*Original Article*

JCBFM

Journal of Cerebral Blood Flow & Metabolism
2021, Vol. 41(9) 2311–2328

DOI: 10.1177/0271678X21999553
journals.sagepub.com/home/jcbfm


# Transcriptome analysis reveals sexual disparities in gene expression in rat brain microvessels

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## Abstract

Sex is an important determinant of brain microvessels (MVs) function and susceptibility to cerebrovascular and neurological diseases, but underlying mechanisms are unclear. Using high throughput RNA sequencing analysis, we examined differentially expressed (DE) genes in brain MVs from young, male, and female rats. Bioinformatics analysis of the 23,786 identified genes indicates that 298 (1.2%) genes were DE using False Discovery Rate criteria (FDR; $p < 0.05$), of which 119 (40%) and 179 (60%) genes were abundantly expressed in male and female MVs, respectively. Nucleic acid binding, enzyme modulator, and transcription factor were the top three DE genes, which were more highly expressed in male than female MVs. Synthesis of glycosylphosphatidylinositol (GPI), biosynthesis of GPI-anchored proteins, steroid and cholesterol synthesis, were the top three significantly enriched canonical pathways in male MVs. In contrast, respiratory chain, ribosome, and 3 ´-UTR-mediated translational regulation were the top three enriched canonical pathways in female MVs. Different gene functions of MVs were validated by proteomic analysis and western blotting. Our novel findings reveal major sex disparities in gene expression and canonical pathways of MVs and these differences provide a foundation to study the underlying mechanisms and consequences of sex-dependent differences in cerebrovascular and other neurological diseases.

## Keywords

Cerebral microvessels, RNA-Seq, gene expression, proteomics, sexual dimorphism

Received 3 February 2021; Revised 3 February 2021; Accepted 7 February 2021

## Introduction

Recent advances have established the pivotal role of cerebral microvessels (MVs) composed of end-arterioles, capillaries, and venules, in maintaining normal brain functioning and in contributing to development of cerebrovascular and neurological diseases.[1–3] Cerebral MVs are particularly susceptible to aging,[3,4] systemic diseases such as the metabolic syndrome,[5–7] and arterial hypertension.[8] The consequent dysfunction of the microvasculature leads to impaired nutrient supply to neurons and glia,[2] blood-brain barrier (BBB) disruption,[9] and detrimental vascular remodeling.[8] In addition, microvascular function and responsiveness to physiological stress varies considerably between the sexes[10,11] and may account for differential susceptibility and severity of neurological diseases in

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males and females. However, little is known about sex-based differences in gene expression in the cerebral MVs. Even though males and females share essentially the same genetic code, gene expression profiles are different between the sexes.

We previously reported substantial sexual dimorphism in the mitochondrial metabolic protein profiles of young male and female rat MVs using a liquid chromatography/mass spectrometry (LC/MS)-based proteomic approach.[11] Interestingly, we observed that females displayed higher levels of mitochondrial proteins involved in energy production, mitochondrial membrane structure, as well as higher levels of antioxidants than males. Conversely, males had higher expression levels of mitochondria-destructive proteins. This previous study was limited to a specific group of proteins and did not examine the full extent of sexual dimorphism in the cerebral microvasculature.

In the present study, we used a high-throughput RNA sequencing (RNA-Seq) platform to study sexual disparities in the cerebral MVs of young male and female rats and the key findings have been validated by the LC/MS-based proteomic analysis, RT-qPCR and western blotting. RNA-Seq and proteomics are powerful, high-throughput technologies for identifying and quantifying RNA transcripts and proteins, respectively. Although RNA-Seq[12,13] and proteomic[11,14] approaches have been applied independently to the field of microvascular pathophysiology, to date there are no reported studies of sex-dependent differences in rat cerebral MVs by multiomic analyses. Therefore, we performed a comprehensive RNA-Seq characterization of sex-dependent differences in the brain MVs of Sprague-Dawley rats and validated these findings by LC/MS-based proteomic analysis, RT-qPCR and western blotting.

## Materials and methods

### Animals

Age-matched, male and female, Sprague-Dawley rats (10–12-weeks old) were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed according to guidelines of the Institutional Animal Care and Use Committee (IACUC) of Tulane University as well as the National Institutes of Health Office of Laboratory Animal Welfare guidelines. The IACUC Committee of Tulane University approved the protocols, which follow the Animal Research Reporting in Vivo Experiments guidelines. Animals had free access to food and water, ad libitum, and were housed in a facility with a standard light dark cycle.

### Microvessels isolation

Our previous studies[3,6,7,11] of mitochondrial respiration have shown that MVs are viable for ex vivo studies. The MVs were isolated as previously described[11] (Supplementary Material). The purity of the MV preparation was verified by light and electron microscopy, immunostaining, and western blotting.

### Alkaline phosphatase staining

An alkaline phosphatase (AP) staining method was applied to differentiate MVs in arterioles, capillaries, and venules using our previously adopted protocol.[11] Color development was observed under light microscopy with 20X magnification.

### RNA isolation and sequencing

Analysis of RNA-Seq samples was performed using the RNA-Seq Analysis Pipeline designed by the Tulane Cancer Center, Next Generation Sequence Analysis Core. Reads were pseudo aligned to the rat reference transcriptome (rnor6) and transcripts were quantified using kallisto.[15] Transcript abundances were collapsed to gene-level in each sample. Differential gene expression analysis was performed using sleuth, as described by Pimentel and colleagues.[16] Gene-level abundances were used as input for Gene Set Enrichment Analysis (GSEA)[17,18] against several Molecular Signatures Databases (MSigDB). The detailed methods of RNA isolation and sequencing are described in the Supplementary Material.

### Discovery-based quantitative proteomics, data analyses, and bioinformatics

The samples were prepared for discovery-based proteomic analysis, as described previously,[11] and the detailed proteomics approach is described in the Supplementary Material.

### Western blot analysis

Western blot analysis for MVs was performed as described previously,[7,19] and a brief description is included in the Supplementary Methods section.

### Real-time RT-qPCR

RNA isolation, and the gene amplification were performed by following the standard techniques. The list of primers that were used in this study are listed in Supplementary Figure 5. The detailed description is included in the Supplementary method section.

### Statistical analysis

Detailed approaches for analyzing RNA-Seq and proteomic data and criteria for statistical significance are included with the descriptions of these methods. Data from western blots and PCR were expressed as mean ± standard deviation. Male and female data sets for western blots and PCR values were assessed by the Shapiro-Wilke test for normality followed by either unpaired Student's t-test for normally distributed data or in a few cases by the non-parametric Mann-Whitney test. Use of the Mann-Whitney test is indicated in the figure legend. Since the western blot data were merged from two separate blots, we verified the Student's t-test using a two-way analysis of variance. $p < 0.05$ or greater was considered statistically significant.

## Results

### MV characterization

Consistent with our previous reports,[3,6,11] visual inspection by light microscopy (Figure 1(a)) and AP-staining (Figure 1(b)) showed that the isolated MVs from male and female rats contained a mixture of end arterioles, capillaries, and venules. Previous reports[3,6,11] from our group have also shown that MVs stained negative for markers of astrocytes and neurons.

### Gene expression and differentially expressed (DE) gene cluster analysis

A total of 23,786 genes were identified by RNA-Seq analysis in male and female rat MVs (Figure 1(c)). The initial analysis showed that expression was not statistically different in most genes examined but identified 298 (1.2%) critically important DE genes that were significantly (FDR < 0.05) different. The maximum Wald test qval (FDR adjusted p-value) of the significantly expressed gene was 0.049 and the minimum Wald test qval was $1.06 \times 10^{-59}$ (see Supplementary Data 1). More than half (60%; 179/298) of the significant DE genes exhibited greater levels in females and nearly 40% (119/298) were more prevalent in males (Figure 1(c)). The DE genes found by differential screening were those clustered according to the signal values of each gene in the sample. A heat map was generated to visualize the top 50 DE genes in each male or female MV subset (Figure 1(d)).

