

Stress-Induced Neuronal Colony Stimulating Factor 1 Provokes Microglia-Mediated Neuronal Remodeling and Depressive-like Behavior

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ABSTRACT

BACKGROUND: Chronic stress exposure causes neuronal atrophy and synaptic deficits in the medial prefrontal cortex (PFC), contributing to development of anxiety- and depressive-like behaviors. Concomitantly, microglia in the PFC undergo morphological and functional changes following stress exposure, suggesting that microglia contribute to synaptic deficits underlying behavioral consequences.

METHODS: Male and female mice were exposed to chronic unpredictable stress (CUS) to examine the role of neuron-microglia interactions in the medial PFC during development of anxiety- and depressive-like behaviors. Thy1-GFP-M mice were used to assess microglia-mediated neuronal remodeling and dendritic spine density in the medial PFC. Viral-mediated knockdown of neuronal colony stimulating factor 1 (CSF1) was used to modulate microglia function and behavioral consequences after CUS.

RESULTS: CUS promoted anxiety- and depressive-like behaviors that were associated with increased messenger RNA levels of CSF1 in the PFC. Increased CSF1 messenger RNA levels were also detected in the postmortem dorsolateral PFC of individuals with depression. Moreover, microglia isolated from the frontal cortex of mice exposed to CUS show elevated CSF1 receptor expression and increased phagocytosis of neuronal elements. Notably, functional alterations in microglia were more pronounced in male mice compared with female mice. These functional changes in microglia corresponded with reduced dendritic spine density on pyramidal neurons in layer 1 of the medial PFC. Viral-mediated knockdown of neuronal CSF1 in the medial PFC attenuated microglia-mediated neuronal remodeling and prevented behavioral deficits caused by CUS.

CONCLUSIONS: These findings revealed that stress-induced elevations in neuronal CSF1 provokes microglia-mediated neuronal remodeling in the medial PFC, contributing to synaptic deficits and development of anxiety- and depressive-like behavior.

Keywords: Colony stimulating factor 1, Depression, Microglia, Neuroimmune, Prefrontal cortex, Stress

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Affective disorders are a major source of disability and cause significant social and economic burdens (1,2). Clinical and basic research shows that anxiety and depressive symptoms arise from synaptic deficits in the medial prefrontal cortex (PFC) and hippocampus (3–5). Similarly, the rodent chronic unpredictable stress (CUS) model causes synaptic loss on medial PFC pyramidal neurons that leads to development of depressive-like behaviors (6–8). Other studies report that repeated stress exposure causes morphological and molecular changes in brain-resident macrophages, termed microglia (9,10). This is pertinent because recent studies show that microglia modulate neuroplasticity, raising the possibility that stress-induced microglia activation contributes to synaptic deficits underlying depressive-like behaviors.

Microglia are acutely sensitive to neuronal homeostasis and influence neuroplasticity by release of neuromodulators (i.e., cytokines) and by activity-dependent elimination of dendrites and synapses (4,11,12). Notably, dysregulation of neuronal

activity and neuronal atrophy in the medial PFC following stress (13,14) may provoke functional alterations in microglia. Indeed, neuronal dystrophy or neuronal damage causes fluctuations in neuron-derived factors that regulate microglia function such as colony stimulating factor 1 (CSF1) (15), fractalkine (CX3CL1) (16), or transforming growth factor β (TGF β) (17). For example, kainic acid-induced neuronal hyperactivity caused upregulation of neuron-derived CSF1, which caused microglia activation and neuronal loss in the hippocampus (18). In another study, sensory neuron injury increased neuronal CSF1, resulting in microglia activation that contributed to neuropathic pain (19). In the context of stress, neuronal atrophy may initiate release of factors that alter microglia function, which may disrupt neuron-microglia interactions and contribute to synaptic deficits underlying depressive-like behaviors (4).

The primary objective of the current studies was to determine the role of microglia in neuronal remodeling and development of depressive-like behavior in mice exposed to CUS.

These studies showed that upregulation of neuronal CSF1 provoked microglia-mediated neuronal remodeling in the medial PFC, leading to development of anxiety- and depressive-like behaviors. These findings revealed a novel cellular mechanism by which microglia contribute to the neurobiology of affective disorders.

METHODS AND MATERIALS

Animals

Transgenic (and wild-type littermate) male and female Thy1-GFP-M mice were obtained from in-house breeders (Jackson Laboratory, Bar Harbor, ME; Tg(Thy1-EGFP)Mjrs/J, #007788). For CSF1 knockdown studies, male wild-type C57BL/6 mice were purchased (C57BL/6J, #000664; Jackson Laboratory). Studies were performed with mice 6 to 12 weeks old. Mice were group housed (3 per cage) in 11.5 × 7.5 × 6-inch polypropylene cages under a 12-hour light/dark cycle with ad libitum access to water and rodent chow.

Chronic Unpredictable Stress

CUS was performed as previously described (6,20). In brief, mice were exposed to random intermittent stressors over 14 days, including cage rotation, isolation, radio noise, food or water deprivation, light on overnight, light off during the day, rat odor, stroboscope overnight, crowding, wet bedding, no bedding, and tilted cage.

Behavioral Testing

The open field (OF) test, forced swim test (FST), sucrose consumption test (SCT), and novelty-suppressed feeding (NSF) test were conducted as previously described (7,20,21). Further details are provided in the *Supplement*.

RNA Isolation and Real-Time Polymerase Chain Reaction

RNA was extracted from whole brain regions (PFC) and N2a cells using TRIzol Reagent according to the manufacturer's protocol (Invitrogen, Waltham, MA). RNA was extracted from microglia using the Single Cell RNA Purification Kit (Norgen Biotech Corp., Thorold, ON, Canada). Samples were reverse transcribed, and real-time polymerase chain reaction was conducted as previously described (22).

Percoll Gradient Isolation of Enriched Microglia

Dissected frontal cortex was passed through a 70-μm cell strainer. Homogenates were centrifuged at 600g for 6 minutes. Supernatants were removed, and cell pellets were resuspended in 70% isotonic Percoll (GE Healthcare, Uppsala, Sweden). A discontinuous Percoll density gradient was layered as follows: 50%, 35%, and 0% isotonic Percoll. The gradient was centrifuged for 20 minutes at 2000g, and enriched microglia were collected from the interphase between the 70% and 50% Percoll layers (23,24).

Immunohistology

Mice were transcardially perfused with sterile phosphate-buffered saline and 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde for 24 hours and incubated in

30% sucrose for an additional 24 hours. Frozen brains were sectioned with a Microm HM550 cryostat (Thermo Fisher Scientific, Waltham, MA). Free-floating sections were washed and then blocked for 1 hour at room temperature. Sections were washed and then incubated with primary antibodies: rabbit anti-Iba-1 (019-19741; Wako, Richmond, VA), mouse anti-CSF1 (AF416; R&D Systems, Minneapolis, MN), mouse anti-CD68 (ab31630; Abcam, Cambridge, MA), goat anti-Iba-1 (NB100-1028; Novus Biologicals, Littleton, CO), and rabbit anti-P2Y12R (AS-55043A; Anaspec, Fremont, CA) overnight at 4°C. Sections were washed and incubated with conjugated secondary antibody overnight at 4°C.

Short-Hairpin RNA and Viral Preparation

CSF1 short-hairpin RNA (shRNA) sequence was designed targeting transcript (25). CSF1 shRNA (5'-TTTGTCTCATC TATTATGTTGACCCCTCCTGTCAGGTACAAGACATAATA GATGAGAAATTTT-3') or scrambled control (Integrated DNA Technologies, Coralville, IA) was ligated into the pEGFP-shRNA construct, designed to coexpress enhanced green fluorescent protein (EGFP) under the cytomegalovirus promoter and ligated shRNA driven by the U6 promoter. The construct was packaged in adeno-associated virus 2 (AAV2) as previously described (6,26).

