Enduring and sex-specific changes in hippocampal gene expression after a subchronic immune challenge

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Data availability: This data is also publicly available on the following databases:
Gene Expression Omnibus (GEO), with GEO accession number GSE126678, and Sequence Read Archive (SRA), with SRA number SRP186132 and BioProject number PRJNA522922

ABSTRACT

Major illnesses, including major surgery, heart attack, and sepsis, can cause long-lasting cognitive impairments, depression, and progressive memory decline that persist well after recovery from the original illness. Similarly, in animal models, immune challenge results in impairments of learning and memory as well as increased depression-like behaviors. Although neuroimmune signaling is now well established as playing a crucial role in the modulation of neural functions, how transient immune signaling mediates enduring changes in cognition, memory, and emotion remains unknown. We have recently demonstrated that a series of intermittent injections of lipopolysaccharides over a two-week period resulted in sex-specific patterns of memory deficits that persist at least several months after the end of the immune challenge. Here, we examined the persistent changes in hippocampal gene expression three months after this subchronic systemic immune challenge in males and in females. We used RNA-sequencing as a large-scale, unbiased approach to identify both persistent changes in gene expression long after an immune challenge, and changes in transcriptional response to a subsequent immune challenge. Males and females differed in number and patterns of gene expression in the hippocampus. In males, we observed enduring dysregulation of gene expression three months after the end of a subchronic immune challenge, in the absence of an additional insult. In contrast, females showed few persistent changes under basal conditions, but striking dysregulation of gene expression in response to an additional acute LPS injection. Striking sex differences in the specific genes, pathways, and biological processes affected were observed both after subchronic immune challenge and after a subsequent insult. Thus, subchronic systemic immune activation has enduring and sex-specific consequences for gene expression and response to subsequent stimuli. Such persistent changes in neural functions further suggest that in both males and females, neuroimmune signaling during illness contributes to subsequent vulnerability or resilience to cognitive decline, memory impairments, and affective disorders.

INTRODUCTION

The ability of biological systems to change as a consequence of experience is a fundamental feature of how individuals adapt to their current environment. In the brain, plasticity at multiple levels including behavior, circuits, synapses, and gene expression are core mechanisms of learning and long-lasting memory that allow animals to change their behavior in accordance with experience. Persistent changes that allow for adaptation to events including stress, drugs of abuse, or major illness may also contribute to vulnerability or resilience to disorders including depression, addiction, and cognitive decline. Here we examined enduring changes in hippocampal gene expression after a systemic, subchronic immune challenge in both sexes, and discuss the implications for persistent dysregulation of cognition, emotion, and memory.

Illness, including sepsis or heart attack and injury result in long-lasting dysregulation of cognition and emotion, with patients of major illness developing depression and cognitive deficits long after recovery from the original sickness (Gharacholou *et al*, 2011; Marra *et al*, 2018; Semmler *et al*, 2007). In rodent models of sepsis, persistent changes in memory and affective processes have been observed after cecal ligation and puncture (Chavan *et al*, 2012; Huerta *et al*, 2016) and after high-dose lipopolysaccharide (LPS) treatment (Anderson *et al*, 2015; Weberpals *et al*, 2009). Such behavioral changes have been attributed to persistent neuroimmune signaling and microglial activation (Fenn *et al*, 2014; Weberpals *et al*, 2009).

Nevertheless, other studies have demonstrated long-lasting memory impairments after immune challenge despite a lack of persistent neuroimmune activation. Several studies have demonstrated that long-lasting memory dysfunction observed in humans and animal models may be due to structural changes including changes in dendritic spine turnover and density or neuronal loss (Kondo *et al*, 2011; Liu *et al*, 2008; Semmler *et al*, 2007; Volpe *et al*, 2015). More recently, we have demonstrated that a lower-dose subchronic systemic immune challenge, that does not result in ongoing neuroimmune activation, also results in memory deficits that emerge and persist at least 8 weeks after the end of the immune challenge (Tchessalova and Tronson, 2019). These persistent changes in behavior, memory, and synaptic structure are likely mediated and maintained by enduring alterations of gene expression and associated transcriptional regulatory mechanisms (Tchessalova *et al*, 2018).

Recent work has demonstrated the persistence of changes in gene expression in the brain after stress or exposure to addictive drugs (Gray *et al*, 2014; Nestler, 2014; Sterlemann *et al*, 2008; Tafet and Nemeroff, 2016). There are also striking sex differences in the precise patterns of gene expression in the brain in the days and weeks after the insult (Hodes *et al*, 2015; Mychasiuk *et al*, 2016). For example, in a chronic unpredictable stress paradigm, males show more changes in gene expression and regulation of different gene networks compared with females (Hodes *et al*, 2015). Changes in transcriptional regulation as a consequence of adverse events contributes to altered responses to later insults. If gene expression is differentially regulated in males and in females, then this may mediate sex-specific vulnerability or resilience to affective and cognitive disorders.

Enduring changes in transcriptional processes is a hallmark of immune system function. During acute activation of the peripheral immune and neuroimmune systems, cytokines increase transcription and production of other cytokines and immune mediators. Activation of the inflammatory response results in persistent transcriptional changes, which encode new antibodies in the adaptive immune system, and training of the innate immune system (Netea and van der Meer, 2017). There is also evidence for such "training" of the innate immune system in the brain, with lasting changes in neuroimmune reactivity after multiple immune challenges (Wendeln *et al*, 2018). Enduring changes in cognition, affective processes, and memory after immune challenge may result from persistent alterations in gene expression and transcriptional regulation

after illness or injury.

Here we examined whether changes in hippocampal gene expression persist long after an immune challenge in males and in females. Given that recent work from our laboratory has demonstrated persistent and sex-specific memory deficits as a consequence of a mild subchronic immune challenge (Tchessalova and Tronson, 2019), we hypothesized that this challenge would also cause enduring and sex-specific changes in gene expression and transcriptional regulation in the hippocampus. We used a next generation RNA-sequencing as a large-scale, unbiased approach to identify long lasting changes in gene expression. We examined both persistent changes in gene expression long after an immune challenge and changes in the transcriptional response to a subsequent immune challenge. We demonstrate that males and females differ in number and patterns of gene expression in the hippocampus both three months after a subchronic immune challenge and in response to a subsequent, acute LPS injection.

METHODS

Animals: Male and female 8-9 week old C57BL/6N mice were purchased from Envigo (Indianapolis, IN). All mice were individually housed with *ad libitum* access to standard mouse chow and water as individual housing in mice prevents fighting-induced stress in males (Meakin *et al*, 2013) and is ethologically appropriate for males and females(Becker and Koob, 2016). The facility is ventilated with constant air exchange (60 m^3/ h), temperature (22 ±1°C), and humidity (55±10%) with a standard 12 h light-dark cycle (Keiser *et al*, 2017). One month prior to collection of tissue, all animals were tested on context fear conditioning anxiety-like behavior, and depression-like behavior. The results from context fear conditioning are published elsewhere (Tchessalova and Tronson, 2019), and we observed no effects of prior LPS challenge on anxiety or forced swim test.

All experimental methods used in these studies were approved by the University of Michigan Committee on the Use and Care of Animals. Sample size was based on minimum number of animals needed per experimental condition for RNA sequencing (3 per condition).
Immune challenge: Lipopolysaccharide (LPS, *Escherichia coli*, serotype 0111:B4; Sigma-Aldrich, St. Louis) was dissolved in saline for a final concentration of 12.5µg/mL. All LPS injections were given intraperitoneally (i.p.) at a dose of 250µg/kg. Vehicle control mice received an equivalent volume of saline (20mL/kg, i.p.) (Cloutier *et al*, 2012; Tchessalova and Tronson, 2019). The subchronic immune challenge consisted of five injections, spaced three days apart (days 1,4,7,10,13). All injections were administered in the morning between 9 and 10am. In the Long-Term condition, tissue collection occurred 12 weeks after the final LPS injection. Separate animals received a subsequent LPS (250µg/kg; or vehicle) injection 12 weeks after the subchronic immune challenge and tissue was collected 6 hours after injection (Long-term + Acute condition). For the Acute condition, animals received a single LPS (250µg/kg, or vehicle) injection and hippocampi were collected 6 hours later.
RNA extraction and sequencing: In order to maintain RNA quality, whole hippocampi were collected and immediately placed in RNALater until processing (Bagot *et al*, 2016, 2017, Cates *et al*, 2017, 2018; Hodes *et al*, 2015; Lorsch *et al*, 2018; Walker *et al*, 2018). One hemisphere (counterbalanced by side between sex and experimental conditions) was selected for RNA extraction while the other was collected for protein analysis. RNA was isolated using Life Technologies PureLink RNA Mini kit (cat. no. 12183018A). Relative RNA quantity and integrity were first analyzed using NanoDrop (ThermoFisher) and gel electrophoresis. Quality and integrity checks were then completed on the Bioanalyzer by the University of Michigan DNA sequencing core, with acceptable RIN values greater than 7. Sequencing was performed using Illumina 4000 High-Seq platform, using single-end, non-strand, Ribo depletion with read

lengths of 50 and sequencing depth of 40 million reads per sample. Total RNA (20ug) was used to construct the mRNA libraries. Barcoded cDNA libraries were constructed from polyadenylated transcripts that were purified, fragmented, and reverse transcribed using random hexameters. Three independent biological replicates were used per experimental condition.

Differential gene expression analyses: Alignment, differential expression analysis, and post-analysis diagnostics were analyzed using the Tuxedo Suite software package. Reads were aligned to the Ensembl *Mus musculus* NCBIM37 reference genome using TopHat and Bowtie. The quality of the raw reads data for each sample was assessed using FastQC to exclude any reads with quality problems.

Expression quantitation, normalization, and differential expression analyses were conducted through Cufflinks/CuffDiff with UCSC mm10.fa reference genome sequence. Differentially expressed genes (DEGs) were determined by multiple comparison correction using FDR > 0.05 cutoff.

DEGs in males and females were visualized using Venn diagrams through Venny 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/).

Metascape (http://metascape.org/gp/index.html#/main/step1) was used to generate gene annotation and gene list enrichment analysis, with a focus on biological pathways and processes. Significance of biological processes was determined through P-values calculated on a hypergeometric distribution (log10). Reference gene lists and annotated information were obtained from the Enrichr web page. Metascape was also used to conduct meta-analysis, with generation of Circos plots to visualize shared genes and pathways amongst the conditions and heatmaps of gene ontology (GO) terms that hierarchically cluster together amongst experimental conditions.

Clusters of functional protein-protein interactions (existing and predicted) between targets in experimental conditions of interest were visualized using the STRING 10.5 software (https://string-db.org/).

