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Fetal CCL2 signaling mediates offspring social behavior and recapitulates effects of prenatal stress

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Abstract

Maternal stress during pregnancy is prevalent and associated with increased risk of neurodevelopmental disorders in the offspring. Maternal and offspring immune dysfunction has been implicated as a potential mechanism by which prenatal stress shapes offspring neurodevelopment; however, the impact of prenatal stress on the developing immune system has yet to be elucidated. Furthermore, there is evidence that the chemokine C-C motif chemokine ligand 2 (CCL2) plays a key role in mediating the behavioral sequelae of prenatal stress. Here, we use an established model of prenatal restraint stress in mice to investigate alterations in the fetal immune system, with a focus on CCL2. In the placenta, stress led to a reduction in CCL2 and *Ccr2* expression with a concomitant decrease in leukocyte number. However, the fetal liver exhibited an inflammatory phenotype, with upregulation of *Ccl2*, *Il6*, and *Lbp* expression, along with an increase in pro-inflammatory Ly6C^{Hi} monocytes. Prenatal stress also disrupted

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Conflict of Interest

The authors declare no competing financial interests.

chemokine signaling and increased the number of monocytes and microglia in the fetal brain. Furthermore, stress increased *Il1b* expression by fetal brain CD11b^{+} microglia and monocytes. Finally, intra-amniotic injections of recombinant mouse CCL2 partially recapitulated the social behavioral deficits in the adult offspring previously observed in the prenatal restraint stress model. Altogether, these data suggest that prenatal stress led to fetal inflammation, and that fetal CCL2 plays a role in shaping offspring social behavior.

Keywords

maternal stress; pregnancy; CCL2; microglia; social behavior

1.1 Introduction

Psychological stress during pregnancy is prevalent and is associated with adverse obstetrical outcomes, including preterm birth and low birth weight (Bussières et al., 2015; Dunkel Schetter and Glynn, 2011; Dunkel Schetter and Tanner, 2015). In addition to impacting the pregnancy, maternal stress has also been linked to increased risk for neurodevelopmental disorders in the offspring (Bale et al., 2010; Glover et al., 2018; Graignic-Philippe et al., 2014; Van den Bergh et al., 2017). Indeed, clinical studies have demonstrated correlations between stress experienced by mothers during pregnancy and autism spectrum disorder-related symptom severity in their children (Ronald et al., 2010; Varcin et al., 2017), while animal models have provided evidence that prenatal stress induces social behavioral deficits in the adult offspring (Chen et al., 2020; Gur et al., 2019; Weinstock, 2016). Although the mechanisms underlying the programming effects of gestational stress are still being defined, the immune system has been implicated as a key mediator (Chen and Gur, 2019; Hantsoo et al., 2019).

There is mounting evidence that maternal stress dysregulates the maternal immune system during pregnancy. Maternal immune function is carefully regulated throughout gestation; thus, disruptions to immune homeostasis and the pro- and anti-inflammatory balance can lead to adverse outcomes such as preterm birth (Cappelletti et al., 2016). In addition to disrupting maternal immune homeostasis, there is also evidence that maternal stress dysregulates the fetal immune system. Both clinical studies and animal studies have demonstrated associations between maternal stress and proinflammatory factors in the placenta (Bronson and Bale, 2014; Gur et al., 2017; Keenan-Devlin et al., 2017; Lian et al., 2017; Marinescu et al., 2014; Miller et al., 2017). Furthermore, maternal stress has also been associated with higher concentrations of the pro-inflammatory cytokines IL-1β and IL-6 in the cord blood (Andersson et al., 2016) as well as increased cytokine production by cord blood mononuclear cells when stimulated *ex vivo* (Wright et al., 2010). While there is mounting evidence that maternal stress impacts offspring immune function later in life (Merlot et al., 2008), the programming effects of stress on offspring peripheral immune function *in utero* is not well understood. To date, completed studies focus on the developing brain, as studies have shown that prenatal stress induces fetal neuroinflammation (Chen et al., 2020; Gur et al., 2019, 2017). There is evidence from studies of psychosocial stress in adults that stress induces peripheral inflammation that activates microglia, the resident

innate immune cell of the brain, to increase production of pro-inflammatory cytokines (Ménard et al., 2017). Although the fetal stress response and immune system is distinct in terms of function from that of adults (Levy, 2007; Sheng et al., 2021), we hypothesize that prenatal stress leads to fetal peripheral inflammation that then enhances neuroinflammation by activating microglia during embryonic development.

C-C motif chemokine ligand 2 (CCL2) is a chemokine that classically functions to recruit leukocytes, including monocytes and dendritic cells, to sites of inflammation (Deshmane et al., 2009). Previous work by our group has specifically implicated CCL2 in mediating sequelae of prenatal stress, including fetal neuroinflammation and adult offspring social behavioral deficits (Chen et al., 2020). However, the specific function of CCL2 in the context of prenatal stress has yet to be fully defined. Although chemotaxis is the most well-known function of CCL2, there is evidence that CCL2 has a broad range of additional effects on myeloid and lymphoid cells, including polarizing macrophages and T cells, promoting survival and proliferation of myeloid cells, and influencing secretion of effector molecules (Gschwandtner et al., 2019; Gu et al., 2000). During embryogenesis, CCL2 has been implicated in promoting the migration of neurons in the lateral hypothalamus in the context of maternal ethanol exposure (Chang et al., 2020) and in the recruitment, activation, and proliferation of immune cells to the fetal choroid plexus following maternal immune activation (Cui et al., 2020). Given the varied roles of CCL2, we aimed to determine the source and function of CCL2 in the context of maternal stress. Furthermore, given that the previous study demonstrated that prenatal stress failed to elicit social behavioral deficits in CCL2 global knockout ($CCL2^{-/-}$) adult offspring (Chen et al., 2020), here we asked the question if gestational CCL2 is sufficient to shape offspring behavior.

Using our well-established mouse model of prenatal restraint stress (Antonson et al., 2020; Chen et al., 2020; Galley et al., 2021; Gur et al., 2019, 2017), we demonstrate in this study that prenatal stress elicits fetal peripheral and central nervous system (CNS) inflammation. Furthermore, using intra-amniotic injections as a method of specifically influencing the fetal compartment during pregnancy, we show that fetal CCL2mediates some elements of offspring social behavior in adulthood, suggesting CCL2 plays a role in the transmission of maternal stress to the developing brain.

1.2 Materials and Methods

Detailed materials and methods can be found in Supplementary Information

1.2.1 Animals and Prenatal Stress

Pregnant C57BL/6 mice were maintained and bred as previously described (Antonson et al., 2020; Chen et al., 2020; Galley et al., 2021). Briefly, pregnant dams underwent restraint stress for 2 hours each day from E10.5 to E16.5 or were left undisturbed. All experiments were conducted according to the principles specified by the National Institutes of Health Guidelines for the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee at the Ohio State University.

1.2.2 Tissue Collection

For the time course study, maternal plasma and spleens, placentas, fetal brains, and fetal tails were collected from dams on E11.5, E13.5, E15.5, E17.5, or P0.5 following CO_2 asphyxiation. Fetal livers were collected from a second cohort on E17.5. Identification of the presence of testes or a uterine horn within the abdominal cavity was used to determine fetal sex, which was confirmed by genotyping of tails for the SRY gene. For fetal tissues, one male and one female sample were collected from each litter. For flow cytometry experiments, fetal livers and fetal brains were pooled per litter separately.

