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ACUTE SYSTEMIC INFLAMMATION TRIGGERS CHANGES IN NEUROPATHOLOGY GENE EXPRESSION WITHIN THE MURINE BRAIN IN AN AGE-, TREM2-, AND SEX-DEPENDENT MANNER

By

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A capstone project submitted for Graduation with University Honors

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ABSTRACT

Microglia are the brain's resident macrophages. In the healthy central nervous system (CNS), they regulate synaptic connections during development and aging. In the presence of systemic inflammation, they become altered in how they do this. Microglia are known to exclusively express the innate immune receptor known as the triggering receptor expressed on myeloid cell 2 (TREM2) in the CNS, which is important for phagocytosis of cellular debris and synapses. In this study, we are interested in how age, sex, and TREM2 deficiency alter the brain's inflammatory response. We use intra-peritoneal lipopolysaccharide (IP-LPS) injections as a systemic immune challenge in mice at three different ages. In our experiment, we analyzed the genetic expression of whole brains within sex (male and female), age (postnatal day 15, 3 month old, 2 year old), genotype (wild-type and TREM2 knockout), and inflammatory condition (naïve or IP-LPS treated), for a total of 24 groups. We isolated whole RNA from the hemisected brains of the aforementioned groups, assayed them using Nanostring's Neuropathology panel, and analyzed the resulting data using nSolver software. We then observed the differentially expressed genes and their respective pathways for up- or down-regulation. Both sexes reacted largely the same, but not identically, in response to systemic inflammation. They also reacted quite differently with TREM2 deficiency in response to systemic inflammation.

ACKNOLWEDGEMENTS

I would like to give special thanks to my mentor, Dr. Monica Carson, and Joseph Valdez for their patience and guidance throughout the years on this project. I would also like to thank Dr. Abdullah Madany for helping introduce me to this project, as well as the other members of the lab for their extraordinary support.

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INTRODUCTION

Systemic inflammation can be caused by an infiltration of bacteria into the body or other environmental insults. Our central nervous system (CNS) also responds to this systemic inflammation, and it is known as neuroinflammation. Microglia are macrophages in the brain and in the healthy CNS, they regulate synaptic connections through phagocytosis during development and aging. They are also important to the brain's inflammatory response. In response to a foreign insult or infection, microglia become activated and produce cytokines to promote an inflammatory response (Xu et. al).

Triggering Receptor Expressed on Myeloid cells 2 (TREM2) is expressed solely on microglia, and TREM2 has been shown to mediate phagocytosis in microglia and more recently, impact the inflammation in the brain. Both TREM2 deficiency and systemic inflammation have been linked to Alzheimer's and/or dementia susceptibility. Our lab has shown that with aging, microglia are phagocytosing less. The loss of the phagocytic function in microglia can lead to a build-up of plaques in the brain that cause neurodegenerative diseases such as Alzheimer's Disease.

It is well understood that females are more susceptible to neurodegenerative diseases that have an onset much later in life, and males are more susceptible to neurodevelopmental disorders that have an onset much earlier in development. There have been some studies that showed morphological differences in microglia at certain points of development between males and females (Hanamsagar), but the specific gene expression differences between the sexes is largely unkown. This is what this project aims to look at; we are interested in looking at how aging, TREM2 deficiency, and sex plays a role in the brain's response to systemic inflammation.

METHODS

Animals

We used C57BL/6J mice as models for human disease. They were purchased from Jackson laboratories and housed in our specific pathogen free (SPF) vivarium. The TREM2 KO (T2KO) mice were graciously donated by Marco Colonna. All mice were bred and housed at the University of California Riverside animal facility with a 12/12-h light/dark cycle (lights on at 06:00 am).

To observe sex differences in response to inflammation, we used an equal number of female (n=3) and male (n=3) mice in each category. We tested three different ages, postnatal day 15 (p15), 3-month-old, and 2-year-old mice, as well as two genotypes, wild-type (WT) and TREM2 deficient (TREM2KO) mice.

Systemic Inflammation Manipulation

To stimulate an immune response, mice were injected intraperitoneally with lipopolysaccharide (IP-LPS). LPS is a component of gram-negative bacteria that allows us to initiate an immune response against bacteria without the complications of infecting with live bacteria. They were given a dose of 5mg/kg body weight, which peaks inflammation between 24-48 hours and resolves by 72 hours.

Brain Dissections, Tissue Harvesting and RNA Isolation, and RNA Quality Check

Mice were euthanized 24 hours following IP-LPS injections. Following euthanasia, the brains were dissected. A sagittal half of the brain was used for Microglial isolation in another experiment, and the other half was used in this experiment for RNA isolation.

