ANIMAL MODELS OF ANXIETY AND DEPRESSION: EXPLORING THE UNDERLYING MECHANISMS OF SEX DIFFERENCES

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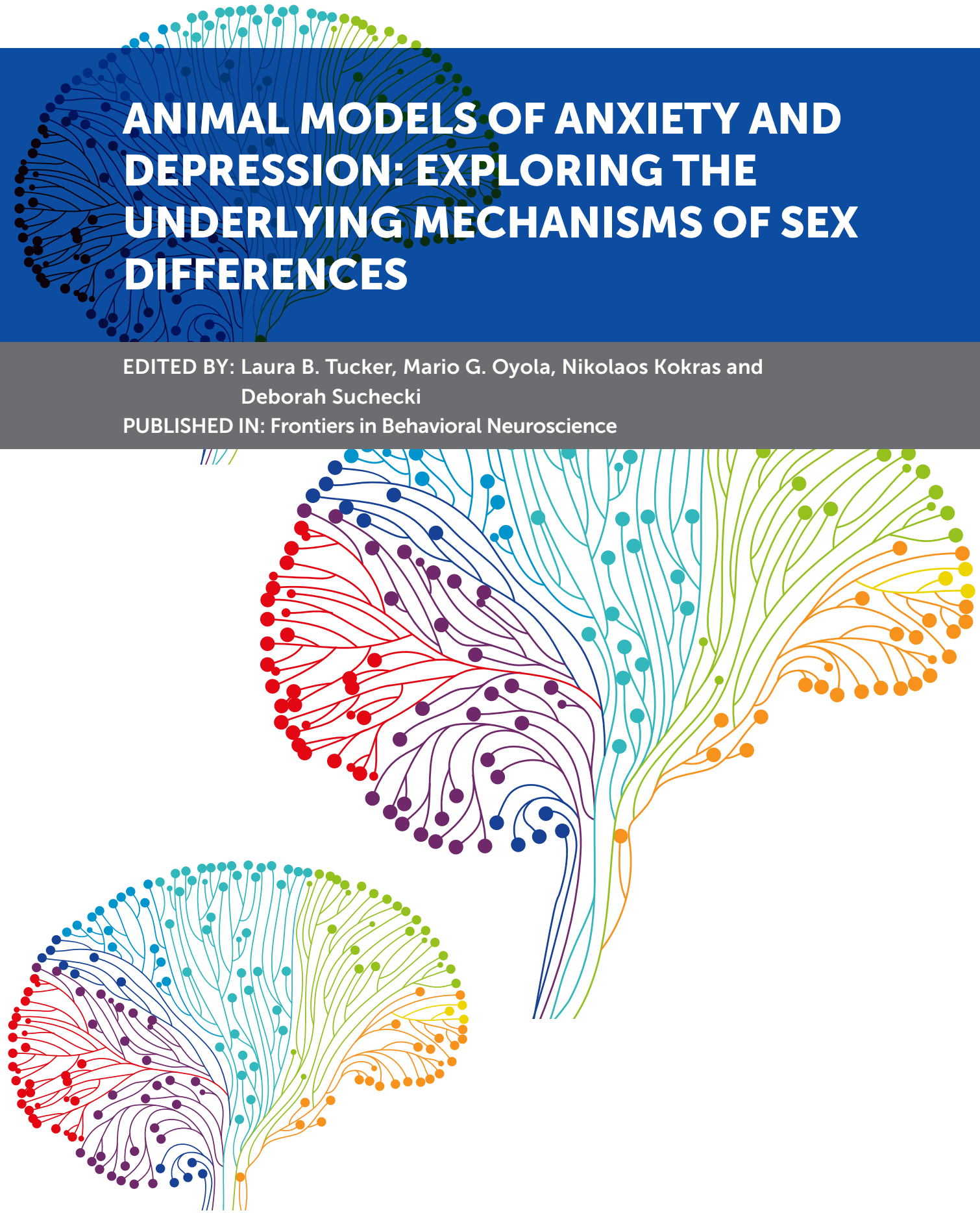

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ANIMAL MODELS OF ANXIETY AND DEPRESSION: EXPLORING THE UNDERLYING MECHANISMS OF SEX DIFFERENCES

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EDITORIAL
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Editorial: Animal Models of Anxiety and Depression: Exploring the Underlying Mechanisms of Sex Differences

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Keywords: sex differences, females, anxiety, depression, gender differences

Editorial on the Research Topic

Animal Models of Anxiety and Depression: Exploring the Underlying Mechanisms of Sex Differences

Anxiety and depression carry a significant burden and disproportionally affect more women than men (Balta et al., 2019). Moreover, men and women differ in symptomatology and responses to psychotropic agents, highlighting the need for a better understanding of the mechanisms leading to these sex differences (Butlen-Ducuing et al., 2021). As the exact neurobiology of these disorders still eludes us, animal models are routinely employed to study anxiety and depression. Although male animals have been traditionally used in pre-clinical studies, the inclusion of both sexes, as recently dictated by NIH policies (Clayton and Collins, 2014), presents an opportunity to explore sex differences in the biological underpinnings, contributions to stress, and other influences that may underlie emotional dysregulation and abnormal performance at behavioral endpoints. Emerging evidence indeed uncovers significant sex differences in most animal models of depression and anxiety, either at baseline or following treatment. Such differences may have substantial implications for translating preclinical to clinical research (Kokras and Dalla, 2014, 2017). Unfortunately, animal models have also yielded inconsistent results and often report greater anxiety- or depressive-like symptoms in male than in female animals or do not show sex differences. For example, a recent study found that the frequently-employed chronic unpredictable mild stress model was more likely to induce depressive-like behaviors in male than in female rats (Iqbal et al., 2020).

In this Research Topic, experiments performed by Eltokhi et al. at two different developmental stages during adolescence revealed strain but no sex differences in a set of depression-related tests, including tail suspension, sucrose preference, and forced swim tests. However, when tested in the anxiety-related hyponeophagia test, male and female mice behaved differently. In continuation, Pitzer et al. showed that, like in adolescent, neither adult C57BL/6N, DBA/2 or FVB/N present significant baseline sex differences in behavioral tests measuring immobility in tail suspension and forced swim tests, as well as anhedonia in the sucrose preference test. However, adult male and female mice showed significantly different results in the baseline apathy-like behaviors depending on the investigated strain. These studies by Eltokhi et al. and Pitzer et al. provide a good baseline characterization of the C57BL/6N, DBA/2 and FVB/N mouse strains regarding the absence of

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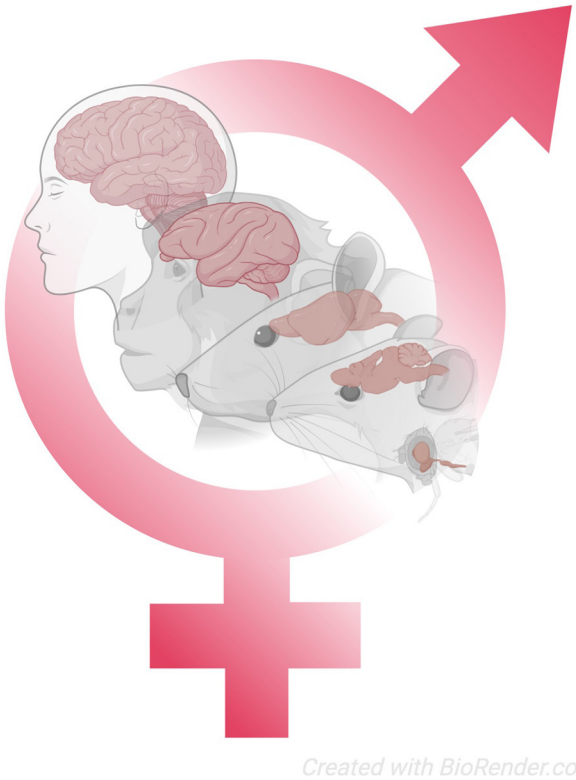

FIGURE 1 | Animal models of anxiety and depression: Exploring the underlying mechanisms of sex differences. Image created with BioRender.com.

sex differences at two different ages in certain tests. Moreover, they highlight the importance of considering several factors, such as strain, age, type of tests, and behavioral outcome when studying sex differences. Such an approach that avoids overlooking critical factors that can influence the planning, conduct and results of studies can increase the reproducibility of preclinical research (Sil et al., 2021). As Eltokhi et al. and Pitzer et al. note, inconsistencies of results between different laboratories investigating rodent models of depression and anxiety call for better standardization and normalization when designing experiments exploring sex differences. In this context, it is important to use appropriate animal models that reproduce specific aspects of the complex clinical manifestations at the behavioral and molecular levels. In this Research Topic Touchant and Lebonte summarize findings from animal models and discuss genome wide transcriptional strategies for such complex clinical manifestations. Such strategies may provide crucial insights into the neurobiological underpinnings of these diseases and the basis of sex-specific molecular responses in experimental animals and humans. Another equally important issue is screening for new psychotropic drugs using both sexes. In this context, Yin et al. studied the effects of Yueju-Ganmaidazao Decoction (YG), a substance with potential antidepressant actions, in relation to NO-cGMP signaling. They found that both YG and escitalopram induce antidepressant-like behavioral responses in both sexes. However, both drugs enhanced CaMKII-nNOS expression in the hippocampus of female mice, in opposition to what was observed in male mice, despite the same behavioral antidepressant response in both sexes. This concept highlights another issue in studying sex differences: that the same behavioral response can be observed in both sexes, but the underlying neurobiological processes that lead to the same behavioral response in male and female brains are not necessarily identical, as also noted previously (Kokras et al., 2011).

On the other hand, brain regions such as the hippocampus and the prefrontal cortex have long been implicated in the neurobiology of stress, anxiety and depression (Duman et al., 1997). Emerging preclinical data identify prominent sexual divergence in these regions as reviewed in this Research Topic by Wallace and Myers, who suggest that chronic stress has sex-specific effects on the rodent infralimbic cortex excitatory/inhibitory balance that may account for sex differences in the prevalence and course of mood disorders. Moreover, McNamara et al. studied sex differences in limbic responses after shock wave exposure, which resulted in a transient blood-brain barrier (BBB) breach of variable severity. Subsequent testing showed sex differences in various behavioral tests of anxiety and depression and in c-Fos expression post-injury. The authors suggest that the increased vulnerability of women to post-traumatic stress disorder could be related to the mild effects of post-injury behavioral and neuronal effects that they observed in the female mice in their study. Sex differences in the BBB is an emerging subject of interest. Dalla et al. summarized preclinical and clinical findings on how sex and sex hormones can influence the activity of BBB transporter systems. They concluded that accumulated evidence supports the existence of several sex differences in expression and activity of BBB transport proteins, which are also modulated by gonadal hormones. As is the case with the BBB, to understand sex differences following stress, we must consider how all cell types within the central nervous system are involved. Indeed Wegener and Neigh in their review discuss the effects of stress and sex steroids on astrocytes and oligodendrocytes. They conclude that studies exploring the mechanisms by which glia are altered by stress and steroids will provide insight into sex differences in animal models. In a similar context Michailidis et al. used the spared nerve injury (SNI) model of neuropathic pain and noted that behavioral depressive-like responses were first observed at different time points in male and female animals. They then proceeded to immunohistochemical analysis and showed that microglial cells were more numerous in female mice in the contralateral ventral anterior cingulate cortex, suggesting that different patterns of glial cell activation may be associated with pain processing and affect in male and female animals. As Gaspar et al. note in their study, microglia, the immune cells of the brain, are involved in the stress-related neuronal and behavioral response, and thus contribute to the development of stress-related psychopathologies. The authors found that following short-term unpredictable chronic mild stress, both male and female rats showed anxiety-like

behavior. However, after longer term chronic stress, male animals demonstrated depression- and anxiety-like behaviors but females demonstrated only the later. Subsequent investigation showed that microglia cells in the dorsal hippocampus and in the nucleus accumbens were found to adapt differently according to duration of stress, brain region studied, and, importantly, sex of the animals.

Finally, early life adversity in humans and rodents is associated with sex-specific emergence of anxious and depressive behaviors, and Ellis and Honeycutt summarized in this Research Topic such findings and suggest the possibility of a combined role of sex hormones and calcium-binding protein parvalbumin expressing neurons driving differences in behavioral outcomes associated with affective dysfunction following early life adversity. The overall message from this Research Topic **(Figure 1)** is that sex differences are observed in many different levels of preclinical research, and as the field of sex differences in neuroscience emerges and accumulates more data, a better understanding of such differences may improve our understanding of depression and anxiety, and lead to better treatments for both diseases.

AUTHOR CONTRIBUTIONS

NK compiled the first draft. All authors made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Latent Sex Differences in CaMKII-nNOS Signaling That Underlie Antidepressant-Like Effects of Yueju-Ganmaidazao Decoction in the Hippocampus

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Previous studies have demonstrated that Yueju-Ganmaidazao (YG) decoction induces rapid antidepressant-like effects, and the antidepressant response is mostly dependent on the suppression of nitric oxide-cyclic guanosine monophosphate signaling in male mice. This study aimed to investigate the sex difference mediated by calcium/calmodulin-dependent protein kinase II (CaMKII)-neuronal nitric oxide synthase (nNOS) signaling involved in the antidepressant-like effect of YG in mice. We found that the immobility times in the tail suspension test (TST) were found to be decreased after the single injection of YG in male and female mice with the same dosage. Additionally, chronic administration for 4 days of subthreshold dosage of YG and escitalopram (ES) also significantly decreased the immobility time in mice of both sexes. Chronic subthreshold dosage of YG and ES in LPS-treated mice and in chronic unpredictable stress (CUS) mice both decreased the immobility time, which was increased by stress. Meanwhile, in CUS-treated mice, sucrose preference test, forced swimming test, and open field test were applied to further confirm the antidepressant-like effects of YG and ES. Moreover, CUS significantly decreased the expression of nNOS and CaMKII, and both YG and ES could enhance the expression in the hippocampus of female mice, which was opposite to that in male mice, while endothelial nitric oxide synthase expression was not affected by stress or drug treatment neither in male mice nor in female mice. Finally, subthreshold dosage of YG combined with 7-nitroindazole (nNOS inhibitor) induced the antidepressant-like effects both in female and in male mice, while the single use of YG or 7-NI did not display any effect. However, pretreatment with KN-93 (CaMKII inhibitor) only blocked the antidepressant-like effect of high-dosage YG in female mice. Meanwhile, in CUS mice, chronic stress caused NR1 overexpression and inhibited cAMP response element binding protein action, which were both reversed by YG and ES in male and female mice, implying that YG and ES produced the

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same antidepressant-like effect in mice of both sexes. The study revealed that chronic treatment with a subthreshold dose of YG also produced antidepressant-like effects in female mice, and these effects depended on the regulation of the CaMKII-nNOS signaling pathway.

Keywords: sex difference, YG, nNOS, CaMKII, CREB

INTRODUCTION

Depression is one of the most common mental disorders that is associated with high morbidity and mortality, and it affects more than 350 million people in the world (Menard et al., 2016). People suffering from depression always display disrupted mood, altered sleep, and memory dysfunction (Benson et al., 2015). According to the World Health Organization (WHO), depression is the leading cause of disability and will become the second most common disease in the world at 2025. Sex is an important factor in influencing the susceptibility to psychiatric illness (Agarwal et al., 2020). Women are more likely to suffer from anxiety disorders, while men are more likely to suffer from substance-use disorders, suggesting that there are some differences in the mechanisms of depression between the sexes. More importantly, the incidence of depression in females is about two to three times of that in males, and this issue has attracted increasing attention in recent years (Bekker and van Mens-Verhulst, 2007; Essau et al., 2010). Furthermore, evidence suggests that the sex-dependent differences have been observed in the antidepressant-like effects of ketamine, which are believed to modulate the NMDA signaling in the brain, and thus potentially indicates that there is a different underlying pathology between men and women (Carrier and Kabbaj, 2013; Franceschelli et al., 2015). Herein, we designed this study to search for clues underlying the sex differences in depression in the response to antidepressants.

Although a number of antidepressants exist, such as widely used selective serotonin reuptake inhibitors (SSRIs), a remarkable population of patients never attain a sustained remission of their symptoms (Zanos et al., 2016). Fast-acting antidepressants like ketamine may be used to treat depression in patients who have no response to SSRIs and to relieve depressive symptoms quickly (Ballard et al., 2015). Unfortunately, addictive and side effects of ketamine limit its clinical application (Wang et al., 2018). Yueju pill and Ganmaidazao decoction are widely used prescriptions of traditional Chinese medicine. YG (Yueju-Ganmaidazao) decoction has also been demonstrated to induce a rapid and lasting antidepressant-like effect after a single administration in male mice (Zhang H. et al., 2020). However, it remains to be shown whether the chronic or a low dose of YG will still reveal the antidepressant-like effects.

Yueju-Ganmaidazao can produce rapid antidepressant-like effects mostly by reducing the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway in the hippocampus of male mice. Meanwhile, we have found that YG can rapidly enhance the expression of cAMP response element binding protein (CREB) signal in the hippocampus of mice. Furthermore, pharmacological experiments show that blocking the NO-cGMP pathway can reverse the rapid antidepressant-like effect of YG after a single administration, suggesting that NO plays a leading role in mediating the effect (Zhang H. et al., 2020). In addition, escitalopram (ES), one of the commonly used clinical antidepressants, showed a safer profile among antidepressants treated in depression patients (Solmi et al., 2020). ES has been found to depend on the NO pathway to produce antidepressant-like effect (Ludka et al., 2013). In the study, we used ES as a positive control. Our study implied that NO signaling may play an important role in promoting antidepressant-like effect of YG or ES. Our previous work has also demonstrated that the NO concentration can be affected by stress or Yueju pill, which induces depressive or antidepressant-like behavior (Wang et al., 2018). However, most existing research mainly focuses on males, while sex differences in the antidepressant-like effects of drugs remain elusive. Thus, it is essential to focus on whether the NO pathway involved in an antidepressant-like effect is dependent on sex. NO is formed by the NO synthase, endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS), and plays an important role in the regulation of antidepressant-like effect in mice. Administration of 7-nitroindazole (7-NI; a nNOS-specific inhibitor) combined with YG can induce antidepressant-like effects in male mice (Zhang H. et al., 2020). It is also shown that nNOS is phosphorylated at Ser847 by calcium/calmodulin-dependent protein kinase II (CaMKII), leading to neuroprotective effects against cerebral ischemia injury (Osuka et al., 2002; Takata et al., 2020). More importantly, nNOS exists in GABAergic inhibitory interneurons where CaMKII co-localizes in the rat hippocampal primary neurons. CaMKII phosphorylates and influences nNOS *via* its specific Ser847 residue (Hayashi et al., 1999; Araki et al., 2020). Meanwhile, like nNOS, CaMKII is a calcium-dependent enzyme (Stein et al., 2020). The CaMKII–nNOS signaling was revealed previously to display neuroprotective effects underlying ischemic preconditioning (Wang et al., 2016), while the action of NO in the adult brain was also demonstrated to be associated with the different expression pattern of CaMKII–nNOS signaling (Packer et al., 2005). Thus, whether the CaMKII–nNOS signaling is participating in the antidepressant-like effect of YG or ES is worth studying clearly.

Based on this, we proposed a hypothesis that the mechanisms involved in sex differentiation of antidepressant-like effects in mice are mediated by the differences in NO changes caused by nNOS, which requires the activation of CaMKII. Here, we aimed to investigate the antidepressant-like response to chronic YG in male and female mice. Then, we determined the expression levels of nNOS as well as eNOS, the potential upstream effector CaMKII and NR1, and examined whether male and female mice

exhibit similar or distinct molecular changes in response to stress exposure as well as YG treatment, with a focus on whether the same molecular pathways are influenced similarly across the hippocampus.

MATERIALS AND METHODS

Animals

Male and female ICR mice (aged 7–8 weeks, 20–25 g) were purchased from Shanghai Sippr BK Laboratory Animals Company. Mice were adapted to animal facilities for 1 week before the experiment. Mice were kept in standard laboratory conditions (temperature: 23 ± 2°C; indoor humidity: 50 ± 10%), with a light/dark cycle of 12:12 h, and allowed free access to rodent chow and drinking water. The procedures complied with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee.

Preparation of Formula

Yueju-Ganmaidazao decoction was processed and purified as described in our previous work, and the quality control demonstrated that the extract was stable for application (Zhang H. et al., 2020). Briefly, the medicinal plants used to prepare YG decoction were *Cyperus rotundus* L. (CR), *Ligusticum chuanxiong* Hort (LC), *Gardenia jasminoides* Ellis (GJ), *Atractylodes lancea* (Thunb) DC (AL), *Massa fermentata* (MF), *Curabitur triticum* (CB), *Licorice* (LR), and *Fructus Ziziphus Jujuba* (FZJ). The usages of single herbs were CR 100 g: LC 100 g: GJ 100 g: AL 100 g: MF 100 g: CB 125 g: LR 250 g: FZJ 375 g. All medicinal Chinese herbs were purchased from the outpatient department of the School of Medicine. The above materials were soaked with water (1:8 ratio) for 30 min and heated for 1 h, then filtered, and collected. This procedure was repeated twice. The yield of YG extraction was 20% and administrated intragastrically. The dosage of each drug followed the Chinese Pharmacopeia.

Animal Treatment

Chronic Drug Treatment in Normal Mice

Female and male mice were randomly divided into three groups, namely, control group, YG (1 g/kg) group, and ES (10 mg/kg) group, with eight mice in each group. YG and ES groups were injected with drugs for 4 consecutive days, while the control group received the same dose of saline.

LPS-Induced Procedure

Female and male mice were randomly divided into three groups, namely, LPS-treated control (1 mg/kg) group, LPS + YG (1 g/kg) group, and LPS + ES (10 mg/kg) group, with eight mice in each group. Lipopolysaccharide (LPS, Aladdin, L118716) was prepared in saline (0.9% sodium chloride). LPS (i.p.) was administered once daily, while YG and ES were given by gavage once a day, and the control group was given the same amount of saline; these above administrations were continued for 4 days. Twenty-four hours after the last drug administration, behavioral tests were performed.

Drugs Interaction Procedure

Female and male mice were pretreated with saline (0.9%) or 7-NI (30 mg/kg, dissolved in 5% Tween 80) or KN-93 (5 mg/kg) for 1 h, and then the mice were treated with the administration of saline, YG (2.5 g/kg, i.g.), or ES (20 mg/kg). After an hour, the tail suspension test (TST) was performed to measure the despair behavior of mice. All drugs were administered i.p. except YG solution.

Chronic Unpredictable Stress

The CUS experimental protocol was followed as described in Tang et al. (2015). Mice lived alone and received unpredictable stress for 3 weeks. Stress was given in a random, unpredictable order every day: food deprivation 24 h, drink deprivation 24 h, 45°cage tilt for 24 h, cage shaking (high-speed horizontal shaking, 200 rpm) for 40 min, cage wet (200 ml water per cage) for 20 h, overnight illumination and being bound in a 50-ml tube for 6 h. All CUS mice were single-housed until the end of the experiment, and the control mice were normally reared for comparison.

Tail Suspension Test

The tail of the mouse was taped to the hanging hook, and the tip of the tail was about 1 cm from the tape. Mice were placed in a universal sound-proof behavior box so that they were hung upside down with their heads 20 cm from the bottom of the box. The mice struggled to overcome the abnormal posture, and animal activities were recorded. Any-maze software was used to record the activity of mice for 6 min. The immobility time during the last 4 min was analyzed.

Forced Swimming Test

The mice were placed in a 10-cm-deep beaker (10cm × 30cm) filled with warm water (25 ± 2°C). Mice were observed for 6 min, and the immobility time during the last 4 min was recorded. When mice floated passively without struggling, they were considered immobile. The immobility time was recorded by using the Any-maze software.

Sucrose Preference Test

All mice were single-housed and adapted to a sucrose solution (2%) for 72 h according to previous work (Xue et al., 2016). After 18 h of fasting and no drinking, the mice were separated in a single cage and presented with two bottles: one bottle of 2% sucrose solution and one bottle of pure water. The sucrose consumption was observed in 2 h. Sucrose preference was calculated by the formula: sucrose consumption/% = [(sucrose intake) / (sucrose intake + water intake)] × 100%, and was standardized by the weight of each animal.

Western Blot

Mice were sacrificed, the brains were rapidly removed, and the hippocampus was dissected out and dissolved in a RIPA buffer containing a protease inhibitor and a phosphatase

inhibitor. Brain tissues were homogenized, and western blot analyses were carried out. Protein lysates were separated by 8% SDS–PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% BSA for 1 h, the membrane was blocked with primary antibodies for eNOS (Millipore, 1: 1000), nNOS (Millipore, 1: 1000), CaMKII and NR1 (Cell Signaling Technology, 1: 1000), and β-tubulin (Proteintech, 1: 5000) and incubated at 4°C for 12 h, and then the membrane was incubated with the secondary antibody for 1 h at room temperature. The blots were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.). All target proteins were normalized to β-tubulin. All experiments were performed at least three times.

Statistical Analysis

Multiple comparisons were made using one-way ANOVA or two-way ANOVA followed by Bonferroni *post hoc* tests. All data are indicated as the mean ± SEM and are statistically significant at the 0.05 level.

RESULTS

Single Administration of Effective Dosage of YG Displayed the Same Antidepressant-Like Effect in Both Male and Female Mice

The TST was used to screen rapid antidepressant-like effects of YG in female and male mice. Twenty-four hours after a single administration of YG, the immobility time was significantly reduced in female mice [**Figure 1A**, one-way ANOVA, $F(3,28) = 23.88$, $p < 0.0001$] both at the dosage of 2.5 g/kg ($p < 0.001$) and at the dosage of 2.0 g/kg ($p < 0.001$), and 2.0 g/kg was equivalent to the human clinical dose. Meanwhile, in male mice, the immobility time [**Figure 1B**, one-way ANOVA, $F(3,36) = 15.04$, $p < 0.0001$] was significantly decreased only at the dosage of 2.5 g/kg ($p < 0.001$). The results showed that YG produced the same antidepressant-like effect in female and male mice, but the effective dosage range was wider in female mice.

Subthreshold Dosage of YG Could Also Induce Antidepressant-Like Effect in LPS-Induced Model Both in Male and in Female Mice

Previous studies have focused on the role of the rapid antidepressant-like action of YG, but the chronic treatment with the formula is more common clinically. The subchronic mice with LPS-injection for 4 consecutive days were used to evaluate the antidepressant-like effect of YG and ES. LPS is a well-known proinflammatory drug and is widely employed to induce the depressive-like behavior (Ali et al., 2020); 1 g/kg of YG ($p < 0.001$) and 10 mg/kg of ES ($p < 0.001$) both produced antidepressant-like effects in female mice [**Figure 2A**, left hind paw: one-way ANOVA, $F(2,23) = 103.9$, $p < 0.0001$] after chronic injections. Meanwhile, immobility times of male mice during the TST [**Figure 2B**, left hind paw: one-way ANOVA, $F(2,23) = 30.24$, $p < 0.0001$] were significantly decreased in YG-treated group ($p < 0.001$) and ES-treated group ($p < 0.001$).

After chronic treatment with YG ($p < 0.001$) or ES ($p < 0.001$) in LPS-treated mice, the immobility time was significantly decreased compared to that of the control group [**Figure 2A**, right hind paw: one-way ANOVA, $F(2,21) = 17.93$, $p < 0.0001$]. In male mice [**Figure 2B**, right hind paw: one-way ANOVA, $F(2,21) = 15.73$, $p < 0.0001$], chronic treatment of YG ($p < 0.001$) and ES ($p < 0.001$) both induced significant antidepressant-like effects. After treating with LPS, the immobility time was significantly increased both in female ($p < 0.001$) and in male ($p < 0.001$) mice. This effect was not affected by sex [two-way ANOVA, female interaction: $F(2,44) = 0.7460$, $p = 0.4802$, male interaction: $F(2,44) = 2.686$, $p = 0.0793$]. Collectively, the antidepressant-like effects of chronic YG and ES showed no significant sex differences.

Chronic Administration of Subthreshold Dose YG Could Also Induce Antidepressant-Like Effect in CUS Female and Male Mice

Both female and male mice received CUS for 2 weeks. Then, mice were given either saline or YG (1 g/kg) or ES (10 mg/kg)

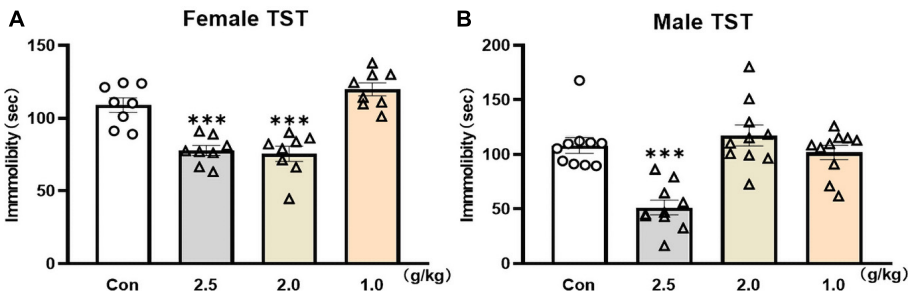

FIGURE 1 | Fast screen of potential rapid antidepressant-like effect of YG in TST of male and female mice. **(A)** Immobility time was measured for the last 4 min during the total 6-min testing time in female mice after different dosages of YG treatments (1.0, 2.0, and 2.5 g/kg). **(B)** Immobility time was measured for the last 4 min during the total 6-min testing time in male mice after different dosages of YG treatments (1.0, 2.0, and 2.5 g/kg). Independent mouse was used to test the behaviors at each time point. ***$p < 0.001$, compared with the control group, one-way ANOVA, n = 8–10/group.

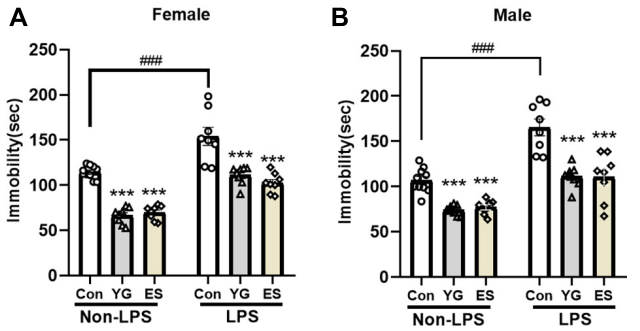

FIGURE 2 | Chronic YG (1 g/kg) and ES (10 mg/kg) displayed significant effects on immobility times in the TST both in **(A)** female and in **(B)** male mice as well as in non-LPS treatment or LPS treatment. $^{***}p < 0.001$, compared with the control group in non-LPS or LPS treatment, one-way ANOVA, n = 8–10/group. The interaction between non-LPS and LPS treatment was analyzed by two-way ANOVA. $^{###}p < 0.001$ compared to non-LPS control group.

for 5 weeks after the establishment of CUS model. After the last administration of saline or drug, the immobility time in the TST and in the forced swimming test (FST) was measured in both sexes, and the sucrose preference test (SPT) was used to evaluate anhedonia-like stage of the mice (**Figure 3A**). The results showed that, compared with the saline group, the preferences for sucrose solution of female mice [**Figure 3B**, one-way ANOVA, $F(3,35) = 17.99$, $p < 0.0001$] and male mice [**Figure 3B**, one-way ANOVA, $F(3,39) = 14.08$, $p < 0.0001$] were significantly reduced (female: $p < 0.001$, male: $p < 0.001$). After 5 weeks of treatment, both YG ($p < 0.001$) and ES ($p < 0.001$) could rescue the anhedonia phenomena induced by CUS. The total solution (containing sucrose or not) was not affected by stress or drugs treatment (**Supplementary Figure 1**). In the TST, the immobility time was significantly increased after stress [**Figure 3C**, one-way ANOVA, female: $F(3,24) = 6.090$, $p < 0.01$, $p < 0.05$ compared to the control group; male: $F(3,28) = 34.00$, $p < 0.0001$, $p < 0.001$ compared to Con group], while YG (female: $p < 0.01$, male: $p < 0.001$) and ES (female: $p < 0.01$, male: $p < 0.001$) significantly decreased the immobility time compared to the CUS group. In the FST, the CUS group displayed higher immobility time, while YG group (female: $p < 0.001$, male: $p < 0.05$) and ES group (female: $p < 0.001$, male: $p < 0.05$) showed lower immobility times in both male and female mice [**Figure 3D**, one-way ANOVA, female: $F(3,32) = 13.19$, $p < 0.0001$; male: $F(3,26) = 4.355$, $p < 0.05$]. Meanwhile, the locomotor activity was not affected by stress or drugs in total distance [**Figure 3E**, one-way ANOVA, female: $F(3,38) = 2.399$, $p = 0.0830$; male: $F(3,43) = 0.7807$, $p = 0.5112$] or in traveling time in center area [**Figure 3F**, one-way ANOVA, female: $F(3,38) = 0.5968$, $p = 0.6210$; male: $F(3,43) = 1.656$, $p = 0.1907$] in the open field test (OFT). We found no significant sex differences after stress or drug treatments [two-way ANOVA, **Figure 3B**: $F(3,74) = 0.9566$, $p = 0.4179$; **Figure 3C**: $F(3,52) = 3.211$, $p = 0.0304$; **Figure 3D**: $F(3,58) = 1.271$, $p = 0.2928$; **Figure 3E**: $F(3,81) = 0.5919$, $p = 0.6221$; **Figure 3F**: $F(3,81) = 1.211$, $p = 0.3110$].

Subthreshold Dosage of YG Combined With 7-NI Could Induce an Antidepressant-Like Effect on Female and Male Mice

The role of nNOS in YG's antidepressant-like action was assessed by using 7-NI, a nNOS inhibitor. 7-NI (30 mg/kg) inhibited the antidepressant-like effect of YG (1 g/kg) in female mice [two-way ANOVA, main effect of treatment, $F(1,30) = 23.88$, $p < 0.001$; pretreatment, $F(1,30) = 4.071$, $p < 0.05$; treatment × pretreatment interaction, $F(1,30) = 20.38$, $p < 0.001$, **Figure 4A**]. 7-NI (30 mg/kg) also inhibited the antidepressant-like effect of YG (1 g/kg) in male mice [two-way ANOVA, the main effect of treatment, $F(1,28) = 14.91$, $p < 0.001$; pretreatment, $F(1,28) = 20.65$, $p < 0.001$; treatment × pretreatment interaction, $F(1,28) = 7.205$, $p < 0.05$, **Figure 4B**]. Results displayed that YG combined with 7-NI could produce antidepressant-like effects both in female and in male mice (Zhang H. et al., 2020). Furthermore, in the FST, 7-NI (30 mg/kg) inhibited the antidepressant-like effect of YG (1 g/kg) in female mice [two-way ANOVA, the main effect of treatment, $F(1,34) = 10.46$, $p < 0.01$; pretreatment, $F(1,34) = 12.25$, $p < 0.01$; treatment × pretreatment interaction, $F(1,34) = 6.625$, $p < 0.05$, **Figure 4C**].

nNOS and CaMKII Showed Different Expression Patterns After Stress or Drug Treatment in Male and Female Mice

Our previous work found that the antidepressant-like effect of YG was dependent on NO-cGMP pathway. In this study, we investigated whether the NOS expression was affected by chronic YG or ES in mice of different sexes. After chronic treatment with YG or ES in mice, neither in female mice [**Figure 5A**, left hind paw, one-way ANOVA, $F(2,9) = 0.1409$, $p = 0.8705$] nor in male mice [**Figure 5B**, left hind paw, one-way ANOVA, $F(2,9) = 0.5718$, $p = 0.5838$], nNOS expression was not affected by the drugs. In LPS-treated mice, we also found that the expression of nNOS was not affected by YG or ES in female mice [**Figure 5A**, right hind paw, one-way ANOVA, $F(2,9) = 0.05090$, $p = 0.9506$] or in male mice [**Figure 5B**, right hind paw, one-way ANOVA, $F(2,9) = 0.1868$, $p = 0.8327$]. Neither LPS treatment nor sex showed differences in nNOS expression by using two-way ANOVA [female interaction: $F(2,18) = 0.1887$, $p = 0.8297$; male interaction: $F(2,18) = 0.4441$, $p = 0.6483$]. Meanwhile, CaMKII expression was similar to the nNOS expression in the hippocampus [**Figure 5C**, left hind paw, one-way ANOVA, $F(2,9) = 0.1161$, $p = 0.8917$; right hind paw, one-way ANOVA, $F(2,9) = 0.03030$, $p = 0.9703$; **Figure 5D**, left hind paw, one-way ANOVA, $F(2,9) = 0.05090$, $p = 0.9506$; right hind paw, one-way ANOVA, $F(2,9) = 0.3785$, $p = 0.6953$; two-way ANOVA, female interaction: $F(2,18) = 0.1660$, $p = 0.8483$; male interaction: $F(2,18) = 0.2262$, $p = 0.7998$]. Furthermore, after CUS exposure, the expression of nNOS [**Figure 5E**, one-way ANOVA, $F(3,17) = 13.84$, $p < 0.0001$] was significantly decreased ($p < 0.01$), which was reversed by YG ($p < 0.01$) and ES ($p < 0.001$) treatment in female mice. However, in

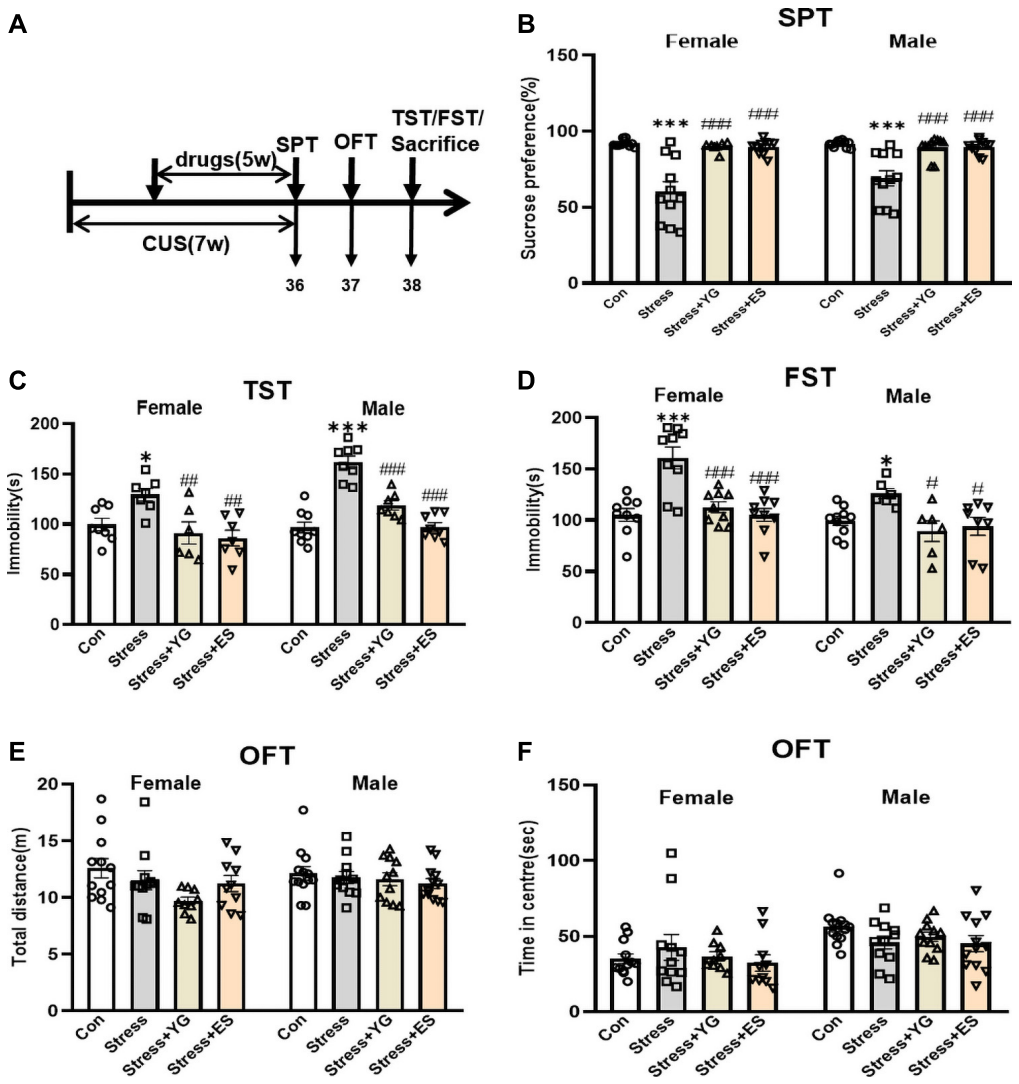

FIGURE 3 | Antidepressant-like behaviors following chronic YG or ES in CUS mice. **(A)** Mice were exposed to chronic unpredictable stress (CUS) and received treatment of YG or ES. Control (Con) mice were not exposed to stress but received saline treatment. Behaviors were tested after the last drug administration. **(B)** Mice were tested in the SPT after the last drug administration. **(C)** Mice were tested in the TST in 2 days after the last drug administration. **(D)** Mice were tested in the FST in 2 days after the last drug administration. Mice were tested in the OFT in 1 day after the last drug administration: **(E)** total distance and **(F)** time in the center. $^{*}p < 0.05$, $^{***}p < 0.001$, compared with the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with the CUS group. One-way ANOVA, n = 6–13/group.

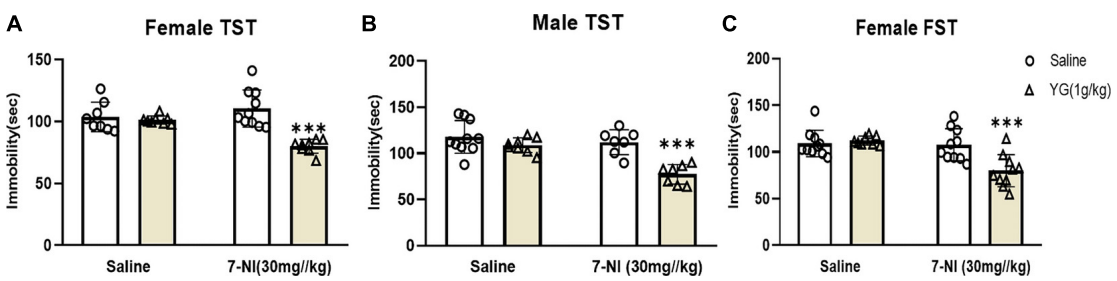

FIGURE 4 | Antidepressant-like effects of YG following nNOS intervention. The effect of 7-NI (30 mg/kg, i.p.) pretreatment on the antidepressant response of YG (1 g/kg, i.g.) in the TST of **(A)** female and **(B)** male mice. Meanwhile, **(C)** FST was also used to evaluate the interaction between YG and 7-NI. $^{***}p < 0.001$, compared with Control + Saline. Two-way ANOVA, n = 7–10/group.

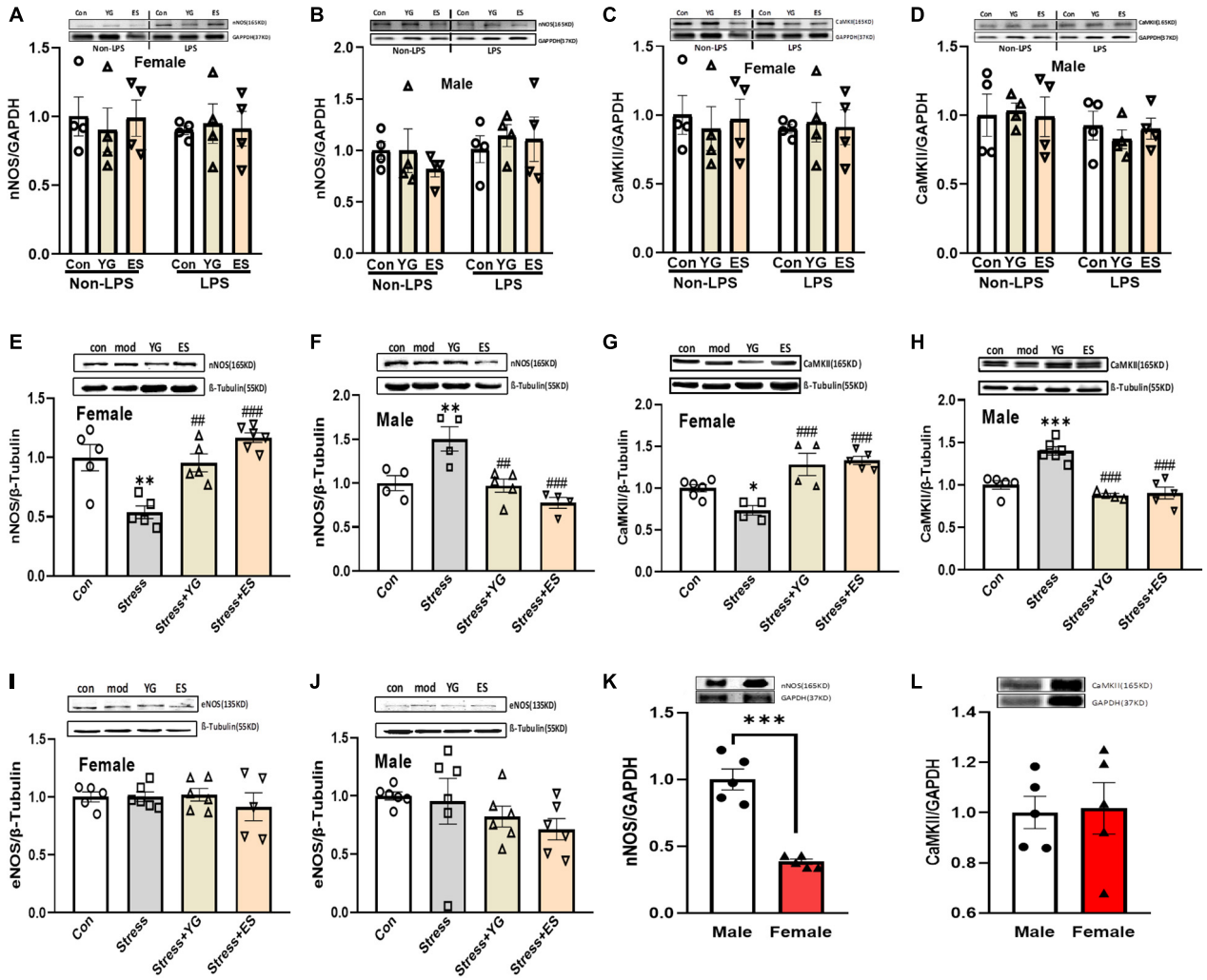

FIGURE 5 | CaMKII, nNOS, and eNOS expression in the hippocampus after chronic administration of stress, YG and ES in female and male mice. **(A)** nNOS expression in female mice in non-LPS or LPS treatment. **(B)** nNOS expression in male mice in non-LPS or LPS treatment. **(C)** CaMKII expression in female mice in non-LPS or LPS treatment. **(D)** CaMKII expression in male mice in non-LPS or LPS treatment. **(E)** nNOS expression in female mice after CUS or drug treatment. **(F)** nNOS expression in male mice after CUS or drug treatment. **(G)** CaMKII expression in female mice after CUS or drug treatment. **(H)** CaMKII expression in male mice after CUS or drug treatment. **(I)** eNOS expression in female mice after CUS or drug treatment. **(J)** eNOS expression in male mice after CUS or drug treatment. **(K)** nNOS expression in normal female and male mice. **(L)** CaMKII expression in normal female and male mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with the CUS group, one-way ANOVA or two-way ANOVA, n = 4–6/group.

male mice [**Figure 5F**, one-way ANOVA, $F(3,13) = 10.60$, $p < 0.001$], chronic stress ($p < 0.01$) significantly increased nNOS expression, while YG ($p < 0.01$) and ES ($p < 0.001$) decreased the overexpression of nNOS. Meanwhile, CaMKII protein expression was found to be similar to nNOS in female and male mice [**Figure 5G**, one-way ANOVA, $F(3,15) = 14.21$, $p < 0.0001$; **Figure 5H**, one-way ANOVA, $F(3,16) = 23.13$, $p < 0.0001$]. We also investigated whether the eNOS participated in the antidepressant-like effect of YG and ES. The results showed that eNOS was not affected by stress or drugs neither in female [**Figure 5I**, one-way ANOVA, $F(3,18) = 0.4326$, $p = 0.7322$] nor in male mice [**Figure 5J**, one-way ANOVA, $F(3,20) = 1.196$, $p = 0.3367$]. These results suggested that although female and male mice exhibited a consistent phenotype under stress or drug action, their potential molecular mechanisms might be different and might be associated with CaMKII-nNOS signaling pathways. In the normal mice, the protein expression levels of nNOS and CaMKII at the basal level were also measured, and the results showed that only nNOS expression in female mice was lower than that in male mice (**Figure 5K**, t-test, $p < 0.001$; **Figure 5L**, t-test, $p = 0.8938$).