Statistical analysis: Criteria for a differentially expressed gene included a fold change greater than 1.5, and false discovery rate greater than 5% (fold change $\geq \pm 1.5$ and FDR ≤ 0.05). All comparisons of DEGs between control and experimental conditions were made within sex using unpaired *t* tests (two-tailed) with Benjamini correction to account for multiple comparisons to determine the effect of immune challenge in males and in females separately. DEGs in the hippocampus of males and females at baseline were compared using unpaired *t* tests (two-tailed) between vehicle treated males and vehicle treated females. Groups were compared as shown in Table 1.

Data availability: To allow all interested parties to explore and utilize our processed data, we have made our data publicly available through user-friendly databases, including the Gene Expression Omnibus (GEO), with accession number GSE126678, and Sequence Read Archive (SRA), with SRA number SRP186132 and BioProject number PRJNA522922.

Link to GEO: (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126678)
Link to SRA: (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP186132&go=go)

RESULTS

Subchronic immune challenge induces persistent changes in gene expression

We observed striking changes in gene expression in the hippocampus 12 weeks after subchronic immune challenge in males, with fewer changes observed in females. Of over 20,000 genes detected, there were 230 DEGs in the hippocampus of males and 26 DEGs in the hippocampus of females. In males, 183 genes were significantly upregulated and 47 significantly downregulated. In females, 7 genes were significantly upregulated and 18 downregulated. Five of these genes were differentially expressed in both males and females, with *Npas4* and *fos* downregulated in males and upregulated in females, and *Ifit1, Spp1* (*Opn*), and *Coch* upregulated in males and downregulated in females (Figure 1A; Table 1; Table 2).

Overall, in males, neuroimmune-related genes showed persistent upregulation, whereas neuroplasticity-related genes showed downregulation months after the subchronic immune challenge. In females, 5 although few DEGs were identified, they were consistently related to dopaminergic/monoaminergic signaling.

Biological pathway and process enrichment analysis revealed top clusters in males were: extracellular matrix organization (log10(P) = -15.41), vascular development (log10(P) = -13.85), response to growth factor (log10(P) = -11.53), regulation of MAPK signaling (log10(P) = -10.2), and response to hormone (log10(P) = -8.87) (Figure 1B). In females, top biological processes included response to amphetamine (log10(P) = -8.49), regulation of defense response (log10(P) = -4.76), and regulation of membrane potential (log10(P) = -3.06) (Figure 1C). Importantly, DEGs in males and females pertain to distinct biological pathways, with neuroplasticity- and MAPK-associated signaling in males and monoaminergic signaling and innate immune-related signaling in females.

Functional protein-protein interaction analysis (STRING) identified groups of DEGs with functional relationships. In males, these clusters included synaptic plasticity and memory-related genes, extracellular matrix targets including collagens and matrix breaking enzymes, growth factors and their receptors, and immediate early genes. There were also several immune-related targets involved in innate immune responses, including regulators of MAPK signaling, complement genes and their regulators, and MHC II-related targets. Notably, some regulators of immune signaling, including *NR4a1/2, Spp1,* and *Dusp1/6* are also plasticity related genes. Smaller clusters of genes included targets associated with organic anion/cation solute carriers, and potassium and chloride channels involved in inhibitory transmission (Figure 1D; Table 1).

In females, STRING analysis identified two clusters: one including dopaminergic signaling, adenosine signaling, adrenergic signaling, and G protein-coupled signaling; and other genes involved in interferon-mediated signaling (Figure 1E; Table 2). Importantly, the clusters identified in females did not overlap with those identified in males.

Prior subchronic immune challenge alters hippocampal gene expression in response to a later acute LPS injection in a sex-specific manner

We examined the long-lasting effect of prior subchronic immune challenge on transcriptional regulation to a subsequent acute LPS injection (Long-term+Acute condition). In males, we observed 58 DEGs, whereas in females, 432 genes were differentially expressed compared with acute immune challenge in previously naïve mice. Twenty-one genes showed differential expression in both sexes under these conditions, with 15 genes (e.g. *Gpr151, Chrna/b3, Ptprv, Slc5a7, Tac, Six3, Penk, Drd2, Adora2a, Foxp2, Syt6, Lrrc55, Drd1a, Susd2*) upregulated in both sexes, one gene (*Lcn2)* downregulated in both sexes, and five were dysregulated in opposite directions (e.g. *Slc35a3, AW551984, Arhgap6, Dlk1, Gpx3*) (Figure 2A).

Biological pathway and process enrichment analysis revealed diverging pathways in males and females. In males, we observed changes in gene expression related to cholinergic synaptic transmission (log10(P) = -11.94), hormone secretion (log10(P) = -10.09), neuropeptide signaling (log10(P) = -7.45), response to nicotine (log10(P) = -6.01), and organic hydroxy compound transport (log10(P) = -6.00) (Figure 2B). In females, the top biological pathways and processes included regulation of hormone levels (log10(P) = -15.39), extracellular matrix organization (log10(P) = -8.1), hormone metabolic processes (log10(P) = -8.41), regulation of cell adhesion (log10(P) = -7.91), and MAPK cascade (log10(P) = -6.68) (Figure 2C).

Functional protein-protein interaction analysis (STRING) in males identified clusters associated with neurotransmission and neuroplasticity, including receptors important for dopaminergic and adenosine receptor-associated signaling and neuropeptides/neuropeptide receptors, a large cluster of transcription factors, and smaller clusters of calcium-activated chloride channels, and neural differentiation (Figure 2D; Table 3). In females, we found more immune-related clusters than in males, including MHC II signaling, interferon-mediated

signaling, cholinergic signaling, and stress hormones. There were also immune and plasticity-related targets, including extracellular matrix and cell adhesion molecules. There were clusters with monoaminergic signaling including dopamine, serotonin, noradrenaline, and adenosine receptors, neuropeptide, and hormone receptors. Smaller clusters included channels, solute carriers important for neuronal inhibition, and metabolic functions (Figure 2E; Table 4).

Hippocampal gene expression in response to an acute immune challenge

We examined the hippocampal transcriptional response to a single, peripheral acute immune challenge in males and females. In males, we found 176 DEGs, and in females, 406 genes were differentially expressed (Figure 3A). Biological pathway and gene enrichment analysis in males revealed gene categories for vascular development (log10(P) = -12.6), MAPK cascade (log10(P) = -11.02), anion transport (log10(P) = -10.63), response to growth factor (log10(P) = -10.43), and negative regulation of MAPK cascade (log10(P) = -7.65) (Figure 3B). In females, most of the DEGs were related to cell adhesion and immune function. These included targets involved in extracellular matrix organization (log10(P) = -11.65), cell chemotaxis (log10(P) = -9.34), cytokine production (log10(P) = -8.86), regulation of cell adhesion (log10(P) = -8.57), copper ion transport (log10(P) = -8.08), positive regulation of MAPK cascade (log10(P) = -7.31), and acute inflammatory response (log10(P) = -5.78) (Figure 3C).

Protein-protein interactions from STRING analysis in males revealed associations between mostly plasticity-related genes including a large cluster related to neuroplasticity with activity-dependent transcription factors and kinases, and solute carriers. There were also changes in targets related to neurotransmission, containing monoaminergic, neuropeptide, and hormone targets; in collagens; and in growth factors including insulin-like growth factor and bone-morphogenetic signaling. There were also immune-associated clusters, including genes related to MHC II signaling, and regulation of immune signaling (Figure 3D; Table 5). In females, we observed clusters of interferon-mediated signaling, negative regulation of cytokine signaling, complement signaling, and immune cell activation. Plasticity-related targets included clusters of cell adhesion molecules and neurotransmission (Figure 3E; Table 6).

Differentially expressed genes between male and female hippocampus

We also examined sex differences in baseline gene expression in the hippocampus. We observed 220 genes differentially expressed in the hippocampus of males compared and females, 95 of which are more strongly expressed in males and 125 in females. As expected, Y-chromosome genes including *Ddx3y* were abundant in males but not evident in females, whereas genes that escape from x-inactivation, including *Xist,* were more abundant in females.

Biological processes for male-biased gene expression included response to hormone (log10(P) = -9.1), response to growth factor (log10(P) = -8.1), negative regulation of catalytic activity (log10(P) = -7.5), cellular response to calcium ion (log10(P) = -6.9), and negative regulation of nuclear transcribed mRNA poly(A) tail shortening (log10(P) = -5.1) (Figure 4A). Female-biased gene expression included biological processes such as extracellular matrix organization (log10(P) = -10.1), neurotransmitter transport (log10(P) = -9.3), response to interferon gamma (log10(P) = -7.5), cell adhesion (log10(P) = -7.5), drug transport (log10(P) = -5.2), regulation of defense response (log10(P) = -4.5), complement and coagulation cascades (log10(P) = -4.1), and cellular metabolic process (log10(P) = -3.9) (Figure 4B).

Protein-protein interaction analysis (STRING) revealed that targets more strongly expressed in the hippocampus of males are involved in G protein and calcium signaling, protein phosphorylation, as well as immediate early genes and DNA-methylation modifiers (Figure 4C; Table 7). Targets that more strongly expressed in the hippocampus of females included

extracellular matrix receptor genes, growth factor, neurotransmitter receptors, neurotransmitter transporter, protein phosphorylation, immune signaling, and complement-associated genes (Figure 4D; Table 7).

To examine the contribution of initial sex differences in gene expression on the sex-specific changes after subchronic immune challenge, we examine the impact of prior immune challenge on genes more strongly expressed in males at baseline ("male-biased" genes) and those more strongly expressed in females ("female-biased" genes). Overall, in males, male-biased genes tended to be downregulated and female-biased genes upregulated long after immune challenge. Here, of the 46 genes higher in males at baseline, 34 were downregulated and only 12 upregulated; and of the 42 genes higher in females at baseline 41 were upregulated in males and only one downregulated long after subchronic immune challenge. Conversely, female-biased genes tended to be downregulated (13 of 13) and male-biased genes were more likely to be upregulated (4 of 4) in females long after subchronic immune challenge.

Specifically, the male-biased genes downregulated in males long after immune challenge represented biological pathways including long-term memory (*Arc, Egr1, Npas4*), p38MAPK cascade (*Gadd45b/g, Per2*), and positive regulation of cell death (e.g. *Atf3, Dusp1, Ptgs2*). Female-biased genes upregulated in males included those belonging to biological pathways transport of bile salts and organic acids, metal ions, and amine compounds (e.g. *Slc6a12, Slc22a6, Sphk1*), collagen chain trimerization (e.g. *Bmp7, Col9a2*), and prostaglandin biosynthesis (e.g. *Cd74, Ptgds*) (Table 8).

In the Long-term+Acute group, female-biased genes were more likely to be upregulated in males and downregulated in females. Interestingly, whereas female-biased genes were strongly differentially expressed in both sexes, male-biased genes were not differentially expressed in either sex. In males, 6 of 6 female-biased genes were upregulated. In females, 53 of 75 were downregulated, including MHC class II (e.g. *H2-Aa, Cd74, Spp1*), cellular hormone metabolism (e.g. *Bmp6, Ttr*), and extracellular matrix organization (e.g. *Cdh1, Col8a1, Enpp2*) (Table 9).

