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ARTICLE

Adolescent stress sensitizes the adult neuroimmune transcriptome and leads to sex-specific microglial and behavioral phenotypes

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Adolescent exposure to chronic stress, a risk factor for mood disorders in adulthood, sensitizes the neuroinflammatory response to a subsequent immune challenge. We previously showed that chronic adolescent stress (CAS) in rats led to distinct patterns of neuroimmune priming in adult male and female rats. However, sex differences in the neuroimmune consequences of CAS and their underlying mechanisms are not fully understood. Here we hypothesized that biological sex would dictate differential induction of inflammation-related transcriptomic pathways and immune cell involvement (microglia activation and leukocyte presence) in the hippocampus of male and female rats with a history of CAS. Adolescent rats underwent CAS (six restraint and six social defeat episodes during postnatal days 38–49), and behavioral assessments were conducted in adolescence and adulthood. Neuroimmune measures were obtained following vehicle or a systemic lipopolysaccharide (LPS) challenge in adulthood. CAS led to increased time in the corners of the open field in adolescence. In males, CAS also increased social avoidance. As adults, CAS rats displayed an exaggerated enrichment of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway and chemokine induction following LPS challenge, and increased number of perivascular CD45$^+$ cells in the hippocampus. However, CAS females, but not males, showed exaggerated glucocorticoid receptor (GR) pathway enrichment and increased microglial complexity. These results provide further insight to the mechanisms by which peripheral immune events may influence neuroimmune responses differentially among males and females and further demonstrate the importance of adolescent stress in shaping adult responses.

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INTRODUCTION

Appreciation has grown for the plausible role of neuroimmune sequalae to contribute to sex differences in neuropsychiatric diseases and stress-related disorders [1]. These sex differences emerge with the onset of puberty, thus highlighting adolescence as a possible critical period for diverging consequences of stress exposure among males and females. In humans, stress during adolescence is associated with a subsequent pro-inflammatory phenotype that may be linked to psychiatric illnesses [2, 3]. In addition, chronic stress plays an instrumental role by exaggerating immune reactivity in brain regions involved in regulating the stress response, including the hippocampus [4, 5]. Transcriptomic studies reveal excessive immune signaling in the hippocampus of depressed individuals [6] as well as rodents that have experienced chronic stress [7, 8]. Furthermore, some of these differences are sex-specific [9], mirroring the influence of sex on transcriptomic profiles of humans with depression [10]. There are also baseline sex differences in immune-related genes [11], suggesting a potential basis for sex differences in the stress-regulated neuroimmune transcriptome. Using a chronic adolescent stress (CAS) model in rats, here we sought to elucidate transcriptional pathways differentially impacted by CAS in male and female rats, with a focus on inflammatory and endocrine pathways known to be altered by chronic stress.

Using RNA-Seq, we assessed changes to nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and glucocorticoid receptor (GR) signaling pathways—primary mediators of the innate immune system and HPA axis, respectively [12],—to probe their potential involvement in CAS-induced neuroimmune priming [4]. Deficits in GR signaling, which are implicated in stress-related disorders including depression [13], allow excessive inflammatory signaling to occur [12]. In turn, exaggerated NFκB signaling has been linked to the pathophysiology of early life stress [14, 15], bipolar disorder [16], post-traumatic disorder [15, 17], and major depressive disorder [14, 16]. Furthermore, building on our previous work demonstrating differential peripheral immune sensitization in males and females [18], we additionally sought to assess the sex-specific influence of CAS on promoting immune-to-brain cellular traffic. The neuroimmune network hypothesis proposes that early life adversity strengthens immune-to-brain

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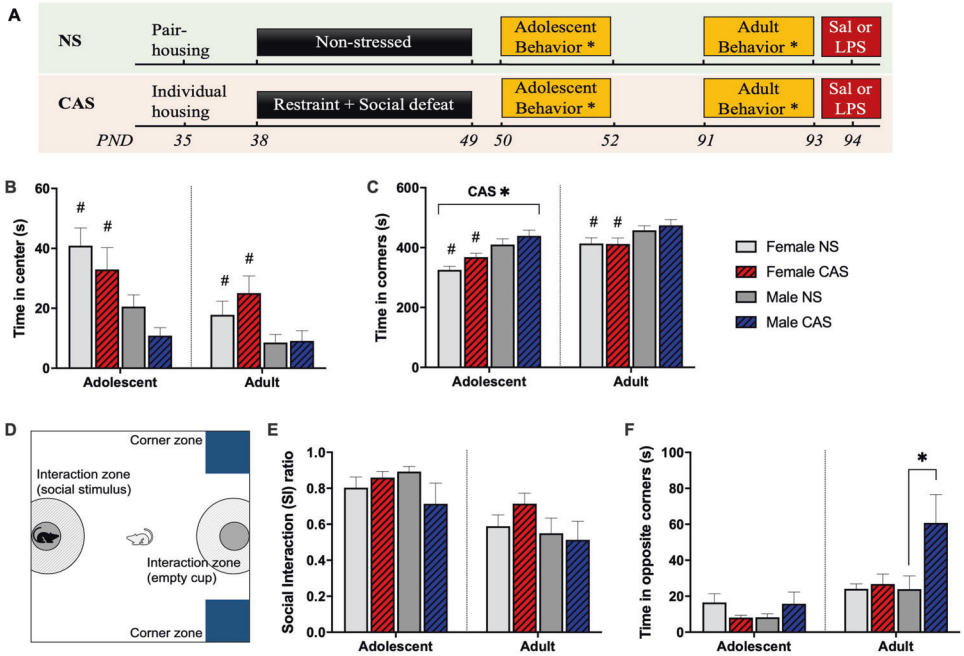

Fig. 1 Chronic adolescent stress (CAS) led to anxiety-like behavior and male-specific social avoidance. A Female and male rats of NS or CAS background were behaviorally tested once in adolescence on postnatal day (PND)50, and again in adulthood on PND91. **B** Adolescent CAS rats displayed no change in time spent in the center, but (**C**) a significant increase in time spent in the corners ($p < 0.05$). **D** Social interaction arena setup is depicted. **E** CAS did not impact SI ratio in either sex when assessed in adolescence or adulthood. **F** CAS led to a significant increase in time spent in opposite corners in males ($p < 0.05$), but not in females. Data are presented as mean ± SEM. CAS*, main effect of CAS ($p < 0.05$); *, effect of CAS ($p < 0.05$) within males; # main effect of sex. CAS Chronic Adolescent Stress, NS Non-stressed, SI social interaction.

crosstalk via sensitizing immune cells of the brain as well as myeloid cells in the periphery to subsequent immune activation [19]. Both the brain's resident immune cells, microglia [20, 21], as well as peripheral monocytes and macrophages of the spleen [22, 23] and bone marrow [24] have been reported to assume a hyper-inflammatory and primed phenotype following various forms of stressors. Although stress-induced infiltration of leukocytes to the brain has been reported to occur in female mice [25–27], and microglia from stressed adult male and female rats display distinct morphological profiles [28], currently it is not clear whether there are sex differences in the extent to which adolescent stress dysregulates immune cells in the brain. Therefore, we also aimed to determine whether CAS differentially impacts the number, composition, and activation of immune cells in the hippocampus of males and females using complementary approaches.

