at -80℃. For experiment 1, tissue was sectioned onto chromium-gelatin subbed slides. For experiment 2, tissue was sectioned onto pre-charged superfrost plus slides. All tissue and slides were handled in a RNase-free environment.

VI. Fluorescent *In Situ* Hybridization (FISH)

One tissue series was labeled for AVP mRNA using FISH to confirm AVP cell expression. Tissue was fixed in 4% paraformaldehyde, washed in 2× saline-sodium citrate (SSC), acetylated in an acetic anhydride/triethanolamine solution, rinsed in DEPC H2O, washed in acetone/methanol solution (1:1), and again washed in 2× SSC. Then, tissue was incubated in hybridization buffer (50% Formamide, 5X SSC, 1 mg/mL Torula RNA, 100 μg/mL Heparin, 1X Denhart's, 0.1% Tween 20, 0.1% CHAPS, 10 mM EDTA, H_2O) only, followed by hybridization buffer plus digoxygenin-labeled riboprobe to the vasopressin gene (NCBI accession # NM_027106.4) overnight at 65 ℃. After hybridization, tissue

was placed through SSC washes to reduce non-specific binding. First, the tissue was washed in heated 2x SSC, then room temperature 0.5x SSC, and finally 1x SSC. After these washes, the tissue was labeled with a peroxidase-conjugated anti-digoxygenin antibody (1:5000), which was then visualized using TSAplus-fluorescin kit (Perkin Elmer), as detailed in Rigney et. al. (2019).

VII. Image Analysis

Bilateral images of the PVN were taken with two fluorescent microscopes (both were ZEISS Axio Imager M2) at 10x magnification. The pixel measurement was quantified at different magnitudes for the two experiments, because two different fluorescent microscopes were used. PictureFrame was used for taking images in Experiment 1, and StereoInvestigator was used for taking images in Experiment 2, due to availability. Using a mouse brain atlas (Paxinos & Franklin, 2012), the PVN (0.6-0.95mm caudal to Bregma; 0.25mm lateral to midline) in these images was manually outlined in ImageJ. ImageJ quantified the fluorescent intensity of pixels in this selected area. The fluorescent intensity is a measure of the fluorescently labeled AVP mRNA-expressing cells. ImageJ outputs mean pixel intensity, the area selected for each image, and integrated density (the product of mean pixel intensity and area) for each image. Average area (Figures 1 & 3) is the total area of the PVN for a given brain divided by the number of sections that were measured for that brain. This is a value used as a control to ensure that the overall area of AVP expression has not changed upon the induction of sickness. Average integrated density is the total integrated density of the PVN for a given

brain divided by the number of sections that were measured for that brain. Fluorescent intensity (Figures 2 & 4) for each brain is the average integrated density divided by average area.

VIII. Statistical Analysis

The time of day construct was eliminated in experiment 2, because the intensity of expression was not significantly different based on time of day overall, day vs night ($p=0.5503$), or for either group individually, Saline ($p=0.7992$) and LPS ($p=0.6248$). Thus, day and night data were analyzed together for each chronic inflammation experimental group, Saline and LPS, respectively. For both experiments, an independent samples t-test statistical analysis (RStudio) was used to analyze group differences and determine whether PVN AVP expression is significantly different for the saline- and LPS- treated groups.

RESULTS

Experiment 1: Acute Inflammation

After acute LPS administration, the average area of PVN AVP expression was not significantly different ($p = 0.90$) in mice treated with LPS compared to saline-treated mice (Figure 1). In this experiment, the LPS group had significantly greater fluorescent intensity ($p = 0.016$) of AVP expressing cells in the PVN than the saline control group (Figure 2). This suggests that the mice administered an acute dosage of LPS did show an increase in AVP mRNA.