Surgery and Cortical Infusion

Mice were anesthetized with ketamine/xylazine (100/10 mg/kg). Bilateral viral infusions in the medial PFC (1 μL; 0.1 μL/minute) were performed with coordinates (from bregma) +2.0 mm anterior-posterior, ±0.2 mm medial-lateral, −2.8 mm dorsal-ventral (27). Incisions were closed with sutures, and mice received intraperitoneal injection of carprofen (5 mg/kg) immediately after surgery and daily for the next 2 days.

Quantitative Immunofluorescence

Confocal images were captured with a Hamamatsu high-resolution digital camera (ORCA-ER; Hamamatsu City, Japan) using Fluoview (FV1000) software on an Olympus BX61WI microscope (Olympus, Tokyo, Japan). Quantification of immunolabeling and dendritic spine density was performed as previously described (28,29). Further details are provided in the *Supplement*.

Statistical Analysis

Data were subjected to statistical analyses with GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Significant main effects and interactions were determined using one-way analysis of variance (genotype or treatment) or two-way analysis of variance (genotype × treatment). Differences between group means were evaluated with Fisher's least significant difference test.

Study Approval

Animal use and procedures were in accordance with the National Institutes of Health guidelines and approved by the Yale University Animal Care and Use Committees.

RESULTS

Male and Female Mice Develop Anxiety- and Depressive-like Behaviors Following CUS

Male and female mice were exposed to 14 days of CUS and then assessed in the OF test, FST, SCT, and NSF test on subsequent days (Figure 1A). In the OF test, male and female mice showed similar locomotor activity; however, male mice showed an increase in latency to enter the OF ($F_{1,31} = 3.49, p < .07$; Figure 1B, C). Both male and female mice had decreased time spent in the OF following CUS ($F_{1,31} = 8.21, p < .007$; Figure 1D). CUS significantly increased FST immobility in both male and female mice ($F_{1,30} = 16.04, p < .0004$; Figure 1E). There was an overall decrease in sucrose consumption ($F_{1,31} = 5.05, p < .03$), but post hoc analyses showed a significant reduction only in male mice compared with control mice (Figure 1F). Similarly, there was a general increase in latency to feed in the NSF test for both male and female mice following CUS ($F_{1,30} = 4.45, p < .04$), but only male mice were significantly different from control mice (Figure 1G). Thus, anxiety- and depressive-like behaviors were observed in both male and female mice after CUS, with male mice demonstrating greater susceptibility to CUS.

CUS Altered PFC Expression of Genes That Regulate Microglia Function

Coincident neuronal atrophy and microglia activation in the PFC suggest that neuron–microglia interactions may be dysregulated (4). To test this hypothesis, male and female mice were exposed to 14 days of CUS, and 2 hours after the final stressor the PFC was dissected for messenger RNA (mRNA) analyses (Figure 2A). CUS exposure caused robust upregulation of *Csf1* expression ($F_{1,19} = 19.75, p < .0003$), with a modest increase in *Cx3cr1* expression ($F_{1,20} = 6.91, p < .02$).

Several markers, including *Csf1r* ($F_{1,19} = 12.12, p < .003$), *Tgfb1r* ($F_{1,19} = 13.28, p < .002$), and *Tnfa* ($F_{1,19} = 5.09, p < .04$), were significantly reduced following CUS (Figure 2B). Other regulatory factors, such as *Iil34*, *Tgfb*, and *Iil1b*, were not altered by CUS exposure (Figure 2B).

To further examine CUS-induced neuroimmune alterations, male and female mice were exposed to CUS, and 2 hours after the final stressor the frontal cortex was dissected and enriched microglia were obtained via Percoll gradient isolation (23,24) (Figure 2C). Gene expression of several neuroimmune markers was examined, including *Csf1*, *Iil34*, *Csf1r*, *Tgfb*, *Cx3cr1*, *Iil1b*, and *Tnfa* (Figure 2D). In enriched microglia from the frontal cortex of male and female mice, *Csf1r* ($F_{1,15} = 31.84, p < .0001$), *Tgfb* ($F_{1,15} = 22.02, p < .0003$), and *Cx3cr1* ($F_{1,15} = 13.02, p < .003$) were upregulated after CUS (Figure 2D). Of note, enriched microglia had increased expression of immunomodulatory receptors in a sex-dependent manner, with male mice showing a greater fold change of *Csf1r* ($F_{1,15} = 4.49, p < .05$), *Tgfb* ($F_{1,15} = 3.08, p = .10$), and *Cx3cr1* ($F_{1,15} = 5.93, p < .03$) compared with female mice. The expression of proinflammatory cytokines *Iil1b* ($F_{1,16} = 33.59, p < .0001$) and *Tnfa* ($F_{1,16} = 41.44, p < .0001$) was robustly decreased in both male and female mice following CUS. These findings indicate that CUS exposure significantly altered gene expression of immunomodulatory receptors and cytokines in a sex-dependent manner (Figure 2D).

To examine whether CSF1 was modulated in clinical samples, mRNA analyses were performed on dorsolateral PFC samples obtained from individuals diagnosed with major depressive disorder (MDD) and assessed in a prior publication (6). Consistent with the rodent model, CSF1 mRNA was upregulated in the postmortem dorsolateral PFC of MDD samples ($F_{1,48} = 4.36, p < .04$; Figure 2E), with men showing significant upregulation of CSF1 compared with control subjects ($p < .051$; Figure 2E). Notably, these

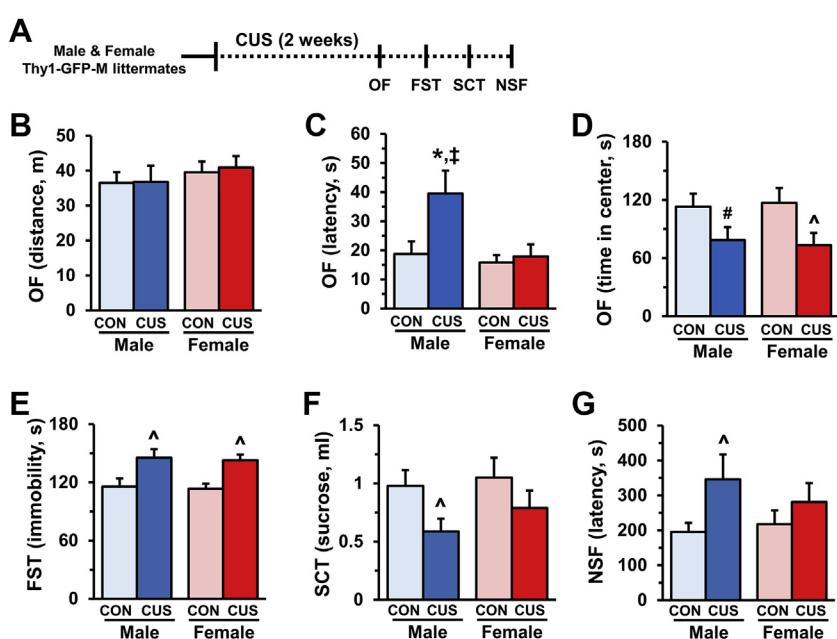


Figure 1. Male and female mice develop anxiety- and depressive-like behaviors following chronic unpredictable stress (CUS). Male and female Thy1-GFP-M littermates were exposed to 14 days of CUS and then assessed in the open field (OF) test, forced swim test (FST), sucrose consumption test (SCT), and novelty-suppressed feeding (NSF) test on subsequent days ($n = 8\text{--}10/\text{group}$). (A) Schematic showing experimental approach and timeline. (B–D) On the final day of CUS, activity in the OF test was assessed; total distance (B), latency to enter the OF (C), and time spent in the center of the OF (D) are shown. (E) The following day, immobility in the FST was measured; immobility in minutes 2 to 6 is shown. (F) On the subsequent day, the SCT was performed; total sucrose consumed is shown. (G) Last, the NSF test was administered; latency to feed is shown. Bars represent the mean \pm SEM. Means significantly different from the respective control (CON) group based on analysis of variance are denoted (main effect: * $p < .05$ or # $p = .08$; interaction: ^ $p < .05$). Means significantly different from the respective female experimental group based on analysis of variance are noted by ^ $p < .05$.