KN-93 Only Block the Antidepressant-Like Effect of YG and ES in Female Mice

To explore whether the antidepressant-like effect of YG or ES depends on CaMKII expression, KN-93 (a specific CaMKII

inhibitor) was used to pretreat the mice before YG or ES. In the normal mice, high dosage of YG (2.5 g/kg, $p < 0.001$) and ES (20 mg/kg, $p < 0.001$) significantly decreased the immobility time after single injection both in female [**Figure 6A**, left hind paw, one-way ANOVA, $F(2,21) = 18.90$, $p < 0.0001$] and male mice [**Figure 6B**, left hind paw, one-way ANOVA, $F(2,22) = 21.62$, $p < 0.0001$]. However, after KN-93 pretreatment for 1 h before YG or ES administration, the antidepressant-like effect induced by YG or ES was blocked by KN-93 in female mice [**Figure 6A**, right hind paw, two-way ANOVA, interaction: $F(2,40) = 10.88$, $p = 0.0002$]. Meanwhile, in male mice, KN-93 [**Figure 6B**, right hind paw, one-way ANOVA, $F(2,21) = 13.05$, $p < 0.0001$] could not blunt the antidepressant-like effect of YG ($p < 0.001$) or ES ($p < 0.001$).

YG and ES Could Regulate the NR1-CREB Signaling Pathway to Display the Same Antidepressant-Like Behavior Phenotype in Female and Male

To find the potential mechanism underlying the antidepressant-like effect in female and male mice after YG and ES treatments, we first detected NR1 protein expression in mice. After administration with a subthreshold dose of YG [**Figure 7A**, one-way ANOVA, $F(3,14) = 12.15$, $p < 0.001$], NR1 expression ($p < 0.01$) was significantly reduced compared to the CUS group, which was significantly increased compared to the control group, and this result was similar to the result of ES administration ($p < 0.001$) in female mice. In male mice, NR1 protein expression showed a similar decreasing tendency [**Figure 7B**, one-way ANOVA, $F(3,19) = 15.50$, $p < 0.0001$]. Meanwhile, we further measured the phosphorylation CREB and total CREB expressions, which were considered as factors affecting by NR1 signaling. After the CUS procedure, pCREB expression was significantly decreased ($p < 0.001$), and YG ($p < 0.001$) and ES ($p < 0.001$) up-regulated pCREB expression in female mice [**Figure 7C**, one-way ANOVA, $F(3,16) = 17.41$, $p < 0.0001$]. In male mice, pCREB [**Figure 7D**, one-way ANOVA, $F(3,16) = 15.31$, $p < 0.0001$] displayed the same trend as in female mice. Meanwhile, total CREB was not affected by stress or drug treatment in female mice [**Figure 7E**, one-way ANOVA, $F(3,16) = 0.5959$, $p = 0.6268$] or male mice [**Figure 7F**, one-way ANOVA, $F(3,16) = 0.07369$, $p = 0.9732$]. Meanwhile, the ratio of pCREB/CREB was significantly decreased compared to control group both in female mice [one-way ANOVA, $F(3,15) = 9.325$, $p = 0.001$] and in male mice [one-way ANOVA, $F(3,15) = 9.544$, $p < 0.001$], and YG and ES could rescue the deficit. Also, we measured the baseline of pCREB and total CREB in mice, and the results showed that pCREB (**Figure 7G**, $p < 0.01$) displayed higher expression in female mice, while the total CREB (**Figure 7H**) showed no difference in mice of both sexes.

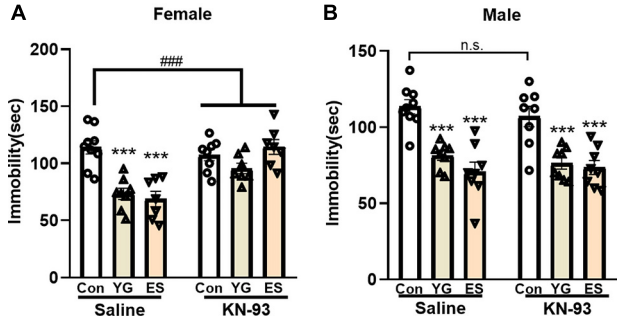

FIGURE 6 | The effect of pretreatment with saline or KN-93 on single YG (2.5g/kg) or ES (20mg/kg) administration during the TST paradigm in **(A)** female and **(B)** male mice. *** $p < 0.001$, compared with the control group of saline pretreatment, one-way ANOVA, $n = 7$-9/group. The interaction between saline and KN-93 pretreatment was analyzed by two-way ANOVA. ### $p < 0.001$ compared to non-LPS control group.

DISCUSSION

In the present study, we have characterized the antidepressant-like response of YG in male and female mice as well as the potential neurobiological mechanisms in both sexes. We found that: (1) single treatment with a high dose of YG could produce antidepressant-like effects both in female and in male mice, while the effective dosage in female mice was wider than that in male mice; (2) chronic administration of a subthreshold dose of YG or ES could produce antidepressant-like effects both in female and in male mice as well as in normal or LPS-treated mice; (3) a subthreshold dose of YG and ES could reverse the depressive behavior induced by chronic mild stress without sex differences; (4) nNOS expression showed different change patterns in female and male mice after stress or drug treatment, and 7-NI (nNOS inhibitor) combined with a subthreshold dose of YG could effectively induce antidepressant-like effects both in female and in male mice, indicating that YG revealed nNOS activity-dependent antidepressant-like effects in both sexes; (5) CaMKII expression also displayed the different change pattern in female and male mice after stress or drug treatment; however, KN-93 (CaMKII inhibitor) only blocked the antidepressant-like effect of YG or ES in female mice, implying that YG and ES produced antidepressant-like effects, which mostly depended on the CaMKII-nNOS pathway in female mice; (6) YG and ES could repair the abnormal expression of NR1-CREB signaling caused by stress and thus formed consistent antidepressant-like phenotypes in mice of both sexes.

Our group previously demonstrated that the ethanol extract of Yueju pill induced a rapid and long-lasting antidepressant-like effect in male mice (Xue et al., 2013, 2016; Tang et al., 2015). However, higher dose of ethanol extract of Yueju pill possibly caused side effects in patients compared to the one of the water extracts. A meta-analysis of GM (Ganmai Dazao) decoction in patient for depression suggested that GM showed an antidepressant action without side effects. GM in combination with regular antidepressants significantly reduced the side effects and enhanced the antidepressant efficacies (Yeung et al., 2014). Another study also revealed that modified GM induced an equivalent efficacy to melitracen-flupentixol in climacteric depression (Ma et al., 2014). Subchronic injection of GM reduced CUS-induced depressive-like behavior in rats, and this was

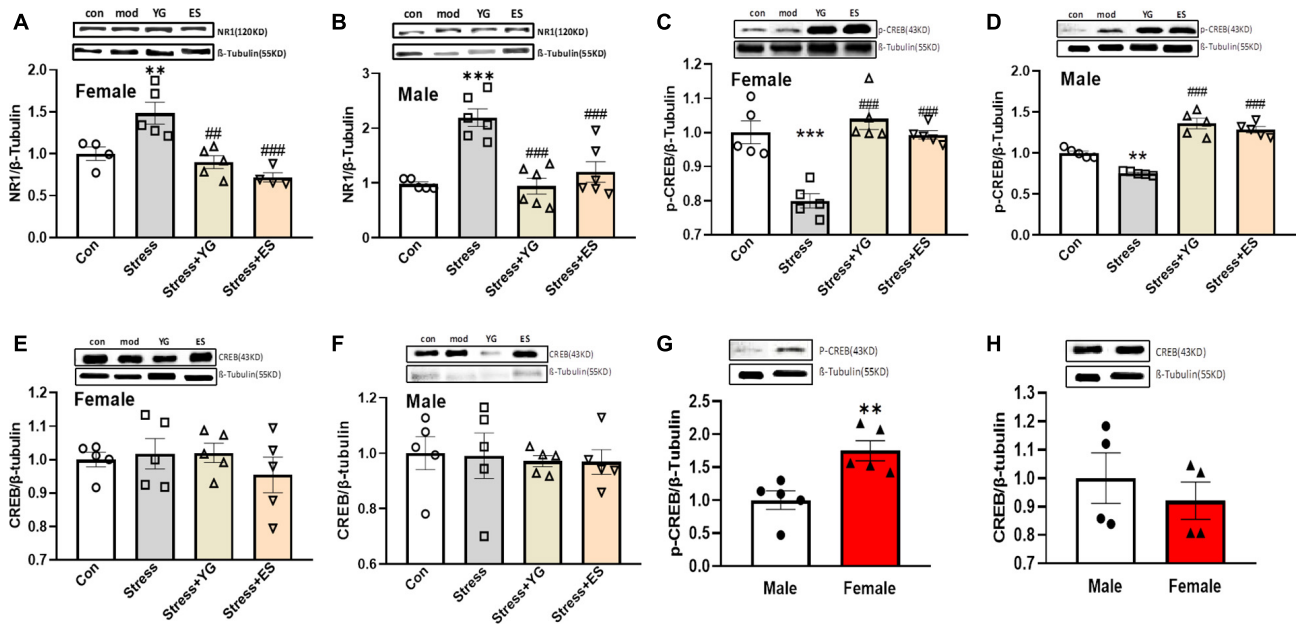

FIGURE 7 | NR1, pCREB, and CREB expression in the hippocampus after chronic subthreshold dosage administration of YG and ES in CUS mice. **(A)** NR1 expression in female mice after CUS or drug treatment. **(B)** NR1 expression in male mice after CUS or drug treatment. **(C)** pCREB expression in female mice after CUS or drug treatment. **(D)** pCREB expression in male mice after CUS or drug treatment. **(E)** CREB expression in female mice after CUS or drug treatment. **(F)** CREB expression in male mice after CUS or drug treatment. **(G)** pCREB expression in normal female and male mice. **(H)** CREB expression in normal female and male mice. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with the CUS group, one-way ANOVA, $n = 4$–6/group.

associated with a reduction in glutamate levels and increased the expression of NR2A and NR2B in the hippocampus (Lou et al., 2010; El-Alfy et al., 2012). Thus, the water extraction of Yueju pill combined with GM, which has been widely used in clinical traditional Chinese medicine treatment without significant side effects, showed significant antidepressant-like effects after a single injection in male mice. In the present study, we further illustrated that YG displayed antidepressant-like effects in female mice with the same dosage as in male mice. Lower dose of drugs means less risk of side effects, and we investigated whether a low dose of YG induced antidepressant-like effects. Interestingly, in the normal mice, LPS-induced mice, and CUS-treated mice, a low dose of YG as well as the positive control ES all produced the antidepressant-like effects without sex differences. It is implied that lower doses of drugs can safely be used to treat depression such as YG and ES. Previous research showed that the therapeutic effects of a low dose of antidepressants merely disappeared, instead of becoming aversive or toxic (Fitzgerald et al., 2020). However, chronic treatment with antidepressants was more popular. Although a high dose of YG was not found to have side effects, the lower dose of drug means a less risk of side effects. In our study, chronic treatment with a low dose of YG was demonstrated to have the same effect as a high dose of YG.

To find the possible mechanisms of the antidepressant-like effects of YG, we investigated nNOS expression, which was reported to show sex-dependent effects after stress. First, mice were treated with a single treatment of YG, and the effective doses of YG in male and female mice were determined. The subthreshold dose of YG (1 g/kg) combined with 7-NI induced antidepressant-like effects in female and male mice. These results indicated that there were sex differences in nNOS expression after stress or drug treatment. Moreover, the eNOS expression in female and male mice was not affected by stress or drug treatment. These results suggested that nNOS was the key to the antidepressant-like effect of YG and led to the sex differences, rather than eNOS. This is consistent with Hu et al.'s (2012) statement that there is a significant sex difference in NO levels catalyzed by nNOS in the hippocampus after stress exposure. Meanwhile, in the normal mice, nNOS expression was lower in female mice than that in male mice, and the result might supply a clue for the susceptibility to depression in female mice.

To investigate how nNOS is involved in the antidepressant-like effects of YG, we also used ES as the positive control. We measured the expression of nNOS and eNOS in the hippocampus of mice, and the results showed that the depressive-like behaviors in both sexes were significantly improved when compared with the CUS group, and in male mice, YG and ES decreased the expression of nNOS, but the eNOS expression did not change significantly. Previous studies also reported that conventional antidepressants like ES inhibited nNOS activity in male mice or rats (Angulo et al., 2001; Ulak et al., 2008; Krass et al., 2010). However, there are few reports regarding female rodents. In female mice, YG and ES increased the expression of nNOS, but did not change the expression of eNOS. Both YG and ES have the same effects on the depressive-like behavior. This evidence indicates that nNOS is an important factor contributing to the antidepressant-like effects of YG and ES treatment in depressive male and female mice.

Furthermore, we explored the NMDA-CaMKII signal pathway which is upstream of nNOS. As a downstream signal factor of the NMDAR subunit, CaMKII is an extremely abundant protein kinase in brain tissue that participates in a variety of signaling cascade reactions and is an important mediation center for the regulation of learning and memory (Zhang C. et al., 2020). CaMKII has been shown to reposition within the NMDAR complex in response to non-ionotropic NMDAR signaling (Aow et al., 2015; Stein et al., 2020). Our results showed that the expression of CaMKII in the hippocampus of male mice decreased, while in female mice the protein expression increased compared to the control group. The expression of nNOS revealed a similar trend in mice of both sexes after stress. Meanwhile, after YG or ES treatment, both CaMKII and nNOS expression was significantly adjusted to the baseline level. We speculated that the differences between male mice and female mice were mostly dependent on nNOS differential expression, which was activated by CaMKII. CaMKII is known to activate and translocate from the cytoplasm to the synaptic density where nNOS is predominantly located (Araki et al., 2020). Some reported that phosphorylation of nNOS at Ser847 by CaMKII attenuated the NO synthesis activity of nNOS *in vitro* and in cells (Hayashi et al., 1999; Komeima et al., 2000). CaMKII phosphorylates at Ser741 could also lead to a reduction of nNOS activity by blocking the binding of Ca^{2+}/CaM (Song et al., 2004; Takata et al., 2020). nNOS is a calcium-dependent enzyme, and we find that nNOS requires downstream of CaMKII signaling in both sexes, and the results are similar to those reported by Stein et al. (2015). Furthermore, the antidepressant-like effects of a high dose of YG and ES were prevented by the presence of KN-93 (CaMKII inhibitor) in female mice but not in male mice, while KN-93 alone showed no effect on the immobility time in mice. After corticosterone exposure, the increase in AMPAR surface trafficking can be pharmacologically modulated by tianeptine in a CaMKII-dependent mechanism (Zhang et al., 2013). In this study, there were no significant differences in CaMKII expression between male and female mice. A previous study reported that CaMKII activity was required for the expression and not initiation of E2-induced synaptic potentiation in female mice (Jain et al., 2019). Thus, this study clearly emphasized that the antidepressant-like effects of YG and ES mostly depended on the CaMKII-nNOS pathway in female mice.

The molecular mechanisms of the antidepressant-like effects of YG and ES are different between the sexes, but the antidepressant-like phenotypes in both sexes are consistent. The expression of NR1, which is one of the important upstream regulators of nNOS, was decreased in the hippocampus of male and female mice after YG and ES treatment when compared to the CUS mice. Additionally, our results showed that, compared to the control group, the expressions of phosphorylation CREB were both decreased in the hippocampus of mice after stress and were reversed by YG and ES without the sex differences. The pathology of depression was caused by the decreases in neuroplasticity in emotion-related brain regions (Hu et al., 2012), and the stress decreased the expression of CREB in the hippocampus of mice. Inhibition of CaMKIIβ-ERK1/2-CREB signaling mediates the chronic ketamine use-associated cognitive impairments by restraining synaptic signaling (Luo et al., 2020). Supplementation of curcumin increases the ratio of pCREB to CREB and corrects the depressive-like behaviors successfully in CUS-treated rats (Liao et al., 2020). CREB signaling, which is inhibited by over-activated GluN2B, participates in the antidepressant-like effects of ketamine, and extrasynaptic CaMKIIα is also involved in the CREB signaling. Studies have indicated that CREB plays an important role in the antidepressant-like effect in rodents. We also found that pCREB expression revealed a basic difference between female and male mice. These results indicate that NR1-CREB displays similar patterns without sex differences after stress or antidepressants, but the potential mechanism might be different.

CONCLUSION

In summary, we first confirmed that YG decoction induced stable antidepressant-like effects both in male and in female mice by using a subthreshold effective dosage in different CUS mice. We speculated that the antidepressant-like effects of YG worked through the nNOS pathway, which also had the function of improving downstream synaptic plasticity, and the changes of nNOS expression showed significant sex differences. Finally, we have illustrated that nNOS is modulated by CaMKII but not NR1 in mice after chronic treatment with YG or ES. The CaMKII-nNOS signaling pathway could enhance CREB activity to induce the same antidepressant-like effects in female mice. Furthermore, we want to investigate which subtype of CaMKII (α, β, γ, and δ) plays a dominant role in the mechanism underlying the antidepressant-like effects of YG or ES as well as a potential specific role of estrogen in regulating CaMKII expression in female mice.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Nanjing University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

WW and WX conceived and designed the experiments. SQ, YY, YC, YS, YL, YFY, JL, ZW, XY, RG, and JH performed the experiments. SQ, YY, YC, WW, and WX analyzed the data. SQ, YY, YC, DS, HW, WW, and WX contributed to the writing of the manuscript. Special thanks to Professor LL for revising this manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.640258/full#supplementary-material

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REVIEW
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Effects of Biological Sex and Stress Exposure on Ventromedial Prefrontal Regulation of Mood-Related Behaviors

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The ventral portion of the medial prefrontal cortex (vmPFC) regulates mood, sociability, and context-dependent behaviors. Consequently, altered vmPFC activity has been implicated in the biological basis of emotional disorders. Recent methodological advances have greatly enhanced the ability to investigate how specific prefrontal cell populations regulate mood-related behaviors, as well as the impact of long-term stress on vmPFC function. However, emerging preclinical data identify prominent sexual divergence in vmPFC behavioral regulation and stress responsivity. Notably, the rodent infralimbic cortex (IL), a vmPFC subregion critical for anti-depressant action, shows marked functional divergence between males and females. Accordingly, this review examines IL encoding and modulation of mood-related behaviors, including coping style, reward, and sociability, with a focus on sex-based outcomes. We also review how these processes are impacted by prolonged stress exposure. Collectively, the data suggest that chronic stress has sex-specific effects on IL excitatory/inhibitory balance that may account for sex differences in the prevalence and course of mood disorders.

Keywords: coping, depression, gonadal hormones, infralimbic cortex, reward, sociability, valence

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INTRODUCTION

Negative mood states are a feature of numerous psychiatric conditions, including anxiety and depressive disorders. Furthermore, major depression, characterized by sadness, reduced motivation, and anhedonia, is the leading cause of years lived with disability worldwide (Friedrich, 2017). Although females are disproportionally impacted by mood disorders, preclinical studies have historically focused on male neural regulation of depression-related behaviors (Kuehner, 2017). However, recent policy and methodological advances have led to the discovery of significant sex differences in the neurobiology of mood. Here, we examine recent studies exploring sex differences in the prefrontal regulation of coping, reward, and sociability.

Clinical neuroimaging studies associate activity in the ventral medial prefrontal cortex (vmPFC) with depressive disorders, and emotional regulation broadly. The vmPFC is also essential for goal-directed and contextually-appropriate behaviors, mood, and stress responding (Nestler et al., 2002; Krishnan and Nestler, 2008; McKlveen et al., 2015). Further, in studies of males and females vmPFC activity is linked with reward processing and positively correlates with the severity of anhedonia (Keedwell et al., 2005; Green et al., 2019). In particular, the vmPFC subregion Brodmann Area 25 (BA25) has decreased volume in MDD patients across sexes, and is a target for deep

brain stimulation in treatment-resistant depression (Drevets et al., 1997, 2008; Crowell et al., 2019; Sankar et al., 2019). A meta-analysis of imaging studies using males and females revealed BA25 is responsive to reward and emotional processing (Beckmann et al., 2009), as well as social exclusion (Vijayakumar et al., 2017). Though these studies indicate BA25 activity associates with depressive disorders, results examining BA25 function in depression are varied. Mixed sex studies have reported both reduced metabolic activity in MDD patients (Drevets et al., 1997), as well as hyperactivity in treatment-resistant depression measured by cerebral blood flow (Mayberg et al., 2005). Yet, both conventional antidepressant treatment and deep brain stimulation reduce BA25 activity (Mayberg et al., 2005, 2013). The heterogeneity of neural populations in BA25 may contribute to these divergent results. BA25 is principally composed of excitatory pyramidal neurons that project throughout limbic and brainstem nuclei, with a smaller but diverse population of inhibitory interneurons (Beckmann et al., 2009). Mounting evidence indicates that changes to the excitation/inhibition balance of the vmPFC relate to depressive symptomology, but the contributions of specific neural populations to behavior are difficult to address clinically (Fogaça and Duman, 2019; McKlveen et al., 2019). Further, although clinical studies commonly include both males and females, few analyze outcomes for sex differences (Vijayakumar et al., 2017). However, recent advances in neurobiology have allowed studies to establish casual roles for specific genetically-defined cell populations for processing and regulating behavior across sexes.

The infralimbic cortex (IL) is the rodent anatomical homolog of primate BA25 and is well-positioned for behavioral regulation based on projections throughout the limbic system (Vertes, 2004; Wood et al., 2018). The IL contains glutamatergic projection neurons with inhibitory interneurons providing local network regulation (McKlveen et al., 2015, 2016; Wood et al., 2018). While pharmacological and lesion studies have linked IL activity with depression-relevant behaviors, advances in cell-type specificity have identified sex-dependent roles in stress, reward, and social processes.

NEGATIVE VALENCE: STRESS COPING

Coping with negatively-valenced stimuli involves coordinated behavioral and physiological responses to address real or perceived stressors. Ultimately, stress exposure initiates a neurohormonal cascade that leads to the synthesis of adrenal glucocorticoids that then provide feedback to the brain at glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) to promote behavioral and physiological adaptation (McKlveen et al., 2013; Myers et al., 2014; Herman et al., 2016). An increasing number of studies have identified a role for the IL in stress coping (**Table 1**), with similarities and differences between male and female rodents.

The IL is acutely stress-responsive, as identified by histological markers of neuronal activation, and expresses both GR and MR in multiple cell types (Granholm et al., 1985; Reul and De Kloet, 1986; Cintra et al., 1994; Cullinan et al., 1995; McKlveen et al., 2013, 2016, 2019). Knockdown of male IL GR expression increases passive coping in the forced swim test (FST) and glucocorticoid responses to acute stress (McKlveen et al., 2013). However, pharmacological manipulations of male IL activity have yielded mixed results on coping behaviors. For instance, IL inactivation via $GABA_A$ receptor activation reduces FST immobility in males, an antidepressant-associated phenotype (Slattery et al., 2011). Similarly, non-specific synaptic blockade in the male IL reduces passive coping in the FST (Scopinho et al., 2010), as does long-term knockdown of IL glutamatergic output in males (Pace et al., 2020). However, both the NMDA antagonist ketamine and the muscarinic antagonist scopolamine increase IL activity and reduce male FST immobility (Fuchikami et al., 2015; Navarria et al., 2015). Further, deep brain stimulation in male rodents reduces passive coping in the FST (Hamani et al., 2010) and increases open arm time in the elevated plus maze (EPM), an anxiolytic-like phenotype (Shimizu et al., 2018). The mixed outcome of pharmacological interventions highlights the need to determine endogenous neural activity patterns during behavior, as well as more temporally- and genetically-specific modulation of neural activity.

In vivo recordings indicate the male IL processes anxiogenic stimuli and acute stressors. IL neural activity, measured by multiunit electrodes, increases in the seconds preceding entry into the open arms of the EPM (Shimizu et al., 2018). More generally, multiunit electrode array recordings of male mPFC neurons at the border between the IL and prelimbic (PL) area have variable activity in response to FST. Although many neurons are inhibited during FST, a large portion have selectivity for immobile vs. mobile periods and the majority of those increase activity during mobile periods (Warden et al., 2012). The heterogeneity of cellular responses underscores the need to examine the contributions of specific IL neural populations. Advances in optogenetics have permitted temporally-precise and cell-type specific modulation of IL activity. Activation of male IL glutamatergic neurons 24 h before testing reduces FST passive coping (Fuchikami et al., 2015), suggesting IL stimulation induces pyramidal neuron plasticity. Although, it remains to be determined how this stimulation may regulate IL efferent activity. Optogenetic modulation also permits synaptic stimulation, which indicates output targets differentially influence behavioral outcomes. For instance, Warden et al. stimulated male mPFC (IL and PL) glutamatergic neurons without affecting FST behavior. However, evoked glutamate release from mPFC terminals in the dorsal raphe increased active coping, while projections to the lateral habenula decreased active coping (Warden et al., 2012). Taken together, these results indicate differing behavioral outcomes from modulating male IL activity, likely relating to the cellular specificity of interventions and/or the differential engagement of output targets.

In line with regulating coping behavior, the IL also mediates physiological responses to acute challenges. Viral-mediated knockdown of male pyramidal neuron vesicular glutamate transporter 1 (vGluT1) reduces glutamate release and increases hypothalamic-pituitary-adrenal (HPA) axis, heart rate, and blood pressure responses to restraint (Myers et al., 2017;

TABLE 1 | Negatively valenced stimuli.

References	Sex	Species	IL Manipulation	Timing	Stressor	Outcome
McKlveen et al., 2013	Male	Spraque-Dawley rats	shRNA GR knockdown	5–6 weeks before testing	FST and restraint stress (RS)	FST immobility ↑ RS corticosterone ↑
Slattery et al., 2011	Male	Spraque-Dawley rats	Muscimol	10 min before testing	FST	Immobility ↓
Scopinho et al., 2010	Male	Wistar rats	$CoCl_2$ synaptic blockade	10 min before testing	FST	Immobility ↓
Pace et al., 2020	Male	Spraque-Dawley rats	siRNA vGluT1 knockdown	6 weeks before testing	FST	Immobility ↓
Fuchikami et al., 2015	Male	Spraque-Dawley rats	Ketamine and muscimol CaMKII-ChR2 stimulation: 15 ms, 10 Hz, 5 mW	24 h before testing	FST	Ketamine: immobility ↓ Ketamine + muscimol: no change ChR2 stim: immobility ↓
Navarria et al., 2015	Male	Spraque-Dawley rats	Scopolamine and muscimol	24 h before testing	FST	Scopolamine: immobility ↓ Scopolamine + muscimol: no change
Hamani et al., 2010	Male	Spraque-Dawley rats	Electrical stimulation	After first FST and prior to second FST	FST	Immobility ↓
Shimizu et al., 2018	Male	Spraque-Dawley rats	Electrical stimulation	During behavior	EPM	Open arm time ↑
Warden et al., 2012	Male	Long-Evans rats	CaMKII-ChR2 stimulation: 5 ms, 20 Hz, 10–20 mW	During behavior	FST	mPFC stim: no change mPFC to DRN: immobility ↓ mPFC to habenula: immobility ↑
Myers et al., 2017	Male	Spraque-Dawley rats	siRNA vGluT1 knockdown	6 weeks before testing	RS	ACTH ↑ Corticosterone ↑
Schaeuble et al., 2019	Male	Spraque-Dawley rats	siRNA vGluT1 knockdown	6 weeks before testing	RS	Heart rate ↑ Blood pressure ↑
Wallace et al., 2021	Male and Female	Spraque-Dawley rats	CaMKII-ChR2 stimulation: 5 ms, 10 Hz, 3 mW	During behavior	RS and novel environment (NE)	**Males:** RS: corticosterone and glucose ↓ NE: heart rate and blood pressure ↓ **Females:** RS: glucose ↑ NE: heart rate ↑

IL effects on coping during acute stressors.

Schaeuble et al., 2019). Similarly, optogenetic stimulation of male IL glutamatergic neurons reduces both corticosterone and glucose responses to restraint stress, as well as heart rate and blood pressure responses to a novel environment. In contrast, optogenetic stimulation of female IL glutamate neurons increases glucose responses to restraint and heart rate reactivity to a novel environment (Wallace et al., 2021). Collectively, these data suggest that male IL glutamatergic neurons are both necessary and sufficient to reduce autonomic and neuroendocrine responses to stress, while female IL glutamate neurons facilitate stress reactivity. It remains to be determined what mechanisms account for sex differences in IL function. While IL c-Fos expression following FST is similar in males and females, males have greater activation following acute restraint, suggesting differences in stress reactivity may be stimuli-specific (Sood et al., 2018). Further, ovarian hormones may be involved as lateral ventricle infusion of corticotrophin releasing hormone leads to negative correlations between IL c-Fos expression and grooming behavior in both male and diestrus female rats. However, IL activity positively associates with grooming in proestrus females (Wiersielis et al., 2016). Overall IL neural populations signal distinct aspects of stressors, while male IL glutamatergic neural activity constrains the physiological stress response and bidirectionally regulates coping style depending upon projection sites. In contrast, female IL glutamatergic neural activity facilitates the physiological stress response and divergent IL responses to stressors may relate to ovarian hormone signaling.

POSITIVE VALENCE: REWARD

The IL has a prominent role in coordinating context-appropriate reward-seeking behaviors (**Table 2**). Pharmacological inhibition of the male rat IL with combined $GABA_A$ and $GABA_B$ agonists reduces inhibitory control in a food reward-seeking task (Capuzzo and Floresco, 2020), as well as extinction and renewal of context-conditioned food reward (Eddy et al., 2016). Moorman and Aston-Jones (2015) used a similar pharmacological approach and found that IL inhibition reduces both lever presses to a reward-associated stimulus and extinction of reward-seeking after the stimulus is no longer paired with reward. Furthermore, male IL multiunit potential recordings found that putative pyramidal neurons heterogeneously respond

(significantly increase or decrease activity) to cue-evoked reward-seeking and extinction (Moorman and Aston-Jones, 2015). Additionally, male IL neurons have prolonged firing in response to rewarded but not unrewarded operant responses and IL inhibition increases the latency to reward acquisition (Burgos-Robles et al., 2013). IL pyramidal neuron regulation of midbrain dopamine signaling may be important for effects on reward and motivation. Ferenczi et al. utilized a stable-step function opsin (SSFO) to optogentically increase male IL glutamate neuron excitability and found reduced sucrose preference. In females, the SSFO approach also reduces the rewarding quality of ventral tegmental area stimulation in a real-time place preference assay (Ferenczi et al., 2016), suggesting IL inhibition of dopamine signaling may contribute to anhedonia. In contrast, SSFO-induced increases in the excitability of IL GABAergic/vasoactive intestinal peptide (VIP)-expressing interneurons reduces high-calorie palatable food consumption without impacting food reward motivation or low-calorie chow intake (Newmyer et al., 2019). Overall, these studies indicate that male IL neurons signal multiple aspects of food reward acquisition, including contextually-appropriate reward-seeking and behavioral inhibition. Although, specific IL cell populations likely have opposing effects on hedonic feeding.

Fewer publications have investigated the role of the female rodent IL in reward seeking and motivational behaviors. To date, evidence suggests that the female IL may have more limited involvement in reward processing and positive affect. For instance, optogenetic activation of the glutamatergic IL to nucleus accumbens shell (NAcSh) pathway following conditioned taste aversion reduces aversive taste reactivity in males but not females. However, both sexes lever press for IL-NAcSh stimulation and a prior history of IL-NAcSh stimulation increases sucrose preference in males and females (Hurley and Carelli, 2020). Further, optogenetic stimulation of IL glutamatergic neurons induces a real-time place preference in males without affecting place preference or aversion in females, suggesting a positive valence to IL glutamatergic activity in males but not females (Wallace et al., 2021). These studies collectively indicate that, in males, IL activity is necessary for contextual appraisal during reward acquisition and that glutamatergic activity has positive valence. In contrast, current evidence suggests that female IL glutamatergic activity does not affect place preference or conditioned aversion, although activity in specific projections may be rewarding.

SOCIAL BEHAVIOR

Reduced sociability is a common symptom of mood disorders. Additionally, decreased motivation for social interaction further worsens the course of depressive illness (Kupferberg et al., 2016). Consequently, determining how neural circuits encode and regulate social behavior is a critical area for investigation. Growing evidence indicates that male IL neural output regulates the affective and motivational processes that underly social interaction (**Table 3**), possibly through descending limbic integration (Vertes, 2004; Wood et al., 2018). Indeed, pharmacological inactivation of the IL reduces both the frequency and duration of social play in adolescent male rats (Van Kerkhof et al., 2013). Furthermore, Minami et al. (2017) conducted electrical recordings of IL activity during social behavior and found that male IL neurons increase firing during the termination of social behavior, an effect absent in isolation-reared rats suggesting experience-dependent social encoding. SSFO enhancement of male IL glutamatergic neuron excitability reduces social interaction with a juvenile interactor (Ferenczi et al., 2016). In contrast, acute optogenetic stimulation of male IL glutamate neurons increases conspecific social motivation (Wallace et al., 2021), providing further evidence for contextual factors impacting IL-mediated behaviors. Increasing the excitability of male IL GABAergic VIP interneurons reduces novel social investigation, as well as novel object interactions (Newmyer et al., 2019). Other interneuronal investigations examined GABAergic parvalbumin (PV) neurons in the male and female mouse mPFC and found that PV neural activity increases in both sexes during social interactions compared to novel object interactions, a phenotype missing in the CNTNAP2 knockout autism model. Further, SSFO-increased excitability of PV interneurons rescues social deficits in the CNTNAP2 model, without impacting sociability in wildtype mice (Selimbeyoglu et al., 2017). Ultimately, these studies highlight the need to further investigate how specific interneuronal populations within the mPFC differentially encode and modulate social behavior.

Currently, evidence suggests the female IL may have a different role in social behavior. Optogenetic stimulation of IL glutamatergic neurons does not alter female social motivation or social novelty prefrence (Wallace et al., 2021). Further, the female IL appears to be less responsive to social interaction with conspecifics as female rats have less c-Fos expression compared to males after social interaction (Mikosz et al., 2015). Moreover, male rats have greater c-Fos responses to a previously-stressed interactor than an unstressed conspecific, an effect that does not occur in females. This sex difference may be independent of ovarian hormones as both intact cycling females and ovariectomized (OVX) females have similar IL c-Fos following social interaction (Mikosz et al., 2015). However, specific projection-defined IL neurons are necessary for social motivation. Huang et al. (2020) chemogenetically inactivated female basolateral amygdala-projecting IL neurons and abolished social preference in a 3-chamber social test. Overall, the data suggest that the male IL is more responsive to conspecific social interaction and that increasing male IL glutamatergic activity can bidirectionally modulate social motivation, dependent upon the method of stimulation, while interneuron stimulation produces opposing effects. Additionally, the female IL has less neural activity than males after conspecific interaction and stimulation of female IL glutamate neurons does not alter sociability. Although, amygdala-projecting female IL neurons are necessary for social preference.

While the female IL may be less involved in conspecific interaction, Pereira and Morrell (2020) demonstrated that the IL plays a critical role in maternal behaviors. Using a conditioned place preference paradigm, new mother rats spend equivalent time in chambers associated with cocaine reward

TABLE 2 | Positively valenced stimuli.

References	Sex	Species	IL manipulation	Timing	Reward	Outcome
Eddy et al., 2016	Male	Wistar rats	Baclofen/muscimol	30–45 min before testing	Food pellet	Response in rewarding condition ↓ Response in extinct condition ↑
Capuzzo and Floresco, 2020	Male	Long-Evans rats	Baclofen/muscimol	10 min before testing	Sucrose pellet	Inhibitory trial success ↓
Moorman and Aston-Jones, 2015	Male	Sprague-Dawley rats	Electrical recording Baclofen/muscimol	Immediately prior	Sucrose	Recording: IL neural activity during sucrose acquisition ↑ Baclofen/muscimol: Lever press for reward ↓ Extinction ↓
Anthony Burgos-Robles et al., 2013	Male	Sprague-Dawley rats	Electrical recording Muscimol	30 min before testing	Sucrose pellet	Recording: IL neural activity during sucrose acquisition ↑ Muscimol: Reward collection latency ↑
Ferenczi et al., 2016	Male and Female	**Male:** Sprague-Dawley rats **Female:** Long-Evans TH-ChR2 rats	**Male:** CaMKII-SSFO: continuous, 4× over 6 h testing **Female:** CaMKII-SSFO: continuous	During behavior	**Male:** Sucrose **Female:** Dopamine stimulation	**Males:** Sucrose preference ↓ **Females:** Preference for dopamine stimulation ↓
Newmyer et al., 2019	Male	VIP-Cre transgenic mice	Cre-dependent SSFO: continuous	5 min before testing	Palatable high-calorie diet	Palatable food intake ↓
Hurley and Carelli, 2020	Male and Female	Sprague Dawley rats	IL-NAcSh CamKII-ChR2 stimulation: 5 s, 20 Hz, 10 mW	During behavior	Sucrose and stimulation	**Males but not females:** Aversive taste response ↓ **Both sexes:** Respond for stimulation ↑
Wallace et al., 2021	Male and Female	Spraque-Dawley rats	CaMKII-ChR2 stimulation: 5 ms, 10 Hz, 3 mW	During behavior	Real-time place preference	**Males:** Time in stimulation side ↑ **Females:** No preference

IL effects on reward behavior.

TABLE 3 | Social behavior.

References	Sex	Species	IL manipulation	Timing	Test	Outcome
Van Kerkhof et al., 2013	Male	Wistar rats	Baclofen/muscimol	5 min before testing	Free interaction	Social play ↓
Minami et al., 2017	Adolescent Male	Spraque-Dawley rats	Electrical recordings	During behavior	Free interaction	IL neural activity during interaction termination ↑
Ferenczi et al., 2016	Male	Sprague-Dawley rats	CaMKII-SSFO: continuous, 4× during testing	2 days prior and on testing day	Male juveline in homecage	Social interaction ↓
Selimbeyoglu et al., 2017	Male and Female	PV-Cre C57BL/6J mice	Border of IL and PL GCaMP6f photometry	During behavior	Free interaction	**Both sexes:** PV neural activity during social interaction ↑
Newmyer et al., 2019	Male	VIP-Cre transgenic mice	Cre-dependent SSFO: continuous	5 min before testing	Conspecific in homecage	Social interaction ↓
Wallace et al., 2021	Male and Female	Spraque-Dawley rats	CaMKII-ChR2 stimulation: 5 ms, 10 Hz, 3 mW	During behavior	3-chambered social test	**Males:** Social motivation ↑ **Females:** No change
Huang et al., 2020	Female	C57BL/6J mice	Cre-dependent inhibitory DREADD hM4Di in IL CAV2-Cre in BLA	CNO 30 min before testing	3-chambered social test	Social preference ↓
Pereira and Morrell, 2020	Female	Sprague Dawley rats	Bupivacaine hydrochloride	5 min before testing	Pup-associated conditioned preference	Time in pup-associated zone ↓ Pup retrieval ↓

IL effects on social behavior.

and pups. However, blockade of sodium conductance in the female IL leads to an exclusive preference for the cocaine-paired chamber. Furthermore, IL inactivation reduces maternal behaviors including nest building and retrievals. In fact, none of the IL-inactivated mothers fully retrieved all pups, while all vehicle-treated females did (Pereira and Morrell, 2020). Further,

histological evidence indicates that the female IL shows greater activation following exposure to newborns than juvenile pups (Pose et al., 2019). Thus, the female IL may be more tuned to facilitate pup rearing than conspecific social interactions.

GONADAL HORMONE INFLUENCES

The impact of gonadal hormones on neural activity may contribute to sexually divergent IL function. Gonadal hormones influence circuit regulation through both organizational effects in development as well as activational effects in adulthood. The importance of gonadal hormones for mPFC development, synapse formation, and pruning has been recently reviewed (Premachandran et al., 2020; Delevich et al., 2021). In adult rodents, estrogen receptors α and β (ERα and ERβ) are present in the male rodent mPFC (**Figure 1**), distributed broadly across cortical layers in both pyramidal and non-pyramidal putative interneurons (Montague et al., 2008). Further, ultrastructural analysis found ERα, ERβ, and G protein-coupled estrogen receptor 1 (GPER1) in the female mPFC. Interestingly, GPER1 is expressed at over twice the levels of ERα and ERβ, suggesting a significant fast-acting component to ER signaling (Almey et al., 2014). Moreover, ER localization is largely extranuclear, with most receptors located axonally (Almey et al., 2014). Growing evidence suggests that ER signaling plays an important role in mPFC regulation of female behavior. For instance, 17β-estradiol (E2) localized to the female PL/IL junction shifts the cognitive strategy used for maze navigation (Almey et al., 2014). Further, ERα and ERβ agonist treatment in diestrus females potentiates the antidepressant-like effect of ketamine (Dossat et al., 2018). Recent evidence from OVX females also indicates that E2 increases the excitability of IL pyramidal neurons in slice and enhances extinction of reward-seeking (Yousuf et al., 2019). While more research is needed to determine the mechanisms by which estrogens regulate prefrontal function, these studies suggest that cyclic fluctuations in intrinsic network activity impact depression-related behaviors. Considerably less is known about the effects of cortical progesterone signaling. The female rodent frontal cortex expresses both progesterone receptor a (PRa) and b (PRb), with PRb levels decreasing during estrus (Guerra-Araiza et al., 2003). Although there are no reports, to our knowledge, of PR expression in males, repeated progesterone administration increases $GABA_A$ receptor subunit α1 expression in the mPFC of both sexes (Andrade et al., 2012). Thus, cyclic increases in progesterone likely affect mPFC E/I balance.

Androgens may also play a role in IL functional differences as androgen receptors (AR) are expressed in the frontal cortex of male and female rodents (DonCarlos et al., 2006). Expression is higher in males than females and current evidence indicates little to no astrocyte expression, though this could be age-dependent (Feng et al., 2010). Further, AR expression in midbrain-projecting neurons suggests putative pyramidal expression (Aubele and Kritzer, 2012; Low et al., 2017). In addition, androgens regulate dopamine (DA) inputs to the male rodent mPFC. Orchiectomy increases DA axonal density and extracellular DA levels within the mPFC, an effect reversed by testosterone administration (Kritzer, 2003; Aubele and Kritzer, 2012). Further, a large portion of ventral tegmental area-projecting IL neurons express AR. Taken together, these results suggest a bidirectional interaction between androgen signaling and mesocortical DA circuitry that may influence IL network excitability as well as mood and behavior. Further evidence for gonadal hormone regulation of mPFC activity comes from studies indicating that androgens and estrogens have opposing effects on mPFC metabolism of DA, norepinephrine, and serotonin during a novel environment stressor (Handa et al., 1997). Collectively, this evidence suggests gonadal hormones modify IL function. Due to the widespread expression of these receptors in mPFC cell populations and varied actions on neural activity, considerable work remains to understand how hormonal fluctuations across the lifespan impact prefrontal network function.

CHRONIC STRESS IMPACTS

The two greatest predictors of depressive outcomes are cumulative lifetime traumas and severe life stressors (Cassileth et al., 1984), indicating that the neural consequences of repeated or severe stress dictate disease burden. Mood-related symptoms, including negative affect, anhedonia, despair, and social withdrawal, are also frequently initiated and/or exacerbated by prolonged stress (Kennedy and Adolphs, 2012; Lupien et al., 2009). Accordingly, chronic stress exposure has been a primary preclinical paradigm for studying depression and mood disorders in animal models. In recent years, there has been growing interest in the sexual basis of chronic stress impacts on limbic structures. Multiple excellent reviews have covered the topic in-depth (McLaughlin et al., 2009; Bourke et al., 2012; Shansky and Woolley, 2016; Shors, 2016; Shepard and Coutellier, 2018; Fogaça and Duman, 2019; Moench et al., 2019; Page and Coutellier, 2019). Here, we review IL-specific effects.