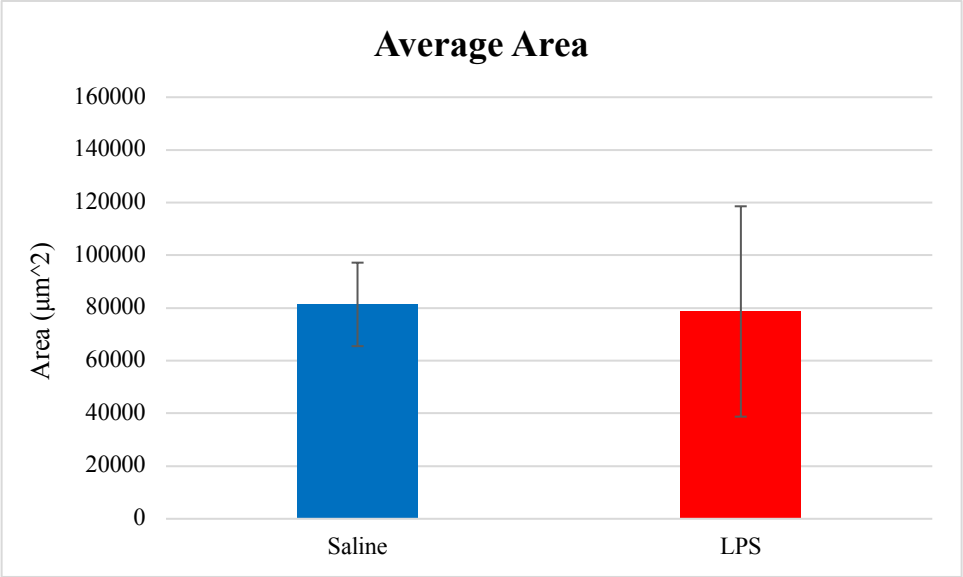

Figure 1: Average Area of AVP expression in the PVN after Acute Inflammation

Area (in μm^2) +/- SEM of measured PVN of saline (blue, n=4) and LPS (red, n=3) treated animals. p = 0.90, t = -0.14.

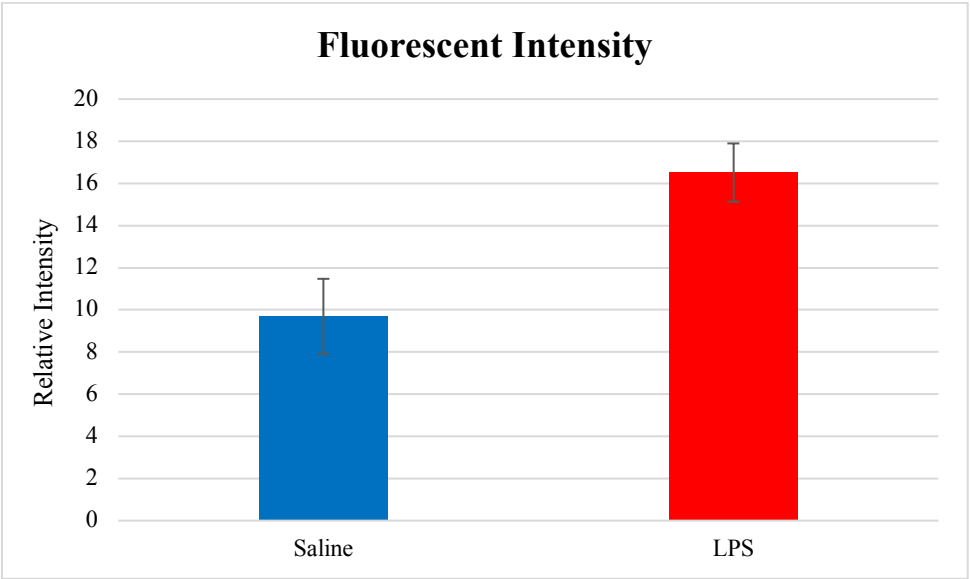

Figure 2: Average Relative Fluorescent Intensity After Acute Inflammation

Fluorescent intensity (arbitrary units) +/- SEM of measured PVN of saline (blue, n=4) and LPS (red, n=3) treated animals. p = 0.016, t = 3.6.

Experiment 2: Chronic Inflammation

AVP Expression:

After chronic inflammation, there was no significant difference ($p = 0.81$) in the average area of AVP expression in the PVN between Saline and LPS groups (Figure 3). In this experiment, the LPS group had significantly greater fluorescent intensity ($p = 0.0072$) of AVP expressing cells in the PVN than the saline control group (Figure 4). This suggests that the mice administered a chronic dosage of LPS did show an increase in AVP mRNA.

Anxiety and Social Behaviors:

The open field test, which quantified total distance traveled (Figure 5a) and proportion of this distance traveled in the center of an open field (Figure 5b) as measures of anxiety-like behavior, did not result in significant differences between mice treated with LPS compared to saline-treated mice ($p = 0.33$; $p = 0.30$). The social interaction test is another measure of anxiety-like behavior. Mice treated with a chronic dosage of LPS showed a not significantly different ($p = 0.50$) number of interactions with a same-sex mouse compared to the saline control group (Figure 6a). The total time of interaction for the LPS administered mice was not significantly different ($p = 0.50$) than the saline control group (Figure 6b).