METHODS AND MATERIALS

Animals

Male and female Wistar rats from Charles Rivers (Durham, NC) were bred to generate litters. Rats were housed on a 14:10 reverse light/dark cycle with standard rat chow and water available ad libitum. Litters were culled to four male and four female pups on postnatal day (PND) 3, and rats were weaned on PND 21. One hundred and seventeen male and female Wistar rats were used for RNA-sequencing ($n = 47$ total; 5–6/group) and quantitative PCR studies ($n = 70$ additional; 6–8/group) at Emory University (see Table S1). A separate group of rats raised from timed-pregnant dams (Charles River, Durham, NC) at Virginia Commonwealth University was used for behavioral ($n = 52$ total; 12–14/group), immunohistochemistry, and flow cytometry experiments ($n = 49$ total; 6–7/group). Throughout all experiments, rats were housed in AAALAC (American Association for Accreditation of Laboratory Animal Care)-accredited facilities, and all studies were approved by the Institutional Animal Care and Use Committees at the respective universities.

Chronic adolescent stress (CAS)

CAS was performed as detailed previously [4, 18, 29–36]. On PND35, rats were randomly assigned to non-stressed (NS) or CAS groups. CAS rats were individually housed, and underwent CAS per experimental timeline in Fig. 1A. The mixed-modality chronic stress paradigm consisted of six random exposures to social defeat and restraint stress each, which took place across 12 days spanning mid-adolescence in the rat (PND38–49). For restraint stress, each Wistar rat was placed in a clear plexiglass restraint tube (Braintree Scientific, Braintree, MA) for 1 h. In the social defeat paradigm, each Wistar rat was placed in the home cage of a same-sex, adult Long Evans rat that is trained to demonstrate aggressive behavior toward the experimental rat. During the first 2 min of social defeat (habituation), the two rats were separated by a perforated, clear barrier that allows visual and olfactory cues. The barrier was subsequently removed, and rats were observed for 5 min of physical interaction or three pins, whichever came first, followed by an additional 25 min separated by the barrier. Upon completion of the stress paradigm (a total of 12 restraint/social defeat sessions), rats were allowed to mature into adulthood without further stressor exposure.

Behavioral assessments

Rats underwent open field and social interaction (SI) tests immediately following the end of the CAS paradigm (PND50–52), and again in adulthood (PND91–93) to assess anxiety-like and social avoidance behaviors as described in Supplementary. For open field test, the outcomes assessed were time spent in the center, and the corners of the open field. For SI test, the outcomes assessed were time spent in interaction zone of the cup containing a social stimulus and time spent in corners opposite from it.

Immune challenge in adulthood with lipopolysaccharide (LPS)

On PND94, 45 days after stress exposure concluded, all rats received a single intraperitoneal injection of either saline or LPS

(L3880, Sigma Aldrich) (0.25 mg/kg; 750,000 Endotoxin Units). At 2 or 4 h after injection rats were anesthetized with Euthasol (150 mg/kg), and transcardially perfused with ice-cold PBS to prevent peripheral immune cells and inflammatory markers present in the blood from confounding outcomes measured in brain tissue (see experimental design in Fig. 1A).

RNA-Seq and quantitative PCR

Bulk RNA-Sequencing was performed by the Nonhuman Primate Genomics Core at Yerkes National Primate Research Center and analysis conducted as detailed in Supplementary. Briefly, the edgeR package was used for normalization and differential expression analysis [37]. Log_2 expression of each gene was modeled as a function of sex, stress, LPS stimulus, and batch (date of sequencing). The Quasi-likelihood F Test was performed to test for differences in gene expression between these factors. The effect of CAS at baseline was assessed within each sex using the pairwise contrasts (1) Male-CAS-Saline (M.CAS.Sal) versus Male-NS-Saline (M.NS.Sal) and (2) Female-CAS-Saline (F.CAS.Sal) versus Female-NS-Saline (F.NS.Sal). Differentially expressed genes (nominal $p < 0.05$ and fold change > 1.2) from these pairwise comparisons were then used to perform GeneGo MetaCore (St. Joseph, MI, USA) pathway enrichment analyses in order to identify biological pathways differentially enriched at baseline (FDR < 0.05).

Moreover, pairwise contrasts of "LPS versus Saline" were conducted within each of (1) Female-NS, (2) Female-CAS, (3) Male-NS, and (4) Male-CAS groups to identify differentially expressed genes. To assess how a background of CAS impacts LPS-induced pathway enrichment compared to NS, Gene Set Enrichment Analysis (GSEA; Broad Institute) was conducted [38, 39]. To perform GSEA, we used the Molecular Signatures Database's (MSigDB) Hallmark (H) collection, which includes 50 hallmark pathways representing well-defined biological processes, with the addition of the GR regulatory network (PID_REG_GR_PATHWAY) from MSigDB's C2 curated collection: Pathway Interaction Database (NCI, NIH and Nature Publishing Group). Tables S2 and S3 list genes contained in GSEA pathways. qPCR was conducted as previously published [18] and described in Supplementary. Primer sequences are provided in Table S4.

Flow cytometry

To assess leukocyte infiltration to the hippocampus, flow cytometry was conducted as described in Supplementary and Fig S1.