Chronic stress-induced IL pyramidal neuron dendritic hypotrophy has been consistently reported in male rodents, though this varies based on projection targets (Cerqueira et al., 2005; Goldwater et al., 2009; Shansky et al., 2009; Luczynski et al., 2015; Czéh et al., 2018). Further, measurement of the long-term activation marker ΔFosB indicates the male IL is responsive to chronic stress exposure, an effect not present in other frontal regions such as the anterior cingulate or orbital cortices (Flak et al., 2012; Pace et al., 2020). However, studies of chronic stress effects on male IL glutamatergic excitability have yielded mixed results, contributing to opposing hypothesis of either hyper- or hypo-inhibition. McKlveen et al. (2016) found that IL pyramidal neurons of male rats exposed to a 2-week variable stress paradigm had increased inhibitory currents and more GABAergic synaptic appositions, suggesting increased inhibition of IL glutamatergic neurons. GR was also reduced specifically in PV interneurons (McKlveen et al., 2016), pointing to the importance of glucocorticoid feedback for regulating local inhibition. In support of hyper-inhibition, chemogenetic inhibition of male mouse IL PV interneurons during CVS reduces passive coping in FST

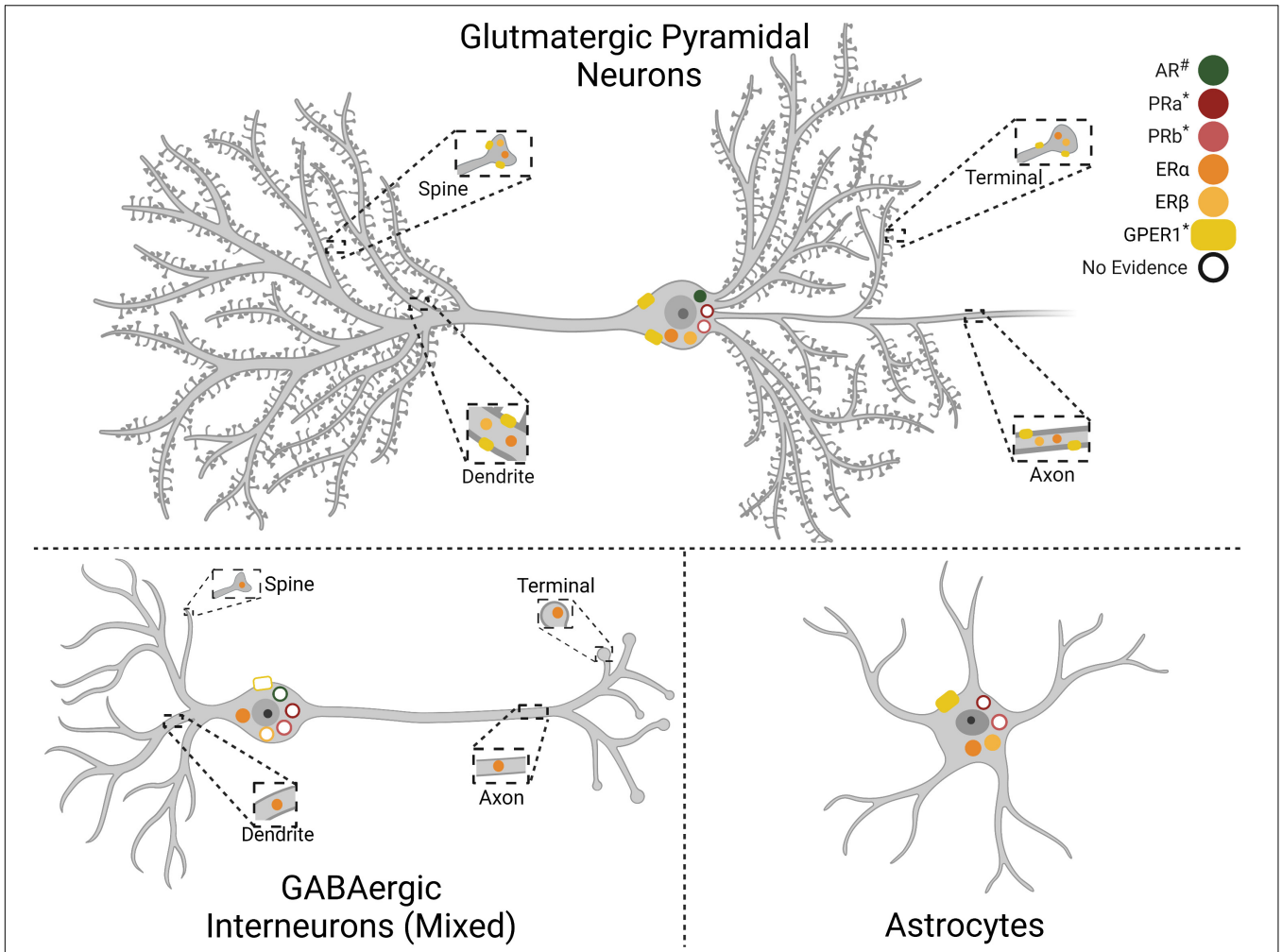

FIGURE 1 | Gonadal hormone receptor expression in infralimbic cortex (IL) cell types. Depiction of gonadal hormone receptor expression and localization. Filled symbols indicate evidence for expression in the cell type and cellular compartment. Empty symbols denote lack of information as to the presence or absence of the receptor. Progesterone receptor cell-type specifity has not been reported. Androgen Receptor (AR), Progesterone Receptor A (PRa), Progesterone Receptor B (PRb), Estrogen Receptor α (ERα), Estrogen Receptor β (ERβ), G protein-coupled estrogen receptor 1 (GPER1). #AR expression is higher in males than females. *PRa, PRb, and GPER1 expression has only been reported in females. Created with BioRender.com.

(Nawreen et al., 2020). Furthermore, chronic stress increases GAD67 mRNA in the male mouse IL (Shepard et al., 2016). Additionally, 3 weeks of daily restraint stress in male mice increases dendritic arborization of GAD67-positive interneurons but reduces GAD67-positive somas (Gilabert-Juan et al., 2013). In support of hypo-inhibition, Czéh et al. (2018) found reductions in both IL interneuron populations and inhibitory currents in an anhedonic subpopulation of male rats exposed to 9 weeks of variable stress (Czéh et al., 2018). Similarly, 3 weeks of chronic unpredictable stress reduces GAD67 mRNA in male rats, although vGluT1 mRNA is also reduced (Ghosal et al., 2020). Thus, there are data to support both increased and decreased inhibition of male IL pyramidal neurons after chronic stress. Multiple factors including differences in methodology, stress paradigms, and temporal factors may contribute to the discrepant findings. Further, how these post-mortem changes affect neural network activity and behavioral outcomes remains to be determined. *In vivo*, electrophysiology studies indicate male IL neurons increase firing during a shock-predicting cue; however, this effect is not present after repeated stress (Wilber et al., 2011). Taken together, chronic stress reduces male IL glutamatergic dendritic complexity and spine density, yet the chronic stress effects on inhibitory neural populations are mixed and have yet to reach a consensus.

Numerous female studies suggest that estrogen may be protective against chronic stress effects on IL function. Though not specific to the IL, Wei et al. (2014) found that repeated restraint stress reduced mPFC miniature excitatory postsynaptic current (mEPSC) amplitude and frequency in males but not females. The decreased excitability was accompanied by a male-specific reduction in glutamate receptor surface proteins. However, ER antagonism in females unmasked stress effects on mEPSC frequency and glutamate receptors, suggesting ER prevents excitatory hypofunction following stress. Intriguingly,

estrogen delivery in males is sufficient to prevent stress effects on mEPSC amplitude and frequency, as well as partially restore glutamate receptor surface protein expression (Wei et al., 2014). Moreover, female IL neurons generally do not show stress-induced dendritic remodeling. However, female IL pyramidal neurons projecting to the basolateral amygdala have estrogen-dependent increases in dendritic branching after repeated restraint stress (Shansky et al., 2010). Further, repeated stress increases spine density in this projection regardless of estrogen treatment (Shansky et al., 2010). Others have reported sex-specific effects of chronic variable stress based on IL projection target. Here, chronically-stressed female mice have increased EPSCs in the IL-NAc projection, while males have greater loss of dendritic complexity in VTA-projecting IL neurons. Additionally, chemogenetic inhibition of NAc-projecting IL neurons rescues chronic stress-induced behaviors only in females (Bittar et al., 2021). In contrast to pyramidal cells, PV interneurons appear to be more stress susceptible in females than males. Female IL PV mRNA increases following 2 weeks of daily stress exposure, with a further increase at 4 weeks. Females at 4 weeks also have increased PV neuron density and reduced IL c-Fos expression following open-field, effects that are absent in males (Shepard et al., 2016). However, both male and female mice have increased c-Fos in PV cells (Page et al., 2019), indicating increased interneuron activity. Although, chemogenetic activation of IL PV interneurons induces anxiety-like behavior only in females. Overall, these results indicate that estrogen is protective for female IL glutamatergic neurons, sex differences in chronic stress effects are projection-dependent, and interneuron populations are more susceptible to chronic stress in females.

CONCLUSION

The increased attention on females in preclinical research and the rapid development of neurobiological techniques with enhanced genetic and temporal specificity have isolated sex-specific regulatory roles of IL neural populations. Manipulations that induce long-term changes in pyramidal E/I balance (SSFO-mediated hyperexcitability or lentiviral knockdown of glutamate release) can lead to divergent and sometimes contradictory behavioral outcomes. However, acutely increasing activity of IL glutamate neurons regulates numerous stress coping, motivational, and social behaviors in male rodents. The aggregate data suggest these cells promote active coping, context-appropriate reward-seeking, motivation, and sociability. Although, specific projections may have differing or even opposing actions. Less work has examined stress coping in females, but IL glutamate neurons have sexually divergent effects on physiological stress responses. Female IL pyramidal neurons may also play a smaller role in reward-seeking and motivational behaviors. In terms of sociability, the female IL seems less involved in conspecific interactions, with significant involvement in maternal behaviors. Many of these differences may be mediated by gonadal hormone signaling in different components of the IL neural and glial network. Generally, estrogens seem to protect glutamate neurons from the effects of chronic stress while androgens modulate cortical dopamine function. The sex-specific functions of the numerous IL interneuron subtypes remain to be determined. Further, the effects of chronic stress on IL cellular excitability are mixed for both sexes. Ultimately, complex interactions between sex and stress impact many aspects of vmPFC local networks and, consequently, brain-wide synaptic signaling. Determining the mechanistic basis of E/I balance in these cell groups is likely to significantly push forward our understanding of mood disorders and identify sex-specific treatment options to improve health outcomes.

AUTHOR CONTRIBUTIONS

Both authors contributed to the conceptualization of the review. TW performed the initial literature search, wrote the first draft of the manuscript, and created the illustrations in BioRender. BM contributed additional literature and revised the manuscript. Both authors approved the submitted version.

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Baseline Depression-Like Behaviors in Wild-Type Adolescent Mice Are Strain and Age but Not Sex Dependent

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Depression is a major neuropsychiatric disorder, decreasing the ability of hundreds of millions of individuals worldwide to function in social, academic, and employment settings. Beyond the alarming public health problem, depression leads to morbidity across the entire age including adolescence and adulthood. Modeling depression in rodents has been used to understand the pathophysiological mechanisms behind this disorder and create new therapeutics. Although women are two times more likely to be diagnosed with depression compared to men, behavioral experiments on rodent models of depression are mainly performed in males based on the assumption that the estrous cycles in females may affect the behavioral outcome and cause an increase in the intrinsic variability compared to males. Still, the inclusion of female rodents in the behavioral analysis is mandatory to establish the origin of sex bias in depression. Here, we investigated the baseline depression-like behaviors in male and female mice of three adolescent wild-type inbred strains, C57BL/6N, DBA/2, and FVB/N, that are typically used as background strains for mouse models of neuropsychiatric disorders. Our experiments, performed at two different developmental stages during adolescence (P22–P26 and P32–P36), revealed strain but no sex differences in a set of depression-related tests, including tail suspension, sucrose preference and forced swim tests. Additionally, the 10-day interval during this sensitive period uncovered a strong impact on the behavioral outcome of C57BL/6N and FVB/N mice, highlighting a significant effect of maturation on behavioral patterns. Since anxiety-related behavioral tests are often performed together with depression tests in mouse models of neuropsychiatric disorders, we extended our study and included hyponeophagia as an anxiety test. Consistent with a previous study revealing sex differences in other anxiety tests in adolescent mice, male and females mice behaved differently in the hyponeophagia test at P27. Our study gives insight into the behavioral experiments assessing depression and stresses the importance of considering strain, age and sex when evaluating neuropsychiatric-like traits in rodent models.

Keywords: depression, sex differences, tail suspension test, sucrose preference test, forced swim test, hyponeophagia test

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INTRODUCTION

Depression is a long-lasting heterogeneous neuropsychiatric disorder and one of the most common mental diseases, which places a significant economic burden on public health and decreases individuals' quality of life (Wittchen et al., 2011; Lim et al., 2012; Murray et al., 2013; Whiteford et al., 2013). It affects approximately 4.4% of the world's population with an incidence rate above the rate of global population growth (Flux and Lowry, 2020). The symptoms include sadness, helplessness, guilt and loss of appetite, sexual desire, and interest in activities that once were pleasurable (for at least 2 weeks) along with recurrent thoughts of suicide (McCarter, 2008; American-Psychiatric-Association [APA], 2013). Because of the unique and complex features in addition to the subjective symptoms of human depression, the generation of valid and insightful depression models for the development of new therapeutic drugs is not straightforward. Still, different rodent models have shown high face validity to human depression and are used in preclinical studies. These depression-like behaviors can be induced in rodents by several means including genetic, environmental, chemical and pharmacological manipulation and brain lesions [for recent reviews, see (Flux and Lowry, 2020; Becker et al., 2021)]. Several tests are usually performed to assess distinct components of depression in rodent models. For example, the tail suspension and forced swim tests measure despair, the splash test measures apathy, while the sucrose preference test evaluates anhedonia (Eltokhi et al., 2018; Becker et al., 2021). Additionally, tests that measure anxiety including open field, elevated plus maze and light/dark compartment are often performed complementary to depression tests for the full characterization of rodent models of neuropsychiatric disorders (Belovicova et al., 2017).

The prevalence and clinical characteristics of depression differ between women and men with women suffering from depression nearly twice as frequently as men during lifetime, independently of race or ethnicity (Weissman and Klerman, 1977; Cyranowski et al., 2000; Andrade et al., 2003; Ford and Erlinger, 2004; Patten et al., 2006; Bromet et al., 2011; Salk et al., 2017). However, male rodent models have traditionally been used in genetic and pharmacological studies of depression. Performing behavioral experiments on male rodents is linked to the assumption of estrous-linked changes in the baseline behavioral activity in females. This concern may require testing female rodents at each of the four stages of the estrous cycle to generate reliable data, which may complicate the experimental design. On the other hand, neglecting the sex difference in depression-related experiments may provoke false interpretations of the results and prevents a successful translation of experimental data into the clinic. Thus, it is essential incorporating female rodents in behavioral, molecular and electrophysiological analyses to decipher the biology behind the sex bias of depression as a prerequisite for improving therapeutics. Unfortunately, so far, rodent models have yielded inconsistent results in different studies and often reported more depression-like symptoms in males than females (Kokras et al., 2009; Dalla et al., 2010; Barkus, 2013; Van den Hove et al., 2013; Najjar et al., 2018; Becker et al., 2021). By standardization of behavioral experiments and by taking into consideration factors that affect behavioral outcomes, the variability in results on sex differences can be likely reduced. Additionally, the sex differences of depression rodent models may also be induced by differences in the general performance and baseline activity of male and female wild-types in depression-related behavioral tests. To test these hypotheses, we investigated the baseline depression-like behaviors of both male and female wild-type mice in three standard behavioral tests, tail suspension, sucrose preference and forced swim tests. We performed our analysis in three inbred strains, C57BL/6N, DBA/2, and FVB/N, well-established to affect the behavioral outcome (McFadyen et al., 2003; Brooks et al., 2005; Moy et al., 2007; Peleh et al., 2019; Eltokhi et al., 2020, 2021), and typically used as background strains in mouse models of neuropsychiatric disorders. Furthermore, although behavioral studies are frequently performed in adult mice taking the advantage of easy handling and complex behaviors, we performed our analysis at two different developmental stages during adolescence since the onset of neuropsychiatric symptoms emerges mainly during adolescence, with more than 50% of adults with neuropsychiatric disorders receiving a diagnosis before 15 years of age (Kim-Cohen et al., 2003; Paus et al., 2008). Because behavioral results are known to be sensitive to small developmental progress during adolescence (Peleh et al., 2019; Eltokhi et al., 2020), we compared the results of these behavioral experiments between these two developmental stages in mice differing only by a few days in age.

Our work indicates that the performance of mice in depression-related behavioral tests during adolescence is mainly strain and age dependent with no obvious effect of sex. This outlines the drawbacks of using an individual strain in genetic and pharmacological studies of depression and highlights the benefits of using adolescent mice in characterizing rodent models of depression, which may reduce inconsistency of results between different laboratories.

MATERIALS AND METHODS

Animals and Housing Conditions

Animals and housing conditions were similar to our previous studies (Eltokhi et al., 2020, 2021). The experiments were conducted in strict compliance with national and international guidelines for the Care and Use of Laboratory Animals. The behavioral analysis was carried out following the ARRIVE guidelines and was approved by the animal ethic committee of the (Regierungspräsidium Karlsruhe) Government of Baden Württemberg (G-101/16).

Experimental Design and Groups

Depression and anxiety-related behavioral tests were carried out during the daylight cycle starting at 7 a.m. Mice were habituated to the behavioral room for half an hour before the start of the tests. We analyzed the depression-like behaviors in 2 cohorts of group-housed mice of both sexes with one cohort starting at P22 till P26 and the second starting at P32 till P46. The

TABLE 1 | Mouse cohorts, number and age of the adolescent mice used in the behavioral test battery.

Cohorts	Strains	Mice (#)		Behavioral test at postnatal day (P#)
		Male ♂	Female ♀	
P22–26	C57BL/6N	11	7	Tail suspension (P22)
	DBA/2	11	12	Sucrose preference (P22–25)
	FVB/N	7	14	Forced swim (P26)
P32–36	C57BL/6N	10	14	Tail suspension (P32)
	DBA/2	9	9	Sucrose preference (P32–35)
	FVB/N	7	11	Forced swim (P36)
P27	C57BL/6N	7	8	
	DBA/2	11	12	Hyponeophagia test
	FVB/N	6	6	
P37	C57BL/6N	15	10	
	DBA/2	5	6	Hyponeophagia test
	FVB/N	7	11	

number of mice per cohort and the type of the behavioral experiments are listed (**Table 1**). The tail suspension test was the first test to be applied to each cohort. Starting the same day, we performed the sucrose preference test for 4 consecutive days. On day 5 the forced swim test was performed. For the anxiety-related hyponeophagia test, two other cohorts of mice were tested at P27 and P37.

The Behavioral Test Battery

The Tail Suspension Test

This test is useful in the screening of potential antidepressants and assessing depression-like behaviors in mice (Can et al., 2012). Each mouse was suspended to a rod by its tail with an adhesive tape at 55 cm above the surface. The latency for the first immobility and total immobility duration were measured during 6 min. An increased immobility duration or a reduced latency to first immobility are indicative of a depression-like phenotype. The test was videotaped and immobility time was analyzed by an independent observer.

The Forced Swim Test

This test was first introduced as a behavioral test to screen antidepressants (Porsolt et al., 1977; Porsolt, 1997). Mice were placed into a glass cylinder (20 cm in height, 14 cm in diameter) filled with water (24 $\pm$ 1°C) to a level that allowed mice to swim or float without their hind limbs or tails touching the bottom of the cylinder. The behavior of mice was monitored with the SYGNIS video tracker system (Sygnis Tracker 3) for 6 min and the immobility duration between 2 and 6 min was measured. Immobility was defined as a lack of swimming with only minimal movement of one hindlimb that was necessary to keep the head above water.

The Sucrose Preference Test

On day 1, the test was performed at P22 or P32 on single-housed mice in cages with two water bottles each. On the following day (day 2), both bottles were removed and changed with a bottle filled with water and a second one filled with a 1% sucrose solution. Both bottles were weighed before placing them into the cage. On day 3, bottles were weighed to determine the liquid consumption during the previous 24 h. Bottles were then refilled and weighed and placed into the cage with an alternated position of the sucrose vs. water bottle to avoid place preference. On day 4, bottles were weighed. The sucrose preference index was calculated as the average consumed sucrose across the last 2-day period divided by the average volume of total consumed liquid (average water plus average sucrose solution).

The Hyponeophagia Test

The hyponeophagia test measures the reduction in feeding in response to a novel environment and a portion of new food or drink, since they induce anxiety, resulting in a delayed food intake. Therefore, this test can be used for the assessment of emotionality and anxiety. To perform the test, we followed the protocol suggested by Deacon (2011) with minor modifications. 1 day prior to testing, food was rationed, and mice were given small pellets of 1 g per mouse. A food well filled with 1:1 milk diluted with water was placed under an inverted transparent jar (15 cm diameter). The mouse was carefully placed under the jar facing away from the food well. The latency to lick the milk continuously for >2–3 s during 2 min was measured. After finishing trial 1, the mouse was placed back into its home cage. After 3 min in the home cage, the mouse was placed again under the jar for another 2 min as trial 2, and the latency to lick the milk was measured.

Statistical Analysis

Two-way ANOVA was used with sex and genotype as the two factors. This was followed by Tukey's *post hoc* test for multiple comparisons to determine differences between the three strains C57BL/6N, DBA/2, and FVB/N and Bonferroni correction to check differences between males and females within each strain. To compare the two developmental stages (P22–26) and (P32–36) within each strain, two-way ANOVA was used with sex and age as the two factors. A P value ≤ 0.05 was considered statistically significant. To unravel the effect of the interaction between strain, sex, and age, three-way ANOVA was performed using a confidence interval of 95% and a tolerance of 0.0001. A $Pr(>F)$ less than 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism 7 and

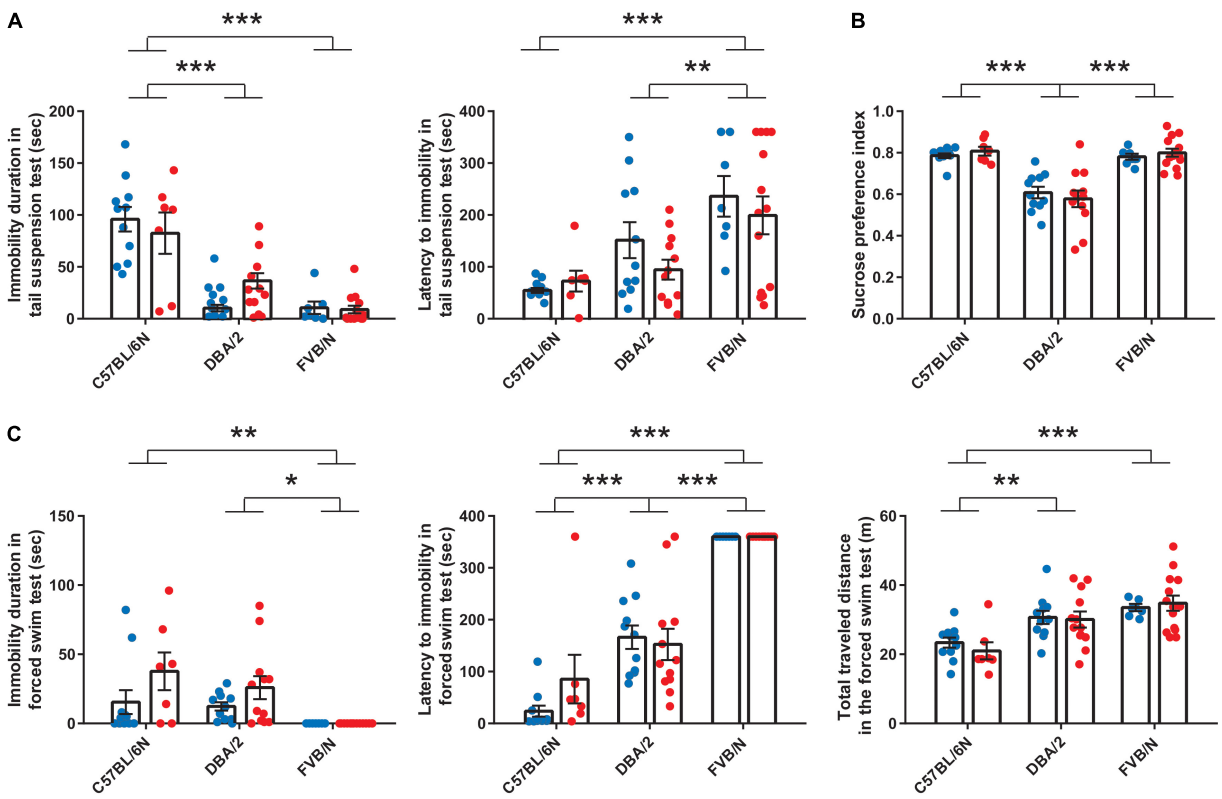

FIGURE 1 | Baseline depression-like behaviors in P22–26 mouse cohort. **(A)** In the tail suspension test, C57BL/6N mice showed a significant increase in the baseline immobility duration compared to both DBA/2 and FVB/N mice. Additionally, FVB/N mice showed a significant increase in the latency to first immobility compared to both C57BL/6N and DBA/2 mice. **(B)** In the sucrose preference test, DBA/2 mice showed a lower sucrose preference index than both C57BL/6N and FVB/N mice. **(C)** In the forced swim test, FVB/N mice showed lower immobility duration and increased latency to first immobility than C57BL/6N and DBA/2 mice. C57BL/6N showed a decreased total traveled distance compared to DBA/2 and FVB/N mice. In **(A–C)**, no sex difference was revealed within any of the aforementioned strains. Blue and red dots represent males and females, respectively. Two-way ANOVA followed by Tukey *post hoc* test,*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$. Error bars indicate the standard error of the mean (SEM).

Microsoft Office Excel including the XLSTAT software. The respective numbers of male and female mice per cohort are described in **Table 1**.

RESULTS

Baseline Depression-Like Behaviors in P22–26 Mouse Cohort

In the first set of experiments, we tested the baseline depression-like behaviors in the wild-type mice using three tests that are well known to assess depression in rodent models. Both forced swim (Porsolt et al., 1977; Porsolt, 1997) and tail suspension tests (Steru et al., 1985) measure the immobility of rodents as an indication of despair when they cannot escape from an aversive situation. Additionally, we performed the sucrose preference test as a reward-based test to assess anhedonia or decreased ability to experience pleasure, as a core symptom of depression (Willner et al., 1987; Papp et al., 1991).

We started our experiments by investigating the depression-like behaviors in male and female mice from a very young cohort (P22–P26). At P22, the tail suspension test revealed a significant increase in the baseline immobility duration of C57BL/6N mice compared to both DBA/2 and FVB/N mice ($P < 0.0001$ vs. DBA/2 and DBA/2; **Figure 1A**). Additionally, FVB/N mice showed increased latency to first immobility compared to both C57BL/6N and DBA/2 mice ($P < 0.0001$ vs. C57BL/6N, $P = 0.007$ vs. DBA/2; **Figure 1A**). For the sucrose preference test, DBA/2 mice showed an increased baseline anhedonia-like behaviors by having a significantly decreased sucrose preference index compared to both C57BL/6N and FVB/N mice ($P < 0.0001$ vs. C57BL/6N and FVB/N; **Figure 1B**). In the forced swim test at P26, FVB/N mice showed no immobility at all, and C57BL/6N mice showed a significant decreased latency to first immobility and total traveled distance compared to both DBA/2 and FVB/N mice (Immobility duration: C57BL/6N vs. DBA/2: $P = 0.78$, C57BL/6N vs. FVB/N: $P = 0.004$, DBA/2 vs. FVB/N: $P = 0.015$; Latency to first immobility: C57BL/6N vs. DBA/2: $P < 0.0001$, C57BL/6N vs. FVB/N: $P < 0.0001$, DBA/2 vs. FVB/N: $P < 0.0001$; Traveled

distance: C57BL/6N vs. DBA/2: $P = 0.001$, C57BL/6N vs. FVB/N: $P < 0.0001$, DBA/2 vs. FVB/N: $P = 0.16$; **Figure 1C**). Interestingly, comparing the performance of male and female mice within each strain revealed no significant difference in their behaviors in any of the aforementioned tests (**Table 2**).

Baseline Depression-Like Behaviors in P32–36 Mouse Cohort

Similar to the results at P22 (**Figure 1A**), the tail suspension test at P32 revealed a significant increase in the baseline immobility duration of C57BL/6N mice compared to both DBA/2 and FVB/N mice ($P < 0.0001$ vs. DBA/2 and FVB/N; **Figure 2A**). Additionally, C57BL/6N mice showed a significantly decreased latency to first immobility compared to DBA/2 and borderline decreased latency compared to FVB/N mice ($P = 0.0001$ vs. DBA/2, $P = 0.09$ vs. FVB/N; **Figure 2A**). For the sucrose preference test, DBA/2 mice showed an increased baseline anhedonia-like behaviors by having a significantly decreased sucrose preference index compared to both C57BL/6N and FVB/N mice ($P < 0.0001$ vs. C57BL/6N and FVB/N; **Figure 2B**). Moreover, FVB/N mice showed an increased sucrose preference index compared to C57BL/6N mice ($P < 0.0001$). In the forced swim test at P36, C57BL/6N mice showed an increased immobility duration, a decreased latency to first immobility and a decreased total traveled distance compared to both DBA/2 and FVB/N mice (Immobility duration: C57BL/6N vs. DBA/2: $P = 0.001$, C57BL/6N vs. FVB/N: $P < 0.0001$, DBA/2 vs. FVB/N: $P = 0.73$; Latency to first immobility: C57BL/6N vs. DBA/2: $P < 0.0001$, C57BL/6N vs. FVB/N: $P < 0.0001$, DBA/2 vs. FVB/N: $P = 0.004$; Traveled distance: C57BL/6N vs. DBA/2: $P < 0.0001$, C57BL/6N vs. FVB/N: $P < 0.0001$, DBA/2 vs. FVB/N: $P = 0.09$; **Figure 2C**). Similar to the P22–26 mouse cohort, the P32–36 mouse cohort did not reveal a significant sex difference in the depression-like behaviors in any of the aforementioned tests (**Table 2**).

Comparison Between the P22–26 and P32–36 Mouse Cohorts Within C57BL/6N, DBA/2, and FVB/N Strains

To test whether the age difference of 10 days can affect the baseline depression-like behaviors during adolescence, results of the behavioral test battery were compared between the two cohorts. In the tail suspension test, P32 C57BL/6N and P32 FVB/N mice showed a significantly increased immobility duration compared to P22 C57BL/6N and P22 FVB/N mice, respectively (C57BL/6N: $P < 0.0001$, FVB/N: $P < 0.0001$; **Figure 3A**). In contrast, P32 C57BL/6N and P32 FVB/N mice showed a significantly decreased latency to first immobility compared to P22 C57BL/6N and P22 FVB/N mice, respectively (C57BL/6N: $P = 0.0005$, FVB/N: $P = 0.0004$). On the other hand, no difference in the immobility duration ($P = 0.17$) or latency to first immobility ($P = 0.78$) was found between P32 and P22 DBA/2 mice (**Figure 3A**). This discrepancy in the effect of age on strain is evident by the significant $\Pr(>F)$ value in the three-way ANOVA test [Strain*Age: $\Pr(>F) < 0.0001$ for both the immobility duration and latency to first immobility] (**Table 3**).

For the sucrose preference test, FVB/N mice showed an increased sucrose preference index in older compared to younger mice with no effect of age on C57BL/6N and DBA/2 strains (C57BL/6N: $P = 0.96$, DBA/2: $P = 0.32$, FVB/N: $P < 0.0001$; **Figure 3B**), suggesting a sensitivity of the strain effect to mouse age [Strain*Age: $\Pr(>F) < 0.0001$] (**Table 3**). In the forced swim test, P36 C57BL/6N and P36 FVB/N mice showed a significantly increased immobility duration compared to P26 C57BL/6N and P26 FVB/N mice, respectively (C57BL/6N: $P = 0.008$, DBA/2: $P = 0.52$, FVB/N: $P = 0.002$; **Figure 3C**). For the latency of the first immobility, only P36 FVB/N mice showed a significantly decreased latency to the first immobility compared to P26 mice ($P < 0.0001$; **Figure 3C**). These results suggest different effects of age on the behavioral outcome in the forced swim test depending on which strain is used [Immobility duration: Strain*Age: $\Pr(>F) = 0.01$], latency of the first immobility: Strain*Age: $\Pr(>F) = 0.004$ (**Table 3**). In contrast, all three strains showed a similar decrease in the traveled distance in older compared to younger age (C57BL/6N: $P < 0.0001$, DBA/2: $P = 0.04$, FVB/N: $P = 0.04$; **Figure 3C**), and suggesting no interaction between strain and age [Strain*Age: $\Pr(>F) = 0.785$] (**Table 3**).

Anxiety-Related Hyponeophagia Test at P27 and P37

Since anxiety-related behavioral tests are often performed together with depression tests in mouse models of neuropsychiatric disorders (Cryan and Mombereau, 2004; Millstein and Holmes, 2007; Krishnan and Nestler, 2011; Jung et al., 2014; Belovicova et al., 2017; Sokolowska et al., 2021), we extended our study and investigated sex differences in an anxiety test, the hyponeophagia test. Consistent with our previous study revealing sex differences in other anxiety tests in adolescent mice (Eltokhi et al., 2020), female C57BL/6N and DBA/2 mice showed an increased latency to drink the diluted milk compared to male mice at P27 (Trial 1: C57BL/6N: $P = 0.006$, DBA/2: $P = 0.041$ FVB/N**:** $P = 0.99$; Trial 2: C57BL/6N: $P = 0.014$, DBA/2: $P = 0.027$, FVB/N**:** $P = 0.55$; **Figure 4A** and **Table 2**). In contrast, this sex effect was not present at P37, highlighting the effect of a 10-day interval effect on the behavioral outcome (**Figure 4B** and **Table 2**). At both P27 and P37, no strain difference was found in the latency to drink the diluted milk (P27 trial 1: C57BL/6N vs. DBA/2: $P = 0.75$, C57BL/6N vs. FVB/N: $P = 0.20$, DBA/2 vs. FVB/N: $P = 0.44$; P27 trial 2: C57BL/6N vs. DBA/2: $P = 0.75$, C57BL/6N vs. FVB/N: $P = 0.16$, DBA/2 vs. FVB/N: $P = 0.37$; P37 trial 1: C57BL/6N vs. DBA/2: $P = 0.95$, C57BL/6N vs. FVB/N: $P = 0.23$, DBA/2 vs. FVB/N: $P = 0.24$; P37 trial 2: C57BL/6N vs. DBA/2: $P = 0.62$, C57BL/6N vs. FVB/N: $P = 0.78$, DBA/2 vs. FVB/N: $P = 0.33$).

DISCUSSION

The global increased prevalence of depression in women compared to men suggests that the differential risk is highly dependent on biological sex differences rather than race, culture or other potentially confounding social and economic factors (Albert, 2015). As the onset of depression

TABLE 2 | List of the *P* values of the comparison between male and female mice of C57BL/6N, DBA/2, and FVB/N strains in the behavioral test battery.

	C57BL/6N	DBA/2	FVB/N	C57BL/6N	DBA/2	FVB/N
	P22–26 cohort			P32–36 cohort		
Tail suspension test: Immobility duration	0.979	0.087	>0.999	0.160	>0.999	>0.999
Tail suspension test: Latency to immobility	>0.999	0.470	>0.999	>0.999	>0.999	0.321
Sucrose preference index	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999
Forced swim test: Immobility duration	0.117	0.422	>0.999	0.401	>0.999	>0.999
Forced swim test: Latency to immobility	0.243	>0.999	>0.999	>0.999	>0.999	>0.999
Forced swim test: Total traveled distance	>0.999	>0.999	>0.999	0.581	0.996	>0.999
	P27 cohort			P37 cohort		
Hyponeophagia test trial 1: Latency to drink	*0.006*	*0.041*	>0.999	>0.999	>0.999	>0.999
Hyponeophagia test trial 2: Latency to drink	*0.014*	*0.027*	0.550	>0.999	0.721	>0.999

The italic indicates significant results.

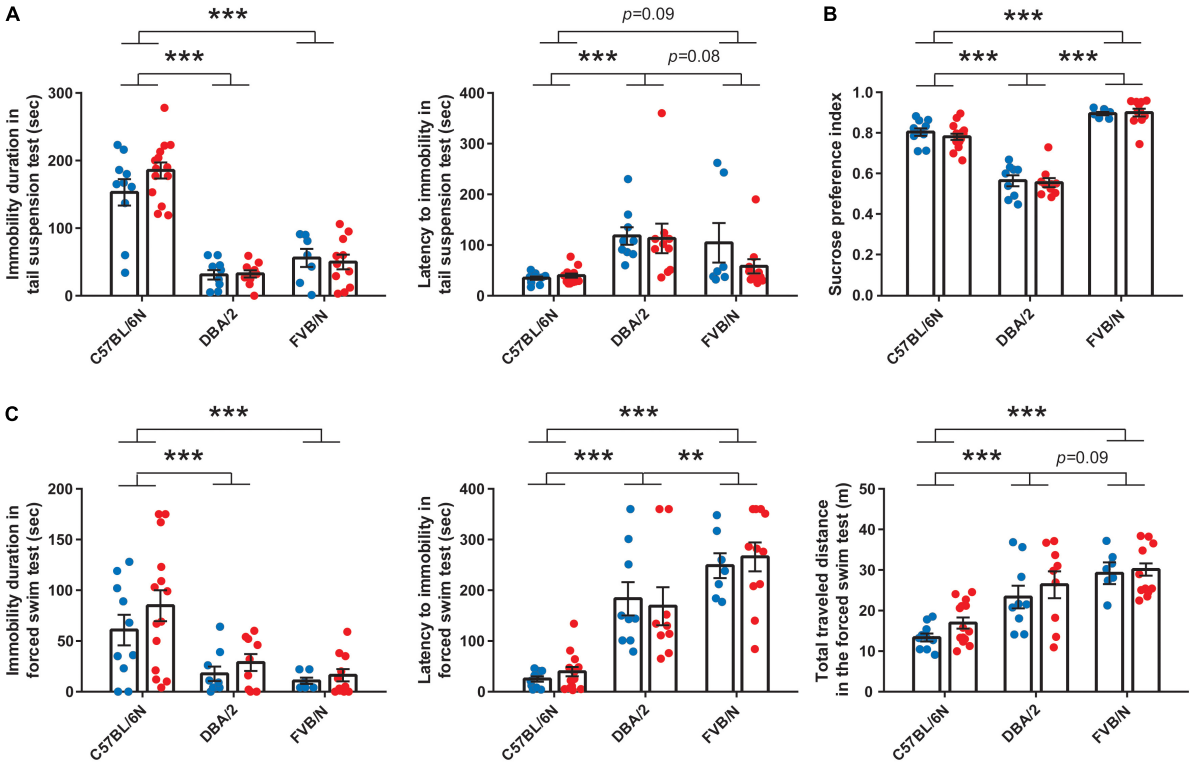

FIGURE 2 | Baseline depression-like behaviors in P32–36 mouse cohort. **(A)** In the tail suspension test, C57BL/6N mice showed a significant increase in the baseline immobility duration compared to both DBA/2 and FVB/N mice. C57BL/6N mice showed a significantly decreased latency to first immobility compared to DBA/2 and borderline decreased latency compared to FVB/N mice. **(B)** In the sucrose preference test, DBA/2 mice showed an increased baseline anhedonia-like behaviors by having a significantly decreased sucrose preference index compared to both C57BL/6N and FVB/N mice. Moreover, FVB/N mice showed an increased sucrose preference index compared to C57BL/6N mice. **(C)** In the forced swim test, C57BL/6N mice showed an increased immobility duration, a decreased latency to first immobility and a decreased total traveled distance compared to both DBA/2 and FVB/N mice. In **(A–C)**, no sex difference was revealed within any of the aforementioned strains. Blue and red dots represent males and females, respectively. Two-way ANOVA followed by Tukey *post hoc* test, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Error bars indicate the standard error of the mean (SEM).

in women peaks in their reproductive years, the increased prevalence of depression may be explained in part by sex hormones. Indeed, the female hormonal fluctuation during puberty, menstruation, pregnancy, and menopause is a trigger for depression (Albert, 2015). The risk of depression increases during the perimenopausal transition (Cohen et al., 2006), with hormone replacement therapy being effective in the prevention of postmenopausal depression in women

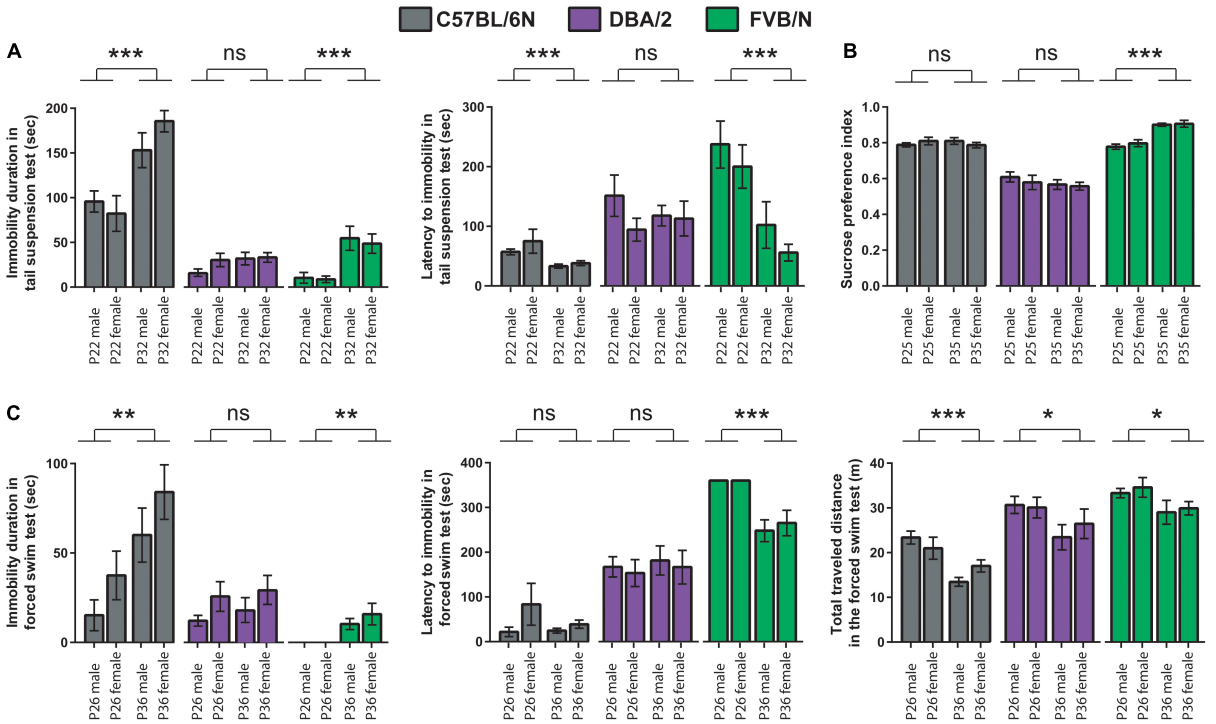

FIGURE 3 | Comparison between the P22–26 and P32–36 mouse cohorts within C57BL/6N, DBA/2, and FVB/N strains. **(A)** In the tail suspension test, P32 C57BL/6N and P32 FVB/N mice showed a significantly increased immobility duration and decreased latency to first immobility compared to P22 C57BL/6N and P22 FVB/N mice, respectively. **(B)** In the sucrose preference test, FVB/N mice showed an increased sucrose preference index in older compared to younger mice with no effect of age on C57BL/6N and DBA/2 strains. **(C)** In the forced swim test, P36 C57BL/6N and P36 FVB/N mice showed a significantly increased immobility duration compared to P26 C57BL/6N and P26 FVB/N mice, respectively. For the latency to the first immobility, only P36 FVB/N mice showed a significantly decreased latency to the first immobility compared to P26 mice. For the total traveled distance, all three strains showed a decreased traveled distance in older compared to a younger age. Two-way ANOVA followed by Tukey *post hoc* test,*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$. Error bars indicate the standard error of the mean (SEM).

(Gordon and Girdler, 2014). Androgens seem to have anxiolytic properties whereas estrogens receptors (ER) activation has opposite consequences with ERα having largely anxiogenic-like properties and ERβ serving to generate anxiolytic-like effects (Borrow and Handa, 2017).

Here we evaluated depression-like behaviors in adolescent mice at two different developmental stages. Our previous studies unraveled that sex differences during adolescence are apparent in certain strains and behaviors such as general activity, anxiety and cognitive function (Eltokhi et al., 2020) as also shown for adult mice (Tarantino et al., 2000). Given the divergent susceptibility of males and females to depression, there is an urgent need for tackling the effect of sex in rodents on behavioral tests assessing depression-like behaviors. However, in all the tests commonly used to assess depression-like behaviors in rodents, we did not find any sex difference in C57BL/6N, DBA/2, or FVB/N strains at these two developmental stages during adolescence. Since we investigated the baseline depression-like behaviors only in young mice before puberty corresponding to P42 in mice (Taft et al., 2006; Dutta and Sengupta, 2016), with no severe effect of sex hormones, we interpret the finding that the effect of sex may become apparent only in adult mice. Indeed, a sex difference in the prevalence of patients with major depressive disorder appears mainly after puberty (Bebbington et al., 1998; Merikangas et al., 2010; Mehta et al., 2013). In adult C57BL/6J mice, the baseline immobility durations in the tail suspension tests were lower in males than females (Liu and Gershenfeld, 2001). Thus, the biological maturation occurring during puberty along with the intensification of sex-specific social roles may be a major key of sex differences in depression (Dalla et al., 2010). On the other hand, a more recent piece of evidence suggests that the sex difference in depression begins in childhood and becomes more pronounced during adolescence (Breslau et al., 2017). Irrespective of these analyses in humans, inconsistent results for depression and anxiety-like behaviors have been reported when comparing male and female adolescent rodents to adults (Slawecki, 2005; Doremus et al., 2006; Hefner and Holmes, 2007; Doremus-Fitzwater et al., 2009; Martínez-Mota et al., 2011). Thus, further developmental and longitudinal studies in rodents should be performed to assess the baseline depression in males and females along different developmental stages. Since the onset of puberty is strain dependent (Pintér et al., 2007), a rough dating of puberty of rodent models should be employed using various markers including the vaginal opening, first vaginal cornification, onset of cyclicity in females and balanopreputial separation in males.