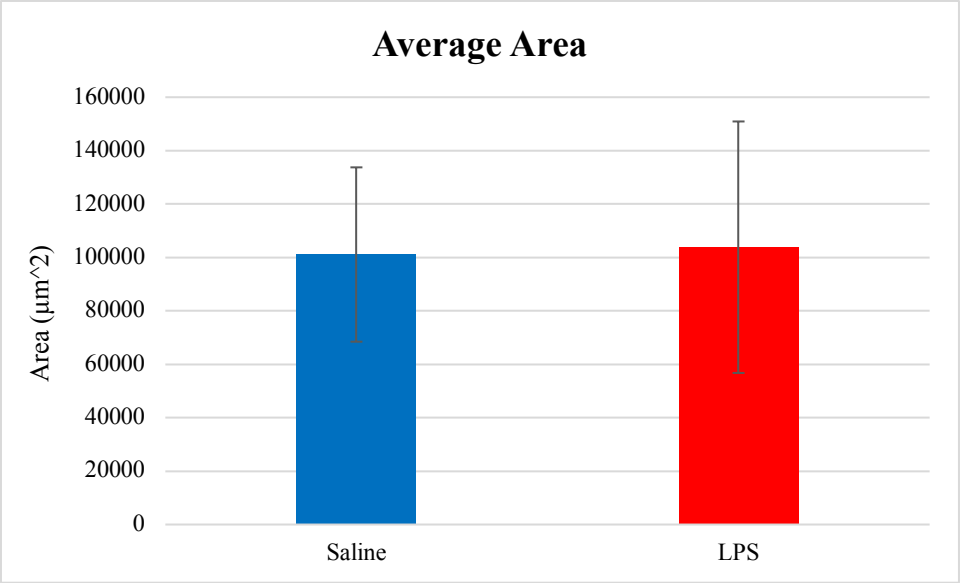

Figure 3: Average Area of AVP expression in the PVN after Chronic Inflammation

Area (in μm^2) +/- SEM of measured PVN of saline (blue, n=5) and LPS (red, n=6) treated animals. p = 0.81, t = 0.25.

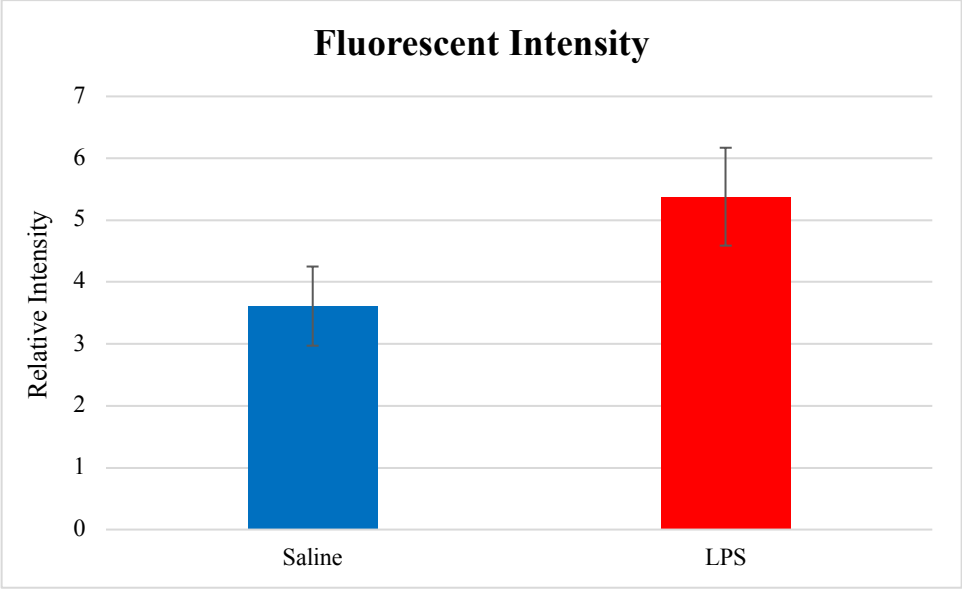

Figure 4: Average Relative Fluorescent Intensity After Chronic Inflammation

Fluorescent intensity (arbitrary units) +/- SEM of measured PVN of saline (blue, n=5) and LPS (red, n=6) treated animals. p = 0.0072, t = 3.5.

