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Xinyi Jiang April 10, 2024

Investigating the Effect of Surgical Menopause on Gene Expression Changes in Retinal Ganglion Cells and Astrocytes

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Abstract

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Purpose: Menopause is one of the risk factors for glaucoma. Animal studies have shown significantly worse visual function in ovariectomized (OVX) rodents after optic nerve injury. There is evidence the estrogen may interaction with retinal ganglion cell (RGC) and retinal astrocytes. However, the gene expression changes in RGC and retinal astrocytes after loss of estrogen by menopause have not been explored.

Method: Young (2-4months) female Brown Norway rats (Charles River) equally divided into naïve (n=6) and OVX (n=6) groups. The retinas have been dissected and dissociated. RGC and astrocytes were sorted, and their mRNA were isolated for bulk RNA sequencing. Principle Component analysis (PCA) was used for verifying sample purity. DESeq2 was used for finding differentially expressed genes between OVX and control group in RGC and Astrocyte separately. Gene Ontology (GO) Enrichment Analysis and (Kyoto Encyclopedia of Genes and Genomes) KEGG pathway analysis were performed.

Results: Samples are clustered and separated by cell type along PC1, capturing 39.5% of the variation. RGC samples exhibited significantly higher counts for RGC specific markers and no photoreceptor marker expression. The RGC OVX samples showed 135 upregulated genes (0.91%) and 130 downregulated genes (0.87%) compared to the RGC control group. The Astrocyte OVX group had 143 upregulated genes (0.96%) and 54 downregulated genes (0.36%) compared to the astrocyte control group (adjusted $p < 0.01$, $\log_2$ fold change > 0.5). Both groups showed a significant increase in immune receptor activity and purinergic nucleotide receptor activity (adjusted $p < 0.05$, $\log_2$ fold change > 0.5). Analysis of the top 40 gene pathways revealed distinct clusters correlated within RGC (22 pathways) and astrocyte groups (13 and 7 pathways), (adjusted $p < 0.01$, $\log_2$ fold change > 0.5). Overlapped pathways between RGC and astrocytes include the TNF pathway, NFκB pathway, IL-7 pathway, MAPK pathway, and pathways in cancer. In the TNF pathway, 17 genes overlapped between the two groups, playing roles in leukocyte recruitment and activation, inflammatory cytokine production, and cell survival. In the NFκB pathway, 11 overlapping genes are responsible for apoptosis, inflammation, and cell survival.

Conclusion: Our results suggest that OVX induces significant changes in gene expression and pathway activations related to immune and inflammatory responses in RGCs and astrocyte cells. These alterations after OVX suggest a systemic adjustment in the retinal environment, potentially affecting the response of these cell populations to injury or disease. Future research should be conducted in vitro to better understand the transcriptomic changes affected by estradiol (E2).

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Introduction and Background

Glaucoma

Glaucoma, the primary cause of irreversible blindness globally, is expected to affect over 112 million people by 2040 (Tham et al., 2014). While glaucoma is a group of heterogeneous eye conditions (Jonas et al., 2017), it manifests with a specific pattern of visual field decline that begins with progressive loss of peripheral vision. In its advanced stages, there are notable physical changes at the posterior eye region known as the optic nerve head (ONH). These changes include posterior bowing of the lamina cribrosa as well as thinning of the retinal nerve fiber (RNFL) and retinal ganglion cell (RGC) layers in the retina (Smith, 2003). The axons of the RGCs exit the eye and form the optic nerve, which relays visual information from the eye to the brain; it is the loss of these cells which leads to the visual impairments observed in glaucoma.

In the USA, Europe, Africa, and Australia, primary open-angle glaucoma (POAG) is the most common subtype of glaucoma (Zhang et al., 2021), (Stamper et al., 2009). A major risk factor of POAG is elevated intraocular pressure (IOP), with ocular hypertension, defined as IOP over 21 mmHg, being the most significant causal risk for POAG. The only current treatment to slow the progression of vision loss in glaucoma is lowering IOP, regardless of the initial IOP of a patient (Weinreb et al., 2014).

Sex as an Important Aspect in Glaucoma

Additional risk factors of POAG include: demographics (ie. age), genetics, and vascular diseases (Smith, 2003). While sex is currently not considered a risk factor for POAG (Smith, 2003), (Grzybowski et al., 2020), it is important to explore how differences in sex may be related to the onset of glaucoma. The National Eye Institute (NEI) states that in the USA, women have a higher prevalence of all glaucoma types compared to men (NEI, 2010). Currently, an estimated 59% of the global glaucoma patient population is female, which equates to roughly 41.3 million patients (Quigley, 2006). In addition, women with glaucoma often report more severe visual

impairment, and they are 24% less likely than men to seek treatment (Quigley, 2006). This indicates that glaucoma's impact on women may be underrepresented in the clinical population. While age increases glaucoma risk, and older women exhibit a higher prevalence than men, life expectancy doesn't fully account for this trend, as middle-aged women also show a slightly higher prevalence. Thus, there may be sex-specific risk factors worth focusing on in glaucoma research (Douglass et al., 2023).