1.2.3 Genotyping

Briefly, genomic DNA was isolated from fetal tails using a lysis buffer and isopropanol. PCR was performed using the following primers: SRY forward (CCA CTC CTC TGT GAC ACT TTA GCC CTC CGA) and SRY reverse (TTG TCT AGA GAG CAT GGA GGG CCA TGT CAA). Gel electrophoresis was then performed and gels were imaged to determine absence or presence of the SRY gene in samples.

1.2.4 Microglia Isolation

Fetal brains were mechanically disrupted using a Potter-Elvehjem tissue grinder and myelin was removed using a 30% isotonic Percoll solution. $CD11b^+$ cells were isolated using CD11b Microbeads and MS columns, per manufacturer's instructions.

1.2.5 Quantitative Real-Time PCR

RNA was isolated from whole placental, fetal brain, and fetal liver tissues using Trizol reagent per the manufacturer's protocol. For microglia, RNA was extracted using the Direct-zol RNA Microprep Kit. cDNA was synthesized, PCR reactions were performed, and data was analyzed as previously described (Chen et al., 2020). The primers used are listed in Supplementary Table 1.

1.2.6 ELISA

Placentas, fetal brains, and fetal livers were homogenized by sonication as previously described (Chen et al., 2020). Amniotic fluid samples were centrifuged to remove cells and supernatant was collected for analysis. CCL2 was measured in tissue lysates, plasma samples, and amniotic fluid using the Mouse CCL2/JE/MCP-1 Duoset ELISA.

1.2.7 Flow Cytometry

For fetal livers and brains, samples were triturated and strained. Red blood cells were lysed, and cells were stained.

For the fetal livers, the following antibodies were used: purified CD16/CD32 Fc blocking antibody (BD Biosciences, San Jose, CA, 1 μL), FITC CD45 (BioLegend, San Diego, CA; 1.5 μL), PE Ly6C (BioLegend, San Diego, CA; 1 μL), PerCP-Cy5.5 Ly6G (BD Bioscience, San Jose, CA; 1 μL), APC F4/80 (BioLegend, San Diego, CA; 1 μL), AF700 CD11b (BioLegend, San Diego, CA; 2 μL), and LIVE/DEAD Fixable Near-IR Dead Cell Stain (Invitrogen, Carlsbad, CA; 0.5 μL). Cells were fixed in 10% neutral buffered formalin. Cells

were then resuspended in FACS buffer and 123 count eBeads (Invitrogen, Waltham, MA) were added to each sample. A LIVE/DEAD compensation control and an unstained control was prepared using pooled cells from the fetal liver samples.

For the fetal brains, the following antibodies were used: purified CD16/CD32 Fc blocking antibody (BD Biosciences, San Jose, CA; 1 μL), V450 CD45 (BD Biosciences, San Jose, CA; 1 μL), FITC Ly6C (BioLegend, San Diego, CA; 1 μL), PE CCR2 (R&D Systems, Minneapolis, MN; 10 μL), APC F4/80 (BioLegend, San Diego, CA; 1 μL), AF700 CD11b (BioLegend, San Diego, CA; 2 μL), and LIVE/DEAD Fixable Near-IR Dead Cell Stain (Invitrogen, Carlsbad, CA; 0.5 μL). Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA) for, and intracellular staining was performed with PerCP Cy5.5 CD206 (BioLegend, San Diego, CA; 1.5 μL. Cells were then resuspended in FACS buffer and 123 count eBeads (Invitrogen, Waltham, MA) were added to each sample. A LIVE/DEAD compensation control, an unstained control, and an FMO control for the intracellular stain was prepared using pooled cells from the fetal brain samples.

Flow cytometry was run, and data was analyzed as described in the supplemental materials.

1.2.8 Immunohistochemistry

One cohort of dams were euthanized using CO_2 and perfused transcardially with PBS, followed by 4% paraformaldehyde. Placentas were post-fixed in 4% paraformaldehyde, and then processed for CD45 immunofluorescence staining and imaging. Antibodies used for immunofluorescence staining include rat anti-mouse CD45 primary antibody (1:300; Bio-Rad, Hercules, CA; Catalog # MCA1388) and Alexa Fluor 488 donkey anti-rat secondary antibody (1:1000; Abcam, Cambridge, MA; Catalog # R37119).

1.2.9 Intra-amniotic Injections

Pregnant dams were anesthetized on E16.5 and mini-laparotomies were performed to expose the gravid uterine horns. Recombinant mouse CCL2 or a saline control was injected into the amniotic sac of each fetus. One cohort of animals were euthanized on E17.5 for tissue collection. A second cohort of animals underwent parturition and offspring underwent behavioral testing at 10 weeks of age.

1.2.10 Behavior

Sociability was assessed using the three-chamber social behavior paradigm, with social approach index calculated as previously described (Chen et al., 2020; Gur et al., 2019). Anxiety-like behavior was assessed using the light-dark preference test (Chen et al., 2020) and the open field test. Tests were performed on separate days, with a day without testing between two tests to minimize confounding effects of stress. The open field test was performed first, followed by a rest day, then the three chamber social behavior test, followed by a rest day, and finally the light dark test. The number of litters used for each experiment are listed in Supplementary Table 2. Of note, the same animals/litters were assessed for anxiety-like behaviors and social behavior; the discrepancy in numbers is due to an issue

with the video recording software for the social behavioral paradigm for the second cohort of animals.

1.2.11 Statistical Analysis

Statistical tests were performed using Graphpad Prism 9. For all data, multi-factorial analysis of variance (ANOVA) tests followed by Tukey's post hoc tests corrected for multiple comparisons were performed to determine main effects and interaction effects of sex. For data in which there was no significant effect of sex, data was averaged by litter to account for litter effects, and data was analyzed using two-tailed unpaired *t*-test.

1.3 Results

1.3.1 Prenatal stress restricts maternal gestational weight gain and placental mass

We have previously demonstrated that prenatal restraint stress restricts maternal body mass gained during pregnancy, as measured on embryonic day (E)16.5, (Antonson et al., 2020). To further understand how stress impacts the fetal compartment, pregnant dams underwent restraint stress from E10.5 to E16.5 for 2 hours each day, and tissues were collected on E11.5, E13.5, E15.5, and E17.5 (Fig 1a).

Replicating our previous findings, restraint stress reduced maternal body mass over the course of the stressor exposure (Fig 1b, $p=0.0015$). This decrease in maternal body mass was not due to pre-existing differences in maternal body mass prior to breeding or prior to stress, differences in litter size or resorptions, or alterations in food consumption (Supp Fig 1a–e). While placental mass increased over gestation, as expected, stress reduced placental mass in addition to restricting maternal body mass gain (Fig 1c, embryonic day: $p<0.0001$; stress: $p=0.021$).

Spleen mass was also determined over the course of gestation and the stressor, intended as a systemic measure of inflammation. In accordance with our previous findings, there was a main effect of stress in reducing spleen mass (Supp Fig 2a, $p=0.042$). When normalized to individual dam body mass, there was no difference between the control and stress conditions (Supp Fig 2b, $p=0.541$), suggesting that the reduction in splenic mass is likely due to maternal weight restriction. However, spleen mass (Supp Fig 2a, $p<0.0001$; Supp Fig 2b, $p<0.0001$) and the concentration of the chemokine CCL2 in circulation (Supp Fig 2c, $p=0.007$) decreased independent of stress as the pregnancy progressed, reflecting the anti-inflammatory course of late gestation.