Strain differences in behavioral tests assessing neuropsychiatric-like phenotypes in adolescent mice were

TABLE 3 | List of the Pr(>*F*) after performing three-way ANOVA test to assess the effect of the interaction between strain, sex and age on the depression-like behavior.

Tail suspension test: Immobility duration		**Forced swim test: Immobility duration**	
Strain*Age	*<0.0001*	Strain*Age	*0.010*
Strain*Sex	0.447	Strain*Sex	0.372
Age*Sex	0.447	Age*Sex	0.901
Tail suspension test: Latency to immobility		**Forced swim test: Latency to immobility**	
Strain*Age	*<0.0001*	Strain*Age	*0.004*
Strain*Sex	0.295	Strain*Sex	0.313
Age*Sex	0.699	Age*Sex	0.672
Sucrose preference index		**Forced swim test: Total traveled distance**	
Strain*Age	*<0.0001*	Strain*Age	0.785
Strain*Sex	0.616	Strain*Sex	0.991
Age*Sex	0.655	Age*Sex	0.195

The italic indicates significant results.

repeatedly reported by our group (Peleh et al., 2019; Eltokhi et al., 2020, 2021). The contribution of genetic factors specifically in the depression-related behaviors of adult inbred rodents as well as in the efficacy of antidepressant drug treatment has been previously shown (Vaugeois et al., 1997; Liu and Gershenfeld, 2001; Lucki et al., 2001). In our study, the baseline depression-like behaviors differed between different strains. In both tail suspension and forced swim tests, C57BL/6N mice showed the highest immobility duration and the lowest latency to first immobility compared to DBA/2 and FVB/N. Interestingly, FVB/N mice at P26 did not show any immobility in the forced swim test. This behavior can be explained by their severe hyperactivity as previously reported (Eltokhi et al., 2020). These data are consistent with a former study showing adult FVB/NJ mice having the least baseline immobility duration in the forced swim test, DBA/2J being intermediate and C57BL/6J showing the highest immobility duration (Lucki et al., 2001). On the other hand, adolescent FVB/N mice showed the highest sucrose preference index, highlighting the suitability of this test to be performed on mice with FVB/N background. In contrast, DBA/2 mice showed low ability to perform the sucrose preference test and had a high baseline anhedonic level, which may mask the depression behavior in mouse models of neuropsychiatric disorders and should be taken into consideration in designing the experiments and analyzing the data. Nonetheless, DBA/2 can be a good strain to test the effect of anti-depressant drugs. Thus, the choice of the strain should be related to the specific scientific question being asked.

As our previous studies uncovered strong changes in behavioral outcomes including activity, anxiety and social interaction in a small developmental window during adolescence (Eltokhi et al., 2020, 2021), we here extended these findings assessing despair by tail suspension and forced swim tests. Both C57BL/6N and FVB/N mice showed an increase in immobility within 10 days of adolescence. For the sucrose preference test, the effect of the 10-day interval was only apparent in FVB/N mice. Interestingly, DBA/2 mice did not show any difference in the behavioral outcome at both time points, highlighting a direct correlation of the effect of age to specific strains. One limitation of our study is that the same mice were tested in a series of depression-related tests, which may have caused earlier tests to affect subsequent performance in later tests. To mitigate this possibility, we ordered the tests in such a way that the two stressful tests, tail suspension and forced swim tests, were not consecutive but separated by the sucrose preference test that was performed in the mouse homecage. Still, the single housing during the sucrose preference test may have also induced some levels of stress on mice, which may have affected their behaviors in the forced swim test. However, we believe that the possible effect of single housing was not strong as indicated by the similar pattern of behaviors in both tail suspension and forced swim tests at both developmental stages, with C57BL/6N mice showing increased immobility durations in both tests. Our findings suggest that taking the effect of age and strain into account will decrease variabilities and inconsistencies and increase the reproducibility of behavioral results between different laboratories.

Anxiety-related behavioral tests are often performed together with depression tests in mouse models of neuropsychiatric disorders since anxiety usually co-morbids with depression (Cryan and Mombereau, 2004; Millstein and Holmes, 2007; Krishnan and Nestler, 2011; Jung et al., 2014; Belovicova et al., 2017; Sokolowska et al., 2021). Several behavioral tests based on the conflict between competing behaviors of exploring novel environments and avoiding potential threatening situations have been validated to assess anxiety, including the elevated plus maze (Pellow et al., 1985; Lister, 1987), open field (Treit and Fundytus, 1988), and light/dark box (Crawley and Goodwin, 1980; Crawley, 1981; Blumstein and Crawley, 1983), where our previous studies revealed sex differences in the open field and elevated plus maze tests with female C57BL/6N and FVB/N mice being less anxious (Eltokhi et al., 2020). To unravel whether different anxiety paradigms tax distinct aspects of anxiety, we here investigated the sex difference in another anxiety-related test, the hyponeophagia test. Female C57BL/6N and DBA/2 but not FVB/N mice showed higher latency to consume the unfamiliar drink in an unfamiliar arena compared to males, reflecting increased bait shyness as an indication of anxiety. This discrepancy of results of males vs. females in different anxiety tests suggests that a battery of different tests should be used in

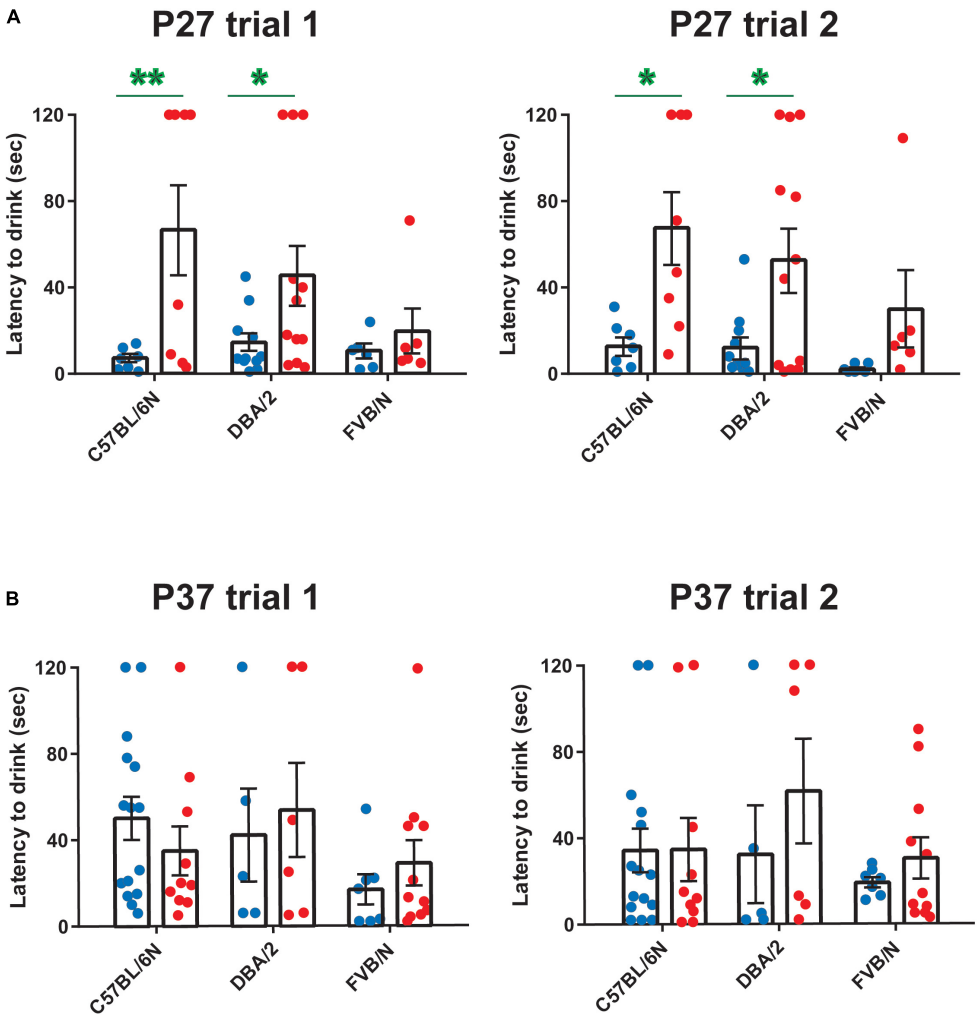

FIGURE 4 | Anxiety-related hyponeophagia test at P27 and P37. Female C57BL/6N and DBA/2 mice showed an increased latency to drink the diluted milk compared to male mice in both trials 1 and 2 at P27 **(A)** but not at P37 **(B)**. At both P27 and P37, no strain difference was found in the latency to drink the diluted milk. The green asterisk indicates a significant difference between males and females. Two-way ANOVA followed by Tukey *post hoc* test,*$p \leq 0.05$, **$p \leq 0.01$. Blue and red dots represent males and females, respectively. Error bars indicate the standard error of the mean (SEM).

studies of anxiety-related behaviors to draw the full picture of mouse phenotype (van Gaalen and Steckler, 2000).

Taken together, since depression is a complex disorder with several endophenotypes including despair, anhedonia and apathy, a complete test battery combining the well-established, robust behavioral tests should be employed when testing rodent models of neuropsychiatric disorders or investigating the effect of anti-depressant drugs. Sex, strain and age are suggested to have different effects on distinct behavioral tests. Therefore, performing a complete test battery will provide important information per animal/drug that cannot be covered in separate behavioral studies. Notably, there should be at least a 1-day rest period between each of the tests in the behavioral battery, with some rodent strains requiring even longer durations (Lad et al., 2009). Some paradigms currently used for assessing antidepressant and/or depression-like behaviors in mice are still questionable. For example, in recent years, there is a trend to interpret the transition from swimming to immobility in the forced swim test as a coping mechanism with inescapable stressors, rather than an indication of despair (Molendijk and de Kloet, 2019). However, the majority of researchers still qualify the rodent's floating response as a depression-like behavior since the persistence of coping with inescapable stressors may indeed enhance the vulnerability to depression (Molendijk and de Kloet, 2019). These different scientific perspectives confirm the necessity of performing a complete test battery in order to draw a full picture of the depression-like behavior.

Finally, we want to touch on the issue of reproducibility of behavioral results in depression-related studies. One potential factor of discrepancy is the slightly different protocols between laboratories. Worth noticing, behavioral tests may cause stress and put unwanted burdens on rodents. To this end, the order of tests on a specific cohort can also play a role in the variabilities

between the results of different laboratories. Moreover, different rodent strains and sex dependence of the stress response frequently result in apparent discrepancies in published data. However, our study, exemplified by trials 1 and 2 in latency to drink (**Figure 4**), demonstrated that consistency of results can be easily achieved by adequate test settings. Furthermore, during adolescence, no sex differences were found. This finding does not exclude an impact of sex in other developmental stages. However, in studies where sex differences are suggested to provoke an additional complication in depression analysis, adolescent mice may open a window for clearly identifying such effects since the general performance and baseline depression activity of male and female wild-type mice are similar.

Thus, strain, age and sex should be taken into account when analyzing neuropsychiatric disorders in mouse models. Without question, optimization and standardization of depression-related tests in rodent models will help in understanding pathophysiological mechanisms and in identifying novel targets for depression treatment.

CONCLUSION

To our knowledge, this is the first study investigating sex, age and strain effects on the baseline depression-like behaviors. We confirmed that genetic strain differences and even small differences in developmental stage are important determinants of depression-related behavioral outcomes. We suggest using adolescent mice, at least in the three investigated depression-related behavioral tests, to reduce variability and inconsistency between different laboratories. Nonetheless, sex differences in mice still need a thorough evaluation throughout their lifetime. Taken together, our behavioral studies in adolescent mice can be used as a guiding platform for the choice of the most suitable combinations of assays and appropriate strain, age and sex selection in mouse models of neuropsychiatric disorders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by The animal Ethics Committee of the Government of Baden Württemberg, Regierungspräsidium Karlsruhe, (G-101/16).

AUTHOR CONTRIBUTIONS

AE: conceptualization, data curation, formal analysis, project administration, supervision, validation, visualization, and writing–original draft, review and editing. BK: methodology, data curation, and formal analysis. CP: conceptualization, project administration, supervision, and validation, review and editing. All authors contributed to the article and approved the submitted version.

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Characterizing Sex Differences in Depressive-Like Behavior and Glial Brain Cell Changes Following Peripheral Nerve Injury in Mice

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Chronic pain and depression are intimately linked; the combination of the two leads to higher health care costs, lower quality of life, and worse treatment outcomes with both conditions exhibiting higher prevalence among women. In the current study, we examined the development of depressive-like behavior in male and female mice using the spared nerve injury (SNI) model of neuropathic pain. Males displayed increased immobility on the forced-swim test – a measure of depressive-like behavior – 2 weeks following injury, while females developed depressive-like behavior at 3-week. Since the pathogenesis of chronic pain and depression may involve overlapping mechanisms including the activation of microglial cells, we explored glial cell changes in brain regions associated with pain processing and affect. Immunohistochemical analyses revealed that microglial cells were more numerous in female SNI mice in the contralateral ventral anterior cingulate cortex (ACC), a brain region important for pain processing and affect behavior, 2-week following surgery. Microglial cell activation was not different between any of the groups for the dorsal ACC or nucleus accumbens. Analysis of astrocyte density did not reveal any significant changes in glial fibrillary acidic protein (GFAP) staining in the ACC or nucleus accumbens. Overall, the current study characterized peripheral nerve injury induced depression-like behavior in male and female mice, which may be associated with different patterns of glial cell activation in regions important for pain processing and affect.

Keywords: chronic pain, depressive-like, microglia, astrocytes, forced swim test and tail suspension test

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INTRODUCTION

Chronic pain is one of the most prevalent and debilitating conditions affecting as many as 20% of the population worldwide (Goldberg and McGee, 2011). It is the foremost cause of long-term sick leave, and disability imposing a profound health care burden that not only affects the individual but also permeates throughout all avenues of social, work, and family life (Lynch, 2011; Gaskin and Richard, 2012). However, despite the prevalence of chronic pain, it remains challenging to treat, in part because it is associated with a high incidence of comorbid conditions, including anxiety and depression (Nicholson and Verma, 2004). Approximately 50% of patients diagnosed with major depressive disorder reportedly suffer from chronic pain, and the risk of developing comorbid

depression in chronic pain patients is greater among women (Radat et al., 2013). Further, patients suffering from chronic pain and depression have a poorer prognosis than patients suffering from each illness alone (Arnow et al., 2006; Munce and Stewart, 2007). Clinically, it is well known that chronic pain and depression are related; however, the neurobiological changes responsible for the co-occurrence of these conditions are not well-understood, and the sex-specific mechanisms that might help explain the high prevalence of these conditions in women remain unknown.

Microglia, a principal immune cell, comprise approximately 5–12% of the glial cell population and emerge from either erythromyeloid precursors of the embryonic yolk sac or invade the central nervous system (CNS) via myeloid progenitors and proliferate during embryonic and postnatal development (Hristovska and Pascual, 2015). Microglia interact with and prune synapses during healthy brain development to modify their structure and function (Chen et al., 2018b). As such, in a healthy resting state, microglia actively sense the environment to maintain normal physiological conditions; however, following tissue damage or inflammation, microglia transition from an inactive ramified state to an active amoeboid state – characterized by enlarged cell bodies and shortened processes and increased expression of microglial markers such as ionized calcium-binding adapter molecule 1 (IBA-1) and CD11b (Ohsawa et al., 2004; Ji et al., 2013; Chen et al., 2018b). A few studies have demonstrated that chronic pain hypersensitivity is dependent on microglia in male but not female rodents (Sorge et al., 2015; Chen et al., 2018b; Mapplebeck et al., 2018). Further, spinal microglial activation promotes BDNF release and pain hypersensitivity in male but not female rats – an effect that is conserved in humans (Dedek et al., 2021).

An abundance of research has focused on spinal mechanisms; however, there is evidence to suggest that brain microglia may contribute to the development and maintenance of chronic pain and its associated comorbidities. A study by Miyamoto et al. (2017) showed that intracerebroventricular injections of minocycline – a broad-spectrum antibiotic known to inhibit glial cell activity – reduced microglial activation in the anterior cingulate cortex (ACC) and reversed tactile allodynia. In a rat model of diabetic neuropathy, ammoxetine, a novel, potent serotonin, and norepinephrine reuptake inhibitor, reversed mechanical allodynia and depressive-like behavior that coincided with decreased spinal microglial activation (Zhang et al., 2018). Furthermore, a recent study using a rodent model of neuropathic pain, called chronic constriction injury (CCI), revealed that male mice developed affective disorders, albeit at a delayed time point, which was associated with activation of microglia characterized by increased numbers and cell body size in the prefrontal cortex, amygdala, and hippocampus (Barcelon et al., 2019).

There is also evidence to suggest that brain astrocytes may be involved in regulating pain sensitivity and affect following injury (Chen et al., 2018b). Several studies have demonstrated that activated astrocytes in the ACC and hippocampus are involved in the maintenance of chronic pain and the onset of depression (see Li et al., 2019 for a review). Narita et al. (2006) demonstrated that peripheral nerve injury in mice increased glial fibrillary acidic protein (GFAP) expression in the ACC, which was associated with increased affective behaviors. Similarly, Ikeda et al. (2013) used a mouse model of inflammatory pain and showed increased astrocytic expression in the ACC resulted in increased affective behavior that was attenuated with the administration of L-α-aminoadipate (L-AA), an astroglia toxin. Further, the administration of antidepressant drugs inhibits hippocampal astrocyte activation and reduces nerve injury-induced pain hypersensitivity (Zhu et al., 2009). However, there seems to be a paucity of research on astrocytes in chronic pain and depression, necessitating further investigation.

The primary goal of the present study was to identify whether sex influenced the development of depressive-like behavior in mice following spared nerve injury (SNI). A secondary goal was to characterize whether brain glial activation was increased in a time-, sex-, and-, brain region-dependent manner following injury. Based off clinical studies showing a greater degree of comorbid depression in female chronic pain patients (Munce and Stewart, 2007), we hypothesized that female mice would display greater and more robust signs of depressive-like behavior, which would be associated with increased glial activity. We used a battery of behavioral tests and examined two early time points (2 and 3 weeks) following nerve injury. Microglia and astroglia in the ACC (ventral and dorsal compartments) and the nucleus accumbens were quantified given data supporting a role for these regions in clinical anhedonia and response to treatment (Pizzagalli et al., 2001; Wacker et al., 2009; Carl et al., 2016).

MATERIALS AND METHODS

Mice

Outbred CD-1 male and female mice (6–8 weeks old) obtained from Charles River were used and maintained in the University of Toronto Mississauga's Animal Care Facility. All mice were housed in non-ventilated cages in groups of 2–4, maintained in a temperature-controlled (20 ± 1°C) environment with 12:12 h light: dark cycle (lights on at 7 am and off at 7 pm) with access to food (Harlan Teklad 8604) and water *ad libitum*. Experiments were conducted only during the light period. All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Toronto Animal Care Committee.

Behavioral Measures

The automated von Frey, forced swim test (FST), tail suspension test (TST), open field, and sucrose preference assays were used on three independent cohorts of mice. In all experiments, mice were habituated to the testing environment for at least 1 h before the experiment. The first cohort was used to assess mechanical sensory thresholds and forced swim behavior at the indicated time points. Following baseline von Frey measurement, mice were randomly assigned to either the SNI or sham group and the 2-week or 3-week post-surgery timepoint groups. FST was only tested once for each animal on its respective timepoint (i.e., at 2-week post-surgery or 3-week post-surgery). If a mouse was assigned to the 3-week time point, its mechanical

threshold was measured at 2-week post-surgery and 3-week post-surgery, while FST was only measured at 3-week post-surgery. Following the FST, mice were sacrificed, and brain tissue was collected for immunohistochemical analysis. The second cohort was tested for tail suspension and open-field behavior at the indicated time points, while a third cohort was tested for sucrose preference weekly.

Automated von Frey

An automated von Frey test (Ugo Basile Dynamic Plantar Aesthesiometer) was used to assess mechanical nociceptive thresholds in all mice. Mice were placed in custom-constructed Plexiglas cubicles (6.3 cm × 5.5 cm × 10 cm) on a perforated metal floor and allowed to habituate for 1 h before testing. A blunt probe was raised toward the plantar surface of the hind paw, upon which pressure was gradually increased until the mouse withdrew its hind paw; the maximal pressure displayed at that point was then recorded. Five consecutive measures were taken on both hind paws. In all experiments, von Frey measurements were taken in both ipsilateral and contralateral hind paws before surgery (baseline), 2-week, and 3-week post-surgery. However, only data from the hind paw ipsilateral to the injury are presented as no significant differences on the contralateral paws were observed.

Spared Nerve Injury

The spared nerve injury (SNI) model, an experimental nerve injury, was used to produce neuropathic pain in mice (Decosterd and Woolf, 2000). Mice were randomly assigned to one of two surgery groups, sham or SNI. Briefly, mice were anesthetized with isoflurane (4% for induction; 2.5% for maintenance), and the biceps femoris muscle was bluntly dissected to expose the sciatic nerve. The common peroneal and tibial branches of the sciatic nerve were ligated with silk sutures (7.0 silk, Ethicon), and a 1 mm portion of the nerve was removed below the suture, leaving the sural nerve intact. The muscle and skin were then stitched with sutures (6.0 coated vicryl, Ethicon). The sham surgery consisted of a similar blunt dissection of the biceps femoris muscle without dissection of the nerve. Mice were allowed to recover in their home cages for 2 weeks following surgery.

Forced Swim Test

The forced swim test was used as a measure of behavioral despair. All mice were placed in glass cylinders (25 cm × 14.6 cm) filled with water (24 ± 1°C) 15 centimeters deep for 6 min and recorded using standard Sony video recording devices. Solid brown dividers were placed between each glass container to prevent mice from observing each other. Videos were uploaded to a tracking software (EthoVision, Noldus) which automatically calculated the time spent immobile in seconds.

Tail Suspension Test

In a subset of mice, the tail suspension test was used as a measure of learned helplessness. This test monitors the amount of time spent immobile when suspended by the tail over 6 min. The mouse was securely fastened by the end of the tail to a flat surface that was suspended in a Plexiglas box (40 cm × 40 cm × 40 cm). Behavior was recorded using a video camera and analyzed for immobility using Noldus EthoVision.

Open Field Test

Mice were placed in a Plexiglas box (40 cm × 40 cm × 40 cm) and videotaped for offline analysis. The open field was divided into a 4 by 4 grid. The four center squares in the grid were considered the "open area," while the 12 perimeter boxes were analyzed as "wall areas." All videos were recorded and analyzed using Noldus EthoVision.

Sucrose Preference Test

Mice were habituated to a 2% sucrose solution in their home cage (150 ml) for 2 days. Two bottles were then attached to the home cages of individually housed mice to ensure sucrose preference before starting the experiment. Mice were then randomly divided into the sham or SNI condition. Mice were then given free access to two bottles, one containing a 2% sucrose solution (150 ml) and the other water (150 ml) for 24 h. Following the 24 h interval, the amount of liquid consumed was then measured, and sucrose preference was calculated as the average of the daily amount of sucrose solution consumed divided by the total liquid consumed from both bottles. Food and water were restricted for 17 h (4:00 pm – 9:00 am), preceding a two-bottle choice as previously done (Chu et al., 2016). During the 2-bottle choice, mice had free access to food.

Immunohistochemistry

Following behavioral testing, mice were deeply anesthetized with pentobarbital and transcardially perfused with cold phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA). Immediately following transcardial perfusions, whole brains were isolated and post-fixed in 4% PFA for 4 h at 4°C and then cryoprotected in 30% PBS-sucrose until sectioning. Brains were hemisected along the sagittal axis and then sectioned using a cryostat (−13°C to −20°C) into 40 μm coronal sections and stored in tissue storage buffer until needed for staining. Sections were washed with PBS for 5 min and then washed in 0.1% PBS-T three times for 5 min. Slices were blocked with goat serum for 2 h and washed three times for 5 min in 0.1% PBS-T. Finally, slices were incubated using two primary antibodies [Anti-Iba1, Red Fluorochrome (635)-conjugated, Wako Chemical; and Anti-GFAP, Cy3 Conjugate, Millipore Sigma] for 48 h at 4°C. Following a post-incubation period of 48 h, slices were washed with PBS for 5 min and then mounted onto Superfrost slides and imaged using the Cytation 5 Cell Imaging Multimode Reader (BioTek, Winooski, VT, United States) at 20x objective. Image acquisition settings were identical for all slices across all time points. Negative controls omitting the primary antibody resulted in a complete absence of positive staining. Immunohistochemistry was run in batches of slices with sections from each group/condition included in each run.

Glial Cell Quantification

Microglia and astrocytes were measured in the contralateral and ipsilateral dACC (n = 5–6/sex/timepoint), vACC (n = 5–6), and Nac (n = 5–6). Briefly, microglia numbers were imaged and counted across three brain slices per region of interest (ROI) for each hemisphere. Images were taken using the Cytation 5 Imaging Multi-Mode Reader (BioTek, Winooski, VT,

United States) at 4X for regional identification and 20X for cell counting. For each slice, a z-stack projection of 40 μm was generated, and each region of interest was outlined and measured using Gen5 software (BioTek, Winooski, VT, United States). Cells expressing fluorescence were counted manually by an experimenter who was blind to experimental conditions using the same area settings for each brain region. Cells were considered microglia based on colocalization between Iba-1 and DAPI. Astrocytes were quantified by measuring GFAP fluorescence signal in the ROI using the GEN5 software. GFAP intensity was calculated to be the GFAP-integrated intensity minus the background-integrated intensity for each ROI. All GFAP images were acquired using the same exposure settings. In all instances, the individual performing this analysis was blind to the condition.

Statistical Analyses

SPSS Statistics 24 (IBM, Chicago, IL, United States) software was used to perform all analyses using three- or four-way ANOVAs as appropriate. Unless otherwise stated, a p-value of less than 0.05 was considered statistically significant. *Post hoc* testing was performed using paired or independent t-tests between sham and SNI mice within sex and time point.

RESULTS

Depressive- and Anxiety-Like Behavior Is Expressed Differently in Male and Female Mice Following Spared Nerve Injury

To determine the effect of chronic pain on depressive-like behavior in mice, we used the SNI model of neuropathic pain (Decosterd and Woolf, 2000). Following surgery, SNI mice demonstrated a significant increase in hind paw mechanical hypersensitivity compared with sham controls when tested 2- and 3-week post-surgery [three-way repeated measures (RM) ANOVA, main effect of surgery: $F_{1,28} = 262.45$, $p < 0.001$; main effect of sex: $F_{1,28} = 3.47$, $p = 0.08$; main effect of time (RM): $F_{2,27} = 30.79$, $p < 0.001$; surgery × time interaction: $F_{2,27} = 75.28$, $p < 0.001$, **Figure 1A**]. There was no difference in hypersensitivity between male and female SNI mice (surgery × sex interaction: $F_{1,28} = 0.007$, $p > 0.05$). To determine whether neuropathic pain altered behavioral despair, mice were tested for immobility on the forced swim test (FST). Male and female mice with SNI displayed overall greater immobility on the FST. However, immobility was greater for male SNI mice 2-week following surgery, while female SNI mice showed more immobility 3-week following surgery when compared with sham controls (three-way ANOVA, main effect of surgery: $F_{1,55} = 27.29$, $p < 0.001$; main effect of sex: $F_{1,55} = 1.868$, $p = 0.17$; main effect of time: $F_{1,55} = 2.01$, $p = 0.16$; surgery × sex × time interaction: $F_{1,55} = 5.42$, $p = 0.02$; **Figure 1B**). Using a separate cohort of mice, we used the tail suspension test (TST) to determine whether the effects of SNI on male and female mice extrapolated to another measure of behavioral despair. In line with the results of our FST, SNI mice displayed greater immobility on the TST with female mice showing greater signs of behavioral despair 3-week following surgery (three-way ANOVA, main effect of surgery: $F_{1,56} = 23.52$, $p < 0.001$; main effect of sex: $F_{1,56} = 12.72$, $p < 0.001$; main effect of time: $F_{1,56} = 0.57$, $p = 0.32$; sex × time interaction: $F_{1,56} = 9.56$, $p = 0.003$; **Figure 1C**). This same cohort of mice was also tested for time spent in the center of the open field as a general measure of anxiety. Female SNI mice showed reduced time spent in the center area of the open field 3-week following surgery, indicating an anxiety-like phenotype at this time point (three-way ANOVA, main effect of surgery: $F_{1,56} = 5.92$, $p = 0.018$; main effect of sex: $F_{1,56} = 6.95$, $p = 0.011$; main effect of time: $F_{1,56} = 1.33$, $p = 0.25$; surgery × sex × time interaction: $F_{1,56} = 8.44$, $p < 0.01$; **Figure 1D**). Walking distance in the open field was not significantly different between the groups indicating that differences on these tests were not caused by mobility issues (three-way ANOVA, All $F_{1,56}$ values < 1.88, and all p values > 0.17; *data not shown*). Finally, we tested a third cohort of mice on the sucrose preference assay to measure whether SNI induced a state of anhedonia in male and female mice. SNI did not alter sucrose preference in male or female mice following surgery. There was only a slight sex difference with male mice displaying a greater sucrose preference (three-way ANOVA, main effect of surgery: $F_{1,26} = 0.003$, $p = 0.95$; main effect of sex: $F_{1,26} = 4.24$, $p = 0.05$; main effect of time: $F_{3,78} = 1.187$, $p = 0.32$; **Figure 1E**).

Microglial Expression in the Ventral Anterior Cingulate Cortex Is Different Between the Sexes Following Spared Nerve Injury

Next, to understand whether the expression of microglia in brain regions associated with depressive-like behavior may be associated with the sex-specific effects on the FST and TST, we examined microglial cell numbers in the dorsal and ventral ACC, and nucleus accumbens. To analyze microglia cell number in each brain structure, four-way mixed ANOVAs [hemisphere (RM) × surgery × sex × timepoint] were conducted on each region. For the ventral ACC, microglial cells were more numerous in the contralateral (i.e., right) hemisphere in female SNI mice compared with sham female mice at the 2-week timepoint [main effect of hemisphere (RM): $F_{1,34} = 1.26$, $p = 0.26$; main effect of surgery: $F_{1,34} = 7.28$, $p = 0.011$; main effect of time: $F_{1,34} = 6.06$, $p = 0.02$; main effect of sex: $F_{1,34} = 2.71$, $p = 0.11$; hemisphere (RM) × condition × sex × time interaction: $F_{1,34} = 4.43$, $p = 0.043$; **Figures 2A,B**]. However, analysis of the dorsal ACC did not reveal any significant effects [main effect of hemisphere (RM): $F_{1,34} = 3.52$, $p = 0.07$; main effect of surgery: $F_{1,34} = 1.86$, $p = 0.18$; main effect of sex: $F_{1,34} = 2.17$, $p = 0.14$; main effect of time: $F_{1,34} = 3.35$, $p = 0.08$; **Figure 2C**]. Finally, analysis of the nucleus accumbens revealed an effect of hemisphere [main effect of hemisphere (RM): $F_{1,34} = 4.38$, $p = 0.044$; main effect of surgery: $F_{1,34} = 1.001$, $p = 0.32$; main effect of sex: $F_{1,34} = 1.71$, $p = 0.2$; main effect of time: $F_{1,34} = 0.29$, $p = 0.59$; hemisphere × surgery × sex × time interaction: $F_{1,34} = 3.38$, $p = 0.074$; **Figure 2D**].

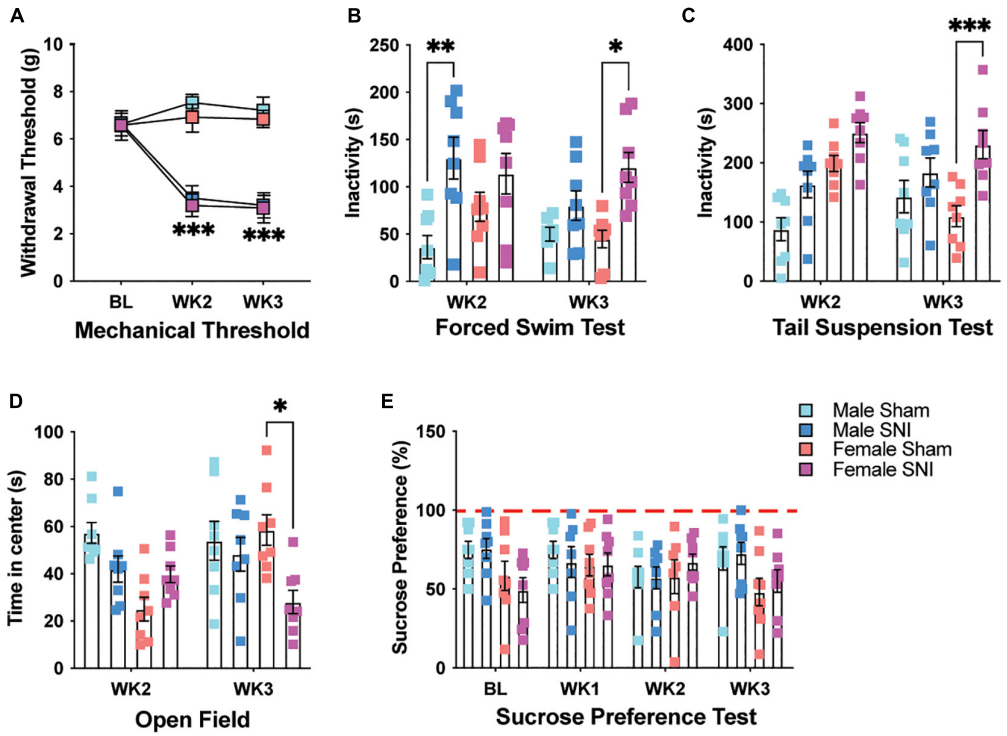

FIGURE 1 | SNI induces mechanical sensitivity and depressive-like behavior. **(A)** Mechanical allodynia is evident in male and female mice following SNI, but not sham surgery, 2- and 3-week post-injury (n = 16 for BL and WK2; n = 8 for WK3/sex/condition). **(B)** SNI increases immobility on the forced swim test in male mice 2-week post-surgery, while immobility increases in female SNI mice 3-week post-surgery (n = 8/sex/condition). **(C)** SNI increases immobility on the tail suspension test in female mice 3-week post-surgery (n = 8/sex/condition). **(D)** Female SNI mice spend less time in the center area of the open field 3-week post-surgery (n = 8/sex/condition). **(E)** SNI did not alter sucrose preference in male and female mice at any of the tested timepoints (n = 7–8/sex/condition). *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$.

Astrocyte Density in Male and Female Mice Following Spared Nerve Injury

Since astrocytes have been shown to contribute to depressive-like behavior (Zhang et al., 2020) and are increased in the brain following nerve injury (Zhu et al., 2009), we measured the fluorescence intensity of GFAP$^+$ staining in the same brain regions as microglial analysis. Fluorescence intensity was used for astrocyte quantification due to their larger size and lack of clear definition, which made it difficult to determine whether branches were from one astrocyte or several. There was a significant hemispheric effect for the ventral ACC with greater intensity of GFAP+ staining in the ipsilateral hemisphere that was independent of surgery condition (four-way ANOVA, main effect of hemisphere: $F_{1,34} = 8.11$, $p < 0.01$; main effect of surgery, $F_{1,34} = 0.2$, $p = 0.66$; main effect of sex: $F_{1,34} = 0.038$, $p = 0.54$; main effect of time: $F_{1,34} = 0.09$, $p = 0.76$, **Figure 3A**). We did not observe any significant effects for GFAP staining in the dorsal ACC (four-way ANOVA, all F's < 1.499; all p's < 0.23, **Figure 3B**). In the nucleus accumbens, there was a slight main effect for sex with males exhibiting overall higher GFAP staining and an interaction between surgery condition and sex that was due to higher GFAP staining in male SNI mice (four-way ANOVA, main effect of hemisphere: $F_{1,34} = 0.36$, $p = 0.55$; main effect of surgery: $F_{1,34} = 1.83$, $p = 0.18$; main effect of sex: $F_{1,34} = 4.23$, $p = 0.04$; main effect of time: $F_{1,34} = 0.06$, $p = 0.8$; surgery × sex interaction: $F_{1,34} = 4.379$, $p = 0.044$; **Figures 3C,D**).

DISCUSSION

The current study used a mouse model of chronic neuropathic pain and investigated the development of depressive-like behavior in male and female mice. We also characterized changes in microglia number and astrocyte density as a measure of activation following injury. Overall, there were sex differences in the onset of depressive-like behaviors in male and female mice such that males displayed immobility on the forced swim test at an earlier time point than females, and only female mice developed immobility on the tail suspension task following nerve injury. In the contralateral vACC, microglia cell number was significantly greater for female SNI mice than sham. No other changes in glial cells were apparent.

The onset of the depressive-like phenotype was different in male and female mice, which mirrors the human literature on the prevalence of depression and shows different onset rates for each sex (Romans et al., 2007). In our study, males developed the depressive phenotype 2-week following injury, while females developed depressive- and anxiety-like behavior at 3-week,

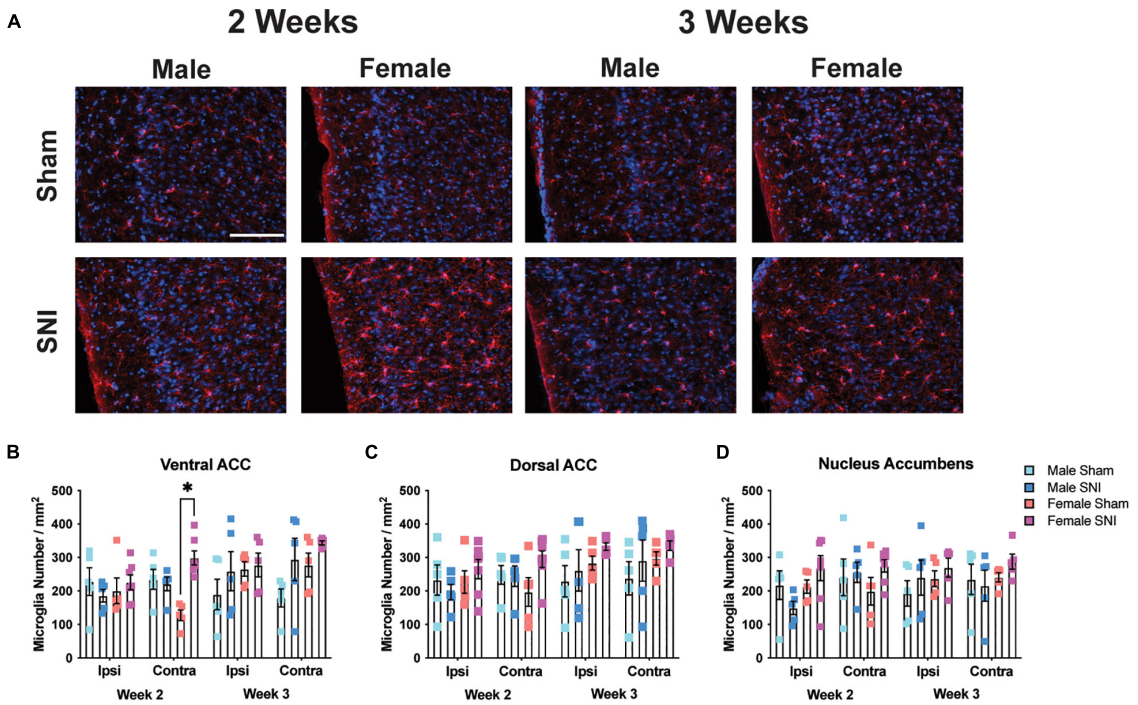

FIGURE 2 | Microglia changes in selected brain regions following SNI. **(A)** Representative fluorescent micrographs taken from the contralateral ventral ACC for male and female mice across surgery condition and timepoints. Scale bar = 100 μM. **(B)** SNI increases microglial number in the contralateral ventral ACC 2-week post-surgery. Microglia remained elevated in female mice 3-week post-surgery but was no longer significant from the sham condition. SNI did not alter microglia numbers in the **(C)** dorsal ACC, or **(D)** nucleus accumbens (n = 5–6/sex/condition, three tissue samples per mouse). *$p < 0.05$.

suggesting that initially, females may be more resilient than males, or changes in affect may take longer to manifest in females. However, depressive-like behaviors have been shown to manifest in male mice at later time points (i.e., 8-week following injury) when the chronic constriction injury model of neuropathic pain was used (Barcelon et al., 2019). We do not believe that the effects of nerve injury on FST immobility are related to movement because nerve injury did not affect walking or general movement in the OFT. However, the possibility exists that depending on sex, behavioral tests such as the FST and TST might be more sensitive for the detection of subtle behavioral differences. As another behavioral measure, we assessed anhedonia using the sucrose preference test; however, nerve injury did not alter sucrose intake. We do not believe that the lack of effect for the sucrose preference test was related to our testing procedures because during pilot studies sucrose intake was decreased when we used a positive control (i.e., restraint stress, *data not shown*). Anhedonia is challenging to induce in mice, and various pain models fail to cause a robust change in sucrose preference following injury (Schwartz et al., 2014). The sucrose preference test did reveal lower baseline preference for female than male mice. This is interesting because there have been reported sex differences in sucrose preference, but they typically are in the opposite direction with female rodents showing a greater preference (McCall et al., 2013). However, a few studies have shown that behavioral taste responses to dilute sucrose solutions are decreased by estrogen, whereas ovariectomy abolishes this effect (Curtis et al., 2004). This suggests that higher levels of estrogen in female mice may elevate the threshold for gustatory detection of sweet taste, a possibility that has been previously suggested (Curtis et al., 2005). Thus, sex differences in this assay may depend on estrogen-modulation of taste responses to specific concentrations of sucrose in these testing procedures.

Human studies are far more complicated than the current mouse experiments and the prevalence of comorbid depression with pain may depend on how and where the patients were assessed and the criteria for depression used, such as severity, assessment method and sample selection (Okifuji and Turk, 2016). While many studies have examined the prevalence of comorbid pain and depression (Tunks et al., 2008; Rayner et al., 2016), few studies fail to specifically examine coping strategies when patients suffer from both conditions as well as recovery time for pain and/or depression. A human study by Rovner et al. (2017) demonstrated that although the severity of chronic pain was the same for females and males, females were more accepting of the pain, remained more active, and reported fewer mood disturbances than males, supporting the idea that initially, females are better able to cope with chronic pain. In humans, females are more likely to endorse rumination (Meints et al., 2017) and rely on social support (Rovner et al., 2017) than males. These coping strategies may be the result of female patients reporting greater levels of pain dismissal (Igler et al., 2017), which may contribute to delayed-onset depression. Unfortunately, no study using a human population has examined whether there is a

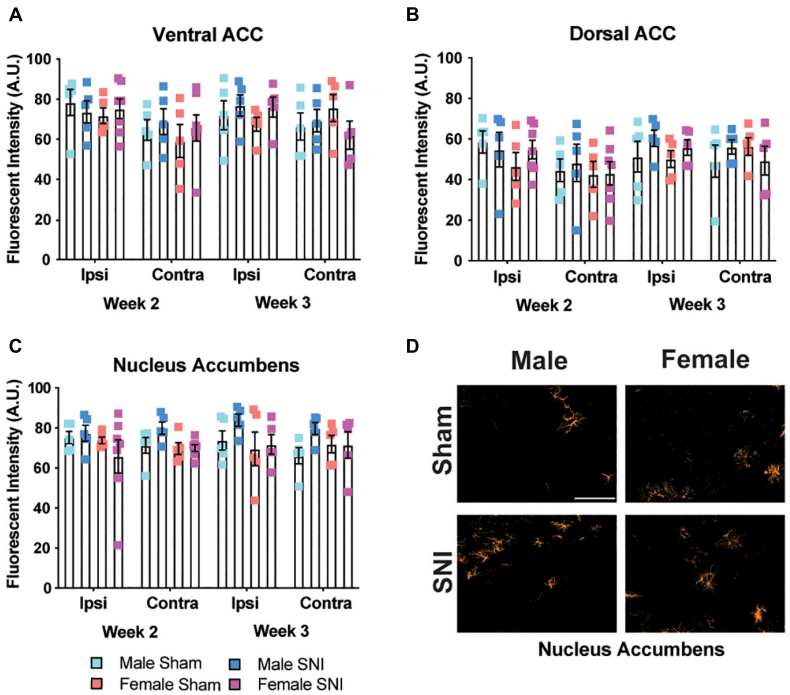

FIGURE 3 | Astrocyte changes in selected brain regions following SNI. Astrocyte expression is not changed following SNI in the **(A)** ventral ACC, **(B)** dorsal ACC, or **(C)** nucleus accumbens in a time and sex-dependent manner (n = 5–6/sex/condition, three tissue samples per mouse). **(D)** Representative images taken from the contralateral nucleus accumbens show astrocyte expression for male and female mice at the 2-week time point. Fluorescence is presented as arbitrary units.

sex-difference in the relationship between chronic pain duration and the onset of depression. Thus, it is possible that female patients are likely to cope with pain better than males, but when coping strategies fail, such as lack of social support the consequence may be increased prevalence of depression.

In addition, pain severity, pain duration and number of pain locations are associated with the recurrence of depressive and anxiety disorders (Gerrits et al., 2014; Sharpe et al., 2017). To our knowledge, no single study has examined whether male patients recover from comorbid depression quicker than females; however, inflammatory markers are predictive of depression in men, but not in women suggesting that the etiology of depressive disorders within the patient population are different between genders (Ernst et al., 2021). In our study, increased inflammation may have contributed to the early behavioral signs of depression in male mice, however, we did not measure general inflammation at the 2-week time point and cannot be certain that this was the precise mechanism. Further, male mice recovered from the depressive phenotype at 3-week, suggesting that males may adapt to chronic pain and eventually recover from affect-related disorders. These results are in line with studies in mice (Sellmeijer et al., 2018) and may explain why there is a higher incidence of depression in women with chronic pain than men (Munce and Stewart, 2007).