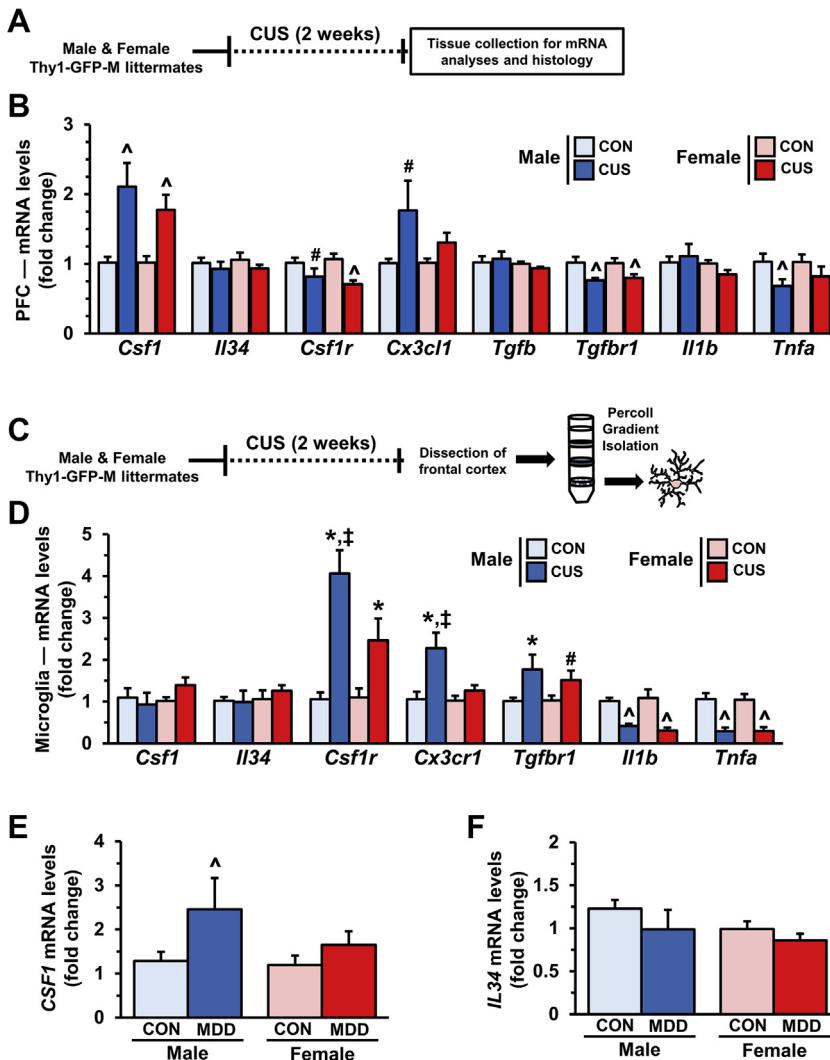


Figure 2. Chronic unpredictable stress (CUS) altered gene expression of factors that mediate neuron-microglia interactions in the prefrontal cortex (PFC). **(A)** Male and female Thy1-GFP-M littermates were exposed to 14 days of CUS, and 2 hours after the final stressor the PFC was dissected for messenger RNA (mRNA) analyses. **(B)** Relative fold changes of mRNA levels in the PFC of male and female mice are shown for *Csf1*, *Il34*, *Csf1r*, *Cx3cr1*, *Tgfb*, *Tgfb1r*, *Il1b*, and *Tnfa* ($n = 5$ –7/group). **(C)** In separate cohorts, male and female wild-type littermates of Thy1-GFP-M mice were exposed to 14 days of CUS. Following the final stressor, the frontal cortex was dissected and microglia were enriched through Percoll gradient isolation. **(D)** Gene expression of *Csf1*, *Il34*, *Csf1r*, *Cx3cr1*, *Tgfb1r*, *Il1b*, and *Tnfa* in enriched microglia is shown ($n = 4$ or 5/group). **(E, F)** Isolated mRNA from the postmortem dorsolateral prefrontal cortex was obtained from control (CON) subjects and patients with major depressive disorder (MDD). Relative fold changes of *CSF1* (**E**) and *IL34* (**F**) mRNA levels are shown ($n = 8$ –14/group). Bars represent the mean \pm SEM. Means significantly different from the respective CON group based on analysis of variance are denoted (main effect: * $p < .05$ or # $p = .08$; interaction: * $p < .05$). Means significantly different from the respective female experimental group based on analysis of variance are noted by † $p < .05$.

samples did not show significant changes in *IL34* mRNA levels (Figure 2F).

Divergent Sex-Dependent Microglia Activation Is Associated With Reduced Dendritic Spine Density in the Medial PFC After CUS

Several studies demonstrate that microglia undergo morphological changes in the medial PFC following stress exposure (9,10), which may represent alterations in gene transcription as well as function (30). To examine the coincidence of microglia alterations and neuronal atrophy, we used Thy1-GFP-M mice, which have a subset of PFC pyramidal neurons that express GFP enabling dendritic and synaptic analyses (Figure 3A). Male and female Thy1-GFP-M mice exposed to 14 days of CUS were perfused 2 to 4 hours later for immunohistology. Representative images of Iba-1 (microglia marker) immunofluorescence in control or CUS male mice (Figure 3B) and female mice (Figure 3C) are shown. Proportional area analyses of Iba-1 showed that CUS caused modest elevations in Iba-1

immunolabeling in the medial PFC ($F_{1,15} = 2.58$, $p = .12$; Figure 3D), with male mice showing elevations in Iba-1 proportional area ($p < .059$; Figure 3D). The number of Iba-1+ microglia in the medial PFC was modestly increased following CUS ($F_{1,15} = 3.26$, $p < .09$), particularly in male mice ($p < .03$; Figure 3E). Dendritic spine analyses were performed on the same samples processed above. CUS significantly reduced dendritic spine density in the medial PFC ($F_{1,12} = 5.10$, $p < .04$), and post hoc analyses revealed that synaptic deficits were more pronounced in male mice ($p < .03$; Figure 3F, G). Taken together, these findings show that CUS caused sex-dependent morphological alterations in microglia that were associated with synaptic deficits in layer 1 of the medial PFC.