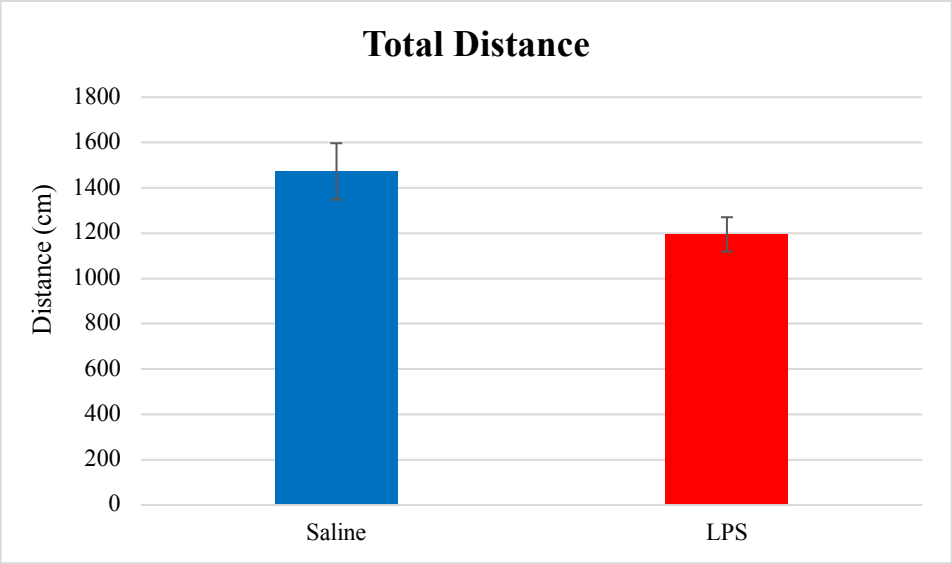

Figure 5a: Total Distance Traveled in the Open Field

Distance (in centimeters) +/- SEM traveled in the open field by saline (blue, n=8) and LPS (red, n=8) treated animals. p= 0.33, t = -1.0.

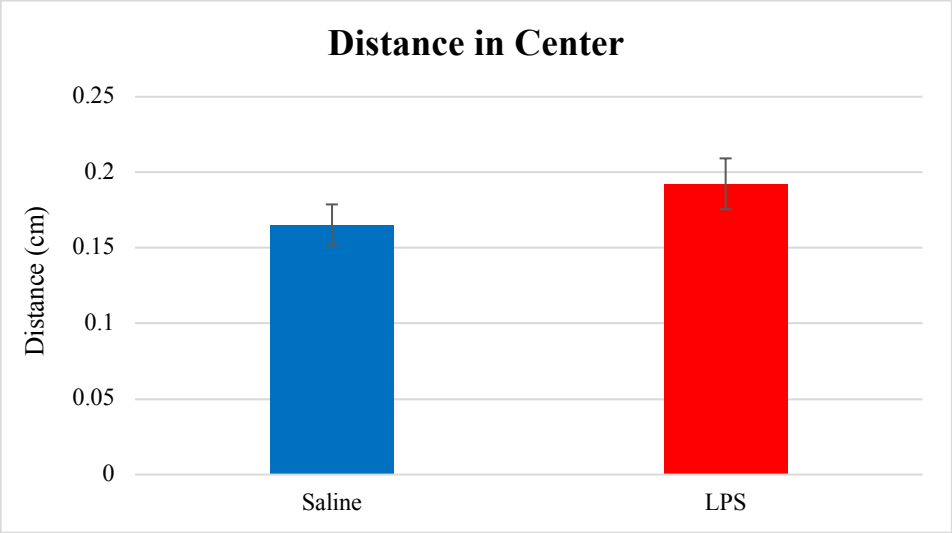

Figure 5b: Proportion of Total Distance Traveled in the Center of an Open Field

Distance (in centimeters) +/- SEM traveled in center of an open field in proportion to the total distance traveled in an open field by saline (blue, n=8) and LPS (red, n=8) treated animals. p= 0.30, t = 1.09.