### Nucleic acid binding, enzyme modulator, and transcription factor were the top three gene functions in male MVs by PANTHER classification

Gene ontology (GO) analysis was used to identify the functional annotating genes engaged in the enrichment of biological processes, cellular components, and molecular functions. The overlapping DE genes in GO classification are presented in the Supplementary Table 1. Our analysis indicated that 17, 14, and 8 categories were involved in biological process, cellular component, and molecular function, respectively. The significant DE genes appeared in multiple categories for each classification. Next to each gene name there was a number in parentheses which showed the number of categories that the gene appears in. Using PANTHER classification, the distribution map of the 23 most significant gene functions showed that the regulation of these gene functions was more highly expressed in male MVs (Figure 2(a)). Nucleic acid binding, enzyme modulators, and transcription factors were the predominant gene functions in male MVs, indicated by the PANTHER classification (Figure 2(a)). The gene cluster analysis supported the data generated by PANTHER classification (Figure 2(c) to (e)). The gene cluster analysis indicated that the 38 genes involved in recombinational repair (Figure 2(c)) and the 16 genes participating in homologous recombination (Figure 2(d)), were abundantly expressed in male MVs. These two gene clusters usually influence the function of nucleic acid binding activity. Similarly, another gene cluster analysis revealed that, except for genes GTF2F1, TAF13, and TBPL2, most of the other 30 genes, basal transcription factors, were also highly expressed in male MVs (Figure 2(e)). Using the same PANTHER classification, the distribution map of the 17 most significant gene functions showed that the regulation of these gene functions was more highly expressed in female MVs (Figure 2(b)). Cytoskeletal protein, extracellular matrix protein, and transcriptional regulator were the top three gene functions in female MVs (Figure 2(b)).

### Significant gene functions were supported by LC-MS/MS-based proteomic analysis

As previously reported,[11] 1,871 proteins were quantified by proteomic analysis from male and female rat MVs. The initial analysis identified 198 (10.6%) proteins in significantly ($p < 0.05$) different quantities. Eighty-four percent (166/198) of the proteins exhibited greater levels in males and only 16% (32/198) were more prevalent in females (Figure 3(a)). The molecular functions of significant DE proteins are presented in Figure 3(b). As with RNA-Seq data, the nucleic acid binding (both DNA and RNA) was the top molecular function of the significant DE proteins. Nearly 32% (64/198) of the significant DE proteins were associated with nucleic acid binding. Interestingly, 86% (55/64) of the nucleic acid binding

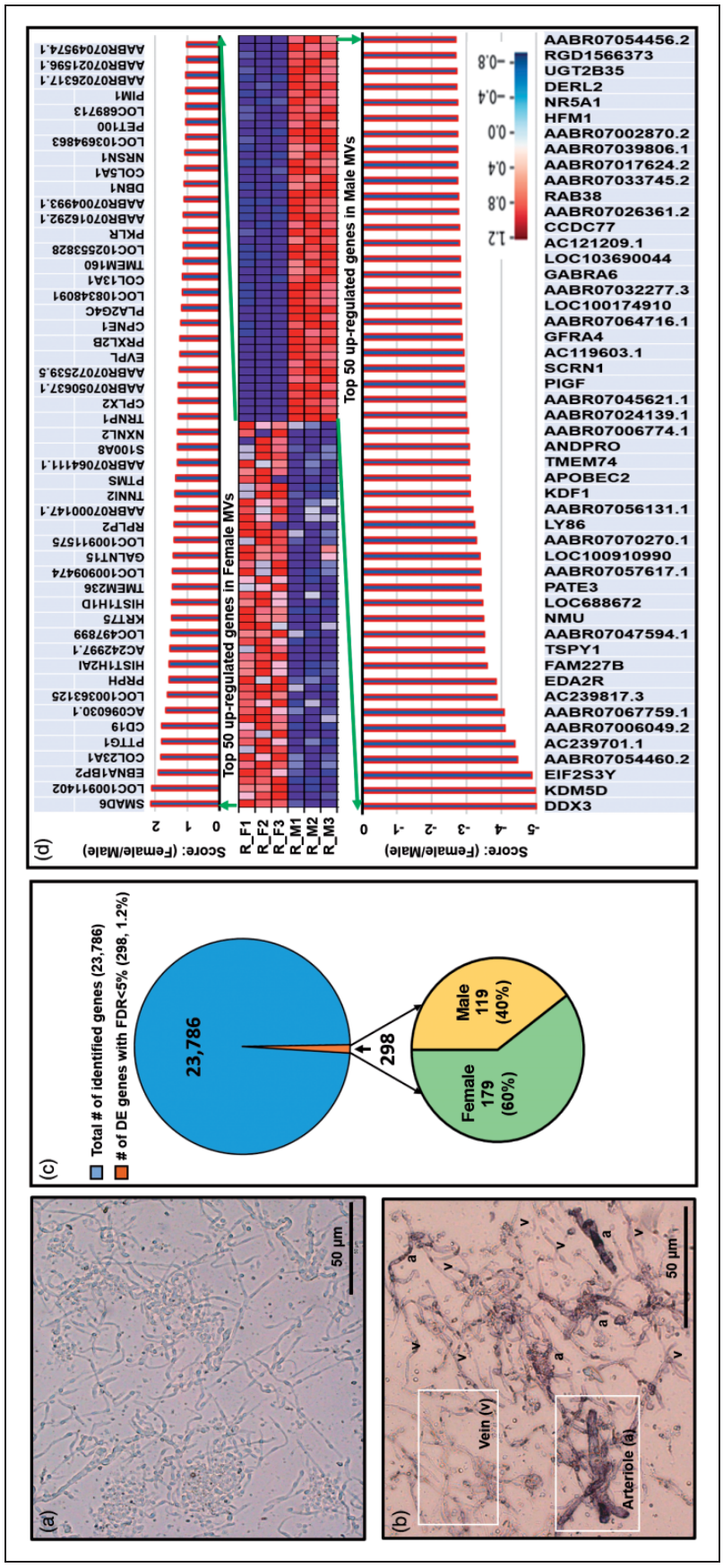


**Figure 1.** Isolation, characterization, and RNA-Seq analysis in brain MVs from male and female rats. (a) A representative image of the MVs isolated from Sprague-Dawley rats at 20X magnification. The isolated MVs contained of end arterioles, capillaries, and venules. (b) A representative image of alkaline phosphatase staining legitimate distinction between the arteriole (a) and venule (v) segments. (c) The diagram represents the total and significant (false discovery rate, FDR $< 5\%$) number of differentially expressed (DE) genes identified in female and male rat brain MVs. (d) The heat-map of the top 50 up-/down-regulated genes in female and male rat brain MVs. The dendrogram of DE genes were ranked in their scores determined by a computational Gene Set Enrichment Analysis (GSEA) method. The top panel represented the DE genes higher in female MVs and the bottom panel indicated the DE genes high in male MVs. Red denotes up-regulation, and blue denotes down-regulation of the DE genes. R: rat brain MVs. F: Female, and M: Male.