Menopause signals a shift in sex hormones, including estrogen, in women, but it is scarcely researched as a specific glaucoma risk factor. Intraocular pressure (IOP), the main modifiable glaucoma risk, is affected by menopause. Postmenopausal women have IOP levels 1.5-3 mmHg higher than their premenopausal counterparts (Weinreb et al., 2014; Panchami et al., 2013), while those on hormone replacement therapy (HRT) had 0.5-3 mmHg lower IOPs (Vajaranant et al., 2016; Altintaş et al., 2004; Affinito et al., 2003).

Preclinical research further indicates a gap in glaucoma research in females, with 78% of studies of drug effects on rodent behavior exclusively using male subjects (Hughes, 2019). Such male-centric research hinders the understanding of potential sex differences in disease causes or drug responses. Studies on both sexes could reveal differences in disease development, progression, and treatment (Douglass et al., 2023). Recent evidence suggests that there may be sex-specific factors for developing glaucoma (Douglass et al., 2023).

Dr. Allen and Dr. Feola used an optic nerve crush (ONC) model to induce a mild injury to the RGC axons in rats to assess how surgical menopause via ovariectomy (OVX) impacted visual acuity as measured by the optomotor response. They found that 12 weeks after ONC injury, OVX animals exhibited a 31.7% decrease in visual acuity relative to the Sham animals. RGC function was measured by examining the scotopic threshold response (STR), a part of the

dark-adapted full-field electroretinogram response. ONC notably impacted the function of the retinal ganglion cell, with a 50.9% reduction in the negative STR amplitude (Allen et al., 2021); however, there was no significant difference between OVX and Sham animals. Dr. Feola also found that ONC correlated with thinning of the RNFL (Feola et al., 2019). Additionally, there is a study showing that 10β,17β-dihydroxyestra-1,4-dien-3-one (DHED), which is a 17β-Estradiol (E2) eye drop, significantly lowers the number of apoptotic RGC cells in treated mice compared to sham mice (Prokai-Tatrai et al., 2013). Additionally, OVX rats show better visual function after receiving DHED eye drops, which indicates the neuroprotective effects of E2 on OVX eyes (Feola et al., 2023). These results indicate that OVX and estrogen likely play a role in visual function and RGC survival after injury. However, we currently do not know the underlying mechanism of how OVX affects the retina on transcriptomic level.

Retinal Ganglion Cell (RGC) and Retinal Astrocytes

Retinal Ganglion Cell (RGC) viability is essential for visual function, and the loss of these cells is one of the main focuses in glaucoma studies. Elevated IOP obstructs axonal transport at the optic nerve head, limiting protein delivery from the RGC to the axon and decreasing neurotrophic supply to the cell body. This reduction can impair RGC function and lead to cell death in glaucoma (Smith, 2003).

RGCs are directly supported by retinal astrocytes, a type of glial cell that helps maintain homeostasis in the central nervous system (CNS) (Verkhratsky & Nedergaard, 2018). Retinal astrocytes release ciliary neurotrophic factor (CNTF), which has been studied for its potential for neuroprotection, including cell neuronal cell differentiation, neurite outgrowth, and sustaining cell viability (Pasquin et al., 2015; Yoo et al., 2021). They aid RGCs by releasing growth factors, reabsorbing neurotransmitters, and maintaining ionic balances.

Estrogen, Astrocytes and RGC

Astrocytes express membrane-associated ERα and ERβ receptors as well as the G protein-coupled estrogen receptor (GPR30) (Garcia-Segura et al., 1999). Both ERα and ERβ receptors can be activated, allowing calcium flux in cells via the phospholipase C (PLC)/inositol trisphosphate (IP_3) pathway. GPR30, signaling by estrogen in astrocytes, has been shown to help facilitate the neuroprotective effects of estrogen (Chaban et al., 2004). Additionally, estrogen plays an anti-inflammatory role in astrocytes, reducing inflammatory markers like IL-6 and controlling neuroendocrine-regulating molecules (Fuente-Martin et al., 2013). Besides the effect of estrogen on astrocytes, recent findings suggest that astrocytes may protect RGCs via EGFR signaling, potentially promoting RGC survival. Notably, EGFR signaling in astrocytes also mediates the up regulation of complement C3, which shields RGCs from high IOP (Yoo et al., 2021).

Therefore, the loss of estrogen after menopause may induce changes in the retina that make the eye more susceptible to developing glaucoma. However, the impact of menopause on astrocytes remain unexplored, which could offer glaucoma treatment insights, especially for postmenopausal women.

Motivation and goal

Current studies have pointed the way toward interaction among estrogen, astrocytes, and RGC. We hypothesize that the loss of estrogen via menopause influences the underlying cellular gene expression profile of astrocytes and RGC in the retina. We use bulk RNA-sequencing on sorted RGC and astrocytes from rats to explore pathway gene expression changes, with particular focus on changes in the inflammatory, apoptosis, and estrogen signaling pathways.

Methods and Material

Animals

All protocols conform to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Atlanta VA Medical Center IACUC. We used 12 young aged (3-4 months) female Brown Norway rats (Charles River), equally divided into naïve (n=6) and ovariectomized (OVX; n=6) groups.

Surgical Menopause

OVX was used to surgically induce a post-menopausal state. To perform the procedure, animals were given isoflurane (2–4%) for sedation and then positioned on a warm pad. A minor cut was made on the dorsal side, allowing for the isolation of both fallopian tubes and ovaries. The tubes were then sealed using cauterization, and subsequently, the ovaries were excised (Feola et al., 2019). Animals were aged 8-10 weeks after OVX before tissue collection. Naïve animals were aged a similar amount of time to ensure cohorts were age-matched.