Half-brains were homogenized in 2.5 mL TRIzol (Millipore-Sigma, cat. R2020) using a mechanical homogenizer (Ultra-Turrax T25). The supernatant was then processed with chloroform (Millipore-Sigma, cat. C2432) and the top layer of nucleic acids was carefully collected and added to isopropanol (Millipore-Sigma, cat. I9516) to precipitate the RNA. The isopropanol was removed, and the pellet was washed three times with 80% ethanol. The RNA was then reconstituted with molecular grade water (Millipore-Sigma, cat. W4502).

We checked our samples by using a spectrophotometer and calculating the concentration of RNA based on measured A_{260} values. We further checked the quality and quantity by Nanodrop 2000, and also with an Agilent RNA 6000 Nano Kit (CAT: PN 5067-1511) on an Agilent 2100 Bioanalyzer. Samples with an RNA integrity number less than 7 were excluded from our analysis.

Gene Expression Analysis

The isolated RNA was hybridized with Nanostring's Neuropath probe set panel, which quantifies the number of transcripts for 770 genes. The hybridized RNA samples were loaded into Nanostring nCounter. To analyze this data, the nSolver analysis software (version 4.0.70) was used. The data was inspected for quality control metrics, and we determined threshold values and housekeeping genes to be used for analysis.

Statistics

Pathway scores were calculated using nanoString's Advanced Analysis feature. The 3-month-old naive condition was used as the reference group for pathway analysis. Statistically significant differences within pathway score analysis were done using GraphPad Prism 7. We then

conducted an all-pairwise comparison with a correction for multiple comparisons using the Bonferroni method. Differential expression was determined by comparing each group's LPS stimulated gene expression to that of the naïve. To correct for multiple comparisons, the Benjamini-Yekutieli method was used. Samples were considered significant when their corrected p-value was <0.05.

RESULTS

Housekeeping genes and endogenous gene counts were above threshold and consistent across all samples.

The quality and quantity of our isolated RNA in all samples passed our quality control metrics. The threshold value was determined to be 41 counts after averaging the counts for our spiked-in negative controls for each sample and adding two times the standard deviation. Housekeeping genes Aars, Cnot10, Csnk2a2, Fam104a, Lars Mto1, and Supt71 were used for normalization, and Asb7, Ccdc127, and Tada2b were dropped.