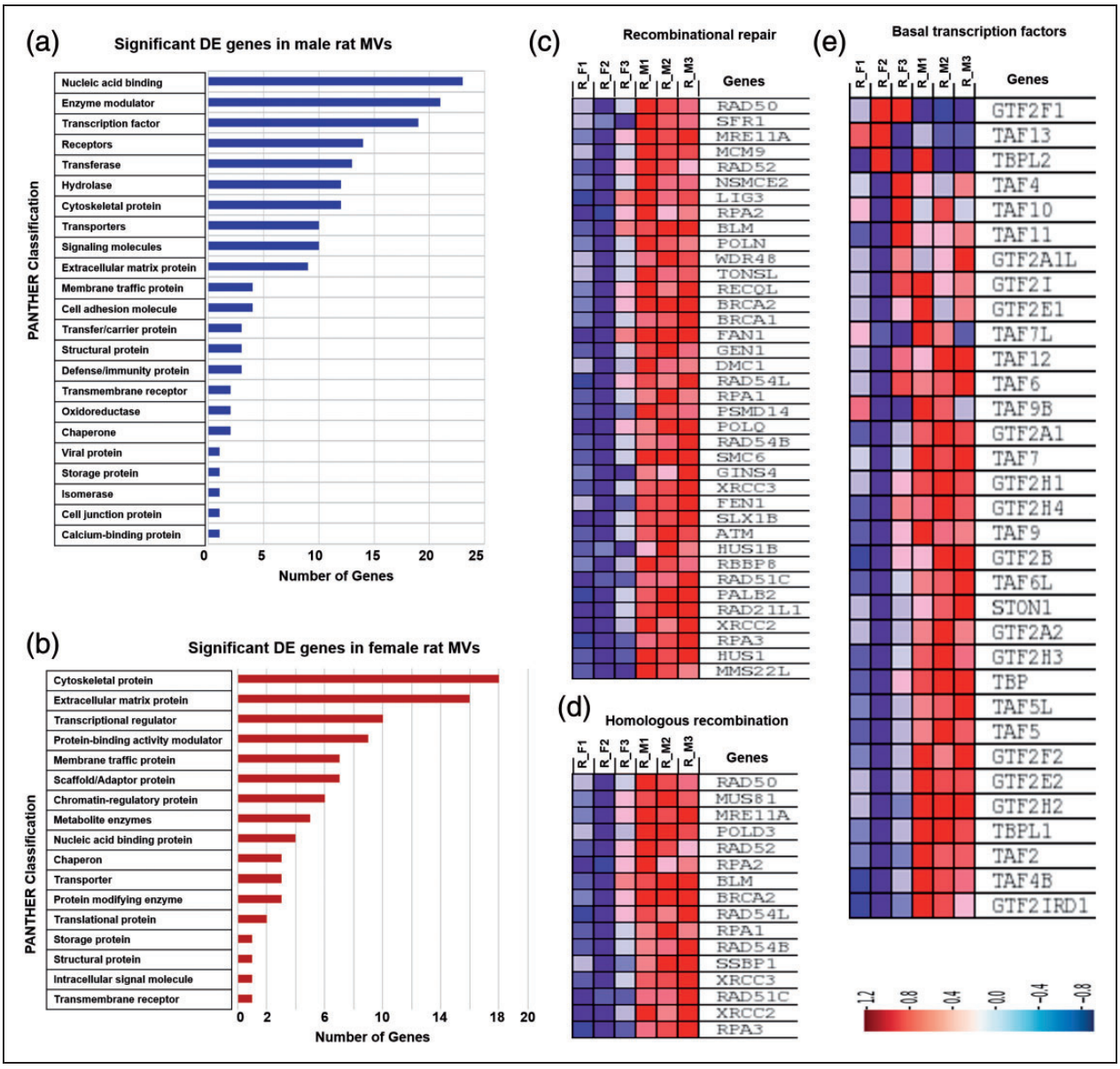


**Figure 2.** The functional classification of significant DE genes in rat MVs. (a) PANTHER classification system analyzed the gene functions of DE genes in male rat MVs. The top 23 gene functions and the number of DE genes in male in each category are presented. (b) Similarly, PANTHER classification system analyzed the gene functions of DE genes in female rat MVs. The top 17 gene functions and the number of DE genes in female in each category are presented. Heat map illustrating the expression pattern of DE genes in three different gene functions: (c) recombinational repair, (d) homologous recombination, and (e) basal transcription factors. These three gene functions were mostly belonging to the 'Nucleic acid binding' PANTHER class. R: rat brain MVs. F: Female, and M: Male. Red: up-regulated genes, Blue: down-regulated genes.

proteins were abundantly expressed in male MVs (Figure 3(b) and (c)). Similarly, the enzyme modulator was the 2nd highest gene function in male MVs (Figure 2(a)) and was further supported by the proteomic data, which indicated that a majority (89%, 25/28) of the enzyme modulator-associated significant DE proteins were highly expressed in male MVs (Figure 3(b)).

## *Kyoto Encyclopedia of Genes and Genomes (KEGG) database*

The KEGG database was used to systematically analyze the relationship between genes and their functions on a genome-wide basis, as an entire network; and the threshold for the significant pathways involved with the DE genes was set using Fisher's exact test and

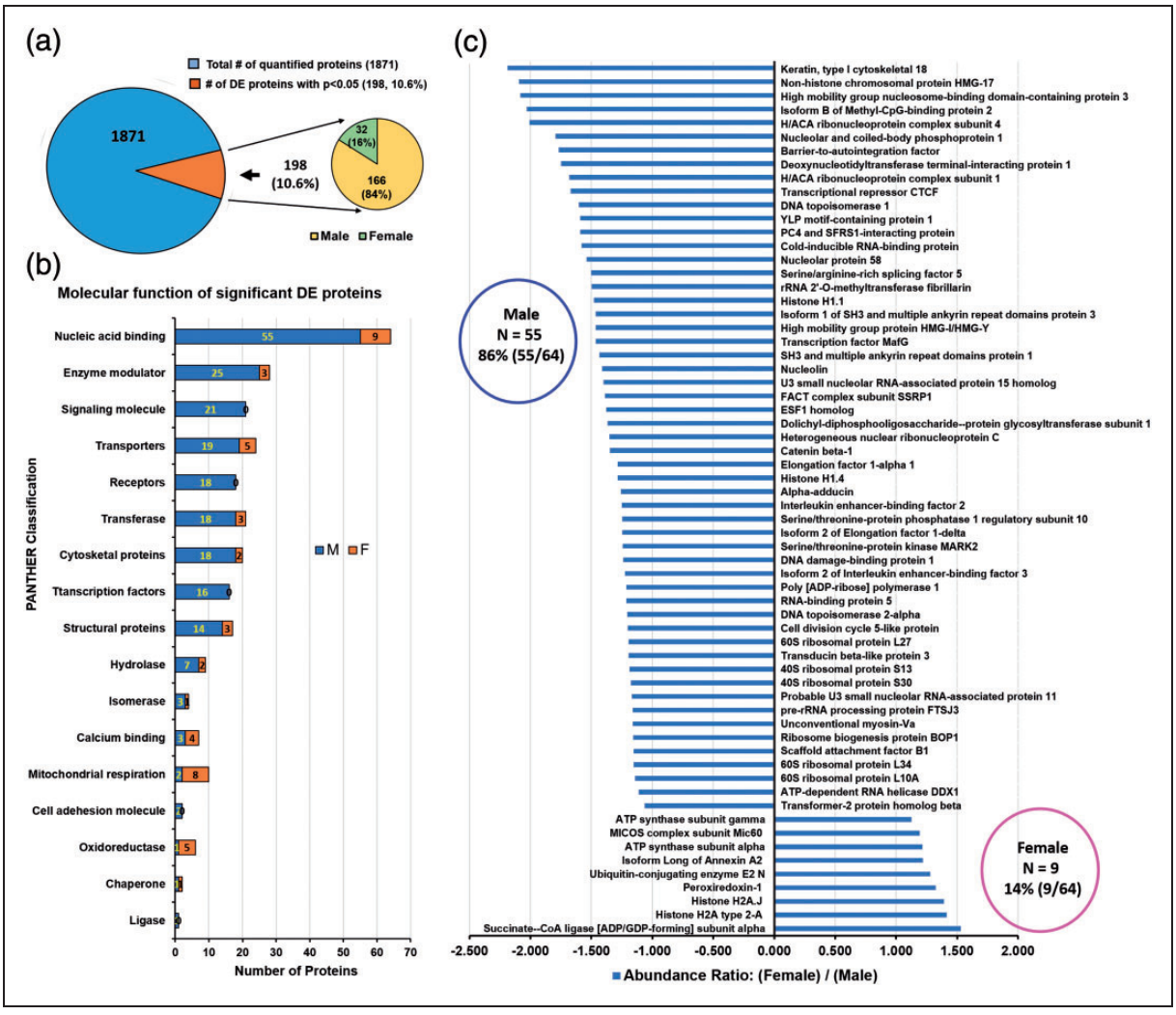


**Figure 3.** The expression and functions of significant proteins identified by LC/MS-based proteomics analysis in male and female rat MVs.[11] (a) The diagram represents the total number of identified proteins and the significant ($p < 0.05$) number of proteins identified in female and male rat brain MVs. (b) PANTHER classification system analyzed the top 17 protein functions in rat MVs. The significant DE proteins involved in each PNTHER class are indicated by the numeric numbers. (c) Sex-dependent differences of significant DE proteins involved in Nucleic acid binding in rat MVs. The x-axis represents the abundance ratio of an average quantity of proteins present in female MVs compared with male MVs (presented as abundance ratio: female/male). The y-axis represents up- or down-regulated nucleic acid binding-related proteins.

Chi-square test, with $p < 0.05$. The distribution map of the 20 most significant gene pathways was generated for male (Figure 4) and female (Figure 5(a) and Supplementary Figure 2) rat MVs.

### *Glycosylphosphatidylinositol (GPI), GPI anchored protein synthesis, and GPI anchor biosynthesis were the most prominent gene-centric canonical pathways expressed in male MVs*

The top three enriched canonical pathways in male rat MVs involved glycosylphosphatidylinositol (GPI), biosynthesis, and post-translational modification of GPI-anchored proteins (Figure 4(a)). Twenty-three genes were involved in either GPI synthesis or GPI anchor protein biosynthesis (Figure 4(b)) and were abundantly expressed in male MVs. For example, phosphatidylinositol glycan anchor biosynthesis class C (PIGC), one of the many PIG-genes, which are abundant in male MVs, encodes an endoplasmic associated protein involved in GPI anchor biosynthesis.