CUS Increased Microglia-Mediated Neuronal Remodeling in Layer 1 of the Medial PFC

To determine whether microglia contribute to synaptic deficits in the medial PFC, as reported in the hippocampus (31), neuron-microglia interactions were examined in the same

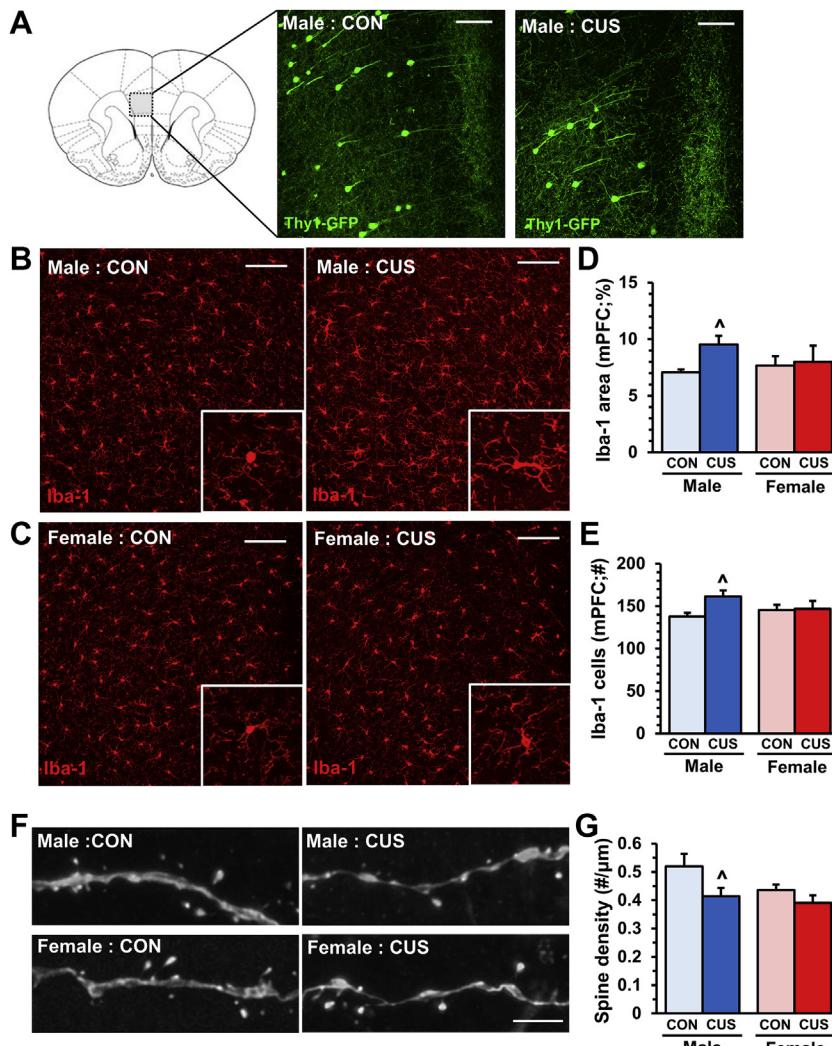


Figure 3. Divergent sex-dependent microglia activation is associated with reduced dendritic spine density in the medial prefrontal cortex (mPFC) after chronic unpredictable stress (CUS). Male and female Thy1-GFP-M mice were exposed to 14 days of CUS, and 2 to 4 hours after the final stressor mice were perfused and brains were collected for immunohistology. **(A)** Representative images of GFP expression in the mPFC of control and CUS mice. **(B, C)** Representative images of Iba-1 immunofluorescence in the medial PFC of control (CON) or CUS male **(B)** and female **(C)** mice. White scale bar represents 100 μm. Enlarged images of representative microglia are shown. **(D)** Quantification of Iba-1 proportional (%) area in medial PFC. **(E)** Number of Iba-1 microglia per field in the medial PFC ($n = 4\text{--}6/\text{group}$). In the same samples, apical dendrites were identified in layer 1 of the medial PFC, and dendritic spine density was analyzed. **(F)** Representative confocal images of layer 1 apical dendrites in the medial PFC of CON and CUS male and female Thy1-GFP-M mice. White scale bar represents 5 μm. **(G)** Quantification of average dendritic spine density in the medial PFC of CON and CUS male and female Thy1-GFP-M mice ($n = 5$ or 6/group). Bars represent the mean ± SEM. Means significantly different from the respective CON group based on analysis of variance are denoted (main effect: $\Delta p < .05$).

samples of male and female Thy1-GFP-M mice used for histological analyses of dendritic spine density (Figure 3). Confocal images of individual microglia were taken in the medial PFC, and stacks were analyzed for neuron–microglia interactions. Representative images show that microglia in both control and CUS mice had processes closely apposed GFP+ neuronal elements (Figure 4A, B, arrowheads). Further analyses showed that a portion of microglia in layer 1 of the medial PFC showed GFP+ inclusions (Figure 4B, rounded arrowhead). The proportion of microglia with GFP+ inclusions was increased by CUS exposure in both male and female mice in layer 1 of the medial PFC ($F_{1,15} = 11.10, p < .004$; Figure 4D). Male mice had significantly more GFP+ inclusions per microglia compared with control and female mice exposed to CUS ($F_{1,15} = 33.07, p < .0001$; Figure 4E), which coincided with an increased frequency of microglial phagocytic cups ($F_{1,16} = 10.00, p < .006$; Figure 4F). Within microglia, GFP+ inclusions colocalized with the lysosomal marker CD68, indicating that these neuronal elements are phagocytosed in layer 1 of the medial PFC (Supplemental Figure S1). In addition, all

microglia (Iba-1+) in the medial PFC colocalized with the resident microglia-specific marker P2Y12 receptor (Supplemental Figure S2). Furthermore, microglia in medial PFC layer 5 or in somatosensory cortex layer 1 did not show increased engulfment of neuronal elements (Supplemental Figure S3), indicating that resident microglia mediate neuronal remodeling in a laminar- and region-specific manner.

CSF1 Knockdown in the Medial PFC Normalized Neuroimmune Responses Following CUS

Experiments using viral-mediated CSF1 knockdown were performed to test the hypothesis that CSF1 signaling in the medial PFC promotes microglia-mediated neuronal remodeling in male mice. Constructs were designed to coexpress a shRNA targeting CSF1 and an EGFP reporter (Figure 5A). The pEGFP-CSF1shRNA construct significantly reduced CSF1 mRNA levels compared with scrambled control (pEGFP-SCR) in transfected N2a cells (Figure 5A; $p < .0001$). Importantly, pEGFP-CSF1shRNA did not significantly change *Cx3cl1*, *Il34*,

or *Tgfb* mRNA levels in transfected N2a cells ([Supplemental Figure S4A](#)). Following viral packaging, wild-type C57BL/6 male mice received bilateral infusion of AAV2^{SCR} or AAV2^{CSF1shRNA} in the medial PFC, and after recovery mice were subjected to CUS ([Figure 5A](#)). After behavioral testing, one subset of mice was perfused and brains were collected for immunohistology. Representative images of AAV2^{SCR} and AAV2^{CSF1shRNA} virus infection (EGFP reporter) in the medial PFC are shown ([Figure 5B](#)). In a separate subset of mice, the medial PFC was dissected and mRNA analyses were performed. As expected, CUS significantly increased *Csf1* mRNA levels in mice that received AAV2^{SCR} ($F_{1,12} = 10.18, p < .008$). Moreover, mice that received AAV2^{CSF1shRNA} showed reductions of *Csf1* mRNA levels in the medial PFC ($F_{1,12} = 10.20, p < .008$). Post hoc analyses showed that AAV2^{CSF1shRNA}/control mice had decreased *Csf1* mRNA levels compared with AAV2^{SCR}/control mice ($p = .07$), and *Csf1* mRNA levels were attenuated in AAV2^{CSF1shRNA}/CUS mice compared with AAV2^{SCR}/CUS mice ([Figure 5C](#)). Similar to previous results, *Il34* mRNA levels were not significantly changed following CUS, and AAV2^{CSF1shRNA} did not influence *Il34* expression ([Figure 5D](#)). AAV2^{CSF1shRNA} did alter some neuroimmune factors given that *Tgfb* ($F_{1,12} = 8.755, p < .01$), *Il1b* ($F_{1,12} = 15.04, p < .002$), and *Tnfa* ($F_{1,12} = 15.23, p < .002$) were significantly reduced in AAV2^{CSF1shRNA}/control mice ([Supplemental Figure S4A, B](#)), suggesting that reduced CSF1 may elicit adaptations in neuron-microglia signaling factors. Additional immunohistology showed that CUS-induced elevation of CSF1 protein in AAV2^{SCR}-infected neurons was blocked in AAV2^{CSF1shRNA}-infected neurons ($F_{1,11} = 7.41, p < .02$; [Figure 5E, F](#)).