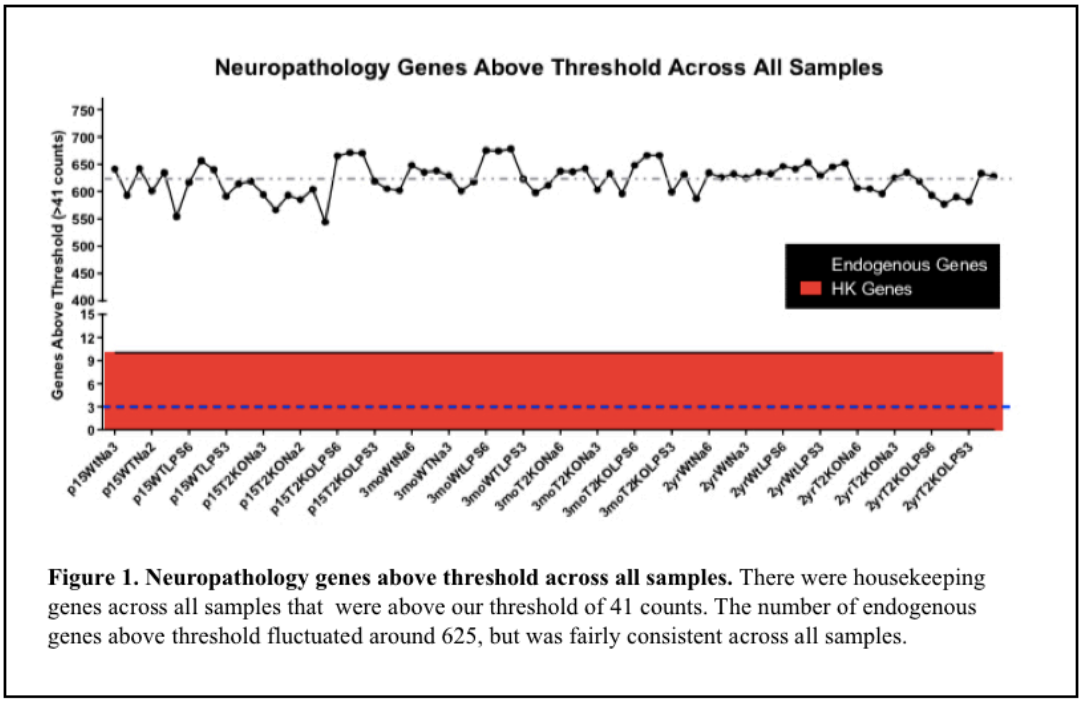


Figure 1. Neuropathology genes above threshold across all samples. There were housekeeping genes across all samples that were above our threshold of 41 counts. The number of endogenous genes above threshold fluctuated around 625, but was fairly consistent across all samples.

WT female mice show more differential regulation of genes in response to LPS stimulation than WT male mice at ages p15 and 3-month-old.

The heatmaps in **Figure 2** generated using nSolver's Advanced Analysis give a general impression of the data. Each group is made up of triplicates and there are no clear outliers, so there is good reproducibility. In **Figure 2A**, the female p15 naïve mice were more differentially

regulated (as seen by the brighter orange and blue colors) than their LPS stimulated counterpart, and they had distinct oppositely regulated groups of genes (as shown in the red box). 3-month-old and 2-year-old naïve mice had largely the same gene expression, as did 3-month-old and 2-year-old LPS stimulated mice. They differed from p15 mice naïve and LPS stimulated gene expression, respectively. In **Figure 2B**, the male p15 and 3-month-old mice did not have much of an LPS-mediated response, as seen by the similar gene expression patterns within each age. However, male 2-year-old mice did, as seen by the brighter orange and blue colors under 2-year-old LPS compared to 2-year-old naive. Male mice had distinct patterns of gene expression as they aged, whereas female mice had more similar regulation as they aged. Aged mice (2-year-old) in both sexes had similar gene expression profiles within their naïve or LPS condition.

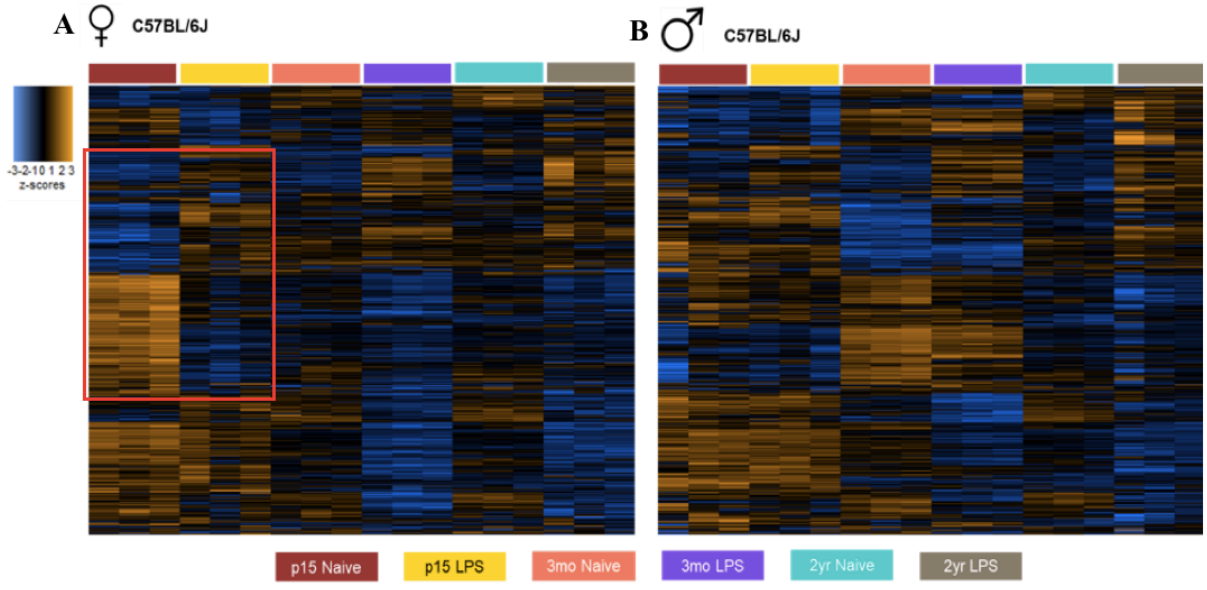


Figure 2. Normalized data heat maps for WT mouse samples. In females (A), comparing within age and between conditions shows more variation in the gene expression than in males (B). In males, we see similar groups and levels of expression within ages and between conditions. Genes are listed in the same order for both heatmaps. A positive z-score (orange) indicates upregulation and a negative z-score (blue) indicates downregulation. Black indicates little to no change in gene expression. The data is grouped by females and males, and they are further grouped by age (p15, 3-month-old, 2-year-old) and condition (naïve or LPS stimulated), as denoted by the colored key underneath the maps.