Immunohistochemistry

Following saline perfusion, one hemisphere of the brain was post-fixed in 4% PFA for 48 h. Coronal sections (40 uM thickness) were stained with IBA-1 (Wako Chemicals USA Inc., Richmond, VA) to assess microglial count and morphology. Separately, sequential immunofluorescent staining with CD45 (Santa Cruz, Dallas, TX USA), RECA-1 (Abd Serptec; Hercules, CA, USA), and IBA-1 (Wako, Richmond, VA, USA) was performed to assess the perivascular presence of $CD45^+$ leukocytes in the hippocampus as detailed in Supplementary.

Microglial count and morphology

The number of microglia in the hippocampus was estimated using unbiased stereology and microglial morphology was assessed using ImageJ (NIH) as described in Supplementary and Fig S2.

Statistical analysis

Analyses were conducted in SPSS 25.0, and detailed in Supplementary. Behavior, flow cytometry, stereology, qPCR, and perivascular CD45 data were analyzed via three-way ANOVAs modeling the main effect of sex, stress, and stimulus and their interactions.

Behavioral data from adolescence and adulthood were analyzed separately in order to assess the immediate and enduring impact of CAS. Significant interactions from ANOVA were followed up with Holm-Sidak post-hoc test. Microglial morphology data were analyzed via generalized estimating equation models to accommodate the clustered data structure (up to 24 cells analyzed per rat). Gene expression data are presented as mean fold change ± SEM. All other data are expressed as mean ± SEM. Significance threshold was set to $\alpha = 0.05$ for all analyses.

RESULTS

CAS leads to anxiety-like behavior in the open field in adolescence

In adolescence, CAS rats displayed no change in time spent in center ($F_{(1,48)} = 1.93$, $p = 0.171$) (Fig. 1B) but a significant increase in time spent in the corners ($F_{(1,48)} = 4.73$, $p = 0.035$) (Fig. 1C). The effect of CAS was no longer observed in adulthood (center: $F_{(1,48)} = 0.85$, $p = 0.363$; corners: $F_{(1,48)} = 0.17$, $p = 0.679$). Female rats spent more time in the center and less time in the corners of the open field compared to males in both adolescence (center: $F_{(1,48)} = 13.68$, $p < 0.001$; corners: $F_{(1,48)} = 22$, $p < 0.001$) and adulthood (center: $F_{(1,48)} = 8.88$, $p = 0.004$; corners: $F_{(1,48)} = 8.33$, $p = 0.006$).

CAS males display increased social avoidance

The SI test arena is shown in Fig. 1D. In adolescence, SI ratio was not impacted by sex ($F_{(1,46)} = 0.16$, $p = 0.69$), CAS ($F_{(1,46)} = 0.78$, $p = 0.38$), or their interaction ($F_{(1,46)} = 2.85$, $p = 0.098$), and similar results were observed in adulthood (sex: $F_{(1,46)} = 2.1$, $p = 0.15$; stress: $F_{(1,46)} = 0.29$, $p = 0.59$, sex*stress: $F_{(1,46)} = 0.97$, $p = 0.33$) (Fig. 1E). Time spent in opposite corners, a metric of social avoidance [40], was not impacted by sex ($F_{(1,46)} = 0.004$, $p = 0.95$), CAS ($F_{(1,46)} = 0.01$, $p = 0.95$), or their interaction ($F_{(1,46)} = 3.55$, $p = 0.066$) in adolescence (Fig. 1F). However, in adulthood while sex did not significantly impact time spent in opposite corners ($F_{(1,46)} = 3.09$, $p = 0.085$), CAS significantly increased time spent in opposite corners ($F_{(1,46)} = 4.19$, $p = 0.046$) (Fig. 1F). Follow-up tests revealed that CAS led to increased time spent in opposite corners in males ($t_{(46)} = 2.81$, $p = 0.014$), but not females ($t_{(46)} = 0.19$, $p = 0.851$).

CAS sex-specifically alters baseline and LPS-induced enrichment of transcriptional pathways

Differential expression analysis was conducted using edgeR by modeling the main effects of LPS, sex, and stress. Expression heatmaps of the top ten genes from each of the main effects are shown in Fig. 2A–C. Furthermore, pairwise comparisons assessed baseline (unstimulated) differences in gene expression between CAS rats and their NS litter mates, by sex (Fig. 2D). Female, CAS, and saline-injected rats (F.CAS.Sal) had 437 upregulated and 982 downregulated genes compared to Female, NS, saline rats (F.NS.Sal). Male, CAS, saline rats (M.CAS.Sal) had 559 upregulated and 82 downregulated genes compared to Male, NS, saline rats (M.NS.Sal). CAS-associated genes at baseline (Saline) were largely distinct between males and females with similar numbers of upregulated genes across males and females and greater number of downregulated genes in female rats. Genes differentially regulated by CAS in female rats enriched pathways related to signal transduction, histone deacetylases, and cytoskeleton remodeling. Genes differentially regulated by CAS in male rats were related to G protein-coupled receptor signaling, Notch signaling, and NMDA receptor trafficking. A complete list of genes from each contrast, along with the pathways they enrich, are shown in Table S5A–C.

LPS challenge led to differential expression of 357 genes in Female NS, 393 genes in Female CAS rats, with 173 genes common between the groups. In males, LPS challenge led to differential expression of 343 genes in NS, 738 genes in CAS rats, with 171 genes common between the groups (Fig. 2E). A complete list of genes from each contrast are shown in Table S6. To identify transcriptional pathways sensitized to immune activation by a background of CAS, we used Gene Set Enrichment Analysis (GSEA)