While several research groups have used microglial cell body size to quantify microglia activation (Taylor et al., 2017; Guneykaya et al., 2018), we used microglial cell number as a less subjective metric (Barcelon et al., 2019). In the contralateral vACC female SNI mice displayed significantly more microglia cell bodies than sham controls whereas SNI mice did not display overall changes in astrocyte intensity. Previous research has indicated that sex differences exist for microglia in the brain, but following nerve injury, the degree of microglia and astrocyte activation is similar between the sexes (Chen et al., 2018a). A possible explanation for this is that following nerve injury, cortical microglia may not be activated in the same way that spinal microglia have been characterized (Coull et al., 2005). Recent evidence suggests that there is an interaction between the spinal dorsal horn and the ACC in pain modulation, but mechanisms in the spinal cord do not necessarily transfer to cortical mechanisms (Tsuda et al., 2017). Previous reports characterizing brain microglia have mainly used CCI-induced nerve injury in male mice, suggesting that injury type may impact microglial or astrocyte activation in the brain differently between the sexes (Taylor et al., 2017).

We used Iba1 and GFAP as molecular markers for activated microglia and astrocytes, respectively, because of their roles in demonstrating morphological features at different microscopic levels (Shapiro et al., 2008; Sofroniew and Vinters, 2010). However, limitations do exist with these two markers and should be noted. Iba1 is not only a marker for activated microglia but also other macrophages that are recruited during nerve injury as well as other subpopulations of microglia (i.e., ramified microglia) (Ohsawa et al., 2004; Shapiro et al., 2009). So, Iba1 immunohistochemical expression may not be limited to just activated microglia and thus may affect analysis and

interpretation of results. It is challenging to acquire quantitative data with the IHC techniques used in the current paper. While some, but not all papers complement their IHC results with qPCR for verification, we did not do this and is a limitation of the present study. In addition, GFAP does not always label healthy CNS astrocytes (Sofroniew and Vinters, 2010), which could alter the difference observed in astrocyte density between nerve-injured and control mice. Further, we considered using Sholl analysis to capture astrocyte complexity; however, in our staining the larger size of the astrocytes and their lack of clear definition, made it difficult to determine whether branches were from one astrocyte or many. The center point of many astrocytes was also not always evident, making other quantification methods difficult. Thus, it would be important to consider the use of complementary techniques or other activated microglia and astrocyte markers, such as TMEM-119 (a transmembrane protein found only on microglia) (Bennett et al., 2016) and SOX9 (expressed exclusively by astrocytes) (Sun et al., 2017), respectively. These markers may be better suited for detecting active glia states and understanding reactive changes in glial cells.

Since, spinal nociceptive afferents are expected to innervate the contralateral side of the brain, our analysis considered whether lateralization occurred in any of the brain regions following injury as previously shown (Taylor et al., 2017). Our results stand in contrast to Taylor et al. (2017), where robust lateralization and regional differences were uncovered; however, we did find a lateralization effect for the number of microglia in the vACC of female SNI mice. Here, microglia cell number per square millimeter was significantly greater in SNI versus sham mice. Notably, there were minimal changes in microglia, and astrocytes even though several other studies have shown microglia changes in the thalamus, amygdala, ventral tegmental area (VTA), nucleus accumbens, bed nucleus of the stria terminalis, and periaqueductal gray following peripheral nerve injury (Taylor et al., 2015; Ni et al., 2016; Liu et al., 2017). Given that we found minimal differences, we did not pursue a mechanistic line of inquiry and we do not know whether glial changes in the ventral ACC are related to the depressive-like behavior in female SNI mice. As with some of our behavioral results, comparing between the 2- and 3-week time points is difficult because there may be inherent baseline expression differences between the mice. There may also be inherent sex differences in microglial expression as previously shown with male microglia being more numerous in the cortex, hippocampus, and amygdala (Guneykaya et al., 2018).

Further investigations may want to explore whether microinjections of glial inhibitors into either the ipsilateral or contralateral hemisphere reverse the pain or depressive phenotype. In line with this, a previous study showed that microglia and astrocytes were increased in the ACC of nerve injured mice and microinjections of minocycline into the contralateral ACC partially reversed mechanical allodynia (Cooper et al., 2018). However, the side of nerve injury (i.e., left vs. right) plays a big role in whether functional pain responses are altered by brain region and hemispheric manipulations. For instance, inactivation of the right or bilateral central amygdala (CeA) attenuates mechanical allodynia and hyperalgesia when SNI is performed on the left side of the body, while inactivation of the left CeA has no effect. The same paper also showed that following right-sided SNI, mechanical allodynia was attenuated only by inactivation of the left CeA, while mechanical hyperalgesia was reduced by left, right and bilateral inactivation of the CeA (Cooper et al., 2018). There is also evidence showing that overproduction of interleukin-1β, a cytokine that activates microglia is a common mechanism underlying the generation of neuropathic pain, memory deficits, and depressive-like behavior in mice (Gui et al., 2016). Thus, a potential strategy may be to target upstream activators of microglia, rather than focus on direct glial inhibition.

Overall, the most important aspect of the current study was the demonstration that SNI induced depressive- and anxiety-like behavior differently in male and female mice. However, this study encourages further research on comorbid pain and depression using both sexes as there are clear behavioral sex differences and understanding the sex-specific mechanisms should be further explored.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Toronto Animal Care Committee.

AUTHOR CONTRIBUTIONS

LM and VM conceived and designed the experiments and wrote the article. VM and CC performed the experiments. LM supervised the acquisition of results. VM, NL, and LM analyzed the data. All authors edited and commented on the final version of the manuscript.

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REVIEW
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Sex Differences in Affective Dysfunction and Alterations in Parvalbumin in Rodent Models of Early Life Adversity

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The early life environment markedly influences brain and behavioral development, with adverse experiences associated with increased risk of anxiety and depressive phenotypes, particularly in females. Indeed, early life adversity (ELA) in humans (i.e., caregiver deprivation, maltreatment) and rodents (i.e., maternal separation, resource scarcity) is associated with sex-specific emergence of anxious and depressive behaviors. Although these disorders show clear sex differences in humans, little attention has been paid toward evaluating sex as a biological variable in models of affective dysfunction; however, recent rodent work suggests sex-specific effects. Two widely used rodent models of ELA approximate caregiver deprivation (i.e., maternal separation) and resource scarcity (i.e., limited bedding). While these approaches model aspects of ELA experienced in humans, they span different portions of the pre-weaning developmental period and may therefore differentially contribute to underlying mechanistic risk. This is borne out in the literature, where evidence suggests differences in trajectories of behavior depending on the type of ELA and/or sex; however, the neural underpinning of these differences is not well understood. Because anxiety and depression are thought to involve dysregulation in the balance of excitatory and inhibitory signaling in ELA-vulnerable brain regions (e.g., prefrontal cortex, amygdala, hippocampus), outcomes are likely driven by alterations in local and/or circuit-specific inhibitory activity. The most abundant GABAergic subtypes in the brain, accounting for approximately 40% of inhibitory neurons, contain the calcium-binding protein Parvalbumin (PV). As PV-expressing neurons have perisomatic and proximal dendritic targets on pyramidal neurons, they are well-positioned to regulate excitatory/inhibitory balance. Recent evidence suggests that PV outcomes following ELA are sex, age, and region-specific and may be influenced by the type and timing of ELA. Here, we suggest the possibility of a combined role of PV and sex hormones driving differences in behavioral outcomes associated with affective dysfunction following ELA. This review evaluates the literature across models of ELA to characterize neural (PV) and behavioral (anxiety- and

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depressive-like) outcomes as a function of sex and age. Additionally, we detail a putative mechanistic role of PV on ELA-related outcomes and discuss evidence suggesting hormone influences on PV expression/function which may help to explain sex differences in ELA outcomes.

Keywords: early life adversity, parvalbumin, sex differences, estrogens, testosterone, anxiety, depression, development

INTRODUCTION

Adversity in early life is widespread (Finkelhor et al., 2013) and places individuals at an increased risk for developing later-life psychiatric disorders, such as anxiety and depression (Gatt et al., 2009; Nugent et al., 2011; Heim and Binder, 2012). Further, experiencing adverse environmental stressors during early development and/or childhood has been linked to impaired cognitive function and maladaptive behavioral outcomes (Chapman et al., 2004; Krugers et al., 2017; Vaiserman and Koliada, 2017), with onset often occurring in a protracted manner, years after the adverse experience (Spertus et al., 2003; Hagan et al., 2015; Russell et al., 2018). Early-life adversity (ELA) manifests in a variety of instances and includes both physical and sexual abuse, emotional/psychological abuse, adverse family circumstances, neglect, poverty, and other environmental factors (Felitti et al., 1998; Kuhlman et al., 2020). The 2021 report released by the National Child Abuse and Neglect Data System (NCANDS) found that approximately 4.4 million children in the United States received a Child Protective Services referral of suspected abuse or neglect in 2019, with over 650,000 identified victims of abuse/neglect (U.S. Department of Health and Human Services, Administration for Children and Families, Administration on Children, Youth and Families, Children's Bureau, 2021). It is important to note that these numbers are likely lower than the actual number of abuse or neglect cases, as most instances of child abuse or neglect go unreported. Early life experiences play a significant role in shaping short- and long-term outcomes regarding both cognitive-behavioral and neural development (Kundakovic and Champagne, 2015; Chen and Baram, 2016). While it is clear that a history of ELA is a significant risk factor in the development of affective disorders (Hoppen and Chalder, 2018), the underlying mechanism(s) by which ELA confers this risk remain largely unknown. Therefore, it is critical that we leverage findings from preclinical models to identify putative neurobiological drivers of sex-specific risk following ELA to reveal windows of opportunity for individualized intervention and/or treatment.

Several rodent models of ELA exist to approximate distinct aspects and types of adverse experiences to elucidate the role of adversity on neural and behavioral consequences across the lifespan. One widely used model leverages maternal separation (MS) as an analog of early caregiver deprivation during the postnatal and pre-weaning periods. MS protocols typically involve the removal and isolation of pups from dam and littermates for a designated period of time over a series of days, typically ranging from 3–4 h per day from postnatal day (P) 2 to 20 (e.g., Grassi-Oliveira et al., 2016; Coley et al., 2019; Köhler et al., 2019; Honeycutt et al., 2020; Drastichova et al., 2021), though some research groups only maintain separations for the first 14 days of life (e.g., Uchida et al., 2010; Callaghan and Richardson, 2011; Teissier et al., 2020). This ELA model is widely used as it closely models early life caregiver deprivation seen in institutionalized rearing (Kundakovic and Champagne, 2015), and MS in rodents also shows outcomes comparable to those in humans with a history of abuse (Teicher et al., 2006; Nemeroff, 2016). Importantly, this model approximates early life psychosocial neglect which is one of the most prevalent forms of ELA in the United States, accounting for approximately 78% of mistreatment cases (National Scientific Council on the Developing Child, 2012). Another widely utilized model of ELA is the limited bedding (LB) paradigm, which aims to model resource scarcity (Molet et al., 2014). Increasing evidence suggests that the LB model results in disruption of maternal behavior, thereby leading to fragmented, abuse-like, and unpredictable maternal care (Ivy et al., 2008; Rice et al., 2008; Walker et al., 2017). While these are both models of ELA, it is clear that the *type* of adversity model used (and therefore the specific type of adversity experienced) impacts both neural and behavioral outcomes (Murthy and Gould, 2018; Brenhouse and Bath, 2019; Demaestri et al., 2020).

There is undeniable evidence suggesting that biological sex plays an important—and alarmingly understudied—role in both short- and long-term outcomes following adversity in both humans (e.g., Altemus et al., 2014; Colich et al., 2017; LoPilato et al., 2019) and rodent models (e.g., Bath, 2020; Eck et al., 2020; Honeycutt et al., 2020). In humans, women are more likely than men to develop anxiety-related disorders in their lifetime (Kessler et al., 1994), with anxiety in women more likely to be clinically significant (McLean et al., 2011). Because ELA is associated with an increased risk of anxiety-related outcomes in both humans and rodent models, it is important that we understand the disparate sex-specific outcomes to better approach individualized risk assessment and treatment. Preclinical findings suggest that male mice with a history of MS show no changes in social interaction following ELA, while MS females show increased social interaction and increased anxiety-like behaviors (Bondar et al., 2018). Interestingly, in this same study MS males exhibited significant variability in locomotor activity, which may account for some of the effects observed. Despite clear evidence for sex differences in affective disorders, most studies examining affect-and, in fact, most studies across behavioral neuroscience-have looked only at males, neglecting to include females or to explicitly consider sex as a biological variable (SABV; Shansky, 2019). As such, more research is needed to understand sex differences following

ELA to: (1) better model mental illness in preclinical assays; (2) address glaring sex differences in symptom onset and patient outcomes; and (3) determine neurobiological drivers of affective dysfunction in an attempt to identify putative targets for intervention and treatment. In this review, we shed light on sex differences as observed in preclinical ELA models (specifically, MS and LB models) and discuss their possible interactions with identified neural markers of pathological risk and circuit dysfunction.

There are several neural changes thought to contribute to deleterious behavioral outcomes associated with anxiety and depression: two affective disorders that show increased risk of emergence following exposure to ELA (Nugent et al., 2011; Pagliaccio and Barch, 2016). One widely observed neural change involves alterations in overall inhibitory/GABAergic function (Page and Coutellier, 2019; Prévot and Sibille, 2021), which are likely exacerbated by adverse experiences (Maguire, 2014). Parvalbumin (PV), a calcium-binding protein expressed within a specific subset of GABAergic neurons, is thought to be involved in affective dysregulation characteristic of anxiety (e.g., Page et al., 2019; Xiao et al., 2020) and depression (e.g., Perova et al., 2015; Thaweethee-Sukjai et al., 2019). Indeed, reduced PV levels are associated with increased anxiety-like behavior and affective dysfunction in rodent studies (Godavarthi et al., 2014; Lussier and Stevens, 2016; Xu et al., 2016; Todorović et al., 2019; Vojtechova et al., 2021) and indirectly in human studies looking at Tourette syndrome, which is thought to be closely related to anxiety (Kalanithi et al., 2005; Kataoka et al., 2009). A multitude of studies have also found a reduction in PV interneurons in the hippocampus (HPC), prefrontal cortex (PFC), and basolateral amygdala (BLA), all of which are thought to be important for affective regulation following ELA (Leussis et al., 2012; Wieck et al., 2013; Ganguly et al., 2015; Grassi-Oliveira et al., 2016; Gildawie et al., 2021).

The goal of the present review is to synthesize the limited amount of prior work examining PV outcomes at the intersection of ELA and sex, to identify patterns that might explain how these factors contribute to affective outcomes. Specifically, we address disparate observations in PV outcomes following ELA that might be mediated by sex hormones, adversity type, and/or the timing of adversity/tissue collection. A discussion on the developmental time course of PV outcomes alongside changes in sex hormone and receptor levels is also presented to evaluate a possible relationship that may help to explain the observed sex-specific effects of ELA.

Parvalbumin

PV is a calcium-binding protein that supports the fast-spiking phenotype of PV-expressing neurons, a property that ideally positions them for synchronizing the activity of surrounding cells (Sohal et al., 2009; Chen et al., 2017; Kawaguchi et al., 2019). PV-containing neurons are the most abundant subtype of GABAergic interneurons in the central nervous system, accounting for ∼40% of all neocortical GABAergic neurons (Rudy et al., 2011). These PV cells are characterized as fast-spiking with low input resistance, leading to a rapid sequence of action potentials (Kawaguchi and Kubota, 1997; Woodruff and Sah, 2007). The high frequencies of action potentials, in addition to their perisomatic synapses on target cells, allow for the synchronization of electrical activity by orchestrating the timing of principal neuron spiking (Freund and Buzsáki, 1996; Woodruff and Sah, 2007). This synchronization plays an important role in the excitatory/inhibitory (E/I) tone of individual neurons as well as regional activity, which is thought to be altered by ELA (Singh-Taylor et al., 2015; Ohta et al., 2020). There are two distinct subtypes of PV-expressing cells: basket cells, which target proximal dendrites and their soma, and chandelier cells, which target synapses on the axon initial segment (Kawaguchi and Kubota, 1997). Both subtypes significantly contribute overall E/I tone in target neurons/regions (Ferguson and Gao, 2018), and therefore are well-positioned to orchestrate neuronal ensembles of activity. There is mounting evidence suggesting that ELA in rodent models leads to a decrease in PV cells in various regions of the brain, particularly the PFC (Brenhouse and Andersen, 2011; Leussis et al., 2012; Wieck et al., 2013; Holland et al., 2014; Ganguly et al., 2015; do Prado et al., 2016; Grassi-Oliveira et al., 2016), the HPC (Murthy et al., 2019), and the BLA (Gildawie et al., 2020). Given the orchestrating role of PV cells, these alterations in PV expression and/or function may contribute to some of the aberrant cognitive and neurobehavioral outcomes of ELA associated with neuronal inhibition and affective dysfunction, as seen in depression, schizophrenia, and anxiety (Brown et al., 2015; Gonzalez-Burgos et al., 2015; Zou et al., 2016; Perez et al., 2019; Murthy and Gould, 2020). However, it is noteworthy to underscore the variability in PV outcomes following ELA that are likely mediated by methodological differences in ELA application (i.e., MS vs. LB), age of tissue collection, species, and sex. We have provided an overview of PV outcomes in **Table 1** that details these findings with an emphasis on implemented methodology and PV levels, as well as related behavioral outcomes.

In rodent models, ELA generally leads to a decrease in PV-expression in the PFC in rats (e.g., Brenhouse and Andersen, 2011; Leussis et al., 2012; Wieck et al., 2013; Holland et al., 2014; Ganguly et al., 2015; do Prado et al., 2016; Grassi-Oliveira et al., 2016; Lukkes et al., 2017), the orbitofrontal cortex in mice (Goodwill et al., 2018), the HPC in both rats and mice (e.g., Katahira et al., 2018; Murthy et al., 2019), and the BLA in rats (e.g., Lukkes et al., 2017, 2018; Gildawie et al., 2020), all of which are regions considered to be key mediators of anxiety- and depressive-like behaviors (Kent and Rauch, 2003; Bannerman et al., 2004; Bertoglio et al., 2006; Pandya et al., 2012; Huang et al., 2018). These decreases in PV cells in the HPC (e.g., Murthy et al., 2019) have also been associated with increases in anxiety-like behaviors within the elevated plus maze (EPM) in male mice. The work outlined in **Table 1** constitutes all relevant research, to our knowledge, that has looked at PV outcomes following ELA (specifically, MS or LB). Of note, out of all studies looking at ELA induced effects on PV (n = 22), only 10 included both males and females in their analyses (with an additional two studies looking only at female subjects), underscoring the need for ELA studies to use SABV in methodological approaches to understand how sex mediates adversity-related outcomes.

TABLE 1 | PV and behavioral outcomes as a function of sex, age, and ELA type.

Study	Sex	Species	Type of ELA	Age of ELA	Age of Brain Collection	PV Meas.	PV Outcome		Anxiety and Depressive Behaviors	
							Male	Female	Male	Female
Short-Term Maternal Separation (1–12 days) and/or Early Weaning										
Murthy et al. (2019)	M	Mouse	MS +Early weaning (P17)	P2–16	P60-P70	IHC	↓HPC (ventral)	-	↑ anxiety (EPM) ↑ activity (NE)	-
Katahira et al. (2018)	M	Mouse	MS: 1 day, 24 hs	P4	P4, P5, P14, P28	IHC	↓(left HPC on P14 and P28)	-	-	-
Aksic et al. (2021)	M	Rat	MS: 1 day, 24 h	P9	P60	IHC	↓(CA1, PFC)	-	-	-
Giachino et al. (2007)	M	Rat	MS: 12 days 3 h/day	P2–14	P35	IHC	↑(LA) n.c. (HPC, BLA)	-	↓ (SI)	-
Richardson et al. (2021)	M + F	Rat	MS: 12 days 3 h/day	P2–14	P18	IHC	n.c. (PFC)	n.c. (PFC)	-	-
Long-Term Maternal Separation (18–19 days)										
Soares et al. (2020)	M + F	Rat	MS: 18 days 4 h/day	P2–20	P20	IHC	n.c. (PFC, CA1, DG) ↓(BLA, CA3)	n.c. (PFC, CA1, DG) ↓(BLA, CA3)	-	-
Gildawie et al. (2020)	M + F	Rat	MS: 18 days 4 h/day	P2–20	P20, P40, P70	IHC	↓(BLA at P40) n.c. (PFC)	n.c. (PFC, BLA)	-	-
Gildawie et al. (2021)	M + F	Rat	MS: 18 days 4 h/day	P2–20	P85	IHC	n.c. (PFC)	n.c. (PFC)	n.c. (EZM)	n.c. (EZM)
Brenhouse and Andersen (2011)	M	Rat	MS: 18 days 4 h/day	P2–20	P25, P40	WB, IHC	↓(PFC at P40)	-	↓Working memory (W/S)	-
Lukkes et al. (2017)	F	Rat	MS: 18 days 4 h/day	P2–20	P41	WB	-	↓(PFC, BLA, DR)	-	↑ depression (LH)
Lukkes et al. (2018)	F	Rat	MS: 18 days 4 h/day	P2–20	P41	WB	-	↓(Amygdala, PFC)	-	n.c. (LH)
Wieck et al. (2013)	M	Rat	MS: 18 days 4 h/day	P2–20	P40	WB, IHC	↓(PFC)	-	-	-
Ganguly et al. (2015)	M	Rat	MS: 18 days 4 h/day	P2–20	P43	IHC	↓(PFC)	-	↑ anxiety (EPM, OFT)	-
Leussis et al. (2012)	M + F	Rat	MS: 18 days 4 h/day	P2–20	P40, P100	WB, IHC	↓(PFC at P40)	↓(PFC at P40)	↑ depression (LH)	↑ depression (LH)
Holland et al. (2014)	M + F	Rat	MS: 18 days 4 h/day	P2–20	P25–27 or P42–45	WB	↓ (PFC in adolescence)	↓ (PFC in juvenility)	↓ (SI in adolescence)	↓ (SI in juvenility)
do Prado et al. (2016)	M + F	Rat	MS: 18 days 4 h/day	P2–20	P56	WB	↓(PFC)	n.c. (PFC)	↓Working memory (W/S)	-
Grassi-Oliveira et al. (2016)	M + F	Rat	MS: 18 days 4 h/day	P2–20	P40	IHC	↓(PFC)	n.c. (PFC)	↓Working memory (W/S)	↓Working memory (W/S)

(Continued)

TABLE 1 | Continued

Study	Sex	Species	Type of ELA	Age of ELA	Age of Brain Collection	PV Meas.	PV Outcome		Anxiety and Depressive Behaviors	
							Male	Female	Male	Female
Kim et al. (2020)	M	Rat	MS: 19 days 3 h/day	P2–21	Adolescent	WB, IHC	WB: n.c. (HPC) IHC: ↓(HPC)	-	↓ anxiety (OFT) ↑ depression (FST)	-
Limited Bedding Model (7–10 days)										
Manzano-Nieves et al. (2020)	M	Mouse	LB: 7 days	P4–11	P16, 21, 28, 50, 75	IHC	↑(BLA at P21; PFC at 75)	-	n.c. (EZM)	n.c. (EZM)
Bath et al. (2016)	M	Mouse	LB: 7 days	P4–11	P16, P21, P28	IHC	↑(PFC)	-	Accelerated contextual fear suppression	-
Goodwill et al. (2018)	M + F	Mouse	LB: 7 days	P4–11	P8, P12, P16, P21	IHC	n.c. (OFC) n.c. (PFC)	↓(OFC); n.c. (PFC)	n.c. Cognitive Function (S/S)	↓ Cognitive Function (S/S)
Guadagno et al. (2020)	M + F	Rat	LB: 10 days	P1–10	P28–29	IHC	n.c. (BLA)	n.c. (BLA)		

The included studies are all those that have explicitly examined PV outcomes following ELA in brain regions associated with affective dysfunction. Here, we report each study and identify the sex of subjects (male (M) or female (F); species (Rat or Mouse); type of ELA (maternal separation (MS) or limited bedding (LB) and adversity postnatal (P) day timeframe); the age of brain tissue collection; brain regions examined [basolateral amygdala (BLA), hippocampus (HPC) and HPC subfields where specified (CA1, CA3, DG), lateral amygdala (LA), orbitofrontal cortex (OFC), and prefrontal cortex (PFC)]; and method of PV quantification [western blot (WB) or immunohistochemistry (IHC)]. PV outcomes are divided based on subject sex, with no change (n.c.) between ELA and controls indicated if a lack of significant effect was observed. Directionality of behavioral outcomes related to PV changes are also detailed based on sex, as well as assay used [elevated zero maze (EZM), forced swim test (FST), novel environment (NE), social interaction (SI), set shifting (S/S), and win/shift (W/S)]. Directionality of behavioral and neural effects are represented as a decrease (↓), increase (↑), or no change (n.c.) compared to controls.

Indeed, in some studies, ELA leads to marked differences in PV expression that are sex-specific, and it is important to note that PV expression and/or staining intensity reportedly varies by sex across brain regions including the PFC, BLA, and HPC (e.g., Blurton-Jones and Tuszynski, 2002; Wu et al., 2014; Soares et al., 2020), with some reports showing similar developmental trajectories in control rats (e.g., Gildawie et al., 2020). In typical mice, there are some documented differences in the developmental trajectory of PV between males and females. In both the dorsal HPC and the ventral HPC females see a continuous increase in PV from week 3 to week 12 of age. This consistent increase is not observed in male mice, whose PV levels appear to remain constant from week 3 to week 12 after an initial pre-weaning surge (Wu et al., 2014; Ueda et al., 2015). However, sex differences in PV development have not been particularly well characterized; see **Figure 1** for a generalized normative trajectory of PV protein expression across age in typically developing rodents. In relation to ELA, male rats are more likely to have a decrease in PV cells in the BLA (Gildawie et al., 2020) and the PFC (do Prado et al., 2016; Grassi-Oliveira et al., 2016). There also may be a difference in timing, as males experience a decrease in PV cells in the PFC during adolescence while females show a decrease in PV expression in the PFC during juvenility following ELA (Holland et al., 2014). Sex differences also appear in the developmental trajectory of PV (Wu et al., 2014; Du et al., 2018), which may explain some of the variability in results. Again, however, few studies have looked at the difference in PV development between males and females, particularly as it relates to ELA. Taken together, prior work suggests that PV likely plays a significant role in the outcomes associated with ELA and is, therefore, a key protein to further characterize within this context. It is possible that alterations in PV expression and/or function significantly contribute to ELA-related affective dysfunction across the lifespan and are influenced by sex hormones to drive sex-specific individual outcomes following adversity, which will be further discussed in this review.

Adversity Type

The type of adversity impacts acute and chronic outcomes—spanning molecular to functional domains—following ELA, with evidence clearly borne out in recent work for review (see Brenhouse and Bath, 2019). Here, we focus on two widely used models of ELA: MS and LB, with MS being most closely associated with caregiver deprivation and LB being most closely associated with resource scarcity and infant maltreatment. Evidence suggests that there are differences in ELA type on later PV outcomes. Specifically, MS has been associated with a decrease in PV cells in the HPC (Katahira et al., 2018; Murthy et al., 2019), the BLA (Lukkes et al., 2017; Gildawie et al., 2020), and the PFC (Leussis et al., 2012; Wieck et al., 2013; Ganguly et al., 2015). Conversely, LB has been associated with an increase in PV in the BLA (Manzano-Nieves et al., 2020), PFC (Manzano-Nieves et al., 2020), and HPC (Bath et al., 2016). However, decreased PV following LB has been observed in the OFC of females but not males (Goodwill et al., 2018). The mechanisms underlying these opposing findings in response to

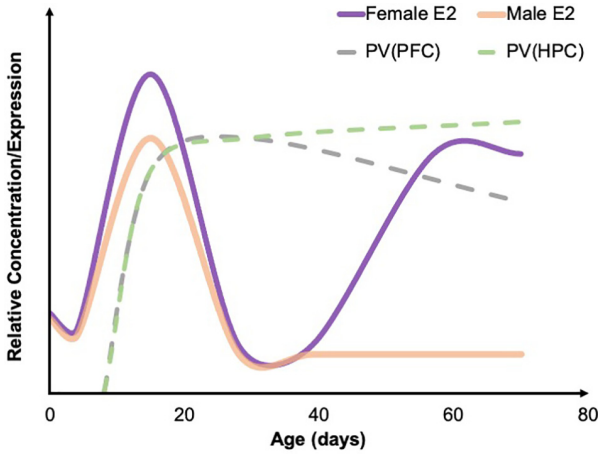

FIGURE 1 | Developmental trajectories of estradiol and PV. A summary of the developmental trajectory of estradiol (E2) concentrations in male (solid orange) and female (solid purple) rodents across early postnatal development through young adulthood, as well as the general developmental trajectory of PV levels in the PFC (dashed gray) and HPC (dashed green). Both males and females see a spike in E2 around P15. PV concentrations across development have not been well studied; however, existing developmental work suggests that PV levels in the HPC steadily increase through early adulthood, while PV levels remain relatively stable in the PFC. Figure adapted from data presented in Bell (2018), de Lecea et al. (1995), and Du et al. (2018). PFC, prefrontal cortex; HPC, hippocampus; PV, parvalbumin.

different models of ELA remain unknown but may be influenced by length and timing of experience. Typically, LB models last for 7 days, while MS models last for 18 days, and it is, therefore, possible that the difference in duration of adversity contributes to the disparity observed between adversity types. MS stress may lend itself more to isolation stress, while LB may lead to more aversive stress, as modified LB methods have been related to abusive maternal care (e.g., Lewin et al., 2019). It is possible that the types of stress elicited by the models differ, leading to unique changes in PV.

Sex may also be a factor to consider when evaluating the impact of adversity type. One study looking at sex differences following ELA using the LB model found that cognitive ability, *via* rule shifting, was impaired more in females than in males (Goodwill et al., 2018). This same study also revealed that female rats have a decrease in PV levels in the OFC following LB, while male rats see no change. This is a different pattern of results than seen in studies that use an MS model, which often find that males experience a long-term decrease in PV following adversity while females do not (e.g., Holland et al., 2014; do Prado et al., 2016; Grassi-Oliveira et al., 2016). This suggests that the type of adversity may also affect males and females differently. However, it is worth noting that there are few studies that have directly compared males and females in this way, and therefore this sex disparity may be biased due to a lack of relevant research.

While the type and timing of ELA appear to markedly influence later life outcomes when presented during the pre-weaning period, the length of the experience during that time may also be important. Prolonged ELA experience, as modeled by MS up until weaning, confers increased risk of PV decreases and/or dysfunction, with most of the past research reporting decreased PV levels following ELA *via* MS (see **Table 1**). This general decrease in PV expression after ELA is also associated with concomitant changes in anxiety- and depressive-like behaviors (e.g., Leussis et al., 2012; Grassi-Oliveira et al., 2016), suggesting a possible link between these outcomes.

Developmental Age

An important factor that warrants consideration when evaluating PV expression following ELA is the age at which the animal experiences ELA, as well as the age of tissue collection and evaluation. In rodents experiencing adversity in early development (P0–20) or even juvenility (P20–35) there were generally decreases in PV levels in the PFC and HPC, particularly after MS (see **Table 1**). However, this was true for when brain tissue was collected in adolescence (approx. P35–50) or young adult/adulthood (approx. P50-P70) following ELA, but not during juvenility (Holland et al., 2014; do Prado et al., 2016; Grassi-Oliveira et al., 2016). This indicates that both the timing of adversity and the age of brain collection are important factors to consider. Adult rodents, however, were found to have increased PV levels following chronic stress in adulthood in the PFC (Shepard et al., 2016; Shepard and Coutellier, 2017), suggesting that age may have differing effects on the brain's response to stress. It is possible that stress occurring after the brain has fully developed elicits a different response than stress that occurs while the brain is still undergoing major development (Romeo and McEwen, 2007). PV cell density increases in the PFC throughout juvenility and adolescence before decreasing again in adulthood (Ueno et al., 2017). Therefore, stress that occurs during juvenility or adolescence may impact the development of PV cells, while stress in adulthood may have a compensatory effect and leads to an increase in PV cells.

There is also evidence suggesting that ELA, specifically MS, may have a delayed impact on PV cell density. Brains that were collected immediately (or within a few days) after ELA experience generally had no significant differences in PV levels compared to control-rearing (Giachino et al., 2007; Brenhouse and Andersen, 2011; Soares et al., 2020; Richardson et al., 2021), while brains collected later in life (in adolescence and/or P40 and older) generally had decreased PV levels (Leussis et al., 2012; Wieck et al., 2013; Ganguly et al., 2015; Lukkes et al., 2017, 2018; Kim et al., 2020; Aksic et al., 2021). Since PV cells have been found to increase significantly in number from juvenility to adolescence in brain regions such as the PFC (Caballero et al., 2014; Wu et al., 2014), it is possible that ELA may inhibit the maturational time course of PV cells. However, this regulation may also be regionally dependent, as some groups have identified decreases in HPC PV across development from juvenility to adolescence in typical rats (e.g., Honeycutt et al., 2016), making the dynamic nature of PV protein expression even more apparent. Furthermore, developmental timing of other insults (for instance, exposure to NMDA antagonists which appear to disproportionately impact PV cell functionality; (Kinney et al., 2006; Abekawa et al., 2007), also mediate PV outcomes in an age-dependent manner (e.g., Honeycutt and Chrobak, 2018).

This research suggests that there may be a compounding effect of ELA and downstream neural changes that lead to the decrease in PV levels following adversity. Furthermore, as males and females have slightly different developmental timelines, it is important to consider sex as a variable when looking at developmental age and ELA effects.

Sex Hormones

An important area of consideration, which has more recently been gaining traction in the field, for ongoing and future research is to systematically investigate sex differences in relation to ELA. The majority of ELA research (and admittedly, research in general) has focused on the investigation of male subjects. However, males and females have different physiological and behavioral responses to ELA (Donner and Lowry, 2013; Maeng and Milad, 2015; Perry et al., 2021), as previously mentioned. For example, juvenile male rats did significantly worse on non-spatial and spatial memory tasks following MS, while female rats had no impairment (Frankola et al., 2010). Social and aggression-related behavior following ELA also differs between males and females (Farrell et al., 2016). Prior work in rodents has shed some light on these differences, suggesting that MS in mice leads to opposite effects in adult offspring, with MS males showing decreased latency to attack a resident intruder, and lactating MS females showing decreased latency in the same task (Veenema et al., 2007), with ELA also leading to increased aggression/play-fighting in male rats (Veenema and Neumann, 2009). Therefore, it is important to consider how sex hormones may be interacting with underlying putative mechanisms, including PV cells, to identify possible relationships that may drive sex-specific risk and/or resilience following ELA.

Estrogens and Aromatase

Estrogens may be a potential explanation for the sex differences observed in PV levels following ELA. Estrogens play many important roles in brain function, including the modulation of neurotransmitters (Herbison, 1997; Rubinow et al., 1998), cognition (Sherwin and Henry, 2008; Albert and Newhouse, 2019), and synaptic plasticity (Albert and Newhouse, 2019). Estrogen receptors (ER) are crucial in the development and behavioral outcomes in both males and females (e.g., Hess and Cooke, 2018), including behaviors such as fear extinction and aggression (Ogawa et al., 1997; Scordalakes and Rissman, 2003; Graham and Milad, 2014). Estrogens have been implicated in contributing to anxiety-related outcomes (Borrow and Handa, 2017) and depression (Albert and Newhouse, 2019), with decreased levels of estrogens in the PFC (Shansky et al., 2004), amygdala (Walf and Frye, 2006), and HPC (Xu et al., 2015, 2016) associated with the development of anxiety- and depressive-like behaviors following stress.

Low levels of estrogens and ERs are associated with increased anxiety behavior, both in rodents (e.g., Walf and Frye, 2006; Borrow and Handa, 2017) and in humans (e.g., Wittchen and Hoyer, 2001; Almeida et al., 2005; Solomon and Herman, 2009; Holsen et al., 2011). Estradiol and ERs, specifically ERα and ERβ, have been found to have protective effects against anxiety- (Lund et al., 2005; Walf and Frye, 2006, 2010; Filova et al., 2015) and depressive-like (Galea et al., 2001; Walf et al., 2009; Österlund, 2010) behaviors. These protective effects may be due to the role of estradiol and ERs in preventing cell death by promoting the release of anti-inflammatory proteins (Behl et al., 1995; Patrone et al., 1999; Simpkins and Dykens, 2008; Smith et al., 2011) and promoting axonal sprouting by increasing axodendritic synapse formation (Matsumoto and Arai, 1979; Kadish and Van Groen, 2002). Estradiol may also play a role in neuronal regeneration by stimulating the release of glial apolipoprotein and enhancing antioxidant mechanisms (Sudo et al., 1997; Stein, 2001; Struble et al., 2007). Finally, estradiol and ERs may also increase synaptic transmission (Garcia-Segura et al., 2001), perhaps due to the antioxidant properties of estradiol molecules (Behl et al., 1997) and the neuroprotective signal cascades that begin with binding to ERs (Sohrabji et al., 1994; Lagrange et al., 1997) or interactions with estrogens (Sohrabji et al., 1995; Singer et al., 1999; Singh et al., 1999).

This has led to the use of estrogens in several human studies as a successful treatment for anxiety and depression in women, with perimenopausal women receiving treatments of estrogens experiencing significantly lower levels of anxiety and depression (Schmidt et al., 2000; de Novaes Soares et al., 2001; Grigoriadis and Kennedy, 2002; Ancelin et al., 2007; Misra et al., 2013). While not a common treatment for anxiety and depression, the results of these studies suggest that estrogens might have potential as a successful treatment, and therefore warrants further investigation to understand the mechanism(s) by which it mediates affective function. In line with these human findings, ovariectomized female rodents show significantly more anxiety- and depressive-like behaviors, but these effects are significantly reduced following replacement of estrogens (Estrada-Camarena et al., 2003; Shansky et al., 2004; Walf et al., 2008, 2009; Walf and Frye, 2010; Kiss et al., 2012; Daendee et al., 2013; Furuta et al., 2013; Tian et al., 2013; Xu et al., 2015). Therefore, the use of estrogens, especially in women with low baseline levels of estrogens or older women who have experienced a decrease in estrogens following menopause, may be important in the treatment of anxiety and depression.

Furthermore, increasing evidence suggests that both artificial and natural increases in estrogens and ERs lead to increases in PV levels (Ross and Porter, 2002; Wu et al., 2014; Bunratsami et al., 2015). Specifically, ovariectomized rats had a decreased number of ERα and ERβ receptors as well as a decrease in PV levels. This effect was reversed following estradiol replacement (Bunratsami et al., 2015). In typical rats, the developmental trajectory of parvalbumin is very similar to the developmental trajectory of estradiol in females (**Figure 1**; Wu et al., 2014). There is also colocalization of ERβ and PV in inhibitory neurons located in the amygdala, HPC, cortex, and basal forebrain (Blurton-Jones and Tuszynski, 2002) and colocalization of ERα and PV in the dorsal HPC of mice (Wu et al., 2014). Therefore, a strong connection can be drawn between estrogens and PV. Additionally, the developmental time course of PV expression lines up closely with the developmental timing of estrogens fluctuations (Alcántara et al., 1993; del Río et al., 1994; de Lecea et al., 1995; Bell, 2018). In both male and female rodents, estrogens are fairly low in concentration until peaking around

P15 before decreasing again and staying at relatively similar levels until females experience a pubertal increase again at P39 (Bell, 2018). In rodents, PV protein expression in most brain regions begins to emerge around P10 and typically peaks in density and intensity around P15 (Alcántara et al., 1993; del Río et al., 1994; de Lecea et al., 1995). In a large number of neocortical regions, as well as in the HPC, adult levels of PV have been observed around P21 (Alcántara et al., 1993; de Lecea et al., 1995). In females, PV levels remain consistent in the PFC throughout adolescence (Du et al., 2018). However, in males, there is a significant increase in PV in both the infralimbic and prelimbic areas of the PFC during adolescence (Du et al., 2018). One explanation for this difference is pubertal timing. Males typically begin puberty around P42, which is when an increase in PV was observed while females begin puberty earlier and experience an earlier increase in PV (Du et al., 2018). Of note, ELA *via* MS in rats has been shown to impact pubertal timing, accelerating pubertal onset in females, while delaying in males (Cowan and Richardson, 2018). However, in an LB rat model, sex hormone levels, but not pubertal onset, were impacted (Eck et al., 2020). It is therefore prudent to consider the impact of ELA-induced alterations in development as a possible driver of sex-specific neural and behavioral outcomes.

Estrogen may have specific implications for the timing of PV decreases and anxiety- and depressive-like behaviors associated with ELA. Females experience the emergence of these changes as early as juvenility, while males experience these alterations in PV expression beginning in adolescence and into adulthood (Holland et al., 2014; do Prado et al., 2016; Grassi-Oliveira et al., 2016). During puberty, females exhibit a surge in estradiol, which may explain why females exhibit altered PV levels before puberty but not after. It is possible that, in females who have experienced adversity, the estradiol surge results in a subsequent surge in PV, while also protecting against the emergence and continuation of anxiety and depression. It is important to note that males also experience a surge in estradiol during puberty; however, most of this estradiol is converted to testosterone by aromatase so the circulating levels of estradiol remain relatively low in males throughout puberty (Oyola and Handa, 2017; Bell, 2018)

While some research has looked at the role of estrogens in development, fear extinction, and aggressive behaviors in males (Ogawa et al., 1997; Scordalakes and Rissman, 2003; Graham and Milad, 2014; Hess and Cooke, 2018), few studies have considered the role of estrogens and stress in males. Interestingly, Tsuda et al. (2014) observed that ERβ knockout mice exhibit increased anxiety-like behavior, whereas male knockouts showed increased aggression that was lessened by MS. Further, MS has been associated with alterations in Erβ gene methylation in male mice (Wang et al., 2013), suggesting a role of altered estrogenic function in behavioral outcomes in both sexes. However, an important consideration is the function of aromatase, which is responsible for the conversion of testosterone to estrogens (Eck et al., 2020). This is particularly important during early-life development, as well as with the development of sexual behaviors during puberty (McCarthy, 2008; Bell, 2018). As estrogen levels are higher in males following ELA, it is possible that ELA leads aromatase to be more efficient in converting testosterone to estradiol (Eck et al., 2020), which may have a protective effect (Wei et al., 2014). Additionally, the aromatase inhibitor letrozole administered in juvenility has been associated with increased anxiety-like behavior in rats (Borbélyová et al., 2017), which suggests that decreased levels of estrogens may be an important consideration in males as well. However, more research is needed to directly determine how ELA impacts the levels and functionality of aromatase, as well as the interaction between aromatase and PV, which could be an important aspect of the sex differences that emerge after ELA.

Testosterone

In addition to estrogens, testosterone may also play an important preventative role in the development of anxiety and depression (Aikey et al., 2002; Buddenberg et al., 2009; Roohbakhsh et al., 2011; Giltay et al., 2012; Hodosy et al., 2012; McHenry et al., 2014). Testosterone is a hormone typically found in higher levels in males than females (Fahey et al., 1976). Males experience a surge in testosterone right before birth and maintain a moderate level of testosterone throughout juvenility until experiencing another surge during puberty (Bale and Epperson, 2017; Bell, 2018). ELA has been found to lead to a change in testosterone levels, but it is unclear whether it leads to an increase (e.g., Veenema et al., 2006; Zito et al., 2017) or decrease (e.g., Llorente et al., 2011; Tsuda et al., 2011). Interestingly, decreases in plasma testosterone were shown to be associated with less aggressive behavior in male mice, though these findings were dependent on age in 5–9 week old mice (Tsuda et al., 2011).

Testosterone has been observed to have anxiolytic and antidepressant effects in both males and females (Goldstat et al., 2003; Miller et al., 2009; Zarrouf et al., 2009; McHenry et al., 2014). Males with hypogonadism, which leads to a decrease in testosterone levels, have significantly higher rates of anxiety and depression (Shores et al., 2004; Zarrouf et al., 2009; Wainwright et al., 2011; Aydogan et al., 2012). Furthermore, hormone replacement therapy in men with a testosterone deficiency prevents or alleviates anxiety and depression (Wang et al., 1996; Seidman and Rabkin, 1998; Seidman et al., 2001; Pope et al., 2003; Kanayama et al., 2007; Zarrouf et al., 2009; Jung and Shin, 2016). Similarly, gonadectomized male rodents exhibit increased anxiety and depressive-like behavior, which is reversed following testosterone replacement treatment (Frye and Seliga, 2001; Edinger and Frye, 2005; Carrier and Kabbaj, 2012; Carrier et al., 2015). While little research has been conducted looking at testosterone therapy in women, a small number of studies found promising evidence that low-dose testosterone treatment led to a decrease in depressive-like behavior in SSRI treatment-resistant women of various ages with major depressive disorder (Miller et al., 2009) and reduced fear potentiated startle in healthy women (Hermans et al., 2006). Furthermore, low levels of salivatory testosterone may lead to an increased risk of females developing anxiety and depression (Carrier et al., 2015).

Despite the potential role of testosterone in anxiety and depression, little research has found clear interactions between PV and testosterone, which is in contrast to the overlap that is seen with PV and estrogens. One study looking at canaries found that an increase in testosterone was associated with higher

levels of PV in the HVC, robust nucleus of the arcopallium, and Area X, which are all regions associated with bird song (Cornez et al., 2020). Another study in mice found no interaction between PV and testosterone (Wu et al., 2014), which may explain why males see a decrease in PV but females do not, following ELA. As previously discussed, PV colocalizes extensively with ERs, suggesting that higher availability or ERs within key brain regions may be capable of modulating PV function through ER signaling (Blurton-Jones and Tuszynski, 2002; Wu et al., 2014), whereas testosterone may not have the ability to modulate PV function this robustly. However, the anxiolytic and antidepressant roles of testosterone, specifically in promoting neuroplasticity, may explain differences in anxiety and depressive-like behavior between sexes (Carrier and Kabbaj, 2012; Wainwright et al., 2016; Walther et al., 2019), although these differences may depend on the behavioral assays used (Polanza, 2001; Scholl et al., 2019).

