Early life stress causes sex-specific changes in adult fronto-limbic connectivity that differentially drive learning

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Abstract

It is currently unclear whether early life stress (ELS) affects males and females differently. However, a growing body of work has shown that sex moderates responses to stress and injury, with important insights into sex-specific mechanisms provided by work in rodents. Unfortunately, most of the ELS studies in rodents were conducted only in males, a bias that is particularly notable in translational work that has used human imaging. Here we examine the effects of unpredictable postnatal stress (UPS), a mouse model of complex ELS, using high resolution diffusion magnetic resonance imaging. We show that UPS induces several neuroanatomical alterations that were seen in both sexes and resemble those reported in humans. In contrast, exposure to UPS induced fronto-limbic hyper-connectivity in males, but either no change or hypoconnectivity in females. Moderated-mediation analysis found that these sex-specific changes are likely to alter contextual freezing behavior in males but not in females.

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Introduction

Childhood maltreatment (CM) is a broad term used to define a heterogenous group of early adversities that range from severe bullying to physical, emotional, and/or sexual abuse (White and Kaffman, 2019b, Teicher and Samson, 2016). Exposure to CM increases the risk for the development of multiple psychopathologies and medical conditions over the lifespan (Anda et al., 2006, Nemeroff, 2016, Teicher and Samson, 2016, Kaffman and Meaney, 2007), with an estimated annual economic burden of $2 trillion in the United states alone (Peterson et al., 2018 2018). While CM is recognized as a significant risk factor for abnormal brain development in industrialized countries, a thorough understanding of how CM impacts neurodevelopment and psychopathology in males and females is lacking. Given that 30–40% of the adult population have experienced some form of CM (Agorastos et al., 2019), clarifying these issues is necessary in order to effectively diagnose and treat the enormous clinical and economic burden associated with CM (White and Kaffman, 2019c).

The study of CM-associated outcomes is generally framed from a cumulative risk perspective, where the risk of negative outcomes raises linearly with the number of maltreatment exposures (Kessler et al., 1997, Anda et al., 2006, Chen et al., 2010, Evans et al., 2013). Newer models have expanded this framework to highlight that different forms of CM fall along multiple dimensions, and this in turn more directly impacts

later outcomes than number of CM instances per se (McLaughlin et al., 2014, McLaughlin and Sheridan, 2016). However, neither of these models have addressed the potential role that sex plays in moderating CM-associated outcomes. Further much of the focus in the field to date has been on the role that sex plays in influencing the rate of psychopathology with little consensus and/or replication of findings (White and Kaffman, 2019c). Briefly, some studies report similar outcomes in males and females, some found females to be more sensitive to CM, while others see effects only in males (White and Kaffman, 2019c). A more nuanced approach suggests that sex-specific effects may depend on genetic vulnerability, the circuit involved, and the developmental stage when the stress occurred or outcomes assessed (White and Kaffman, 2019a).

Importantly, similar clinical presentation may be driven by different underlying mechanisms in males and females as reported for depression (Labonte et al., 2017) and neuropathic pain (Sorge et al., 2015). These findings suggest that sex-specific interventions might be needed to more effectively treat psychiatric and medical consequences of CM (White and Kaffman, 2019c). Promising progress on this issue comes from advanced imaging techniques (Lerch et al., 2017, Le Bihan, 2003, Biswal, 2012, Moldrich et al., 2010). The most reproducible findings are from structural MRI showing reduced hippocampal and corpus callosum volume in those exposed to CM, with a hint that these effects are larger in males compared to age-matched females (Teicher and Samson, 2016). A recent multi-center study using a large cohort of 3,036 individuals did not find any significant changes in hippocampal size but noted reduced caudate size in females that was not seen in males exposed to CM (Frodl et al., 2017). Few studies have had sufficient power to directly test for CM by sex interactions on task mediated fMRI activation, and connectivity using resting state fMRI (rsfMRI), or diffusion MRI (dMRI) (White and Kaffman, 2019a). Even within these few studies, there is no agreement with regard to a specific pattern of task-mediated neuronal activation (Crozier et al., 2014, Colich et al., 2017, Dannlowski et al., 2012) or connectivity (Herringa et al., 2013, Ohashi et al., 2019).

This ambiguity highlights the difficulty of studying long-term consequences of complex adversity in human populations where the nature and severity of the CM may be different between and within studies and findings may be confounded with other psychological or medical co-morbidities, for a thorough review on this topic, see (Herringa, 2017). Preclinical studies in rodents can bypass many obstacles faced in clinical research by precisely controlling the genetic composition of the animals, and the severity, duration, and complexity of the stress. Human imaging techniques, such as high-resolution MRI, dMRI, and rsfMRI, can be used in rodent models to characterize structural and functional outcomes that can be more directly compared to humans. Further, the contribution of structural and functional changes, measured via imaging, to behavioral alterations can be rigorously tested using optogenetics and chemogenetic tools (Kaffman et al., 2019). Although, rodents and non-human primates exposed to early life stress (ELS) show similar behavioral and physiological outcomes reported in human studies, the vast majority of work has been done only in males (White and Kaffman, 2019a). Similarly, the few studies using imaging to characterize outcomes in rodent models of ELS were done in males and report inconsistent findings (Johnson et al., 2018a, Bolton et al., 2018, Molet et al., 2016, Carlyle et al., 2012, Yan et al., 2017, Guadagno et al., 2018c). The need to extend these findings to females is highlighted by a report of considerable neuroanatomical sex differences in the postnatal mouse brain (Qiu et al., 2018) and a recent study showing that maternal separation leads to sex-specific effects in amygdala connectivity with the prefrontal cortex in adolescent rats (Honeycutt et al., 2020).

Our lab has modified and built upon the widely used limited nesting and bedding paradigm (LBN) developed by Tallie Baram's lab (Walker et al., 2017) by extending the time frame dam and pups are exposed to LBN (from PND2-9 to PND0-25) and adding brief, 1 hr, bouts of maternal separation on an unpredictable schedule, i.e. PND 14, 16, 17, 21, 22, & 25, followed by nest disruption. This ELS paradigm is termed unpredictable postnatal stress (UPS) and was designed to represent both a cumulative and multidimensional subtype of CM that allows us to test how complex adversity can alter neurodevelopment and behavioral outcomes in both male and female mice (White and Kaffman, 2019b). We have previously shown that UPS produces an elevated anxiety phenotype in both adolescence and adulthood (Johnson et al., 2018b). Further, using rsfMRI we found increased fronto-limbic connectivity in UPS male mice that included increased amygdala-prefrontal cortex and amygdala-hippocampus connectivity, the strength of which was highly correlated with anxiety-like behaviors (Johnson et al., 2018a). Interestingly, females did not show

an elevated anxiety phenotype, suggesting that UPS may affect males and females differently. This original study did not include females in rs-fMRI analysis and is thus unable to speak to potential ELS by sex interactions on fronto-limbic connectivity patterns.

The current study aimed to replicate and extend our understanding of UPS-induced behavioral phenotypes and brain connectivity patterns in adult male and female mice. Our first objective was to replicate our previous findings that UPS increases anxiety-like behavior in male, but not female mice and to examine the effects of UPS, sex and their interaction on other exploratory behaviors (i.e. novel object) and contextual and cued fear conditioning. The second goal was to assess the effects of UPS and sex on local volumetric changes, microstructural alterations in white matter tracts, and structural connectivity using high resolution dMRI. Finally, we sought to replicate our fronto-limbic hyper connectivity rsfMRI findings in UPS male mice using dMRI tractography and to test whether similar changes would also be seen in female mice exposed to UPS.

Methods

Animals

BALB/cByj mice (Stock # 001026, Jackson Laboratories) were housed in standard Plexiglas cages and kept on a standard 12:12 hr light-dark cycle (lights on at 7:00AM), with food provided *ad libitum* and constant temperature and humidity (23 $\pm$ 1^{o}C and 43% $\pm$ 2). Background noise in the room was kept at (dB: 56.5) which was significantly lower compared to levels recorded(dB: 65.9) in our previous studies (Johnson et al., 2018a). All studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Yale University and were conducted in accordance with the recommendations of the NIH Guide for the Care and the Use of Laboratory Animals.

Early-life stress models

Thirty females and 7 male BALB/cByj mice, 8-10 weeks old, were purchased from the Jackson Laboratory and allowed to acclimate for 12 days in our facility. Breeding cages were set up using a 2:1 female to male harem in standard mouse Plexiglas cages with 2 cups corncob bedding and no nesting material. Visibly pregnant dams were transferred to 'maternity cages' containing 2 cups corncob bedding with no nesting material and 3 chow pellets on the floor. New females were added to the harem cages. On postnatal day (P0) litters were culled to 5–8 pups and randomized to either control (CTL) or unpredictable postnatal stress (UPS) conditions. Mice raised under CTL condition were provided with 2 cups corncob bedding and one 5 x 5 cm nestlet per cage. Bedding for CTL condition was changed on P14 and P21. UPS litters were provided with 1/2 cup of corncob and no nesting material from P0–25 with bedding changes on P7, P14, & 21. In addition to limited bedding and nesting material, UPS litters were separated from their dam for 1 h on P14, P16, P17, 21, 22, & 25 (Figure 1A). During the separation period, the dam was transferred to a new cage followed by the individual transfer of the pups to a different cage containing clean corncob bedding. During the separation period, the home cage was briefly shaken to evenly spread the bedding and disrupt the nest. At the end of the 1 h separation, the pups were individually returned to their home cage followed by the return of the dam. All animals were weighed at P14, P26, and at the time of tissue collection. At the time of weaning (P26) animals were group-housed with same sex littermates with 2 cups corncob bedding and no nesting material. Cages were changed weekly but were otherwise left undisturbed until behavior testing commenced at P70.

