Published in final edited form as:

J Neurosci Res. 2020 December ; 98(12): 2541–2553. doi:10.1002/jnr.24724.

Sex-specific peripheral and central responses to stress-induced depression and treatment in a mouse model

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Abstract

Major depressive disorder (MDD) affects ~20% of the world population and is characterized by strong sexual dimorphism with females being 2–3 times more likely to develop this disorder. Previously, we demonstrated that a combination therapy with dihydrocaffeic acid (DHCA) and malvidin-glucoside (Mal-gluc) to synergistically target peripheral inflammation and stress-induced synaptic maladaptation in the brain was effective in alleviating chronic social defeat stress (CSDS)-induced depression-like phenotype in male mice. Here, we test the combination therapy in a female CSDS model for depression and compared sex-specific responses to stress in the periphery and the central nervous system. Similar to male mice, the combination treatment is also effective in promoting resilience against the CSDS-induced depression-like behavior in female mice. However, there are sex-specific differences in peripheral immune responses and differential gene regulation in the prefrontal cortex to chronic stress and to the treatment. These data indicate that while therapeutic approaches to combat stress-related disorders may be effective in both

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Author contributions: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Conceptualization*, S.J.R. and J.W.; *Methodology*, K.D., S.J.R. and J.W.; *Investigation*, K.D., K.L.C., H.L., K.L., L.F.P., F.C., K.B.L., M.E.F., L.L., H.A., C.G. and J.W.; *Formal Analysis*, Q.W., H.C., and J.W.; *Resources*, K.H., B.Z., S.J.R. and J.W.; *Writing - Original Draft*, K.D., Q.W., K.H. and J.W.; *Writing - Review & Editing*, K.L.C, F.C., L.L., H.A., B.Z., S.J.R.; *Visualization*, Q.W., H.C. and J.W.; *Supervision*, K.H., B.Z., S.J.R. and J.W.; *Funding Acquisition*, K.H., B.Z., S.J.R. and J.W.

Conflict of Interest statement: The authors declare no conflict of interest of any type

sexes, the mechanisms underlying these effects differ, emphasizing the need for inclusion of both sexes in preclinical studies using animal models.

Introduction

The World Health Organization (WHO) estimates, as of 2017, that 322 million people worldwide are affected by Major Depressive Disorder (MDD), and the prevalence of this severe mood disorder is on the rise ¹. Despite the broad use of antidepressants, they exhibit limited efficacy and can produce undesirable side effects ². Although newer antidepressants have been developed such as monoamine reuptake inhibitors, including selective serotonin reuptake inhibitors (SSRIs) and selective serotonin and norepinephrine reuptake inhibitors (SNRIs), the therapeutic efficacy has not improved ³. Moreover, antidepressants must be administered for weeks to months before any clinical benefit is noted ³. Thus, there is an urgent need for the development of new classes of antidepressants.

A vast literature documents the strong sexual dimorphism seen in depression and related stress disorders. Epidemiologic studies of depression consistently show sex differences in prevalence rates with depression being two to three times as common in women as in men ⁴⁻⁷. When compared to men diagnosed with depression, women report more frequent symptoms and greater symptoms severity ^{6,8,9}. Given the differences in the presentation, coping strategies and responses to stress and to treatment ^{6,7,8,9,4,10}, further investigation into the biological mechanisms modulating these pathways in males and females is warranted.

Depression is a multifaceted disease, the etiology of which is believed to be a combination of interactions between genetic and environmental elements. Depression carries genetic risks, although the search for specific genes responsible for its manifestation and the consensus on contributing genes are still unfolding ^{11,12}. Non-genetic risk factors including stressful life events and physical and emotional trauma are implicated in the etiology of depression. Recently, dysregulation of immune response has also been linked to depression. While it is unclear whether inflammation can be a causative agent, several studies support the theory that it plays a contributing role in the development of depression in a subset of patients ¹³⁻¹⁵. Leukocyte elevation has been observed in patients with depression, possibly explaining the increases in pro-inflammatory circulating cytokines such as tumor necrosis factor- α (TNF- α), IL-1 β , and IL-6 detected in this patient population ^{13,14}. Two recent meta-analyses found significantly increased peripheral TNF- α and IL-6 levels in MDD patients when compared to healthy controls ^{13,16}. Investigations into IL-6 suggest it can cross the blood-brain barrier (BBB) and may have some influence on the modulation of microglia and other central nervous system (CNS) immune cells, and perhaps even some role in neuronal plasticity ^{17,18}. Stress-induced structural and functional changes of synaptic plasticity have been reported in the hippocampus ¹⁹⁻²², the prefrontal cortex (PFC) ²³⁻²⁶ and subcortical limbic structures such as the amygdala and nucleus accumbens (NAc) ²⁷⁻³² in various animal models of stress. In humans, reduced volume in select brain regions are observed in patients with MDD ³³⁻³⁵ and select depression phenotypes such as anhedonia are correlated with a reduction in NAc volume and its response to reward stimuli ^{36,37}.