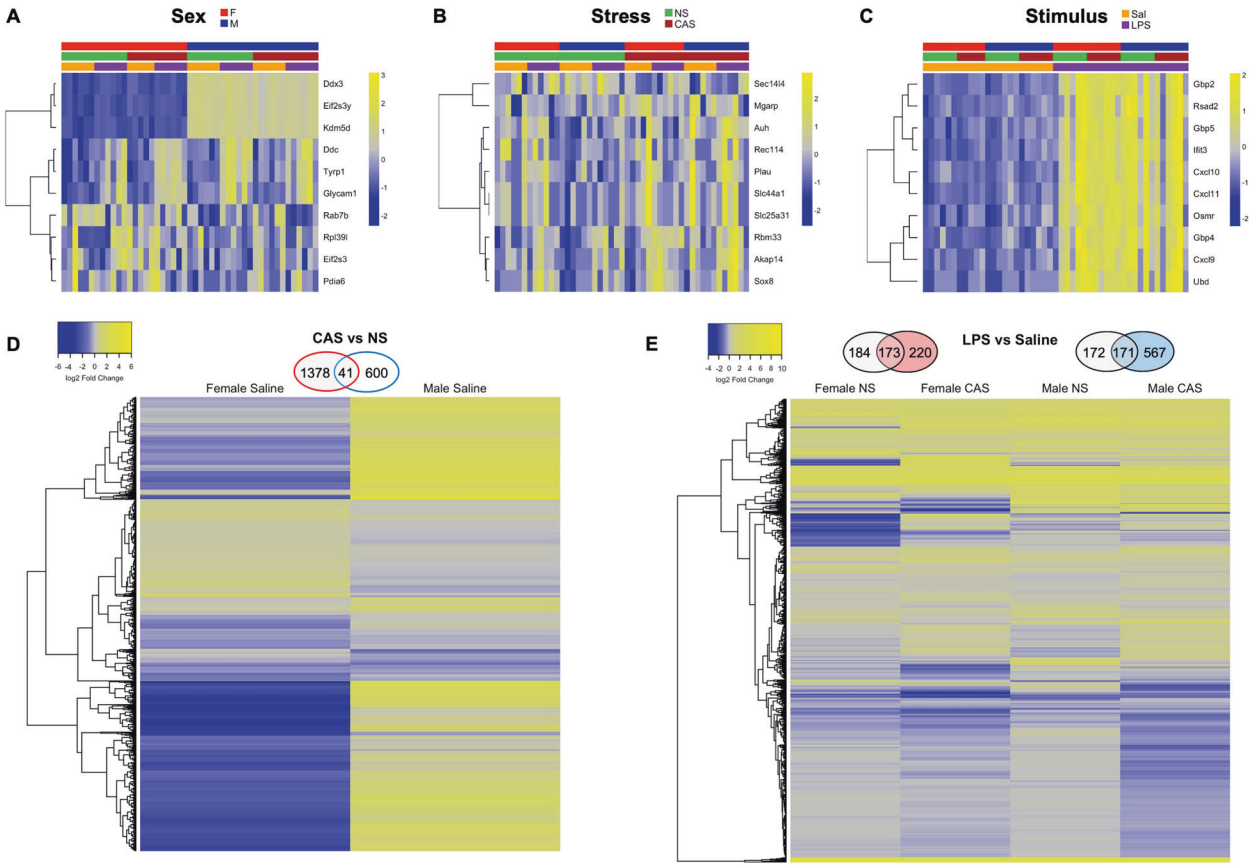

Fig. 2 Chronic adolescent stress (CAS)-associated genes were largely distinct between females and males at baseline. Female and male rats of NS or CAS background received a systemic injection of either saline or LPS in adulthood. Four hours later, hippocampal tissue was collected for bulk RNA-Seq. Differential expression analysis on the hippocampal transcripts was performed using the package edgeR in Bioconductor. **A–C** Expression of top ten DEGs are shown for the main factors of sex, stress, and LPS. **D** The impact of CAS on baseline (unstimulated) gene expression was suassessed within each sex using the contrasts "F.CAS.Sal versus F.NS.Sal" and "M.CAS.Sal versus M.NS.Sal." The number of differentially expressed genes (DEG) from each paired contrast is displayed in Venn diagrams above the heatmaps. CAS-associated genes were largely distinct between males and females with a greater number of upregulated genes in female rats and similar numbers of downregulated genes across males and females. Subsequent pathway analyses showed that genes differentially regulated by CAS in female rats enriched pathways related to signal transduction, histone deacetylases, and cytoskeleton remodeling. Genes differentially regulated by CAS in male rats were related to G protein-coupled receptor signaling, Notch signaling, and NMDA receptor trafficking (Tables S5–6). **E** LPS-induced changes in gene expression were assessed within each of the four groups (Female-NS, Female-CAS, Male-NS, Male-CAS) using the contrast "LPS versus Sal." Similar clusters of genes were differentially regulated by LPS across the four groups, with substantial DEG overlap between NS and CAS conditions within each sex. See GSEA pathway results in detail in Table 1, S7–10 and Fig S1. CAS Chronic Adolescent Stress, NS Non-stressed, Sal Saline.

[41]. In GSEA, we contrasted LPS-challenged and saline-treated rats within each of Female CAS, Female NS, Male CAS, and Male NS backgrounds. Acute immune challenge led to significant enrichment (nominal $p < 0.05$ and FDR q value < 0.1) of 21 pathways in female NS, 24 in female CAS, 23 in male NS, and 24 in male CAS (See Tables S7–10 for a list of pathways significantly enriched by LPS in each of the four groups). A qualitative comparison of the normalized enrichment scores (NES) of LPS-induced pathways across stressed and NS rats revealed that genes regulated by NFκB in response to Tumor Necrosis Factor (pathway name "TNFA_SIGNALING_VIA_NFκB") was among the Top 10 pathways enriched to a greater extent in CAS compared to NS rats of either sex (Table 1). Pathways related to adipogenesis, myogenesis, apical junction, and MYC targets were also enriched to a greater extent in CAS rats of either sex. In CAS females, LPS challenge led to greater enrichment of the GR ("PID_REG_GR_PATHWAY"), early estrogen response, TGF-beta signaling, and p53 pathways compared to Female-NS rats. In CAS males, LPS challenge led to greater enrichment of interferon alpha and gamma response as well as metabolic pathways (glycolysis and xenobiotic metabolism) compared to NS males.

CAS exaggerates LPS-induced enrichment of the NFκB signaling pathway and expression of NFκB subunits and regulators

As indicated by the GSEA results in Table 1, the magnitude of TNFA_SIGNALING_VIA_NFκB pathway enrichment following LPS challenge revealed a greater cumulative increase in CAS male and female rats (NES of 2.87 and 2.71) compared to same-sex NS controls (NES of 2.45 and 2.48). Several members of the NFκB transcriptional complex including *Nfkbia*, *Nfkb1*, and *Nfkb2* were among the "leading-edge" genes driving the enrichment of the TNFA_SIGNALING_VIA_NFκB pathway. Because prior exposure to stressors or stress hormones can alter the inflammatory response to an immune challenge [42], we assessed a time course of NFκB response to LPS via targeted qPCR, focusing on mRNA expression of canonical (*Nfkbia*, *Nfkb1*, *Rela*) and non-canonical (*Nfkb1*, *Nfkb2*) cascade subunits. LPS stimulus induced the mRNA expression of *Nfkbia* ($F_{(2,105)} = 103.84$, $p < 0.001$), *Rela*

Table 1. Top ten gene sets enriched to a greater extent in Chronic Adolescent Stress (CAS) rats compared to Non-Stressed (NS) rats following an acute immune challenge with LPS in adulthood.