Behavioral Testing

General strategy. Based on our previous work we estimated that roughly 13 mice per rearing condition and sex are needed to achieve a power of 0.8, using an effect size (Cohn's d= 1.15) and alpha= 0.05 (two tails)

(Johnson et al., 2018a). Using these a priori power calculations we conducted behavioral testing in a cohort of 12-23 mice per group from 4-6 litters, for a total of 4 groups (i.e. CTL-males, UPS-males, CTL-females, UPS-females, Fig 2). To identify robust behavioral outcomes, we repeated the behavioral work using a second cohort consisted of n = 9-19 mice per group from 4-6 litters. Behavioral data for cohort 1, and cohort 2 are summarized in the Supplemental Information, Table S1.

Handling. All animals were handled each morning for 3 days prior to the beginning of behavioral testing. Animals were individually removed from the cage and placed onto the back of the hand of the experimenter, and allowed to explore the experimenter hand for 30 s. The animal was placed into a holding cage until all animals from that home cage had been handled, at which time all animals were returned to the home cage. At the end of 3rd day of handling, animals were placed into the behavioral room and allowed to acclimate for 2 h prior to the onset of behavioral testing. Behavioral testing was conducted between 1230-1700 h daily.

Exploratory behaviors. Exploratory behavior was tested using the open-field test, the elevated plus maze, and exploration of novel objects. In the open-field test, mice were allowed to explore a 50 x 50 cm arena (lux 60) for 5 min during which the distance traveled and the time spent in the inner 15 cm-area were measured using the EthoVision tracking system (Noldus Information Technology). For the elevated plus maze (EPM), the mice were placed in the middle of a standard elevated plus maze (each arm is 10 x 50 cm long; open arm lux 120 & closed arm lux 60) facing an open arm and allowed to explore the maze for 5 min. The time spent exploring the open and closed arms were determined using the EthoVision tracking system. To assess approach behaviors in a novel context, mice we placed at the right-hand corner of a 50 x 25 cm box (lux 100) with corncob bedding covering the bottom and two identical objects located at the opposite end of the box. Animals were allowed to explore for 5 minutes, the sessions were recorded, and an experimenter blind to sex and condition scored the amount of time each animal spent in direct contact with the objects for the first 3 minutes of the trial.

Fear-Conditioning. On training day, animals are placed into a Med Associates' fear conditioning chamber (Part # VFC-008 with interior dimensions of 29.53 x 23.5 x 20.96 cm) with a grid floor (Part # ENV-005FPU-M: 29.21 x 29.21 x 6.05 cm), a 2% lemon scent diluted in 70% EtOH. Animals were allowed 300 s to explore the chamber before the onset on tone-shock pairings. Animals were presented with 30, 0.5 s tones (7500 Hz, 80 Db) and a 1 s 0.65 mA foot shock that co-terminates with the last tone. This pattern was repeated for a total of 5 tone-shock pairings with a variable inter-trial interval (30 s —180 s). There was a 30 s period at the end of the session following the last pairing. Freezing behavior was recorded using Med Associates' Video Freeze software (V2.6.5.81; Med Associates Inc, St. Albans, VT.). Animals were then returned to their home cage until testing. The apparatus was cleaned with 70% EtOH between each session. Twenty-four hr following training, animals were returned to the testing room and placed into the same context (grid floor with a 2% lemon scent diluted in 70% EtOH); freezing behavior was monitored for 10 min and the first 5 min were scored. On the 3rd day of testing, animals were placed into a novel context (walls were replaced with an opaque plastic sheet to round off the walls, a thick mesh netting covered the grid floor, and animals were exposed to a 2% peppermint scent diluted in 70% EtOH). The same 5-tone presentation schedule from training was employed in the absence of the shock, and freezing behavior was continually monitored.

Tissue Collection/Processing

All tissue was collected between (1030-1230) to minimize changes associated with circadian rhythm and was done after completing the behavioral testing. In brief, mice were anesthetized with chloral hydrate (100 mg/kg) and a cardiac puncture was performed to collect roughly 100uL of blood in tubes containing heparin.

These were then spun down (5000 x g for 5 min) to collect serum and assess corticosterone levels. Mice were then transcardially perfused using cold a PBS/heparin (50 u/ml) solution followed by 10% formalin (polyScience). Adrenal glands were then dissected and weighed. After perfusion, mice were decapitated, and intact skulls were post-fixed for 24 hrs at 4 degC in 10% formalin, then transferred to sterile 1x PBS (ph 7.4) and left at 4degC until transfer to the imaging facility at NYU for ex-vivo dMRI studies (n = 6 mice per condition and sex). These numbers were based on an effect size of (Cohn's d = 2.0), alpha = 0.05 (two tails), and power = 0.8 (Johnson et al., 2018a). Corticosterone levels were determined using ELISA (Cat. # K014-H1, Arbor Assays, Ann Arbor, MI).

dMRI studies

Upon arrival at the NYU imaging facility, brains were equilibrated with Gadolinium (0.2mM) in PBS for 1 week at 4degC and scanned by an experimenter blind to the sex and rearing condition. Scanning was conducted overnight using a 7-Tesla MR system equipped with a 4-channel cryogenic probe for enhanced sensitivity (Ratering et al., 2008). Images were acquired using a modified 3D GRASE sequence (Wu et al., 2013) with the following parameters: echo time (TE)/repetition time (TR)=33/400 ms, 100 um isotropic resolution, two non-diffusion weighted images (b_0s) and 60 diffusion weighted images (DWIs) with a diffusion weighting (b) of 5,000 s/mm^2, and a total imaging time of 12 hours. DTIStudio (www.mristudio.org) was used to align all DWIs to the average of b_0s to remove small sample displacements due to vibrations during the long scan and compute average DWI (aDWI) and fractional anisotropy (FA) maps using the diffusion tensor model. The aDWI and FA maps were normalized to an MRI-based atlas (Chuang et al., 2011, Arefin et al., 2019) using the dual-channel (aDWI+FA) large deformation diffeomorphic metric mapping (LDDMM) (Ceritoglu et al., 2009). To assess global and regional amygdala connectivity, we transferred 14 structural labels (i.e. 7 structures in each hemisphere, e.g., cortex, hippocampus, cerebellum, etc. as defined in the Allen mouse brain atlas) to the subject images using the inverse mapping from LDDMM. The transferred structural labels were visually examined and showed good agreement with the corresponding structures in the subject images. For each pair of ipsilateral structures, random seeds were first assigned to a structure (e.g., the amygdala). Next, the number of seeds proportional to the volume of the structure, and streamlines from the seeds passing through the target structure (e.g. hippocampus) were reconstructed using a probabilistic tractography method implemented in MRtrix (www.mrtrix.org) as described previously (Wu and Zhang, 2016). The numbers of streamlines between regions were used as a measure of the connection strength. GRETNA software (Wang et al., 2015) was used to quantify global and regional properties of the structural connectome using principles of graph theory (Bullmore and Sporns, 2009). Global features included measurement of global efficiency (Geff) and the small-worldness (SW) and were normalized with respect to 1000 simulated random networks with equal distribution of edge weight and node strength as reported previously (Rubinov and Sporns, 2011, Schlemm et al., 2017). Furthermore, local graph parameters, such as, nodal clustering coefficient (NCp), nodal efficiency (Neff), and degree centrality (Dcent) (Watts and Strogatz, 1998, Rubinov and Sporns, 2010) were calculated to capture alterations in the vicinity of the left and right amygdala.

Moderated-mediation analysis

A moderated mediation regression analysis was conducted using Preacher and Hayes' (2008) bootstrapping procedure (5,000 bootstrap resamples) and associated SPSS macro (Model 58) to test the moderating impact of sex on UPS-effects on the number of streamlines between the amygdala and ventral hippocampus and the relationship between these projections and freezing behavior in the contextual fear conditioning test (see Fig. 7A for visual depiction of path model). For this analysis, rearing condition (dummy-coded; 0 = CTL-reared; 1 = UPS-reared) was entered into the model as the independent variable (X variable), the number of amygdala-ventral hippocampus streamlines (continuous, mean-centered) was entered into the model as the mediator (M variable), sex (dummy-coded; 0 = male, 1 = female) was specified as the moderator (W

variable), and percent freezing was included as the outcome variable (Y variable; log-transformed to correct positive skew, per convention for regression analyses).

Statistical Analysis

Statistical analyses were done using SPSS (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY; IBM Corp.) and visualized with GraphPad Prism version 8.1.0 for MacOS, GraphPad Software, La Jolla California USA. Animals that were > 2 s.d. above or below the mean were eliminated from behavioral analysis. Data were examined using a two-way ANOVA with rearing condition (CTL or UPS) and sex as fixed factors. A 2 X 2 ANOVA, written in matlab, was used to assess the effects of UPS, sex and their interaction on maps of local volumetric changes and FA using voxel-based analysis (Ashburner and Friston, 2000). The minimum number of voxels used to define a volumetric cluster and levels for false-base discovery rate correction for multiple comparisons differ depending on whether main effects of rearing, sex or interaction where tested, and these are specified in the results section. Repeated measures ANOVA was used to assess the effect of laterality as a within-subject variable and sex and rearing as between subject variables on the following 5 projections: 1) amygdala-prefrontal cortex, 2) amygdala-ventral hippocampus, 3) amygdala-dorsal hippocampus, 4) ventral hippocampus- prefrontal cortex. Significant interactions were followed by post-hoc comparisons using Tukey's HSD or Sidak's test. These same procedures were used to analyze laterality in both global and amygdala connectivity.