### *Steroid and cholesterol biosynthesis pathways were abundantly expressed in male MVs*

Steroid and cholesterol biosynthesis pathways were the next most upregulated pathways in male MVs (Figure 4 (a)). The gene cluster analysis indicated that all of the

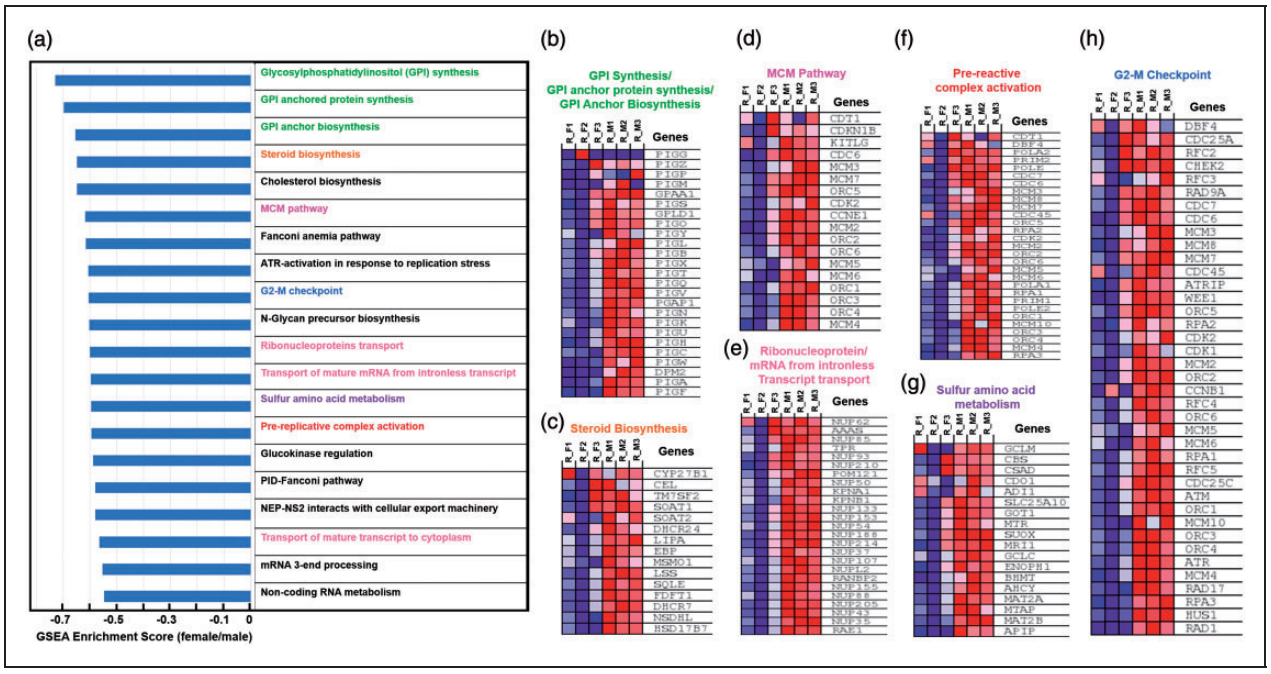


**Figure 4.** Enriched canonical pathways and the expression of different DE genes that were involved in different canonical pathways in male MVs. (a) Histogram of significant pathways that were upregulated in male MVs. The ordinate represents the name of the canonical pathways, and the abscissa indicates the GSEA enrichment score (female/male). (b–h) Heat maps are shown demonstrating the expression pattern of different DE genes involved in different canonical pathways. R: rat brain MVs. F: Female, and M: Male. Red: up-regulated genes, Blue: down-regulated genes.

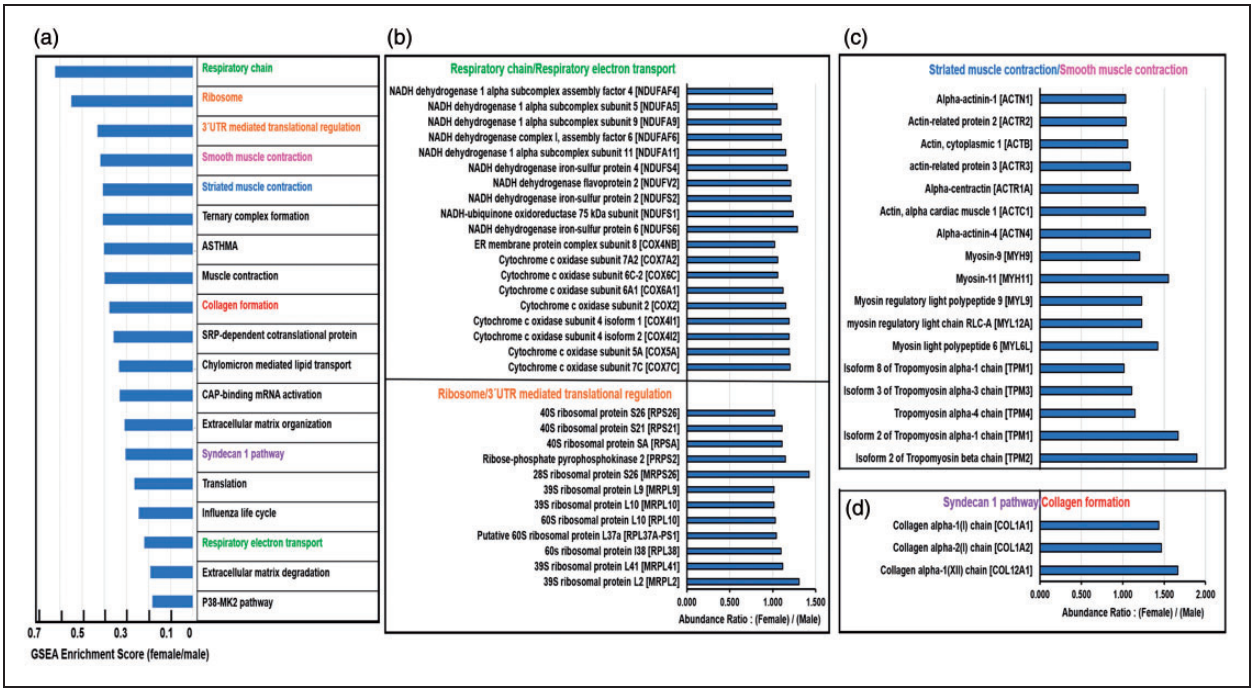


**Figure 5.** Enriched canonical pathways (by RNA-Seq) and the detection of different pathway-related DE proteins (by LC/MS analysis) in female MVs. (a) Histogram of significant pathways that were upregulated in female MVs. The ordinate represents the name of the canonical pathways, and the abscissa indicates the GSEA enrichment score (female/male). (b to d) The most abundant proteins involved in determining the different canonical pathways are presented. The x-axis signifies the abundance ratio of an average quantity of proteins present in female MVs compared with male MVs (presented as abundance ratio: female/male). The y-axis represents up-regulated proteins involved in different canonical pathways.

15 genes involved in steroid biosynthesis were highly expressed in male MVs (Figure 4(c)). For example, 17-beta-hydroxysteroid dehydrogenase VII (HSD17B7) functions as an enzyme involved in the biosynthesis of sex hormones or in the biosynthesis of cholesterol.

### *Pathways linked with genomic DNA replication were abundant in male MVs*

The minichromosomal maintenance protein complex (MCM) pathway (Figure 4(a)), and its associated genes (Figure 4(d)), were abundant in male MVs. Interestingly, the G2-M checkpoint pathway (Figure 4(a)) and its related genes (Figure 4(h)) were also largely upregulated in male MVs. Unwinding occurs at specific sites along the chromosomes, the so-called DNA replication origins. This process requires a sophisticated multi-protein complex which allows recruitment of the putative replicative helicase, the MCM2-7 (Figure 4 (f)) protein complex, in an ATP-dependent manner.[20,21] This reaction is currently known as pre-reactive complex formation, which was abundant in male MVs (Figure 4(a) and (f)). Ataxia-telangiectasia mutated and Rad3-related (ATR), a master regulator of the DNA damage response, especially during DNA replication, are crucial for biological processes. ATR controls and coordinates DNA replication origin firing, replication fork stability, cell cycle checkpoints, and DNA repair.[22] The pathways related to ATR-activation in response to replication stress as well as the Fanconi anemia pathway, a biochemical network that assists in DNA repair, DNA replication, and other cellular processes were significantly higher in male than female MVs (Figure 4(a)).