Shared targets and pathways amongst experimental conditions:

We conducted meta-analyses for both males and females to examine similarities and differences between persistent, acute, and long-lasting transcriptional changes in the hippocampus. In males, 103/230 the DEGs (purple lines) in the Long-term condition were also differentially expressed in response to an acute LPS injection, demonstrating a persistent change in immune processes 3 months after subchronic immune challenge. In contrast, only one DEG was common to both Long-term condition and animals in the Long-term+Acute, demonstrating that the DEGs that are altered at baseline, and those that are differentially regulated in response to another immune challenge reflect different pathways or processes. In addition, 6/230 genes were dysregulated in both the Long-term+Acute and Acute conditions, demonstrating that the acute neuroimmune response in hippocampus is largely unchanged by prior subchronic immune challenge (Figure 5A).

In females, only 1/26 DEGs in the Long-term condition were also dysregulated after acute immune challenge. One gene is also commonly dysregulated between Long-Term and Long-term+Acute conditions suggesting that baseline changes in gene expression likely contribute to dysregulation of subsequent neuroimmune response. Consistent with this idea, more than 188 DEGs in the Long-term+Acute condition were shared with those in the Acute conditions, demonstrating an exaggerated – or dysregulated – acute immune response long after a previous subchronic immune challenge (Figure 5C).

This analysis also compares biological pathways and processes between groups (blue lines).

In males, 15 of the top 20 biological pathways are shared between the Long-term and Acute conditions. Most of these shared pathways show similar degrees of enrichment in both conditions. The exception here is extracellular organization processes, which is more highly enriched in the Long-term vs Acute conditions, indicating more changes in extracellular organization processes in long-term conditions. Between Long-term and Long-term+Acute conditions, only 3 biological pathways are shared, again demonstrating that baseline changes in gene expression long after immune challenge regulate processes other than response to acute challenge. Similarly, only 3 biological processes substantially differ between Long-term+Acute and Acute groups, demonstrating the overwhelming similarity of the acute response, with or without prior subchronic immune challenge. (Figure 5B)

In females, 6 biological pathways are shared between Long-term and Acute conditions. Interestingly, even in overlapping pathways, the Acute condition is much more enriched for immune-related processes including cytokine production, whereas Long-term condition results in enriched categories related to catecholaminergic signaling. Unlike males, females showed a strong overlap between Long-Term and Long-term+Acute conditions, with 9 overlapping pathways; and 15 shared pathways in Long-term+Acute and Acute conditions, demonstrating the strongly increased transcriptional response to an acute challenge after prior immune experience. (Figure 5D).

Comparing these males and females in these analyses demonstrates the diverging impact of prior immune challenge on different components of immune function. Whereas males show persistent alterations in baseline expression of immune-related genes also activated during acute immune challenge, females show a fewer DEGs and less overlap with acute immune regulation. In contrast, males show little impact of a prior immune challenge on the acute transcriptional response in the hippocampus, whereas females that have experienced a prior immune challenge show a grossly exaggerated response to an acute inflammation.

DISCUSSION

Here we demonstrated long-lasting consequences of subchronic immune challenge on gene expression in the hippocampus of males and females. In males, we observed enduring dysregulation of gene expression three months after the end of a two-week immune challenge. In contrast, females showed few persistent changes at baseline, but striking changes in gene expression in response to an additional acute LPS injection three months after the subchronic immune challenge. These findings suggest that sex-specific changes in transcriptional regulation may mediate sex- and gender-differences in vulnerability to cognitive decline (Lobo *et al*, 2018) and affective dysregulation (Bjerkeset *et al*, 2005) long after illness or injury. For example, in patients of heart attack, women were at increased risk of anxiety or depression (Bjerkeset *et al*, 2005). Animal models have also shown persistent emotion and memory, and recent findings from our laboratory demonstrate sex differences in vulnerability to memory impairments long after subchronic immune challenge (Tchessalova and Tronson, 2019). The findings described in this paper therefore set the foundation for future studies of how the specific genes, pathways, and processes dysregulated long after subchronic immune challenge contribute to cognitive and affective processes and to vulnerability to further insult in males and females.

Males and females differed in both the magnitude of gene expression changes, and in the specific genes and pathways differentially expressed in the hippocampus long after immune challenge. In males but not females, we observed clear changes in both immune-related pathways and in genes and clusters related to neural plasticity. Immune-related pathways and genes, including MAPK signaling (e.g., *Spp1*, *Dusp1/6*), response to interferon-gamma (e.g., *Ifitm3, Ifit1, Oasl2*), complement signaling (e.g., *C2, Cfh, Col8a1, Col8a2*), and MHC II

signaling (e.g., *H2-Eb1, Cd74*) were significantly dysregulated in males. We also observed changes in expression of several pathways that are important for neuroplasticity. This includes extracellular matrix organization (e.g., *Col4a1, Pcolce, Sulf1*) and cell adhesion molecules (e.g., *Cdh1/3*) that are important for synaptic organization, as well as immediate early genes (e.g., *Fos, Arc, Egr1, Nr4a1, Nr4a2, Junb, Atf3*) that are involved in activity-dependent transcriptional changes necessary for synaptic plasticity (Hawk and Abel, 2011; Minatohara *et al*, 2016). Several genes (*Fos, NR4a1/2, Egr, Spp1, Dusp1/6*) and pathways (MAPK signaling) are notably required for both immune- and neuroplasticity-related functions (Borja-Cacho and Matthews, 2008; Donzis and Tronson, 2014; Nisticò *et al*, 2017; Stephen *et al*, 2017). Alterations in these and other immune-associated targets may therefore also contribute to long-lasting changes in neural function.

In females, the dominant changes were pathways and genes related to monoaminergic signaling and its regulation, including adenosine, dopamine, and adrenergic receptor (e.g., *Adora2a*, *Drd2*, *Adr1b*), and its downstream signaling (e.g., *Ppp1r1b*). Given the importance of dopaminergic and adrenergic signaling for memory modulation, motivational processes, and affective responses (Badgaiyan *et al*, 2010; Stone *et al*, 1999; Strange and Dolan, 2004; Wassum *et al*, 2011). These findings demonstrate striking sex differences in the persistent changes in basal gene expression after a subchronic immune challenge. (Importantly, this suggests that differential vulnerability to cognitive decline, memory impairments, and affective disorders (Himanen *et al*, 2006; Hogue *et al*, 2003; Lavoie *et al*, 2017; Léveillé *et al*, 2017; Liossi *et al*, 2009; Niemeier *et al*, 2007; Rainville and Hodes, 2018; Suarez *et al*, 2015) after illness or injury may be mediated by sex differences in the changes in gene expression and transcriptional regulation that persist long after immune challenge.

Differences in persistent changes in gene expression are likely mediated, in part, by sex differences in the initial cascade of events during the acute immune response. We observed that females showed more changes in hippocampal gene expression after an acute LPS injection compared with males. These findings are consistent with previous findings of sex differences in the neuroimmune response, with differential activation of glial cells (Acaz-Fonseca *et al*, 2015; Santos-Galindo *et al*, 2011), and differences in pattern and timing of cytokine production in the hippocampus (Acaz-Fonseca *et al*, 2015; Santos-Galindo *et al*, 2011; Speirs and Tronson, 2018; Tonelli *et al*, 2008). Surprisingly, stronger regulation of gene expression during acute immune challenge in females did not correspond to more enduring dysregulation of baseline gene expression in females. Nevertheless, three months after subchronic immune challenge, females showed strikingly different transcriptional responses to an acute challenge compared with previously naïve mice. In contrast, males showed similar responsiveness to an acute challenge regardless of prior immune experience. Together, these findings demonstrate that a subchronic immune challenge results in a persistent shift in hippocampal gene expression in males, with few changes in response to a subsequent immune challenge. In contrast, females show little change in gene expression under baseline conditions months after a subchronic immune challenge, but an exaggerated transcriptional response to a subsequent immune challenge.

There were also changes in gene expression that showed notable similarities between the sexes across several conditions. One example is extracellular matrix-related genes, including collagens (e.g., *Col4a1, Col8a1*), matrix regulatory enzymes (e.g., *Pcolce, Sulf1, Adamts1*), and others such as *Otx2*, *Spp1*, and *Cyr61*. Extracellular matrix, and the more specialized perineuronal nets, are of particular interest for their regulation by immune stimuli and their permissive or limiting roles in neuronal plasticity (Beurdeley *et al*, 2013; Carulli *et al*, 2010). As such, extracellular matrix proteins and perineuronal nets contribute to enduring changes in plasticity associated with memory and affective behaviors (Riga *et al*, 2017; Thompson *et al*,

2018). Several of the extracellular matrix genes were differentially regulated months after subchronic immune challenge in males (e.g., *Otx2, Cyr61*) and in females both after a secondary insult and after an acute challenge (e.g. *Otx2, Col9a3*). Such similarities across conditions and sex suggests that extracellular matrix organization may be fundamental to mediating persistent effects of immune challenge and other environmental stimuli (Li *et al*, 2013) on neuronal plasticity and function.

Enduring changes in gene expression after immune challenge is consistent with many studies demonstrating that a variety of experiences cause long-lasting changes in the brain and in behavior. Stress or drugs, for example, induce persistent alterations at the behavioral, physiological, and transcriptional level that impact how an individual will respond to subsequent stressors or novel stimuli (Becker and Le Merrer, 2016; Gray *et al*, 2014; Greenwood *et al*, 2014; Krishnan *et al*, 2007; Mychasiuk *et al*, 2016; Nestler, 2014). As such, it is not surprising that an environmental insult such as an immune challenge results in persistent transcriptional changes that may induce long-lasting alterations in neural function, cognition, and emotion. Our findings are also consistent with previous demonstrations that males and females show strikingly different patterns of gene expression in the brain after stress or drugs of abuse (Finn *et al*, 2018; Hodes *et al*, 2015; Randesi *et al*, 2018) and further demonstrate that the consequences of environmental insults including immune challenge have sex-specific implications for enduring changes in gene expression and function.

Changes in gene expression that persist long after an environmental event mediate adaptive responses to later experiences, and thereby alter vulnerability and resilience to future stressors. Here we observed sex-specific transcriptional changes to a subsequent immune challenge. Males showed similar patterns of gene expression after an acute LPS injection regardless of whether or not they had previously experienced a subchronic challenge. In contrast, females previously exposed to a subchronic immune challenge showed markedly different patterns of gene expression after an acute injection compared with previously naïve mice. Together with the sex-specific changes in baseline gene expression, where males but not females showed changes 12 weeks later, these findings suggest that males and females have different patterns of vulnerability and resilience to future stressors, immune challenge, and other environmental events (Tchessalova *et al*, 2018).