1.3.2 The placenta does not increase production of CCL2 following prenatal stress

Based on our previous study demonstrating that CCL2 mediates prenatal stress-induced neuroinflammation and offspring social deficits (Chen et al., 2020), we next aimed to investigate the role of the placenta in producing or responding to CCL2 following stress. For all analyses in offspring tissue, main effects and interaction effects of sex were assessed; in the absence of sex effects, data were averaged by litter to account for litter effects.

At the E17.5 timepoint, there was a trending decrease in CCL2 protein levels in placental lysates with stress compared to controls (Fig 2a, $p=0.081$) and no change in placental *Ccl2*

gene expression (Fig 2b, p=0.600) due to stress. In terms of CCL2 signaling, expression of *Ccr2*, the primary receptor for CCL2, was reduced by stress exposure in the placenta (Fig 2c, p=0.018). Since CCL2 classically functions to recruit immune cells, primarily monocytes, to sites of inflammation or injury (Deshmane et al., 2009), we then assessed the density of total immune cells in the placenta by quantifying $CD45^+$ cells by immunohistochemistry. In accordance with the decrease in *Ccr2* expression, stress decreased the density of placental $CD45^+$ leukocytes (Fig 2d, quantified in Fig 2e, p=0.032). This suggests that stress reduces recruitment of immune cells to the placenta.

1.3.3 Prenatal stress increases Ccl2 expression in the fetal liver and induces inflammation

To further examine the impact of stress on intrauterine immune function, we interrogated expression of *Ccl2* in the fetal liver on E17.5 following exposure to stress, as the liver serves as the primary site of hematopoiesis during embryonic development from E12.5 to parturition (Mikkola and Orkin, 2006; Zon, 1995). Stress increased expression of *Ccl2* in the fetal liver (Fig 3a, p=0.050) without impacting *Ccr2* expression (Fig 3c, p=0.970). However, CCL2 protein was not impacted by stress (Fig 3b, p = 0.506).

Given that stress increased *Ccl2* in the fetal liver and our previous findings that gestational stress induced fetal neuroinflammation, we hypothesized that, in addition to the neuroinflammation, stress increases peripheral inflammation, and thus expression of pro-inflammatory cytokines *Il6* and *Tnf* in the fetal liver. Prenatal stress increased expression of *Il6* (Fig 3d, p=0.019), but not *Tnf* (Fig 3e, p=0.913). Additionally, we examined expression of the gene encoding for lipopolysaccharide-binding protein, *Lbp*, which is an acute phase protein produced by the liver in response to inflammation and found that stress increased fetal liver *Lbp* (Fig 3f, p=0.0098). Altogether, these data suggest that prenatal stress induces liver, and thus peripheral, inflammation in the fetus. In accordance with these findings, there was an increase in classical pro-inflammatory $Ly6C^{Hi}$ monocytes in the fetal liver (Fig 3j, p=0.017) and a decrease in alternative $Ly6C^{Lo}$ monocytes (Fig 3l, p = 0.029) with no differences in transitioning $Ly6C^{Int}$ monocytes (Fig 3k, p=0.284) or in the overall $Ly6G^{Lo}SSC^{Lo}$ monocyte population (Fig 3i, p = 0.515), $Ly6G^{Hi}$ neutrophils (Fig 3h, p=0.187), or $CD11b^+F4/80^+$ macrophages (Fig 3g, p=0.284). Gating strategy for the fetal liver flow cytometry experiments is depicted in Supp Fig 3a, with representative images for control and stress liver monocytes depicted in Supp Fig 3a–b.

1.3.4 Prenatal stress enhances chemokine signaling and myeloid cell recruitment in the developing brain

To determine the role of CCL2 in the developing brain following exposure to stress, we first extended our findings by showing that stress increased *Ccl2* gene expression (Fig 4a, p=0.007) and CCL2 protein (Fig 4b, p=0.006) in the brain on the first postnatal day. However, *Ccr2* expression was not impacted (Fig 4c, p = 0.633). In addition to CCL2, we also assessed expression of *Cx3cl1*, or fractalkine, which plays a role in modulating activation of microglia through the receptor *Cx3cr1*. While *Cx3cl1* expression was not altered by stress (Fig 4d, p=0.376), there was a trending increase in *Cx3cr1* expression (Fig 4e, p = 0.065), potentially indicating increased microglia within the developing brain

(Wolf et al., 2013). Despite the disruption in chemokine signaling, there was no difference in expression of *Tnf* at P0.5 (Fig 5f, p=0.522).

We next aimed to assess the function of stress-induced elevations in CCL2 in the developing brain. Since we have observed increased CCL2 protein and *Ccl2* expression as early as E17.5(Chen et al., 2020), we assessed number and relative composition of monocytes and microglia within the fetal brain at E17.5 by flow cytometry. Consistent with the increase in CCL2 in the brain, we observed a trending increase in the number of $CD11b^{+}CD45^{Hi}$ monocytes due to stress (Fig 4g, p=0.06). Furthermore, consistent with the increase in *Cx3cr1* expression, stress increased the number of $CD11b^{+}CD45^{Int}$ microglia in the brain (Fig 4h, p=0.040), though the relative composition of monocytes and microglia was not altered by stress (Fig 4i–j, $CD11b^{+}CD45^{Hi}$ monocytes: p=0.413; $CD11b^{+}CD45^{Int}$ microglia: p=0.447). Gating strategy for the fetal brain flow cytometry experiments is depicted in Supp Fig 3c.

As CCL2 has been implicated in activation of myeloid cells in addition to recruitment, we isolated $CD11b^{+}$ microglia and monocytes from the fetal brain at E17.5 using a magnetic bead separation protocol and isolated RNA to assess expression of the pro-inflammatory cytokine *Il1b*. Stress increased expression of *Il1b* in $CD11b^{+}$ microglia and monocytes (Fig 4k, p=0.042).

1.3.5 Intra-amniotic injections of recombinant CCL2 specifically increases CCL2 protein in the fetal compartment

Based on our observations that CCL2 was increased specifically in the fetal compartment following prenatal stress, we aimed to determine whether increasing CCL2 protein in the fetal compartment is sufficient to induce behavioral alterations in the offspring. Mini-laparotomies were performed on pregnant dams on E16.5 and injections of mouse recombinant CCL2 protein or a saline control were performed into the amniotic sac (Fig 5a). The dose of CCL2 was chosen to induce a comparable increase in fetal brain CCL2 levels as we have previously shown with our restraint stress model (Chen et al., 2020). To validate our paradigm, tissues were collected on E17.5, 24 hours following the intra-amniotic injection, and assessed for CCL2 protein by ELISA. As expected, CCL2 protein was increased in the amniotic fluid (Fig 5b, p=0.0005), but not the maternal plasma (Fig 5c, p=0.891). Interestingly, placental CCL2 was not altered (Fig 5d, p=0.540), though CCL2 was increased in the fetal plasma (Fig 5e, p=0.0006), the fetal liver (Fig 5f, p = 0.015), and the fetal brain (Fig 5g, p=0.018). Altogether, these data indicate that our intra-amniotic injection paradigm specifically elevates CCL2 protein concentration within the fetal, but not maternal, compartment at this specific timepoint.