TREM2KO amplifies sex differences but dampens LPS mediated differences.

The heatmaps in **Figure 3** generated using nSolver's Advanced Analysis give a general impression of the data. Each group is made up of triplicates and there are no clear outliers, so there is good reproducibility. In **Figure 3A**, the female mice did not have a strong LPS-mediated response. When looking at naïve versus their LPS stimulated counterpart within ages, we can see that there is not much difference in the level of regulation or in the genes that were regulated. However, differences between ages is amplified, especially seen in female 2-year-old mice. The genes from the top to bottom were upregulated then shifted to downregulated, while female p15 and 3-month-old mice were downregulated then upregulated. In **Figure 3B**, the male p15 mice oppositely regulated groups of genes in response to LPS stimulation. In male 3-month-old, there is some differential regulation between naïve and LPS stimulated genes, but it is not as much as we see in p15 mice. And in 2-year-old males, there is not much difference in the level of regulation or in the genes that were regulated in response to LPS stimulation. Therefore, we can see as TREM2KO male mice age, their genes become less differentially regulated in response to LPS stimulation. Between females (**Figure 3A)** and males (**Figure 3B**), only the p15 Naïve groups showed similar regulation of genes. All other categories between females and males (p15 LPS, 3-month-old Naïve, 3-month-old LPS, 2-year-old Naïve, 2-year-old LPS) had opposite regulation. Where females were downregulated, males were upregulated and where females were upregulated, males were downregulated.

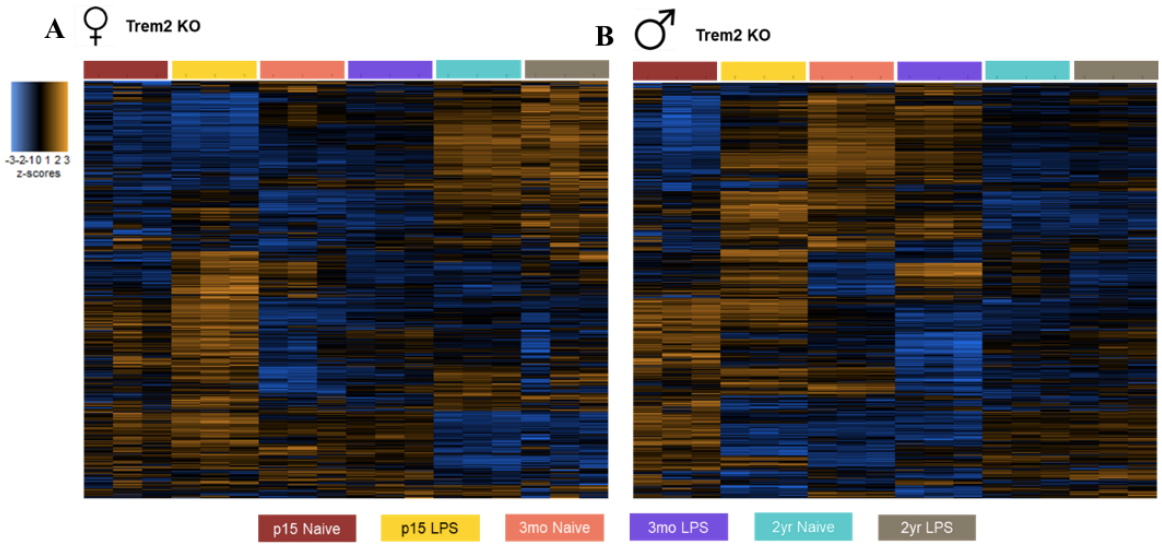


Figure 3. Normalized data heat maps for TREM2KO mouse samples. TREM2KO amplifies sex differences but dampens LPS mediated differences. Genes are listed in the same order for both heatmaps. A positive z-score (orange) indicates upregulation and a negative z-score (blue) indicates downregulation. Black indicates little to no change in gene expression. The data is grouped by females and males, and they are further grouped by age (p15, 3-month-old, 2-year-old) and condition (naïve or LPS stimulated), as denoted by the colored key underneath the maps.