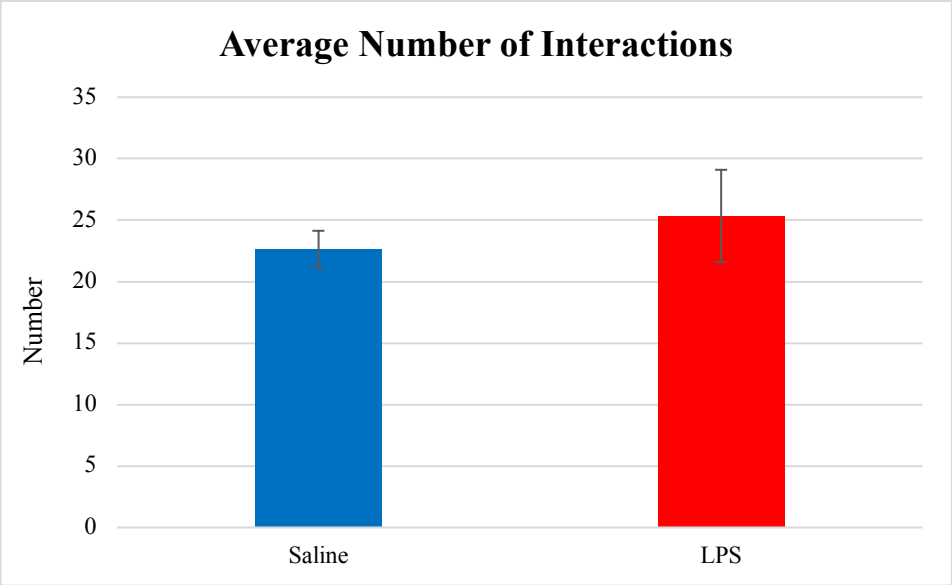

Figure 6a: Average Number of Interactions per 10 Minutes with Same Sex Stimulus Mouse

Number +/- SEM of interactions per 10 minutes between either saline control mice (blue, n=8) or LPS (red, n=8) treated mice and same sex stimulus mouse. p = 0.50, t = 0.71.

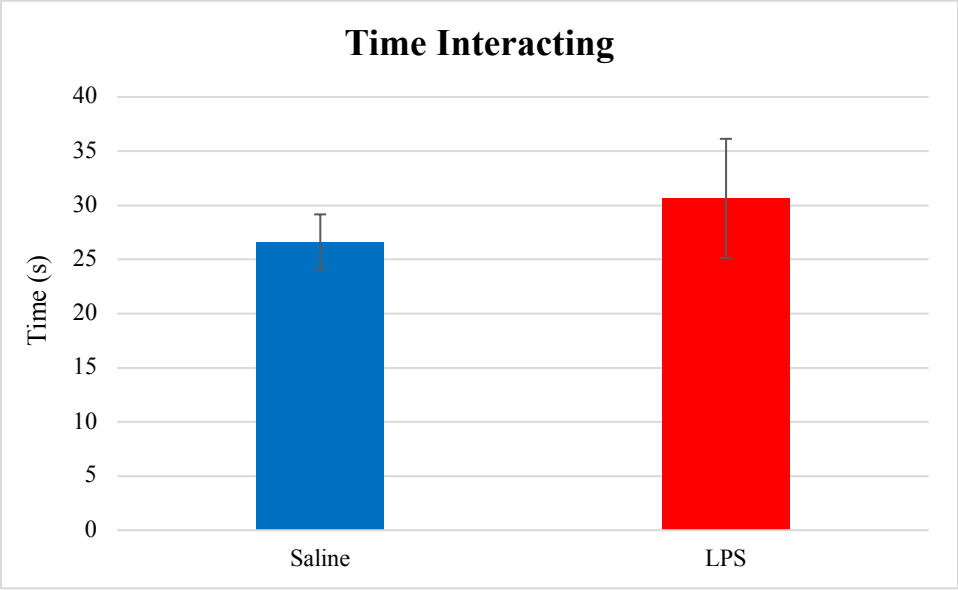

Figure 6b: Duration of Social Investigation with Same Sex Stimulus Mouse

Total time (in seconds) +/- SEM of interaction between either saline control mice (blue, n=8) or LPS (red, n=8) treated mice and same sex stimulus mouse. p = 0.50, t = 0.70.

DISCUSSION

The purpose of this project was to better understand the relationship between acute or chronic inflammation, AVP expression in the PVN, and sickness behavior in female mice. We administered LPS systemically to induce an inflammatory response. It was found that acute LPS administration increased PVN AVP expression, but did not affect the area of this expression (Figure 1 & 2). These results support the hypothesis that LPS enhances AVP expression. Likewise, this is consistent with the findings of previous literature that suggest an acute dosage of LPS can be correlated with features of sickness behavior, such as decreased locomotion and reduced social exploration (Clark et al., 2015; Zhao et al., 2019).