Sex-specific patterns of differential gene expression are consistent with the differential patterns of memory deficits observed in males and females after immune challenge or illness. We have recently demonstrated that males but not females show deficits of fear memory, but that both sexes exhibit impairments of object recognition memory several months after subchronic immune challenge (Tchessalova and Tronson, 2019). Sex-specific patterns of memory deficits are also observed in patients, where women are more vulnerable to disruption of visuospatial tasks months after surgery or injury (Hogue *et al*, 2003; Liossi *et al*, 2009) whereas men show more progressive memory decline over the following years (Himanen *et al*, 2006). Determining the long-lasting changes in gene expression in males and in females is therefore important for identifying sex differences in the contribution of environmental insults to the vulnerability or resilience to cognitive decline, memory impairments, and affective disorders.

How baseline sex differences in hormonal levels (Koss and Frick, 2017; McEwen and Milner, 2017), memory processes (Keiser *et al*, 2017; Keiser and Tronson, 2015), emotion (Pitychoutis and Papadopoulou-Daifoti, 2010), and gene expression (Vied *et al*, 2016) mediate differential vulnerability to immune-triggered memory decline remains unknown. Here, we examined the relationship between sex-biased gene expression at baseline and differential regulation of those genes after immune challenge in males and females. We observed that most of the genes that were higher in the hippocampus of males at baseline were downregulated in the

male hippocampus and upregulated in the female hippocampus long after immune challenge. Similarly, we found that female-biased genes were downregulated in females and upregulated in males after immune challenge. This pattern suggests that baseline differences in hippocampal gene expression contribute to their regulation after immune challenge. The pattern of regulation may be important for predicting which genes and pathways may be differentially vulnerable between the sexes to changes as a consequence of illness or stress.

Our main finding, that immune challenge causes long-lasting changes in transcriptional regulation follows a wealth of data demonstrating that LPS induces both short-lasting tolerance and long-lasting priming of the immune response. Extensive previous work has demonstrated tolerance to consecutive doses of LPS (Norden *et al*, 2016; Seeley and Ghosh, 2017). However, additional LPS injections several months later show no lingering tolerance (Wendeln et al., 2018). In contrast, transient immune activation can result in life-long priming or exaggerated responses to subsequent immune challenge (Hoeijmakers *et al*, 2016) although whether this effect also occurs for immune activation during adulthood is less well-studied. Our findings suggest, at least for immune activation during adulthood, females may be more vulnerable to long-term priming effects compared with males, although this requires additional study. Importantly, although it is tempting to think about such changes of gene expression, as potentially detrimental to neural function, it is equally likely that these changes represent an adaptive or protective response (e.g., *Spp1* (Yu *et al*, 2017). Future work will assess the role of sex-specific persistent transcriptional changes in vulnerability and resilience to immune-mediated cognitive and affective dysregulation across the lifespan.

The findings described here provide critical new insight into the long-lasting impact of immune-related signaling on gene expression in the brain. Understanding the acute and long-lasting contributions of neuroimmune signaling to neuromodulation and plasticity is particularly important given the growing recognition that many environmental events, including stress (Frank *et al*, 2017; McKim *et al*, 2016; Serrats *et al*, 2017; Weber *et al*, 2015; Wohleb *et al*, 2015) and drugs of abuse (Crews *et al*, 2015; Hofford *et al*, 2018), recruit neuroimmune signaling pathways. Here we demonstrated sex-specific patterns of gene expression months after a subchronic immune challenge, where males showed a persistent shift in baseline gene expression and females showed a markedly different response to subsequent stimulation. Enduring changes in genes and pathways that mediate plasticity-related processes, in addition to immune-related genes, suggests that transient inflammatory signaling in the brain has important implications for neural function. As such, identifying the sex-specific changes in transcriptional regulation will be of importance for predicting vulnerabilities to cognitive decline, memory impairments, and affective disorders, and in the future, identifying new, sex-specific biomarkers and therapeutic targets.

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Table 1. Clusters of functional Protein-protein interactions (PPI) between targets in males 3 months after subchronic immune challenge, as shown interactions in Figure 1D.

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
Neuroplasticity				
	Fos	FBJ osteosarcoma oncogene	-4.76	0.0038
	Fosl2	fos-like antigen 2	-1.14	0.0038
	Junb	jun B proto-oncogene	-2.28	0.0038
	Nr4a1	nuclear receptor subfamily 4, group A, member 1	-1.96	0.0038
	Nr4a2	nuclear receptor subfamily 4, group A, member 2	-1.38	0.0038
	Atf3	activating transcription factor 3	-1.8	0.0038
	Egr1	early growth response 1	-1.91	0.0038
	Egr2	early growth response 2	-4.92	0.0038
	Egr3	early growth response 3	-1.51	0.0038
	Egr4	early growth response 4	-2.6	0.0038
	Gadd45b	growth arrest and DNA-damage-inducible 45 beta	-0.75	0.0038
	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	-1.64	0.0038
	Spp1	secreted phosphoprotein 1	1.16	0.0038
	Dusp1	dual specificity phosphatase 1	-1.61	0.0038
	Dusp6	dual specificity phosphatase 6	-0.78	0.0038
	Cdh1	cadherin 1	1.44	0.0038
	Cdh3	cadherin 3	1.67	0.0151
Extracellular matrix organization				
	Col3a1	collagen, type III, alpha 1	1.04	0.0038
	Col4a3	collagen, type IV, alpha 3	1.53	0.0038
	Col4a4	collagen, type IV, alpha 4	1.24	0.0038
	Col4a6	collagen, type IV, alpha 6	0.9	0.0327
	Col8a2	collagen, type VIII, alpha 2	1.56	0.0038
	Col9a3	collagen, type IX, alpha 3	1.23	0.0038
	Col1a1	collagen, type I, alpha 1	0.98	0.0038
	Col1a2	collagen, type I, alpha 2	0.87	0.0038
	Col17a1	collagen, type XVII, alpha 1	1.77	0.0038
	Krt8	keratin 8	1.56	0.0038
	Krt18	keratin 18	1.69	0.0038
	Fbln1	fibulin 1	0.69	0.0038
	Pcolce	procollagen C-endopeptidase enhancer protein	1.39	0.0038
	Pcolce2	procollagen C-endopeptidase enhancer 2	0.68	0.0099

	Adamst1	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1	-0.9	0.0038
Interferon-mediated signaling				
	Ifit1	interferon-induced protein with tetratricopeptide repeats 1	0.84	0.0286
	Ifitm3	interferon induced transmembrane protein 3	0.70	0.0038
	Islr	immunoglobulin superfamily containing leucine-rich repeat	0.97	0.0038
	Oasl2	2'-5' oligoadenylate synthetase-like 2	0.80	0.0307
	Bub1b	BUB1B, mitotic checkpoint serine/threonine kinase	-0.91	0.0038
	Cenpa1	centromere protein A	-0.95	0.0175
	Tuba1c	tubulin, alpha 1C	1.33	0.0175
Growth factor signaling				
	Igf2	insulin-like growth factor 2	1.55	0.0038
	Igfbp2	insulin-like growth factor binding protein 2	1.33	0.0038
	Tgfbi	transforming growth factor, beta induced	1.10	0.0038
	Bmp6	bone morphogenetic protein 6	1.04	0.0038
	Bmp7	bone morphogenetic protein 7	0.97	0.0038
MHC Class II signaling				
	H2-Aa	histocompatibility 2, class II antigen A, alpha	1.11	0.0038
	H2-eb1	histocompatibility 2, class II antigen E beta	1.1	0.0125
	H2-q1	histocompatibility 2, Q region locus 1	1.22	0.0366
	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	1.08	0.0038
	Fap	fibroblast activation protein	1.14	0.007
Complement signaling				
	C2	complement component 2 (within H-2S)	0.98	0.0099
	Cd59a	CD59a antigen	1.09	0.0038
	Cfh	complement component factor h	0.6	0.0038
	Serping1	serine (or cysteine) peptidase inhibitor, clade G, member 1	0.92	0.0038
Solute carriers				
	Slc13a3	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	0.78	0.0038
	Slc22a2	solute carrier family 22 (organic cation transporter), member 2	1.45	0.0347
	Slc22a6	solute carrier family 22 (organic anion transporter), member 6	1.03	0.0038
	Slc47a1	solute carrier family 47, member 1	1.19	0.0099
Tight-junctions				
	Cldn1	claudin 1	1.06	0.0038
	Cldn2	claudin 2	2.18	0.0038
	Cldn9	claudin 9	2.09	0.0403

Table 2. Clusters of functional Protein-protein interactions (PPI) between targets in females 3 months after subchronic immune challenge, as shown interactions in Figure 1E.

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
Monoaminergic signaling				
	Ppp1r1b	protein phosphatase 1, regulatory (inhibitor) subunit 1B	-0.69	0.0038
	Adora2a	adenosine A2a receptor	-1.57	0.0038
	Drd2	dopamine receptor D2	-1.10	0.0125
	Adra1b	adrenergic receptor, alpha 1b	-0.76	0.0125
	Gpr88	G-protein coupled receptor 88	-0.88	0.0038
Immune signaling				
	ifit1	interferon-induced protein with tetratricopeptide repeats 1	-0.85	0.0175
	Irgm2	immunity-related GTPase family M member 2	-0.64	0.0476
	Gbp4	guanylate binding protein 4	-1.02	0.0038

Table 3. Clusters of protein-protein interactions (PPI) differentially regulated after an acute LPS challenge in males previously exposed to a subchronic immune challenge, as shown in Figure 2D.

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
Neurotransmission				
	Adora2a	adenosine A2a receptor	0.85	0.0286
	Drd1a	dopamine receptor D1	0.69	0.0099
	Drd2	dopamine receptor D2	0.94	0.0440
	Penk	preproenkephalin	0.96	0.0038
	Sstr5	somatostatin receptor 5	1.80	0.0265
	Tac1	tachykinin 1	1.05	0.0038
	Tac1r	tachykinin receptor 1	0.79	0.0385
Transcription factor				
	Isl1	ISL1 transcription factor, LIM/homeodomain	3.63	0.0385
	Eomes	eomesodermin	2.24	0.0038
	Barhl2	BarH-like 2 (Drosophila)	2.16	0.0221
	Six3	sine oculis-related homeobox 3	0.99	0.0385
	Foxp2	forkhead box P2	0.81	0.0151
Cholinergic signaling				
	Chrna3	cholinergic receptor, nicotinic, alpha polypeptide 3	1.84	0.0038
	Chrmb3	cholinergic receptor, nicotinic, beta polypeptide 3	1.78	0.0038
	Crhmb4	cholinergic receptor, nicotinic, beta polypeptide 4	1.06	0.0307
Calcium activated chloride channels				
	Ano1	anoctamin 1, calcium activated chloride channel	0.93	0.0038
	Ano2	anoctamin 2	0.74	0.0151
Neural differentiation				
	Dlk1	delta-like 1 homolog (Drosophila)	0.65	0.0038
	Peg10	paternally expressed 10	0.59	0.0038

Table 4. Clusters of protein-protein interactions (PPI) differentially regulated after an acute LPS challenge in females previously exposed to a subchronic immune challenge, as shown in Figure 2E.