The 770 genes in Nanostring's Neuropathology panel are categorized by Nanostring into 23 fundamental pathways for neurodegeneration, which are listed under "Pathways" in **Table 1** and **Table 2**. Pathway scores of naïve groups were compared to LPS stimulated groups, and the difference was determined to either be statistically significant or not. We can see that TREM2KO dampens LPS mediated differences. There are less significantly regulated pathways in both TREM2KO females and males (**Table 2**) compared to their WT counterparts (**Table 1**).

IP-LPS/Naive Comparison's

Pathways	Female			Male		
	p15	3mo	2yr	p15	3mo	2yr
Activated Microglia	<0.0001	<0.0001	0.0008	0.0002	<0.0001	0.0001
Cytokines	<0.0001	<0.0001	<0.0001	0.0114	<0.0001	<0.0001
Angiogenesis	<0.0001	0.0004	0.001	0.0069	<0.0001	0.0021
Unfolded Protein Response	<0.0001	<0.0001	<0.0001	0.0046	<0.0001	<0.0001
Apoptosis	<0.0001	<0.0001	<0.0001	ns	<0.0001	<0.0001
Transcription and Splicing	0.0086	<0.0001	<0.0001	ns	0.0243	<0.0001
Matrix Remodeling	0.0207	0.0012	ns	ns	<0.0001	0.0094
Chromatin Remodeling	ns	<0.0001	<0.0001	ns	<0.0001	<0.0001
Lipid Metabolism	ns	0.0001	0.0023	ns	0.0001	0.0003
Myelination	ns	<0.0001	<0.0001	ns	<0.0001	0.0012
Tissue Integrity	ns	0.0027	0.0255	ns	ns	0.0165
Axon and Dendrite Structure	<0.0001	<0.0001	0.0002	ns	0.0087	0.0237
Carbohydrate Metabolism	<0.0001	<0.0001	0.0007	ns	0.0001	0.0063
Growth Factor Signaling	<0.0001	<0.0001	<0.0001	ns	<0.0001	<0.0001
Neural Connectivity	<0.0001	<0.0001	<0.0001	ns	0.0012	0.0044
Neural Cytoskelaton	<0.0001	0.0003	0.0027	ns	0.0014	0.0063
Oxidative Stress	<0.0001	<0.0001	<0.0001	ns	<0.0001	<0.0001
Trophic Factors	<0.0001	<0.0001	<0.0001	ns	<0.0001	<0.0001
Vesicle Trafficking	<0.0001	<0.0001	0.0002	ns	0.0082	0.0169
Autophagy	<0.0001	<0.0001	<0.0001	ns	ns	ns
Transmitter Response and Reupatke	<0.0001	<0.0001	0.0016	ns	ns	ns
Transmitter Syntheis and Storage	<0.0001	0.0005	0.0063	ns	ns	ns
Transmitter Release	<0.0001	ns	ns	ns	ns	ns

Table 1. Significantly regulated neuropathology pathways in WT mice. LPS stimulated and naive groups were analyzed within both female and male groups. These depict a pairwise comparison with a correction for multiple comparisons using the Bonferroni method. Statistical Significance is indicated if $p<0.05$. Pathways not significantly regulated are denoted by "ns."

IP-LPS/Naive Comparison's

Pathways	Female			Male		
	p15	3mo	24m	p15	3mo	24m
Activated Microglia	<0.0001	0.0134	ns	<0.0001	<0.0001	<0.0001
Cytokines	<0.0001	0.0038	ns	0.0021	<0.0001	0.0004
Angiogenesis	<0.0001	ns	ns	ns	<0.0001	0.0005
Unfolded Protein Response	0.0329	0.0152	ns	<0.0001	<0.0001	0.0009
Apoptosis	0.0002	ns	ns	<0.0001	ns	ns
Transcription and Splicing	ns	ns	ns	<0.0001	<0.0001	ns
Matrix Remodeling	ns	ns	ns	ns	<0.0001	ns
Chromatin Remodeling	0.0002	ns	ns	<0.0001	ns	ns
Lipid Metabolism	0.0308	0.0263	ns	<0.0001	0.016	ns
Myelination	ns	0.0367	ns	0.0396	<0.0001	ns
Tissue Integrity	0.0015	ns	ns	ns	0.0004	0.0002
Axon and Dendrite Structure	0.007	ns	ns	<0.0001	0.0051	ns
Carbohydrate Metabolism	ns	ns	ns	<0.0001	ns	ns
Growth Factor Signaling	0.0006	ns	ns	<0.0001	ns	ns
Neural Connectivity	0.0038	ns	ns	<0.0001	0.0074	ns
Neural Cytoskelaton	<0.0001	0.0427	0.0188	ns	<0.0001	ns
Oxidative Stress	ns	ns	ns	<0.0001	<0.0001	ns
Trophic Factors	0.0188	ns	ns	<0.0001	ns	ns
Vesicle Trafficking	0.0036	ns	ns	<0.0001	ns	ns
Autophagy	ns	ns	ns	<0.0001	ns	ns
Transmitter Response and Reupatke	0.0287	ns	ns	<0.0001	ns	ns
Transmitter Syntheis and Storage	ns	ns	ns	<0.0001	ns	ns
Transmitter Release	0.0075	ns	ns	<0.0001	ns	ns