Both peripheral inflammation and synaptic function play an important role in the pathogenesis of depression. We initiated a high throughput *in vitro* drug screening study and identified two phytochemicals: Dihydrocaffeic acid (DHCA) can reduce IL-6 generations and malvidin-3-*O*-glucoside (Mal-gluc) can modulate synaptic plasticity³⁸. We hypothesized that simultaneously targeting both mechanisms may increase the likelihood of efficacy. Because of the lack of female defeat stress model, in previous studies, we only tested these two compounds in male mice and found that DHCA/Mal-gluc promote resilience against depression-like behaviors in following chronic social defeat stress (CSDS). We found that the two phytochemicals exert their anti-depressant activity through inhibition of peripheral inflammation and prevention of stress-induced synaptic modifications in the brain³⁸. Given the aforementioned sex differences in depression, in this study, we tested the effect of DHCA/Mal-gluc using a recently developed female model of CSDS³⁹ and compared the responses to stress and treatment in the periphery as well as the CNS with male mice.

Materials and Methods

Animals and treatment:

Seven-week-old female C57BL/6 (The Jacksons Laboratory) were randomly assigned to one of the four groups: vehicle treatment without exposure to CSDS, DHCA/Mal-gluc treatment without exposure to CSDS, vehicle treatment with exposure to CSDS, and DHCA/Mal-gluc treatment with exposure to CSDS. DHCA (5mg/kg-BW/day, Sigma-Aldrich) and Mal-gluc (0.5µg/kg-BW/day, Extrasynthese, Genay Cedex, France) were administered through their drinking water for 14 days prior to the CSDS and throughout CSDS. All animals had access to regular chow *ad lib* and were maintained on a 12:12-h light/dark cycle with lights on at 07:00 h in a temperature-controlled (20 ± 2 °C) vivarium. All procedures were approved by the Institutional Animal Care and Use Committee.

Generation of male aggressors for female CSDS:

Male aggressors were generated by breeding homozygous *Esr1* (ERa)-Cre males on a C57BL/6J background (The Jackson Laboratory, Bar Harbor, Maine) with wild type CD-1 females (Charles River Laboratories)³⁹. This breeding strategy ensured that all F1 inherited heterozygous *Esr1*-Cre alleles that was confirmed by random genotyping of the offspring.

Viral surgery for DREADD expression in male aggressors for female CSDS:

Three to four months old heterozygous *Esr1*-Cre male mice were anesthetized with intraperitoneal (i.p.) injection of ketamine HCl (100 mg/kg) and xylazine (10 mg/kg). The head was shaved and an incision was made down the midline of the head to expose the skull. The AAV vector encoding AAV2/hSyn-DIO-hM3D(Gq)-Cherry (Gq-DREADD; University of North Carolina, Chapel Hill, NC) was bilaterally injected into the ventromedial hypothalamus (VMHvl) at coordinates (AP, -1.5; ML, ± 0.7; DV, -5.7 mm from bregma) at a controlled rate of 0.3 µl/3 minutes per side³⁹. Following injection, the needle remained in the targeted brain area for 5 minutes and then was withdrawn slowly to reduce backflow. Mice were then returned to their home cage following the surgery for 4 weeks to allow for recovery and expression of the virus.

Aggressor screening and selection for female CSDS:

One month after surgery, the F1 heterozygous *Esr1-Cre* male mice with Gq-DREADD viral injection were screened for aggressiveness toward female mice after chemogenetic stimulation. Specifically, the F1 males were i.p. injected with 1.0 mg/kg clozapine-N-oxide (CNO) thirty minutes before screening on each screening day to activate DREADDs expressed in VMHv1 neurons. One 7-week old female C57BL/6J “screener” was introduced into the aggressor’s home cage for 3 minutes. The aggressor’s attack latency was recorded each day for 3 consecutive days. Aggressors that demonstrated aggressive behavior during the 3 minutes of intrusion for at least 2 out of the 3 screening days were deemed aggressive and were used for subsequent social defeat experiments. Aggressors that did not demonstrate aggressiveness following CNO injection were used as target mice during social interaction experiments.

CSDS protocol for female defeat:

The female CSDS protocol recently established by our lab was used for this experiment³⁹. Briefly, the aggressors were i.p. injected with 1.0 mg/kg CNO thirty minutes before initiation of the social defeat bout. Each defeat bout was carried out for 5 minutes over 10 consecutive days, where the vehicle or DHCA/Mal-gluc treated females were introduced into the home cage of a novel aggressor each day. If the male aggressor did not initiate aggressive behavior or initiated sexual behavior toward the intruder during the 5-minute encounter, the intruder female was removed and then introduced into the home cage of another novel aggressor. During the 10-day protocol, each female encountered a novel male aggressor to avoid habituation. All females were returned to their home cage with cage mates (4–5 females per cage) following each defeat bout and remained group-housed in their home cage following the last defeat day.