### *Transport of ribonucleoproteins, mature mRNAs, and transcript related pathways were abundant in male MVs*

A cluster of 25 genes related to the transport of ribonucleoproteins, mature mRNAs, and mRNA transcripts were largely upregulated in male MVs (Figure 4(e)). For example, members of the nucleoporin (NUP)-family, which encodes proteins involved in forming the nuclear pore complex between the inner and outer nuclear membranes, are more prevalent in male than female MVs.

### *Sulfur amino acid metabolism pathway was abundant in male MVs*

We observed that a cluster of 18 genes related to diverse aspects of the sulfur amino acid metabolism pathway were upregulated in male MVs (Figure 4(a) and (g)). For example, glutamate-cysteine ligase (GCLM) is the first, rate-limiting step in glutathione synthesis. In addition, apoptotic protease activating factor 1 (APAF1) is an enzyme involved in the methionine salvage pathway.

### *The mitochondrial respiration pathway was abundant in female MVs*

Supporting our previous findings using proteomics,[11] expression of genes involved in the mitochondrial respiratory chain/electron transport chain complexes was higher in female than male MVs (Figure 5(a) and (b)). Our RNA-Seq data indicated that a cluster of 25 genes involved in mitochondrial respiration or respiratory electron transport were significantly higher in female than male MVs (Supplementary Figure 2A).

### *Ribosomal biogenesis, viral RNA transcription, and 3′UTR-mediated translational regulation pathways were significantly high in female MVs*

We observed that 12 ribosomal proteins (Figure 5(b)), 21 ribosomal protein small subunit (RPS), and 24 ribosomal protein large subunit (RPL) genes involved in ribosomal biogenesis/3′UTR-mediated translational control were significantly high in female MVs (Supplementary Figure 2B). For example, ribosomal protein lateral stalk subunit P2 (RPLP2) gene encodes a ribosomal phosphoprotein, a component of 60S subunit, which is involved in the elongation step of protein synthesis.

### *Striated and smooth muscles contraction pathways were abundant in female MVs*

We observed a cluster of genes involved in either the striated (Supplementary Figure 2C) or the smooth muscle contraction pathways (Supplementary Figure 2D), which were predominant in female MVs (Figure 5(a) and (c)). For example, the caldesmon 1 (CALD1) gene, which is expressed more in female than male MVs, encodes a calmodulin- and actin-binding protein that plays an essential role in the regulation of smooth muscle contraction.

### *The syndecan-1, collagen formation, muscle contraction, and the extracellular matrix organization and degradation pathways were all abundantly expressed in female MVs*

We observed a cluster of genes involved in syndecan-1 (Supplementary Figure 2E) or collagen formation pathways (Supplementary Figure 2F) were predominant in female MVs (Figure 5(a) and (d)). For example, the collagen type VI alpha 2 chain (COL6A2) gene

codes for synthesis of the alpha2 chain of type VI collagen, a flexible protein found in the space surrounding cells, which contributes to structural support, tissue strength, and flexibility.

### *Various gene-centric canonical pathways were supported by the LC/MS-based proteomic analysis*

The relationship between gene expression and corresponding protein levels are complex due to many factors.[11] Therefore, we compared RNA-Seq and proteomic results to discover similarities and differences. RNA-Seq showed that PIGU, PIGS, and PIGZ genes are involved in GPI and GPI biosynthesis pathways (Figure 4(a) and (b)), and proteomic analysis confirmed higher protein abundance in male than female MVs (Supplementary Figure 1). Similarly, eight proteins involved in the MCM pathway, the pre-reactive complex activation, or the G2-M checkpoint pathway showed abundant male MVs (Supplementary Figure 1). Interestingly, RNA-Seq data indicated that the ribonucleoprotein transport pathway was higher in males, but most of the related proteins were higher in females (Supplementary Figure 1). It is noteworthy that female MVs, RNA-Seq data associated with different canonical pathways, were supported by proteomic data. Mitochondrial respiration, ribosome, and 3UTR mediated translational regulation pathways were the top canonical pathways in female MVs (Figure 5(a) to (c)). Interestingly, nearly 19 mitochondrial respiration pathway-related proteins and 12 ribosome/3′UTR mediated, translational regulation pathway-related proteins were abundantly expressed in female MVs (Figure 5(b)). Similarly, proteins involved in striated muscle contraction/smooth muscle contraction pathways (Figure 5(c)) and syndecan-1/collagen formation pathways (Figure 5(d)) were abundantly expressed in female MVs.

### *Validation of RNA-Seq analysis of host genes by western blotting*

We analyzed protein levels of several significant DE genes whose expression in male and female rat MVs are unknown, including DDX3, EIF2S3Y, KDM5D, MCM2, CDK2, NDUFA9, COX5, and COL23A1 (Figures 6 and 7). To validate the reproducibility of our methods, we performed two separate western blots for the same 8 proteins using samples from a total of 9 different males and 9 different females. In our RNA-Seq data, we observed that DDX3, EIF2S3Y, and KDM5D were more highly expressed in male than female MVs (Figure 1(d)). Western blot data indicated that the protein expression of KDM5D tended to be higher in males but was not significant and EIF2S3Y was significantly higher in male than female MVs (Figure 6(e)). Although DDX3 was the highest transcribed gene in male MVs, the western blot result indicated that the DDX3 protein level was significantly higher in female MVs (Figure 6(e)). The discrepancy between mRNA and protein expression of DDX3 might be due to abundant expression of a gene cluster involved either in RNA degradation (Supplementary Figure 3A), in post-translational modification (Supplementary Figure 3B), or in ubiquitin mediated proteolysis (Supplementary Figure 3C) in male MVs. We also validated the protein expression of genes involved in different canonical pathways. The MCM2 gene was highly expressed in male MVs (Figure 4(d), (f), and (h)) and the protein expression was higher in males (Figure 7(e)). In contrast, CDK2 gene expression was elevated in males (Figure 4(d), (f), and (h)), but protein levels for CDK2 were significantly ($p < 0.05$) higher for female MVs (Figure 7(e)). It is noteworthy that the gene expression of NDUFA9 (Figure 5(b)) was higher in female MVs and this finding was supported by western blot data (Figure 7(e)). However, despite differential gene expression of COL23A1 (Figure 1(d) and Supplementary Figure 2F), there were no significant differences in protein levels between male and female MVs (Figure 7(e)). The results for statistical significance of Figures 6(e) and 7(e) using merged western blot data were identical whether we used the Student's t-test or two-way analysis of variance.

### *Validation of RNA-Seq analysis of host genes by RT-qPCR*

Since there was a discrepancy between mRNA expression and protein expression especially for DDX3 and CDK2, we validated the top three DE genes in male (DDX3, KDM5D, and EIF2S3Y) and female (SMAD6, EBNA1BP2, and COL23A1) MVs by RT-qPCR. The RT-qPCR results were in agreement with the RNA-Seq data (Supplementary Figure 4). We observed that the RNA expression of DDX3, KDM5D, and EIF2S3Y was significant higher in male than female MVs (Supplementary Figure 4A-C). Similarly, the RNA expression of SMAD6, EBNA1BP2, and COL23A1 was significant higher in female than male MVs (Supplementary Figure 4D-F). The primer sequences for these genes are presented in Supplementary Figure 5.

## Discussion

The major findings show that there are extensive, previously undocumented, sex differences in gene expression in rat MVs that might significantly influence function and pathophysiology of the neurovascular