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
Extracellular matrix proteins				
	Col4a3	collagen, type IV, alpha 3	-1.89	0.0038
	Col4a4	collagen, type IV, alpha 4	-2.17	0.0038
	Col8a1	collagen, type VIII, alpha 1	-2.02	0.0038
	Col8a2	collagen, type VIII, alpha 2	-1.71	0.0038
	Col9a3	collagen, type IX, alpha 3	-1.28	0.0038
	Col18a1	collagen, type XVIII, alpha 1	-0.76	0.0038
	Col23a1	collagen, type XXIII, alpha 1	-0.67	0.0038
	Col24a1	collagen, type XXIV, alpha 1	1.34	0.0038
	Col17a1	collagen, type XVII, alpha 1	-1.46	0.0038
	Fbnl1	fibulin 1	-0.77	0.0038
	Krt8	keratin 8	-1.48	0.0038
	Lgals1	lectin, galactose binding, soluble 1	-0.67	0.0070
	Pcolce	procollagen C-endopeptidase enhancer protein	-1.16	0.0038
Monoaminergic signaling				
	Adora2a	adenosine A2a receptor	2.95	0.0038
	Adra1b	adrenergic receptor, alpha 1b	1.23	0.0038
	Drd1a	dopamine receptor D1	1.38	0.0038
	Drd2	dopamine receptor D2	2.54	0.0038
	Gpr6	G protein-coupled receptor 6	2.33	0.0038
	Gpr88	G-protein coupled receptor 88	2.32	0.0038
	Oxtr	oxytocin receptor	-0.71	0.0038
	Trhr2	thyrotropin releasing hormone receptor 2	1.79	0.0070
Immune signaling				
	Ifg2	insulin-like growth factor 2	-1.69	0.0038
	Igfbp2	insulin-like growth factor binding protein 2	-1.47	0.0038
	Igfbp3	insulin-like growth factor binding protein 3	-0.75	0.0038
	Igfbp6	insulin-like growth factor binding protein 6	0.63	0.0070
	Spp1	secreted phosphoprotein 1	-1.03	0.0038
	Tgfbi	transforming growth factor, beta induced	-0.69	0.0125
MHC Class II signaling				
	Cd4	CD4 antigen	3.59	0.0038
	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	-1.15	0.0038

	H2-Aa	histocompatibility 2, class II antigen A, alpha	-1.28	0.0038
	H2-Ab1	histocompatibility 2, class II antigen A, beta 1	-1.22	0.0038
	H2-Eb1	histocompatibility 2, class II antigen E beta	-1.05	0.0099
	H2-Q1	histocompatibility 2, Q region locus 1	-1.45	0.0458
Cell adhesion molecules				
	Icam	intercellular adhesion molecule 1	-1.10	0.0038
	Vcam1	vascular cell adhesion molecule 1	-0.71	0.0038
	Cdh1	cadherin 1	-1.28	0.0038
	Cdh3	cadherin 3	-1.83	0.0038
Interferon-mediated signaling				
	Ifi44	interferon-induced protein 44	-1.20	0.0038
	Ifitm1	interferon induced transmembrane protein 1	-1.36	0.0038
	Ifitm3	interferon induced transmembrane protein 3	-0.81	
	Irf7	interferon regulatory factor 7	-0.98	0.0038
	Oasl2	2'-5' oligoadenylate synthetase-like 2	-0.68	0.0265
Neuronal inhibition				
	Best3	bestrophin 3	-1.13	0.0243
	Clic6	chloride intracellular channel 6	-2.09	0.0038
	Gabrd	gamma-aminobutyric acid (GABA) A receptor, subunit delta	0.83	0.0038
	Slc6a13	solute carrier family 6 (neurotransmitter transporter, GABA), member 13	-0.63	0.0125
Tight junction				
	Cldn1	claudin 1	-1.66	0.0038
	Cldn2	claudin 2	-2.94	0.0038
Gap junction				
	Gjb1	gap junction protein, beta 1	0.87	0.0038
	Gjb2	gap junction protein, beta 2	-0.67	0.0038
	Gjc2	gap junction protein, gamma 2	0.78	0.0038
Stress hormone				
	Crhr2	corticotropin releasing hormone receptor 2	-1.28	0.0038
	Pomc2	pro-opiomelanocortin-alpha	2.14	0.0038

Table 5. Clusters of functional Protein-protein interactions (PPI) in males after an acute LPS injection, as shown in Figure 3D.

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
Neuroplasticity				
	Camk2d	calcium/calmodulin-dependent protein kinase II, delta	0.83	0.0038
	Gadd45b	growth arrest and DNA-damage-inducible 45 beta	-0.87	0.0038
	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	-1.86	0.0038
	Clic6	chloride intracellular channel 6	1.67	0.0038
	Kcne2	potassium voltage-gated channel, Isk-related subfamily, gene 2	1.73	0.0038
	Kcnj2	potassium inwardly-rectifying channel, subfamily J, member 2	-1.69	0.0038
	Slc37a2	solute carrier family 37 (glycerol-3-phosphate transporter), member 2	0.88	0.0038
	Slc17a6	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6	0.84	0.0038
	Tmem27	transmembrane protein 27	1.73	0.0476
Regulation immune signaling				
	Ccl3	chemokine (C-C motif) ligand 3	-2.14	0.0327
	Nfkbiz	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, zeta	-0.80	0.0038
	Ppp1r15 A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	-0.91	0.0038
	Ptgs2	prostaglandin-endoperoxide synthase 2	-1.80	0.0038
	Krt8	keratin 8	1.18	0.0038
	Krt18	keratin 18	1.24	0.0038
Growth factor				
	Igf2	insulin-like growth factor 2	1.08	0.0038
	Igfbp2	insulin-like growth factor binding protein 2	0.96	0.0038
	Igfbp6	insulin-like growth factor binding protein 6	0.62	0.0198
	Bmp6	bone morphogenetic protein 6	0.71	0.0070
	Bmp7	bone morphogenetic protein 7	0.75	0.0038
Extracellular matrix protein				
	Col4a4	collagen, type IV, alpha 4	0.89	0.0494
	Col6a3	collagen, type VI, alpha 3	-0.75	0.0175
	Col8a1	collagen, type VIII, alpha 1	1.59	0.0038
	Col8a2	collagen, type VIII, alpha 2	1.28	0.0038
	Col9a3	collagen, type IX, alpha 3	0.91	0.0038
Immune cell activation				
	Cyr61	cysteine rich protein 61	-2.43	0.0038
	Lbp	lipopolysaccharide binding protein	1.01	0.0038
	Ly6a	lymphocyte antigen 6 complex, locus A	0.80	0.0038

MHC Class II signaling				
	H2-Aa	histocompatibility 2, class II antigen A, alpha	0.97	0.0243
	H2-Ab1	histocompatibility 2, class II antigen A, beta 1	1.15	0.0038
	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	1.07	0.0038
Complement signaling				
	A2m	alpha-2-macroglobulin	0.77	0.0198
	Cd59a	CD59a antigen	0.97	0.0038
Neurotransmission				
	Gabra6	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 6	-2.53	0.0038
	Htr2c	5-hydroxytryptamine (serotonin) receptor 2C	0.76	0.0038
	Ntsr1	neurotensin receptor 1	0.62	0.0458

Table 6. Clusters of protein-protein interactions (PPI) between targets in females after an acute LPS injection, as shown in Figure 3E.

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
Immune cell activation				
	Lbp	lipopolysaccharide binding protein	2.00	0.0038
	Tlr2	toll-like receptor 2	1.25	0.0038
	Lgals3	lectin, galactose binding, soluble 3	1.00	0.0366
	Lgals3bp	lectin, galactoside-binding, soluble, 3 binding protein	0.89	0.0038
	Lgals9	lectin, galactose binding, soluble 9	0.72	0.0038
	Cd4	CD4 antigen	-1.45	0.0038
	Bcl3	B cell leukemia/lymphoma 3	1.26	0.0347
	Socs3	suppressor of cytokine signaling 3	0.86	0.0151
Interferon-mediated signaling				
	Ifi35	interferon-induced protein 35	0.81	0.0265
	Ifi44	interferon-induced protein 44	1.42	0.0038
	Ifim1	interferon induced transmembrane protein 1	0.87	0.0151
	Ifitm3	interferon induced transmembrane protein 3	1.15	0.0038
	Irf7	interferon regulatory factor 7	1.67	0.0038
	Oas2	2'-5' oligoadenylate synthetase 2	0.96	0.0366
	Oasl2	2'-5' oligoadenylate synthetase-like 2	0.69	0.0175
	Zbp1	Z-DNA binding protein 1	2.61	0.0038
Monoaminergic signaling				
	Htr2c	5-hydroxytryptamine (serotonin) receptor 2C	1.40	0.0038
	Cckbr	cholecystokinin B receptor	-0.67	0.0038
	Trhr	thyrotropin releasing hormone receptor	1.17	0.0038
	Enpp2	ectonucleotide pyrophosphatase/phosphodiesterase 2	2.75	0.0038
	Enpp6	ectonucleotide pyrophosphatase/phosphodiesterase 6	-0.76	0.0151
Complement activation				
	A2m	alpha-2-macroglobulin	2.96	0.0038
	Ccr2	chemokine (C-C motif) receptor 2	1.12	0.0476
	Ccr5	chemokine (C-C motif) receptor 5	0.68	0.007
	Cd59a	CD59a antigen	1.41	0.0038
	Cd55	CD55 molecule, decay accelerating	0.75	0.0198
Cell adhesion molecules				
	Cldn1	claudin 1	0.89	0.0038
	Cldn2	claudin 2	4.55	0.0038
	Col4a4	collagen, type IV, alpha 4	2.17	0.0038
	Col8a1	collagen, type VIII, alpha 1	3.35	0.0038

	Col8a2	collagen, type VIII, alpha 2	2.45	0.0038
Metabolism				
	Steap1	six transmembrane epithelial antigen of the prostate 1	4.01	0.0038
	Steap2	six transmembrane epithelial antigen of prostate 2	1.69	0.0038
	Ucp2	uncoupling protein 2 (mitochondrial, proton carrier)	0.90	0.0038
	Xdh	xanthine dehydrogenase	1.1	0.0038
Negative regulation cytokine signaling/growth factor signaling				
	Igf2	insulin-like growth factor 2	1.06	0.0038
	Igfbp2	insulin-like growth factor binding protein 2	1.14	0.0038
	Slc22a8	solute carrier family 22 (organic anion transporter), member 8	-0.83	0.0038

Table 7. Clusters of protein-protein interactions (PPI) between targets that show differential expression in male and female hippocampi, as shown in Figure 4D.