CSDS protocol for male defeat:

CSDS was performed as previously described^{65, 68}. CD-1 mice were screened for aggressive characteristics prior to the start of social defeat experiments based on previously described criteria⁶⁵, and housed within the social defeat cage (26.7w × 48.3d × 15.2h cm; Allentown Inc) 24 hours prior to the start of defeats on one side of a clear, perforated Plexiglass divider (0.6 × 45.7 × 15.2 cm; Nationwide Plastics). Briefly, male C57BL/6 mice subjected to CSDS were exposed to a novel CD-1 aggressor mouse for 10 minutes once per day, over 10 consecutive days. Following the 10 minutes of interaction, the experimental mice were removed to the opposite side of the social defeat cage and allowed sensory contact with the CD-1 during the following 24 hour period. Control mice without CSDS were housed two mice per cage, on opposite sides of the perforated divider, rotated daily in a manner similar to the defeat group, but never exposed to aggressive CD-1 mice.

Social interaction (SI) test—Twenty-four hours after the last defeat, the SI test was performed in all mice. The SI test was completed in a red-light cubicle containing an open field. The defeated mice were habituated to the SI room outside of the SI cubicles for 1 hour prior to testing. Non-aggressive F1 that had never encountered the defeated mice were used as social targets. The SI protocol consisted of a control and an experimental session. In the

control session of the SI test, the test mouse was placed in the center of an open field (42cm (w) × 42 cm (d) × 42cm (h)) with an small empty wire enclosure (10cm (w) × 6.5 cm (d) × 42 cm (h)) within the interaction zone (8 cm wide corridor surrounding the social target) on one side of the open field. The mouse was allowed 150 seconds to explore. The experimental sessions of the SI test were conducted in a similar manner but with a non-aggressive F1 social target mouse placed in the wire enclosure. The mouse's exploratory behavior was recorded by a CCD camera located above the open field. Video-tracked behaviors were analyzed using Ethovision 3.0 (Noldus Information Technology). Mice were returned to their home cages following the SI test and the open field and wire enclosures were cleaned after each trial. The SI ratio of the mouse was determined by dividing the time spent in the interaction zone with the social target present by the time spent in the same zone without a social target. Mice with an SI ratio greater than 1 were classified as resilient and socially interactive, whereas mice with an SI ratio less than 1 were deemed susceptible and socially avoidant.

Sampling of whole blood following defeat and brain tissue collection upon sacrificing—Eighteen hours after the first bout of defeat, blood samples from all mice were collected via the submandibular vein into heparin-lithium coated tubes (Eppendorf). Blood was centrifuged for 10 minutes at $375 \times g$ at 4°C. Plasma was collected and stored at -80°C until analysis. Upon sacrificing, mouse brains were removed and punches of the PFC were collected, snap frozen, and stored at -80 °C until further processing.

Plasma cytokine measurement—Plasma cytokines were measured using the Multiplex MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore) as previously described³⁸. Specifically, plasma samples were incubated with multiplex MAP magnetic beads at 4 °C overnight, washed twice and incubated with detection antibodies for 1 hour at room temperature followed by a 30-minute incubation with streptavidin-phycoerythrin. The samples were washed twice and subjected to the Luminex® 200™ assessments.

RNA isolation, RNA-seq and qRT-PCR—Total RNA from the PFC punches were isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). All RNA preparations were confirmed with integrity numbers >8.0 by a Bioanalyzer before proceeding to library construction. Libraries were created from pooled mRNA samples (n=3–4 mice/library and 4–5 libraries per test condition). Library construction (LncRNA library, Ribo-Zero), quality assessment and Illumina Novaseq 6000 with 150-pb paired-end reads were conducted by Novogene. The same RNA samples were reverse transcribed and subjected to real-time qRT-PCR as previously described³⁸.

RNA-seq data analysis and bioinformatics—Quality control was carried out on the raw sequence data coming from the sequencer using the FastQC software⁴⁰. This assesses total sequence, reads flagged as low quality, read length, GC content, per base and per tile quality, per sequence quality score, per base content distribution, per sequence GC content, per base N content, sequence length distribution, sequence duplication levels, overrepresented sequence, adaptor content, and Kmer content. Reads of universal low base quality were discarded, and reads with certain low quantity bases were trimmed. Following

QC, RNA reads were mapped to mouse reference genome (mm10) with STAR aligner⁴¹. Gene-level read counts in every gene model and all exons were derived using STAR. We removed genes or exons that were not expressed in any sample, where we define expression as at least 20 mapped reads in the entire data set. Read counts within samples and between samples were then normalized using the edgeR R-package⁴². Limma model⁴³ was used to examine the differential expression level (log-transformed) between groups.