Gene set	Female CAS		Female NS		ΔNES
	NES	FDR	NES	FDR	(CAS - NS)
ADIPOGENESIS[a]	1.52	0.021	0.83	1	0.69
APICAL_JUNCTION[a]	1.55	0.018	0.93	0.672	0.62
ESTROGEN_RESPONSE_EARLY	1.34	0.1	0.87	0.758	0.47
TGF_BETA_SIGNALING	1.43	0.049	0.98	0.744	0.45
MYOGENESIS*	1.35	0.098	0.95	0.671	0.4
PID_REG_GR_PATHWAY	1.88	0.001	1.57	0.015	0.31
UV_RESPONSE_UP	1.65	0.009	1.37	0.079	0.28
TNFA_SIGNALING_VIA_NFKB[a]	2.71	0	2.48	0	0.23
MYC_TARGETS_V2[a]	1.51	0.024	1.29	0.142	0.22
P53_PATHWAY	1.86	0.001	1.72	0.003	0.14
Gene set	**Male CAS**		**Male NS**		**ΔNES**
	NES	FDR	NES	FDR	(CAS - NS)
ADIPOGENESIS[a]	1.62	0.009	0.59	1	1.03
MYOGENESIS[a]	1.83	0.001	0.95	0.709	0.88
MYC_TARGETS_V2[a]	1.58	0.013	0.93	0.727	0.65
INTERFERON_GAMMA_RESPONSE	3.6	0	3.12	0	0.48
TNFA_SIGNALING_VIA_NFKB[a]	2.87	0	2.45	0	0.42
KRAS_SIGNALING_UP	1.94	0.001	1.52	0.027	0.42
GLYCOLYSIS	1.45	0.035	1.07	0.505	0.38
XENOBIOTIC_METABOLISM	1.84	0.001	1.49	0.035	0.35
APICAL_JUNCTION[a]	1.66	0.007	1.31	0.114	0.35
INTERFERON_ALPHA_RESPONSE	3.27	0	2.95	0	0.32

Using the Molecular Signatures Database's Hallmark (H) gene set in Gene Set Enrichment Analysis (GSEA), differential enrichment of 50 "hallmark" pathways representing well-defined biological processes as well as a gene set for glucocorticoid receptor regulatory network from Pathway Interaction Database (NCI, NIH and Nature Publishing Group) was assessed between LPS-challenged and saline-treated rats of Female CAS, Female NS, Male CAS, and Male NS backgrounds. Of the significantly enriched gene sets, Top 10 pathways with the largest difference in NES (ΔNES) between CAS and NS groups are shown for each sex.
CAS chronic adolescent stress, *NS* non-stressed, *NES* normalized enrichment score, *FDR* false discovery rate.
[a]Pathways whose enrichment is exaggerated by CAS in both males and females.

($F_{(2,105)} = 15.21$, $p < 0.001$), *Nfkb1* ($F_{(2,105)} = 38.66$, $p < 0.001$), and *Nfkb2* ($F_{(2,105)} = 120.24$, $p < 0.001$) (Fig. 3A–D). There was no effect of sex on *Nfkbia*, *Rela*, *Nfkb1*, and *Nfkb2* ($p > 0.177$). CAS rats displayed exaggerated hippocampal mRNA expression of *Nfkbia* ($F_{(1,105)} = 4.21$, $p = 0.043$), *Rela* ($F_{(1,105)} = 5.26$, $p = 0.024$), and *Nfkb2* ($F_{(1,105)} = 8.19$, $p = 0.005$) compared to NS rats. CAS did not impact the expression of *Nfkb1* ($F_{(1,105)} = 2.72$, $p = 0.102$).

CAS potentiates GR signaling in the hippocampus of female rats

Per Table 1, the magnitude of the GR regulatory network pathway (PID_REG_GR_PATHWAY) enrichment following LPS challenge was greater in CAS females (NES = 1.88) compared to that of NS females (NES = 1.57) (Table 1). However, the GR regulatory network was not enriched to a greater degree in CAS males (NES = 0.84) compared to NS males (NES = 1.56). We examined expression of several GR-inducible genes via qPCR to assess post-LPS time course of CAS-mediated changes in GR transcriptional activity. These included the anti-inflammatory gene *Dusp1* (encoding dual specificity phosphatase 1 which inhibits the MAPK signaling pathway [43]), and GR regulator gene *Fkbp5* (FK506-binding protein 5) which is implicated in major depressive disorder [44]. Additional GR-inducible genes were *Tsc22d* (glucocorticoid-induced leucine zipper), *Sgk1* (Serum/Glucocorticoid Regulated Kinase 1), and *Nr3c1* (GR). LPS increased the expression of *Dusp1* ($F_{(2,105)} = 59.32$, $p < 0.001$), *Fkbp5* ($F_{(2,105)} = 17.09$, $p < 0.001$) (Fig. 3E, F), as well as *Tsc22d3* ($F_{(2,105)} = 15.98$, $p < 0.001$), *Sgk1* ($F_{(2,105)} = 36.4$, $p < 0.001$), and *Nr3c1* ($F_{(2,105)} = 6.83$, $p = 0.002$). There was no effect of sex on *Dusp1*, *Fkbp5*, *Tsc22d3*, *Sgk1*, or *Nr3c1* ($p > 0.62$). CAS increased expression of *Dusp1* ($F_{(1,105)} = 4.17$, $p = 0.044$), but not *Fkbp5* or *Tsc22d3*, *Sgk1*, or *Nr3c1* ($p > 0.266$).