Table 2. Significantly regulated neuropathology pathways in TREM2KO mice. LPS stimulated and naive groups were analyzed within both female and male groups. These depict a pairwise comparison with a correction for multiple comparisons using the Bonferroni method. Statistical Significance is indicated if $p<0.05$. Pathways not significantly regulated are denoted by "ns."

In order to better understand sex differences in regulation of fundamental pathways for neurodegeneration, we further categorized the statistically significant pathways into upregulated (**Figure 4**) and downregulated (**Figure 5**) pathways within their respective groups. One of the fundamental themes of neurodegeneration as categorized by Nanostring is "Neuroinflammation," and it consists of three pathways: Activated Microglia, Matrix Remodeling, and Cytokines. We can see that all three of these pathways are upregulated in both WT male (**Figure 4A**) and WT female (**Figure 4B**) mice, with the exception of Matrix Remodeling in male p15 and Matrix Remodeling in female 2-year-old. The same 7 pathways were upregulated between WT males and WT females; however, females are more consistent as they age (as seen by the majority of pathways being common across all three ages) and males increase in the number of pathways upregulated as they age. 3-month-old WT males and females were exactly the same in upregulated pathways. In TREM2KO mice, upregulation of these "Neuroinflammation" pathways was confined to p15 and 3-month-old mice. Activated Microglia and Cytokines were upregulated in p15 and 3-month-old males (**Figure 4C**) and in p15 females (**Figure 4D**) only. In

both sexes of TREM2KO mice, the number of upregulated pathways decreased with age. Unlike WT male and female mice that had the same 7 pathways upregulated, only p15 TREM2KO male and female mice had any similar pathways. TREM2KO mice had a larger variety of pathways upregulated than WT mice, but the pathways were mostly inconsistent between sexes. This suggests that males and females have different response mechanisms to having inactivated microglia during neuroinflammation.