Additionally, we confirmed that the intra-amniotic injections of CCL2 did not overtly impact obstetrical outcomes. There were no differences in maternal body mass prior to breeding, prior to the injections, or 24 hours following the procedure (Supp Fig 4a–c). Furthermore, injections of CCL2 did not alter litter size, gestational length, or maternal spleen mass (Supp Fig 4d–f).

1.3.6 Intra-amniotic injections of recombinant CCL2 induces social behavioral deficits in adult female offspring without impacting anxiety-like behaviors

To determine the effect of increasing CCL2 in the fetal compartment on offspring behavior, we performed intra-amniotic injections of recombinant mouse CCL2 or a saline control on E16.5, allowed the dams to undergo parturition, and assessed behavior in the adult offspring at 10 weeks of age. The open field test and the light dark test were used to assess anxiety-like behavior, as prenatal stress has previously been shown to have anxiogenic effects on the adult offspring (Gur et al., 2017; Weinstock, 2016). However, CCL2 administration did not alter time spent in the center (Fig 6a, CCL2: p=0.814; sex: p=0.777; interaction: p=0.564), latency to enter the center (Fig 6b, CCL2: p=0.514; sex: p=0.286; interaction: p=0.189), or distance traveled (Fig 6c, CCL2: p=0.392; interaction: p=0.530) in the open field test, indicating that intrauterine CCL2 does not induce anxiety-like behavior. This was further supported by observations that CCL2 did not alter time spent in the light (Fig 6d, CCL2: p=0.995; sex: p=0.844; interaction: p=0.867) or latency to enter the dark (Fig 6e, CCL2: p=0.406; interaction: p=0.987) in the light dark test, though male offspring had a higher latency compared to females (Fig 6e; sex: p=0.045).

To assess social behavior, we used the three chamber social approach paradigm as we have previously demonstrated that prenatal stress decreases social approach behavior in the adult offspring in a CCL2-dependent manner using this paradigm (Chen et al., 2020). The total duration of time spent in the social side of the behavioral apparatus was similar for the offspring exposed to CCL2 *in utero* compared to saline, for both female and male offspring (Fig 6f, CCL2: p=0.894; sex: p=0.059; interaction: p=0.976). Additionally, there was no difference in social approach index due to CCL2, though there was a main effect of sex with a decreased social approach index in male offspring compared to female offspring (Fig 6g, CCL2: p=0.572; sex: p=0.014; interaction: p=0.282). However, while female offspring exposed to saline spent more time in the social chamber compared to the chamber with the object (Fig 6h, p=0.00029), the female offspring exposed to CCL2 did not exhibit this same preference (Fig 6h, p=0.134), suggesting that CCL2 reduced sociability in female offspring. Neither the male offspring exposed to saline or CCL2 exhibited a preference for the social chamber (Fig 6i, saline: p=0.682; CCL2: p=0.834). Importantly, the alterations observed with the female offspring was not due to hyperactivity as CCL2 treatment did not impact distance traveled during the sociability test (Fig 6j, CCL2: p=0.342; interaction: p=0.141), though male offspring were more active compared to females (Fig 6j, sex: p=0.027), similar to what was observed in the open field test (Fig 3c, sex: p=0.020). Together, these data indicate that exposure to increased levels of CCL2 specifically in the fetal compartment induces a female-specific deficit in sociability, without impacting anxiety-like behaviors.

1.4 Discussion

Stress during pregnancy is associated with neurodevelopmental and behavioral deficits in the offspring (Bale et al., 2010; Glover et al., 2018; Graignic-Philippe et al., 2014; Van den Bergh et al., 2017). While the mechanisms underlying the fetal programming effects of stress are still being elucidated, the immune system has been increasingly implicated as a key mediator (Chen and Gur, 2019; Hantsoo et al., 2019). Building on prior studies by our

group that prenatal restraint stress induces neuroinflammation and social behavioral deficits in the adult offspring in a CCL2-dependent manner (Chen et al., 2020), we hypothesized that stress induces maternal peripheral inflammation that signals through the placenta to cause fetal neuroinflammation and monocyte recruitment to the brain, ultimately shaping offspring social behavior. However, here, we demonstrate that increasing CCL2 specifically in the fetal compartment partially recapitulates the social behavioral deficits induced by prenatal stress. This is the first time, to our knowledge that fetal CCL2 signaling has been shown to alter offspring behavior.

Similar to our previous study (Antonson et al., 2020), we show in this study that restraint stress reduces maternal body mass and is observed as early as E11.5. This is also consistent with studies using restraint stress in pregnant rats, revealing reductions in body mass (Baker et al., 2008; Fujita et al., 2010; Van den Hove et al., 2014). Furthermore, we extended the findings, showing that restraint stress reduces placental mass. We confirmed that the reduction in body mass was not due to differences in body mass prior to breeding or initiating stress, food consumption, or differences in litter size – indeed, the reduction was observed despite an *increased* litter size in the stress group. Instead, it is possible that this phenotype is due to glucocorticoid signaling since administration of corticosterone to pregnant mice has been shown to reduce both maternal body mass and placental mass (Vaughan et al., 2012) and we previously demonstrated that restraint stress increases maternal circulating corticosterone (Antonson et al., 2020).

We next turned our attention to the fetal compartment, as clinical and pre-clinical studies indicate that prenatal stress induces inflammation in the intrauterine environment (Bronson and Bale, 2014; Chen et al., 2020; Gur et al., 2017; Hantsoo et al., 2019; Lian et al., 2017; Miller et al., 2017). The placenta has been implicated in mediating the programming effects of stress by integrating signals from the maternal circulation and initiating a response in the fetal circulation (Bronson and Bale, 2016) and is capable of producing cytokines and chemokines, including CCL2 (Deshmane et al., 2009; Khyzha et al., 2019; Renaud et al., 2009; Shirasuna et al., 2016). Maternal restraint stress did not impact CCL2 protein or *Ccl2* gene expression in the placenta. However, expression of *Ccr2*, which is the primary receptor for CCL2, was decreased by stress exposure. The primary function of CCL2, as a chemokine, is to recruit circulating $CCR2^+$ monocytes to infiltrate sites of active inflammation and mature into tissue macrophages (Deshmane et al., 2009); thus, we examined the density of $CD45^+$ leukocytes in placental tissue using immunohistochemistry. Consistent with the decrease in *Ccr2* expression, stress decreased the number of $CD45^+$ cells per mm^2 of placental tissue, which is similar to previous findings from our lab showing decreased mononuclear cells in the placenta by flow cytometry with stress (Antonson et al., 2020). This may suggest that the placenta is unable to maintain immunological homeostasis in the context of the maternal stressor, though additional methods of assessing placental immune homeostasis are warranted given the limited analysis in this study.