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
MALE BIASED GENE EXPRESSION				
Neuroplasticity				
	Fos	FBJ osteosarcoma oncogene	-5.57	0.0038
	Fosl2	fos-like antigen 2	-1.44	0.0038
	Atf3	activating transcription factor 3	-1.74	0.0038
	Nr4a2	nuclear receptor subfamily 4, group A, member 2	-1.20	0.0038
	Jun	jun proto-oncogene	-0.69	0.0038
	Junb	jun B proto-oncogene	-2.48	0.0038
	Egr1	early growth response 1	-2.12	0.0038
	Egr3	early growth response 3	-1.48	0.0038
	Egr4	early growth response 4	-2.85	0.0038
	Ier2	immediate early response 2	-1.73	0.0038
	Arc	activity regulated cytoskeletal-associated protein	-2.44	0.0038
	Gadd45b	growth arrest and DNA-damage-inducible 45 beta	-0.98	0.0038
	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	-2.04	0.0038
	Per1	period circadian clock 1	-0.83	0.0038
	Irs2	insulin receptor substrate 2	-0.61	0.0038
	Nfil3	nuclear factor, interleukin 3, regulated	-0.69	0.0070
	Kl	Klotho	-0.91	0.0038
	Cyr61	cysteine rich protein 61	-2.42	0.0038
	Dusp1	dual specificity phosphatase 1	-1.73	0.0038
	Dusp6	dual specificity phosphatase 6	-1.10	0.0038
	Ppp1r15a	protein phosphatase 1, regulatory (inhibitor) subunit 15A	-0.86	0.0038
	Sgk1	serum/glucocorticoid regulated kinase 1	-0.70	0.0038
	Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1	-1.23	0.0265
	Tiparp	TCDD-inducible poly(ADP-ribose) polymerase	-1.27	0.0038
Neural inhibition				
	Gabra6	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 6	-2.29	0.0038
	Clic6	chloride intracellular channel 6	-1.08	0.0038
	Kcne2	potassium voltage-gated channel, Isk-related subfamily, gene 2	-2.52	0.0038
	Kcnj2	potassium inwardly-rectifying channel, subfamily J, member 2	-1.35	0.0038
Neurotransmission				
	Chrm5	cholinergic receptor, muscarinic 5	-1.49	0.0175
	Trhr	thyrotropin releasing hormone receptor	-0.81	0.0099
Coagulation				
	F5	coagulation factor V	-1.93	0.0038
	Folr1	folate receptor 1 (adult)	-2.11	0.0038
FEMALE BIASED GENE EXPRESSION				
MHC II signaling				
	H2-Aa	histocompatibility 2, class II antigen A, alpha	1.01	0.0198
	H2-Ab1	histocompatibility 2, class II antigen A, beta 1	1.20	0.0038

	H2-D1	histocompatibility 2, D region locus 1	0.63	0.0038
	H2-K1	histocompatibility 2, K1, K region	0.71	0.0038
	H2-Q1	histocompatibility 2, Q region locus 1	1.50	0.0038
	H2-Q4	histocompatibility 2, Q region locus 4	0.93	0.0038
	H2-Q6	histocompatibility 2, Q region locus 6	2.57	0.0070
	Cd4	CD4 antigen	1.38	0.0175
	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	1.10	0.0038
	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	0.68	0.0327
	Myoc	Myocilin	0.66	0.0038
	Mrc1	mannose receptor, C type 1	0.93	0.0038
	Mrc2	mannose receptor, C type 2	0.75	0.0038
Interferon signaling				
	Ifi44	interferon-induced protein 44	1.30	0.0099
	ifi47	interferon gamma inducible protein 47	1.36	0.0307
	ifit1	interferon-induced protein with tetratricopeptide repeats 1	0.94	0.0099
	Ifitm3	interferon induced transmembrane protein 3	0.73	0.0038
	Isg15	ISG15 ubiquitin-like modifier	0.95	0.0440
	Irgm2	immunity-related GTPase family M member 2	0.76	0.0125
	Gbp2	guanylate binding protein 2	1.10	0.0038
	Gbp3	guanylate binding protein 3	0.88	0.0038
	Gbp4	guanylate binding protein 4	1.47	0.0038
	Oasl2	2'-5' oligoadenylate synthetase-like 2	0.88	0.0038
Monoaminergic signaling				
	Adora2a	adenosine A2a receptor	0.86	0.0099
	Adra1b	adrenergic receptor, alpha 1b	0.72	0.0366
	Cckbr	cholecystokinin B receptor	0.78	0.0038
	Drd1a	dopamine receptor D1	0.87	0.0038
	Drd2	dopamine receptor D2	1.17	0.0038
	Gpr88	G-protein coupled receptor 88	0.97	0.0038
	Ntsr1	neurotensin receptor 1	0.66	0.0286
	Ptgds	prostaglandin D2 synthase (brain)	1.40	0.0038
	Ptgfr	prostaglandin F receptor	1.39	0.0070
Extracellular matrix organization				
	Aebp1	AE binding protein 1	1.01	0.0038
	Col3a1	collagen, type III, alpha 1	1.45	0.0038
	Col4a6	collagen, type IV, alpha 6	0.94	0.0243
	Col1a1	collagen, type I, alpha 1	1.38	0.0038
	Col1a2	collagen, type I, alpha 2	1.07	0.0038
	Efemp1	epidermal growth factor-containing fibulin-like extracellular matrix protein 1	0.93	0.0038
	Fmod	Fibromodulin	1.50	0.0038
	Islr	immunoglobulin superfamily containing leucine-rich repeat	1.05	0.0038
	Ogn	Osteoglycin	0.89	0.0038
Solute carriers				
	Slc13a3	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	0.99	0.0038
	Slc22a2	solute carrier family 22 (organic cation transporter), member 2	2.06	0.0038

	Slc22a6	solute carrier family 22 (organic anion transporter), member 6	1.33	0.0038
	Slc22a8	solute carrier family 22 (organic anion transporter), member 8	0.69	0.0038
	Slc47a1	solute carrier family 47, member 1	1.62	0.0038
Complement signaling				
	C2	complement component 2 (within H-2S)	1.26	0.0038
	Cfh	complement component factor h	0.72	0.0038
	Spp1	secreted phosphoprotein 1	1.71	0.0038
	Serping1	serine (or cysteine) peptidase inhibitor, clade G, member 1	0.94	0.0038
	Serpinf1	serine (or cysteine) peptidase inhibitor, clade F, member 1	0.62	0.0151
Growth factor				
	Cdh1	cadherin 1	1.71	0.0038
	Bmp6	bone morphogenetic protein 6	0.64	0.0125
	Bmp7	bone morphogenetic protein 7	0.89	0.0038
	Foxc2	forkhead box C2	1.69	0.0038

Table 8. Impact of subchronic immune challenge on expression of genes more strongly expressed in males ("male-biased") or females ("female-biased") at baseline.

MALE-BIASED GENES				
	MALES		**FEMALES**	
	Pathway	**Genes**	**Pathway**	**Genes**
Upregulated	**Inorganic cation transport**		n/a	
		Steap1		
		Slc39a4		
		Kcne2		
Downregulated	**Positive regulation of cell death**			
		Atf3		
		Egr1		
		Fos		
		Nr4a1		
		Ccn1		
		Gadd45b		
		Ptsg2		
		Dusp1		
		Gadd45g		
		Dusp6		
		Per1		
		Pppr1r15a		
		Sik1		
	p38 MAPK cascade			
		Gadd45b		
		Gadd45g		
		Dusp1		
		Per1		
	Long-term memory			
		Arc		
		Egr1		
		Npas4		
FEMALE-BIASED GENES				
	MALES		**FEMALES**	
	Pathway	**Genes**	**Pathway**	**Genes**

Upregulated	**Collagen chain trimerization**		n/a
		Aebp1	
		Aldh1a2	
		Bmp7	
		Col1a1	
		Col1a2	
		Col3a1	
		Col4a6	
		Col9a2	
		Cd74	
		Efemp7	
		Emilin1	
		Ifitm3	
		Islr	
		Mrc2	
		Ptgdr	
		Serping1	
		Slc22a6	
	Immunoglobulin mediated immune response		
		Sned1	
		Bmp7	
		C2	
		Col3a1	
		Emilin1	
		H2-Ab1	
		Ifit1	
	Transport of bile salts and organic acids, metal ions and amine compounds		
		Serping1	
		Slc6a12	
		Slc6a13	
		Slc22a6	
		Slc47a1	
		Slc6a20a	
		Slc13a3	
	Prostaglandin biosynthesis		
		Sphk1	

		Aldh1a2		
		Bmp6		
		Cd74		
		Ptgds		
		Sphk1		
Downregulated	n/a		**Response to amphetamine**	
				Adora2a
				Adra1b
				Cckbr
				Coch
				Drd2
				Gbp4
				Irgm2
				Scn4b
				Spp1
			Response to virus	
				Coch
				Gbp4
				Ifit1

Table 9. Impact of subchronic + Acute immune challenge on expression of genes more strongly expressed in males ("male-biased") or females ("female-biased") at baseline.

MALE-BIASED GENES				
	MALES		**FEMALES**	
	Pathway	**Genes**	**Pathway**	**Genes**
Upregulated	n/a		n/a	
Downregulated	n/a		n/a	

FEMALE-BIASED GENES				
	MALES		**FEMALES**	
	Pathway	**Genes**	**Pathway**	**Genes**
Upregulated	**Regulation synaptic transmission**		**Neurotransmitter transport**	
		Adora2a		*Adora2a*
		Drd1		*Cckbr*
		Drd2		*Cd4*
		Sphk1		*Cobl*
	Learning			*Dpp4*
		Adora2a		*Drd1*
		Drd1		*Drd2*
		Drd2		*Gpr88*
		Foxp2		*Rims3*
				Scn4b
				Slc6a12
				Syt2
				Trhr2
			Regulation synaptic vesicle exocytosis	
				Adora2a
				Drd1
				Drd2
				Rims3
				Syt2
			Leukocyte cell-cell adhesion	
Downregulated	**n/a**			*Adora2a*
				Cd4
				Dpp4

		Antigen processing and presentation of exogenous peptide antigen via MHC class II	
			Bmp7
			Cdh1
			Cd74
			Cldn2
			Col8a1
			Cyp1b1
			Enpp2
			Gbp2
			H2-Aa
			H2-Ab1
			H2-Q1
			Ifitm3
			Krt18
			Mpzl2
			Mrc1
			ASpp1
		Negative regulation cellular proliferation	
			Aldh1a2
			Bmp7
			Cdh1
			Cyp1b1
			Dlk1
			H2-Aa
			H2-Ab1
			Ifitm3
			Npr3
			Ptdgs
		Extracellular matrix organization	
			Bmp7
			Cdh1
			Col8a1
			Cyp1b1
			Fol1r
			Pcolce
			Spp1

			Ttr
		Cellular hormone metabolic process	
			Aldh1a2
			Bmp6
			Crym
			Cyt1b1
			KI
			Mc4r
			Spp1
			Ttr
		Regulation viral life cycle	
			Cd74
			Ifitm3
			Oasl2
		Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor	
			F5
			Itih2
			Spp1

FIGURE LEGENDS

Figure 1. Subchronic, peripheral LPS challenge induces changes in hippocampal gene expression 12 weeks after last injection. (A) Males (purple) show a greater number of DEGs than females (yellow). Males showed 183 upregulated and 47 downregulated genes, whereas females showed 7 and 18 up- and down-regulated genes, respectively. (B,C) Biological pathways and processes enriched in gene set (B) in males and (C) in females were generated through Metascape. Distinct biological pathways are observed in males and females, with greater plasticity and immune-related targets in males and greater monoaminergic signaling in females. (D, E) Protein-protein interaction (PPI) networks of targets were generated using STRING 10.5 and clustered by biological function (D) in males and (E) in females. Edges between nodes are color coded for relationship type. Blue: known interactions; Pink experimentally determined interactions; Black: Co-expression of targets; Purple: protein homology; Green, yellow, and dark blue: predicted interactions.