CAS potentiates chemokine signaling and increases perivascular myeloid cell presence in the hippocampus

Several of the leading-edge genes driving the exaggerated enrichment of the HALLMARK_TNFA_SIGNALING_VIA_NFκB pathway in CAS rats were genes related to chemokine signaling (e.g., *Cxcl11*, *Ccl4*, *Ccl2*, *Cxcl10*, *Ccrl2*) which play a key role in recruiting peripheral immune cells to sites of injury or inflammation. Therefore we measured the mRNA expression of *Ccl2* and *Cxcl11*—two chemokines which have been implicated in stress-related pathology [4, 45, 46]. LPS strongly induced mRNA expression of *Ccl2* ($F_{(2,105)} = 83.02$, $p < 0.001$) and *Cxcl11* ($F_{(2,105)} = 237.22$, $p < 0.001$) (Fig. 3G, H). There was no effect of sex on expression of either chemokine ($p > 0.657$). CAS exaggerated the hippocampal mRNA expression of both *Ccl2* ($F_{(1,105)} = 7.04$, $p = 0.009$) (Fig. 3G) and *Cxcl11* ($F_{(1,105)} = 4.2$, $p = 0.043$) (Fig. 3H).

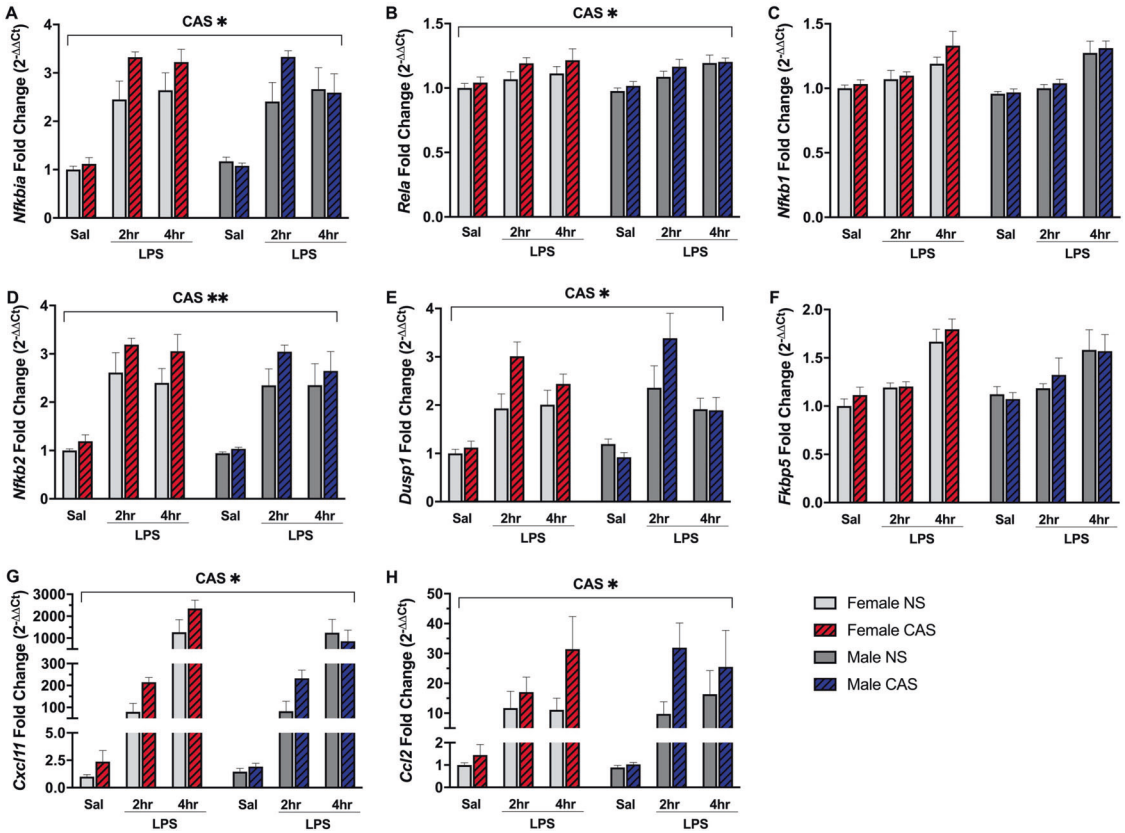

Fig. 3 Chronic Adolescent Stress (CAS) exaggerated the expression of NFκB subunits and chemokines. Female and male rats of NS or CAS background received a systemic injection of either saline or LPS in adulthood. Two and four hours later, mRNA expression of NFκB pathway members *Nfkbia*, *Rela*, *Nfkb1*, and *Nfkb2* (**A–D**), GR targets *Dusp1* and *Fkbp5* (**E–F**), and the chemokines *Ccl2* and *Cxcl11* (**G–H**) were assessed via quantitative PCR. NFκB pathway: CAS rats displayed exaggerated hippocampal mRNA expression of (**A**) *Nfkbia*, (**B**) *Rela*, and (**D**) *Nfkb2* compared to NS rats (all $p < 0.05$). CAS did not significantly impact the expression of (**C**) *Nfkb1*. GR pathway: CAS potentiated the expression of (**E**) *Dusp1* ($p < 0.05$). CAS did not alter the expression of (**F**) *Fkbp5*. Chemokines: CAS exaggerated the expression of (**G**) *Ccl2* and (**H**) *Cxcl11* (all $p < 0.05$). Data are mean fold change ± SEM. Main effect of CAS is denoted by CAS* ($p < 0.05$) or CAS** ($p < 0.01$). CAS Chronic Adolescent Stress, NS Non-stressed, LPS lipopolysaccharide, Sal Saline.

The extent of leukocyte infiltration was measured via flow cytometry and expressed as percent of $CD11b^{+}/CD45^{high}$ myeloid cells, which include monocytes and neutrophils, relative to $CD11b^{+}$ cells (gating shown in Fig S1). The proportion of infiltrating leukocytes was not impacted by sex ($F_{(1,40)} = 1.78$, $p = 0.189$), CAS ($F_{(1,40)} = 0.22$, $p = 0.64$), or LPS ($F_{(1,40)} = 0.09$, $p = 0.771$) (Fig. 4A). Immunohistochemical experiments revealed that CAS increased the number of $CD45^{+}$ cells in the perivascular area ($F_{(1,36)} = 8.52$, $p = 0.006$) (Fig. 4B, C). There was no main effect of sex ($F_{(1,36)} = 8.52$, $p = 0.006$) or stimulus ($F_{(1,36)} = 8.52$, $p = 0.006$) on the number of perivascular $CD45^{+}$ cells.