Knockdown of Neuron-Derived CSF1 Blocked Microglia-Mediated Neuronal Remodeling in the Medial PFC Following CUS

To examine the role of neuron-derived CSF1 in stress-associated changes in microglia function, male mice were infused with AAV2^{SCR} or AAV2^{CSF1shRNA} in the medial PFC and then exposed to CUS. After behavioral analyses, mice were perfused and brains were collected for immunohistology. CUS-induced increase in Iba-1+ proportional area were attenuated in mice that received AAV2^{CSF1shRNA} ($F_{1,11} = 12.36, p < .005$; [Supplemental Figure S5A-C](#)). The modest increase in the number of Iba-1+ microglia ($F_{1,11} = 2.84, p = .12$) after CUS was also blocked in mice that received AAV2^{CSF1shRNA} ($F_{1,11} = 6.463, p < .03$; [Supplemental Figure S4D](#)). These findings indicate that knockdown of neuronal CSF1 with AAV2^{CSF1shRNA} prevented CUS-induced alterations in morphology and number of Iba-1+ microglia.

The EGFP reporter of AAV2^{SCR} and AAV2^{CSF1shRNA} enabled further analyses of microglia interactions with pyramidal neuron apical dendrites in layer 1 of the medial PFC ([Figure 6A, B](#)). Consistent with prior results ([Figure 4](#)), CUS significantly increased the proportion of microglia in layer 1 of the medial PFC with EGFP+ inclusions, and these microglia responses were attenuated in mice that received AAV2^{CSF1shRNA} ($F_{1,11} = 7.55, p < .02$; [Figure 6C](#)). Moreover, CUS increased the average number of EGFP+ inclusions in microglia and AAV2^{CSF1shRNA} reduced the number of EGFP+

inclusions per microglia after CUS ($F_{1,11} = 7.18, p < .02$; [Figure 6D](#)). These results demonstrate that neuron-derived CSF1 promotes microglia-mediated phagocytosis of dendritic elements in the medial PFC after CUS.

Knockdown of Neuron-Derived CSF1 Prevented CUS-Induced Anxiety- and Depressive-like Behaviors

Behavioral effects of neuronal CSF1 knockdown were examined in male mice exposed to 14 days of CUS ([Figure 7A](#)). In the OF, no significant differences in total distance traveled were observed ([Figure 7B](#)); however, in mice that received AAV2^{SCR}, CUS increased the latency to enter the center ($F_{1,27} = 3.05, p = .09$; [Figure 7C](#)) and reduced time spent in the center ($F_{1,27} = 2.64, p = .11$; [Figure 7D](#)). In both metrics, mice that received AAV2^{CSF1shRNA} had blunted responses following CUS and showed a relative increase in time spent in the center ($F_{1,27} = 4.25, p < .05$; [Figure 7D](#)). In the FST, CUS increased immobility in mice that received AAV2^{SCR}, and these effects were prevented in mice that received AAV2^{CSF1shRNA} ($F_{1,26} = 8.42, p < .008$; [Figure 7E](#)). Similarly, in the SCT, CUS-induced reductions in sucrose consumption were blocked in mice that received AAV2^{CSF1shRNA} ($F_{1,27} = 3.66, p = .07$; [Figure 7F](#)). In the NSF assay, AAV2^{SCR} mice exposed to CUS had increased latency to feed, while mice that received AAV2^{CSF1shRNA} had similar latency to feed as control mice ($F_{1,27} = 7.43, p < .01$; [Figure 7G](#)).

DISCUSSION

Clinical and preclinical research demonstrates that the pathophysiology of MDD includes neuronal atrophy and synaptic deficits in the medial PFC ([3-5](#)). While intrinsic molecular mechanisms that contribute to neuronal atrophy have been studied ([32,33](#)), the roles of other cellular mediators in stress-induced synaptic deficits have not been extensively studied. Recent findings indicate that microglia support homeostatic neuronal function and modulate synaptic plasticity ([11,12,34](#)). Moreover, repeated stress exposure causes morphological and functional alterations in microglia ([4,9,10](#)), implicating microglia in the neurobiology of depressive-like behaviors. Here we show that CUS caused anxiety- and depressive-like behaviors in male and female mice, with changes more pronounced in male mice. These behavioral effects were associated with markers of dysregulated microglia function, including altered expression of neuron-derived factors that modulate microglia activation as well as morphological changes in microglia in the medial PFC.

One of the prominent neuron-derived factors increased in the medial PFC following CUS was CSF1. In addition, consistent with the murine CUS model, CSF1 mRNA levels were elevated in the postmortem dorsolateral PFC obtained from individuals with depression. CSF1 is a key neuron-derived signal that modulates microglia in physiological and pathological conditions ([15](#)). Indeed, studies show that dystrophic neuronal responses in models of kainic acid-induced excitotoxicity or sensory nerve injury increased CSF1 expression, leading to microglia activation ([18,19](#)). Consistent with these findings, CUS-induced CSF1 expression in the medial PFC corresponded with increased expression of immunomodulatory receptors, including CSF1 receptor (CSF1R), in enriched