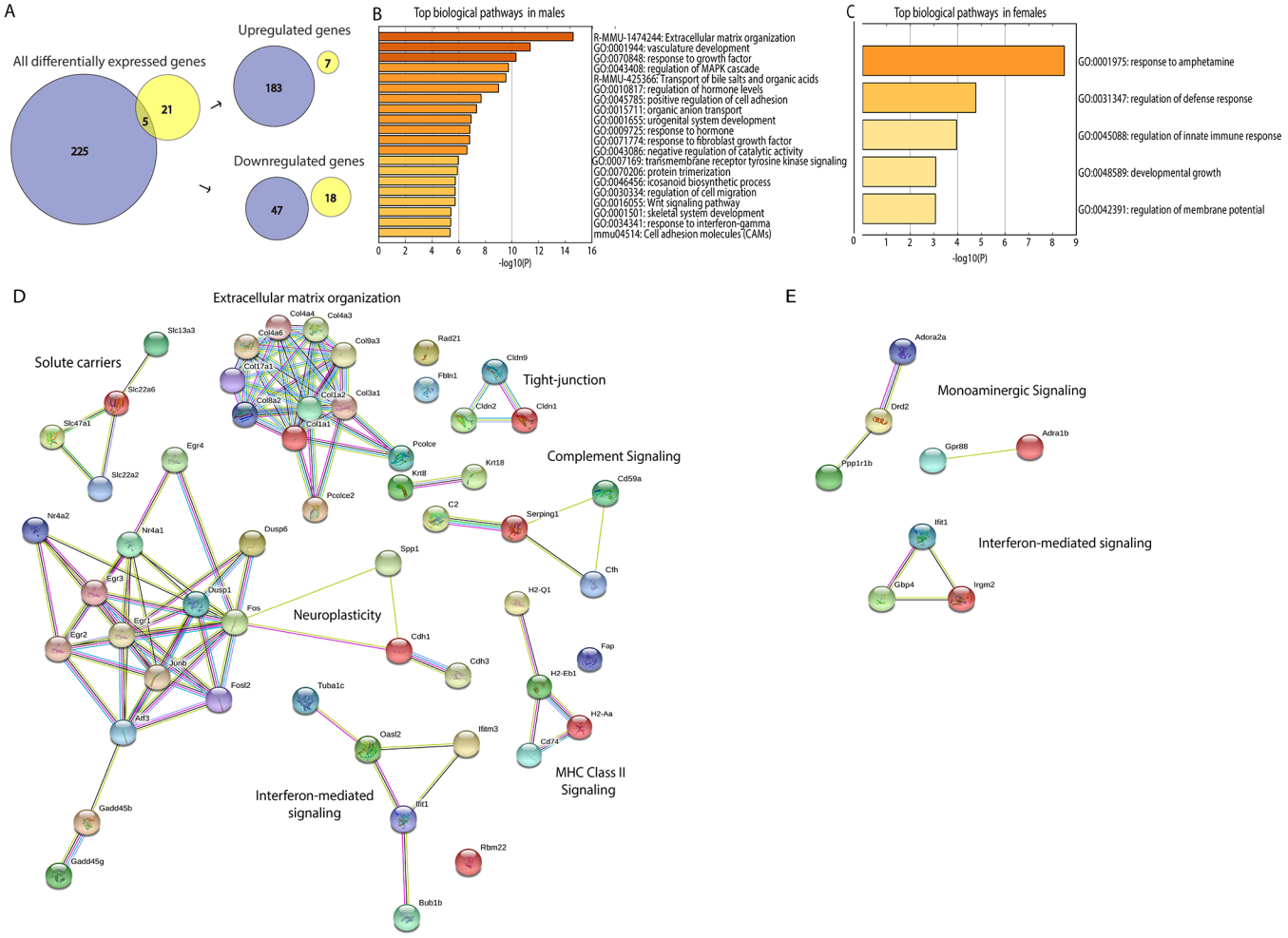
Figure 2. Prior subchronic, peripheral LPS challenge alters hippocampal gene expression in response to a subsequent, acute challenge in a sex-specific manner. (A) Females (yellow) show a greater number of differentially expressed genes than males (purple). Females show 192 upregulated and 240 downregulated genes, whereas males show 67 up- and 1 downregulated gene compared with the response to acute immune challenge in previously naïve animals. (B,C) Distinct biological pathways and processes are enriched in DEGs of (B) males and (C) females who showed greater numbers of pathways and changes in immune-related pathways. (D,E) Protein interaction (PPI) networks of targets, clustered by biological function (D) in males and (E) in females.

Figure 3. Acute, peripheral immune challenge induces greater changes in the female hippocampus. (A) Previously naïve females (pink) exhibited greater differential gene expression in response to acute LPS challenge than do males (turquoise). Females had 353 genes upregulated and 53 downregulated genes compared with saline treated mice, and males showed 118 upregulated and 54 downregulated after acute immune challenge. (B,C) Biological pathways and processes are enriched in DEGs in (B) males and (C) females. Both sexes show changes in MAPK cascade and extracellular structure organization, yet males show greater changes in growth factor signaling while females shown greater changes in other immune-related pathways. (D, E) Protein interaction (PPI) networks of targets, clustered by biological function (D) in males and (E) in females.

Figure 4. Differential gene expression in hippocampus of males *vs* females prior to immune challenge. (A) All differentially expressed (DEGs) in immune-naïve male *versus* female mice. 95 genes were more highly expressed in male hippocampi whereas 125 genes were more highly expressed in females. (B,C) Biological pathways and processes enriched in (B) males compared with females and (C) in females compared with males. (D,E) Protein interaction (PPI) networks of targets, clustered by biological function (D) in males and (E) in females.

Figure 5. Meta-analysis of differentially expressed genes amongst long-term and acute conditions in males and females. (A) Males. Circos plot of differentially expressed genes amongst Long-term (3 months after subchronic LPS injections) Acute (6 hrs post single LPS injection), and Long-term+Acute (LPS injection 3 months after subchronic challenge) conditions. DEG links shared between experimental conditions are depicted by purple lines

while different genes that share similar biological pathways are depicted in blue. (B) Heatmap of selected enriched gene ontology (GO) terms compared between long-term and acute conditions in males. (C) Females. Circos plot of differentially expressed genes amongst Long-term, Acute, and Long-term+Acute conditions. (D) Heatmap of selected enriched gene ontology (GO) terms compared between long-term and acute conditions in females.



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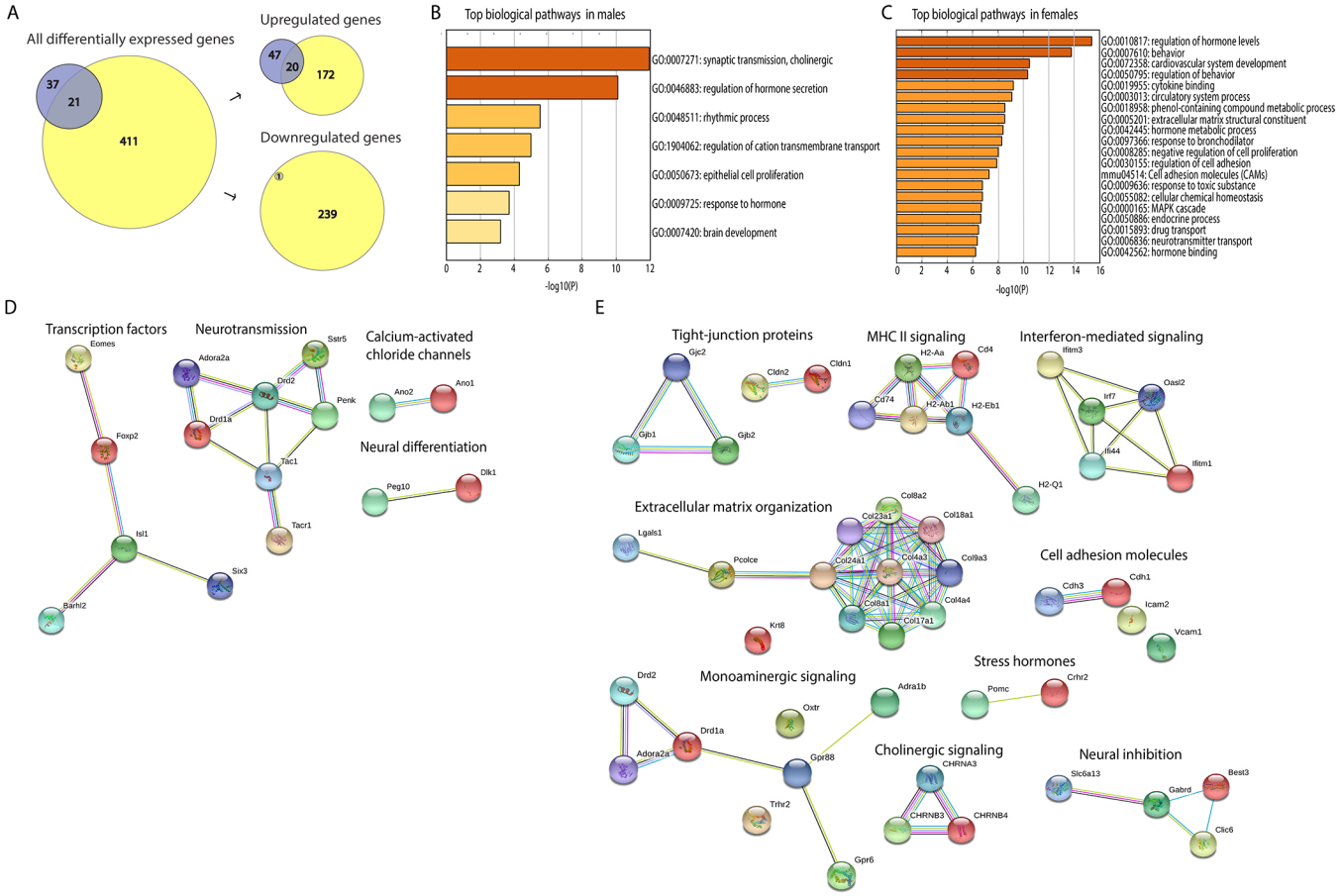