Gene Ontology (GO) analysis—GO analyses were performed on differentially expressed genes (DEGs) from different contrasts by using Fisher's Exact Test (FET) with Benjamini-Hochberg (BH) correction. The annotated genes in the GO biological process were considered as the background. The enriched GO terms with BH-corrected FET p value <0.05 were considered statistically significant.

Power analysis and overall statistics—Group size calculation was based on our previous neurobehavioral studies, employing social avoidance test. Power calculation found that 12 mice/group will have 90% statistical power to detect 25% (0.32 Log2) fold change. All values are expressed as mean and standard deviation (SD). For behavioral (SI) test as well as biochemical analyses comparing control group vs. test group, two-way ANOVAs or one-way ANOVA followed by Post Hoc Bonferroni multiple comparisons. In all studies, outliers (2 SD from the mean) were excluded and the null hypothesis was rejected at the 0.05 level. All statistical analyses were performed using Prism 6 (GraphPad Software, San Diego CA).

Results

DHCA/Mal-gluc treatment promotes resilience in female mice

We injected 45 *Esr1*-Cre heterozygous F1 males with the AAV virus encoding hM3DGq-DREADDs into the VMHvl. The mice were then screened for aggressive behavior toward females following 4-week recovery. We identified 36 mice that showed robust aggressive activity toward female intruders following i.p. injection of CNO and were therefore used as aggressors for the female CSDS experiment (Fig. 1A). To investigate the potential protective effect of DHCA/Mal-gluc in females, we treated female mice with either vehicle or DHCA/Mal-gluc for 14 days prior to and throughout the 10-day CSDS protocol, followed by social interaction (SI) testing (Fig. 1B).

Consistent with previous findings³⁹, we found that 10-day CSDS led to a significant reduction of social interaction compared to the non-stressed mice ($P < 0.001$, Fig. 1C) while treatment with DHCA/Mal-gluc significantly increased the SI ratio compared to the vehicle-CSDS group ($P = 0.0114$, Fig. 1C). There was no significant difference between the DHCA/Mal-gluc-CSDS and the non-stressed groups suggesting DHCA/Mal-gluc treatment rescued the CSDS-induced social avoidance phenotype in female mice. We then examined the corner occupancy during the social interaction test by two-way-ANOVA, and observed a significant interaction between stress and treatment ($P = 0.0006$, Fig. 1D), suggesting that the effect of the treatment differs between non-stressed and stressed mice. Multiple-comparison Post-Hoc tests demonstrated that there were no differences in corner occupancy between the vehicle and the DHCA/Mal-gluc groups in non-stressed mice. However, in the stressed mice, there

were significant differences between the vehicle group and the treatment group (47.6 ± 29.6 seconds vs. 19.6 ± 16.3 seconds, $P=0.0002$, Fig. 1D), further confirming that DHCA/Mal-gluc attenuated the stress-induced social avoidance phenotype (Fig. 1D). Control studies showed that neither stress nor treatment had any effect on the locomotor activity of the mice (Supplementary Fig. 1A and 1B).

We further dissected the behavioral differences between the two socially defeated female groups. Vehicle-CSDS females showed a significant decrease in interaction zone duration in the presence of a social target (two-tailed paired t -test, $t_{13}=2.402$, $P=0.0320$, Supplementary Fig. 2A). We noted a trend toward increased interaction zone duration when the target was present in the DHCA/Mal-gluc treatment group, however it did not reach statistical significance (two-tailed paired t -test, $t_{13}=1.983$, $P=0.0689$, Supplementary Fig. 2B). DHCA/Mal-gluc treatment significantly increased the SI ratio (Supplementary Fig. 2D, two-tailed unpaired t -test, $t_2=3.383$, $P=0.0023$). About 28% of the mice in the vehicle group demonstrated a resilient phenotype (SI ratio ≥ 1) while 71% of the DHCA/Mal-gluc treated mice exhibited the resilient phenotype (Supplement Fig. 2E). Taken together, these data provide strong evidence that DHCA/Mal-gluc is effective in protecting female mice against CSDS-induced social avoidance behavior.