Based on the evidence of disruption of immune homeostasis in the placenta, we next aimed to examine immune function in the fetal compartment, hypothesizing that the fetal liver could be affected as it is the primary site of hematopoiesis from E12.5 until birth (Mikkola and Orkin, 2006; Zon, 1995). Indeed, we observed increased *Ccl2* expression in the fetal

liver following prenatal restraint stress. However, the lack of change in CCL2 protein, *Ccr2* expression, and percent of monocytes in the fetal liver suggests that the increase in *Ccl2* does not serve a chemotactic function in the liver. Although CCL2 has classically been known for its role in monocyte chemotaxis, there is evidence that CCL2 can also influence cytokine production, prime cells to respond to subsequent immune activation or infection, and increase survival and proliferation (Gschwandtner et al., 2019). Given this, in combination with previous observations by our group that restraint stress during pregnancy induces neuroinflammation (Chen et al., 2020), we examined inflammatory cytokine expression in the fetal liver, observing an increase in *Il6* expression, which was similar to our previous finding of increased fetal brain *Il6* with stress (Chen et al., 2020). However, unlike the brain (Chen et al., 2020), *Tnf* was not elevated in the fetal liver. In accordance with these findings, there was an increase in the proportion of proinflammatory Ly6C^{Hi} monocytes (Kratofil et al., 2017), and a concomitant decrease in the patrolling Ly6C^{Lo} monocyte population (Kratofil et al., 2017). As previous studies have shown that monocyte egress from the fetal liver is not dependent on CCL2-CCR2 mechanisms (Hoeffel et al., 2015), it is possible that the *Ccl2* upregulation by the liver is activating the monocytes and increasing pro-inflammatory cytokine expression instead of increasing monocyte recruitment to or egress from the liver. Despite a lack of difference in the macrophage population in the liver following exposure to stress, examination of the macrophages is limited in this study and does not exclude functional differences in liver macrophages. In addition to this proinflammatory profile in the liver, there was also an increase in expression of *Lbp*, or lipopolysaccharide-binding protein, which is an acute-phase protein involved in enhancing innate immune function and is typically induced by cytokines or lipopolysaccharide from gram-negative bacteria (Zweigner et al., 2006). Although it is possible that *Lbp* was upregulated in response the presence of an infectious bacterial agent in the intrauterine environment, the presence of microbes or microbial-related metabolites in the fetal compartment was not investigated in this study and warrants additional research as a potential mechanism.

In addition to fetal systemic inflammation, we observed that prenatal stress induces neuroinflammation that lasted beyond the duration of the stressor. Similar to the liver and to our previously published results (Chen et al., 2020), stress increased *Ccl2* expression and CCL2 protein without impacting *Ccr2* expression, suggesting that CCL2 may serve a function distinct from monocyte recruitment. Indeed, there is evidence that CCR2 is not required for the recruitment of fetal monocytes into tissues (Hoeffel et al., 2015), though CCR2^{+} cells are present on the pial surface of the hypothalamus during embryonic development (Rosin et al., 2021). However, fetal monocytes have a higher proliferative capacity compared to adult monocytes (Hoeffel et al., 2015), and CCL2 has been shown to enhance proliferation of macrophages (Amano et al., 2014) and microglia (Hinojosa et al., 2011), which could explain the increase in the number of monocytes and microglia in the fetal brain observed in this study. These findings are in agreement with another study showing that prenatal restraint stress increases multivacuolated microglia in the fetus by immunohistochemistry (Gumusoglu et al., 2017). The increased expression of *Il1b* by embryonic microglia and monocytes following stress exposure is in line with other studies demonstrating that prenatal stress leads to neuroinflammation in the embryonic brain (Chen

et al., 2020; Gur et al., 2017) and that maternal stress impacts embryonic microglial populations (Rosin et al., 2021). However, additional work is warranted using techniques with more cell-specific precision to disentangle the effects of prenatal stress on embryonic microglia and monocytes.

In addition to disrupting neuroimmune function *in utero*, there is evidence that prenatal stress induces social behavioral deficits in the adult offspring in a CCL2-dependent manner (Chen et al., 2020). Here, we aimed to build upon those findings and to determine whether CCL2 induces social behavioral deficits. Furthermore, as *Ccl2* gene expression and CCL2 protein was specifically increased in the fetal compartment, we performed injections of recombinant mouse CCL2 into the amniotic sac of fetuses during embryonic development. We validated the paradigm by confirming that injection of CCL2 into the amniotic fluid increased CCL2 concentrations in the amniotic fluid, fetal plasma, fetal liver, and fetal brain, but not the maternal plasma or placenta 24 hours after the procedure, demonstrating that we are able to specifically manipulate protein concentrations in the fetal, but not maternal compartment at this timepoint. Furthermore, we demonstrated that these injections did not overtly impact obstetrical outcomes. Using this model, we demonstrated that fetal exposure to CCL2 induces social behavioral deficits in female offspring, but not male. In contrast to our prior study (Chen et al., 2020), social approach index was not altered by CCL2; however, there was a significant difference in duration spent in the chamber with the social conspecific compared to the chamber with the object in the female offspring exposed to saline *in utero*, but not the female offspring exposed to CCL2. Together, this suggests that CCL2 may mediate some aspects of social behavior in female offspring. Additionally, these deficits are specific to social behavior as anxiety-like behavior was not impacted in the open field test or the light dark test. This is also in contrast to our prior study (Chen et al., 2020), which showed that prenatal stress reduced the latency to enter the dark chamber in the light dark test, which was ameliorated in CCL2 knockout. The lack of impact on anxiety-like behavior in this study suggests that, if CCL2 is critical for shaping offspring anxiety-like behavior, it may play a role at a different timepoint. Although other studies have found that CCL2 signaling is necessary for the recruitment of monocytes to the brain and inducing anxiety-like behavior following psychosocial stress in adulthood (Mckim et al., 2018; Miller and Raison, 2016), and that maternal immune activation leads to social behavioral deficits in the offspring that is reversed with administration of a CCL2-neutralizing antibody (Maldonado-Ruiz et al., 2021) this is the first time, to our knowledge, that prenatal exposure to CCL2 has been shown to disrupt offspring behavior in any capacity. However, given the limited behavioral testing in our study, further behavioral analysis is warranted to fully elucidate how prenatal CCL2 exposure impacts offspring behavior and neurodevelopment.

Interestingly, this effect was only observed in female offspring, though this is in part because the saline-treated male offspring did not display a preference for the social side. This is consistent with our prior study showing a stronger effect of prenatal stress in perturbing social behavior in female offspring (Chen et al., 2020). Notably, sex differences were only observed postnatally. All of the prenatal outcomes were examined for main effects and interaction effects of sex; however, as these effects were not significant, the data depicted are averages of one male and one female sample per litter. The implications of these findings are two-fold. First, the lack of sex differences prenatally is in contrast to existing

literature. For example, Bronson and Bale showed that prenatal stress induces male-specific placental inflammation, though this study utilized a chronic variable stress model during early gestation (E1-E7) (Bronson and Bale, 2014). In another study, prenatal restraint stress in rats was shown to increase aromatase activity in the hypothalamus and amygdala in male, but not female, fetuses (Weisz et al., 1982). In a third, prenatal restraint stress in rats decreased BDNF in the neonatal brain (Van den Hove et al., 2006). However, few studies have specifically examined sex differences in fetal immune function following exposure to prenatal restraint stress during late gestation. The fact that sex differences manifest in behavior without differences noted prenatally could indicate a contribution of postnatal factors. For example, colonization of male and female offspring by different maternal commensal microbes could play a role, as prenatal stress has been shown to differentially alter the fecal microbiome of male and female offspring in adulthood (Gur et al., 2019, 2017). It is also possible that a sex difference exists in fetal immune function that was not examined in this study. However, additional work is warranted to further tease apart the mechanisms underlying this sex difference.