Retinal dissection and dissociation

For tissue collection, animals were euthanatized using carbon dioxide, and the eyes were enucleated. The eyes were positioned securely with tweezers, and scissors were employed to sever the anterior segment. The retina was then expelled using another pair of tweezers once the lens had been removed. The retinas from a single rat were combined and immersed in ice-cold pH 7.4 ACSF solution (125 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 25 mM D-glucose with carbogen [95% O2, 5% CO2]). The retinas were kept on ice in ACSF-filled tubes until they were transferred into a prepared Papain solution also oxygenated with carbogen (using the Worthington Papain Dissociation Kit). A single-cell suspension was achieved by dissociating the cells in a 37°C water bath with carbogen for 15

minutes. Figure 1 shows an overview of the experimental design and procedures of this experiment.

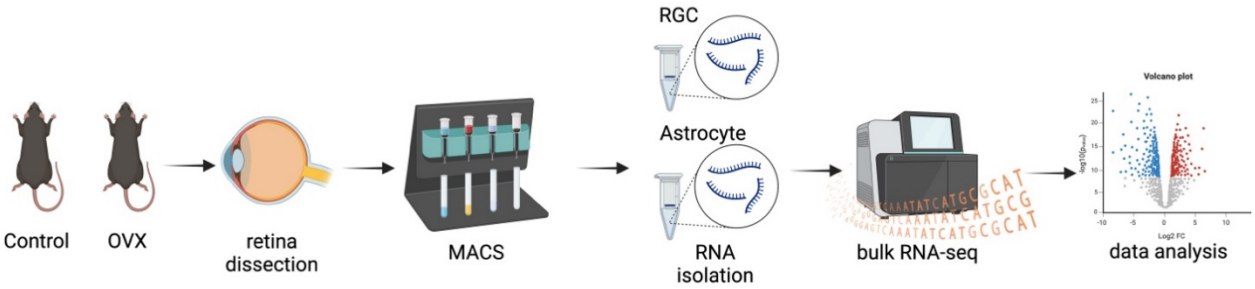

Figure 1: Experimental Workflow: created with BioRender.com. OVX – ovariectomy; MACS - magnetic-activated cell sorting; RGC- retinal ganglion cell.

RGC and Astrocyte isolation

For cell isolation, we used a revised protocol based on Holt et al., 2019. The cell suspension was first incubated at 4°C with 30µL CD90.1 microBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes on a rocker. LS columns were placed on a QuadroMACS Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) and prepared with 0.5% BSA PBS. The surplus microbeads were then cleared by centrifugation at 1200g for 5 minutes after adding 1mL of 0.5% BSA PBS. The supernatant was discarded, and the pellet was resuspended in 3mL of 0.5% BSA PBS and loaded onto the LS columns. The flowthrough was collected in 15mL conical tubes. This washing step was repeated twice to ensure the removal of non-RGC cells. Subsequently, 5mL of 0.5% BSA PBS was applied to the columns, and the RGC cells were pushed into new 15mL conical tubes using MACS plungers.

The collected flow-through was centrifuged for 5 minutes at 1200g, the supernatant was discarded, and the pellet was resuspended in 1mL of 0.5% BSA PBS. The suspension was then incubated with 30µL Anti-GLAST Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°C for 15 minutes on a rocker, followed by an incubation with 30µL ACSA-1 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The procedure was repeated with CD 90.1 (Miltenyi Biotec, Bergisch Gladbach, Germany) for the isolation of the RGCs. The resultant

flow-through containing astrocytes was collected in 15mL conical tubes, and 1mL of Trizol Reagent (Thermo Fisher, Walthma, MA) was added after centrifugation at 1200g for 5 minutes and subsequent supernatant removal. The samples were then stored at -20°C.

RNA isolation

RGC and astrocyte RNA were isolated by using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). The procedures were followed as stated in the RNeasy mini handbook. The samples were diluted by 30 μL RNase-free water. The RNA concentration (30.0 ± 4.2 ng/ μL) was quantified by using spectrophotometry Nanodrop 2000 (Thermo Fisher, Walthma, MA). The purity of RNA was verified based on the A260/A280 ratio (~1.8).

RNA-sequencing

RNA quality was assessed using a TapeStation 4200 (Agilent), and 10 ng of total RNA was used as input for cDNA synthesis. Library preparation were Clontech SMART-Seq v4 Ultra Low Input RNA Kit with Illumina's Nextera XT DNA Library Preparation Kit. The library has been validated by capillary electrophoresis on a Fragment Analyzer (Agilent), pooled at equimolar concentrations. RNA-sequencing was performed by the Emory National Primate Research Center (NPRC) Genomic Core Service for bulk RNA-sequencing on Illumina NovaSeq 6000, yielding 30 million reads per sample on average.

Alignment was performed using STAR version 2.7.3a, and transcripts were annotated using Rnor_6.0 Ensembl release 95. Transcript abundance estimates were calculated internally to the STAR aligner using the htseq-count algorithm. DESeq2 (Love et al., 2014) was used for analysis, producing per gene differential expression statistics and both a normalized read count table and a regularized log expression table.