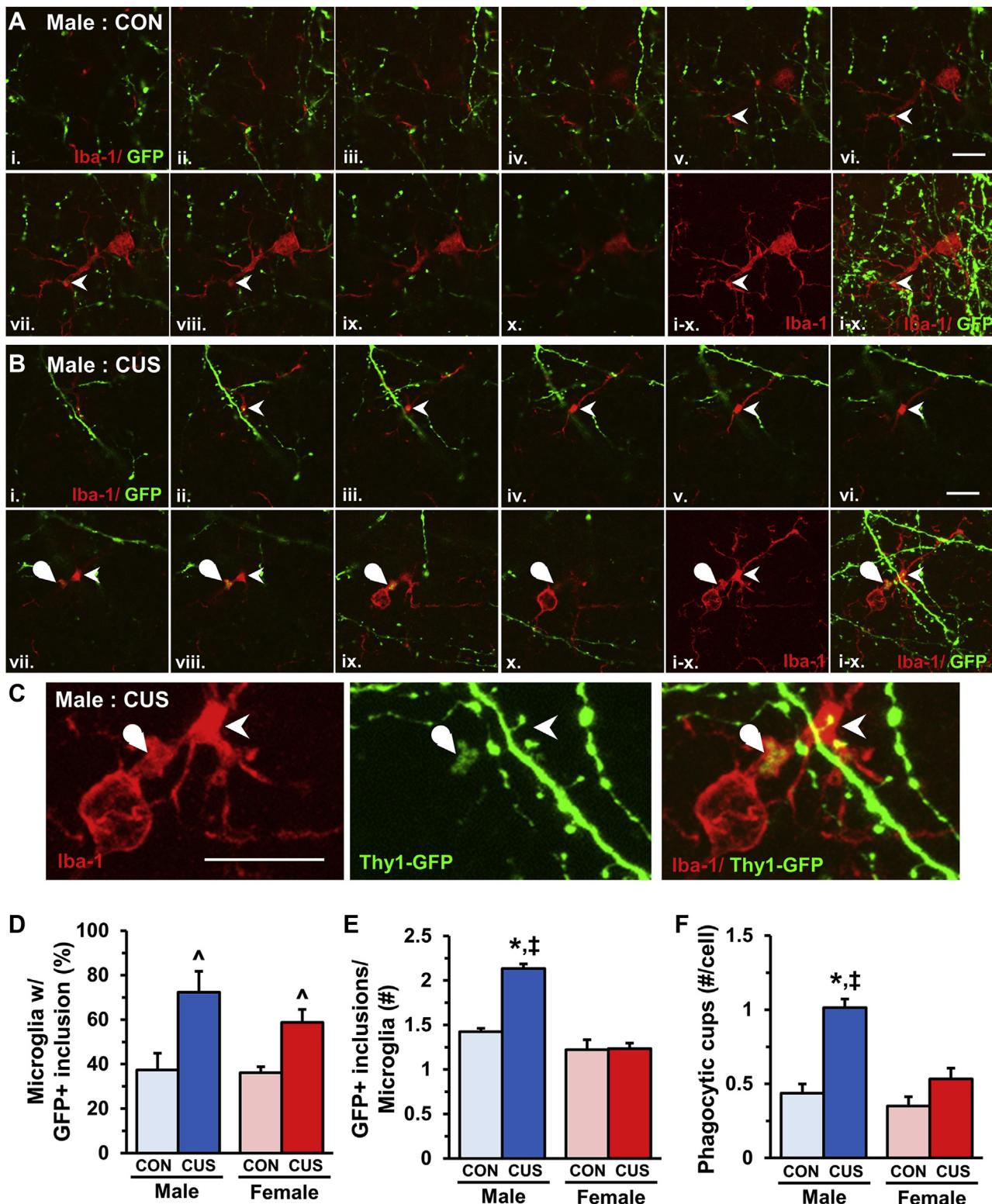


Figure 4. Chronic unpredictable stress (CUS) increased microglia-mediated neuronal remodeling in layer 1 of the medial prefrontal cortex (PFC). Male and female Thy1-GFP-M mice were exposed to 14 days of CUS, and 2 to 4 hours after the final stressor mice were perfused and brains were collected for immunohistology. **(A, B)** Representative sequence of confocal images (i-x) obtained from layer 1 of the medial PFC in control (CON) **(A)** and CUS **(B)** male Thy1-GFP-M mice. Arrowhead shows microglia process in close proximity to dendrite or dendritic spine, and curved arrow notes green fluorescent protein

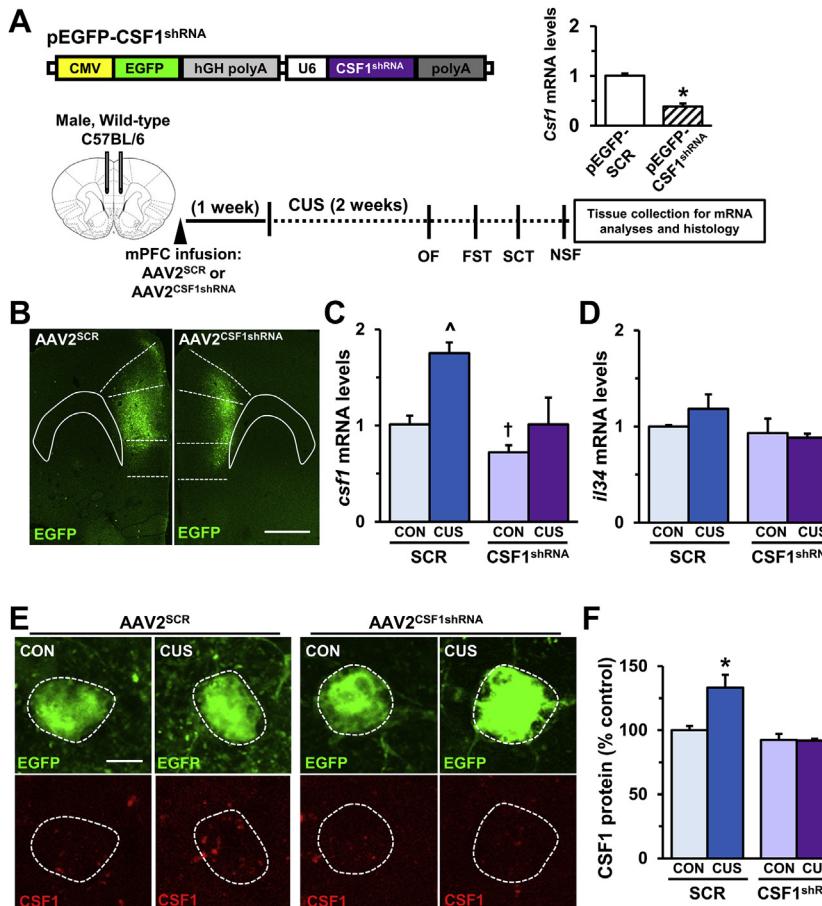


Figure 5. AAV2^{CSF1shRNA} attenuated stress-induced upregulation of neuronal colony-stimulating factor 1 (CSF1) in the medial prefrontal cortex (mPFC). **(A)** Schematic of plasmid design for pEGFP-CSF1^{shRNA}. Relative messenger RNA (mRNA) levels of *csf1* in N2a cells transfected with pEGFP-SCR or pEGFP-CSF1^{shRNA} are shown. Experimental timeline is shown for *in vivo* CSF1 knockout studies. Male wild-type mice received bilateral infusion of AAV2^{SCR} or AAV2^{CSF1shRNA}, and after recovery mice were subjected to 14 days of chronic unpredictable stress (CUS). **(B)** Representative images of AAV2^{SCR} and AAV2^{CSF1shRNA} viral infection in the medial PFC. White scale bar represents 500 μm. Following CUS and behavioral testing, the medial PFC was collected from a subset of mice for mRNA analyses. **(C, D)** Relative mRNA levels of *csf1* (**C**) and *i34* (**D**) are shown ($n = 4$). A subset of control (CON) and CUS male mice infused with AAV2^{SCR} or AAV2^{CSF1shRNA} were perfused and brains were collected for CSF1 immunohistology. **(E)** Representative images of enhanced green fluorescent protein (EGFP)+ neurons with CSF1 colabeling are shown for each experimental group. White scale bar represents 10 μm. **(F)** Quantification of average relative fluorescent intensity for CSF1 immunolabeling is represented as percentage AAV2^{SCR}:CON ($n = 4$). Bars represent the mean ± SEM. Means that are significantly different from the respective CON group based on analysis of variance are denoted (main effect: * $p < .05$ or † $p < .07$; interaction: * $p < .05$). AAV2, adeno-associated virus 2; CMV, cytomegalovirus promoter; FST, forced swim test; hGH poly A, human growth hormone poly A; NSF, novelty-suppressed feeding; OF, open field; SCR, scrambled control; SCT, sucrose consumption test; shRNA, short hairpin RNA; U6, shRNA promoter.

microglia. It is notable that different patterns of gene expression were observed between whole homogenates and enriched microglia in the frontal cortex. In particular, CSF1R and TGF β receptor (TGF β R) expression was decreased in the whole PFC, but expression of these receptors was increased in enriched microglia. These opposing expression patterns may be linked to the differential role of CSF1R and TGF β R in neurons as compared with microglia. For instance, CSF1R and TGF β R signaling in neurons mediates neurodevelopment and promotes neuroplasticity (35,36), while CSF1R signaling in microglia is essential for their viability (15) and TGF β R signaling promotes the unique molecular and functional profile of microglia (17,37). These divergent roles in neurons and microglia may underlie differential expression of CSF1R and TGF β R following stress. In addition, mRNA levels of interleukin-1 β and tumor necrosis factor α were significantly decreased in enriched microglia, and not whole PFC, because these cytokines are enriched in immune cells. Other studies using longer bouts of CUS increased interleukin-1 β and/or

tumor necrosis factor α levels in the hippocampus, which contributed to development of depressive-like behaviors (20,38). These contrasting results may reflect dynamic neuro-immune effects dependent on stress duration, which is consistent with other findings that show brain region-specific microglia responses that elicit divergent neurobiological effects (39–41). Together, the findings indicate that CUS-induced anxiety- and depressive-like behaviors are linked to neuron-derived factors that promote functional and molecular alterations in microglia.