DISCUSSION

ELA is a prevalent issue globally, and its contribution to individual risk of developing later-life psychiatric disorders places an undue burden on society at large. A potential mechanism of ELA-associated outcomes (such as affective dysfunction) may be a reduction in PV expression in the PFC and HPC. However, these changes in PV levels are not ubiquitous and appear to be differentially impacted by adversity type, age, and sex. Here, we detail that the two of the most prominent models of ELA, MS and LB, have markedly different effects on PV outcomes both acutely and in the long-term. LB models have led to increases in PV in the PFC (Bath et al., 2016; Manzano-Nieves et al., 2020) of male mice, and the OFC of female, but not male rats (Goodwill et al., 2018). Conversely, MS leads to a general decrease in PV in the PFC (Leussis et al., 2012; Wieck et al., 2013; Ganguly et al., 2015) and HPC (Katahira et al., 2018; Murthy et al., 2019) of rats, which differs based on sex (see **Table 1**). It is likely that both the duration of the two ELA models (i.e., 7 vs. 18 days), as well as the type of adversity conferred by these models, lead to different neural outcomes.

Age of adversity also plays an important role in PV levels, with pre-weaning adversity generally leading to a decrease in PV (Holland et al., 2014; do Prado et al., 2016; Grassi-Oliveira et al., 2016), and adversity occurring in adulthood typically resulting in an increase in PV cells (e.g., Shepard et al., 2016; Shepard and Coutellier, 2017). The mechanism(s) underlying PV outcomes, as well as manifestations of anxiety- and depressive-like behaviors, as a function of both age and adversity type, remain largely unknown. Interestingly, the research overviewed here suggests males and females may be differentially susceptible to ELA-induced PV pathology, particularly following MS. Specifically, in those studies examining both sexes, female PV outcomes were either comparable to those seen in males (a decrease or no change compared to controls; Leussis et al., 2012; Guadagno et al., 2020; Soares et al., 2020; Gildawie et al., 2021; Richardson et al., 2021), or females showed a lack of PV decrease while males showed a marked decrease in PV expression following ELA (do Prado et al., 2016; Grassi-Oliveira et al., 2016; Gildawie et al., 2020). Even more intriguing, was that Holland et al. (2014) revealed an age-dependent decrease in the PFC after MS, with females showing earlier decreases in PV than males. While this disparity in findings based on sex is not yet understood, it is worth noting that PV neurons show substantial co-expression of ERs (Blurton-Jones and Tuszynski, 2002), which may serve a protective function preventing ELA-induced expression phenotypes. Indeed, administration of 17-β estradiol increased PV levels in *Pvalb* heterozygous mice (Filice et al., 2018), and estradiol administration further protects PV expression outcomes in models of ischemic brain injury (Koh, 2014). Therefore, the presence of estrogens, particularly during key points of PV development (see **Figure 1**), may play a protective role in neural outcomes following ELA in females. However, while this is promising, it does not account for the increased incidence of ELA-related affective dysfunction/aberrant behavior that is often observed in females, raising the possibility that alterations in PV cell function—and not simply reduced PV protein—may also be linked to ELA risk (Murthy and Gould, 2020).

In addition to underscoring the need to increase our understanding of how sex impacts ELA-associated outcomes, we detail compelling data that may suggest an overarching role of PV expression/function on ELA-related affective dysfunction. Indeed, as PV cells are well-positioned to orchestrate local circuit oscillatory patterns, it follows that significant changes in PV protein expression and/or PV neuron function would disrupt the delicate E/I balance within discrete brain regions/circuits. This careful balance of overall E/I tone is critical for mediating behavior, and therefore PV disruption leads to downstream neural and behavioral alterations characteristic of affective dysfunction (Ferguson and Gao, 2018). Taken together, we show that ELA leads to sex-dependent changes in PV outcomes, and that type/age of ELA and age of brain tissue collection further contribute to these observations. Most importantly, we also overview evidence suggesting that estrogens may serve a protective role due to colocalization of ERs on PV cells, perhaps blunting females from some of the PV-associated outcomes seen in males. Given the prevalence of ELA and the increased risk of later-life affective dysfunction, it is essential that the field recognizes that key methodological differences (i.e., age of adversity/tissue collection) and sex contribute to ELA outcomes. By systematically addressing these factors and by including SABV, we can work toward individualized prevention and treatment.

AUTHOR CONTRIBUTIONS

SNE and JAH both wrote the manuscript. JAH provided guidance on literature review and writing and edited the manuscript and prepared it for submission. All authors contributed to the article and approved the submitted version.

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Animal Models of Anxiety and Depression: Incorporating the Underlying Mechanisms of Sex Differences in Macroglia Biology

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Animal models have been utilized to explore the mechanisms by which mood disorders develop. Ethologically based stress paradigms are used to induce behavioral responses consistent with those observed in humans suffering from anxiety and depression. While mood disorders are more often diagnosed in women, animal studies are more likely to be carried out in male rodents. However, understanding the mechanisms behind anxiety- and depressive-like behaviors in both sexes is necessary to increase the predictive and construct validity of the models and identify therapeutic targets. To understand sex differences following stress, we must consider how all cell types within the central nervous system are influenced by the neuroendocrine system. This review article discusses the effects of stress and sex steroids on the macroglia: astrocytes and oligodendrocytes. Glia are involved in shaping the synapse through the regulation of neurotransmitter levels and energy resources, making them essential contributors to neural dynamics following stress. As the role of glia in neuromodulation has become more apparent, studies exploring the mechanisms by which glia are altered by stress and steroids will provide insight into sex differences in animal models. These insights will facilitate the optimization of animal models of psychiatric disorders and development of future therapeutic targets.

Keywords: glia, astrocytes, oligodendrocytes, stress, anxiety, depression, sex differences

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INTRODUCTION

Despite their variety and vast numbers within the central nervous system (CNS), glial cells were initially considered to function only in a supportive capacity to the electrically excitable neurons. However, following decades of research, glial cells have emerged as active contributors to, and modulators of, neuronal activity with functions in neurodevelopment and neuromodulation through the release of gliotransmitters (substances released by glia to facilitate communication) that continuously shape neuronal activity (Allen and Barres, 2005). There are two main classifications of glial cells within the CNS, macroglia and microglia. Two of the major macroglia include astrocytes and oligodendrocytes (Zhou et al., 2020). Astrocytes make up the majority of glial cells within the brain (Eroglu and Barres, 2010). A single astrocyte process may contact up to 100,000 synapses,

allowing for direct contact and the ability to influence synaptic communication. Along with microglia, astrocytes are considered to be essential immune cells within the brain, and are involved in neuroinflammation and neurodevelopment through the promotion and maintenance of synapses (Schwarz and Bilbo, 2012). Oligodendrocytes are responsible for producing myelin within the CNS, providing insulation to axons, and increasing the speed of neuronal action potential propagation (Edgar and Sibille, 2012). Increased understanding of the intimate relationship of glia with the synapse has raised the question of how glia may regulate neuronal abnormalities and the development of disorders, as well as serve as potential therapeutic targets (Halassa et al., 2007).

Given the central role of glial in CNS function, it is essential to develop a complete understanding of their contribution to normal and pathological outcomes. One domain in which glial dysfunctions have been implicated is the development of neuropsychiatric disorders, such as anxiety and depression. This may not be surprising considering that canonical pathways dysregulated in these mood disorders include glutamate homeostasis and inflammation, both of which are tightly regulated by glial cells (Kalivas, 2009; Bilbo et al., 2012). Additionally, glia pathology has been recorded in patients that suffered from major depressive disorder (MDD; Miguel-Hidalgo et al., 2000; Rajkowska and Stockmeier, 2013), and astrocyte-oligodendrocyte communication deficits have been implicated in depression, as shown by decreased glia coupling in postmortem brain tissue of male-depressed suicide victims (Tanti et al., 2019). Although complex neuropsychiatric disorders like anxiety and depression cannot be recapitulated in other animals (Dalla et al., 2010), advances in understanding the neurobiology of depression and anxiety have been facilitated by examination of models of chronic stress, a known catalyst of depression and anxiety in humans (Carr et al., 2013). Therefore, this review will focus largely on the impact of chronic stressors on glia biology and function. Finally, we view glia through the lens of sex differences and examine gaps in understanding. While the number of studies evaluating the role of glia in neuropsychiatric disorders has increased, a full understanding of sex differences within these cell-types requires further examination. Women are twice as likely to suffer from anxiety and depression as men (Albert, 2015). However, neuroscience has extensively utilized male rodents in preclinical studies (Mamlouk et al., 2020), including animal models of anxiety- and depressive-like behaviors. The under-representation of females in both clinical and preclinical research leaves a significant gap in the understanding of the biology of mood disorders. Because the topic of sex differences and microglia has been extensively reviewed elsewhere (Bilbo et al., 2012; Bekhbat and Neigh, 2018; Rainville and Hodes, 2019), this review focuses on the macroglia: astrocytes and oligodendrocytes.

PRIMER ON STEROIDS AND THE NERVOUS SYSTEM

We begin with a brief overview of the influence of steroids in the CNS. Steroids are a class of hormones which are highly lipophilic and are synthesized on demand both by endocrine organs and in the central nervous system (Schmidt et al., 2008; Diotel et al., 2018). The most studied class of steroid receptors are the nuclear receptors which act as transcription factors and thereby can exert profound and long-lasting effects within the brain and periphery (Lösel and Wehling, 2003). Steroids are further subdivided into corticosteroids and sex steroids. The corticosteroids include mineralocorticoids and glucocorticoids and are critical in modulation of circadian functions, such as sleep and feeding behavior (Oster et al., 2006; Dickmeis, 2009), and the response to physical and psychological stressors (Jauregui-Huerta et al., 2010). The sex steroids include progesterone, estrogen, and testosterone. All types of sex steroids exist in both sexes. In addition to the influence of sex steroids over reproductive behaviors and secondary sex characteristics (Morris et al., 2004), sex steroids also influence a wide range of normal and pathological functions unrelated to reproduction.

Chronic and traumatic stress influence the manifestation and progression of mood disorders (McEwen, 2007); therefore, we begin by discussing the interactions among glia and glucocorticoids. Disruptions in homeostasis, whether physical or perceived, activate the hypothalamic-pituitary-adrenal (HPA) axis. This leads to an increase in circulating glucocorticoids (GCs) in the bloodstream that bind to glucocorticoid (GR) and mineralocorticoid receptors (MRs) throughout the organism (McEwen, 2007). HPA axis activation is a normal and healthy response to a perceived threat or physical perturbation; however, continuous HPA axis activation during chronic stress can evoke maladaptive consequences within stress-sensitive brain regions as well as in other organ systems. Although GR binds with lower affinity than MR, GR appears to be more complicit in chronic stress-induced effects. The pervasive impact of GR is at least in part driven by the genomic impact of GR such that activation of the GR can lead to transcriptional changes of up to 10% of genes through glucocorticoid response elements (Jauregui-Huerta et al., 2010). The robust influence of GR is regulated by multiple chaperones and co-chaperones and the effects can be cell-type specific. GR expression has been demonstrated on glia cells within the brain (Vielkind et al., 1990; Bohn et al., 1991). Engagement of the transcriptional influences of steroids is also evident in macroglia. In primary oligodendrocytes cultures isolated from male and female mice, steroid hormones differentially influenced cell survival (Swamydas et al., 2009). Cultured astrocytes respond to corticosterone application with an altered expression of 141 mRNAs, an effect that was attenuated by co-administration with the GR antagonist RU486 (Carter et al., 2012). Additionally, corticosterone treatment in rats resulted in decreased expression of the astrocyte enriched structural protein glial fibrillary acidic protein (GFAP; O'Callaghan et al., 1989; Nichols et al., 1990).

Sex steroids are present in both sexes and differ in concentrations and timing of developmental surges (McCarthy et al., 2002). In addition to the well-known functions of sex steroids in reproductive behaviors and secondary sex characteristics, these steroids catalyze a range of changes in physiology through neuronal actions (McEwen and Parsons,

1982) and influence glial development (McCarthy et al., 2002; Marin-Husstege et al., 2004) and function (Kuo et al., 2010). Importantly, sex steroids and GCs also interact with one another and influence the impact of one another to influence both physiology and behavior (Conrad et al., 2004; Hiroi and Neumaier, 2006) illustrating the centrality of considered sex differences in the examination of stress and related neuropsychiatric disorders. This is particularly important in the context of glial biology because, in addition to the impact of sex steroids on glia through estrogen receptors expressed on both astrocytes and oligodendrocytes (Azcoitia et al., 1999; Takao et al., 2004), glia also influence sex steroid function in that they provide a central source of steroid synthesis. Although peripheral sources of steroids (i.e., ovaries, testes, and adrenals) exert substantial influence in the CNS due to their highly lipophilic nature which facilitates the crossing of the blood-brain barrier, neuroactive steroids can be generated centrally from cholesterol synthesized by astrocytes and oligodendrocytes (Arbo et al., 2016). Thus, sex steroids are positioned to influence a plethora of functions in the brain that are outside the realm of reproductive behaviors.

ASTROCYTES

The role of astrocytes in neural function, pathology, and repair has provided foundational information for studying the possible role of glia in neuropsychiatric disorders. For instance, progesterone and estrogen have demonstrated neuroprotective effects following injury, inflammation, and stress at the level of glia (Garcia-Ovejero et al., 2005; Arbo et al., 2016). Even in the absence of injury, sex steroids influence astrocyte function. The estrous cycle stage of female rats is associated with glia process ensheathment of synaptic terminals within the arcuate nucleus, with ensheathment decreasing during estrus compared to proestrus (Olmos et al., 1989). Further, the relationship between astrocytes and sex steroids is bidirectional. Glial cells express estrogen receptors and can produce estrogen, thus they can be the driver of estrogen's protective effects within models of CNS disease (Arevalo et al., 2010). For example, ERα located on astrocytes are responsible for the protective effects of estrogen within a model of autoimmune encephalomyelitis, not neuronal ERα, as seen by decreased levels of macrophage and T-cells of ERα ligand treated animals (Spence et al., 2011).

Although not as easily defined as neural injury studies, a relationship between astrocytes, steroids, and neuropsychiatric disorders has begun to be established. Altered expression of astrocyte density, as seen in post-mortem human tissue, has been implicated in the development of anxiety and depression (Rajkowska and Miguel-Hidalgo, 2007). A similar relationship has been detailed using animal models. Chemically induced deficits of the astrocyte population within the prefrontal cortex (PFC) using the astrocyte toxin L-alpha-aminoadipic acid (L-AAA) induce depressive-like behaviors in rodents (Banasr and Duman, 2008). In addition, exogenous administration of corticosterone, the primary glucocorticoid in rodents, leads to a decrease in the astrocyte structural protein GFAP mRNA (Nichols et al., 1990) and protein (O'Callaghan et al., 1989) within the hippocampus, a stress-sensitive brain region. Changes in GFAP-immunoreactivity (GFAP-IR) may indicate less coverage of the synapse, suggesting a relationship between glucocorticoids and synaptic activity. Changes in cell proliferation rate within the adult CNS can also contribute to altered expression of GFAP positive cells, as seen in male mice that underwent chronic stress and expressed a decreased number of newborn astrocytes in the hippocampus compared to controls (Dioli et al., 2017). In addition, comparison of GFAP-IR in the male Wistar-Kyoto (WKY) rat, which is commonly used as a model in studies of anxiety-like and depressive-like behaviors due to heightened stress-sensitivity, to male Sprague-Dawley rats, found that overall GFAP expression was lower in the PFC, basolateral amygdala (BLA), and hippocampus of the WKY rats (Gosselin et al., 2009). This lower expression of GFAP in stress-sensitive brain regions of the WKY strain suggests that the magnified stress-induced behaviors observed may be due to basal differences in the astrocyte profile within these regions.

While the preceding studies did not include the variable of sex, differences in astrocyte complexity may vary by sex. Ovariectomized (OVX) female rats given estradiol display an increase in GFAP surface density compared to OVX controls in the hippocampus and globus pallidus (Trangue et al., 1987) demonstrating the ability of estradiol to influence astrocytic proteins. In addition, GFAP-IR in the hippocampus of adult Wistar rats was greater in the CA1 region in females, with males displaying greater reactivity in CA3 (Conejo et al., 2003). These findings demonstrate baseline differences in a marker of astrocyte complexity which may be reflective of sex differences in astrocyte phenotype in stress-sensitive brain regions. Further, animal models of stress show sex differences in astrocyte complexity in the medial PFC (mPFC) following chronic stress, results that were sex steroid dependent (Bollinger et al., 2019). This suggests that sex steroids have direct actions on glia activity and may drive sex differences in the glial response to stress, which could facilitate sex differences in behavioral outcomes following stress exposure.

Astrocytes have an established role in neurotransmitter modulation. Importantly, astrocyte-mediated regulation of glutamate basal tone and dysregulation of glutamate homeostasis may contribute to the potentiation of anxiety and depression (Blacker et al., 2019). To this end, the astrocyte enriched glutamate transporter GLT-1 has decreased expression in postmortem tissue obtained from individuals diagnosed with MDD (Choudary et al., 2005; Rajkowska and Stockmeier, 2013). Similarly, preclinical research suggests a role of GLT-1 in behaviors related to mood disorders. Knockout of the GLT-1 gene in astrocyte using male transgenic GFAP-Cre mice results inincreased anxiety-like behaviors in the elevated plus maze (Jia et al., 2020). However, the effects of GLT-1 are region dependent such that deletion of GLT-1 within the lateral habenula increased depressive-like behavior as measured by social interaction and behavior in the novelty-suppressed feeding

paradigm (Cui et al., 2014). The brain region specific effects of GLT-1 may also be sex specific. Male and female Long-Evans rats exposed to chronic stress displayed reductions in GLT-1 expression (Rappeneau et al., 2016). However, females had decreased GLT-1 and GFAP mRNA within the PFC and nucleus accumbens (NAc), whereas, males displayed reductions in GLT-1 only in the striatum. Furthermore, comprehensive evaluation of overall glutamate homeostasis in male mice that underwent varying lengths of stress suggests that neuronal and glia protein expression are altered (Nasca et al., 2017). Unfortunately, female mice were not included in this study leaving a gap in knowledge regarding sex differences in glutamate homeostasis following stress which will be important to address given that evidence for differential glutamate homeostasis as a function of sex is evident from postmortem human studies. Analysis of ionotropic and metabotropic glutamate receptor expression in postmortem tissue reveals that females with MDD have higher expression of both receptor classes in the dorsolateral PFC, while males with MDD only display variation in the glutamate metabotropic receptor 5 (GRM5) expression (Gray et al., 2015). While this study focused on neuronal proteins, it suggests there may be sex differences in glutamate homeostasis during the clinical state of depression. Given that astroglia are essential in maintaining glutamate homeostasis and astrocyte-enriched proteins that regulate glutamate clearance are differentially altered in males and females following stress, a comprehensive evaluation of the role of astrocyte enriched protein expression in females following stress will be foundational in building an understanding of the role of astrocytes in the regulation of glutamate homeostasis in the context of both normal brain function and neuropsychiatric disorders.

OLIGODENDROCYTES

White matter consists of oligodendrocytes that ensheath axons and allow neurons to propagate action potentials (Bonnefil et al., 2019). Similar to astrocytes, our understanding of oligodendrocytes and their relationship to steroids was bolstered by the study of neural injury. Examination of a model of autoimmune encephalomyelitis demonstrated that estradiol promotes oligodendrocyte myelin protein survival (Offner and Polanczyk, 2006). Again in line with astrocytes, evidence exists to indicate a relationship between oligodendrocytes and neuropsychiatric diseases. Reductions in white matter are visible in patients with MDD using neuroimaging (Coloigner et al., 2019). Analysis of the anterior cingulate cortex from male victims of suicide with a history of active depression found decreased gap junction coupling between oligodendrocytes and astrocytes, compared to controls (Tanti et al., 2019). Although causality cannot be assessed in this type of study, animal models can be leveraged to investigate how social experience may alter oligodendrocytes and myelinationcreating a risk factor for developing a neuropsychiatric disorder (Toritsuka et al., 2015). Chronic stress in male mice caused morphological changes of oligodendrocytes in the corpus callosum, including increased length of the paranode and juxtaparanode regions and upregulation of multiple adhesion molecules (Miyata et al., 2016) suggesting compromised function. Following chronic social defeat of male mice, the percent area in the NAc covered with myelin basic protein (MBP) is decreased along with the altered length of myelin segmentation in the mPFC (Bonnefil et al., 2019). In the same study, a demyelination compound was directly targeted at the mPFC and decreased social preference in male mice demonstrating that decreasing myelination alone can lead to altered social behavior, similarly to the depletion of astrocytes in the PFC leading to depressive-like behaviors (Banasr and Duman, 2008). Further, male mice that underwent chronic variable stress displayed increased depressive-like behaviors and oligodendrocyte transcriptional changes in the PFC, NAc, amygdala, and corpus callosum (Liu et al., 2018). Thus, changes in oligodendrocyte function are not limited to social stress alone—at least for male organisms as all of the studies discussed excluded females from the analysis.

Despite the frequent exclusion of female subjects from research studies, there is substantial evidence for sex differences in oligodendrocytes. The density and proliferation of oligodendrocytes vary between male and female mice, with adult castrated male mice displaying similar proliferation in the corpus callosum as females which supports a causative role of sex steroids in oligodendrocyte proliferation (Cerghet et al., 2006). This influence of sex on oligodendrocyte proliferation has been confirmed *in vitro* as demonstrated by greater expression of genes associated with oligodendrocyte proliferation in primary cultures obtained from neonatal female rats compared to males (Yasuda et al., 2020). In addition to basal differences, sex differences in oligodendrocyte markers have been reported following acute stress with differentially altered MBP levels in male and female mice. Male mice displayedincreased levels of MBP in the amygdala and hippocampus 12 days after the stressor (Breton et al., 2020). Conversely, females did not have altered MBP levels until 2 months after the stressor and exhibited increases in the PFC, amygdala, and hippocampus.Thus, the temporal influence of stress on oligodendrocytes differs between the sexes and couldrepresent an important and understudied mechanism driving sex differences in neuropsychiatric diseases.

ANTIDEPRESSANTS AND MACROGLIA

There is robust evidence to support the role of glial cells in the development of neuropsychiatric disorders as identified in human post-mortem pathology and the rodent studies discussed. This relationship is further supported by evidence that glial cells are responsive to the influence of some antidepressant treatments. For instance, astrocyte enriched gap junction protein connexin 43 is lowered by chronic unpredictable stress, but the administration of fluoxetine or duloxetine recovered expression deficit in male rats (Sun et al., 2012). In addition, the antidepressant clemastine rescues behavioral deficits and restores MBP coverage in the PFC of socially isolated male mice (Liu et al., 2016). However, antidepressants are not universally efficacious in reversing stress effects on macroglia as evidence of the failure of citalopram to prevent

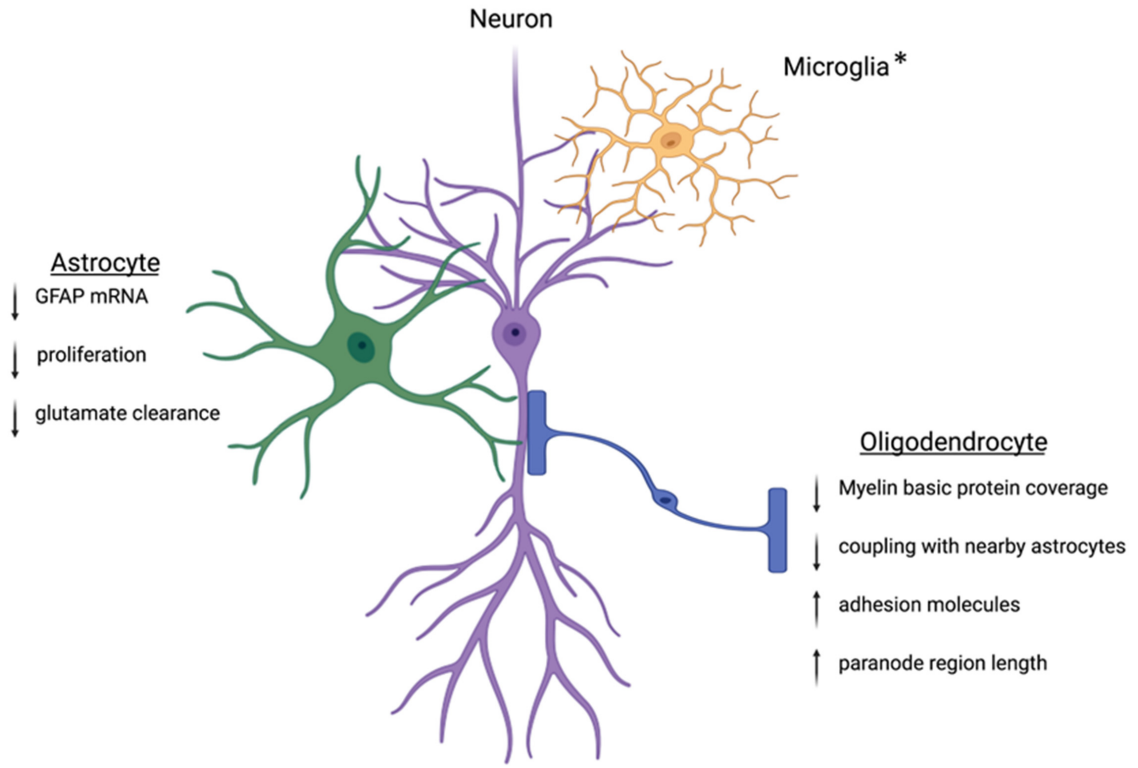

FIGURE 1 | Within the CNS, glia cells are positioned in close contact with neighboring neurons. This positioning allows the glial cells to tightly control the environment and facilitate neuronal communication. Following stress, this regulation can become dysregulated. Key proteins involved in astrocyte function are downregulated leading to decreased cradling at the synapse and altered glutamate homeostasis. Oligodendrocytes will experience downregulation of the key myelin basic protein and altered wrapping at the neuronal axon. Preclinical works suggest protein dysregulation following stress is different between males and females, but more work is needed to understand these mechanisms. *Microglia are intimately associated with the synapse and other nearby glia cells. The interactions between microglia and neurons can be disrupted following stress. While microglia are not discussed in this review, other reviews have extensively outlined the ways microglia contribute to sex differences in the stress response (Bilbo et al., 2012; Bekhbat and Neigh, 2018; Rainville and Hodes, 2019). Created with Biorender.com.

stress-induced reductions of GFAP in astrocytes of male rats (Araya-Callís et al., 2012). These studies did not consider females and illustrate that only partial responsivity of glia to current antidepressants exists. This represents both an opportunity to directly target macroglia in the treatment of mood disorders and the tremendous need to consider females in these assessments.

CONCLUSIONS

The goal of this mini-review was to highlight the importance of sex in the consideration of the role of macroglia in mental health disorders and altered affective-like behaviors. Glia are involved in shaping the synapse through the regulation of neurotransmitter levels and energy resources, making them essential contributors to neural dynamics following stress. We have highlighted astrocytes and oligodendrocytes, both of which are implicated in the progression of MDD (**Figure 1**). To date, studies have focused on the role of glia or neuronal proteins in the progression of anxiety and depression; however, opportunities to identify expression variations in multiple cell types within a single condition or preclinical model could elucidate the overall change in tone at the synapse and provide an important contribution to advancing our understanding. Additionally, assessment of glia-glia communication following stress will be a critical area of future study in order to determine how macroglia change the dynamics of neural resources and overall communication. Furthermore, as the role of glia in neuromodulation continues to be elucidated, studies exploring the mechanisms by which glia are altered by stress and steroids will provide insight into sex differences in animal models and inform our understanding of sex differences in the clinical setting. Finally, clarification of macroglia function in health and disease in both sexes will provide the foundation for novel mechanistic interventions that may have the capacity to treat beyond the symptoms and target the biological nexus from which symptoms manifest.

AUTHOR CONTRIBUTIONS

GN and AW decided on the topic area together. AW drafted the first draft of the review with guidance from GN. GN revised and edited the review. All authors contributed to the article and approved the submitted version.

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The Duration of Stress Determines Sex Specificities in the Vulnerability to Depression and in the Morphologic Remodeling of Neurons and Microglia

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Stress exposure has been shown to induce a variety of molecular and functional alterations associated with anxiety and depression. Some studies suggest that microglia, the immune cells of the brain, play a significant role in determining neuronal and behavioral responses to chronic stress and also contribute to the development of stress-related psychopathologies. However, little is known about the impact of the duration of stress exposure upon microglia and neurons morphology, particularly considering sex differences. This issue deserves particular investigation, considering that the process of morphologic remodeling of neurons and microglia is usually accompanied by functional changes with behavioral expression. Here, we examine the effects of short and long unpredictable chronic mild stress (uCMS) protocols on behavior, evaluating in parallel microglia and neurons morphology in the dorsal hippocampus (dHIP) and in the *nucleus accumbens* (NAc), two brain regions involved in the etiology of depression. We report that long-term uCMS induced more behavioral alterations in males, which present anxiety and depression-like phenotypes (anhedonia and helplessness behavior), while females only display anxiety-like behavior. After short-term uCMS, both sexes presented anxiety-like behavior. Microglia cells undergo a process of morphologic adaptation to short-term uCMS, dependent on sex, in the NAc: we observed a hypertrophy in males and an atrophy in females, transient effects that do not persist after long-term uCMS. In the dHIP, the morphologic adaptation of microglia is only observed in females (hypertrophy) and after the protocol of long uCMS. Interestingly, males are more vulnerable to neuronal morphological alterations in a region-specific manner: dendritic atrophy in granule neurons of the dHIP and hypertrophy in the medium spiny neurons of the NAc, both after short- or long-term uCMS. The morphology of neurons in these

brain regions were not affected in females. These findings raise the possibility that, by differentially affecting neurons and microglia in dHIP and NAc, chronic stress may contribute for differences in the clinical presentation of stress-related disorders under the control of sex-specific mechanisms.

Keywords: chronic stress, microglia morphology, sex differences, dorsal hippocampus, *nucleus accumbens*, neurons morphology

INTRODUCTION

Exposure to stress has a detrimental impact on certain brain functions, depending on the duration, type, and severity of stress. Uncontrollable stress is a contributing factor for major depressive disorder (Iwata et al., 2013), a severe and debilitating psychiatric illness characterized by a significant change in mood and accompanied by symptoms such as anhedonia and disrupted sleeping, eating, and cognitive deficits (Kessler, 2012).

A wide variety of animal models have been used to mimic human depression, but, as a heterogeneous disorder, many of its symptoms (depressed mood, feelings of worthlessness, and suicidal ideation) are hard to be mimicked in laboratory animals and, for an animal model to be valid, it is not necessary to exhibit all the traits of depression, since patients do not manifest every symptoms of the disease (Belzung and Lemoine, 2011).

Unpredictable chronic mild stress (uCMS) protocol is widely used and involves a permanent exposure to a variety of mild stressors in an unpredictable manner. In adult rodents, uCMS is a valid model of depression (Willner, 2005) and induce a variety of behavioral alterations, including anxiety, anhedonia, decreased exploratory behavior, and increased immobility/despair behavior when exposed to stressful environments, as well as impaired spatial cognition (Henningsen et al., 2009; Hill et al., 2012; Bessa et al., 2013; Morais et al., 2014; Patricio et al., 2015).

Stress impact various aspects of immunity that in turn promote stress susceptibility. As innate immune cells of the brain, microglia play an integrative role in maintaining neuronal homeostasis (Salter and Stevens, 2017). These cells are distributed throughout the brain and function as a critical line of defense against injury and pathogenic insults (Hanisch and Kettenmann, 2007). It has been reported that stress induces morphologic changes of microglia (Sugama et al., 2007), namely promoting microglial hyper-ramification in the prefrontal cortex (PFC) (Tynan et al., 2013), which supports the theory that these cells play an important role in modulating stress responses (Reus et al., 2015). In the healthy adult central nervous system (CNS), microglia have a ramified morphology characterized by long and thin processes that support the ability for searching potential threats for local homeostasis (Nimmerjahn et al., 2005; Kettenmann et al., 2011; Xavier et al., 2014; Wu et al., 2015). Some studies have described that when microglia respond to insults, they change their morphology, the processes retract and the cell body enlarges, giving microglia an amoeboid shape (Davalos et al., 2005; Cho et al., 2006). However, in our recent studies we report a diversity of morphologic changes that globally depend on the time of stress exposure (prenatal versus adult stress), on the sex of the animal and on the brain region under study (Caetano et al., 2017; Duarte et al., 2019; Gaspar et al., 2021). Our observations suggest that microglia remodeling upon stress are not limited to the acquisition of an amoeboid phenotype, as previously described (Sugama et al., 2007; Tynan et al., 2010; Kreisel et al., 2014), but instead may vary from different degrees of atrophy to hypertrophy.

In addition to microglial changes, several studies also point toward stress-induced sex differences in neurons morphology (Galea et al., 1997; Garrett and Wellman, 2009; Bock et al., 2011; Breach et al., 2019), although the majority of studies were performed exclusively in males (Magarinos and McEwen, 1995; Lambert et al., 1998; Radley et al., 2006; Bessa et al., 2009a, 2013; Morais et al., 2014; Melo et al., 2015; Patricio et al., 2015). In fact, stress-induced morphologic changes in microglia and neurons are associated with behavioral alterations in rodent models, including anhedonia, anxiety-like behavior and despair-like behavior (Fonken et al., 2018; Liu et al., 2019).

Sexual dimorphism at multiple levels, including cellular, molecular, and immune system in stress response suggest that stress-elicited neuroinflammatory priming may vary between sexes (Couch et al., 2013; Kreisel et al., 2014; Bekhbat and Neigh, 2018; Wohleb et al., 2018). However, little is known about the morphologic adaptation of brain cells in its relation with depression vulnerability between sexes when subjected to stress protocols of different duration. Therefore, in this study, we examined the effects of the exposure to short and long uCMS in both sexes upon behavior and plastic changes of microglia and neurons. We used a set of different behavioral tests to evaluate anxiety- and depression-like profiles of adult rats exposed to uCMS. Using an automated methodology, we quantified how uCMS alters several morphologic properties of microglia and neurons in the dorsal hippocampus (dHIP) and *nucleus accumbens* (NAc), two key brain regions in stress responses.

MATERIALS AND METHODS

The timeline of all procedures is shown in **Figure 1A**.

Animals

Adult male and female rats (Wistar Han), 3-months old (Charles River Laboratories, L'Arbresle, France) were housed and kept under standard laboratory conditions: 22°C, 55% relative humidity, and 12 h light/dark cycle with free access to food and water. A complete timeline of all manipulations and behavioral tests is provided in **Figure 1A**. The handling and health monitoring were performed according to federation of european laboratory animal science associations (FELASA) guidelines. All

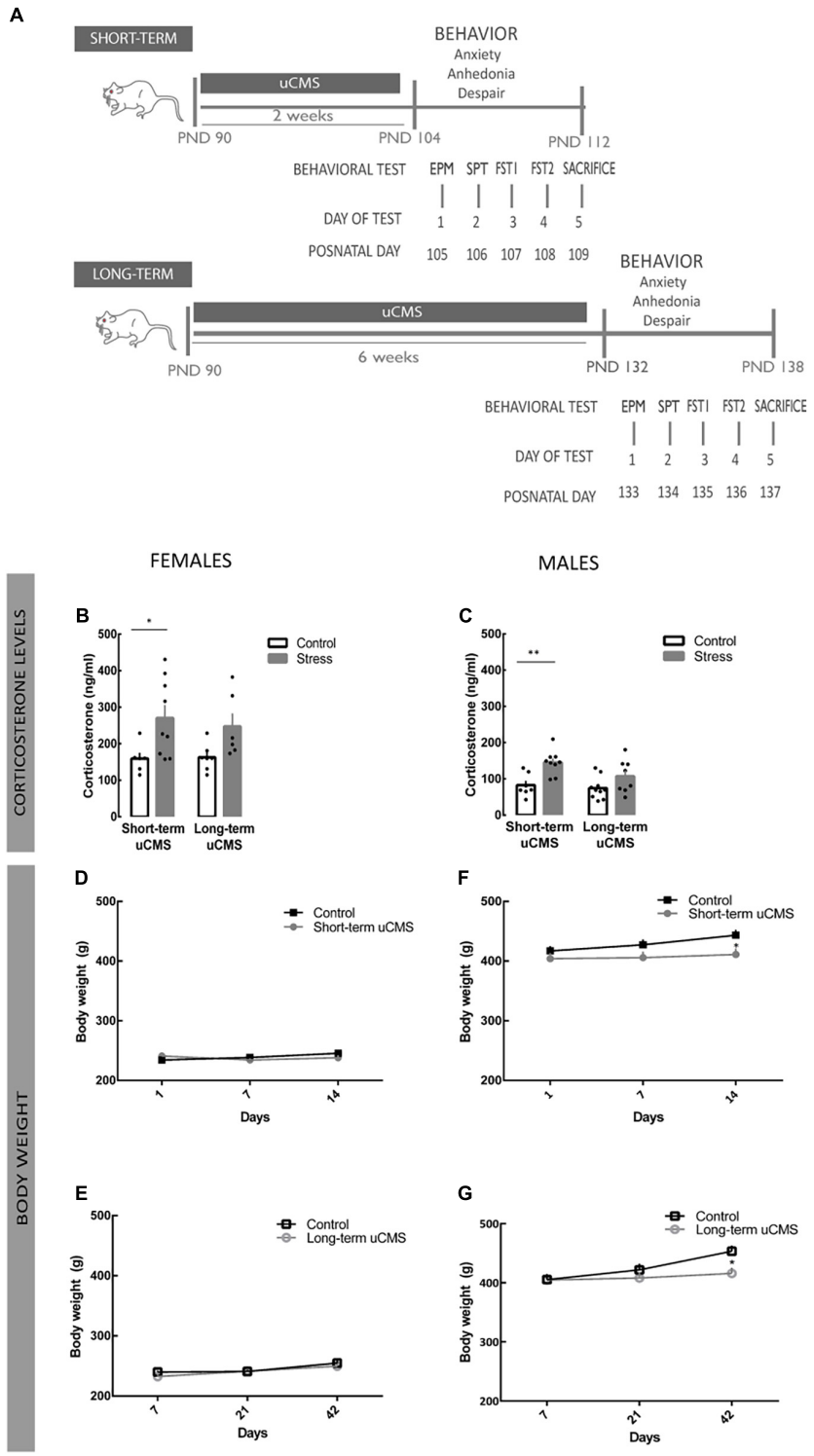

FIGURE 1 | Unpredictable chronic mild stress (uCMS) induces a dysregulation of body weight in males and in the circadian corticosterone secretion pattern in both sexes. **(A)** Schematic drawing of the uCMS protocol. **(B,C)** Corticosterone serum levels measured at 8:00 a.m. in female and male rats exposed to stress in adulthood. **(D,E)** Body weight of female rats exposed to short- or a long-term protocol of chronic mild stress for 2 and 6 weeks, respectively. **(F,G)** Body weight of male rats exposed to short- or a long-term protocol of chronic mild stress for 2 and 6 weeks, respectively. Results are presented as the mean ± SEM of 10–20 animals (body weight) 6–10 animals (corticosterone); comparing with control, calculated using a two-way Analysis of Variance (ANOVA) followed by a Bonferroni *post-hoc* test. $^{*}p < 0.05$ and $^{**}p < 0.01$.

experimental procedures were approved by european union (EU) – Directive 2010/63/EU and the Portuguese National Authority for animal experimentation, Direção-Geral de Animal e Veterinária (DGAV). All protocols were approved by the Ethics Committee of the Life and Health Sciences Research Institute and by DGAV (#19074).

Unpredictable Chronic Mild Stress

At posnatal day (PND)90, animals were randomly divided into four experimental groups and placed in separate rooms: a group of animals exposed to uCMS for 2 weeks (Short-term uCMS – Stress); a group not exposed to uCMS for 2 weeks (Short-term uCMS – Control); a group of animals exposed to uCMS for 6 weeks (Long-term uCMS – Stress); a group not exposed to uCMS for 6 weeks (Long-term uCMS – Control). An adapted version of the previously described and validated uCMS protocol (Willner, 2005; Alves et al., 2017), was applied for two periods of different duration (2 and 6 weeks). The uCMS protocol consisted of a variety of unpredictable mild stressors, including confinement to a restricted space for 1 h, placement in a tilted cage (30°) for 3 h, housing on damp bedding, 15 h of food deprivation followed by exposure to inaccessible food for 1 h, water deprivation for 15 h followed by exposure to an empty bottle for 1 h, exposure to stroboscopic lights during 4 h and reversed light/dark cycle for 48 h, every 7 days. Rats subjected to stress were randomly exposed to 2–4 stressors every day for 2 or 6 weeks (**Supplementary Figure 1**). The controls were left undisturbed under the previously described maintenance conditions. Body weight was monitored weekly to monitor the overall effects of the stress paradigms.

Behavioral Analysis

At the end of the uCMS protocol, a series of behavioral tests were performed in sequence to evaluate anxiety and depressive-like behavior. The Elevated Plus Maze (EPM) and Forced Swimming Tests (FST) were conducted during the light period of animals (9:00 a.m.–5:00 p.m.); the Sucrose Preference Test (SPT) test was performed during the dark period, from 9:00 p.m. to 10:00 p.m.

Elevated Plus Maze

To assess anxiety-like behavior, the EPM test was performed at PND105 (short-term) and at PND133 (long-term). The maze (ENV-560; Med Associates Inc., St. Albans, VT, United States) has two closed (50.8 cm × 10.2 cm × 40.6 cm) and two open arms (50.8 cm × 10.2 cm), raised 72.4 cm above the floor and illuminated by a dim light. Each animal was positioned in the center of this elevated plus-shaped platform for 5 min. The performance of rats in EPM was video-recorded and subsequently analyzed. The ratio of time spent in the open arms *per* total time spent in the open and in close arms was calculated as an index of anxiety-like behavior.

Sucrose Preference Test

This test was performed at PND106 (short-term) and PND134 (long-term). Briefly, after 12 h of food and water deprivation, rats were presented with two pre-weighted bottles containing tap water or a solution of sucrose 2% for 1 h. The liquid intake from each bottle was calculated by comparing the differences in bottle weights before and after the test. The sucrose preference was determined as the percentage of sucrose solution intake that was calculated according to the formula: SP = [sucrose intake/(sucrose intake + water intake)] × 100, as previously described (Bekris et al., 2005). Low sucrose preference represented anhedonia, a core symptom of depression. When the preference test ended, rats were given free access to water and food.

Forced Swimming Test

The test was performed at PND107-108 (short-term) and PND135-136 (long-term) after SPT. On the 1st day, rats were placed individually in a glass cylinder with water (62 cm height; 25.4 cm diameter; depth no less than 50 cm, 23°C) for 5 min. Then, the rats were dried and transported back to their home cages. In the 2nd day, the rats were subjected to one 5-min session of swimming. The test session was video-recorded, and the immobility time of each rat was measured using the EthoVision XT 11.5 tracking system (Noldus Information Tecnhology, Wageningen, The Netherlands). Immobility was defined as floating state in the water, without struggling and making only those movements to keep the head above water. Depressive-like behavior was defined as an increase in the immobility time.

Immunohistochemistry and 3D Morphometric Analysis of Microglia

After completion of stress protocols and behavioral tests, all groups of rats were deeply anesthetized with sodium pentobarbital (20%; Eutasil®, Sanofi, Gentilly, France) and transcardially perfused with 0.9% saline. The brains were removed and one hemisphere from each brain was used for Golgi staining technique and the other for immunohistochemistry for ionized calcium-binding adaptor protein-1 (Iba-1) followed by the 3D reconstruction of microglia cells. The right hemispheres, used for Iba-1 immunohistochemistry, were post-fixed in 4% paraformaldehyde (PFA), cryoprotected in 30% sucrose overnight, and then embedded in Optimal Cutting Temperature compound (OCT, ThermoScientific, Waltham, MA, United States), snap-frozen and stored at −80°C. Coronal sections (50 μm) of the hippocampal dentate gyrus (DG) (stereotaxic coordinates of interaural 5.20 mm and bregma −3.80 mm) and NAc (stereotaxic coordinates of interaural 10.20 mm and bregma 1.2 mm) were further stained to visualize microglia cells. Microglia were visualized using the following protocol: free-floating sections were blocked 2 h with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) + 0.1% Triton X at room temperature (RT) and incubated for 48 h at 4°C with an antibody specific to Iba-1 (1:1,000; Wako Chemicals Inc., Richmond, VA, United States) in 5% BSA/0.1% Triton X/PBS. Iba-1 is constitutively expressed in microglia, being involved in cytoskeletal reorganization, and is up-regulated in response to microglial cell activation. Sections were then rinsed and incubated for 2 h at RT with the

appropriate secondary antibody (donkey anti-rabbit, 1:1,000, Invitrogen, Waltham, MA, United States) and 4',6-diamidino-2-phenylindole (DAPI, 1:5,000). Sections were rinsed and mounted on gelatinized slices, using glycergel (DAKO mounting medium, Santa Clara, CA, United States). Images of 10 random microglial cells from each animal were acquired with a laser scanning confocal microscope LSM 710 META connected to ZEN Black software (Zeiss Microscopy, Oberkochen, Germany) using a 63x objective lens (oil immersed, Plan-Apochromat 63x/1.40 Oil DIC M27). Microglia cells were reconstructed using the Neurolucida software (MBF Bioscience, Williston, VT, United States). Morphometric data related to branch analysis were extracted by the Neurolucida Explorer software (MBF Bioscience, Williston, VT, United States). The parameters analyzed were the total number and the length of cellular processes and their measures *per* branch order, considering processes of order 1 those emerging directly from the cell body, processes of order 2 those arising from processes of order 1, and so forth (Caetano et al., 2017).