CAS increases microglial complexity in the female hippocampus

To assess the impact of CAS on the resident immune cells in the brain, we examined microglial morphology and total number in the hippocampus. There was a significant sex*stress interaction on microglial morphological complexity, as indicated by the number of quadruple points created by intersecting branches (Wald $X^2 = 6.28$, df = 1, $p = 0.012$). Specifically, CAS increased morphological complexity in females (mean difference = 0.67, SE = 0.22, $p = 0.003$), but not males (mean difference = −0.22, SE = 0.27, $p = 0.4$) (Fig. 4D, E). In addition, a sex*stimulus interaction was observed for maximum branch length (Wald $X^2 = 5.63$, df = 1, $p = 0.018$). Specifically, LPS led to a decrease in maximum branch length within males (mean difference = −8.84, SE = 3.82, $p = 0.021$) but not within females (mean difference = 2.92, SE = 3.24, $p = 0.368$) (Fig. 4F). The estimated number of microglia in the hippocampus was not impacted by sex ($F_{(1,40)} = 2.78$, $p = 0.103$), stress ($F_{(1,40)} = 0.74$, $p = 0.396$), or LPS ($F_{(1,40)} = 2.16$, $p = 0.15$).

DISCUSSION

Together, the data presented here indicate that chronic exposure to adolescent stress leads to persistent behavioral and immunophenotypes that manifest differentially in males and females. Previously, CAS has been shown to lead to female-specific depressive-like behavior [31] immediately following CAS which persisted into adulthood. Herein, we demonstrated a complementary anxiety-like phenotype in adolescence as indicated by increased time spent in the corners of the open field. We also found that CAS led to social avoidance in males as indicated by increased time spent in opposite corners from a social stimulus rat during SI [47]. Given that the social stimulus used here was a novel rat of the defeater strain (Long Evans), it is possible that CAS-induced social avoidance in males is context-dependent with regards to the phenotypic characteristics of the defeaters, similar to reduced SI in defeated mice that is specifically induced by a stimulus of the aggressor strain [48]. While SI ratio was not significantly impacted, sex-specific and/or CAS-related changes in social behavior may be investigated via alternative measures of SI, including reduced social approach and increased social vigilance which have been

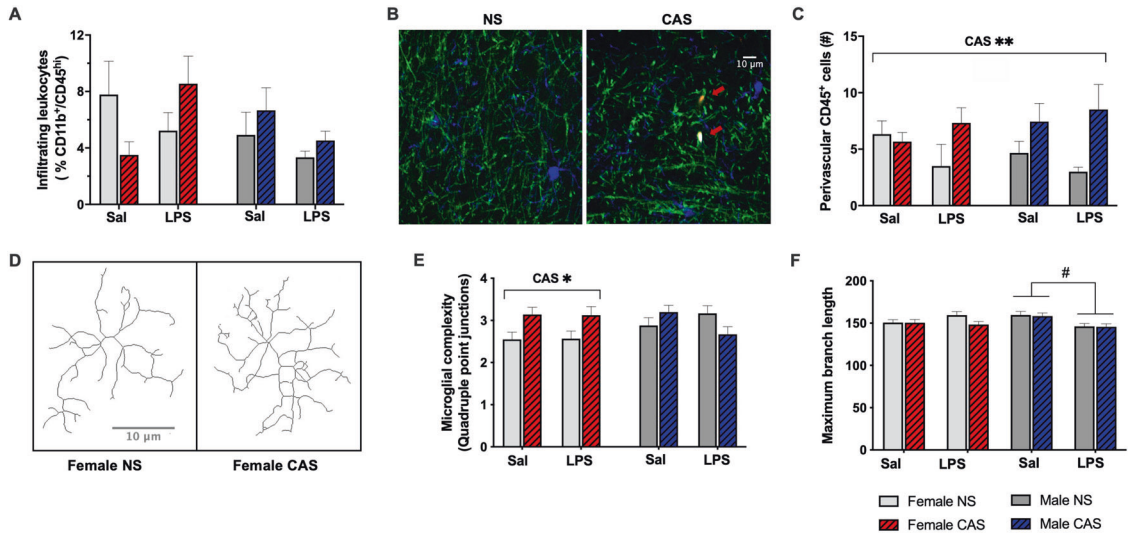

Fig. 4 CAS increased perivascular CD45 presence and sex-specifically alters microglial morphology in the hippocampus. Female and male rats of NS or CAS background received a systemic injection of either saline or LPS in adulthood on PND94. Two hours later, leukocyte infiltration, perivascular CD45 immunoreactivity, and microglial morphology were assessed in the hippocampus. **A** The percent of $CD11b^{+}$/$CD45^{high}$ infiltrating myeloid cells was not impacted by sex, stress, or LPS stimulus. **B** Representative CD45 staining (red, indicated by arrow) in close proximity to blood vessels (green) and distinct from microglia (blue) in the hippocampus of NS and CAS rats. **C** CAS increased CD45 immunoreactivity in the hippocampus ($p < 0.01$). **D** Representative microglia skeletons are shown to illustrate differential microglial complexity between females NS and CAS groups. **E** CAS increased microglial complexity within females ($p < 0.05$), but not males. **F** LPS decreased maximum branch length of microglia in males ($p < 0.05$), but not females. Data are presented as mean ± SEM. **CAS, main effect of CAS ($p < 0.01$); *CAS, effect of CAS ($p < 0.05$) within females; #, LPS effect within males. CAS Chronic Adolescent Stress, NS Non-stressed, LPS lipopolysaccharide, Sal Saline.

demonstrated following social defeat in female and male mice [49, 50].

Several next generation sequencing studies have investigated sex differences in stress-induced changes in the rodent brain [9, 29, 51], but to our knowledge, this is the first study to report long-lasting immune-inducible changes primed by adolescent stress. At baseline, CAS led to largely distinct sets of DEGs in the male and female rat hippocampus, a result consistent with prior transcriptomic studies in depression [10] as well as acute and chronic stress [29, 51, 52]. Following LPS, hippocampal immune transcripts were inducible to a greater extent in CAS rats compared to their NS litter mates. These transcriptomic changes were epitomized by an exaggerated enrichment of the NFκB signaling pathway in both males and females. Our results therefore extend similar findings from male mice subjected to chronic restraint stress [53] and are consistent with data from chronically stressed humans showing upregulated transcripts with response elements for NFκB [54]. In addition, we found that CAS exaggerated induction of several key members of the NFκB transcriptional complex, consistent with previously reported effects of stress on NFκB expression and activity in rodents [55–58] and humans [14, 59].