Data Analysis

Pair-end RNA-seq reads in Fastq format were mapped to the reference genome using with its default settings. The SubRead package's FeatureCounts tool was utilized to generate

gene-level counts. For expression analysis, the DESeq2 R package (Love et al., 2014) was employed. Furthermore, by utilizing the BioMart package (Durinck et al., 2009), gene symbols were converted to ENSEMBL ID aligning with the “rnorvegicus_gene_ensembl” dataset. DESeq2 data was grouped into RGC OVX vs. RGC Control, and Astrocyte OVX vs. Astrocyte Control.

We performed an initial principal component analysis (PCA) for dimensionality reduction on normalized count data using the “prcomp” package in R Studio to observe clustering of cell types. To verify separation of cell types, we examined RGC markers Thy1, POU4F1, and SNCG. While not specific to RGCs, the expression of these markers is expected to be higher in RGCs than in astrocytes. To ensure we did not have contamination of other cell types, we also examined PDE6A that encodes a rod outer segment protein (a marker of photoreceptors), which 0 counts for all samples.

Next, we examined volcano plots to identify the upregulated and downregulated genes of each cell type (OVX compared to Naïve). In brief, we performed DESeq2 analysis (Love et al., 2014) separately for RGC samples and astrocyte samples, referencing their control groups (Naïve cohorts). To understand the profile of differentially expressed genes, volcano plots were used to display individual gene expression differences within each cell type, with a p-value cut-off of 0.01 and a log2 fold change of 0.5. The "org.Rn.eg.db" dataset (Marc, 2019) was used for Gene Ontology (GO) Enrichment Analysis (Thomas et al., 2022). ShinyGo 0.77 was used for (Kyoto Encyclopedia of Genes and Genomes) KEGG pathway analysis (Kanehisa, 2000).

Results

RGC and retinal Astrocytes purity

To ensure successful cell sorting and sample clustering, we performed Principal Component Analysis (PCA) on normalized count data from both RGC and astrocyte RNA-seq results. Samples are clustered and separated by cell type along PC1, which captured 39.5% of the variation (**Figure 2.A**). We examined a series of markers specific to RGCs, including Thy1, Pou4f1, and SNCG (Langer et al., 2018). Even though astrocyte samples still expressed these genes, RGC samples showed significantly higher (p-value<0.05) expression than astrocyte

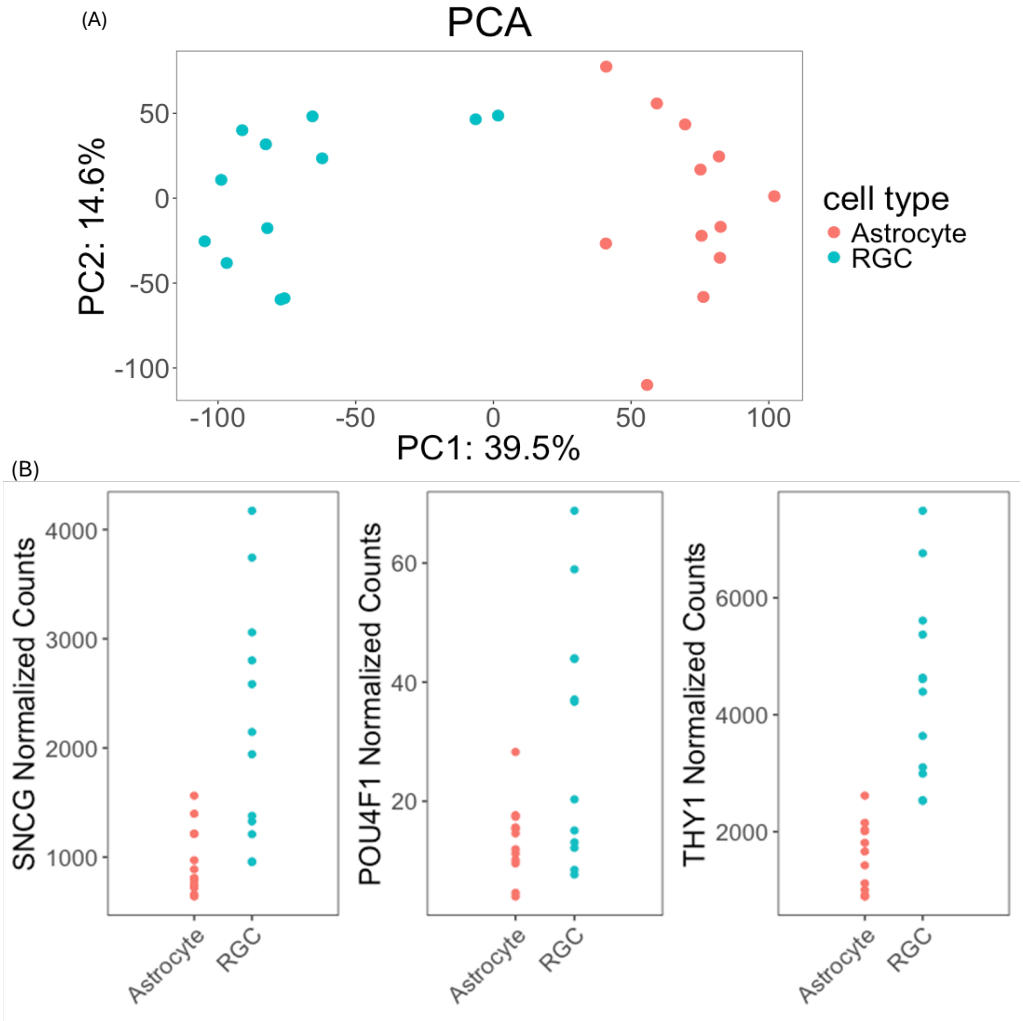

Figure 2: *(A) Principal Components Analysis (PCA) of normalized count data from both RGC group and astrocyte: samples are clustered based on cell type (B) RGC markers: Thy1, POU4F1, and SNCG are express higher in RGC samples.*

samples, indicating a relatively high purity of RGC samples (**Figure 2.B).** Lastly, we found that astrocyte and RGC cell populations had zero expression of the photoreceptor maker PDE6A (data not shown).