Neuronal Morphology

To assess the dendritic morphology of granule neurons of DG and spiny medium neurons of NAc, three-dimensional morphological analysis was performed on Golgi-Cox stained material. The left hemispheres were immersed in a Golgi-Cox solution (1:1 solution of 5% potassium dichromate and 5% mercuric chloride diluted 4:10 with 5% potassium chromate) for 14 days, cryoprotected with 30% sucrose solution for 72 h, and sectioned at 200 μm in a vibratome in a 6% sucrose solution. Brain sections were mounted on gelatin-coated slides, lightly pressed and kept in moist container until developed, clarified, and then cover slipped. For each selected neuron, dendritic branches were reconstructed at 1,000× (oil) magnification, using a motorized microscope (Axioplan 2; Carl Zeiss, Oberkochen, Germany) and Neurolucida Neuron Tracing Software (MBF Bioscience, Williston, VT, United States). For each animal, approximately 10 neurons were analyzed in the dHIP and in the NAc. Data for process length was obtained using Neurolucida explorer (MBF Bioscience, Williston, VT, United States). Measurements from individual neurons from each animal were averaged. Total dendritic length was compared among the experimental groups. Branching of the neurons was evaluated using 3D Sholl analysis; for this, the number of dendritic intersections with concentric circles positioned at radial intervals of 20 μm was determined.

Corticosterone Levels Measurement

For all animals, serum corticosterone levels were measured using a commercially available ELISA kit (Abcam, Cambridge, United Kingdom), according to the manufacturer's instructions. Blood sampling (tail venipuncture) was performed during the diurnal nadir (N, 8:00–9:00 a.m.) at the end of the stress protocol. Results are expressed as ng of corticosterone *per* ml of serum. Absorbance at 450 nm was determined using a microplate reader and corticosterone concentration (ng/ml) was extrapolated from a standard curve. The coefficient of variation for intra- assay was 5.7% and for inter-assay was 10.2%.

Estrous Cycle Analysis

In the day of sacrifice, vaginal cytology was performed. Exfoliate cytology was examined under light microscope (Leica DM 4000B, Leica, Wetzlar, Germany) with a 10x objective lens (Plan 63x/0.25PH1) and estrous cycle was determined based on the morphology of the cells present in the smear as previously described (Westwood, 2008).

Data Analysis

All data are presented as mean ± standard error of the mean (mean ± SEM). GraphPad Prism 6 Software was employed for statistical analysis. Outliers were identified using GraphPad Prism 6. Two-way Analysis of Variance (ANOVA) followed by a Bonferroni *post-hoc* test was used to assess the effects of stress (Control vs. Stress) and duration of stress (Short- vs. Long-term uCMS). The level of significance for all analysis was a set at $p < 0.05$.

RESULTS

Unpredictable Chronic Mild Stress Induces a Dysregulation of the Circadian Corticosterone Secretion Pattern in Both Sexes and in the Body Weight of Males

It is known that stress impairs the activity of the hypothalamus-pituitary-adrenal (HPA) axis and results in disrupted secretion of corticosteroids (Pariante and Lightman, 2008; Willner et al., 2013). In this work, we exposed animals of both sexes to a well-established uCMS protocol (Willner, 2005; Bessa et al., 2009a; Mateus-Pinheiro et al., 2013) for either 2 or 6 weeks. To validate the uCMS protocol, we measured corticosterone levels as an indicator of HPA axis function. In basal conditions, control females exhibited higher corticosterone levels than males. At the end of short- and long-term uCMS protocol, basal serum corticosterone levels were higher in both sexes exposed to stress [females: **Figure 1B**; $F_{(1,24)} = 10.94$, $p = 0.003$; males: **Figure 1C**; $F_{(1,30)} = 15.28$; $p = 0.0005$], although only statistically significant in the case of the short-term protocol. We also monitored weekly the body weight until the end of the uCMS protocol. In the case of females, short and long uCMS protocols did not significantly affect total body weight [$F_{(1,55)} = 1.79$, $p = 0.19$; **Figures 1D,E**]. Male rats exposed to uCMS displayed a reduction of body weight [$F_{(1,56)} = 12.02$, $p = 0.001$] after completion of short- (*post-hoc* analysis, $p = 0.0277$) or long- term (*post-hoc* analysis, $p = 0.0388$) uCMS protocols (**Figures 1F,G**).

Unpredictable Chronic Mild Stress Induces Anxiety- and Depressive-Like Behavior That Is More Pronounced in Males

Unpredictable chronic mild stress induced anxiety-like behavior in females [$F_{(1,54)} = 19.97$, $p < 0.0001$], as demonstrated

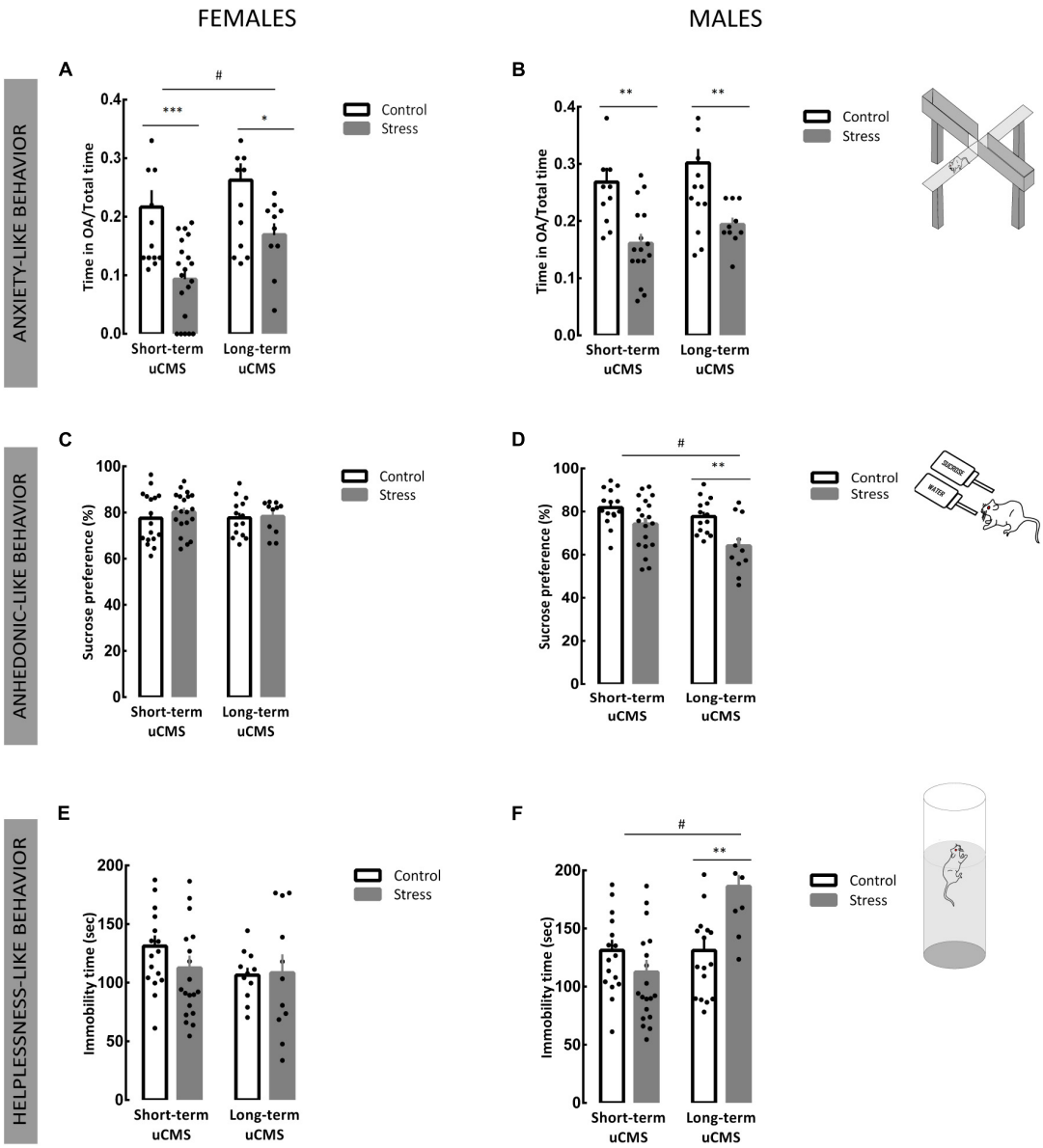

FIGURE 2 | Unpredictable chronic mild stress induces anxiety and depressive-like behavior, an effect more pronounced in males. **(A,B)** Time spent in open arms *per* total time of the elevated plus maze (EPM) test performed to evaluate anxiety-related behavior of females and males. **(C,D)** Anhedonic-like behavior assessed by the preference for sucrose in the sucrose preference test (SPT) in females and males. **(E,F)** Depressive-like behavior assessed by the total time of immobility in the forced swimming test (FST) for females and males. Results are presented as the mean ± SEM of 10–20 animals comparing with control, calculated using a two-way Analysis of Variance (ANOVA) followed by a Bonferroni *post-hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. $^{\#}p < 0.05$ (stress effect).

by the reduced time spent in open arms, after 2 (*post-hoc* analysis, $p = 0.001$) or 6 weeks of uCMS (*post-hoc* analysis, $p = 0.029$; **Figure 2A**). Like females, we also found a significant effect of stress in males [$F_{(1,50)} = 23.40$, $p < 0.0001$]. Males exposed to a short- or long-term uCMS presented anxiety-like behavior (*post-hoc* analysis, $p = 0.002$; $p = 0.003$, respectively; **Figure 2B**).

In the SPT, that evaluates anhedonia, no main effect of stress [$F_{(1,58)} = 0.4701$, $p = 0.4957$] or duration of exposure to uCMS [$F_{(1,58)} = 0.138$, $p = 0.712$] was found in females when assessing the percentage of sucrose solution consumed (**Figure 2C**). In males, we observed a significant stress effect [$F_{(1,55)} = 14.24$, $p = 0.0004$], although only males exposed to a long-term protocol of uCMS showed a decrease in sucrose consumption when compared with controls (*post-hoc* analysis, $p = 0.004$; **Figure 2D**) with an increase in water consumption (**Supplementary Figure 2**).

In the FST, behavioral despair was calculated as time of immobility. In females, no differences in immobility were observed (**Figure 2E**). In males, a main effect of duration of exposure to stress [$F_{(1,61)} = 12.41$, $p = 0.0008$] was found since males exposed to a long-term uCMS showed significantly higher levels of despair behavior, when compared to controls (*post-hoc* analysis, $p = 0.0019$; **Figure 2F**).

The estrous cycle analysis was performed in females and demonstrated that females were distributed by all phases of the estrous cycle (**Supplementary Table 1**).

Unpredictable Chronic Mild Stress Induces Sex-Dependent Morphologic Adaptation of Microglia

Our group described that prenatal stress induces changes in the morphology of microglia (Caetano et al., 2017; Duarte et al., 2019; Gaspar et al., 2021), but the effect of uCMS with different duration and potential sex differences have not been explored. In order to better understand the role of stress in the morphology of microglia, we performed the morphometric analysis of microglia in adult female and male rats in two different brain regions, the dHIP and the NAc. A detailed analysis of microglia, including the number of processes *per* branch order, the total number of branches and the total length of branches was performed.

In the dHIP, short-term uCMS did not induce alterations in microglia morphology in females (**Figures 3A–D** and **Supplementary Table 2**). Conversely, long-term exposure to

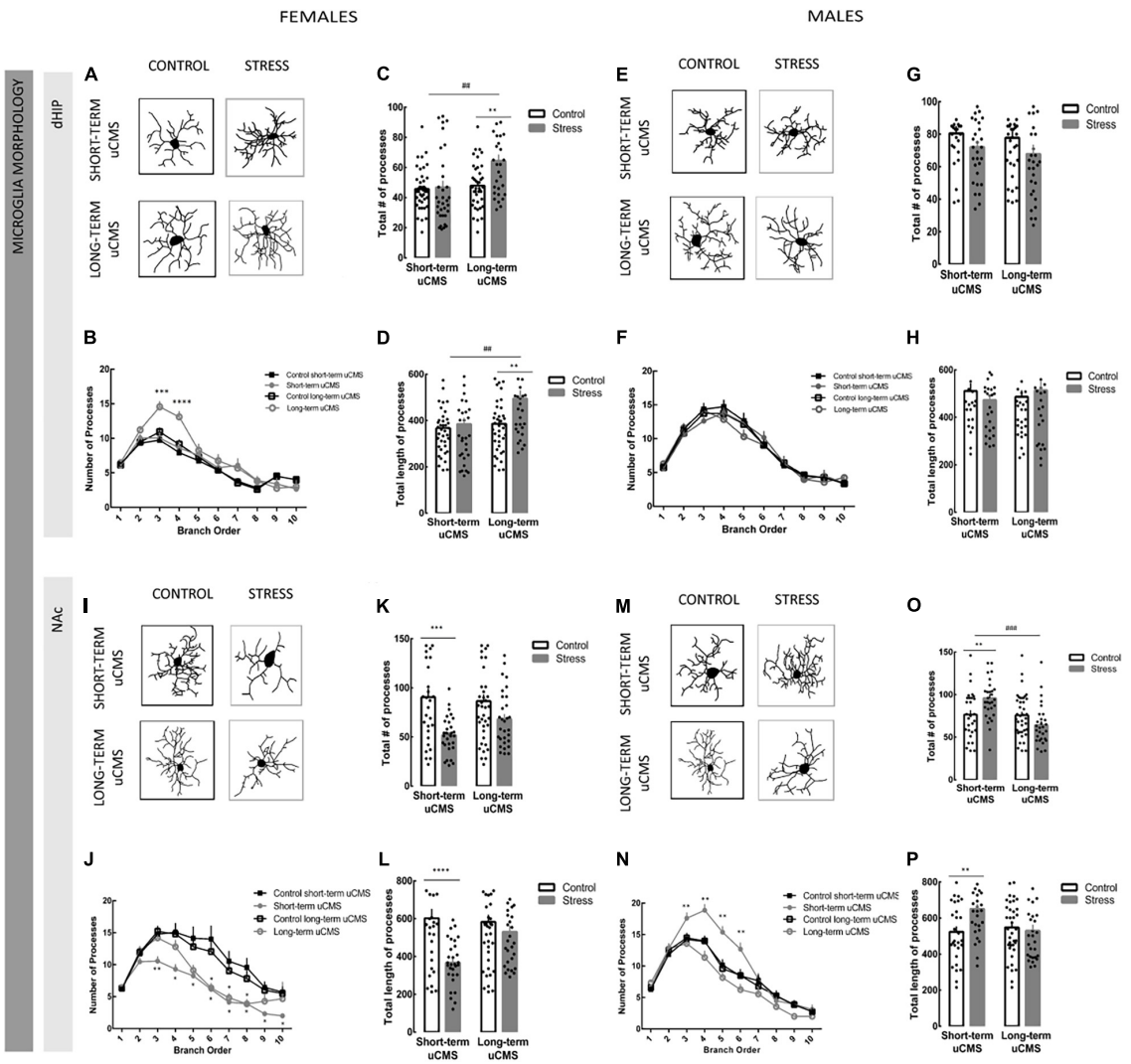

FIGURE 3 | Unpredictable chronic mild stress induces remodeling of microglia, an effect more pronounced in females. Microglial morphometric structure was manually reconstructed in the Neurolucida software based on 3D images of Iba-1 stained microglia. **(A)** Representative microglia cells of the dorsal hippocampus (dHIP) in females. **(B)** Number of processes *per* branch of microglia of the dHIP in females. **(C,D)** Total number and length of microglia cells of the dHIP in females. **(E)** Representative microglia cells of the dHIP in males. **(F)** Number of processes *per* branch of microglia of the dHIP in males. **(G,H)** Total number and length of microglia cells of the dHIP in males. **(I)** Representative microglia cells from of the *nucleus accumbens* (NAc) in females. **(J)** Number of processes *per* branch of microglia of the NAc in females. **(K,L)** Total number and length of microglia cells of the NAc in females. **(M)** Representative microglia cells of the NAc in males of the NAc. **(N)** Number of processes per branch of microglia of the NAc in males. **(O,P)** Total number and length of microglia cells of the NAc in females. Results are presented as the mean ± SEM of 40–50 cells from 4 to 5 animals; comparing with control, calculated using a two-way ANOVA followed by a Bonferroni *post-hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ (stress effect).

uCMS induced a hypertrophy of microglia in the dHIP in females, when compared to control animals, either in the total number of processes (*post-hoc* analysis, $p = 0.002$) and in total length [$F_{(1,140)} = 3.08$, $p = 0.005$; **Figures 3C,D**]. In males, we did not observe any effect of stress (both short or long) in the morphology of microglia either in terms of total number or length (**Figures 3E–H** and **Supplementary Table 2**).

In the NAc, we observed opposite differences between sexes. Short-term uCMS in females induced a general decrease in the total number of processes [$F_{(1,126)} = 19.47$, $p < 0.0001$; **Figures 3I,K**] and in the length [$F_{(1,126)} = 14.23$, $p = 0.0002$; **Figures 3I,L**] (atrophy). Long-term uCMS induced also a decrease of microglia morphology, but only in the number of processes *per* branch order (**Figure 3J** and **Supplementary Table 3**). On the other hand, males exposed to short-term uCMS presented an increase in the total number of processes [$F_{(1,123)} = 0.69$; $p = 0.0088$] and in the length (hypertrophy) [$F_{(1,123)} = 3.49$, $p = 0.0069$] of NAc microglia, but long-term uCMS did not induce alterations in microglia morphology in males (**Figures 3M–P** and **Supplementary Table 3**).

When we compared microglia morphology under physiological conditions in both regions, we observed that microglia cells of females in the NAc exhibited a more complex morphology compared with dHIP. No differences between dHIP and NAc were observed in males microglia (**Supplementary Figures 3A,B** and **Supplementary Table 4**).

Unpredictable Chronic Stress Induces Contrasting Patterns of Neuronal Dendritic Remodeling in the Dorsal Hippocampus and *Nucleus Accumbens* in Males

Neuronal morphology was assessed by three-dimensional morphometric analysis of Golgi impregnated granule neurons in the DG of dHIP and spiny medium neurons in NAc.

Unpredictable chronic mild stress revealed no significant effect in the morphology or in the Sholl analysis of neurons of the dHIP in females (**Figures 4A–C**). In males, exposure to stress induced an atrophy of granule neurons of the dHIP, with a significant decrease in their total dendritic length [$F_{(1,10)} = 59.75$, $p < 0.00001$] as compared with neurons of control animals (**Figures 4D–F**). Both short- (*post-hoc* analysis, $p = 0.0003$) and long-term of CMS (*post-hoc* analysis, $p = 0.0008$) significantly decreased total dendritic length in granule neurons of the dHIP (**Figure 4E**). In Sholl analysis we also observed an effect of stress: males presented a less complex morphology when compared with controls [$F_{(3,120)} = 53.39$, $p < 0.00001$; **Figure 4F**].

We next analyzed the morphological effects of stress in NAc neurons. In females we did not observe any effect of stress in the morphology or in the Sholl analysis of spiny medium neurons (**Figures 4G–I**). Contrarily to what we observed in the dHIP, uCMS induced a hypertrophy in the NAc medium spiny neurons of males, which displayed a significant increase in dendritic length [$F_{(1,10)} = 79.65$, $p < 0.00001$; **Figures 4J,K**]. Both short- (*post-hoc* analysis, $p = 0.0003$) and long-term CMS (*post-hoc* analysis, $p = 0.0001$) significantly increased total dendritic length of medium spiny neurons (**Figure 4K**). Sholl analysis revealed more complex medium spiny neurons in males exposed to long-term uCMS compared to controls [$F_{(3,112)} = 3.122$, $p = 0.028$; **Figure 4L**].

DISCUSSION

The present study explored how short- and long-term uCMS at adulthood alters behavior in males and females and identified changes in the morphology of microglia and neurons of the dHIP and NAc. This issue deserves particular investigation, considering that the process of morphologic remodeling of neurons and microglia is usually accompanied by functional changes with behavioral expression.

The uCMS model is one of the most widely used rodent models to produce behavioral deficits and neuroplastic changes with strong face validity to human depression, that include not only anhedonia, but also anxiety and cognitive impairments in spatial memory and object recognition tasks (Willner et al., 1987; Willner, 1997, 2005; Bessa et al., 2013). However, the differential risk for anxiety and depressive-like behavior between sexes considering a short- (2 weeks) and long-term (6 weeks) uCMS protocol is still not fully elucidated, in particular in what concerns to the characterization of cellular (neurons and microglia) plasticity in an attempt to find a correlation pattern. Considering the marked differences in the prevalence of depression in men and women (Marcus et al., 2005), there has been a considerable interest in sex specificities in anxiety- and depression-like symptoms expressed in animals exposed to stress. Nevertheless, sex differences in the risk and resilience to stress are complex and vary according to the characteristics of the stressor, such as timing, type and severity (Hodes and Epperson, 2019). The basis for these differences is unknown, in part because much of the work in the field is performed mostly in male rodents (Klein et al., 2015), perhaps due to the challenges associated with carrying out experiments influenced by fluctuating gonadal hormones in females (O'Connor and Barrett, 2014).

First, our results showed that body weight is affected (reduced) in males, but not in female rats after short- or long-term uCMS protocols. Although consistent with several studies, showing that chronic stress has a higher impact in reducing male weight gain (Konkle et al., 2003; Mateus-Pinheiro et al., 2013; Patricio et al., 2015), it is important to consider the influence of conditions, such as the type and the intensity of stressor, as well as the age of stress onset. For instance, chronic stress in late adolescent female animals reduces body weight gain (Wulsin et al., 2016).

Assessment of corticosterone levels as an index of the stress response revealed higher levels in both sexes exposed to uCMS comparing to control animals. It is important to note that females have higher basal concentrations of corticosterone and secrete higher levels after stress exposure, as previously described by other authors (Kitay, 1961; Goel et al., 2014; Oyola and Handa, 2017).

In behavioral tests, we showed that male rats are more affected than females by these protocols of stress. Both sexes

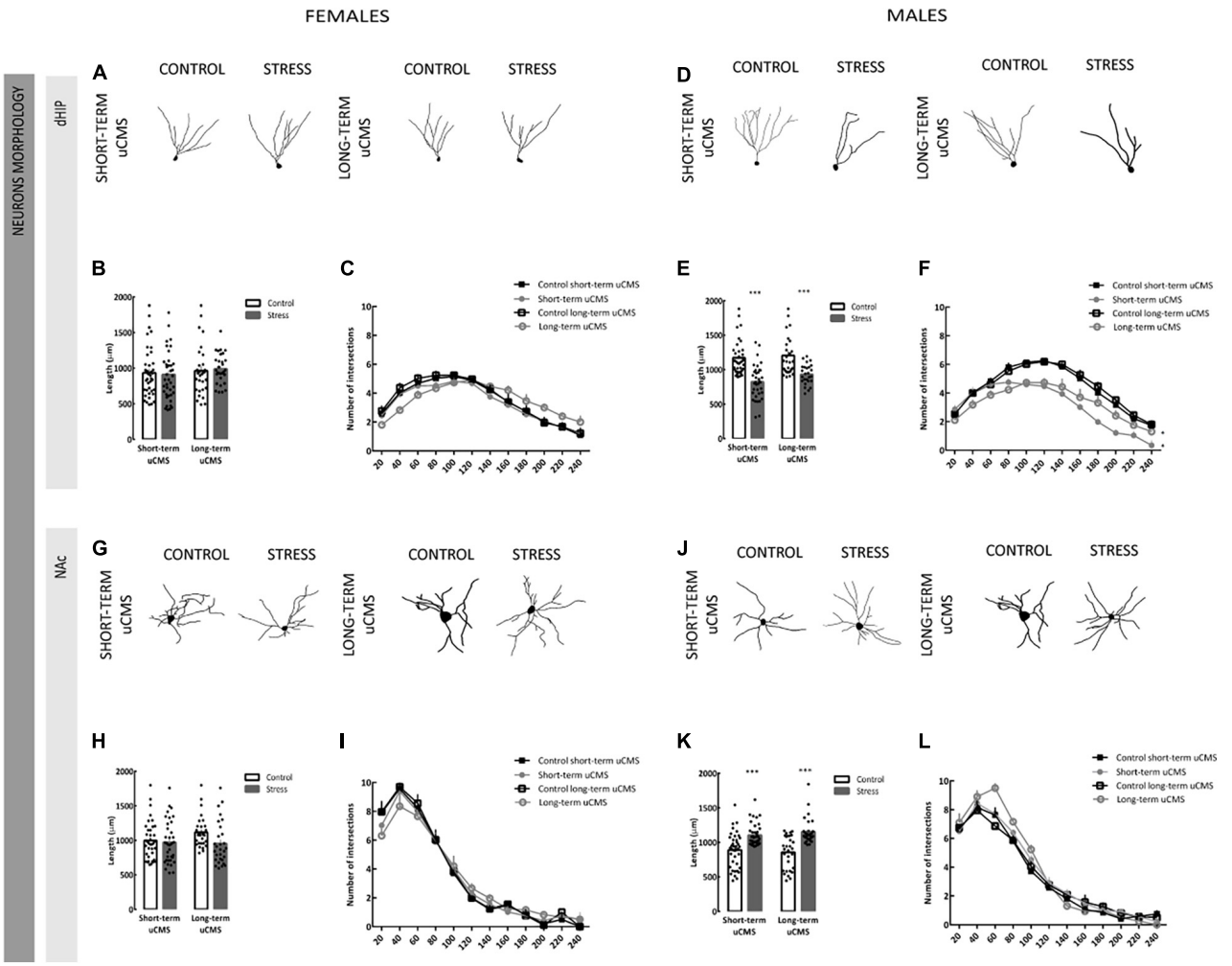

FIGURE 4 | Unpredictable chronic mild stress induces remodeling of neurons only in males. **(A)** Representative manual reconstruction of Golgi-impregned granule neurons of the dorsal hippocampus (dHIP) in females. **(B,C)** Total dendritic length and sholl analysis of dendritic distribution of neurons in the dentate gyrus of the dHIP in females. **(D)** Representative manual reconstruction of Golgi-impregned granule neurons of the dHIP in males. **(E,F)** Total dendritic length and sholl analysis of dendritic distribution of neurons in the dentate gyrus of the dHIP in males. **(G)** Representative manual reconstruction of Golgi-impregned medium spiny neurons of the *nucleus accumbens* (NAc) in females. **(H,I)** Total dendritic length and sholl analysis of dendritic distribution of neurons in the NAc in females. **(J)** Representative manual reconstruction of Golgi-impregned medium spiny neurons of the NAc in males. **(K,L)** Total dendritic length and sholl analysis of dendritic distribution of neurons of the NAc in males. Results are presented as the mean ± SEM of 30–40 cells from 3 to 4 animals; comparing with control, calculated using a two-way Analysis of Variance (ANOVA) followed by a Bonferroni *post-hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

exhibited anxiety-like behavior in response to stress, but only male rats presented anhedonia and despair-like behavior, cardinal symptoms of depression.

Unpredictable chronic mild stress-induced anxiety-like behavior in both sexes is consistent with other studies showing that animals exposed to chronic stress spent less time in the open arms in the EPM test (Kompagne et al., 2008; Yue et al., 2017; Wang et al., 2018).

Furthermore, 6 weeks of uCMS lead to anhedonia and helplessness/despair behaviors in male animals, core symptoms of depression that have been also described as characteristics of stress-related conditions (D'Aquila et al., 1994; Willner et al., 1996; Bekris et al., 2005; Bessa et al., 2009a; Patricio et al., 2015). Stress-induced differences in sucrose consumption between males and females were somehow expected due to sex differences in taste and/or ingestion responses (Clarke and Ossenkopp, 1998; Curtis et al., 2004) or in reactivity to reward (Michaels and Holtzman, 2007). Indeed, other studies support the present observation that stress-induced alterations in sucrose consumption are differently expressed between male and female animals (Dalla et al., 2005, 2008; Pitychoutis et al., 2009). Regarding despair behavior (here assessed by the FST), our data, although in line with other studies [showing that females exposed to CMS cope better and present increased active behavior in the FST, whereas males are more vulnerable (Bielajew et al., 2003; Dalla et al., 2005)] are particularly intriguing because in humans, depression is more prominent in females (Frank et al., 1988; Marcus et al., 2005; Wittchen et al., 2011).

In this study we explored the effect of stress on microglia morphology in the dHIP and NAc, two key brain regions in the control of depressive-like behavior (Di Chiara et al., 1999; Nestler, 2001; Nestler and Carlezon, 2006; Bessa et al., 2013; Alves et al., 2018). Microglia are diverse in shape and function and may present phenotypic differences according to the brain region analyzed (Caetano et al., 2017; De Biase et al., 2017; De Biase and Bonci, 2019; Duarte et al., 2019; Gaspar et al., 2021) and determined by sex (Lenz et al., 2013; Caetano et al., 2017; Villapol et al., 2017; Gaspar et al., 2021). All these variables may contribute to adapted functional responses to different insults (Schwarz et al., 2012; Villapol et al., 2017; Guneykaya et al., 2018; Perkins et al., 2018; Villa et al., 2018). Thus, it is not surprising that chronic stress elicits brain region- and sex-specific alterations in microglial phenotypes that likely contribute to divergent neurobiological and behavioral responses (Hinwood et al., 2013; Kreisel et al., 2014; Milior et al., 2016; Franklin et al., 2018). In the dHIP, microglia from males are not affected by chronic stress (shorter or longer periods of exposure), while females, although requiring a longer period of exposure to stress, present hypertrophied microglia (more and longer cellular processes). In line with these results, our group described that prenatal stress exposure induces a hypertrophy of microglia in females with no differences in males (Gaspar et al., 2021). These findings are consistent with other study reporting the absence of changes in the morphology of microglia in males in the HIP following chronic stress (Lehmann et al., 2016). In the case of NAc, microglia from both sexes is affected by stress, but changes observed after 2 weeks of stress are apparently transient and no longer observed after 6 weeks of stress exposure. To our knowledge, our group described for the first time alterations in microglia morphology in the NAc after stress exposure. Recently we showed that prenatal exposure to stress induced also sex-specific alterations in microglia (atrophy in females and hypertrophy in males) (Gaspar et al., 2021). It is becoming evident that microglia morphology is robustly and differently affected by stress in different brain regions. For example, 21 days of restraint stress increased the complexity of microglia in males, enhancing ramifications in the PFC (Hinwood et al., 2013). Studies from our team have shown that prenatal stress triggers long-lasting sex differences in microglia morphology in the mPFC, dHIP, and NAc (Caetano et al., 2017; Duarte et al., 2019; Gaspar et al., 2021). Given that microglia present sexual dimorphic features, namely density, function, and morphology in several brain regions (Bilbo et al., 2012; Schwarz and Bilbo, 2012; Schwarz et al., 2012; Caetano et al., 2017; Duarte et al., 2019; Gaspar et al., 2021), some of which conserved among species (Simoes-Henriques et al., 2019), sex differences after stress are not surprising.

The morphologic adaptation of neurons to stress has been also studied by several authors. In general, it is accepted that stress induces an atrophy of neurons in the HIP (Watanabe et al., 1992; Magarinos and McEwen, 1995; McLaughlin et al., 2007; Bessa et al., 2009a; Morais et al., 2014; Patricio et al., 2015) and a hypertrophy in the NAc (Bessa et al., 2013; Melo et al., 2015). One of the main goals of this work was to analyze behavior and, in parallel, microglia and neurons morphology. Interestingly, the morphometric analysis of neurons in the dHIP revealed that these cells are morphologically not responsive to stress in the case of females, but males present an atrophic pattern after 2 weeks of stress, an effect that persists until 6 weeks of stress. In the NAc, only males present changes (conversely to the dHIP, a hypertrophy was observed), which are observable after a short protocol of stress and persist after longer periods of exposure.

In summary, neuronal changes in this brain region seem to be exclusive to males and opposite between dHIP and NAc. In

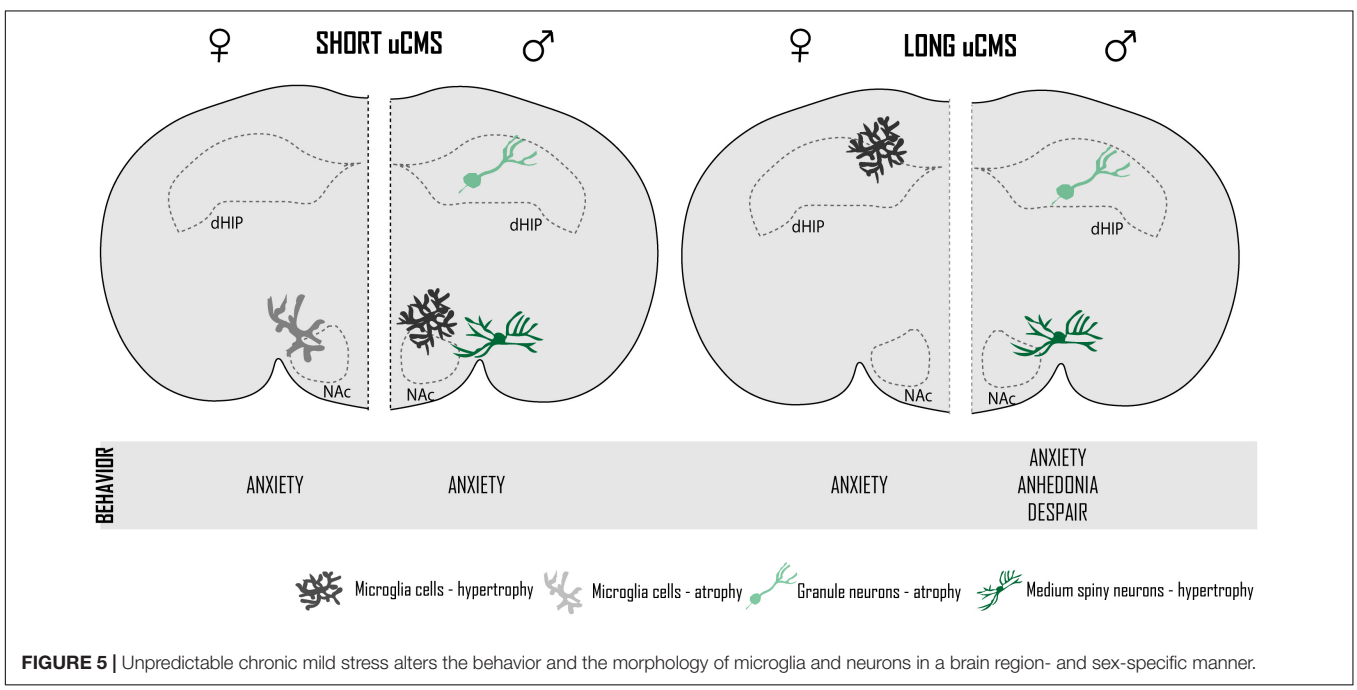

FIGURE 5 | Unpredictable chronic mild stress alters the behavior and the morphology of microglia and neurons in a brain region- and sex-specific manner.

line with our results, some studies (only performed in males) demonstrated that chronic stress caused an atrophy of neurons in the DG of HIP (Bessa et al., 2009b; Morais et al., 2014; Patricio et al., 2015) and in the mPFC (Radley et al., 2005; Shansky et al., 2009; Melo et al., 2015). Interestingly, chronic adult stress triggered a hypertrophy of medium spiny neurons in the NAc, that was associated with a depressive-like phenotype (Bessa et al., 2013; Melo et al., 2015). Thus, the NAc neuronal hypertrophy that we observed in this study can contribute for the depressive-like phenotype that is observed in males. In this framework, the lack of changes in females is in agreement with the absence of a phenotype in the SPT and FST.

CONCLUSION

The present results show that chronic stress significantly alters the behavior and the morphology of microglia and neurons in a brain region- and sex-specific manner: males are more affected by stress, presenting anxiety- and depression-like behaviors, hypertrophy of microglia, and dendritic hypertrophy in the NAc. Females present anxiety-like behavior, but no depression-like behavior, with remodeling of microglia in dHIP (hypertrophy) and NAc (atrophy) (**Figure 5**). Globally, our results show that the morphology of neurons is not affected by chronic stress in females and this morphologic stability is accompanied by a process of microglia remodeling. In the case of males, neurons are affected in both regions, but microglia seem to be only and transiently affected in the NAc. This study led us to question if microglia plasticity is related with the morphologic stability of neurons observed in females, eventually underlying stress resilience, a hypothesis that deserves further investigation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by EU-Directive 2010/63/EU and the Portuguese National Authority for animal experimentation, Direção-Geral de Animal e Veterinária (DGAV). All protocols were approved by the Ethics Committee of the Life and Health Sciences Research Institute and by DGAV (#19074).

AUTHOR CONTRIBUTIONS

RG designed the experiments with CS-C, AR, and CG, performed the experiments, and wrote the manuscript. CS-C, AR, and BC helped to perform behavioral experiments. CG and AR supervised RG, contributed to the design of the experiments, and revised the manuscript. All authors discussed the results, contributed to the article, and approved the final submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.834821/full#supplementary-material

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Sex Differences in Depression-Like Behaviors in Adult Mice Depend on Endophenotype and Strain

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Depression affects women nearly twice as frequently as men. In contrast, rodent models of depression have shown inconsistent results regarding sex bias, often reporting more depression-like behaviors in males. This sex discrepancy in rodents modeling depression may rely on differences in the baseline activity of males and females in depression-related behavioral tests. We previously showed that the baseline despair and anhedonia behaviors, major endophenotypes of depression, are not sex biased in young adolescent wild-type mice of C57BL/6N, DBA/2, and FVB/N strains. Since the prevalence of depression in women peaks in their reproductive years, we here investigated sex differences of the baseline depression-like behaviors in adult mice using these three strains. Similar to the results in young mice, no difference was found between adult male and female mice in behavioral tests measuring despair in both tail suspension and forced swim tests, and anhedonia in the sucrose preference test. We then extended our study and tested apathy, another endophenotype of depression, using the splash test. Adult male and female mice showed significantly different results in the baseline apathy-like behaviors depending on the investigated strain. This study dissects the complex sex effects of different depression endophenotypes, stresses the importance of considering strain, and puts forward a hypothesis of the inconsistency of results between different laboratories investigating rodent models of depression.

Keywords: sex difference, tail suspension test, forced swim test, sucrose preference test, splash test, C57BL/6N, DBA/2, FVB/N

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INTRODUCTION

Depression is one of the most prevalent and life-threatening neuropsychiatric disorders accompanied by a high incidence rate and severe economic burden (Hasin et al., 2018; Greenberg et al., 2021). This heritable disorder is characterized by remarkable interindividual differences in symptoms ranging from weight changes, diminished interest or pleasure in activities, sleep disturbances, feelings of worthlessness or guilt, decreased cognitive ability and recurrent suicidal thoughts (McCarter, 2008; American-Psychiatric-Association, 2013; Pu et al., 2017; Park and Zarate, 2019). It straddles all races and ethnicities and affects different age groups, with more prevalence among elderly people (Sjöberg et al., 2017; Malhi and Mann, 2018). Irrespective of

race or other possible confounding social and economic factors, women suffer from depression nearly twice as frequently as men (Weissman and Klerman, 1977; Cyranowski et al., 2000; Andrade et al., 2003; Ford and Erlinger, 2004; Patten et al., 2006; Bromet et al., 2011; Albert, 2015; Salk et al., 2017). There is evidence suggesting the contribution of biological factors, such as sex hormones, to the increased prevalence of depression in women (Albert, 2015).

Much research on depression has been undertaken, with its progress being coupled with the development of rodent models. Indeed, several features of depression have homologies in mouse and rat behaviors. Accordingly, rodent models have been developed to improve our understanding of the pathophysiological mechanisms of depression. Still, the origin of sex bias in depression is not well established. One reason is the underrepresentation of female rodents in preclinical studies of depression due to the presumption that the estrous cycles may increase the intrinsic variability. Additionally, the comparison between male and female rodent models of depression yielded inconsistent results, with some studies revealing lower depression-like behaviors in females (Alonso et al., 1991; Brotto et al., 2000; Dalla et al., 2005, 2008; Grippo et al., 2005; Brummelte et al., 2006; Chen et al., 2006; Kamper et al., 2009; Martínez-Mota et al., 2011; Bai et al., 2014; Burke et al., 2016), higher depression-like behaviors in females (Konkle et al., 2003; Bhatnagar et al., 2004; Drossopoulou et al., 2004; Pitychoutis et al., 2009, 2011; Dalla et al., 2010; Bourke and Neigh, 2011; Kokras et al., 2012; Xing et al., 2013; Hodes et al., 2015; Page et al., 2016; Goodwill et al., 2019), or no differences between males and females (Poltyrev et al., 2005; Alves et al., 2008; Olivier et al., 2008; Eltokhi et al., 2021). This inconsistency between different laboratories demands optimizing the standardization of experimental settings and consideration of confounding factors possibly causing variability in rodent behaviors. These factors include the rodent species and strain, age, tested endophenotypes, methodology of behavioral tests, and model of depression (genetic, environmental, chemical, pharmacological, etc.). While the aforementioned factors can explain the lack of reproducibility of sex differences between different laboratories, they cannot safely answer why, contrasting to humans, male rodent models often show more depression-like behaviors than their female counterparts.

In this study, we hypothesized that the baseline performance of male and female wild-type mice in depression-related behavioral tests may play a role in the sex differences in rodent models. The suggested baseline differences in performance can mask or exaggerate depression-related genetic or pharmacological-induced effects. To test this hypothesis, we investigated three depression endophenotypes in three wild-type inbred strains, C57BL/6N, DBA/2, and FVB/N, during adulthood. Accordingly, we performed the tail suspension and forced swim tests to assess despair-like behaviors, the sucrose preference test to evaluate anhedonia-like behaviors, and the splash test to measure apathy-like behaviors (Eltokhi et al., 2018; Planchez et al., 2019; Becker et al., 2021). Distinct from our previous study performed in adolescent mice before puberty (Eltokhi et al., 2021), we here used adult mice since biological maturation following puberty and clear sex-specific social roles in adults may be major factors of a sex bias of depression (Dalla et al., 2010).

Our work indicates that sex differences in the baseline depression-related behaviors are present in wild-type mice and depend on the strain and investigated endophenotype. These results may explain the inconsistency of results between laboratories experimenting on different mouse strains as well as the increased depression-like behaviors in males in some studies.

MATERIALS AND METHODS

Animals and Housing Conditions

Male and female C57BL/6N, DBA/2, and FVB/N mice were maintained at the Interdisciplinary Neurobehavioral Core at Heidelberg University as previously described (Peleh et al., 2019). Male and female mice were housed separately in groups of three per cage with free access to food and water under a standard 12-h light/dark cycle (7:00 p.m.–7:00 a.m.) with a regulated temperature of 22°C and at a relative humidity of 40–50%. Behavioral tests were conducted on 14-weeks old male and female mice. Notably, we used new mouse cohorts different from the ones investigated in our previous study performed during adolescence (Eltokhi et al., 2021) to avoid the familiarity of mice with the behavioral tests. The experiments were conducted in strict compliance with national and international guidelines for the Care and Use of Laboratory Animals and carried out following the ARRIVE guidelines. The animal ethic committee of the (Regierungspräsidium Karlsruhe) Government of Baden Württemberg approved the study (G-101/16).

Experimental Design

All behavioral tests were performed during the daylight cycle. Mice were habituated to the behavioral room for half an hour before the start of the tests. We started the behavioral test battery with the tail suspension test. Starting the same day, we performed the sucrose preference test for 4 consecutive days. On day 5, the forced swim test was carried out. Mice were allowed to rest for 3 days before performing the splash test on day 9.

The Behavioral Test Battery

The Tail Suspension Test

The test started by suspending the mouse to a rod by its tail with adhesive tape at 60 cm above the surface. The behavior of the mouse was videotaped, and the latency to first immobility and immobility duration within 6 min were scored manually by an independent observer.

The Forced Swim Test

The test started by placing the mouse in a glass cylinder (26 cm in height, 16 cm in diameter) filled with water (24 ± 1°C, 20 cm in height). The level of water was sufficient to allow the mouse to swim or float without its hind limbs or tail touching the bottom of the cylinder. The swimming path was tracked *via* a top-mounted video camera connected to proprietary high-resolution tracking software (SYGNIS tracker 3 v4.1.14.). The

video-based tracking system was detecting the change in pixel level. A constant illumination of 40–42 lux has been set for all behavior measurements. The total duration of the test was 6 min, and the immobility duration between 2 and 6 min was measured. Immobility was defined as a lack of swimming with only minimal movement of one hindlimb that was necessary to keep the head above water.

The Sucrose Preference Test

On day 1, each single-housed mouse was left in its homecage with two water bottles. On day 2, both bottles were changed with a bottle filled with water and a second one filled with a 1% sucrose solution. Both bottles were weighed before placing them into the cage. On day 3, bottles were weighed to determine the liquid consumption during the previous 24 h. Bottles were then refilled, weighed and placed into the cage with an alternated position of the sucrose vs. water bottle to avoid place preference. On day 4, bottles were weighed. The sucrose preference index was calculated as the average consumed sucrose across the last 2-day period divided by the average volume of total consumed liquid (average water plus average sucrose solution).

The Splash Test

The test started with squirting a 10% sucrose solution on the dorsal coat of the mouse in its home cage. Due to the high viscosity of the sucrose solution, it dirtied the mouse fur, which induced a grooming behavior. After applying the sucrose solution, the latency of first grooming and total durations of grooming were assessed for 5 min as an indication of self-care and motivation.

Statistical Analysis

Two-way ANOVA was used with sex and genotype as the two factors. This was followed by Tukey's *post-hoc* test for multiple comparisons to determine differences between the three strains C57BL/6N, DBA/2, and FVB/N and Bonferroni correction to check differences between males and females within each strain. Statistical analysis was performed using GraphPad Prism 7 and Microsoft Office Excel.