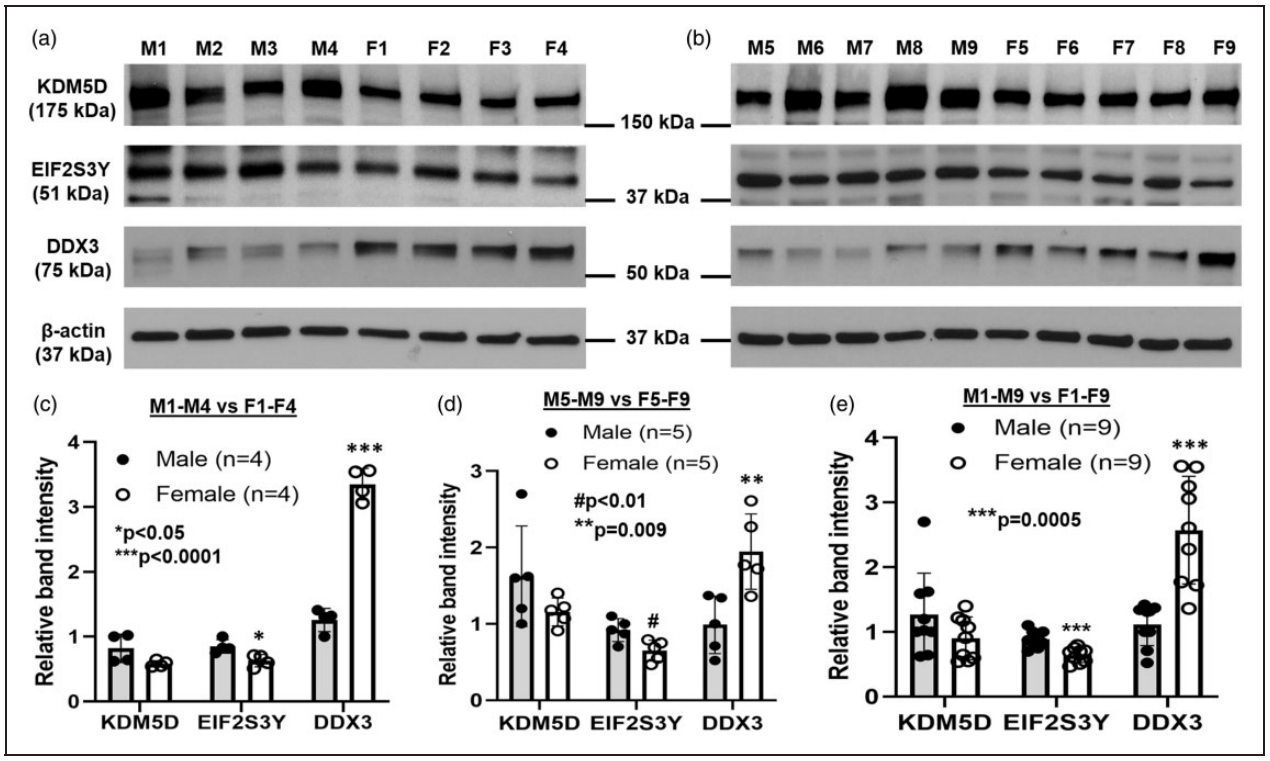


**Figure 6.** Validation of the top three up-regulated genes in male MVs by western blotting. (a and b) MVs from age-matched males and females were isolated at the same time, lysed the MVs, and equal amount (20 μg) of proteins were loaded in the gels and the indicated proteins were detected by immunoblots. We performed two separate western blots using different samples for each protein and analyzed the individual and combined results. (c to e) The proteins of the top three up-regulated genes in male MVs (DDX3, KDM5D, and EIF2S3Y) were qualitatively detected by western blotting by using $\beta$-actin as an internal control. The relative band intensities of individual ($n = 4$, $n = 5$) and combined samples ($n = 9$) were quantified using Image-J softwire (version 1.50). The separate western blots showed the same male/female relationships. Values were mean ± standard deviation and significant changes were presented as indicated. The statistical analysis was performed using GraphPad Prism version 9.0.0 for Windows. KDM5D: lysine demethylase 5 D; EIF2S3Y: Eukaryotic translation initiation factor 2 subunit 3, Y-linked; DDX3: DEAD-box polypeptide 3. All data sets passed the normality test and thus t-tests were used. Values are mean ± SD. The results for statistical significance of (e) using merged western blot data were identical whether we used the Student's t-test or two-way analysis of variance. Level of statistical significance is indicated in the graphs.

unit during health, in aging, and diseases. Although relatively few in number compared with the total number of genes examined, these differentially expressed genes and their associated canonical pathways play vital roles in normal function of MVs and in the resiliency and susceptibility of MVs to physiological and pathological stresses. To our knowledge, this is the first multiomics study to focus on sex-based disparities in normal brain MVs. The most significant findings are discussed below.

## *Glycosylphosphatidylinositol (GPI, male enhancement)*

GPI synthesis, GPI anchored protein synthesis, and GPI anchor biosynthesis were the top gene-centric canonical pathways in male versus female MVs (Figure 4). GPI is a phosphoglyceride that can be attached to the C-terminal end of a protein during posttranslational modification, making it a GPI anchor protein (GPI-AP). Enhanced gene expression involved in post-translational modification is a characteristic of male MVs. The GPI-APs play key roles in different biological processes including receptor coupling, and protein-protein interactions. GPI-anchored proteins also are localized to lipid rafts. The apical-to-basolateral delivery of raft-associated GPI-APs occurs via a transcytotic pathway in polarized cells. Furthermore, transcytosis of macromolecules at the BBB can occur, in part, via tightly regulated caveolae-associated lipid rafts. Directly relating to brain MVs, GPI-APs have been reported to regulate leukocyte adhesion and thereby protect endothelium from damage.[23] Defects in the GPI-anchor synthesis occur in paroxysmal nocturnal hemohemoglobinuria[24] and congenital diseases.[25] Reck, a GPI-AP, plays an