The functional role of microglia has expanded, with seminal reports demonstrating that microglia sculpt neurocircuitry during neurodevelopment in an activity-dependent manner (42,43) and eliminate dystrophic synapses in disease models (44,45). Using Thy1-GFP-M mice, we show that microglia processes in unstressed mice are in close proximity to dendrites and axons in layer 1 of the medial PFC, and subsets of microglia (~40%) contained GFP+ inclusions. This is consistent with other studies showing that microglia regularly

(GFP)+ neuronal elements engulfed in microglia process. White scale bar represents 10 μm. **(C)** Enlarged and merged confocal stacks from panel B are shown. White scale bar represents 10 μm. **(D)** Proportion of microglia in layer 1 of medial PFC with GFP+ inclusions ($n = 18–22$ microglia/sample). **(E)** Average number of GFP+ inclusions in microglia with neuronal elements ($n = 2–20$ microglia/sample). **(F)** Average number of putative phagocytic cups per microglia in layer 1 of the medial PFC ($n = 12$ microglia/sample). Bars represent the mean ± SEM ($n = 4–6$ /group). Means that are significantly different from the respective CON group based on analysis of variance are denoted (main effect: * $p < .05$; interaction: * $p < .05$). Means significantly different from the respective female experimental group based on analysis of variance are noted by † $p < .05$.

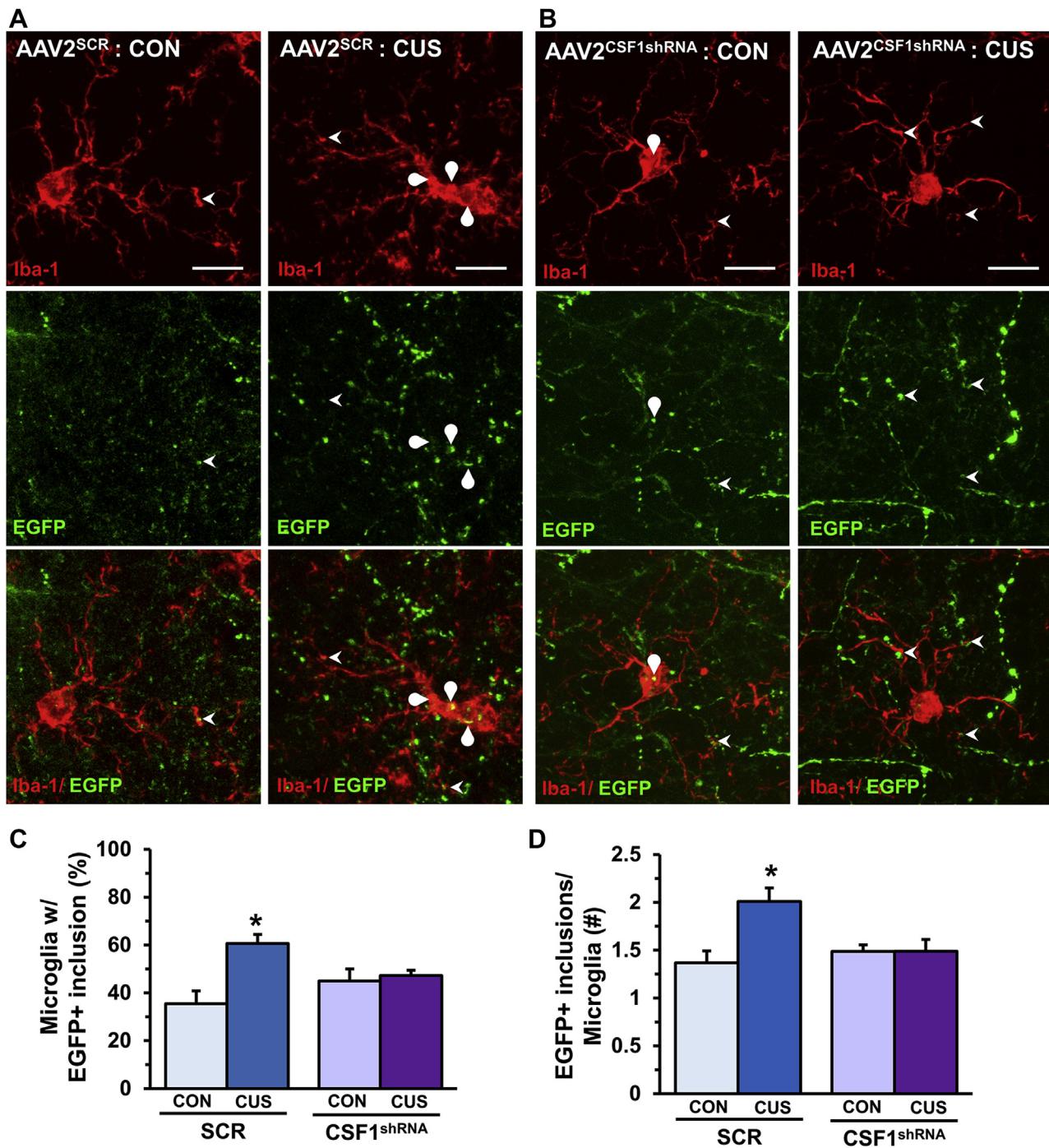


Figure 6. Knockdown of neuron-derived colony stimulating factor 1 (CSF1) blocked microglia-mediated dendritic remodeling in the medial prefrontal cortex (PFC) following chronic unpredictable stress (CUS). Male wild-type mice received bilateral infusion of AAV2^{SCR} or AAV2^{CSF1shRNA}, and after recovery mice were subjected to 14 days of CUS. Mice were perfused and brains were collected for immunohistology 2 to 4 hours after novelty-suppressed feeding. **(A, B)** Representative confocal images obtained from layer 1 of the medial prefrontal cortex (proximal to virus infection) in control (CON) or CUS male mice infused with AAV2^{SCR} (**A**) or AAV2^{CSF1shRNA} (**B**). Arrowhead shows microglia process interacting with dendrite or dendritic spine, and curved arrow notes EGFP+ neuronal elements engulfed in microglia process. White scale bar represents 10 μ m. **(C)** Proportion of microglia in layer 1 of the medial prefrontal cortex with EGFP+ inclusions ($n = 12\text{--}15$ microglia/sample). **(D)** Average number of EGFP+ inclusions in microglia with neuronal elements ($n = 3\text{--}10$ microglia/sample). Bars represent the mean \pm SEM ($n = 4$ /group). Means significantly different from the respective CON group based on analysis of variance are denoted (interaction: $*p < .05$). AAV2, adeno-associated virus 2; EGFP, enhanced green fluorescent protein; SCR, scrambled control; shRNA, short hairpin RNA.