Differentialy Expressed Genes between OVX and Control

Compared to the RGC control group, the RGCs from the OVX group had 135 upregulated genes (0.91%) and 130 downregulated genes (0.87%). In the astrocytes, we found that the astrocyte OVX group had 143 upregulated genes (0.96%) and 54 downregulated genes (0.36%) compared to astrocytes from the control group (**Figure 3**).

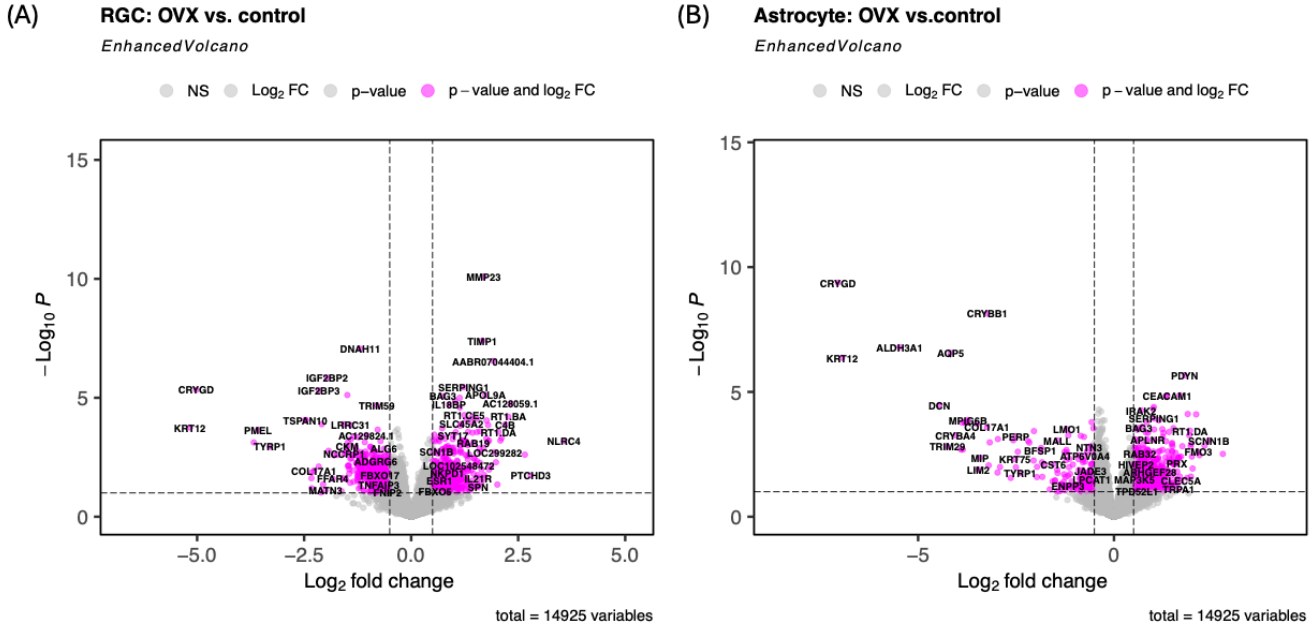

Figure 3: Volcano plots with p-value cut-off =0.01, log_2 fold change > 0.5. (**A**) RGC OVX vs. Control (normalized to RGC Control) (**B**) Astrocyte OVX vs. Control (normalized to Astrocyte Control). RGC – retinal ganglion cell; OVX – ovariectomy.

Pathway Changes

To better understand the functional implication of differential gene expressions, we performed Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

We mapped out the top 20 GO results with an adjusted p-value smaller than 0.05 and a $\log_2$ fold change > 0.5, combining all three types: Biological Processes (BP), Molecular Functions (MF), and Cellular Components (CC). We noticed that both the RGC and astrocyte OVX groups have a significant increase in immune receptor activity and purinergic nucleotide receptor activity (**Figure 4**).