Results

Effects of UPS on body weight and baseline stress levels

A main effect of rearing on body weight was consistently found at P14 ($F\ (1, 106) = 36.66$, $p < 0.001$, $h_p{}^2 = 0.257$ Fig. 1B) and P26 ($F\ (1, 104) = 61.22$, $p < 0.001$, partial eta squared ($h_p{}^2$) $= 0.303$, Fig. 1C) showing that UPS animals were significantly smaller than CTL animals. No significant effects of

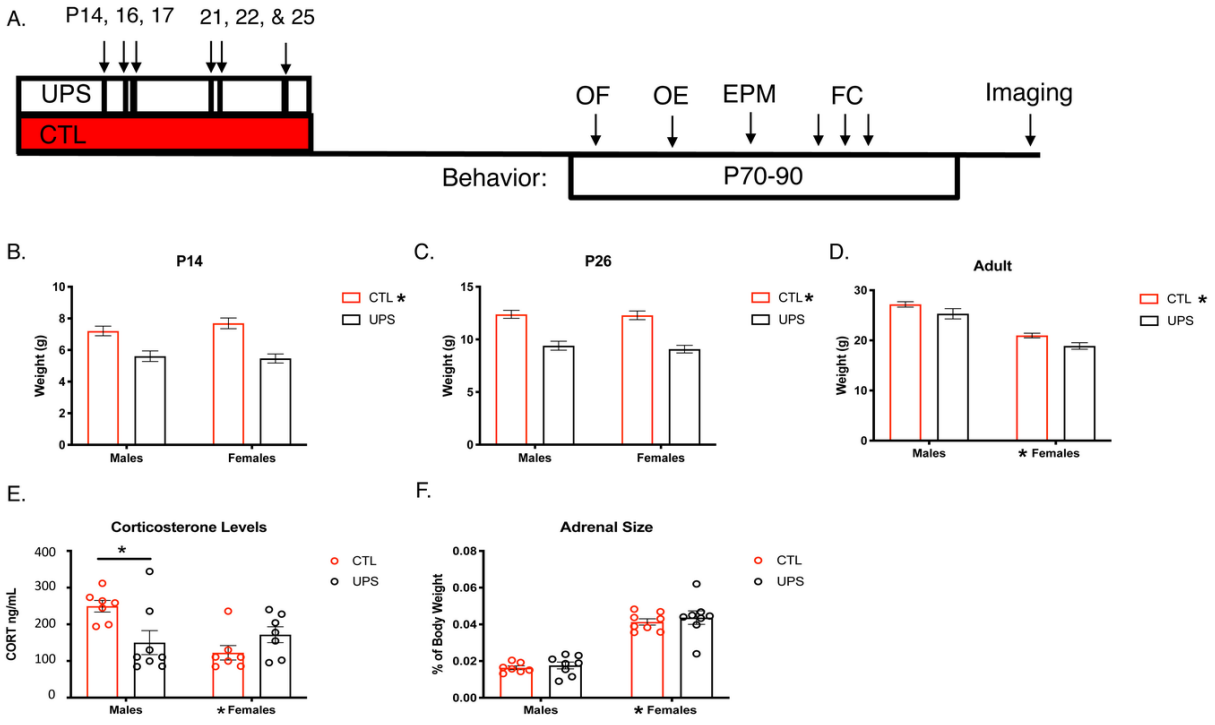

Fig 1. Long lasting effects of UPS on body weight and stress response. (A) Timeline. (B-D) Body Weights across the lifespan (P14 & 26: n = 23-33, Adulthood: n= 8 for each rearing and sex group, see Fig1 source data for raw data). (E) Baseline corticosterone levels in adulthood (n= 7-8 per rearing and sex group). (F) Adult adrenal size normalized to body weight (n= 7-8 per rearing and sex group). UPS: unpredictable early life stress, CTL: Control, OF: Open Field, OE: Object Exploration, EPM: Elevated Plus Maze, FC: Fear Conditioning. Means and SEM, $*p < 0.05$. For raw data, see Fig 1-source data.

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`Figure 1 source Data.xlsx` available at https://authorea.com/users/309157/articles/446313-early-life-stress-causes-sex-specific-changes-in-adult-fronto-limbic-connectivity-that-differentially-drive-learning

sex or an interaction were seen in 14 and 26-day old pups (p's > 0.05). The effect of rearing persisted into adulthood (F (1, 28) = 7.86, $p = 0.009$, $h_p^2 = 0.219$, Fig 1D), with a significant main effect of sex (F (1, 28) = 80.31, $p < 0.0001$, $h_p^2 = 0.741$), but no interaction between sex and rearing (F (1, 28) = 0.03, $p = 0.87$, $h_p^2 = 0.001$). Serum from adult animals was processed for corticosterone levels. Results reveal a significant interaction of sex and rearing on corticosterone level (F (1, 25) = 9.37, $p = 0.005$, $h_p^2 = 0.273$). Sidak's post-hoc analysis revealed a significant reduction in corticosterone levels in UPS compared to CTL males (p = 0.01, Cohn's d = -1.4), that was not seen in females ($p = 0.31$, Cohn's d = 0.9, Fig 1E). Further, there was a significant main effect of sex (F (1, 25) = 4.72, $p = 0.04$, $h_p^2 = 0.159$), but no effect of rearing (F (1, 25) = 1.06, $p = 0.31$, $h_p^2 = 0.041$; Figure 1E). Given the interaction between UPS and sex for corticosterone levels we also assessed the effects of UPS, sex and the interaction on the normalized weight of the adrenal gland (Figure 1F). A significant effect of sex was also found for normalized adrenal gland weight (F (1, 27) = 117.10, $p < 0.0001$, females > males, $h_p^2 = 0.813$), but with no significant effects for rearing (F (1, 27) = 0.60, $p = 0.45$, $h_p^2 = 0.022$), or interaction (F (1, 27) = 0.05, $p = 0.83$, $h_p^2 = 0.002$).

Long-term consequences of UPS on behavior

Two-way ANOVA revealed no significant effects of sex, rearing, or an interaction on time spent in the center of the open field (sex: F (1, 68) = 1.34, $p = 0.25$, $h_p^2 = 0.019$; rearing: F (1, 68) = 0.912, p = 0.34, $h_p^2 = 0.013$; interaction: F (1, 68) = 3.37, $p = 0.31$, $h_p^2 = 0.015$; Fig. 2A), or on total distance traveled (sex: F (1, 68) =0.908, $p = 0.234$, $h_p^2 = 0.013$, rearing: F (1, 68) = 0.626, $p = 0.43$, $h_p^2 = 0.009$; interaction: F (1, 68) = 1.959, $p = 0.17$, $h_p^2 = 0.028$; Fig. 2B). These behavioral outcomes were replicated in an additional cohort, see Supplementary Information Table S1.Two-way ANOVA on time spent in the open arms of the EPM revealed a significant main effect of rearing (F (1, 72) = 7.411, $p = 0.008$, h_p^2 = 0.093), where UPS-reared animals spent significantly more time in the open arms (Fig. 2C). This effect was not replicated in a second cohort suggesting that UPS-induced elevations in exploration of open arms may not be robust. Further, there were no significant effects of sex (F (1, 72) = 0.054, $p = 0.82$, $h_p^2 = 0.001$), or interaction (F (1, 72) = 0.49, $p = 0.48$, $h_p^2 = 0.007$), and no differences were seen in closed arm exploration (sex: F (1, 72) = 0.35, $p = 0.55$, $h_p^2 = 0.005$; rearing: F (1, 72) = 0.83, $p = 0.37$, $h_p^2 = 0.011$; interaction: F (1, 72) = 2.25, $p = 0.14$, $h_p^2 = 0.03$; Fig. 2D). These effects were replicated in a second cohort, see Table S1.

UPS mice spent significantly less time exploring novel objects, rearing: F (1, 70) = 9.77, $p = 0.003$, $h_p^2 = 0.123$ (Figure 2E), but with no significant interaction F (1, 70) = 0.306, $p = 0.58$, $h_p^2 = 0.004$. These effects were seen in an additional cohort (Table S1). In cohort 1, there was also a significant effect of sex: F (1, 70) = 22.53, $p < 0.001$, $h_p^2 = 0.243$, but this finding was not replicated in the additional cohort (Table S1).

Learned freezing behavior was then assessed in a contextual and cued-fear conditioning paradigm. Initial freezing prior to the onset of shocks revealed no inherent differences in baseline activity between sexes or rearing conditions (sex: F (1, 68) = 0.28, $p = 0.60$, $h_p^2 = 0.004$; rearing: F (1, 68) = 2.34, $p = 0.13$, $h_p^2 = 0.033$; interaction: F (1, 68) = 0.02, $p = 0.89$, $h_p^2 < 0.001$), findings that were all replicated in an additional cohort (sex: F (1, 62) = 0.22, $p = 0.64$, $h_p^2 = 0.004$; rearing: F (1, 62) = 0.004, $p = 0.95$, h_p^2

< 0.001; interaction: F (1, 62) = 0.717, $p = 0.40$, $h_p^2 = 0.011$). Repeated measures ANOVA revealed a significant effect of freezing behavior over time (Greenhouse-Geisser: F (2.2686, 272) = 91.38, $p < 0.001$, $h_p^2 = 0.573$; cohort 2: F (2.464, 248) = 70.50, $p < 0.001$, $h_p^2 = 0.532$) where animals froze more as the number of shocks experienced increased regardless of sex or rearing condition. No other significant within-subject effects were noted (p's > 0.1).

Two-way ANOVA revealed a significant main effect of rearing (F (1, 68) = 18.52, $p < 0.001$, $h_p^2 = 0.214$; Fig. 2F) where UPS-reared animals froze significantly less to the original training context compared to CTL-reared counterparts, an effect that was replicated in cohort 2 (Table S1). No significant effect of sex (F (1, 68) = 1.88, $p = 0.18$, $h_p^2 = 0.027$) or an interaction (F (1, 68) = 3.37, $p = 0.07$, $h_p^2 = 0.047$) were seen in cohort 1 or cohort 2 (Table S1). Two-way ANOVA for cue-recall revealed a significant main effect of sex (F (1, 68) = 8.57, $p = 0.005$, $h_p^2 = 0.112$), with females freezing more than males to the cue, an effect replicated in cohort 2 (Table S1). Further, there were no significant effects of rearing condition or an interaction on cue-induced freezing (rearing: (F (1, 68) = 2.204, $p = 0.14$, $h_p^2 = 0.031$; interaction: (F (1, 68) = 3.32, $p = 0.07$, $h_p^2 = 0.047$; Figure 2G), with similar outcomes seen in the second cohort (Table S1).