Peripheral inflammatory responses to CSDS and to DHCA/Mal-gluc treatment

To study peripheral inflammatory responses, whole blood from all mice was collected via the submandibular vein 18 hours after the first defeat bout. Peripheral cytokine levels were analyzed by multiplex enzyme-linked immunosorbent assay (ELISA). Consistent with previous findings in males^{15,38,39}, CSDS induced a significant upregulation of the pro-inflammatory cytokine IL-6 in female mice compared to non-CSDS control mice (Fig. 2A). Moreover, we found a significant elevation of additional pro-inflammatory cytokines including IL-1 α , IL-1 β , IL-12(p70), TNF- α , and granulocyte-colony stimulating factor (G-CSF) (Fig. 2B–2F). We also found a significant increase in select chemokines including monocyte-chemoattractant protein-1 (MCP-1/CCL2) and regulated on activation, normal T cell expressed and secreted (RANTES or CCL5) in the plasma (Fig. 2G–2H). Comparing to the studies we previously conducted in male mice³⁸, we did not find any of these changes in male mice after the first bout of defeat except for increased IL-6 (supplementary Fig. 3). Posthoc comparison between the two female CSDS groups, we found that the DHCA/Mal-gluc group had significantly lower levels of IL-12(p70) ($P=0.0202$, Fig. 2D) and RANTES ($P=0.0081$, Fig. 2H) compared to the vehicle-CSDS group. For all other pro-inflammatory cytokines, the DHCA/Mal-gluc-CSDS group had lower average values compared to the vehicle-CSDS group; however, none reached statistical significance (Fig. 2). In a parallel control study, we did not find any differences in the two non-CSDS groups, suggesting that treatment alone has no effect on peripheral cytokine/chemokine production. We then conducted correlation analyses between plasma cytokine levels and SI behavior in the two groups of mice that were subjected to RSDS. We found that plasma levels of IL-12(p70) ($P=0.0038$, Fig. 3A) and RANTES ($P=0.0003$, Fig. 3B) were positively correlated with corner zone duration. Plasma levels of TNF- α ($P=0.0053$, Fig. 3C) and G-CSF ($P=0.0233$, Fig. 3D) were also positively correlated with corner zone duration. The levels of IL-1 α , IL-1 β , IL-6 and MCP-1 in plasma did not correlate with corner zone duration (Fig. 3E–3H).

Gene expression assessment in the PFC in response to stress and to DHCA/Mal-gluc treatment

The PFC plays an important role in stress response and chronic stress leads to structural changes such as reduced spine density and altered dendritic arborization in the PFC^{44–47}. It is a well established brain region involved in stress responses in mice and in depression in humans^{48–50}. The activity of the PFC is a key determinant of depression phenotype as well as antidepressant responses and transcriptome data from human patients and mouse stress model showed dramatic sex differences in expression profiles^{51,52}. Therefore, we tested whether DHCA/Mal-gluc can modulate stress-induced gene expression changes in the PFC. We collected PFC punches from female mice following the SI test and performed RNA-seq. Heatmaps of transcripts that were significantly regulated are shown in Figs. 4A and 4B. In female mice, there were 1173 stress-induced DEGs (584 stress-upregulated DEGs and 589 stress-downregulated DEGs, Fig. 4A–4D, Supplementary Table 1), and 209 DEGs were reversed by the treatment (Fig. 4C, Supplementary Table 2). Top GO terms enriched with stress-induced upregulated genes were related to response to stimulus, vascular development, cell motility, cell proliferation and cell signaling while stress-induced downregulated pathway are associated with potassium ion transport and regulation of lymphocyte activation (Fig. 4D). Treatment-mediated downregulated pathways are related to tissue development, kinase/phosphatase, response to external stimulus, transcription, and regulation of cell differentiation and cell signaling, and regulation of immune system (Fig. 4E). The stress-induced pathways rescued by DHCA/Mal-gluc treatment were involved in tissue development, cell proliferation, transcription and biosynthesis and regulation of the immune system such as leukocyte differentiation and cytokine production (Fig. 5F).

We also assessed the DEGs in male samples collected from previous studies³⁸, we found there were almost twice as many stress-induced DEGs in the PFC compared to the females (1015 stress-upregulated DEGs and 1020 stress-downregulated DEGs, Fig. 5A–5C, Supplementary Table 3). Among the stress-induced DEGs, 428 DEGs were reversed by the treatment (Fig. 5C, Supplementary Table 4). Biological pathways related to neurogenesis, cell motility, cation and ion transport were significantly enriched in stress-upregulated genes while stress downregulated genes were enriched in pathways associated with nervous system development, adhesion and transcription (Fig. 5D). Treatment-mediated upregulated genes were enriched in pathways related to extracellular structure organization, nervous system development, cytosolic calcium ion, IGF signaling and synapse assembly while treatment-downregulated DEGs were enriched in pathways associated with angiogenesis and oligodendrocyte differentiation (Fig. 5E). Rescue assessment showed that stress-induced downregulated pathways including neurogenesis, guanylate cyclase activity and VEGF signaling were reversed by the treatment (Fig. 5F). Stress-upregulated pathways such as nervous development, response to calcium ion and response to monoamine were reversed by the treatment (Fig. 5F).