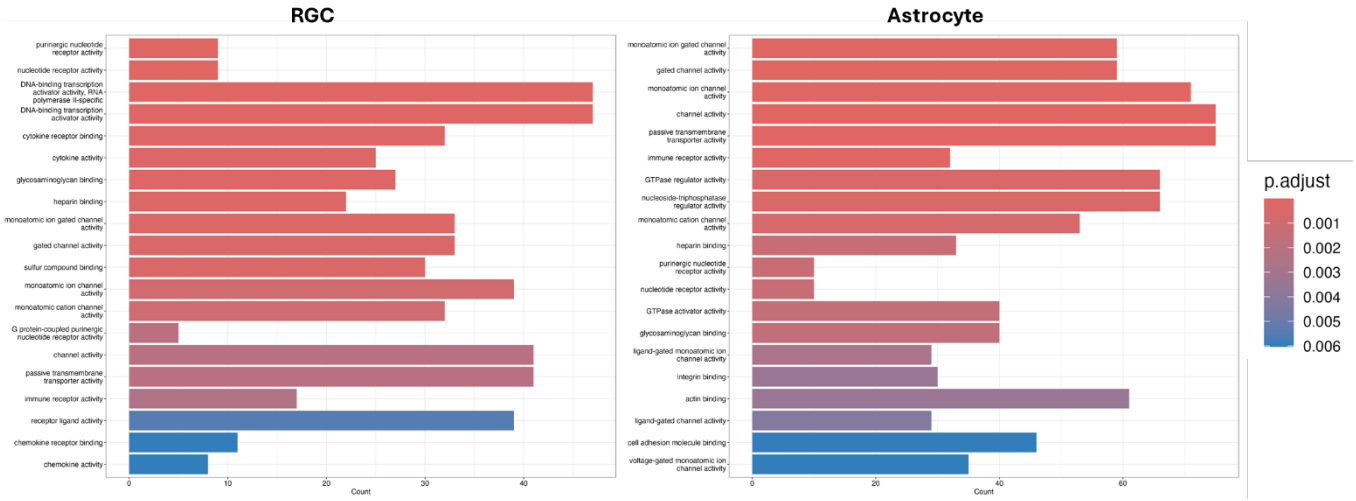

Figure 4: Gene Ontology (GO) analysis: Top 20 differential expressions of combined biological processes, cellular components, and molecular functions annotation. (Left) RGC. (Right) Astrocyte. P-value cut-off =0.05, log2 fold change= 0.5

For a better understanding of the pathways in which differentially expressed genes are involved, we performed a KEGG pathway analysis with an adjusted p-value smaller than 0.01 and a $\log_2$ fold change >0.5. We selected the top 40 pathways and visualized them in a dot plot, with scaled numbers of genes in each pathway and gene expression differentiation (**Figure 5**). After separately comparing OVX samples and Control samples in each group, we identified a cluster of 22 pathways that are correlated in the RGC group as well as a cluster of 13 pathways and a cluster of 7 pathways in the astrocyte group. The network connections among pathways exclude the randomness of single pathway alterations and highlight systemic changes. Several of these shared clusters in both the RGC and astrocyte cell populations after OVX were along the

immune responses and inflammatory responses (**Figure 6**). There are 5 pathways overlapping between the RGC and Astrocyte groups: TNF pathway, NFκB pathway, IL-7 pathway, MAPK pathway, and pathways in cancer. The IL-7 pathway is downstream of the TNF pathway, while the other pathways are directly connected with residuals of the top differentially expressed gene pathways.

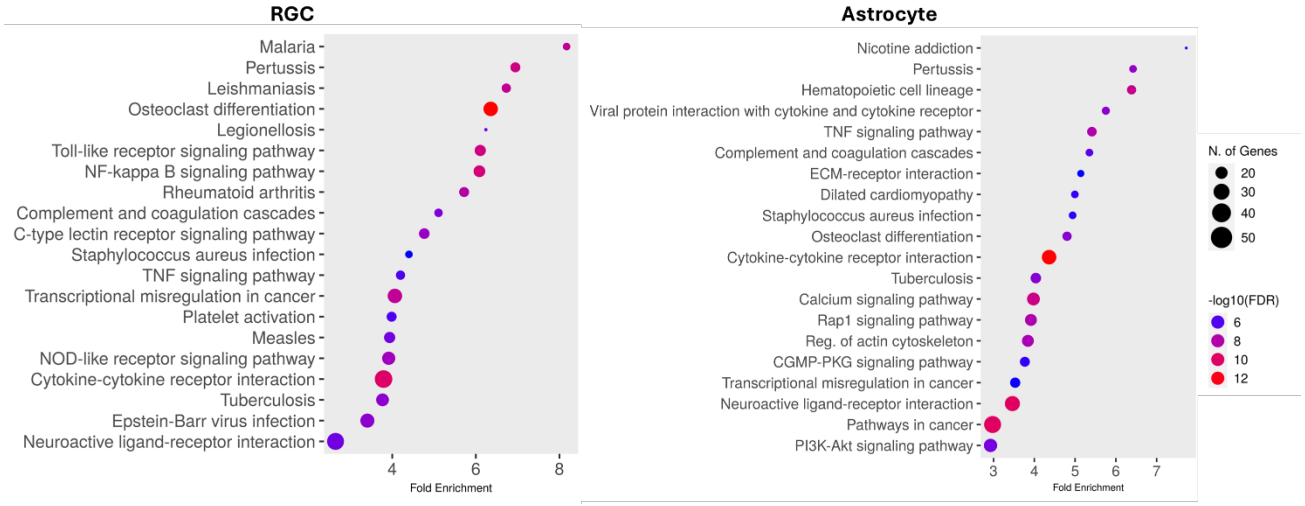

Figure 5: KEGG pathway analysis: Top 20 differential pathways. (Left) RGC. (Right) Astrocyte. P-value cut-off =0.01, log2 fold change= 0.5