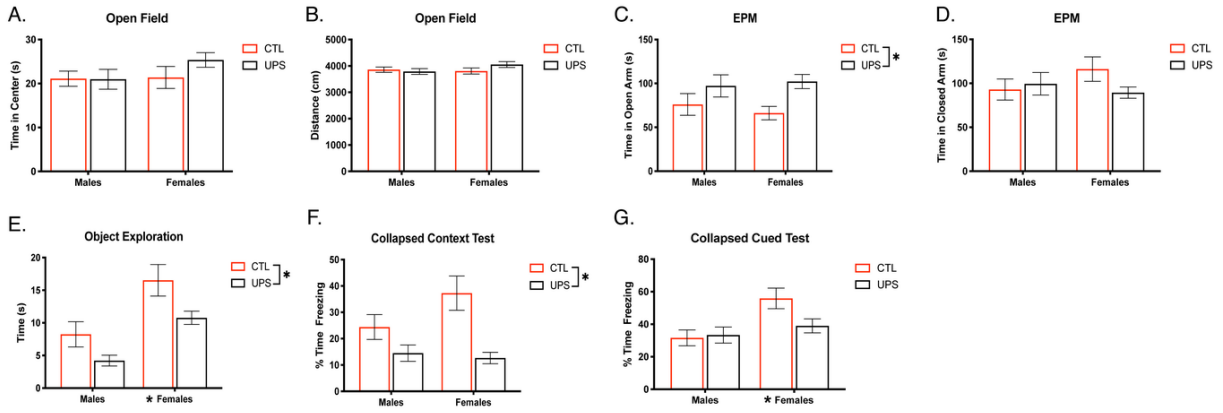

Fig 2. Effects of UPS and sex on adult behavior. Time in the center (A) and distance traveled in the OF (B). Time spent in the open (C) and closed arms (D) of the EPM. Time exploring objects in an arena (D). Average freezing behavior in contextual fear conditioning test (E) and during cue presentations in novel context (F). (n= 12-23 per rearing and sex group). Means and SEM, $*p < 0.05$. See Fig 2-source for raw data.

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`Figure 2 source data.xlsx` available at https://authorea.com/users/309157/articles/446313-early-life-stress-causes-sex-specific-changes-in-adult-fronto-limbic-connectivity-that-differentially-drive-learning

Voxel-based volumetric changes

Next, we obtained high resolution dMRI scans (n= 6 mice per group) and conducted a 2 X 2 whole-brain voxel-based morphometric analysis to identify local volumetric changes affected by UPS, sex and their interaction. Using a minimal voxel cluster of 25 and a false discovery rate of 0.1 ($p< 0.0105$), we identified several volumetric changes that were affected by condition regardless of sex (Fig. 3 and Table S2). Volumetric changes were relatively small (20-30%) and included increased volumes in the nucleus accumbens, olfactory bulb, cingulate cortex, sub-regions within the sensory cortex, ventral hippocampus, and amygdala in UPS mice. UPS mice showed reduced volumes in the frontal cortex, prefrontal cortex, sub-regions of the motor cortex, the sensory cortex, fimbria, striatum, and thalamus when compared to CTL mice. Relatively few

areas showed volumetric changes between males and females and their identification required less stringent false discovery rate of 0.3 ($p< 0.0107$) and minimum cluster size at 25. Using these parameters, males showed increased volumes in sub-regions of the olfactory bulb, dorsal hippocampus, and hypothalamus and reduced volume in parts of the motor cortex, sensory cortex, striatum, and thalamus when compared to the female mice (see supplemental information, Fig. S1). No brain regions showed UPS by sex interaction after correcting for multiple comparison and adjusting the minimal cluster size to more than 20. Therefore, an exploratory analysis was conducted using uncorrected analysis ($p< 0.05$ uncorrected, minimal cluster size $>$ 20) and the results are shown in the supplemental information Figure S2.

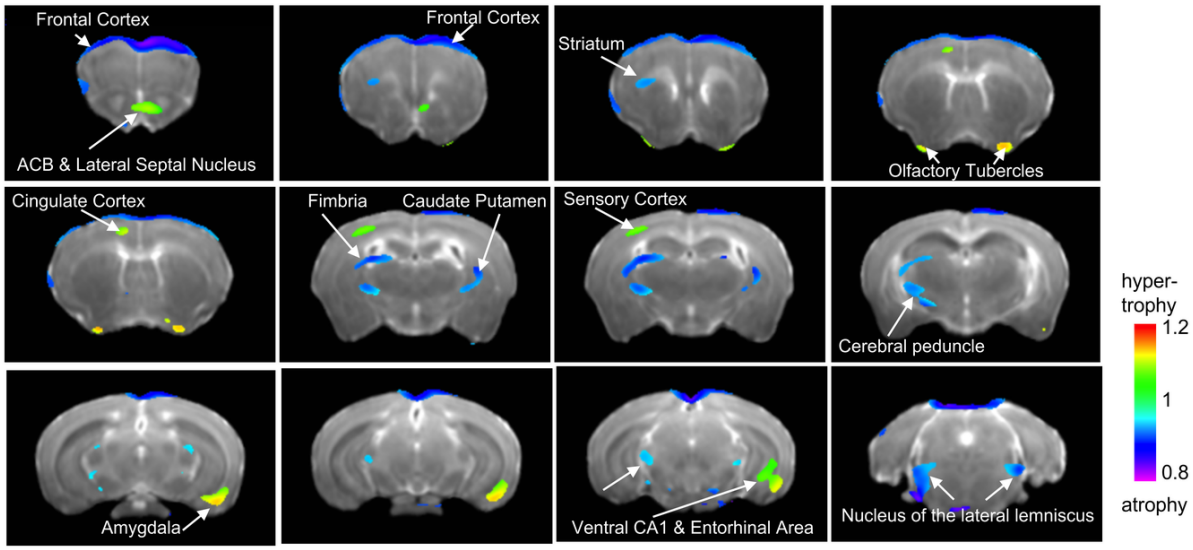

Fig 3. Main effect of UPS on local volumetric changes. Minimal cluster size $>$ 25 voxels, FDR$<$ 0.1, $p< 0.0105$. 1= no change; <1 (blue) reduced volume in UPS; >1 (green) increased in UPS. (n= 6 per rearing and sex group). Nucleus accumbens- ACB. Matlab codes used to conduct the 2 X 2 analyses for figures 3 and 4 are available as Figs 3-4-source data.

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`Figures 3-4 source data.zip` available at https://authorea.com/users/309157/articles/446313-early-life-stress-causes-sex-specific-changes-in-adult-fronto-limbic-connectivity-that-differentially-drive-learning

Fractional anisotropy (FA)

A 2 X 2 ANOVA revealed significant effect of UPS on FA in numerous brain regions (minimum cluster size $>$25, FDR= 0.1, $p< 0.007$) with moderate effect size of 40-50% (Fig. 4). Increased FA in UPS was most pronounced in sub-regions of the sensory cortex, hypothalamus and amygdala with reduced FA was seen in the rostral corpus callosum and stria medularis (Fig. 4). Similar to the volumetric analysis, the effects of sex on FA were subtle requiring lowering the FDR to 0.3 (supplemental info, figures S3) and an exploratory uncorrected characterization of the interaction between UPS and sex interaction is available in the supplemental information, Figure S4.

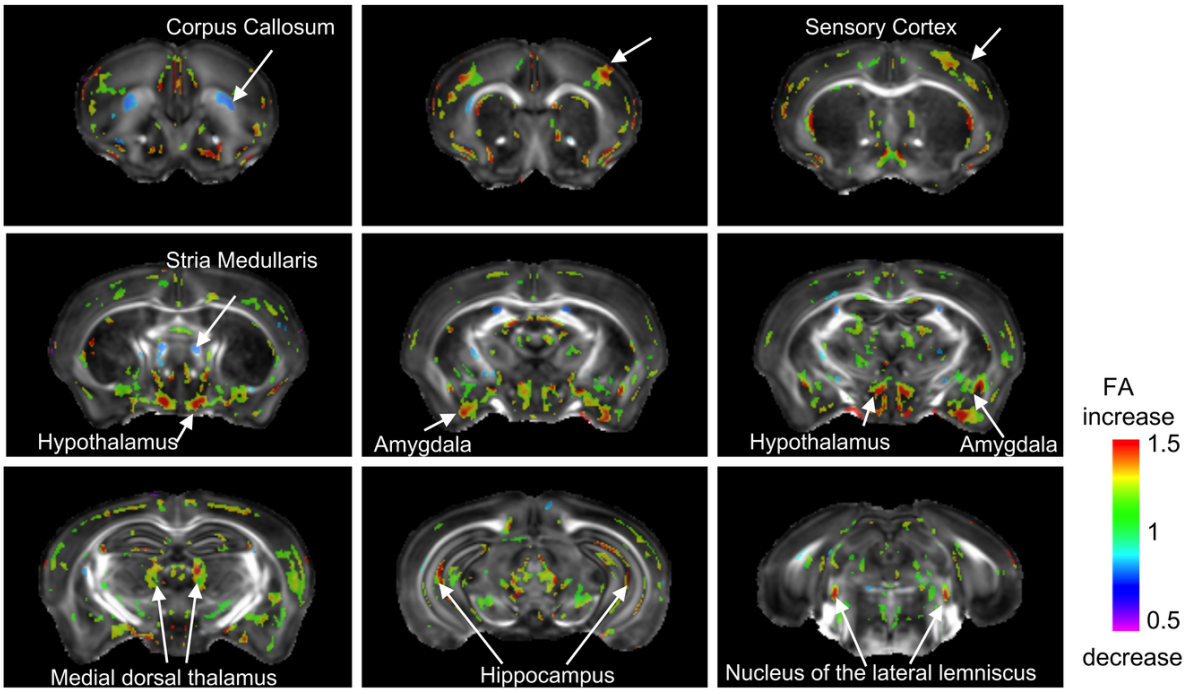

Fig 4. Main effect of UPS on local changes in FA. Minimal cluster size > 25 voxels, FDR< 0.1, p< 0.007. 1= no change; <1 (blue) reduced volume in UPS; >1 (green/red) increased in UPS (n= 6 per rearing and sex group). Matlab codes used to conduct the 2 X 2 analyses for figures 3 and 4 are available as Figs 3-4-source data.