While CAS-driven sensitization of NFκB induction was present in both sexes, it is nonetheless possible that stress-induced NFκB activity exerts different effects on behavioral outcomes in males and females. Indeed, whereas in stressed male rodents NFκB has been shown to be necessary for changes in synaptic strength [60] and neurogenesis [61] underlying behavioral susceptibility, in female mice subjected to chronic unpredictable stress NFκB protected against ovarian hormone-related depressive-like behavior [62]. Interestingly, CAS females exhibited enhanced GR signaling. Although the effects of GR are commonly conceptualized as anti-inflammatory [63], there are several contexts in which GR has been documented to enhance pro-inflammatory processes [20, 64–66]. In contrast, CAS males displayed lower GR pathway induction compared to NS males, which may be consistent with reports of diminished expression of transcripts bearing the GR response element in chronically stressed humans [67–69] and rodents [70].

Here we found that although CAS markedly potentiated chemokine induction following LPS challenge, there was no evidence of peripheral myeloid cell infiltration into the brain parenchyma in rats due to sex, stress, or LPS. Thus, peripheral myeloid cell infiltration could not have contributed to CAS-driven priming of inflammatory genes reported here. Notably, while there is mixed evidence of monocyte trafficking to the mouse brain following social defeat or LPS injection [21, 24, 71, 72], leukocyte infiltration in rats has only been documented following severe inflammatory insults such as ischemia [73], experimental allergic encephalomyelitis [74], traumatic brain injury [75], and glioma [76]. However, in the absence of overt parenchymal infiltration, it is possible that subtly activated peripheral immune cells become associated with blood vessels in the brain. Indeed, under normal circumstances circulating monocytes are known to patrol the brain's vasculature, and give rise to perivascular macrophages upon receiving pro-inflammatory signals [77]. Here we found that CAS increased $CD45^{+}$ cells in the perivascular area. Consistent with the flow cytometry results, obtained using hippocampal tissue after removal of blood vessels, very few $CD45^{+}$ cells were seen in the parenchyma, with the vast majority associated with the endothelium. Consistent with previous reports at similar time points [77], LPS did not increase perivascular CD45 presence.

We also assessed the impact of CAS on microglial morphology to probe if microglia may be involved in CAS-related inflammatory gene expression priming [78–80]. Similar to another index of microglial hypercomplexity identified by Sholl analysis following chronic stress model in rats [81], here we found that CAS led to a more complex microglial morphology—as measured by the number of quadruple points, indicating intersecting branches of a ramified cell—in female rats only. Consistently, microglia have been demonstrated to be critical in neuroinflammatory and behavioral profiles seen in other models of chronic stress [82]. Additional work will be necessary to determine the functional implications of increased ramification of adult microglia following

adolescent stress in females. A potential limitation of the current study is that estrous cycle was not controlled for at the time of tissue collection. Indeed, stress-related neuroinflammatory outcomes such as brain concentrations of the cytokine IL-1 have been shown to differ by estrous stage, with considerable upregulation during diestrus, proestrus, and estrus [83]. Previous studies from our group using the CAS paradigm have shown that plasma values of 17β-estradiol and progesterone were not different at baseline between CAS and NS females in adolescence and in adulthood [4, 31]. While terminal uterine weights, a proxy to estrous cycle stage [84], indicated that CAS females had lower uterine weights compared to NS females, uterine weights did not correlate with experimental endpoints collected on the same day, including microglial morphology, suggesting that estrous cycle stage was not a primary driver of observed differences in CAS females. Another limitation was that the single housing was not only a part of the intended stressor during CAS, but that it extended beyond the specific adolescent stress period. This design was selected because we've previously shown that individual housing alone does not produce equivalent effects to CAS [31] and retaining the individual housing condition eliminates the potential effects of pair-housing-induced noise in the data including social buffering effects (which may also be sex-specific) [85, 86], or heightened stress and potential injury particularly in males due to in-cage fighting. However, because the individual housing condition extended beyond the end of the adolescent stress period, it does represent a continuous stressor for CAS rats. While the current paradigm offers relevance to real-world scenarios whereby chronically stressed individuals are more likely to withdraw from SIs following their stressful experiences [87, 88], it does not allow complete isolation of the individual housing effects to the adolescent period. Consistent with our assessment of sustained individual housing versus CAS-effects [31], we've recently shown that the impact of chronic stressor exposure during adolescence in mice is evident on behavioral and neural outcomes in adulthood even when all groups are exposed to individual housing following the initial adolescent stress period [89].

Rodent studies examining sex differences in stress-related neuroimmune outcomes have found exaggerated pro-inflammatory responses more frequently in males than females [4, 58, 90]. Our results hereby confirm and extend the occurrence of stress-induced immune priming in female rodents. Although the experiments herein utilized LPS to induce immune activation, it would be reasonable to expect that non-pathogenic sources of inflammation, including psychological stressors, would similarly engage the NFκB pathway [91, 92] in the periphery and possibly in the brain. Furthermore, in females an estrogen response pathway was potentiated by CAS following LPS challenge, which could contribute to sex differences presented in the current study, as we've previously reported estrogen receptor-mediated transcriptional changes in CAS females [29]. In males, CAS enriched pathways involved in glycolysis and metabolism, which warrants future investigation into the role of stress in altered energy metabolism in the brain [89]. Taken together, our results provide a novel framework within which mechanisms mediating sex differences in chronic stress-related neuroimmune changes can be further explored.

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AUTHOR CONTRIBUTIONS

MB conceived of, collected, and analyzed behavioral, microglial, flow cytometry, and qPCR data, conducted pathway analysis, and wrote the paper. DM collected and analyzed Iba-1 and CD45 staining data. MMH assisted with collection of Iba-1 and CD45 staining data, and contributed to interpretation of behavioral data. MGD, JCS, and GKT analyzed RNA-Seq data. SDB and JLD assisted with flow cytometry experimental design and data collection. SAR and SDK assisted with behavioral data collection, and contributed to experimental design. ZQ assisted with statistical analysis. MGT advised project design, and assisted with data interpretation. GNN conceived of project, acquired funding, advised project design and data interpretation, and revised the paper.

ADDITIONAL INFORMATION

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