In summary, we demonstrate here that prenatal stress induces fetal peripheral and central inflammation. Furthermore, we provide additional evidence of the role of fetal CCL2 in shaping offspring behavior. Additional research is necessary to identify the signals that directly induce fetal inflammation, as a maternal factor is likely shaping fetal physiology. However, our data is further evidence that the sequelae of maternal stress are evident even at embryonic timepoints, and indicate that fetal CCL2 may be an enticing target for developing novel treatments for preventing the negative outcomes associated with prenatal stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Maternal restraint stress reduces placental mass.
- Despite decreasing the number of leukocytes in the placenta, maternal restraint stress increased the number of fetal brain microglia and monocytes.
- Maternal restraint stress caused fetal liver inflammation.
- Intra-amniotic injections of CCL2 recapitulated deficits in social behavior in female offspring.

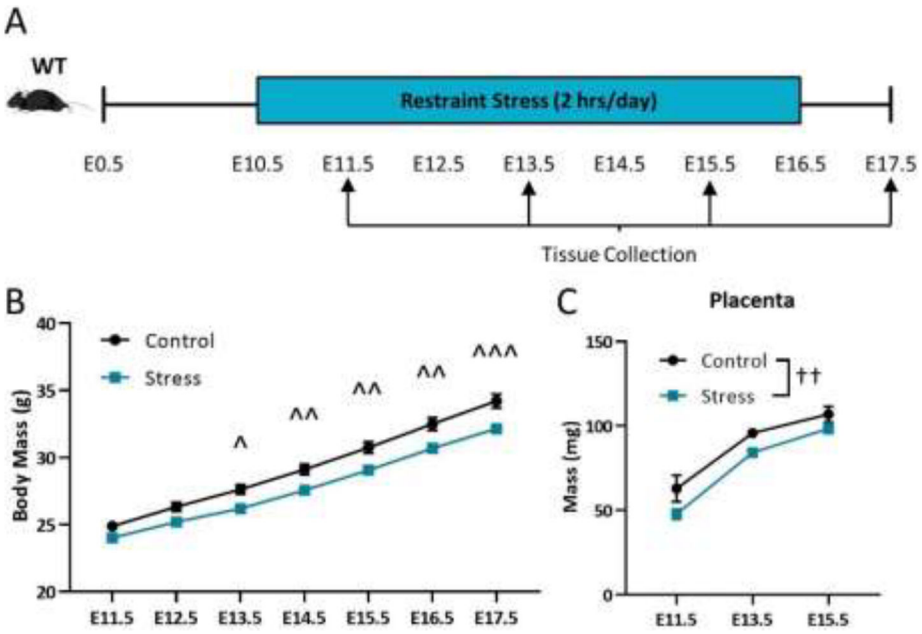

Figure 1: Prenatal stress restricts maternal weight gain and placental mass.

A. Experimental Design: C57BL/6 pregnant mice underwent restraint stress for 2 hours each day from E10.5 to E16.5. Tissues were collected on E11.5, E13.5, E15.5, and E17.5. Restraint stress reduced **B.** body mass gained during gestation (main effect of embryonic day: $f(6,426) = 2101$, $p < 0.0001$; main effect of stress: $f(1,71) = 10.97$, $p = 0.0015$; interaction: $f(6,426) = 8.596$, $p < 0.0001$ with significant differences between control and stress at the different timepoints indicated with carats) and **C.** placental mass (main effect of embryonic day: $f(2,28) = 67.54$, $p < 0.0001$; main effect of stress: $f(1,28) = 12.15$; $p = 0.0016$; no interaction: $f(2,28) = 0.3271$, $p = 0.7237$. Bars represent mean ± SEM. Two-Way ANOVA or Repeated Measures ANOVA: † indicates main effect of stress (†† $p < 0.01$); ^ indicates significant interaction followed by Tukey's post hoc test (^ $p < 0.05$; ^^ $p < 0.01$; ^^^ $p < 0.001$).

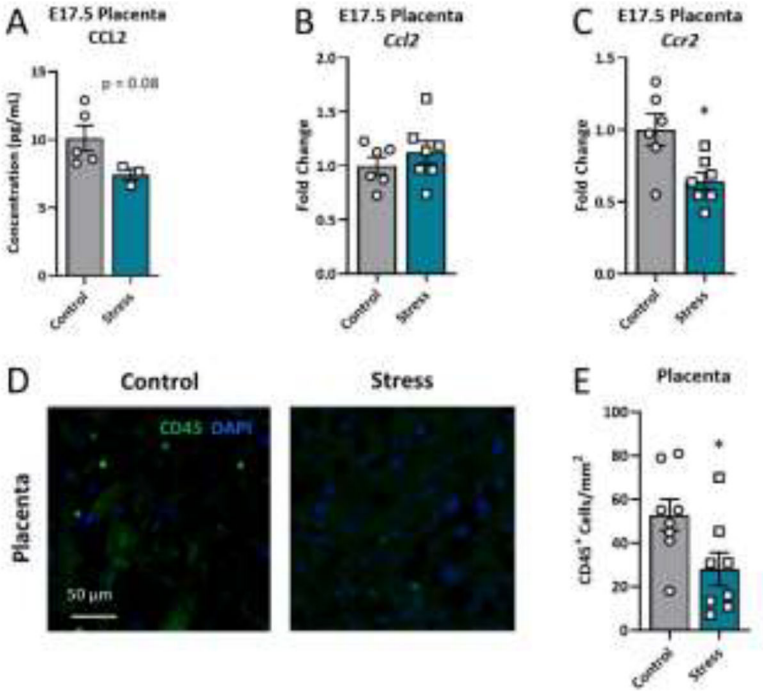

Figure 2: The placenta does not increase CCL2 production following prenatal stress at E17.5.
A. CCL2 protein ($t(6) = 2.100$, $p = 0.081$) and **B.** *Ccl2* gene expression were not altered in the placenta with stress ($t(11) = 0.540$, $p = 0.600$), though **C.** expression of *Ccr2* was reduced ($t(11) = 2.767$, $p = 0.018$). **D.** Representative images of CD45 and DAPI staining in placentas. **E.** Quantification of the images is presented as number of $CD45^+$ cells/mm^2 of placental tissue and reveals a decrease in placental leukocytes ($t(14) = 2.386$, $p = 0.032$). Bars represent mean ± SEM. T Tests: * $p < 0.05$.

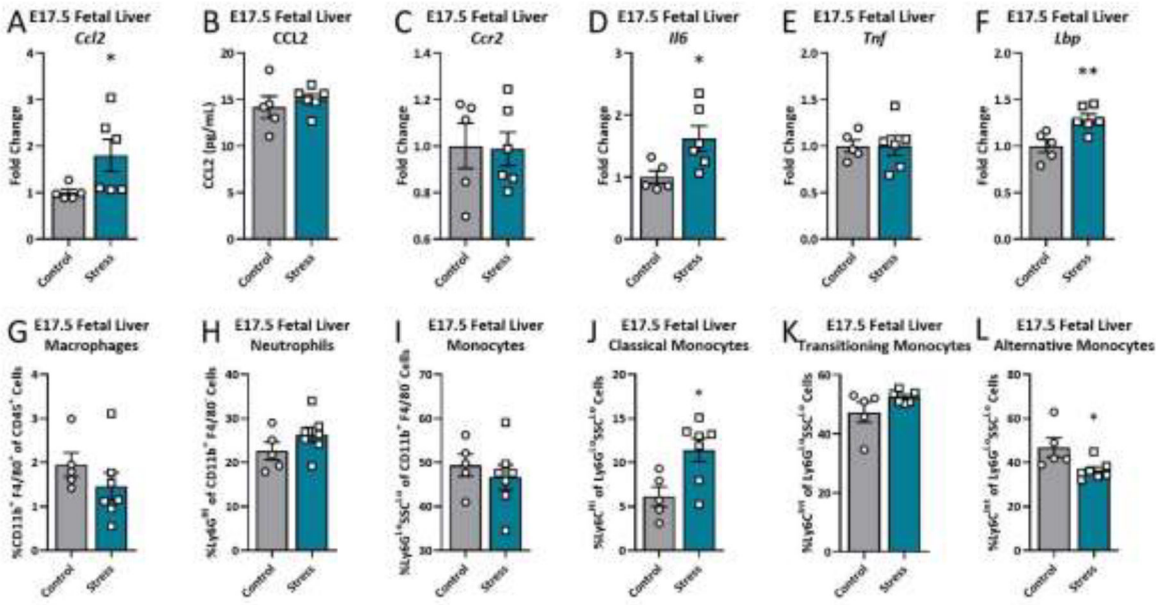

Figure 3: Prenatal stress increases *Ccl2* expression in the fetal liver and induces inflammation.