Chronic LPS administration increased PVN AVP expression, but did not affect the area of this expression (Figure 3 & 4). Mice administered a chronic dosage of LPS did not travel a significantly greater total distance nor travel a greater proportion of the total distance in the center of the open field compared to the control group, suggesting that the mice did not express sickness behaviors (Figure 5a & 5b). Similarly, mice administered a chronic dosage of LPS did not have a significantly greater number of interactions or total time of interaction compared to the control group, suggesting that the mice did not express sickness behaviors (Figure 6a & 6b). While enhanced AVP expression for experiment 2 supports the hypothesis of this study, the lack of behavioral signs of sickness was unexpected. These behavioral results are contradictory to that of previous literature which indicates that a chronic dosage of LPS causes increased anxiety coupled with depressive-like behavior (Berk et al., 2013; Krishna et al., 2016), particularly in female mice (Kubera et al., 2013). However, research has also found that acute LPS-induced sickness behavior can develop into chronic depressive-like behavior, despite stabilization from

the immediate behavioral response post-LPS administration (Frenois et al., 2007; Henry et al., 2008; O'Connor et al., 2009).

Both models of inflammation displayed greater fluorescence of AVP mRNA in the PVN when compared to their respective control groups (Figures 2 & 4). Fluorescent intensity is a metric of AVP mRNA labeling, which is a measure of its expression. Increased AVP mRNA expression could indicate that either AVP expressing cells in the PVN are expressing more AVP mRNA or more cells in the PVN are expressing AVP mRNA. A cell count quantification of the PVN could distinguish this difference in the origin of amplified AVP mRNA expression. Since the area of AVP mRNA expression is not affected by chronic or acute inflammation (Figures 1 & 3), this suggests that PVN AVP cells are expressing more AVP, rather than more PVN cells expressing AVP.

The greatest limitation to the interpretation of the results from this project is that it cannot be concluded whether the change in AVP intensity is related to sickness. A contributing factor is that behavioral testing was not undertaken for experiment 1. Repeated exposure to LPS, such as in the chronic model of sickness, may produce a tolerance effect caused by alterations in peripheral responsiveness (Zhang et. al., 2000), thus explaining the lack of sickness behaviors in these mice. Furthermore, despite the lack of significant evidence for sickness behavior under chronic inflammation, it has been previously validated that an acute model of inflammation produces sickness behaviors such as increased anxiety-related behavior and reduced motor behavior (Whylings et al., 2019). Thus, it could be inferred that AVP from the PVN mediates sickness behaviors, because both models of inflammation generate similar changes in AVP expression in this study. However, a limitation to this interpretation is that this project measured

AVP mRNA expression, rather than gene expression or protein translation, so the results do not directly indicate that there are changes occurring to AVP peptide production.

In the future, this study could be expanded to include a larger number of subjects, which would provide another cohort of acute inflammatory mice that could be tested for behavioral signs of sickness, and limit the influence of extreme observations on the results obtained, in turn producing significant differences in sickness behavior that are more representative of the chronic inflammatory population. Additionally, future studies could measure actual AVP protein level in the PVN by using immunohistochemistry (IHC), as a qualitative method, or western blot, as a quantitative method. IHC can be used to detect the presence of a protein within a tissue sample (Bauman et. al., 2016), while the western blot generates densitometry data that can be used to compare relative changes in protein abundance between different conditions (Butler et. al., 2019). Another future direction to determine whether the changes in PVN AVP mRNA expression can be connected to sickness behaviors could be ablation studies. Ablating AVP expressing cells in the PVN would allow for the characterization of a direct relationship between inflammatory responses and changes in sickness behaviors. This type of study would also help conclude that the changes seen in AVP mRNA levels are not in counterbalance to other immune responses caused by the induction of inflammation.

In conclusion, the data suggests that acute and chronic models of inflammation work in similar ways, despite a lack of behavioral correlation to PVN AVP mRNA expression. Both models of LPS administration enhanced average cell fluorescent intensity of AVP-expressing cells in the PVN without altering the area of PVN AVP expression. The results are not a direct indicator of AVP peptide production, because AVP mRNA expression is measured using FISH in this study. In the future, AVP production could be measured using IHC or western blot. Also,

it cannot be concluded at this time whether a change in AVP mRNA expression can be related to sickness. Future PVN ablation studies could be used to explore the presence of a causative relationship between inflammatory responses and sickness behaviors.

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