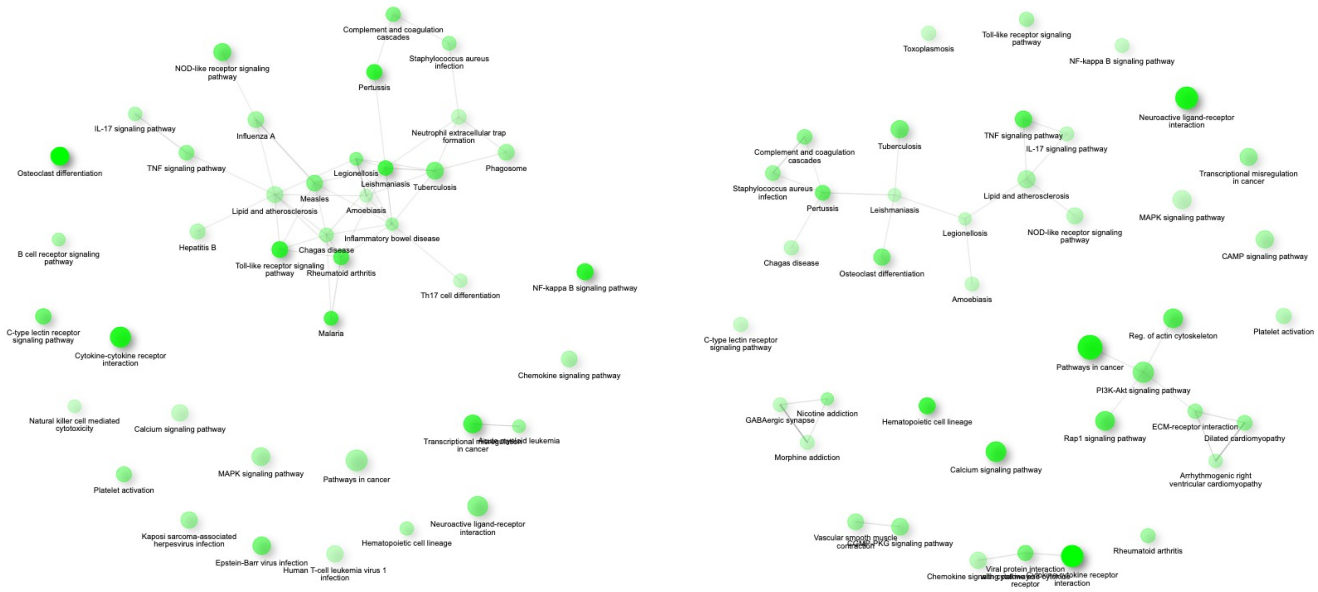

Figure 6: network plot of KEGG pathway analysis (Left) RGC. (Right) Astrocyte.