A. Exposure to stress increases expression of *Ccl2* in the fetal liver ($t(6.11) = 2.436$, $p = 0.050$), **B.** but not CCL2 protein ($t(9) = 0.692$, $p = 0.506$) or **C.** *Ccr2* ($t(9) = 0.039$, $p = 0.970$). **D.** Stress also increased *Il6* expression ($t(9) = 2.841$, $p = 0.019$) and **F.** *Lbp* ($t(9) = 3.264$, $p = 0.0098$), but not **E.** *Tnf* ($t(9) = 0.112$, $p = 0.913$). Stress did not impact the **G.** macrophage ($t(10) = 1.133$, $p = 0.284$), **H.** neutrophil ($t(10) = 1.416$, $p = 0.187$), or **I.** monocyte population in the liver ($t(10) = 0.674$, $p = 0.515$), though within the monocyte population, stress **J.** increased the proportion of proinflammatory classical monocytes ($t(10) = 2.874$, $p = 0.017$) and **L.** decreased the proportion of alternative monocytes ($t(10) = 2.541$, $p = 0.029$) without impacting the proportion of transitioning monocytes ($t(10) = 1.133$, $p = 0.284$). Bars represent mean ± SEM. T Tests: * $p < 0.05$, ** $p < 0.01$.

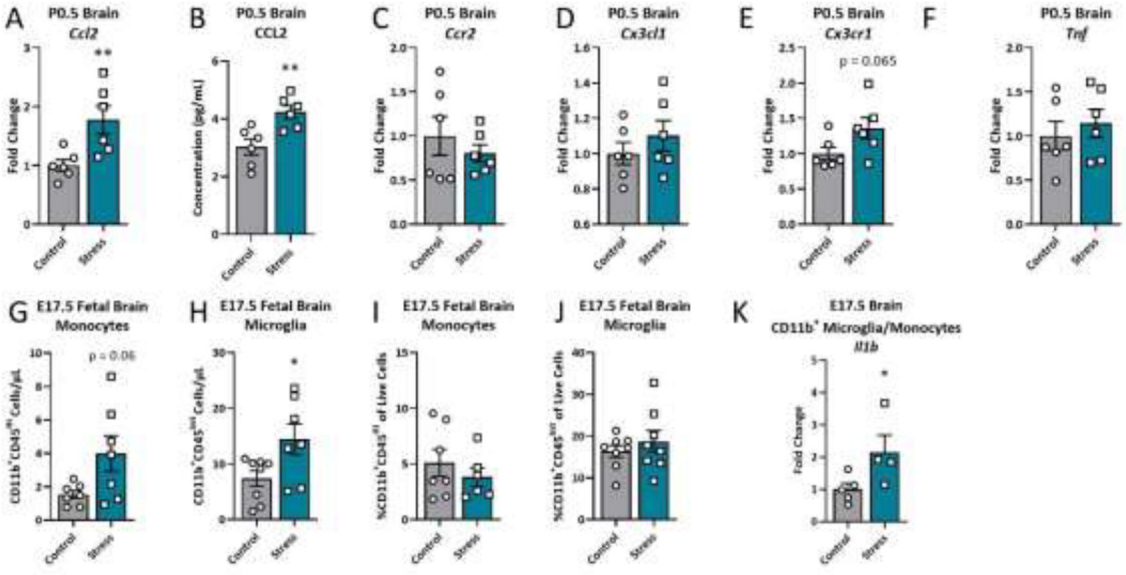

Figure 4: Prenatal stress enhances chemokine signaling and increases myeloid cells in the developing brain.

Prenatal restraint stress increases **A.** *Ccl2* gene expression ($t(10) = 3.351$, $p = 0.007$) and **B.** CCL2 protein in the neonatal pup brain on P0.5 ($t(10) = 3.455$, $p = 0.006$), while not impacting **C.** *Ccr2* expression ($t(10) = 0.492$, $p = 0.633$). Although **D.** *Cx3cl1* expression was not impacted ($t(10) = 0.927$, $p = 0.376$), **E.** there was a trending increase in *Cx3cr1* expression ($t(10) = 2.072$, $p = 0.065$). **F.** Expression of the pro-inflammatory cytokine *Tnf* was not altered by stress ($t(10) = 0.663$, $p = 0.522$). When examining the cellular composition of the fetal brain, there was an increase in number of **G.** monocytes ($t(6.61) = 2.255$, $p = 0.06$) and **H.** microglia in the fetal brain (Mann-Whitney Test: $p = 0.040$), though no changes in relative percentage of **I.** monocytes ($t(11) = 0.850$, $p = 0.413$) or **J.** microglia ($t(14) = 0.783$, $p = 0.447$). **K.** *Il1b* expression was increased in $CD11b^+$ microglia and monocytes in the fetal brain ($t(7) = 2.480$, $p = 0.042$). Bars represent mean ± SEM. T Tests: * $p < 0.05$, ** $p < 0.01$.