Tractography

We previously found increased rsfMRI connectivity between the amygdala and the prefrontal cortex (PFC) and between the amygdala and the hippocampus (both ventral and dorsal) in UPS male mice and wanted to test whether a similar increase in structural connectivity is seen using tractography. Moreover, our volumetric and FA analyses have revealed long-term structural changes associated with UPS, with only subtle effects of sex, and little evidence to support interaction between UPS and sex. Thus, it became important to test whether a similar pattern would be seen using tractography. We quantified ipsilateral streamlines between the amygdala, hippocampus (ventral and dorsal) and the PFC in both the right and left hemispheres. Repeated Measures ANOVA was first conducted to ascertain whether laterality existed in the effect of UPS, sex, and their interaction on connectivity. In most cases there was no significant effect of laterality allowing us to combine streamlines data from the left and right hemispheres; a detailed analysis for each hemisphere is available in the supplemental information, figures S5-S7.

Amygdala-PFC. No significant effects were found within-subjects (p's > 0.05), but a 2 X 2 ANOVA found significant main effects of sex (F (1, 20) = 8.90, $p = 0.007$, $h_p^2 = 0.292$), rearing condition (F (1, 20) = 6.48, $p = 0.02$, $h_p^2 = 0.289$), and interaction (F (1, 20) = 19.269, $p < 0.001$, $h_p^2 = 0.483$) on the number of streamlines between the amygdala and the prefrontal cortex (Fig. 5A). Sidak's post-hoc analysis showed a significant effect of sex within the UPS condition ($p < 0.001$, Cohn's d = -3.5), but not the CTL condition ($p = 0.33$, Cohn's d = 0.5). Further, there was a significant effect of rearing condition for males ($p < 0.001$, Cohn's d = 2.9), but not females ($p = 0.21$, Cohn's d = -0.6). Overall, amygdala-PFC connectivity was significantly increase in UPS-reared males but was not altered in UPS-reared females (Fig 5A).

Ventral Hippocampus - PFC. No significant effects were found within-subjects (p's > 0.05), and there was no significant effect of sex (F (1, 20) = 0.08, $p = 0.78$, $h_p^2 = 0.004$). A significant effect of rearing (F (1, 20) =

7.07, $p = 0.02$, $h_p^2 = 0.261$) and interaction between rearing and sex (F (1, 20) = 18.416, $p < 0.001$, $h_p^2 = 0.479$) were found for the number of streamlines between the ventral hippocampus and the PFC (Fig. 5B). Sidak's post-hoc analysis showed a significant effect of sex within the UPS condition ($p = 0.004$, Cohn's d = -1.9) and the CTL condition ($p = 0.01$, Cohn's d = 1.6) whereby within the CTL condition females have increased number of streamlines over males, but this pattern was reversed in the UPS condition. Further, there was a significant effect of rearing condition for males ($p < 0.001$, Cohn's d = 2.4), but not females ($p = 0.26$, Cohn's d = -0.9). Overall, ventral hippocampal-PFC showed a similar pattern of connectivity to that seen between the amygdala and the PFC, with UPS increasing the number of streamline projections in males but not females (Fig. 5B).

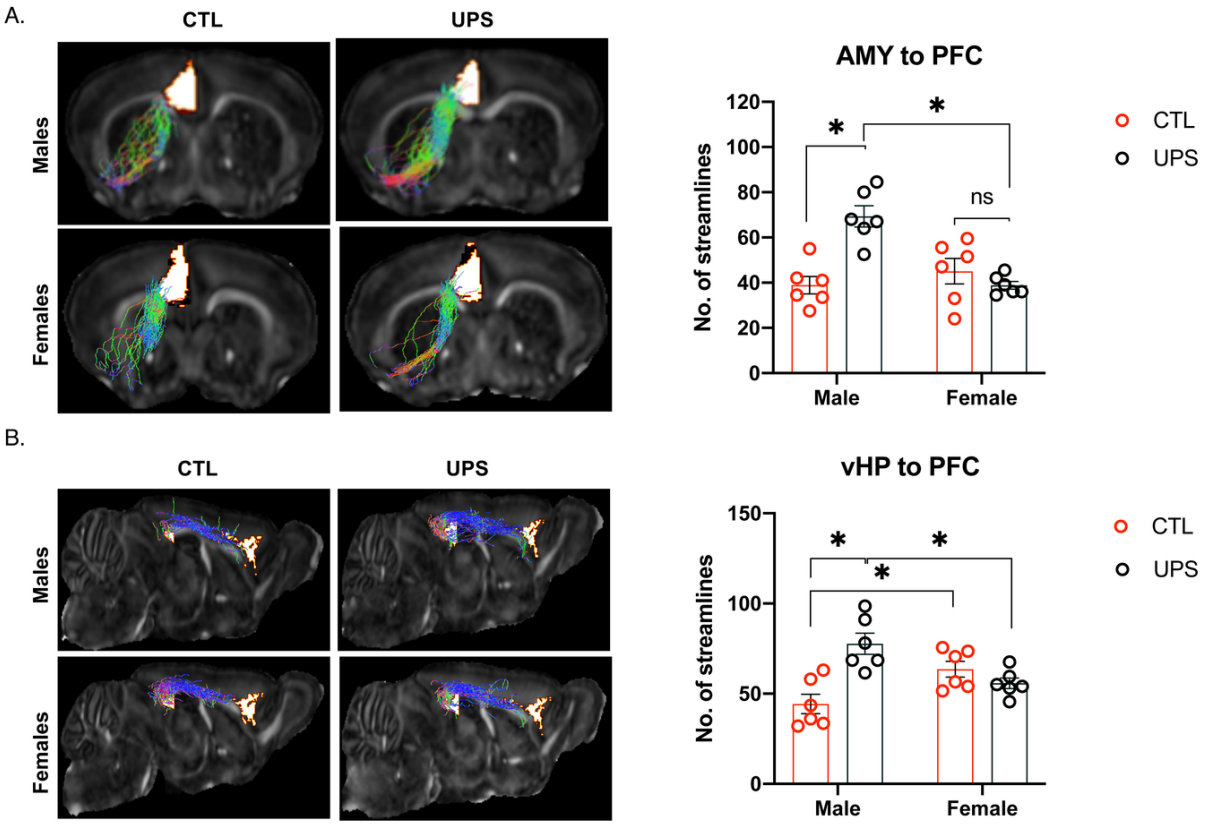

Fig 5. UPS differentially alters PFC connections with the amygdala and ventral hippocampus in males and females. (A) Representative images and quantification of amygdala-PFC tractography. (B) Representative images and quantification of ventral hippocampus-PFC tractography. Amy- Amygdala, PFC- prefrontal cortex, vHP- ventral hippocampus. (n= 6 per rearing and sex group). Means and SEM *$p < 0.05$

Amygdala - Hippocampus. Repeated measures ANOVA revealed a significant within-subject main effect of hemisphere on the number of streamlines between the amygdala complex and the hippocampal formation (Fig. 6A). Specifically, the left hemisphere showed significantly higher connectivity compared to the right hemisphere (Greenhouse-Geisser F (1, 20) = 8.183, $p < 0.01$, $h_p^2 = 0.29$ Supplemental Information Fig S7A-B). Further, there was a significant interaction between sex and hemisphere (Greenhouse-Geisser F (1, 20) = 17.82, $p < 0.01$, $h_p^2 = 0.471$) that when unpacked showed a significant effect of sex within the right hemisphere ($p = 0.001$, Cohn's d = 1.3) but not within the left ($p = 0.29$, Cohn's d = -0.4), and a significant effect of hemisphere for females ($p < 0.001$, Cohn's d = 1.8), but not males ($p = 0.35$, Cohn's d = -0.2), Fig.

S7 A-B. Together, these findings suggest a small reduction in right hemisphere connectivity in females (Fig. S7B). No other significant effects were found within-subjects (p's > 0.05). Between-subject analysis found a significant interaction between sex and rearing condition (F (1, 20) = 18.74, $p < 0.001$, $h_p^2 = 0.484$) that when unpacked, revealed a significant effect of sex within the UPS condition ($p < 0.001$, Cohn's d = 2.3), but not within the CTL condition ($p = 0.16$, Cohn's d = -1.1) where UPS females show a reduced number of streamlines compared to UPS males. Further, a significant effect of rearing condition was found within males ($p = 0.001$, Cohn's d = 2.1) where UPS males showed increased connectivity compared to CTL males, but the pattern was significantly reversed in females ($p = 0.03$, Cohn's d = -1.4; Figure 6A). To further explore this, streamlines were specifically quantified between the amygdala and dorsal versus ventral hippocampus.

Amygdala - Dorsal Hippocampus. No significant effects were found within-subjects (p's > 0.05, Fig. S7 E & G), but significant between-subject effects were seen (Fig 6B). There was no significant effect of sex (F (1, 20) = 0.093, $p = 0.76$, $h_p^2 = 0.005$), but a significant effect of rearing condition (F (1, 20) = 13.62, $p = 0.001$, $h_p^2 = 0.405$) and interaction (F (1, 20) = 5.96, $p = 0.001$, $h_p^2 = 0.444$). Unpacking the interaction revealed a significant increase in connectivity in females ($p < 0.001$; UPS > CTL, Cohn's d = 3.5) but not males ($p = 0.83$; Cohn's d = -0.1 Figure 6B).