Comparing male with female mice, we found 83 upregulated and 106 downregulated genes shared in the PFC of male and female mice (Fig. 6A–6D). Pathways upregulated by stress in male and female mice were those involved in response to steroid hormones, response to oxidative stress, cellular response calcium and response to cytokine while those

pathways involved in G-protein coupled receptor signaling, circadian rhythm and regulation of ion transport were downregulated in both males and females (Fig. 6D). DHCA/Mal-gluc treatment rescued stress-induced changes in pathways associated with response to ROS, stress, and inflammation as well as several of the immunity-associated pathways in both male and female CSDS mice (Fig. 6E). Among the common DEGs shared by male and female mice in response to stress, eleven were upregulated by stressed and rescued by DHCA/Mal-gluc treatment. We then conducted real-time qRT-PCR to validate these RNA-seq findings. Among the eleven DEGs, we were able to measure ten with the exception of ZFP189, the expressions of which were too low to be accurately measured in both male and female mice. In female mice, we confirm that nine DEGs were indeed increased by stress with the exception of JUNB that showed no change by PCR (Supplementary Table 5). The treatment rescued all the stress-induced DEGs except for KCNN which showed no differences between the stressed female mice with or without treatment. In male mice, except for KCNN, the other nine stress-induced DEGs were confirmed to be increased by stress and the treatment rescued all of them. There is no differences in the expression of KCNN between the control, the stressed and the stressed male mice with treatment (Supplementary Table 5).

Discussion

In this study, we tested the efficacy of the recently identified phytochemical combination, DHCA/Mal-gluc, in protecting against stress-induced depression-like behavior in female mice following CSDS. We found that female mice receiving DHCA/Mal-gluc treatment showed significantly less social avoidance behavior in comparison to mice receiving vehicle treatment. Over 70% of the treated mice exhibited a resilient phenotype while only 28% of the vehicle group displayed resilience. Similar to our previous study in male mice³⁸, our present study demonstrated that the combination treatment is also effective in preventing a CSDS-induced social avoidance phenotype in female mice.

Also consistent with our previous studies^{15,53}, both male and female mice had a significant increase in peripheral IL-6 following social defeat stress. However, female mice also showed a much broader inflammatory profile than males that included increased IL-1 α , IL-1 β , IL-12(p70) and TNF- α in the periphery. Chemokines MCP-1 and RANTES were also elevated in female mice following defeat. This is consistent with literature suggesting that there are sex differences in the immune system that might make females more vulnerable to inflammatory diseases^{54–56}. Previously we reported that DHCA/Mal-gluc treatment significantly reduced stress-induced IL-6 production in male mice³⁸, but we did not find any significant decrease of IL-6 in females in the treatment group, nor did we observe any correlation of IL-6 with susceptible or resilient phenotypes in female mice in the present study. However, we found that treatment with DHCA/Mal-gluc significantly reduced the levels of IL-12(p70) and RANTES in plasma. Moreover, plasma levels of IL-12(p70) and RANTES correlated strongly with depression-like phenotype. IL-12 is produced by antigen presenting cells and stimulates the differentiation of T₀ cells toward T helper 1 cells (TH₁)⁵⁷ and is comprised of two subunits: an alpha chain (p35 subunit) and a beta chain (p40 subunit). Co-expression of the independently regulated genes encoding the two subunits yields the biologically active compound, IL-12(p70)⁵⁸. Elevations of peripheral IL-12(p70)

have been associated with depression in humans, but studies of how this specific cytokine influences mood disorders are sparse⁵⁹. RANTES is a chemokine produced by many cell types and acts as a potent chemoattractant for monocytes, NK cells and T cells. Ogłodek et. al. reported in their study that plasma levels of RANTES were significantly higher in both depressed men and women compared to healthy controls and that levels were highest in patients with the most severe level depression⁶⁰. They also observed that women had higher levels of RANTES compared to men⁶⁰. Besides IL-12(p70) and RANTES, peripheral TNF- α levels in the DHCA/Mal-gluc treatment group trended downward and strongly correlated with a reduction in depression-related phenotypes. Peripheral elevations of TNF- α have been reported in depressed patients^{13,16} and treatments targeting peripheral TNF- α , such as infliximab do exhibit antidepressant properties⁶¹. However, in the current study, stressed-induced changes in TNF- α were not seen in male mice, suggesting that there is likely sex-specific cytokine/chemokine cascades in response to stress that need to be considered. Previous studies by Hodes *et al.* reported that stress induced the upregulations of IL-1 β , IL-6, IL-10 and MCP-1 in male mice 20 minutes after the first defeat bout and the expression of IL-6 in the plasma negatively correlated with stress phenotype¹⁵. Our assessments of the plasma cytokine was measured 18 hours after the first defeat and there is a general trend of increase of almost all of the inflammatory cytokines measured however, did not reach statistical significance. It is possible that social defeat triggers an immediate inflammation response following the first bout and subsequently subsides in male mice. Future study will measure cytokine levels in female mice immediately following the first bout of the defeat and to compare with the levels measured in a later time point to assess whether the female mice respond immediately and sustain the levels or the female mice have a delayed immunological response to acute stress. In summary, our study points to a distinction in the immunological processes following stress and the mechanisms underlying stress-related depression between males and females, with females exhibiting a far more robust immune response to stress than males. It is well established that inflammation contributes to the pathogenesis of depression, but the exact mechanisms are not completely understood. Further studies of the influence of differentially regulated cytokines and chemokines on the CNS of males and females may provide insights into how these elevations are implicated in the pathogenesis of depression.