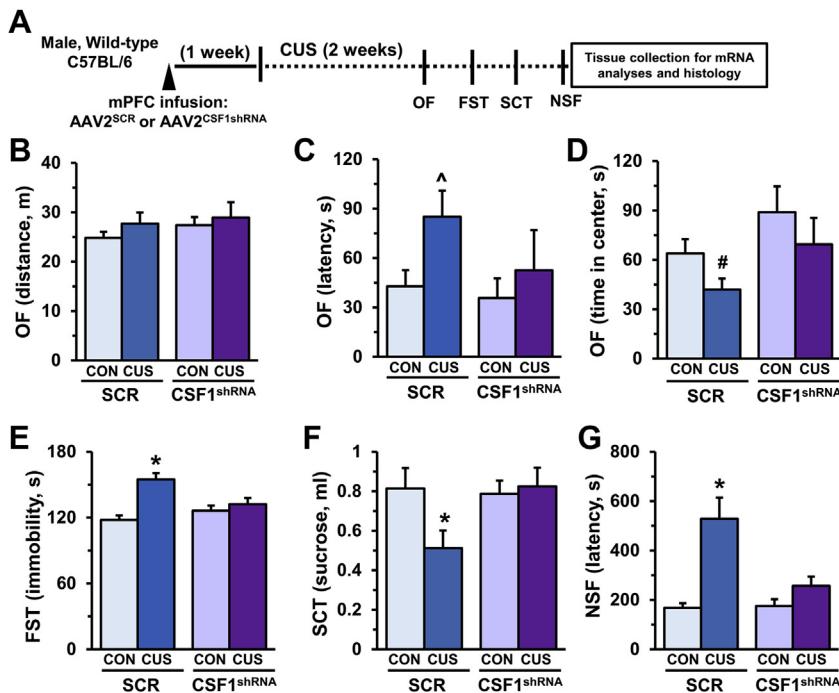


Figure 7. AAV2^{CSF1shRNA} prevented stress-induced anxiety- and depressive-like behaviors. Male wild-type mice received bilateral infusion of AAV2^{SCR} or AAV2^{CSF1shRNA}, and after recovery mice were subjected to 14 days of chronic unpredictable stress (CUS). On subsequent days, behavior was assessed in the open field (OF), forced swim test (FST), sucrose consumption test (SCT), and novelty-suppressed feeding (NSF) test. (A) Schematic showing experimental approach and timeline. (B–D) On the final day of CUS, activity in the OF test was assessed; total distance (B), latency to enter the OF (C), and time spent in the center of the OF (D) are shown. (E) The following day, immobility in the FST was measured; immobility in minutes 2 to 6 is shown. (F) On the subsequent day, the SCT was performed; total sucrose consumed is shown. (G) Last, the NSF test was administered; latency to feed is shown. Bars represent the mean \pm SEM. Means that are significantly different from the respective control (CON) group based on analysis of variance are denoted (main effect: $*p < .05$ or $#p = .08$; interaction: $*p < .05$). AAV2, adeno-associated virus 2; CSF1, colony stimulating factor 1; mPFC, medial prefrontal cortex; SCR, scrambled control; shRNA, short hairpin RNA.

sample synaptic and dendritic elements in the cortex (43). Further analyses revealed that CUS significantly increased the proportion of microglia in layer 1 of the medial PFC ($\sim 75\%$) that contained GFP+ inclusions. CUS also increased the frequency of microglial phagocytic cups in layer 1 of the medial PFC, which is pertinent because these structures are observed in other models of microglia-mediated neuronal remodeling (42,46,47). Further immunohistology showed that GFP+ inclusions were colocalized with CD68+ lysosomal markers within microglia, indicating that these neuronal elements had been internalized via phagocytosis. These functional changes in microglia coincided with decreased dendritic spine density on apical dendrites of pyramidal neurons in the medial PFC. Moreover, microglia (Iba-1+) in the medial PFC of control and CUS mice colocalized with the resident microglia-specific marker, P2Y12 receptor. These results indicate that stress-induced neuronal remodeling can be attributed to resident microglia rather than trafficking peripheral monocytes/macrophages. This is consistent with recent studies showing that trafficking peripheral monocytes/macrophages utilize neurovascular signaling to promote development of social defeat-induced anxiety-like behavior (48–50). Notably, the proportion of microglia with GFP+ inclusions in layer 5 of the medial PFC or layer 1 of the somatosensory cortex did not change following CUS. Altogether, these findings demonstrate that resident microglia mediate neuronal remodeling in a laminar- and region-specific manner, which likely contributes to selective synaptic deficits in layer 1 of the medial PFC following repeated stress (14,51). It is unclear how microglia target specific neuronal elements, and it is possible that GFP+ inclusions in microglia may comprise axonal and dendritic components (31). Further studies will need to use higher-resolution microscopy to assess spatial relationships

between microglia and neuronal elements and examine the specificity of microglia phagocytosis in the medial PFC.

An important finding in these studies was that microglia showed differential sex-dependent responses to CUS, which corresponded with susceptibility to anxiety- and depressive-like behaviors. Microglia from male mice had higher CSF1R expression that corresponded with increased phagocytosis of GFP+ neuronal elements and significant reductions in dendritic spine density following CUS. Because CSF1 was increased in both male and female mice, intrinsic molecular pathways (i.e., CSF1R) may underlie divergent microglia responses after CUS. Our findings are consistent with preclinical studies showing that female subjects are resilient to stress-induced neuronal impairments and behavioral or cognitive deficits (52–54), which may be linked to blunted microglia responses following stress (55). These findings contrast with clinical reports that show higher incidence of MDD in women than in men (56,57). Several factors may contribute to the increased risk of MDD in female individuals, including drastic fluctuations in sex hormones (e.g., during adolescence and pregnancy) (58,59) as well as differential neuroendocrine and immune reactivity to environmental and social stressors (60). In all, our findings suggest that female subjects demonstrate partial resiliency in this stress paradigm, and more pronounced changes in behavior and neuroimmune dysfunction may be unmasked with longer CUS exposure. Further studies into sex-dependent differences in microglia, including paradigms that model fluctuations in sex hormones, and subsequent response to stressors may provide insight into susceptibility for affective disorders.

Prior studies indicate that stress-induced neuroendocrine and neurotransmitter fluctuations mediate morphological and functional changes in microglia following stress exposure

(24,61,62). Here we provide initial evidence that local neuron-derived signals promote functional alterations in microglia, which, in turn, shape neuronal and behavioral responses to stress. Of note, knockdown of neuronal CSF1 attenuated microglia-mediated dendritic remodeling in layer 1 of the medial PFC and prevented development of anxiety- and depressive-like behaviors following CUS. Previous studies implicated microglia in the neurobiology of stress, with minocycline blocking stress-induced microglia activation and neuronal FosB in the medial PFC (63). Furthermore, recent work revealed that microglia phagocytosed neuronal elements in subregions of the hippocampus, which corresponded with synaptic plasticity deficits and anhedonia after CUS (31). Taken together, these findings indicate that microglia actively shape neuronal responses to stress, leading to dendritic remodeling and development of depressive-like behaviors.

In summary, our findings provide further evidence that brain-resident microglia are critical mediators of neuroplasticity and reveal a novel cellular pathway that contributes to the pathophysiology of stress-induced depressive-like behaviors. Moreover, chronic stress exposure elicited divergent neuro-immune responses in male and female mice, demonstrating sex-dependent differences in neuron–microglia interactions. Furthermore, microglia contributed to neuronal remodeling in layer 1, but not layer 5, of the medial PFC, indicating that neuron–microglia interactions are compartmentalized within specific brain regions. While microglia responses are typically directed to restore neuronal homeostasis (64,65), chronic stress exposure causes microglia-mediated neuronal remodeling that contributes to synaptic deficits and anxiety- and depressive-like behaviors. These findings indicate that interventions aimed at normalizing neuron–microglia interactions, such as CSF1 signaling, may provide therapeutic benefits in affective disorders.

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ESW and RSD designed research studies; ESW, RT, and CHD conducted experiments and acquired data; ESW and RSD provided resources and materials; and ESW, CHD, and RSD analyzed data and wrote the manuscript.

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