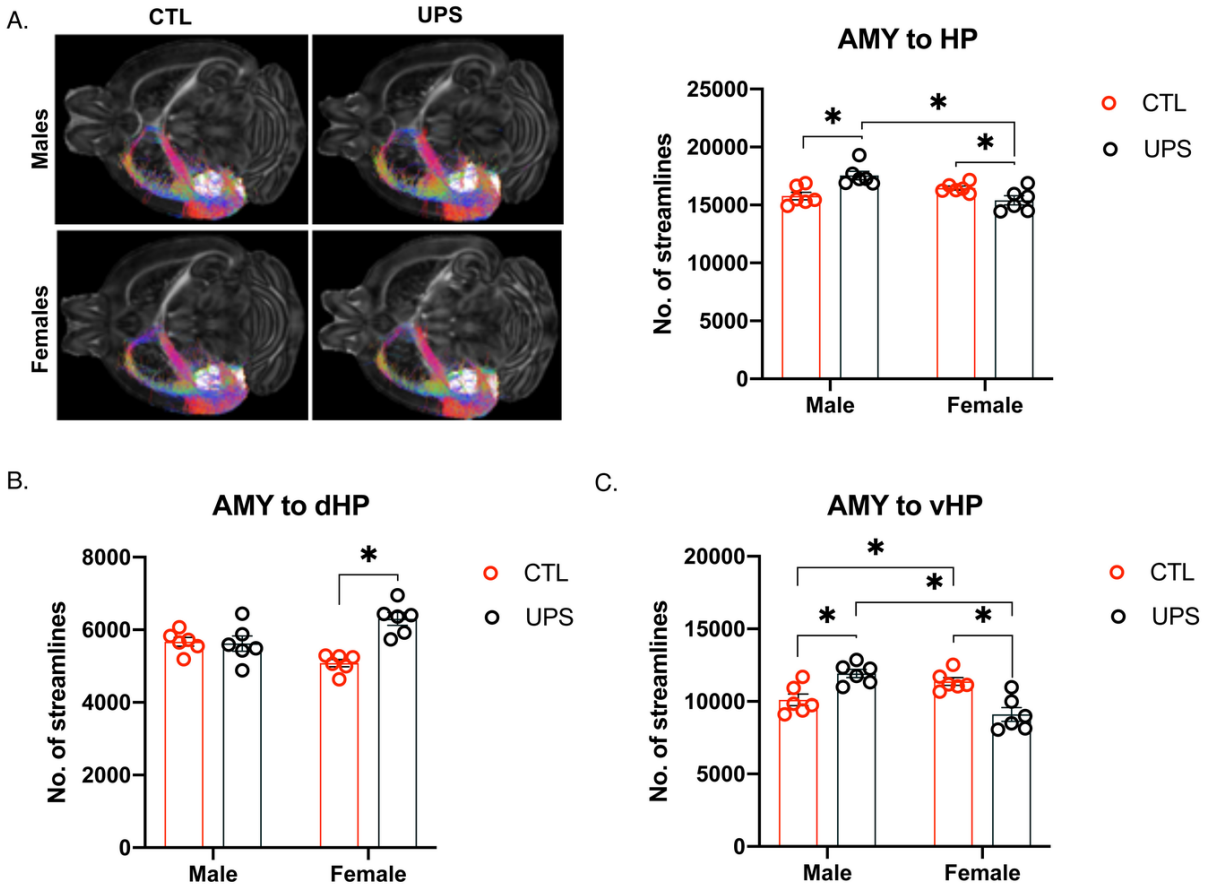

Fig 6. UPS causes sex-specific alterations in Amygdala-Hippocampus connections. (A) Representative images and quantification of the number of streamline connections between amygdala and the entire hippocampus. Number of streamline connections between the amygdala and the dorsal hippocampus (B) and between the amygdala and the ventral hippocampus (C). Amy- Amygdala, dHP- dorsal hippocampus,

vHP- ventral hippocampus. (n= 6 per rearing and sex group). Means and SEM, $^*p < 0.05$.

Amygdala - Ventral Hippocampus. Repeated measures ANOVA revealed a significant within-subject main effect of hemisphere on the number of streamlines with higher connectivity in the left compared to the right hemisphere (Greenhouse-Geisser F (1, 20) = 5.318, $p = 0.03$, $h_p^2 = 0.21$). There was a significant interaction of sex and hemisphere (Greenhouse-Geisser F (1, 20) = 6.93, $p = 0.02$, $h_p^2 = 0.257$) that when unpacked revealed a significant effect of sex on the right ($p = 0.005$, Cohn's d = 1.0) but not left ($p = 0.86$, Cohn's d = -0.1) hemisphere. Further, females show decreased connectivity in the right hemisphere ($p = 0.002$, Cohn's d = -1.0) compared to the left, a pattern not observed in males ($p = 0.82$, Cohn's d = 0.1), Fig. S7 F & H. No other significant effects were found within-subjects (p's > 0.05), but significant between subject effects were seen. Results reveal an insignificant main effect of rearing condition (F (1, 20) = 0.386, $p = 0.54$, $h_p^2 = 0.019$), but a significant effect of sex (F (1, 20) = 4.473, $p = 0.047$, $h_p^2 = 0.183$) and interaction (F (1, 20) = 30.89, $p < 0.001$, $h_p^2 = 0.607$ Fig. 6C). Sidak's post-hoc analysis found enhanced connectivity in UPS compared to CTL males ($p = 0.002$, Cohn's d = 2.1), but reduced connectivity in UPS females compared to CTL females ($p < 0.001$, Cohn's d = -2.4). These suggest that the alterations in connectivity seen in UPS between the amygdala to the whole hippocampal formation (Fig. 6A) are mainly driven by changes in amygdala connectivity with the ventral hippocampus (Fig 6C).

Amygdala-Ventral Hippocampus Tractography and Freezing Behavior

Given the large number of streamline projections between the amygdala and ventral hippocampus (Fig 6C) and their role in footshock consolidation (Huff et al., 2016) and contextual fear learning and retrieval (Fanselow and Dong, 2010, Zhu et al., 2014, Rudy and Matus-Amat, 2005), we conducted a moderated mediation analysis to examine the role that sex plays in impacting the effects of UPS on the number of amygdala-ventral hippocampus streamlines in the left hemisphere (Fig 7A, path A) and its relationship to freezing behavior in contextual fear conditioning (Fig 7A, path B). This analysis revealed that neither rearing condition (b = -160.50, SE = 462.87, t = -.35, p = 0.73, 95% CI [-1126.08, 805.08]), nor sex (b = 80.17, SE = 462.87, t = 0.17, p = 0.86, 95% CI [-885.41, 1045.74]) significantly predicted the number of streamlines between the amygdala and ventral hippocampus (Fig 7A, path A). Consistent with our previous analysis, there was a significant, interaction between rearing and sex, (b = -3966.33, SE = 925.73, t = -4.29, p = 0.0004, 95% CI [-5897.48, -2035.18]). Specifically, for males, the number of amygdala-ventral hippocampus streamlines was greater for UPS animals compared to CTL animals (b = 1822.67, SE = 654.59, t = 2.78, p = 0.01, 95% CI [457.14, 3188.20]) while the opposite effect was found for females (b = -2143.67, SE = 654.59, t = -3.28, p = 0.004, 95% CI [-3509.20, -778.14]). While both the main effects of number of amygdala-ventral hippocampus streamlines (b = -0.0001, SE = 0.0001, t = -2.66, p = 0.02, 95% CI [-0.0002, 0.0001]) and sex (b = 0.34, SE = 0.13, t = 2.63, p = 0.02, 95% CI [-1126.08, 805.08]) significantly predicted time spent freezing, there was also a significant interaction between sex and rearing (b = 0.0003, SE = 0.0001, t = 2.75, p = 0.01, 95% CI [0.0001, 0.0006], Fig 7A, path B). For males, a greater number of amygdala-ventral hippocampus streamlines predicted decreased time spent freezing (b = -0.0003, SE = 0.001, t = -3.61, p = 0.002, 95% CI [-0.0005, -0.0001]), but there was no significant relationship between number of amygdala-ventral hippocampus streamlines and freezing behavior for females (b = 0.0001, SE = 0.0001, t = 0.70, p = 0.49, 95% CI [-0.0001, 0.0002]). In contrast to findings in the left hemisphere, the number of amygdala-ventral hippocampus streamlines in the right hemisphere did not significantly predict freezing behavior for either male or female animals ($p > 0.5$).

Overall, tests of statistical mediation revealed a significant indirect effect of rearing condition on freezing behavior via number of left amygdala-ventral hippocampus streamlines for males (b = -0.54, SE = 0.20, 95% CI [-0.92, -0.11]), but not females (b = -0.11, SE = 0.21, 95% CI [-0.55, 0.37], Fig 7A, paths A and B, and bottom of figure for summary stats). The direct effect of rearing condition on freezing behavior (path C) was no longer significant after controlling for the mediating influence of streamline number and its interaction with sex, (b = 0.05, SE = 0.18, t = 0.27, p = 0.79, 95% CI [-0.33, 0.43]). Together, these results support the notion that reduced freezing behavior seen in UPS males is driven, at least in part, by increased

connectivity between the amygdala and ventral hippocampus in the left hemisphere. In contrast, the reduced number of amygdala-ventral hippocampus projections is not likely to explain the reduced freezing behavior seen in UPS females (Fig 7B).