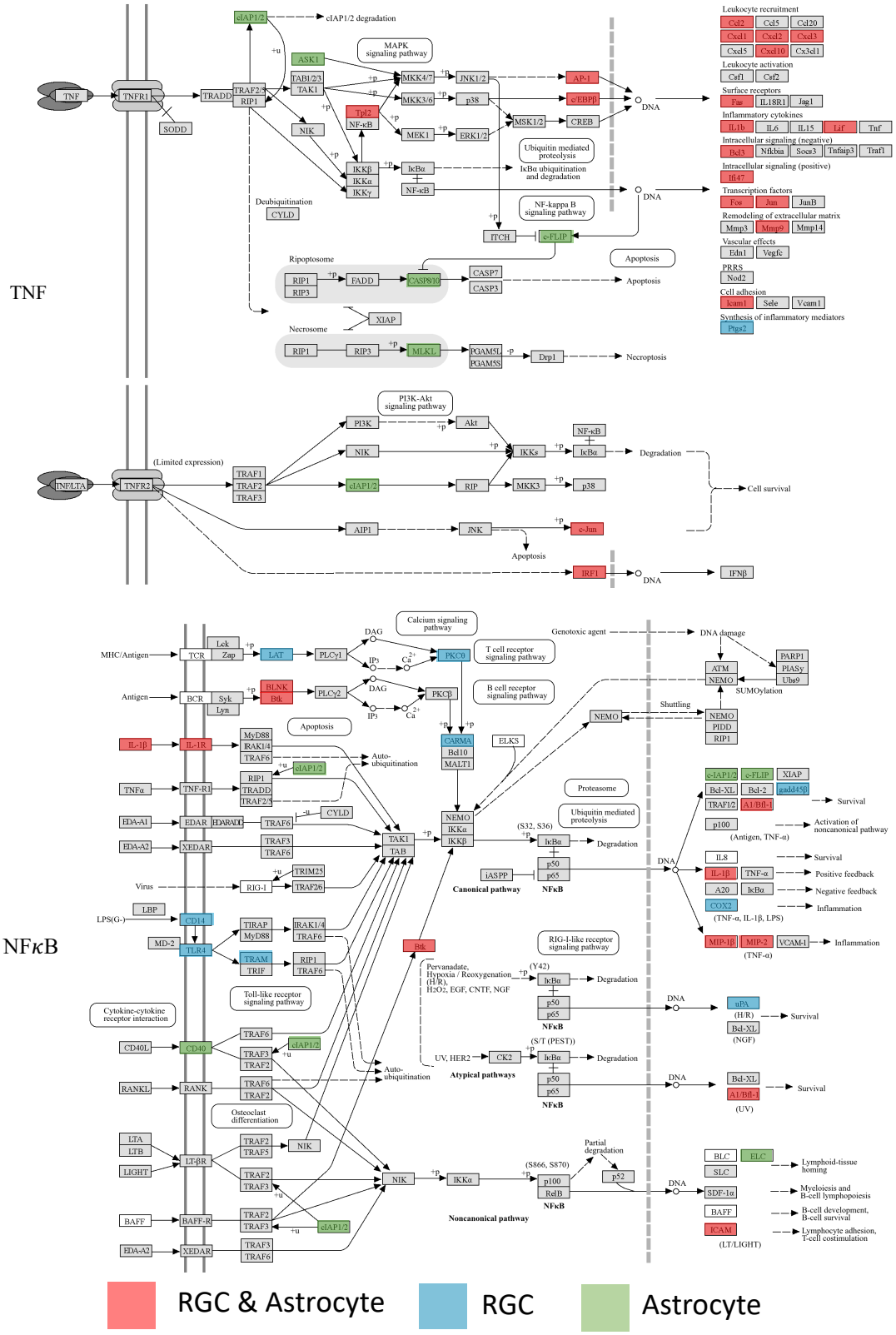

Figure 7: *(*Top*)TNF Pathway. (Bottom) NFκB* Pathway. Red stands for overlapping differentially genes between RGC and Astrocyte. Blue stands for only RGC. Green stands for only Astrocyte

Here, we examined the TNF and NFκB pathways in detail (**Figure 7**). In the TNF pathway, the RGC group had 20 differentially expressed genes, and the astrocyte group had 29. recruitment and activation, inflammatory cytokine production, and cell survival. We observed that the CASP8/10 and MLKL genes in astrocytes, which are responsible for triggering apoptosis and necroptosis, are expressed significantly differently.

In the NFκB pathway, after OVX, the RGCs had 22 differentially expressed genes, while the astrocytes had 19, with 11 overlapping genes responsible for apoptosis, inflammation, and cell survival. It is worth mentioning that the MIP-1β and MIP-2 genes are differentially expressed in both OVX groups.

Discussion

Our study investigated the effects of ovariectomy (OVX) on retinal ganglion cells (RGCs) and astrocytes cell populations through bulk RNA-seq analysis. Our PCA analysis demonstrated gene expression patterns that allowed clustering of RGC and astrocyte cell populations, while DESeq2 analysis revealed significant gene expression changes in both cell types post-OVX.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) unveiled the functional implications of these differentially expressed genes. Both RGC and astrocyte OVX groups exhibited a significant increase in immune receptor and purinergic nucleotide receptor activities, pointing to altered immune responses and potential inflammatory changes within the retina post-OVX.

RGC and astrocyte cells from the OVX groups exhibited significant changes in immune receptor activity. Purinergic nucleotide receptor activity might indicate inflammatory responses and changes in the immune system. This suggests a possible alteration in the regulation of

immune responses post-ovariectomy, potentially leading to inflammatory responses and changes in the immune system within the retina. Cytokine and cytokine receptor activities were also differentially expressed in RGC cells after OVX, which specifically suggests alterations in the regulation of immune responses (Janeway et al., 2001). While changes in immune receptor activity can alter molecular functions, changes in purinergic nucleotide receptor activity not only initiate molecular functions changes but also affect biological processes. Purinergic nucleotide receptors bind nucleotides like ATP and are involved in processes such as neurotransmission and the immune response. The ligand-binding activity can influence cell function, inflammation, and apoptosis (Chen et al., 2014; Burnstock & Boeynaems, 2014).

The identification of correlated pathway clusters in both cell types emphasized systemic changes, particularly in immune and inflammatory responses. Detailed examination of the TNF and NFκB pathways revealed differential expression of genes involved in leukocyte recruitment, inflammatory cytokine production, and cell survival. TNFα is also a potent cytokine that can stimulate the production of chemokines such as MIP-1 β and MIP-2 (Idriss & Naismith, 2000), (Widmer et al., 1993). TNFα can initiate a signaling cascade that leads to the activation of various intracellular signaling pathways, including NFκB(Idriss & Naismith, 2000). Both RGC and astrocyte cells after OVX showed alterations in this signaling pathway (**Figure 6**).

In the OVX group, the astrocyte cell population showed CASP8/10 and MLKL gene upregulation, which are crucial for apoptosis and necroptosis. The presence of differentially expressed MIP-1β and MIP-2 genes in both cell populations after OVX further indicated the effects of OVX on inflammatory and immune response pathways.

In conclusion, our findings demonstrate that OVX induces significant changes in gene expression and pathway activations related to immune and inflammatory responses in RGCs and

astrocyte cells. These alterations suggest a systemic adjustment in the retinal environment, potentially affecting its function and response to injury or disease after OVX. The differences in gene expression after OVX may be the underlying factor for worse visual function after OVX following RGC injury (Allen et. al., 2021). This study provides valuable insights into the molecular effects of surgical menopause on cell populations within the retina, which emphasizes the importance of considering hormonal status in retinal research and potential therapeutic strategies. Further, it is the first study to specifically examine the gene expression profiles of RGCs and astrocytes after menopause.

In the future, we can further support our findings in vitro by culturing primary control and OVX astrocytes. We could apply different dosages of Estradiol E2 and measure mRNA expression changes in selected gene markers identified here to better understand the transcriptomic changes in the retina after OVX and screen potential therapeutic interventions. Additionally, we might combine this with models of optic nerve crush or ocular hypertension to explore RGC and astrocyte gene expression in response to RGC injury after OVX.

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