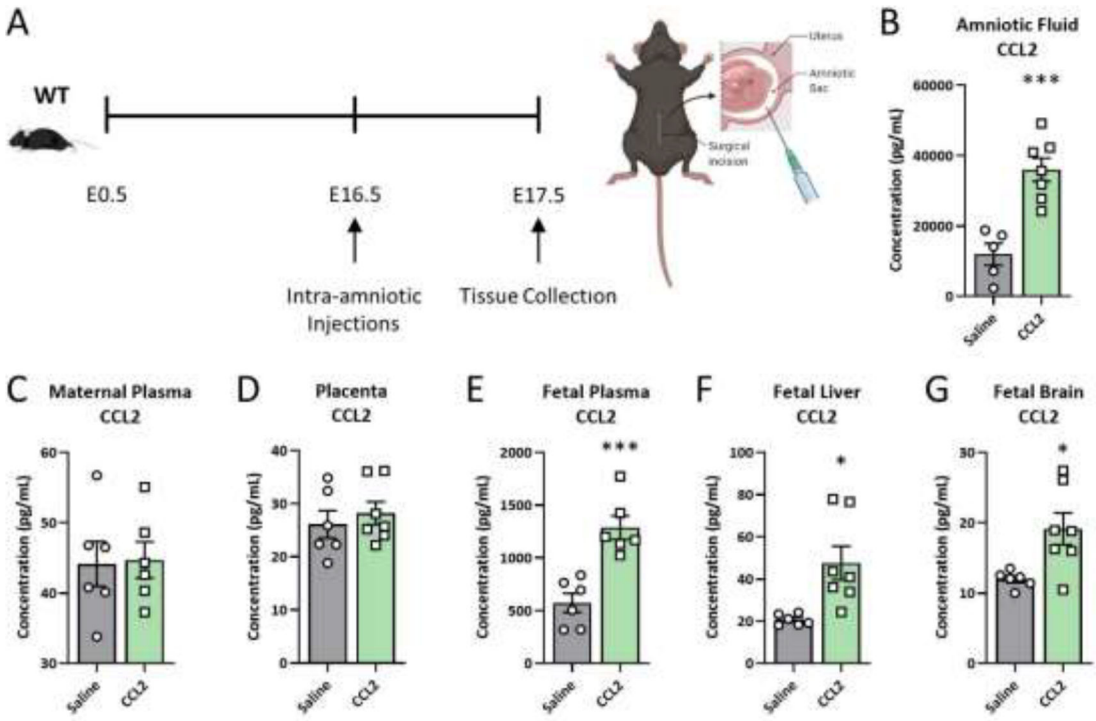

Figure 5: Intra-amniotic injections of recombinant CCL2 specifically increases CCL2 protein in the fetal compartment.

A. Experimental design. Intra-amniotic injections of recombinant CCL2 or saline were performed on E16.5. Tissues were collected 24 hours after the procedure on E17.5 to measure concentration of CCL2 in maternal and fetal tissues. **B.** As expected, CCL2 was increased in the amniotic fluid following injection of CCL2 into the amniotic sac ($t(10) = 5.073$, $p = 0.0005$). CCL2 concentrations were not impacted in the **C.** maternal plasma ($t(10) = 0.140$, $p = 0.891$) or **D.** placenta ($t(11) = 0.632$, $p = 0.540$). However, CCL2 was increased in the **E.** fetal plasma ($t(10) = 4.939$, $p = 0.0006$), **F.** fetal liver ($t(6.208) = 3.338$, $p = 0.015$), and **G.** fetal brain ($t(6.548) = 3.136$, $p = 0.018$). Bars represent mean ± SEM. T Tests: * $p < 0.05$, *** $p < 0.001$.

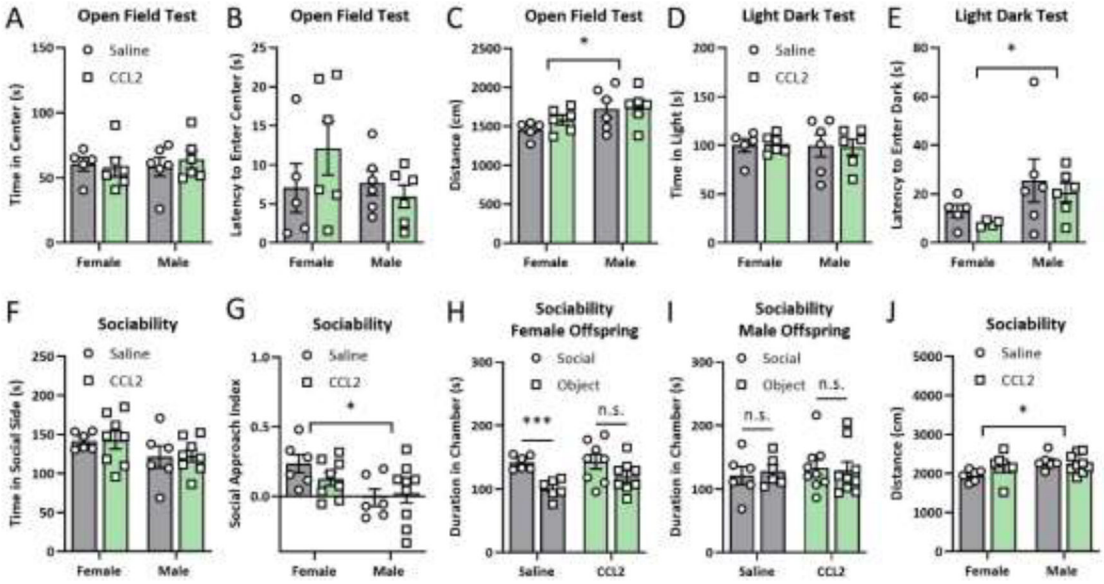

Figure 6: Intra-amniotic injections of recombinant CCL2 induces social behavioral deficits in adult female offspring.

CCL2 exposure *in utero* did not impact A. time spent in the center (no effect of CCL2: $f(1,19) = 0.057$, $p = 0.814$; no effect of sex: $f(1,19) = 0.082$, $p = 0.777$; no interaction: $f(1,19) = 0.345$, $p = 0.564$), **B.** latency to enter the center of the open field test in either female or male offspring (no effect of CCL2: $f(1,19) = 0.443$, $p = 0.514$; no effect of sex: $f(1,19) = 1.205$, $p = 0.286$; no interaction: $f(1,19) = 1.859$, $p = 0.189$), or **C.** distance traveled (no effect of CCL2: $f(1,19) = 0.767$, $p = 0.392$; main effect of sex: $f(1,19) = 6.429$, $p = 0.020$; no interaction: $f(1,19) = 0.409$, $p = 0.530$). Additionally, **D.** time spent in the light (no effect of CCL2: $f(1,19) = 0.000049$, $p = 0.995$; no effect of sex: $f(1,19) = 0.040$, $p = 0.844$; no interaction: $f(1,19) = 0.029$, $p = 0.867$) and **E.** latency to enter the dark compartment in the light dark test was not altered by CCL2 treatment in either female or male offspring (no effect of CCL2: $f(1,17) = 0.727$, $p = 0.406$; main effect of sex: $f(1,17) = 4.672$, $p = 0.045$; no interaction: $f(1,17) = 0.0002$, $p = 0.987$). Although CCL2 treatment did not alter **F.** time spent in the social chamber of the three chamber social behavior test (no effect of CCL2: $f(1,24) = 0.018$, $p = 0.894$; no effect of sex: $f(1,24) = 3.930$, $p = 0.059$; no interaction: $f(1,24) = 0.00091$, $p = 0.976$) or **G.** social approach index (no effect of CCL2: $f(1,25) = 0.329$, $p = 0.572$; main effect of sex: $f(1,25) = 6.950$, $p = 0.014$; no interaction: $f(1,25) = 1.211$, $p = 0.282$), **H.** female offspring exposed to saline spent more time in the social chamber compared to the object chamber ($t(10) = 5.421$, $p = 0.00029$), a preference that was not observed in the female offspring exposed to CCL2 ($t(14) = 1.591$, $p = 0.134$), or **I.** in either condition for the male offspring (saline: $t(10) = 0.422$, $p = 0.682$; CCL2: $t(16) = 0.212$, $p = 0.834$). **J.** These alterations were not due to a difference in activity as distance traveled in the behavioral test was not impacted by CCL2 (no effect of CCL2: $f(1,25) = 0.939$, $p = 0.342$; no interaction: $f(1,25) = 2.311$, $p = 0.141$), though male offspring were more active compared to female (main effect of sex: $f(1,25) = 5.548$, $p = 0.027$). Bars represent mean ± SEM. A-E, H: Two Way ANOVA: * indicates main effect of sex (* $p < 0.05$). F-G: T Tests: *** $p < 0.001$.