Stress-induced synaptic maladaptation has been studied in the male CSDS model^{30,32,62,63}. To better understand the mechanisms of such dysregulation in female mice and the potential benefits of DHCA/Mal-gluc treatment, we examined the transcriptional profile in the PFC, a brain region known to play important roles in stress and depression. We identified specific DEGs and GO pathways that were enriched in stressed female mice and compared to those of male stressed mice. Our studies showed that even though the most highly ranked pathways differ in the PFC between male and female stressed mice, we found some shared DEGs and several enriched pathways that may be common to stress-induced responses in both sexes. Some of the shared pathways, such as inflammatory responses, organic cyclic compounds metabolism and circadian rhythm, have also been reported in human MDD^{64–66}. Though both male and female mice were similarly treated with DHCA/Mal-gluc, we found that the most highly ranked pathways were different. In females, the highest ranked pathways were associated with tissue development and protein phosphorylation while in

male mice, the treatment influenced the extracellular matrix and autonomic nervous system. Assessment of the common rescue enriched pathways shared by male and female mice revealed that in the DHCA/Mal-glu treatment downregulated multiple pathways associated inflammation and immune responses. This is consistent with the role of DHCA as DHCA was originally chosen to target stress-induced inflammatory responses³⁸. The current study tested the prophylactic effect of these two compounds in preventing social defeat induced depressive behavior. Our previous study showed that the two compounds were also effective in treating male mice with established depression phenotype and the efficacy was similar to standard tricyclic imipramine³⁸. We also found that the improvement was associated with normalization of synaptic plasticity³⁸. Future study will test the therapeutic effect of these two compounds in female and male mice following social defeat stress and compare the treatment efficacy and gender specific responses in periphery as well as the CNS. Collectively, our study demonstrates differences in male and female mice in response to social defeat stress and DHCA/Mal-glu treatment highlighting the importance of sexual dimorphisms when studying depressive disorders using animal models. These data are consistent with those from human studies demonstrating intrinsic sex-differences in MDD subjects^{64–67} as well as in other mouse models for the study of depression⁶⁸.

Sexual dimorphism in depressive disorders is well documented. Women and men differ in the prevalence, symptom presentation, and responses to antidepressant treatment. However, basic research and drug discovery have mainly been limited to male models. Currently available antidepressants demonstrate disparate effects in males and females. Our studies compare sex differences in peripheral immunological responses and identify distinct molecular and cellular changes in the brain to stress and to DHCA/Mal-glu treatment, which further advances the goal of elucidating sex differences in depression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

Funding was provided by the P50 AT008661-01 from the National Center for complementary and Integrative Health (NCCIH) and the Office of Dietary Supplements (ODS), R01MH090264 and R01 MH104559 from the National Institute of Mental Health (NIMH). In addition, J.W. holds positions in the Research and Development unit of the Basic and Biomedical Research and Training Program at the James J. Peters Veterans Affairs Medical Center. We acknowledge that the contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the views of the NCCIH, NIH, ODS or the U.S. Department of Veterans Affairs or the United States Government.

Data availability statement:

The RNA-seq data reported in the paper are deposited in GEO with the accession number GSE146845. Other data that support the findings of this study are available from the corresponding author upon reasonable request.

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Significance Statement

In this study, we tested a combination therapy that targets peripheral as well as central pathophysiology of depression in a newly developed female mouse model for the study of depression. We compared the responses to social stress and a novel antidepressant treatment between male and female mice. Our study demonstrated that combination treatment is effective in modulating stress-induced depression phenotype in both male and female mice. We noted strong sex-specific responses to stress-induced inflammatory cytokine/chemokine expression in the periphery and sex-specific transcriptional regulation in the prefrontal cortex. Our findings emphasize the inclusion of both sexes in the preclinical studies.