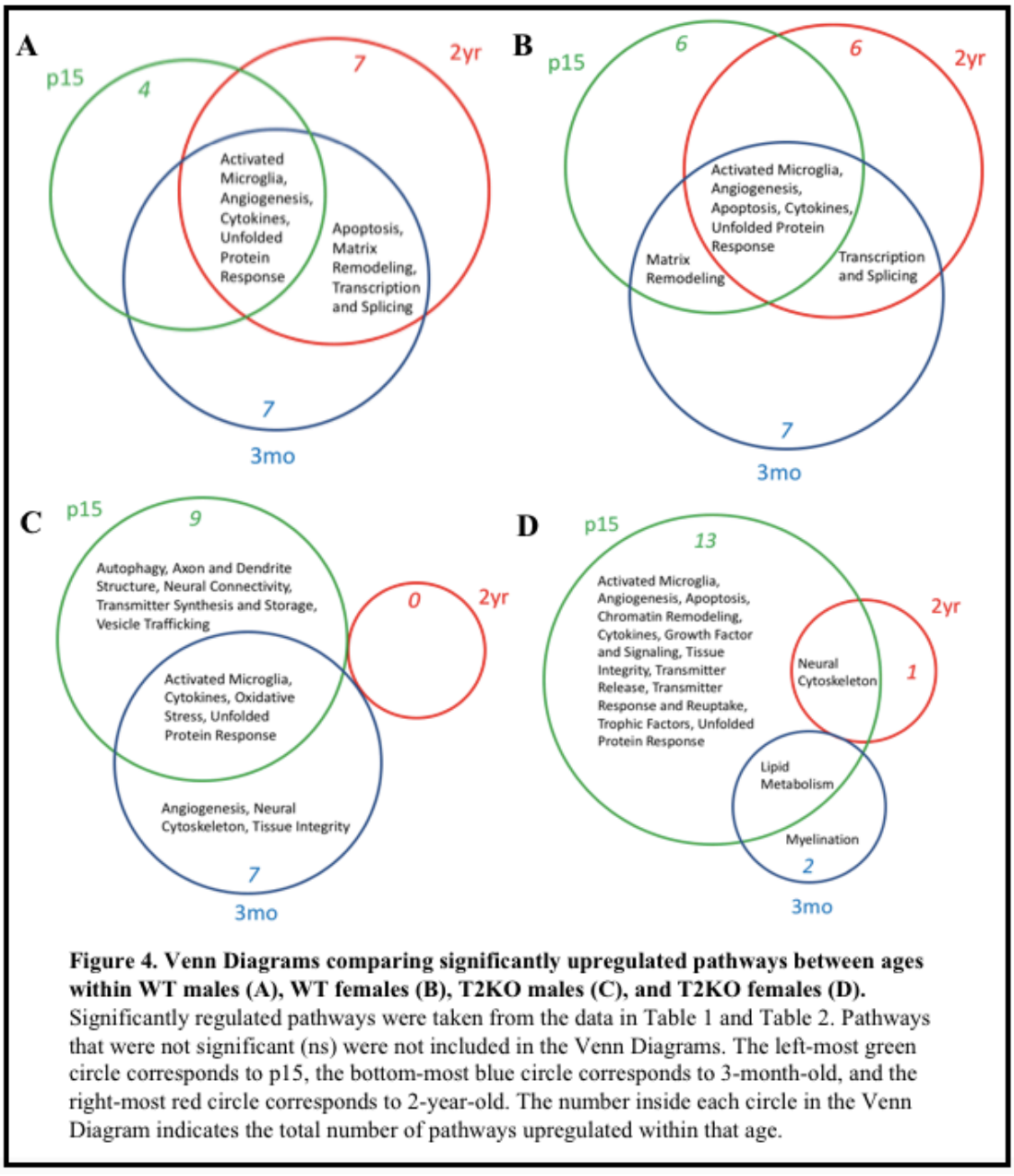


Figure 4. Venn Diagrams comparing significantly upregulated pathways between ages within WT males (A), WT females (B), T2KO males (C), and T2KO females (D). Significantly regulated pathways were taken from the data in Table 1 and Table 2. Pathways that were not significant (ns) were not included in the Venn Diagrams. The left-most green circle corresponds to p15, the bottom-most blue circle corresponds to 3-month-old, and the right-most red circle corresponds to 2-year-old. The number inside each circle in the Venn Diagram indicates the total number of pathways upregulated within that age.

In the WT mice (**Figures 5A-5B**) downregulated pathways, we notice the same trend as with upregulated pathways (**Figures 4A-4B**). WT females (**Figure 5B**) are more consistent as they age (as seen by the majority of pathways being common across all three ages) and males (**Figure 5A**) increase in the number of pathways downregulated as they age. The pathways being downregulated are once again very similar between WT 3-month-old and WT 2-year-old male and female. In TREM2KO mice (**Figures 5C-5D**) downregulated pathways, we once again notice the same trend as with upregulated pathways (**Figures 4C-4D**). The number of downregulated pathways in both male and female TREM2KO mice decrease with age. The patterns of regulation are the same between upregulated (**Figure 4**) or downregulated (**Figure 5**) pathways.