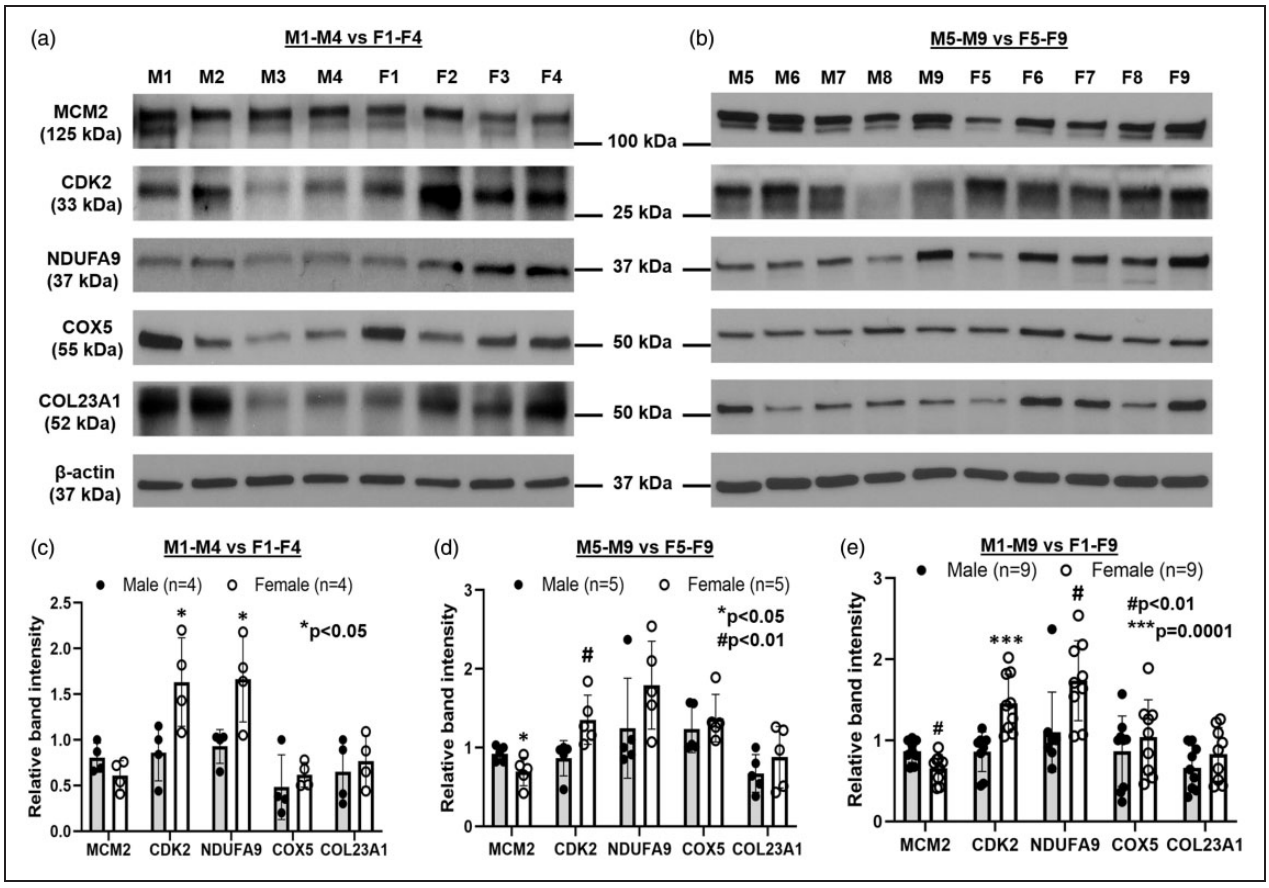


**Figure 7.** Validation of DE genes involved in different canonical pathways by Western blotting. The proteins of MCM2, CDK2, NDUFA9, COX5, and COL23A1 genes were qualitatively detected by western blotting by using $\beta$-actin as an internal control. (a) The experiments were performed with MVs from age-matched four males (M1-M4) and four females (F1-F4), and (b) with MVs from an additional age-matched five males (M5-M9) and five females (F5-F9). (c to e) The relative band intensities of MCM2, CDK2, NDUFA9, COX5, and COL23A1 were compared by the Image-J softwire (version 1.50). The separate western blots showed the same male/female relationships. Values were mean $\pm$ standard deviation and significant changes were presented as indicated. The statistical analysis was performed using GraphPad Prism version 9.0.0 for Windows. MCM2: DNA replication licensing factor MCM2; CDK2: Cyclin Dependent Kinase 2; NDUFA9: NADH: Ubiquinone Oxidoreductase Subunit A9; COX5: ATP Synthase alpha (complex V); COL23A1: Collagen alpha-1(XXIII) chain. Values are mean $\pm$ SD. NDUFA9 ($n = 4$, $n = 5$, $n = 9$), CDK2 ($n = 5$, $n = 9$), and COX5 ($n = 5$) were analyzed using the Mann-Whitney test and all of the other comparisons were done using unpaired t-tests. The results for statistical significance of (e) using merged western blot data were identical whether we used the Student's t-test or two-way analysis of variance. Level of statistical significance is indicated in the graphs.

important role in neurovascular development.[26] In our study, most of the GPI-APs that are highly expressed in males belong to phosphatidylinositol glycan anchor biosynthesis class S (PIG) group (Figure 4, and Supplementary Figure 1), whose mutations are associated with early infantile epileptic encephalopathy.[27]

## *Steroid and cholesterol biosynthesis (male enhancement)*

Steroid and cholesterol biosynthesis gene expressions (Figure 4(c)) were higher in male compared with female MVs and this finding is consistent with our results concerning the interactions between cholesterol-rich lipid rafts and GPI/GPI-anchoring proteins described in the previous section. For vascular physiology, cholesterol is an indispensable component. Elevated vascular levels of cholesterol and disruption of normal cholesterol metabolism; however, have been implicated in the development of atherosclerosis,[28] which occurs more commonly in men than women before middle age, although women lose this protection after menopause.[29] Studies in rats have shown sex differences in neuroactive steroid concentrations in plasma and in brain.[30–32] Brain cholesterol metabolism defects have been implicated in neurodegenerative diseases.[33,34] It has also been reported that hormones can enter cells through specific transporters.[35] In our study,

we observed a high expression of different transporters from gene (Figure 2(b)) and protein (Figure 3(b)) levels in male MVs, which may explain the high abundance of steroid and cholesterol biosynthesis pathway in male MVs.

### *Sulfur amino acid metabolism (male enhancement)*

Genes involved in sulfur amino acid metabolism (Figure 4(g)) were also expressed higher in male MVs. Sulfur containing amino acids (mainly methionine and cysteine) play roles in the maintenance and integrity of cellular systems by manipulating cellular redox state and cellular capacity to detoxify toxic compounds, free radicals, and reactive oxygen species.[36] Methionine and cysteine are precursors for important sulfur metabolites such as $H_2S$, an important signaling compound in the vasculature, and glutathione (GSH), the main cellular redox buffer.[37] $H_2S$ synthesis is higher in male than female mouse brains,[38] and $H_2S$ has potent effects on vascular tone either directly or through nitic oxide.[39] The three genes involved in S-adenosylmethionine synthesis, a key metabolite for cell function, are: *MAT1A*, *MAT2A*, and *MAT2B*.[40,41] We observed that both MAT2A and MAT2B genes were highly expressed in male MVs. The importance of sulfur amino acid metabolism in male MVs is emphasized by the genes promoting post-translational modifications of relevant proteins that regulate function of sulfur-containing proteins or regulate sulfur availability (Supplementary Figure 3). Modifications in sulfur amino acid metabolism are linked with an increased risk of several common late-life diseases, which increases the possibility that metabolism of sulfur amino acids may change during aging. Studies have suggested that homocysteine is a specific risk factor and/or a marker for human cardiovascular disease[42,43] and Alzheimer's patients have shown elevated homocysteine in brain.[44] Hyperhomocysteinemia is an independent risk factor for vascular disease[42] and diseases of the central nervous system.[44]

### *Nucleic acid binding (both DNA and RNA binding) (male enhancement)*

Recent studies have shown that the MCM pathway, which is involved in replication elongation, is essential for maintenance of genomic DNA replication. Various checkpoint pathways target the MCM signaling pathway. Using the PANTHER classification system, our RNA-Seq and proteomic data indicated that both DNA and RNA nucleic acid binding showed that the top gene and protein functions were higher in male than female MVs (Figures 2 and 3). Notably, genes/proteins involved in different pathways linked with genomic DNA replication (Figure 4 and Supplementary Figure 1), and the transport of ribonucleoproteins were also abundant in male MVs. DNA- and RNA-binding proteins control numerous cellular processes.[45] Sex disparities are manifest in human neurological disorders. Males are more prone than females to be diagnosed with certain neurological disorders such as autism spectrum disorder,[46,47] dyslexia,[48] Parkinson's disease,[49] and schizophrenia.[50] However, females are more likely to be diagnosed with Alzheimer's disease[51] or major depressive disorder[52] than males of the same age. In young rats, hippocampal neurogenesis has been reported as sexually dimorphic, and male rats produce more new neurons with lower average survival rates than females.[53] RNA-binding proteins (RBPs) have well-known functions in neurodevelopment and synaptic plasticity[54] and an extensive literature documents the influence of RBPs to neurodegenerative and neurodevelopmental disorders.[55–57]

### *Transcription factors (male enhancement)*

RNA assembles with proteins forming dynamic complexes called ribonucleoproteins (RNPs). The sequence of the transcript, its processing, and the activity of the available RNA-binding proteins (RBPs) determine the composition of the RNPs,[58,59] forming an additional layer of information that decides the fate of the RNA, thereby shaping the transcriptome and proteome. Transcription factors (TF) are among the most fundamental elements regulating gene expression and, with different co-factors, that regulate the transcription.[60] Post-transcriptional modification is attained by a similar combinatorial complexity of RBPs and protein-protein interactions.[61] The brain is a major candidate for studying both transcriptional and post-transcriptional regulation due to its complex functional sub-divisions.[62] Our results suggested that TF-related different forms of GTF2 (the general transcription factors for RNA polymerase II) and TAF (TATA-binding protein-associated factors) genes were predominantly upregulated in male MVs (Figure 2(e)). GTF2 include TF2B, TF2D, TF2E, TF2F, TF2H and TATA-binding protein. The GTF2 genes emerge as strong candidates for the neurological features of Williams syndrome, and Hirota et al.[63] has suggested that GTF2I probably contributes to the difficulty with visual-spatial tasks in Williams syndrome. Taf4b is essential for male and female fertility[64,65] and Taf4b regulates neuronal gene expression.[66] Interestingly, in our study, TAF, and GTF2 genes were abundantly expressed in male MVs (Figure 2(e)).

### Metabolism (female enhancement)

Recently, we reported on sexual dimorphism in the mitochondrial metabolic protein profiles of MVs in young rats.[11] Our proteomic-based study indicated that female rat MVs expressed more mitochondrial proteins involved in energy production, mitochondrial membrane structure, antioxidant enzyme proteins, and those involved in fatty acid oxidation.[11] Our present RNA-Seq data supports our previous findings. We observed a 25 gene cluster involved in mitochondrial respiration or respiratory electron transport pathway (Supplementary Figure 2) that was significantly higher in female than male MVs. The regulation of ribosomal proteins (RP) as well as mitochondrial ribosome proteins (MRP) are crucial to mitochondrial respiration,[67] and thus to cell viability,[68,69] growth[70,71] cellular development,[72,73] and differentiation.[74,75] Knockdown of MRPs caused protein imbalances and impaired mitochondrial respiration.[76] We also found that MRPS26, MRPL2, MRPL9, MRPL10, and MRPL41 were expressed higher in female than male MVs (Figure 5). Knockdown of RP genes has exhibited a variety of axis flaws and abnormalities in the central nervous system.[77] RPs might also play an important role in the maintenance of cognitive functions and high levels of RPL10 are observed in the murine hippocampus, a key site in the brain for promoting memory and learning.[78–80] Interestingly, in our study, we observed that the expression of both the mitochondrial MRPL10 and the cytosolic RPL10 proteins and genes were high in female MVs.