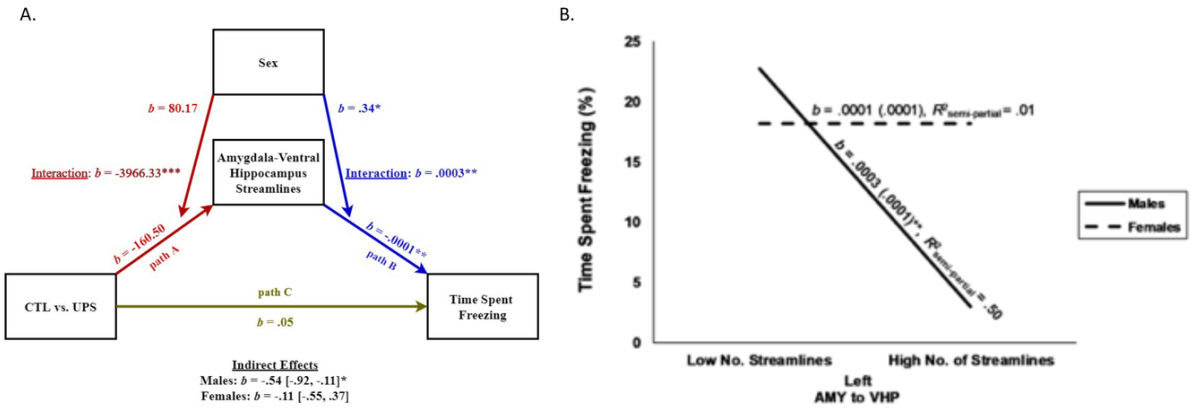

Fig 7. Moderated mediation model of freezing behavior. (A) Path model testing the effects of sex on number of amygdala-ventral hippocampus streamlines (left hemisphere) in mice exposed to UPS (path A) and the moderating effects of sex on the relationship between number of streamlines connections and freezing behavior (path B). Direct effect of UPS on freezing behavior after controlling for the indirect effects of streamline number and its interaction with sex is shown as path C, and the overall significance for the indirect moderating paths are shown for males and females at the bottom. (B) Interaction between number of streamlines (left hemisphere) between amygdala (AMY) and ventral hippocampus (VHP) and sex on freezing behavior. High and low number of streamlines corresponds to one standard deviation above and below the mean of this variable, respectively. Overall, streamlines predict freezing behavior in males but not females. ***p $<$ 0.001, **p $<$ 0.01, *p $<$ 0.05. SPSS syntax used for the moderator analysis is available as Fig 7-source data.

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`Figure 7 source data.txt` available at https://authorea.com/users/309157/articles/446313-early-life-stress-causes-sex-specific-changes-in-adult-fronto-limbic-connectivity-that-differentially-drive-learning

Global and Regional Connectivity

The sex-dependent effects of UPS on fronto-limbic connectivity appear inconsistent with a recent dMRI study in humans showing similar global connectivity outcomes in adult males and females exposed to CM (Ohashi et al., 2019). To test whether similar sex-specific changes are also seen in parameters of global network connectivity we generated a 7 X 7 matrix that included the cortex, hippocampus, striatum, thalamus, hypothalamus, amygdala, and the cerebellum (Fig 8A). This network included 42 tractograms in each hemisphere that were used to calculate global efficiency and small-worldness using Graph Theory (Bullmore and Sporns, 2009). No significant effects of hemisphere were found within-subjects (p's > 0.05) allowing us to test the effects of rearing, sex and their interaction across both hemispheres. A 2 X 2 ANOVA found a main effect of rearing on global network efficiency (F (1, 20) = 15.213, $p = 0.001$, $h_p^2 = 0.432$; Fig. 8B) and small-worldness (F (1, 20) = 18.647, $p < 0.001$, $h_p^2 = 0.482$; Fig. 8C), but with no significant effects of sex or interaction. Consistent with the findings in humans (Ohashi et al., 2019), UPS animals had reduced global network efficiency and increased small- worldness in both males and females, outcomes that

were mainly driven by reduced connectivity between the amygdala and several other brain regions including the striatum, hippocampus, and cerebellum (Fig 8A).

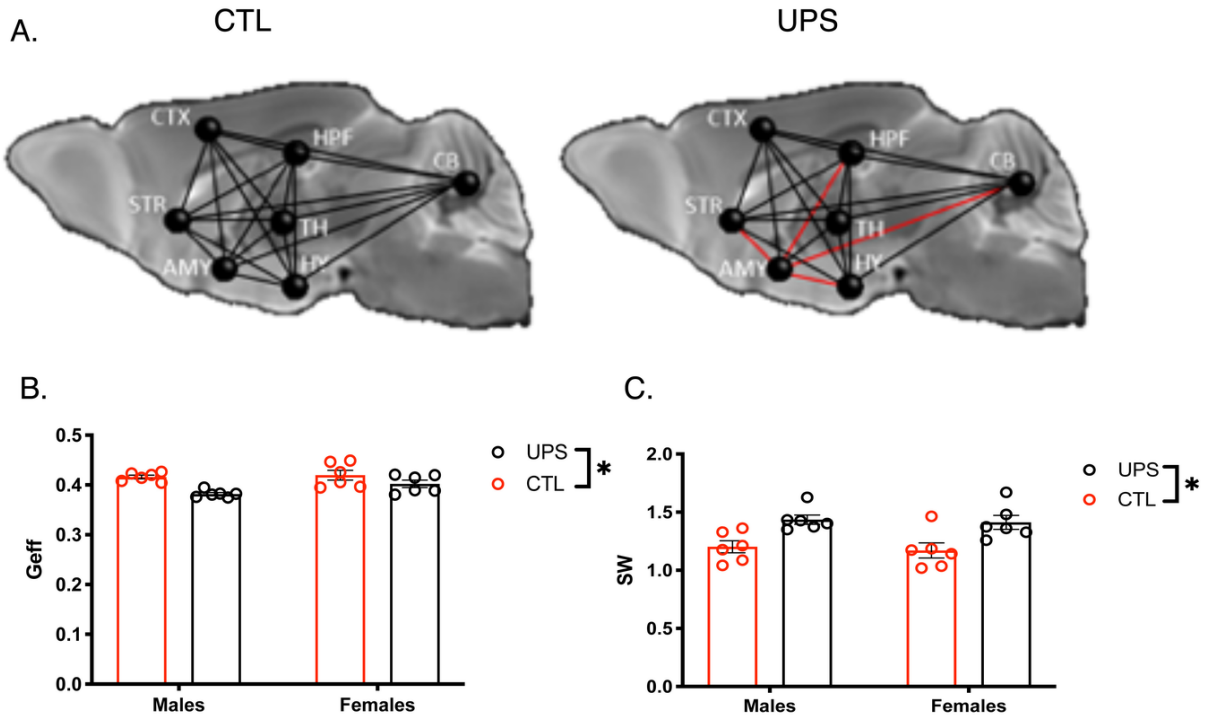

Fig 8. Effects of UPS and sex on global connectivity. (A) Schematic representation of global connectivity for CTL and UPS. Red lines indicate reduced connectivity compared to CTL group. Quantification of Global efficiency (B) and Small-worldness (C). (n= 6 per rearing and sex group). Means and SEM, $^*p < 0.05$. GRETNA codes used for global connectivity analysis are available in Fig 8-source data.

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Previous work in humans has shown that alterations in amygdala's centrality can distinguish between symptomatic and asymptomatic individuals exposed to CM (Ohashi et al., 2019). To test this issue in UPS mice we examined the effects of UPS, sex and their interaction on nodal clustering, efficiency, and degree of centrality for the right and left amygdala (Fig 9A). No significant differences on these outcomes were found between the left and the right amygdala allowing us to collapse across hemispheres. A 2 X 2 ANOVA found a significant effect of rearing on the nodal clustering coefficient ($F\ (1,\ 20) = 6.067$, $p = 0.023$, $h_p^2 = 0.233$), where UPS-reared animals displayed a smaller nodal clustering coefficient compared to CTL-reared animals (Fig. 9B). No significant effects of sex or interaction were found for nodal clustering. Significant effects of rearing ($F\ (1,\ 20) = 29.39$, $p < 0.001$, $h_p^2 = 0.595$), and interaction ($F\ (1,\ 20) = 3.69$, $p = 0.001$, $h_p^2 = 0.406$), but not sex alone ($F\ (1,\ 20) = 0.339$, $p = 0.57$, $h_p^2 = 0.017$) were found for amygdala nodal efficiency (Fig 9C). Follow-up Sidak's post-hoc analysis showed that males and females were different within both the CTL ($p = 0.007$, Cohn's d = 1.9) and UPS conditions ($p = 0.04$, Cohn's d = -1.2). UPS-reared males showed significantly reduced amygdala nodal efficiency compared to CTL-reared males ($p < 0.001$, Cohn's d = -4.1); a difference that was not seen in females ($p = 0.24$, Cohn's d = -0.7).

Further inspection of the connectivity maps clarified that reduced nodal amygdala efficiency seen in male UPS mice is driven by reduced connectivity between the amygdala and other structures such as striatum, hypothalamus and cerebellum (Fig 9A). No effects of rearing, sex or interaction were found for the degree of amygdala centrality (Fig 9D). Together these findings highlight the exquisite specificity by which UPS alters structural connectivity in male and female mice.