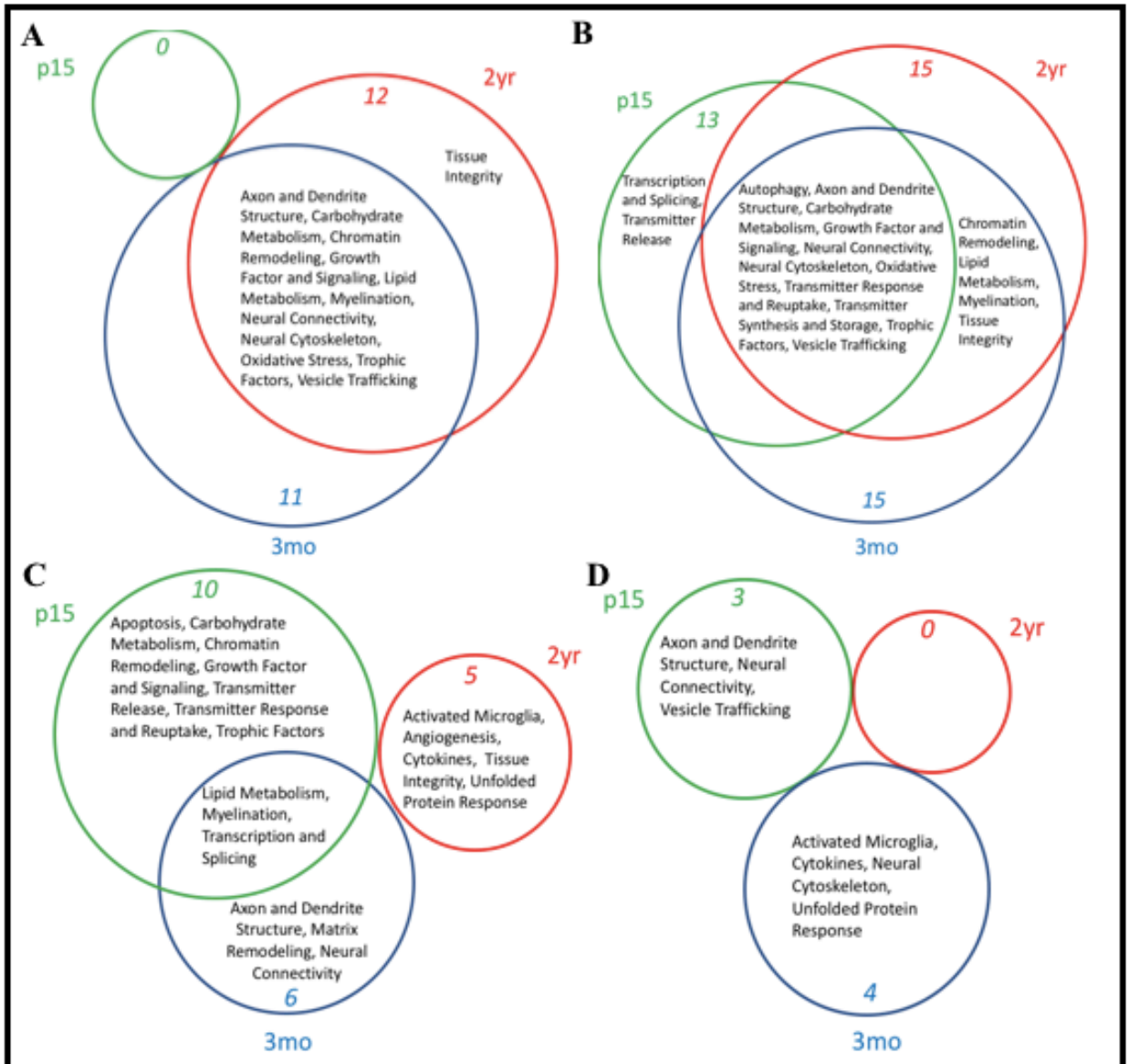


Figure 5. Venn Diagrams comparing significantly downregulated pathways between ages within WT males (A), WT females (B), T2KO males (C), and T2KO females (D). Significantly regulated pathways were taken from the data in Table 1 and Table 2. Pathways that were not significant (ns) were not included in the Venn Diagrams. The left-most green circle corresponds to p15, the bottom-most blue circle corresponds to 3-month-old, and the right-most red circle corresponds to 2-year-old. The number inside each circle in the Venn Diagram indicates the total number of pathways upregulated within that age.

DISCUSSION

It is well-established that neurodegenerative diseases, such as Alzheimer's, which usually arise later in the lifetime are more prevalent in females than in males. Neurodevelopmental disorders, such as autism, arise early in development and are more prevalent in males than females (Hanamsagar). Our findings are consistent with, and may explain, the differential onset of neurological diseases and disorders between sexes. Our gene expression data between wild-type males and females suggest that early in development, the female brain responds more to systemic inflammation than the male brain. It can then be speculated that the male brain may be predisposed to an inflammatory state at baseline than females early in development.

TREM2 is expressed exclusively on microglia in the CNS and deficiency in TREM2 causes a loss of function in microglia. Other studies have shown how loss of TREM2 may keep microglia in a resting state and prevent activation (Mazaheri et al.). We found that there was a lack of significantly regulated genes/pathways in both female and male aged (2-year-old) TRE2KO mice in response to systemic inflammation, which suggests that microglia play a bigger role in responding to systemic inflammation in aged mice than in adult (3-month-old) or juvenile (p15) mice. There are most likely other mechanisms in adult and juveniles that make up for the absence of activated microglia, and further study is needed to understand what exactly those mechanisms and differences between sexes are.

Our results can be used to better understand how males and females respond differently to systemic inflammation at different ages, as well as how the loss of normal microglial function may also affect these responses.

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