### Ribosomal biogenesis and 3′UTR-mediated translational regulation pathways (female enhancement)

Ribosome assembly is a complex mechanism involving 79 ribosomal protein (RPs) subunits.[81] The gene coding for all RPs and for some other proteins involved in the production or function of the translation apparatus are controlled at the translational level. During the last decade, new and unexpected mechanisms of 3′UTR-mediated translational control and their contributions to disease have received increased attention. To facilitate cap-independent translation, different viral mRNAs recruited 40S ribosome to the 3′UTR region. RPs are engaged in construction of either the small 40S or the large 60S ribosomal subunits and, as such, are designated RPS or RPL, respectively.

### Smooth and striated muscle contraction (female enhancement)

Smooth muscle cells associated with end-arterioles and perhaps pericytes and venules were included in our MV preparation. Gene expression of many of the smooth and striated muscle proteins was consistently higher in females than males, perhaps indicating differential control mechanisms of vascular tone and perfusion control into capillary networks. It has been shown that women have better autoregulatory control than men.[82,83] The designation of "striated muscle contraction" in canonical pathways is misleading since most of these proteins have recently been found to also be present in smooth muscle.

### Syndecan-1 (female enhancement)

Syndecan-1 (or CD138) regulates the activity of chemokines, cytokines, integrins, and other adhesion molecules, which play critical roles in the regulation of inflammation. Syndecan-1 also inhibits leukocyte adhesion and migration, possibly by preventing the interactions between leukocyte integrins and endothelial ICAM-1 and VCAM-1.[84] Syndecan-1 provides structural support by binding to extracellular matrix molecules such as collagens.[85,86] Syndecan mutants displayed significantly reduced metabolic rate and reduced mitochondrial respiration.[87] The cytoplasmic domain of syndecan-1 is essential for association with the actin cytoskeleton.[88] The syndecan-1 pathway has been shown to regulate collagen formation, muscle contraction, and extracellular matrix organization and degradation. In this study we observed that the syndecan-1 pathway was up-regulated in female MVs (Figure 5, and Supplementary Figure 2). We also observed that striated and smooth muscle contraction as well as the collagen formation pathway was higher in female MVs. We will explore the significance of these novel observations in our future studies.

### Concurrence and discrepancies between gene expression and resultant proteins

One surprising finding in this study was that despite large differences in the expression of some genes, the resultant protein concentrations were not always consistent with these differences. In particular, there was a greater correspondence between gene expression and protein synthesis in female MVs while, despite relatively large differences in gene expression favoring males, actual protein levels were either not different between the sexes or were opposite of expectations. For example, DEAD-box RNA helicase 3 was the top up-regulated gene in male MVs (Figure 1(d)), but the protein expression of DDX3 by western blotting showed that it was significantly more expressed in female MVs (Figure 6). Moreover, the expression of KDM5D gene was the second highest in male MVs (Figure 1); however, we did not obtain a significant

difference in its protein expression by western blotting. Similarly, the CDK2 gene was highly expressed in male MVs (Figure 4(d), (f), and (h)) but its protein expression was significantly ($p < 0.05$) higher in female MVs (Figure 7). Also, some ribonucleoprotein genes: NUP50, NUP54, NUP62, NUP85, NUP93, NUP107, NUP153, and NUP155 were up-regulated in male MVs (Figure 4) but the proteomic data indicated that the protein expression of these genes were abundant in female MVs (Supplementary Figure 1). On the other hand, in female MVs, the gene expression and its corresponding proteins were more consistent. The difference between the gene and protein expression in male MVs may be due to high gene expression related with RNA degradation, post-translational modification, and/or ubiquitin mediated proteolysis (Supplementary Figure 3). The stability of mRNA influences the dynamics of gene expression and the relationship between mRNA and protein synthesis. The mammalian CCR4–NOT (CNOT) complex is associated with deadenylase activity, which shortens the mRNA poly (A) tail, and contributes to destabilization of mRNA. CNOT6, 6 L, 7, and 10 are expressed nearly ubiquitously, displaying especially high expression in the brain. Interestingly, almost all CNOT genes were highly expressed in male MVs (Supplementary Figure 3). Similarly, post-translational modifications (PTMs) including phosphorylation, glycosylation, ubiquitination, methylation, acetylation, lipidation, and proteolysis play a critical role in the regulation of protein activity involved in almost all aspects of cell structure and function. Our data showed that genes related with PTMs and ubiquitin-mediated proteolysis were expressed higher in male MVs (Supplementary Figure 3B) and probably play an important role on the formation, stability, and functions of various proteins. Thus, our use of multiple approaches including RNA-Seq, proteomics, and western blotting, demonstrating similarities and differences, indicates that a complex interaction occurs among mRNA synthesis and stability, protein synthesis, protein post-translational modifications, and protein degradation in cerebral MVs. Furthermore, as we have completed for large arteries,[6,7,19,89,90] functional studies are needed to document changes in gene or protein levels.

## Limitations of the study

There are several limitations in our study. **First**, our isolated MVs contained different segments of the microvasculature and did not represent specific types (i.e., arterioles, venules, or capillaries). Therefore, we cannot attribute our results to any one segment or cell type of the microvasculature. Still, we do not know which procedures would allow us to separate vascular segments with any degree of confidence and still yield enough protein or RNA for analyses. **Second**, because of a large and diverse amount of data we could validate only a limited number of up- or down-regulated genes by western blotting. **Third**, the data set for RNA-Seq analysis (23,786 identified genes) was much larger than that for the proteomics study (1,871 quantified proteins). **Forth,** the female MVs were not isolated at a fixed time of the estrous cycle.

## Implications

Through similar and different processes involving gene expression, protein synthesis, post-translational modifications, and signaling pathways, male and female blood vessels in regional circulations can meet the needs of tissues and organs specific to their sex under normal conditions. However, a reliance on narrow, sex-determined specific signaling pathways and protein families for vascular control can compromise a regional circulation during periods of stress, disease, or aging. The occurrence and severity of cerebrovascular responses to diseases such as diabetes and arterial hypertension and vascular-related neurological diseases differ in males and females and the underlying mechanisms are not yet defined; however, they may be associated with the findings presented here. We have presented clear evidence that even in healthy, young rats major differences in gene expression and protein synthesis are present that can affect the normal function of brain MVs as well as the responses of MVs to disease and aging. Building upon our previous findings where we used only proteomic analyses, the present study has shown that genes related to OXPHOS are more highly expressed in females than males. Greater OXPHOS capacity provides for a greater ability of cerebral MVs to respond to hormonal fluxes during the estrus cycle and during vascular remodeling which occurs in localized brain areas during pregnancy. Enrichment of glucokinase regulation in male MVs (Figure 4(a)), on the other hand, due to its higher Km than hexokinase, may indicate that glycolytic production of ATP represents an alternative approach to OXPHOS for rapidly meeting increased energy demand in MVs. The enhanced gene expression involved in the MCM pathway, in sulfur amino acid metabolism, and in the G2-M checkpoint may lead to greater resiliency and repair of male MVs against stress and injury, while greater mitochondrial anti-oxidative ability might provide the same protection. The enhanced gene expression of GPI synthesis and anchoring as well as steroid and cholesterol biosynthesis may indicate greater internal organization within MVs associated with protein-protein interactions and signaling pathways but may also place men at a disadvantage

for the development of atherosclerosis. In conclusion, we have provided the first comprehensive RNA-Seq examination of male and female MVs of rats and have shown significant sex-dependent differences in gene expression and canonical pathways. Our study indicates that male and female MVs have significant differences in gene expression and protein synthesis, which may explain the sex-dependent differences in the microcirculation during health and diseases.

## Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the grants NIH HL-093554 (DWB), NIH AG-063345 (DWB), NIH HL148836 (DWB), NS094834 (PVGK), and NS114286 (PVGK). This research was supported in whole or in part by the Louisiana Board of Regents Endowed Chairs for Eminent Scholars program (DWB).

## Acknowledgements

We thank Nancy Busija, MA, CCC-SLP, for editing the manuscript. We thank Dana Liu, Barbara Rider, and Fayez M Mohammedsaleh for technical help.

## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Authors' contributions

P.K.C, and D.W.B. conceived and designed the experiments; P.K.C., S.C., M.C.B., I.R., and J.J.G. performed experiments; P.K.C., M.C.B., and J.J.G analyzed data; P.K.C. interpreted experimental results, and prepared figures; P.K.C., and D.W.B. drafted the manuscript; P.K.C., S.C., M.C.B., I.R., J.J.G., E.K.F., P.V.G.K and D.W.B. edited and revised the manuscript and approved the final version of the manuscript.

## Supplementary material

Supplemental material for this article is available online.

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