Fig. 1. DHCA/Mal-gluc promotes resilience in CSDS female mice.

(A) A schematic of bilateral injections of AAV2-hSyn-DIO-GqDREADD-mCherry into male ER α -Cre mice ($n=45$) and time course of ER α -Cre males surgery, recovery, and screening process for successful aggressive behavior toward female mice ($n=36$). (B) Time course of C57BL/6J female treatment and behavioral tests. (C-D) Social interaction test in mice treated with DHCA/Mal-gluc with or without 10 days CSDS (C) Social interaction distribution. Two-way ANOVA, $F_{1,53}=23.79$, $P<0.0001$ for stress effect; $F_{1,53}=5.26$, $P=0.0259$ for treatment effect. The interaction effect was non-significant, $F_{1,53}=3.30$, $P=0.0752$. Data represent mean $\pm$ SD. $n=13-15$ per group. (D) Time spent in the corner zone during the SI. Two-way ANOVA, $F_{1,53}=27.49$, $P<0.0001$ for stress effect; $F_{1,53}=11.97$, $P=0.0011$ for treatment effect. The interaction effect was significant, $F_{1,53}=13.25$, $P=0.0006$. Data represent mean $\pm$ SD, $n=13-15$ per group.

Fig. 2. Peripheral inflammation assessment 18 hours following first bout of defeat in female mice with or without DHCA/Mal-gluc treatment.

(A-F) Plasma levels of cytokines 18 hours after the first social defeat bout (A) IL-6, One-way ANOVA, $F_{2,38}=3.555$, $P=0.0384$. (B-C) IL-1 α , One-way ANOVA, $F_{2,40}=7.201$, $P=0.0021$ and IL-1 β , One-way ANOVA, $F_{2,42}=4.113$, $P=0.0234$. (D) IL-12(p70), One-way ANOVA, $F_{2,41}=10.96$, $P=0.0002$. (E) TNF- α , One-way ANOVA, $F_{2,40}=8.551$, $P=0.0008$ and (F) G-CSF, One-way ANOVA $F_{2,40}=6.177$, $P=0.0046$. (G-H) peripheral levels of chemokines (G) MCP-1, One-way ANOVA $F_{2,40}=5.718$, $P=0.0065$ and (H) RANTES, One-way ANOVA $F_{2,40}=6.600$, $P=0.0033$. One-way ANOVA Post Hoc Bonferroni multiple comparisons. Data represent mean $\pm$ SD, $n=13-15$ per group.

Fig. 3. Correlation analysis of pro-inflammatory cytokines with CSDS-induced behavior phenotypes in female mice.

(A-D) Peripheral cytokines/chemokines significantly correlated with time spent in the corner zone during the SI test (A) IL-12(p70), (B) RANTES, (C) TNF- α and (D) G-CSF. (E-H) Peripheral cytokines/chemokines do not correlate with time spent in the corner zone during the SI test (E) IL-1 α , (F) IL-1 β , (G) IL-6 and (H) MCP-1. Only mice subjected to RSDS are included in the correlation analysis, n=27–29 per analysis, Solid lines show correlations and dotted lines show the 95% CI.

Fig. 4. Transcriptional assessment of PFC in response to stress and DHCA/Mal-gluc treatment in female mice.

(A) Heatmap of genes differentially regulated by stress compared with the same genes differentially regulated by treatment. (B) Heatmap of genes differentially regulated by treatment in mice following stress compared with the same genes regulated by stress. (C) Venn diagrams of total number of significantly upregulated and downregulated genes in the stressed mice and the total number regulated by treatment. (D-E) Top 10 GO enrichment pathway analysis of DEGs regulated by (D) stress (E) treatment and (F) treatment-rescued according to biological processes.

Fig. 5. Transcriptional assessment of PFC in response to stress and DHCA/Mal-gluc treatment in male mice.

(A) Heatmap of genes differentially regulated by stress compared with the same genes differentially regulated by treatment. (B) Heatmap of genes differentially regulated by treatment in mice following stress compared with the same genes regulated by stress. (C) Venn diagrams of total number of significantly upregulated and downregulated genes in the stressed mice and the total number regulated by treatment. (D-E) Top 10 GO enrichment pathway analysis of DEGs regulated by (D) stress (E) treatment and (F) treatment-rescued according to biological processes.

Fig. 6. Comparison of PFC transcription regulation in response to stress between female and male mice.

(A) Heatmap of stress-mediated DEGs in male compared with the same genes differentially regulated in female. (B) Heatmap of stress-mediated DEGs in male compared with the same genes differentially regulated in female (C) Venn diagrams of total number of significantly upregulated and downregulated genes in the stressed male and female mice. (D-E) GO analysis of common enriched pathways shared by male and female following (D) stress and (E) rescued by the treatment.