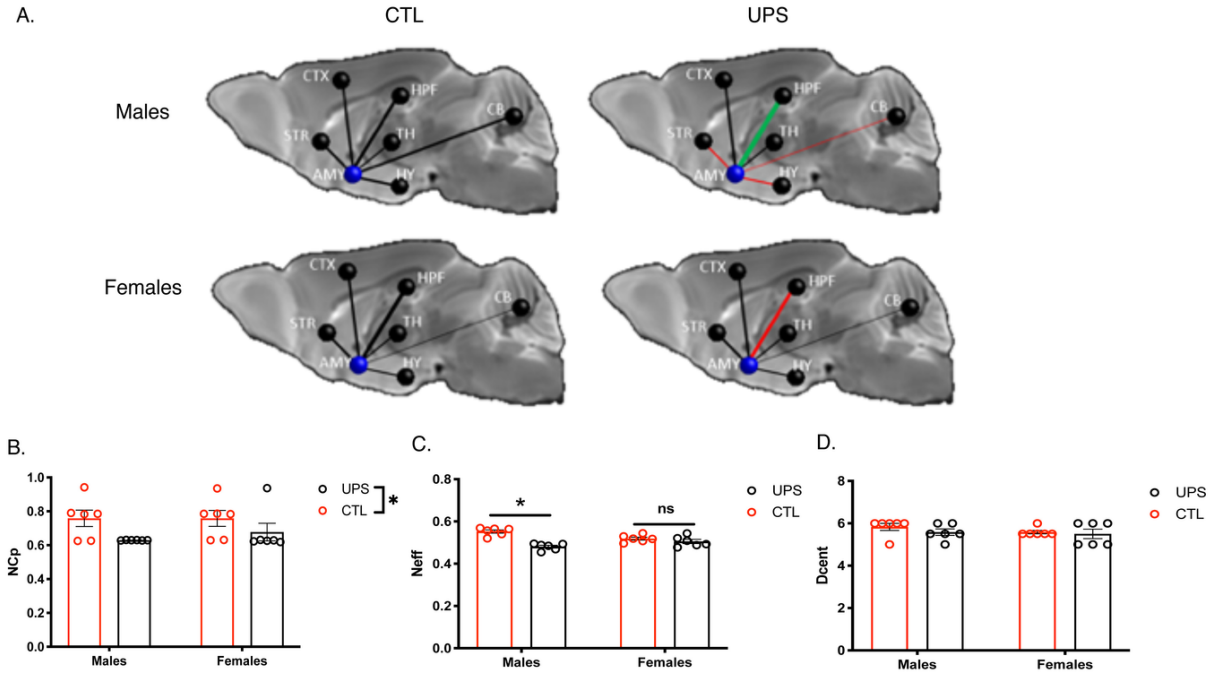

Fig 9. Effects of UPS and sex on amygdala regional connectivity. (A) Schematic representation of amygdala connectivity with other brain regions. Red lines indicate reduced connectivity and green lines indicate enhanced connectivity compared to same sex group. Summary quantification for Nodal centrality (B), Nodal efficiency (C), and Degree of centrality (D). Amy- Amygdala, CB- cerebellum, CTX- cortex, HPF- hippocampus, HY- Hypothalamus, STR- striatum, TH- thalamus. (n= 6 per rearing and sex group). Means and SEM, $*p < 0.05$. GRETNA codes used to assess amygdala connectivity are available in Fig 9-source data.

Discussion

The present study demonstrates that UPS, a complex model of ELS in the mouse, produces long-lasting behavioral and central anatomical changes, some of which are moderated by sex. The role that sex plays in CM-associated outcomes is a growing area of interest (White and Kaffman, 2019c), and it is becoming increasingly clear that the influence of sex may only be seen in certain behaviors, neurodevelopmental processes, and/or specific circuits (White and Kaffman, 2019b). This is in line with findings reported here. For example, here we report similar impact of UPS on body weight, object exploration, contextual conditioning, global connectivity and local volumetric and FA changes in males and females. In contrast, there are robust moderating effects of sex on UPS-induced outcomes in baseline corticosterone levels, fronto-limbic connectivity, and amygdala nodal efficiency.

To the best of our knowledge this is the first paper to utilize high resolution dMRI to assess the effects of ELS on brain structure in both males and females. Using these methods, we demonstrated that males and females exposed to UPS showed several changes that are consistent with those previously reported in

human and animal literature. Specifically, UPS leads to reduced global network efficiency and increased small-worldness that resemble those seen in a large cohort of individuals exposed to CM (Ohashi et al., 2019). Global efficiency, the ability of a network to propagate parallel information, increases during brain development and is associated with improved cognition (Bassett and Bullmore, 2017, Farahani et al., 2019, Huang et al., 2015, Bullmore and Sporns, 2009). The reduced global efficiency seen in UPS mice suggests abnormal maturation of this feature and is consistent with the cognitive deficits seen in the same animals. Small-worldness, a highly conserved network pattern across species, decreases during development (Bassett and Bullmore, 2017, Bullmore and Sporns, 2009, Lupfer et al., 2013). Thus, the increased small-worldness seen in UPS animals is further support of a premature network.

In terms of volumetric changes, UPS mice showed several outcomes that were also reported in humans (summarized in Table S2 in the Supplemental Information). These include, reduced volumes in the frontal cortex (Carrion et al., 2001, De Bellis et al., 2002, Heim et al., 2013), reduced corpus callosum volume (Teicher and Samson, 2016), increased anterior cingulate (Zuo et al., 2019) and increased amygdala volume (Pechtel et al., 2014, Mehta et al., 2009, Tottenham et al., 2010). It is important to note however that increased anterior cingulate and amygdala volumes have not been consistently reported in humans (Teicher and Samson, 2016, Frodl et al., 2017), but increased amygdala volumes is seen in non-human primates (Coplan et al., 2014) and juvenile rats exposed to LBN (Guadagno et al., 2018b). With regard to FA changes, UPS mice showed reduced FA in the corpus callosum which is one of the most robust findings in the human literature (Teicher and Samson, 2016, McCarthy-Jones et al., 2018). In contrast, the increased ventral hippocampal volume seen in UPS mice is diametrically opposite to a large body of work showing reduced hippocampal volume in individuals exposed to CM (Baker et al., 2013, Cohen et al., 2006, Teicher and Samson, 2016). However, a recent analysis using over 3000 subjects found no statistical differences in hippocampal volume (Frodl et al., 2017), underscoring the challenges involved in conducting this work in humans. Inconsistent outcomes have also been reported in rats exposed to LBN, with one report indicating increased volume (Guadagno et al., 2018a) and a second publication noting reduced hippocampal volume (Molet et al., 2016). Additional work is therefore needed to replicate our findings, clarify mechanisms, and the contribution of this expansion in ventral hippocampus to connectivity and behavioral outcomes.

Sex caused volumetric changes in several brain regions including the sensory cortex, hippocampus, thalamus, hypothalamus, and striatum (See Fig S1). These findings are consistent with previous study is C57BL/6 mice (Qiu et al., 2018) and in humans (Gillies and McArthur, 2010, Ruigrok et al., 2014, Goldstein et al., 2001), underscoring the conserved nature of these sexual dimorphic alterations.

Our dMRI scans demonstrate a robust increase in fronto-limbic connectivity in males exposed to UPS (Figs. 5-6). These structural changes are consistent with our previous resting-state fMRI findings in UPS males (Johnson et al., 2018a) and in male rats exposed to LBN (Bolton et al., 2018). The observation that the hyper connectivity is unique to males and is not seen in females, reveals a novel sex-dependent effect of UPS on this specific circuit. Sex-specific alterations in connectivity have been reported in adolescents and young adults exposed to ELS (Crozier et al., 2014, Herringa et al., 2013, Colich et al., 2017) but not in adult subjects (Ohashi et al., 2019, Dannlowski et al., 2012). Nevertheless, increased amygdala connectivity was associated with higher levels of symptomatology including anxiety and depression in adulthood (Ohashi et al., 2019), suggesting that findings in UPS males may have translational utility for some types of ELS in humans. Exposure to maternal separation also induced sex-specific changes in the connectivity between the basolateral amygdala and the PFC in juvenile rats, however these changes appear to be more prominent in females (Honeycutt et al., 2020). Together, these findings highlight intriguing sex-dependent effects of varying ELS paradigms on fronto-limbic connectivity in rodents.

Despite clear differences in UPS-induced fronto-limbic connectivity between males and females, their behavioral profiles were remarkably similar and raise the possibility that alterations in connectivity may lead to different behavioral outcomes in males and females. To address this issue, we conducted a moderated mediation analysis to test whether sex was moderating the relationships between UPS-induced changes in connectivity between the amygdala and the ventral hippocampus and their resulting influence on freezing

behavior. We focused on these connections for several reasons, i.e., UPS induced opposing outcomes in males and females (increased in males and reduction in females, Fig 6D), there were clear volumetric changes in both areas, and these areas play an important role in fear learning. Our analysis suggests that alterations in connectivity in males, but not females, is in part responsible for the reduced freezing behavior seen in UPS mice (Fig 7). Future work should use optogenetic or chemogenetic approaches to further test the conclusions drawn from this moderated-mediation model, but the findings reported here are consistent with other examples showing that similar behavioral outcomes can be driven by different mechanisms in males and females (Labonte et al., 2017, Sorge et al., 2015)

We were unable to replicate our UPS-induced anxiety-like behavior in the open field or the EPM (Johnson et al., 2018b). One possible explanation for this failure to replicate may be due to differences in the implementation of the paradigm. For instance, the current studies were conducted in a room that was roughly 10 times quieter than the room utilized in previous work (see Animals heading within the Methods section) and the work was conducted by different individuals. These outcomes highlight the sensitivity of the OF and the EPM tests to multiple variables such as sex of the experimenter, phase of the light/dark cycle, degree of illumination in the arena, noise, smells, and order of behavioral testing (Tractenberg et al., 2016, Alves et al., 2019, Murthy and Gould, 2018, Lehmann and Feldon, 2000), and underscores the need to develop more robust behavioral tests of anxiety, see (Oberrauch et al., 2019, Meyer et al., 2019) for some encouraging examples. Consistent with our previous work (Johnson et al., 2018a) we found that UPS caused a robust reduction in weight across different ages (Fig 1). Moreover, male and female UPS mice spent less time exploring novel objects and showed reduced contextual freezing, findings that were replicated in two independent cohorts (Fig 2 and Table S1) and in additional studies conducted in the lab (data not shown).

Conclusion

Using high resolution dMRI we show that UPS induces several neuroanatomical changes in the adult rodent brain that mimic those seen in individuals exposed to CM. Some of the most important examples include alterations in global network connectivity, reduced volume and FA in the corpus collosum, frontal cortex atrophy, and increased amygdala size, findings that were seen in both males and females. In contrast, exposure to UPS induced sex-specific changes in fronto-limbic connectivity, raising the possibility that these connections may mediate different behavioral outcomes in males and females. This possibility is consistent with findings from a moderated-mediation analysis indicating that UPS-induced alterations in connectivity between the amygdala and the ventral hippocampus is responsible for reduced contextual freezing behavior in males but not in females. These findings reinforce the continued need for additional research into the moderating effects of sex on CM-induced outcomes in both humans and animals.

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