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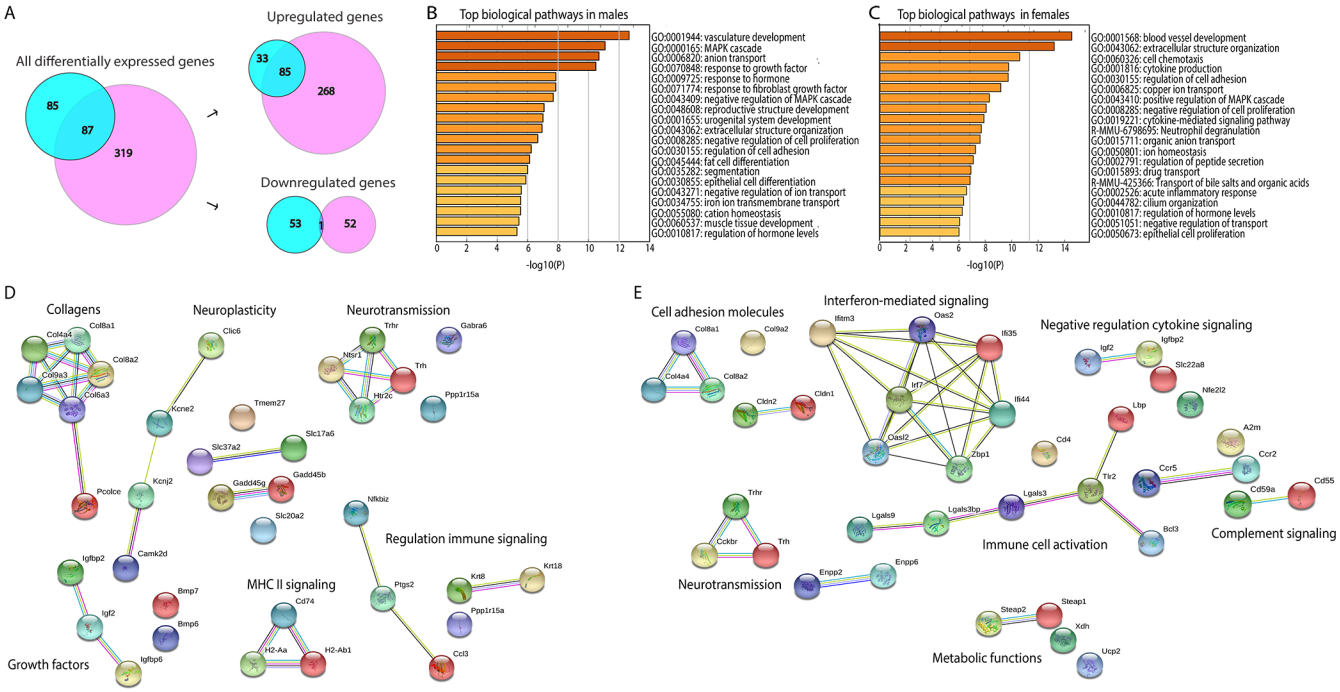

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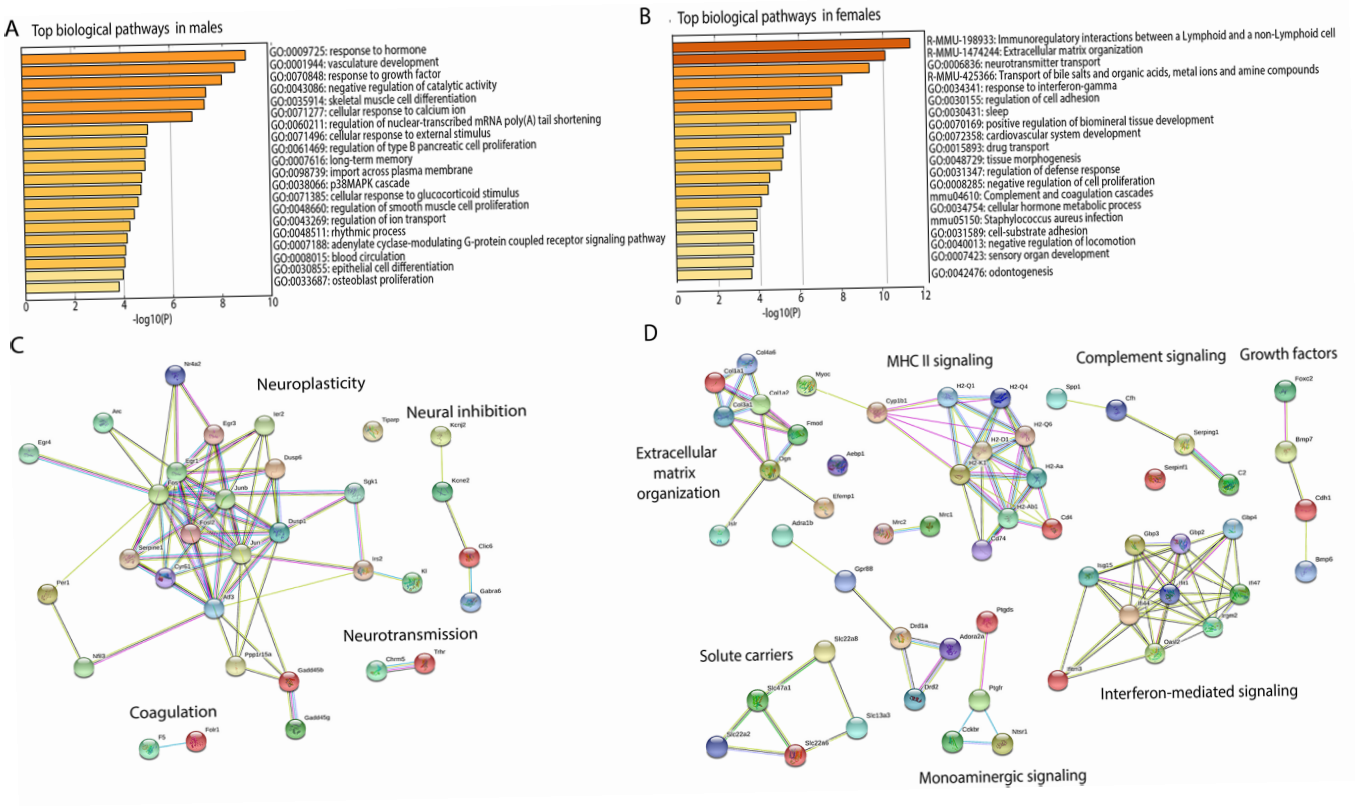

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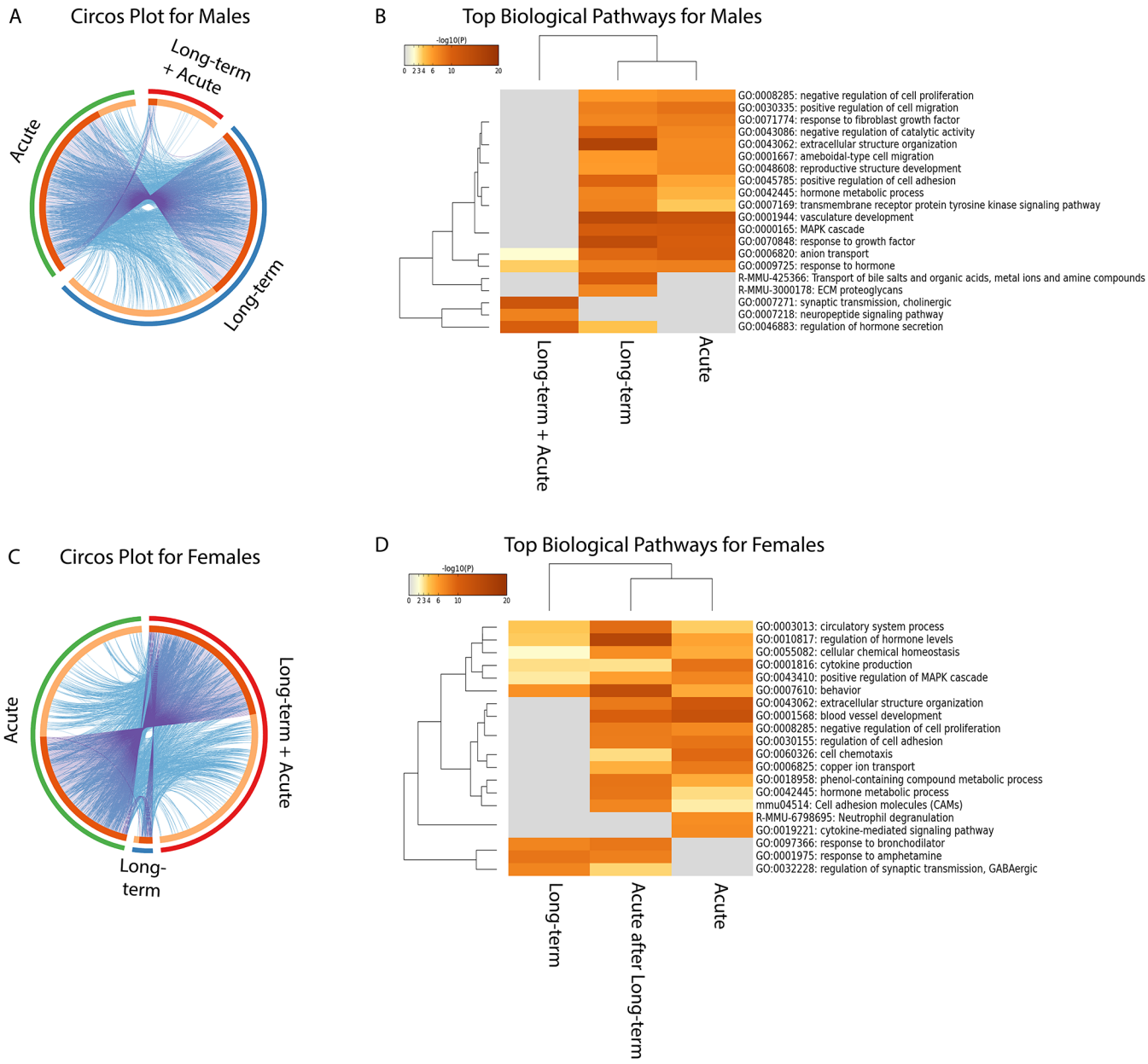

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