RESULTS

Baseline Despair-Like Behaviors in Male and Female C57BL/6N, DBA/2, and FVB/N Mice

We tested the sex difference in the baseline despair-like behaviors in three inbred strains, C57BL/6N, DBA/2, and FVB/N, using the well-known tail suspension (Steru et al., 1985) and forced swim tests (Porsolt et al., 1977; Porsolt, 1997). In the tail suspension test, one FVB/N and two DBA/2 mice climbed up their tails and ran away from the adhesive tape and were excluded from all behavioral tests. Male and female mice within each strain exhibited similar performance in both tail suspension and forced swim tests [Immobility duration in tail suspension test: $F_{(1,57)} = 1.11$, $P = 0.2964$; Latency to immobility in tail suspension test: $F_{(1,57)} = 1.021$, $P = 0.3165$; Immobility duration in forced swim test: $F_{(1,57)} = 0.02646$, $P = 0.8713$; Latency to immobility in forced swim test: $F_{(1,57)} = 0.01275$, $P = 0.9105$; Traveled distance: $F_{(1,57)} = 0.2099$, $P = 0.6486$] (**Figure 1**). The comparison between strains in the tail suspension test revealed a significant increase in the baseline immobility duration of adult C57BL/6N compared to both DBA/2 and FVB/N mice [$F_{(2,57)} = 73.78$, $P < 0.0001$; Interaction between strain and sex: $F_{(2,57)} = 0.2436$, $P = 0.7846$] (**Figure 1A**). On the other hand, the latency to first immobility was higher in adult DBA/2 than C57BL/6N and FVB/N mice [$F_{(2,57)} = 49.51$, $P < 0.0001$; Interaction between strain and sex: $F_{(2,57)} = 0.08017$, $P = 0.9231$] (**Figure 1B**). In the forced swim test, adult C57BL/6N mice showed a higher immobility duration and lower latency to first immobility as well as a decreased total traveled distance compared to adult DBA/2 and FVB/N mice [Immobility duration: $F_{(2,57)} = 16.83$, $P < 0.0001$; Interaction between strain and sex: $F_{(2,57)} = 1.616$, $P = 0.2076$; Latency to first immobility: $F_{(2,57)} = 22.5$, $P < 0.0001$; Interaction between strain and sex: $F_{(2,57)} = 1.903$, $P = 0.1585$; Traveled distance: $F_{(2,57)} = 31.13$, $P < 0.0001$; Interaction between strain and sex: $F_{(2,57)} = 3.041$, $P = 0.0556$] (**Figures 1C–E**).

Baseline Anhedonia- and Apathy-Like Behaviors in Male and Female C57BL/6N, DBA/2, and FVB/N Mice

Considering anhedonia-like behaviors, male and female C57BL/6N, DBA/2, and FVB/N mice showed similar performance in the sucrose preference test [$F_{(1,57)} = 0.05388$, $P = 0.8173$] (**Figure 2A**). The comparison between these strains revealed a lower sucrose preference index in DBA/2 compared to C57BL/6N and FVB/N mice, suggesting an increase in the intrinsic anhedonia-like behaviors in the DBA/2 strain [$F_{(2,57)} = 64.06$, $P < 0.0001$; Interaction between strain and sex: $F_{(2,57)} = 0.08282$, $P = 0.9206$] (**Figure 2A**). In the splash test that assesses apathy-like behaviors, C57BL/6N and FVB/N strains showed significant sex differences. Interestingly, this sex effect depended on the strain, with male FVB/N mice showing a decrease in the duration of grooming and increased latency to the first grooming compared to their female littermates, while male C57BL/6N mice exhibited increased duration of grooming compared to females [Interaction between strain and sex in grooming duration: $F_{(2,57)} = 10.61$, $P = 0.0001$; interaction between strain and sex in the latency of grooming: $F_{(2,57)} = 2.817$, $P = 0.0481$] (**Figures 2B,C**). The comparison between strains revealed a decrease in the duration of grooming in FVB/N compared to C57BL/6N and DBA/2 mice [$F_{(2,57)} = 22.49$, $P < 0.0001$] (**Figure 2B**). On the other hand, C57BL/6N mice showed a decreased latency to first grooming compared to DBA/2 and FVB/N mice [$F_{(2,57)} = 16.76$, $P < 0.0001$] (**Figure 2C**).

DISCUSSION

Depression is a complex non-homogenous pathology with a wide range of core and additional symptoms as well as other comorbidities such as anxiety and social withdrawal (Hasler et al., 2004). This complexity is recapitulated in rodent models of depression by several distinct endophenotypes including

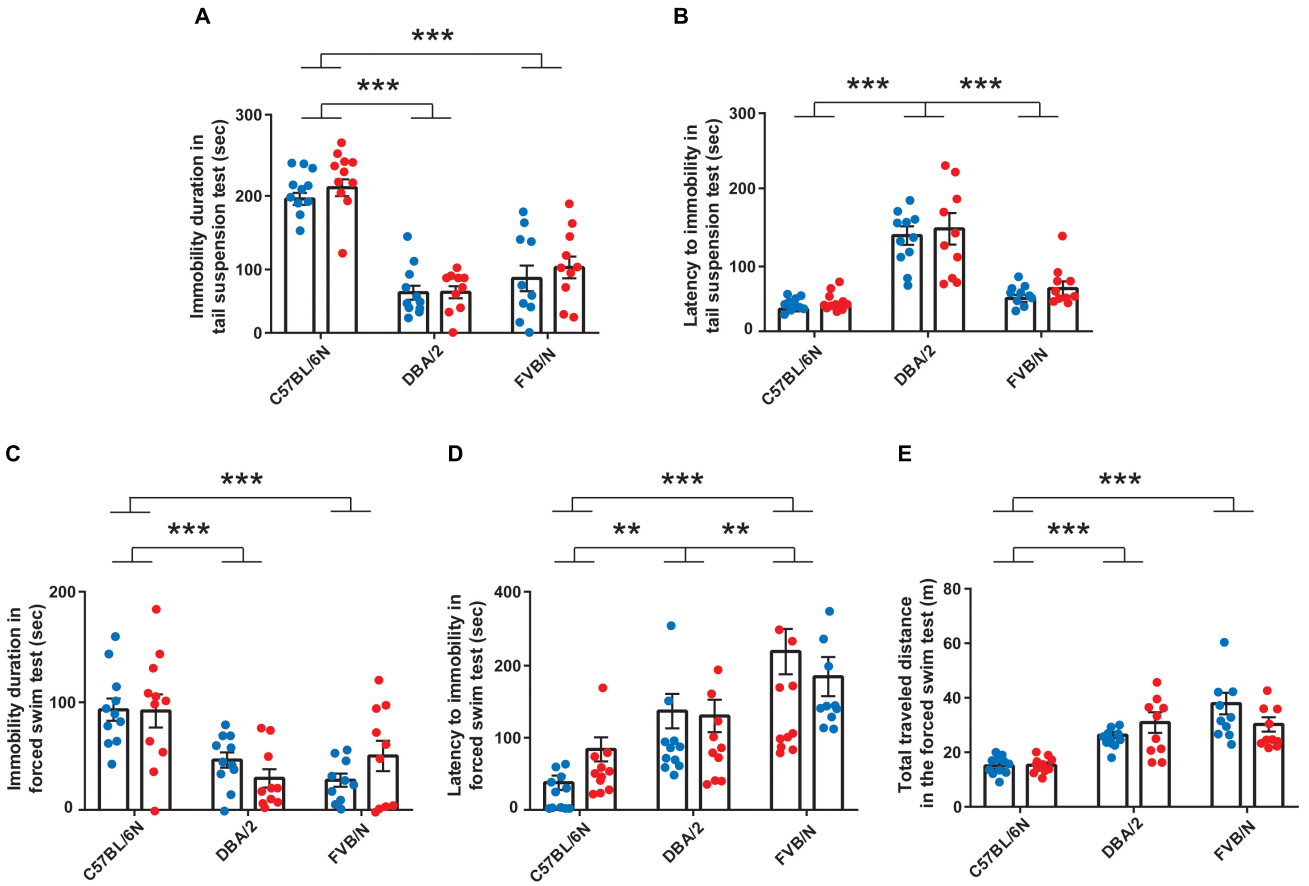

FIGURE 1 | Baseline despair-like behaviors in adult C57BL/6N, DBA/2, and FVB/N mice. **(A)** In the tail suspension test, adult C57BL/6N mice exhibited a higher duration of baseline immobility than DBA/2 and FVB/N mice. **(B)** Adult DBA/2 mice showed a significantly increased latency to first immobility compared to C57BL/6N and FVB/N mice. In the forced swim test, adult C57BL/6N mice showed a higher immobility duration **(C)**, a lower latency to first immobility **(D)** and a lower total traveled distance **(E)** than DBA/2 and FVB/N mice. In panels **(A–E)**, male and female mice within the aforementioned strains showed similar performance in the tail suspension and forced swim tests. Blue and red dots represent males and females, respectively. Two-way ANOVA was used followed by the Tukey's *post-hoc* test for multiple comparisons to determine differences between the three strains, C57BL/6N, DBA/2, and FVB/N (**$p \leq 0.01$, ***$p \leq 0.001$) and Bonferroni correction to check differences between males and females within each strain. Error bars indicate the standard error of the mean (SEM).

despair-, anhedonia- and apathy-like behaviors, which can be assessed using different behavioral tests. In contrast, other depression symptoms like feelings of worthlessness, guilt and suicidal attempts are difficult to be tested in rodents, which limits achieving a complete face validity of rodent models of depression and hinders a full correlation to the clinical condition of individuals with depression (Planchez et al., 2019).

The inconsistent sex differences in the behaviors of rodent models added another layer of complexity in reaching a complete face validity of depression. Several studies in rodents failed to recapitulate the increased susceptibility of women to depression, with some studies revealing even lower depression-like behaviors in female rodents [for a review, see Kokras and Dalla (2014)]. More importantly, contradicting findings between different studies on the sex effect on the behavioral performance of rats were observed in the forced swim and sucrose preference tests (Kokras and Dalla, 2014). Multiple factors may have jointly promoted this inconsistency including different genetic backgrounds, protocols of behavioral tests, models of depression (genetic, environmental, chemical, pharmacological, etc.) and the age of tested rodents. Additionally, small differences in testing conditions can affect the results in the forced swim test including differences in tank dimensions, temperature, water depth, number of sessions, housing, etc. (Ma et al., 2018). Generally, the lack of observing consistent increased depression-like behaviors in female rodents compared to males may point out that some specific behavioral paradigms in rodents are not strictly equivalent to the clinical phenotype of individuals with depression. For example, the transition from swimming to immobility in the forced swim test may not be a measure of despair but rather a coping mechanism with inevitable situations (Molendijk and De Kloet, 2019). However, the persistence of coping with inescapable stressors in the forced swim test may still be a measure of an ultimate vulnerability to depression. Despite the use of the sucrose preference test for assessing anhedonia-like behaviors, the situation is more complex

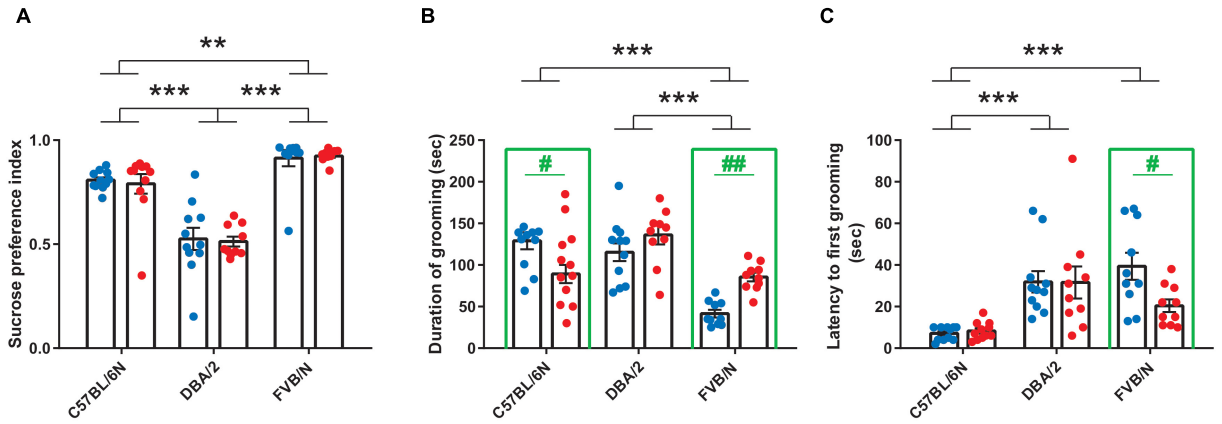

FIGURE 2 | Baseline anhedonia- and apathy-like behaviors in adult C57BL/6N, DBA/2 and FVB/N mice. **(A)** In the sucrose preference test, adult DBA/2 mice showed increased baseline anhedonia-like behaviors by having a lower sucrose preference index than C57BL/6N and FVB/N mice. Additionally, FVB/N mice showed a higher sucrose preference index than C57BL/6N mice. **(B)** The splash test revealed a decreased grooming duration in FVB/N compared to C57BL/6N and DBA/2 mice. **(C)** C57BL/6N mice exhibited a decreased latency to first grooming in the splash test compared to DBA/2 and FVB/N mice. In panel **(A)**, no sex difference was revealed in the sucrose preference test within any of the aforementioned strains. In panel **(B)**, male FVB/N mice showed a decreased duration of grooming, while male C57BL/6N mice showed an increase in the duration of grooming compared to their female littermates. In panel **(C)**, male FVB/N mice showed an increased latency to first grooming compared to their female littermates. Blue and red dots represent males and females, respectively. Two-way ANOVA was used followed by the Tukey's *post-hoc* test for multiple comparisons to determine differences between the three strains C57BL/6N, DBA/2, and FVB/N (**$p \leq 0.01$, ***$p \leq 0.001$) and Bonferroni correction to check differences between males and females within each strain (in green: #$p \leq 0.05$, ##$p \leq 0.01$). A green rectangle indicates a significant difference between sexes within a strain. Error bars indicate the standard error of the mean (SEM).

regarding sadness in depression (Planchez et al., 2019). Apathy-relevant behavioral tests such as splash test and nest building can also be used to assess other behaviors including self-care and social interaction, respectively. Therefore, the results obtained from behavioral tests in rodents should be taken with caution before a direct correlation with complex human behaviors. Notably, the symptoms of patients with depression are very heterogeneous with a high degree of within-disorder variability (Olbert et al., 2014), which may complicate modeling the sex bias of depression in rodents. Another possibility for the discrepancy between rodents and humans in the sex bias of depression is the development of currently used behavioral tests in male rodents, and thus, the results of females may not accurately tap into feminine attributes.

Sex differences in the behaviors of depression in rodent models may be partially induced by a difference in the general and baseline performance of male and female mice. In a previous study, we tested this possibility for despair and anhedonia in three wild-type inbred mouse strains, C57BL/6N, DBA/2, and FVB/N during adolescence. Baseline depression-like behaviors were strain and age dependent, but no sex effect was seen in adolescent mice (Eltokhi et al., 2021). In the current study, we tested the sex difference of depression-like behaviors in adult mice since the increased prevalence of depression in women in their reproductive years suggests that biological maturation following puberty can be a major factor in the sex bias of depression. Additionally, we extended our study using the splash test to evaluate apathy, another endophenotype of depression.

In adult mice, the baseline depression-like behavior showed sex-related effects in C57BL/6N and FVB/N mice in apathy-, but not despair- or anhedonia-relevant behavioral tests. Having investigated only three mouse strains, testing other strains is mandatory to confirm a correlation between strains and specific endophenotypes. Indeed, looking at apathy-like behaviors in the splash test, sex effects were opposite in C57BL/6N and FVB/N strains, with decreased grooming durations in male FVB/N and female C57BL/6N mice, highlighting the high intercorrelation between sex and strain in depression-related behaviors. Even though these results in adult mice complicate the investigation of sex bias in depression in rodent models, accepting this additional complexity is unavoidable. In fact, the apparent sex effect in a specific endophenotype may suggest as well a sex bias in specific symptoms of depression in humans. Gotland's studies postulated male depressive syndrome proposing that men show symptoms of depression different from common depression symptoms among women, which can be assessed by the Gotland Scale of Male Depression (GSMD) (Rutz et al., 1995; Rutz, 1999). These male symptoms include but are not limited to irritability, anger attacks, aggression and alcohol use, which may mask the diagnosis of depression in men and can be the reason for their high suicidal rate (Möller-Leimkühler, 2003; Oquendo et al., 2003). However, the specificity of this male depressive syndrome is still disputed, and remains to be answered whether GSMD, specifically devised for the diagnosis of depression in men, is a reliable screening (Zierau et al., 2002; Möller-Leimkühler et al., 2004; Möller Leimkühler et al., 2007; Rihmer et al., 2009; Innamorati et al., 2011). To this end, whether some depression symptoms in humans show a male/female bias is still an open question.

Strain differences in depression-like behaviors in rodents have been previously reported (Vaugeois et al., 1997; Liu and Gershenfeld, 2001; Lucki et al., 2001). In our study, C57BL/6N

mice showed increased immobility durations and decreased latencies to first immobility compared to DBA/2 and FVB/N mice in both tail suspension and forced swim tests. In the sucrose preference test assessing anhedonia-like behaviors, DBA/2 mice showed the lowest sucrose preference index, which may mask the depression-like behavior in mouse models of neuropsychiatric disorders. Adult mouse strains showed a similar pattern of differences to that of adolescent mice (Eltokhi et al., 2021). This suggests that the effect of genetic differences in strains exceeds that of age differences, which is in line with the results of comparing mouse strains at distinct developmental stages during adolescence (Eltokhi et al., 2021).

One limitation of our study is the lack of examination of females' estrous cycle phases and their effect on the behavioral outcome. Previously, the estrous cycle was assumed to cause an intrinsic variability in female rodents, which resulted in an underrepresentation of females in behavioral tests (Meziane et al., 2007). On the other hand, a meta-analysis reported a comparable variability in male and female mice in several behavioral assays (Prendergast et al., 2014). For depression-relevant behavioral tests, the effect of the estrous cycle of rats on the behavioral outcome in the forced swim test was not conclusive (Kokras et al., 2015). Out of 22 studies between 1990 and 2013, 12 studies revealed no effect of the estrous cycle on behavioral performance. The other 10 studies showed some effects with questionable power and opposite results, suggesting a little influence of the estrous cycle of naturally cycling females. The different effects of the estrous cycle between studies is probably related to different methodological approaches and factors influencing the behavioral responses as suggested in Kokras et al. (2015). Ideally, female rodents in the normal distribution of all phases of the estrous cycle should be used to avoid the chance of over-representation of a particular phase causing skewed results and wrong interpretations of the data. However, this approach will ultimately increase the number of tested rodents in each behavioral study. Several methods of evaluating the estrous cycle are used including visual assessment, vaginal cytology, histological exa mination of the reproductive organs, vaginal wall impedance, and urine biochemistry [for a review, see Ajayi and Akhigbe (2020)]. Although visual assessment of the female estrous cycle is simple, cheap and less stressful to animals, it may cause more handling of female mice than males, which is known to affect the behavioral outcome including the immobility of rodents in the forced swim test (Cannizzaro et al., 2002).

CONCLUSION

In conclusion, the sex difference in the baseline depression-like behaviors in adult mice depends on the investigated endophenotype and strain. These effects can mask or exaggerate the behavioral outcomes in rodent models of depression and may explain the poor data reproducibility of different studies. Thus, the intercorrelation between the investigated endophenotype, strain and sex requires caution when comparing the behavioral results between different laboratories. Additionally, the proper choice of behavioral tests assessing specific endophenotypes should be based on a profound knowledge of behavioral genetics and the specific goals of the study. As a rule, several behavioral tests covering different endophenotypes of depression should be used in characterizing new mouse models of neuropsychiatric disorders. We also urge researchers to standardize sources of variability including using the same apparatuses, standardized operating protocols and testing conditions for better reproducibility of behavioral outcomes. Since the handling of rodents affects their baseline behavior, automated methods using, for example, video tracking systems with minimum handling of rodents may play a role in increasing the reproducibility of results. Ultimately, the optimized characterization of sex differences in the established rodent models of depression will pave the way to decipher the sex-specific mechanisms of depression and further develop sex-specific therapeutics.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of the Government of Baden Württemberg (G-101/16).

AUTHOR CONTRIBUTIONS

CP: conceptualization, project administration, supervision, validation, and review and editing. BK: methodology, data curation, and formal analysis. AE: conceptualization, data curation, formal analysis, project administration, supervision, validation, visualization, and writing original draft, review and editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Sex-Specific Brain Transcriptional Signatures in Human MDD and Their Correlates in Mouse Models of Depression

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Major depressive disorder (MDD) is amongst the most devastating psychiatric conditions affecting several millions of people worldwide every year. Despite the importance of this disease and its impact on modern societies, still very little is known about the etiological mechanisms. Treatment strategies have stagnated over the last decades and very little progress has been made to improve the efficiency of current therapeutic approaches. In order to better understand the disease, it is necessary for researchers to use appropriate animal models that reproduce specific aspects of the complex clinical manifestations at the behavioral and molecular levels. Here, we review the current literature describing the use of mouse models to reproduce specific aspects of MDD and anxiety in males and females. We first describe some of the most commonly used mouse models and their capacity to display unique but also shared features relevant to MDD. We then transition toward an integral description, combined with genome-wide transcriptional strategies. The use of these models reveals crucial insights into the molecular programs underlying the expression of stress susceptibility and resilience in a sex-specific fashion. These studies performed on human and mouse tissues establish correlates into the mechanisms mediating the impact of stress and the extent to which different mouse models of chronic stress recapitulate the molecular changes observed in depressed humans. The focus of this review is specifically to highlight the sex differences revealed from different stress paradigms and transcriptional analyses both in human and animal models.

Keywords: stress, rodents, sexual dimorphism, resilience, susceptibility, behavioral stress responses, transcription profiles/signatures

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INTRODUCTION

Major depressive disorder (MDD) represents one of the top causes of disability worldwide (Vos et al., 2017). Recent studies estimate that more than 20% of the population worldwide will be affected at least once in their life by depressive episodes which ultimately translates into a major burden on modern societies (Alonso et al., 2004). Despite the importance of the disease, little progress has been made in understanding the etiologies of MDD. However, recent progress with fast-acting antidepressant molecules shows promising perspectives in the treatment of this disorder (Berman et al., 2000; Thelen et al., 2016; Mandal et al., 2019; Polis et al., 2019; Ouyang et al., 2021).

From a clinical perspective, MDD is a highly heterogenous disease defined by complex clinical manifestations including depressed mood or irritability, anhedonia, grief, guilt, apathy, self-injury, indecision, and concentration disorders. This also includes psychomotor retardation, vegetative symptoms with sleep, appetite, and stress hormone dysregulation that is associated with either gain or loss of weight, suicidal ideation, and cognitive disorder (American Psychiatric Association, 2003). These clinical features are expressed and shared by both men and women with MDD despite important sex differences (Weissman and Klerman, 1977; Nolen-Hoeksema, 1987; Salk et al., 2017; Eid et al., 2019). Past and recent epidemiological studies show that the prevalence of MDD is about 20% in a lifetime with a higher incidence in women, and females are two-three times more susceptible than males. Women also exhibit earlier age of onset (Kessler et al., 1993), higher symptom severity from childhood (Kessler et al., 2007; Marcus and Flynn, 2008; McLean et al., 2011; Avenevoli et al., 2015), and higher rates of depressive episodes (Bertschy et al., 2016) than men. At the clinical level, men and women diagnosed with MDD express more or less the same symptoms although their prevalence varies in a sex-specific fashion. For instance, aggression, substance abuse, and risk-taking behaviors are more prevalent in males (Martin et al., 2013), while women with MDD exhibit higher rates of comorbid anxiety (Regier et al., 1990; Kessler et al., 1994; Schuch et al., 2014). A higher prevalence of atypical depression is also observed in women. In men and women, this is defined by the expression of reactive mood to environmental cues, increased appetite, hypersomnia, leaden paralysis, and interpersonal rejection sensitivity. While MDD in women is defined by a higher prevalence of internalized disorders such as ruminating and emotionality, externalized symptoms are more common in men including constraint and aggressive behavior (Krueger et al., 2001).

Crucial insights into the molecular and functional mechanisms underlying differences between males and females with MDD have resulted from studies performed in human populations or post-mortem tissue, some of which have forged our pathophysiological conception of the disease (Rajkowska, 2003; Tham et al., 2011; Zhao et al., 2019). For instance, studies have revealed functional, morphological, and molecular changes affecting the activity of several brain regions in MDD (Frodl et al., 2008; Ramezani et al., 2014; Lu et al., 2016; Li et al., 2021). These studies alone have generally provided limited mechanistic insights into the pathophysiological processes underlying the expression of the disease. Mechanistic insights have also been obtained using animal models of stress or depressive-like behaviors. Indeed, past decades have seen the development of several animal models of stress-induced depressive-like behaviors. These models have mostly been developed based on McKinney and Bunney's criteria (McKinney and Bunney, 1969) of external validity that was later refined by Willner (Willner, 1984, 1991) as predictive, face, and construct validity. This has led to the development of a wide variety of mouse models based on physical, psychosocial, and/or genetic paradigms, each reproducing common and distinct aspects of stress and anxiety-like behaviors in humans (Deussing, 2006; Abelaira et al., 2013; Planchez et al., 2019).

With technological developments to map transcriptional profiles induced by different types of stress, these models provide unique insights into the transcriptional programs that underly the expression of complex behavioral phenotypes in MDD. Importantly, by combining human and mouse data, these studies are now providing highly translational insights into the morphological and functional impact of stress and the function of the brain while highlighting some of the molecular mechanisms underlying these effects (aan het Rot et al., 2009; Duman and Voleti, 2012; Penninx et al., 2013). However, most of the research on this topic has been performed in males, predominantly leaving females understudied for years. Several of the most widely used mouse models of stress and anxiety were originally developed in males; only very recently have the models been revisited to include female cohorts (Lopez and Bagot, 2021). This will provide new opportunities to better understand the common but also distinct mechanisms underlying the development and expression of anxiety and depressive-like behaviors in males and females.

In this review, we first elaborate on the behavioral features exhibited by mouse models of stress with an emphasis on their respective validity in both males and females. We then discuss the most recent findings generated by genome-wide transcriptional studies in both human and mouse models. We also review the main findings that describe the transcriptional impact of different types of chronic stress in males and females. Along these lines, we draw important parallels with findings from studies in humans with MDD to evaluate the capacity of these models to reproduce the transcriptional signatures associated with the expression of the human disease in a sex-specific fashion.

ANIMAL MODELS OF DEPRESSION

The clinical heterogeneity of MDD and anxiety has always represented a challenge in selecting appropriate mouse models. According to McKinney and Bunney (1969), animal models should mimic the human condition (face validity), be relevant to human pathological mechanisms (construct validity), and demonstrate drug efficacy (predictive validity). In this context: (1) face validity refers to the capacity of a model to reproduce the phenomenological, behavioral, anatomical, or phenotypic properties observed in human patients; (2) construct validity refers to the stress paradigm (psychosocial, physical, etc.) to explain theoretically what humans experience in real life; and (3) predictive validity refers to the capacity of pharmacological or non-pharmacological treatments to rescue anxiety and depressive-like behaviors as it would in humans (Willner, 1984, 1991; McKinney, 2001; Willner and Mitchell, 2002; Nestler and Hyman, 2010). Additional features have since then been included in these criteria including mechanistic (common underlying mechanisms in humans and animals), homological (adequate species and strains), and pathogenic (challenges triggering the expression of the pathological state) validity (Belzung and Lemoine, 2011). In the following section, we start by describing

some of the most widely used mouse models of anxiety- and depressive-like behaviors to evaluate their respective capacity to achieve high levels of validity in both males and females.

Chronic Unpredictable/Variable Stress

Models of stress based on the administration of physical stressors refer to the idea that low levels of chronic and unpredictable physical stress, mimicking daily-life stress exposure in humans, trigger the expression of anxiety and depressive-like behaviors in individuals (Kendler et al., 1998, 1999; Haroon et al., 2012). Indeed, clinical and epidemiological studies report that mild but repeated stressors throughout life increase vulnerability to anxiety and depression in men and women (Kessler et al., 1985). Examples of these models that rely on the repeated administration of physical stressors include chronic mild stress (CMS; Katz et al., 1981; Katz, 1982; Forbes et al., 1996; Willner, 2016), chronic unpredictable stress (CUS; Monteiro et al., 2015), chronic unpredictable mild stress (CUMS; Frisbee et al., 2015; Burstein and Doron, 2018), and chronic variable stress (CVS; Willner et al., 1992; Hodes et al., 2015; Labonté et al., 2017) models. CUMS/CMS involves continuous (6–8 weeks) unpredictable exposure to stressful stimuli including wet cage, damp bedding, bedding removal, cage tilt, alterations of light/dark cycle, shallow water bath, restraint, and predator sounds/smells (Katz et al., 1981; Willner, 2016). CVS involves daily exposure to mild foot shocks, tail suspension, or tube restraint for 3 weeks (LaPlant et al., 2009; Hodes et al., 2014; Labonté et al., 2017). Importantly, each model induces a complex phenotype defined by anxiety, behavioral despair, and anhedonia in both males and females. Additionally, subchronic CVS (sCVS), consisting in exposing mice to 6 days of stress rather than 21 days, has been shown to induce an anxiety and depressive-like phenotype in females but not males, mimicking variations in stress susceptibility in both sexes (Hodes et al., 2015; Fatma and Labonté, 2019). The chronic restraint stress (CRS) has often been used as an alternative to CUMS or CVS. However, the nature of the paradigm, along with the type of behavioral consequences induced by CRS, challenges its construct and face validity criteria. Males seem to respond to CRS in a time-dependent manner (Selye, 1976; Beck and Luine, 1999, 2002; Gomez et al., 2012; Gomez, 2013), which confirms the allostatic load concept (McEwen and Stellar, 1993), while females demonstrate a resilient phenotype (Bowman et al., 2001; Bowman and Kelly, 2012). Chronic treatment with antidepressants reverses these depressive-like phenotypes (Stone et al., 1984; Ulloa et al., 2010; Yu et al., 2012).

Learned Helplessness

Learned helplessness is a model in which animals are exposed to unpredictable stress, after which they develop behavioral deficits in escaping aversive situations. Subjecting mice to situations in which they have no control (e.g., electroshocks) results in motivational, cognitive, and emotional deficits (Abramson et al., 1978). The behavioral deficits induced by learned helplessness are characterized by anxiety, anhedonia, and behavioral despair in males and females (Caldarone et al., 2000; Anisman and Merali, 2001; Chourbaji et al., 2010) that can be reversed by the administration of fast-acting antidepressants drugs (Ramaker and Dulawa, 2017). Additionally, not all mice in this model display helplessness (22%), with a high percentage (78%) exhibiting resilience regardless of the mice's sex (Kim et al., 2016), further supporting the face validity. However, it should be noted that controversial aspects restrict its usage (Teasdale, 1978). Indeed, it has been suggested that learned helplessness may rely on the motivation to avoid aversive challenges (Maier et al., 1976; Dweck and Wortman, 1982; Kuhl, 1984), rather than inducing a robust emotional response (Beck, 1967, 1987; Abramson et al., 1989; Rose and Abramson, 1992; Possel and Thomas, 2011; Liu et al., 2015). Even though this model reproduces certain behavioral aspects of anxiety and depression in humans, further validation is required to truly reproduce the emotional responses associated with anxiety and MDD in men and women.

Social Isolation

Psycho-social stress refers to any situation that threatens the psychological need of being affiliated with others and to maintain social self (Cannon, 1932). This can range from social evaluation of performance achievement to social devaluation such as bullying (Björkqvist, 2001; Silver and Teasdale, 2005; Brunstein Klomek et al., 2007; Nedg et al., 2011; Vinkers et al., 2014). In animals, this concept has been modeled by different approaches but mainly through prolonged social isolation (SI; Panksepp et al., 1991). SI has a high construct validity and is highly relevant to the study of human depression and anxiety disorders (Costello and Kendrick, 2000; Heinrich and Gullone, 2006; Wallace et al., 2009). SI also achieves good face validity from a behavioral perspective. For instance, losing a partner or chronic SI induces the expression of depressive-like behaviors in monogamous prairie voles, notably anhedonia, with females being more sensitive to isolation (Grippo et al., 2007). Prolonged SI also induces sex-specific depressive and anxiety-like behaviors such as despair, compulsive and obsessive behaviors, and cognitive defects in a wide range of species including mice, rats, flies, birds, and monkeys (Mercier et al., 2003; Cacioppo et al., 2006; Nonogaki et al., 2007; Apfelbeck and Raess, 2008; Cacioppo and Hawkley, 2009; Han and Richardson, 2010; Makinodan et al., 2012; Amiri et al., 2014; Hom et al., 2017; Tan et al., 2019; Rogers et al., 2020). Interestingly, rather than inducing social avoidance, socially isolated mice have been reported to interact more with their congeners (Lefebvre et al., 2020). Furthermore, when returned to social groups, the behavioral alterations induced by SI are rapidly rescued by social interactions (Zhao et al., 2021). Nonetheless, several molecular alterations that reproduce the human condition have been reported in socially isolated animals, further supporting the face validity of this model. Yet, most of these studies have been performed in males (Lu et al., 2003; Liu et al., 2012; Siuda et al., 2014; Cole et al., 2015; Ieraci et al., 2016).

Chronic Social Defeat Stress

The chronic social defeat stress (CSDS) animal model reproduces the context of bullying and excessive competitive behaviors in a social environment. In humans, these stressors are strongly associated with a significant increase in adverse mental

health consequences and elevated suicide rates (Meltzer et al., 2011). CSDS involves submitting a mouse, either male or female, to repeated bouts of physical subordination followed by prolonged sensory stressors (odor, vocalization, intimidation) without physical contact (Berton et al., 2006; Golden et al., 2011; Harris et al., 2018). By design, it represents a model combining physical and psychosocial bases. Given that male mice are naturally not aggressive with female congeners, protocol adaptations have been proposed to study the impact of chronic social stress in females. One involves luring the resident male by masking the females' scent and pheromones (Harris et al., 2018). Consequently, the resident males impose repeated bouts of physical aggression on the intruder females. Another approach involves triggering aggressive behaviors in resident male mice by chemogenetically activating the ventromedial hypothalamus. This results in prolonged aggressive behaviors toward female intruders (Takahashi et al., 2017). Interestingly, male and female mice that endure CSDS develop phenotypes of susceptibility or resilience to social stress. This confirms the high levels of face validity. Susceptibility to social stress in both sexes is defined by the expression of social withdrawal, anhedonia, anxiety, behavioral despair, cognitive impairments, and metabolic alterations (Takahashi et al., 2017; Harris et al., 2018). In contrast, resilient animals do not express social withdrawal nor anhedonia but exhibit anxiety-like behaviors (Krishnan et al., 2007; Golden et al., 2011; Takahashi et al., 2017; Harris et al., 2018). Importantly, susceptibility-related behavioral deficits can be rescued by the administration of conventional and fast-acting antidepressant molecules supporting the predictive validity of this model in both males and females (Hare et al., 2017; Hashimoto, 2019).

It should be noted that susceptibility and resilience to social stress are greatly influenced by the mouse's genetic background (Goyens and Noirot, 1975; Kudryavtseva and Bakshtanovskaya, 1989; Kudryavtseva, 1994; Fuchs et al., 2001; Berton et al., 2006; Huhman, 2006; Miczek et al., 2008; Golden et al., 2011; Laine et al., 2018). The original CSDS protocol (Berton et al., 2006; Golden et al., 2011) was designed with the C57BL/6J mouse strain and reported a rate of resilience to social stress around 30% to 40% (Berton et al., 2006; Golden et al., 2011). However, studies that compared different inbred mouse strains reported varying proportions, with 23% of BALB/c, 19% of 129, and 5% of D2 mouse strains being resilient to CSDS (Dadomo et al., 2011; Razzoli et al., 2011; Savignac et al., 2011; Laine et al., 2018). Together, this suggests that the genetic background in mice has an important impact on the coping strategies with social stress, and more work should be performed with male and female mice to test whether the same conclusions stand.

Vicarious Chronic Social Defeat Stress

Interestingly, CSDS paradigm variations are now used to study the impact of witnessing social defeat in mice. The vicarious CSDS model (Warren et al., 2013; Sial et al., 2016; Iñiguez et al., 2018) consists of having mice witnessing conspecifics during repeated bouts of social defeat. As such, it relies on emotional and psychological stressors with an important social component. The model induces a variety of behavioral alterations including decreased social interaction, anxiety, weight loss, and increased corticosterone levels (Warren et al., 2013; Qi et al., 2022) expressed in a transient but also prolonged fashion. Similar to the CSDS model, susceptible and resilient phenotypes are also produced. Antidepressant treatments improved the depressive-like behaviors (Savignac et al., 2011; Yoshioka et al., 2022).

Social Instability Stress

Another model with a strong psychosocial component is the social instability stress (SIS) model (Schmidt et al., 2008; Green and McCormick, 2013; Scharf et al., 2013; Yohn et al., 2019) where, male and female mice are exposed to unstable social hierarchies every 3 days for 7 weeks, and results in the expression of depressive- and anxiety-like behaviors. Anhedonia is a striking feature of the SIS model while hormonal stress response and novelty response remain unchanged (Dadomo et al., 2011). These effects are reversed by fluoxetine in both sexes (Yohn et al., 2019). This paradigm doesn't discriminate between resilient and susceptible phenotypes.

Early-Life Stress

Models such as maternal separation in mice (Plotsky and Meaney, 1993; Meaney, 2001; Millstein and Holmes, 2007) and variations in maternal behavior in rats (Champagne et al., 2003; Brunelli et al., 2015) are also commonly used to reproduce the impact of early-life stress (ELS) on the capacity to deal with stress later in life. In humans, early life trauma, childhood abuse, and parental neglect have significantly been associated with the development of mood disorders (Negele et al., 2015; Lippard and Nemeroff, 2020) in men and women. In rodents, ELS during postnatal development results in lifelong cognitive and emotional alterations that interfere with animals' ability to react and cope with subsequent stressful events (Everson-Rose et al., 2003). For instance, separated pups are more submissive, and generally seek passive coping strategies later in life (Ménard et al., 2016). Similarly, maternal separation in mice increases the susceptibility to social and physical stress in adulthood in both males and females (Tsuda and Ogawa, 2012; Rana et al., 2015).

Male and female pups raised by mothers that provide low levels of licking and grooming early in life also develop anxious and depressive-like behaviors during adulthood, as opposed to pups raised with high licking and grooming mothers (Liu et al., 1997; Caldji et al., 1998; Zhang et al., 2005). Variations in maternal care can also be induced by either the destruction of the nests or the reduction of nesting material available to the pups (Brunson et al., 2005; Cui et al., 2006; Ivy et al., 2008; Rice et al., 2008). Indeed, these manipulations increase maternal anxiety that trigger deficient and abusive maternal care (Dalle Molle et al., 2012; Murthy and Gould, 2018). Pups raised in these conditions exhibit anxiety- and depressive-like behaviors in adulthood, supporting the translational validity of this approach (Ivy et al., 2008; Wang et al., 2011; Raineki et al., 2012; van der Kooij et al., 2015) although negative results have also been described (Brunson et al., 2005; Rice et al., 2008; van der Kooij et al., 2015).

Environmental and Genetic Constructs

Models based on genetic considerations are also used to study anxiety and depressive-like behaviors in both sexes. For instance, the *Flinders Sensitive* rat strain displays behavioral changes such as diminished appetite, psychomotor retardation, as well as sleep and immune alterations that resemble specific aspects of clinical MDD attributes in males and females (Overstreet et al., 2005; Dalla et al., 2009; Kokras et al., 2009; Kokras and Dalla, 2014). However, these rats do not exhibit anhedonia, one of the main clinical manifestations of MDD (Overstreet and Wegener, 2013). *Wistar Kyoto* rats are hypertensive and exhibit high anxiety-like behavior in control conditions (Will et al., 2003; McAuley et al., 2009). Males exhibit anhedonia, hypophagia, and weight loss/gain, while these characteristics in females are absent (Burke et al., 2016). Similarly, rats with high (bHR) and low (bLR) levels of exploratory activity in novel environments (Clinton et al., 2011) are used to reproduce aspects of internalizing and externalizing behaviors associated with psychiatric conditions such as anxiety and MDD. High responder rats (bHR) are often highly exploratory, disinhibited, hyperactive and aggressive while low responders (bLR) exhibit hypo-locomotion, anxiety, and depressive-like behaviors to novelty (Stead et al., 2006; Flagel et al., 2010, 2014; Stedenfeld et al., 2011; Prater et al., 2017; Birt et al., 2021). Importantly, these features in both strains begin in early developmental phases, supporting both the construct and face validity of this model. However, as for most models, the majority of studies performed with these rat lines have been accomplished in males.

Overall, these models support the idea that distinct stress types induce common behavioral phenotypes but also distinct behavioral responses (i.e., social withdrawal, anhedonia, behavioral despair, etc.; **Figure 1**). It also suggests that no single mouse model can reproduce the full complexity of anxiety and MDD conditions in humans. Rather, one should consider using a model to reproduce one specific aspect, symptom, and/or clinical manifestation of the disease. One also needs to know if these models can reproduce the molecular and transcriptional alterations associated with the human condition. In the next section, we elaborate on the capacity of these models to reproduce not only some of the behavioral features relevant to the disease in humans, but also the transcriptional alterations affecting the brain of men and women suffering from anxiety and depression.

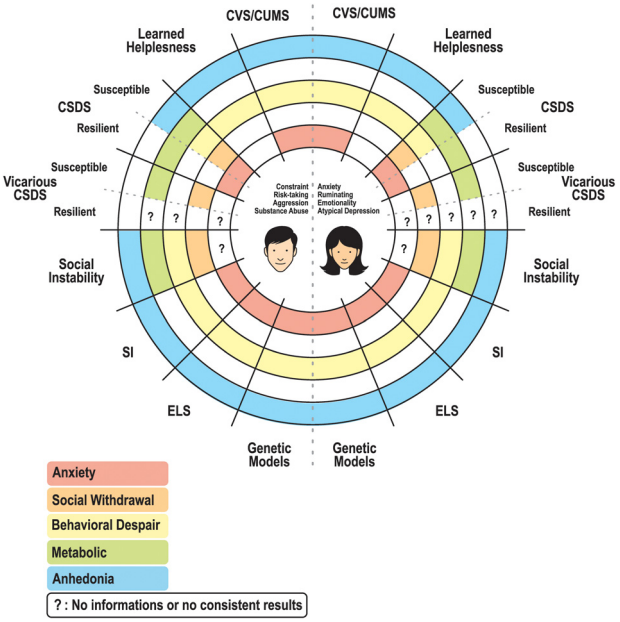

FIGURE 1 | Schematic representation of a circle diagram regrouping the main symptoms (anxiety, social withdrawal, behavioral despair, metabolic dysregulation, and anhedonia) characterizing stress responses in a variety of animal models in males and females. Some unknown results remain, particularly for the resilient group of the vicarious CSDS model, as well as the controversial results concerning the anxiety displayed or not in the social instability model. Abbreviations: CVS/CUMS, chronic variable stress/chronic unpredictable mild stress; CSDS, chronic social defeat stress; SI, social isolation; ELS, early life stress.

SEX-SPECIFIC MOLECULAR ALTERATIONS IN MDD

In addition to its capacity to reproduce behavioral features relevant to a human condition, a model's face validity also relates to its ability to replicate the molecular alterations associated with the disease. This important aspect has been investigated by several groups over the past years, most often using gene candidate approaches (Fatma and Labonté, 2019). Historically, this strategy has been mostly applied to the study of males. Nevertheless, there has been a recent interest in the identification of molecular mechanisms that could underly some aspects of the sexual differences in the expression of anxiety and MDD in men and women. With the availability of genome-wide approaches, combined with the development of highly comprehensive computational strategies, recent studies revealed the transcriptional structures that define stress responses.

Transcriptional Studies in Human Post-mortem Tissue

Global analyses of the male transcriptome in MDD have revealed several gene-related alterations to different pathways including the glutamatergic, GABAergic, serotonergic, and polyaminergic systems across several cortical and subcortical brain regions (Choudary et al., 2005; Sequeira et al., 2007, 2009, 2012; Klempan et al., 2009; Bernard et al., 2011; Duric et al., 2013). Other studies of cortical regions reported alterations in lipid metabolism, immune response, ATP synthesis, regulation of transcription and translation, fibroblast growth factor signaling, and cell proliferation (Evans et al., 2004; Iwamoto et al., 2004; Kang et al., 2007; Tochigi et al., 2008; Klempan et al., 2009; Lalovic et al., 2010). Furthermore, changes in the regulation of the hypothalamic–pituitary–adrenal (HPA) axis and in the control of circadian rhythms have been reported in the hypothalamus (Wang et al., 2008) and cortical/subcortical regions (Li et al., 2013). However, fewer studies have assessed female transcriptional regulation in MDD.

The large majority of these studies adopted a candidate gene approach, showing alterations in brain-derived neurotrophic factor (BDNF), GABAergic, somatostatin (SST), cholinergic, serotonergic, and glutamatergic systems as well as alterations in mitochondrial, energy metabolism, and circadian rhythms in cortical and limbic regions (Boldrini et al., 2008; Szewczyk et al., 2009; Goswami et al., 2010; Lin et al., 2011; Guilloux et al., 2012; Tripp et al., 2012; Bassi et al., 2015; Gray et al., 2015; Seney et al., 2015).

Unfortunately, very few studies directly compared male and female transcriptional profiles. This leaves little comprehension of the molecular mechanisms underlying the expression of the disease in both sexes. The extent to which transcriptional signatures differ between males and females in MDD was assessed by a series of studies published recently. Using RNAseq, Labonté et al. (2017) compared transcriptional signatures across six post-mortem brain regions from men and women with MDD reporting roughly 5%–10% of genes differentially expressed in males and females across all six brain regions. Not only was there a small overlap reported between men and women with MDD, but the directionality of the effects was often opposite in different brain regions. A similar lack of overlap was reported in independent studies also performed on post-mortem brain samples from men and women with MDD (Seney et al., 2018; Girgenti et al., 2021). More recently, analyses of peripheral blood cell samples from MDD patients reported mostly an overlap of the transcripts regulated by the glucocorticoid receptor activation in both men and women. But genetic variants acting on downstream epigenetic and regulatory elements were regulated in a sex-specific manner. This finding was correlated to the transcriptional signatures found in post-mortem brain tissue and the genome-wide association studies (GWAS) analyses showing an enrichment of these variant transcripts associated with MDD (Moore et al., 2021).

These results have been further expanded by the use of network-based approaches. Combined with conventional differential gene expression analyses, network-based approaches provide detailed data-driven molecular classifications associated with specific pathological states such as Alzheimer's disease (Zhang et al., 2013), autism (Parikshak et al., 2013; Willsey et al., 2013), post-traumatic stress disorder (Breen et al., 2015), neurodegenerative diseases (Narayanan et al., 2014), stress in mice (Bagot et al., 2016, 2017; Labonté et al., 2017; Lorsch et al., 2018, 2019; Scarpa et al., 2020; Walker et al., 2022a), and MDD in humans (Labonté et al., 2017; Scarpa et al., 2020). This strategy revealed the existence of male and female MDD-specific gene networks modulating stress susceptibility in a sex-specific fashion *via* the activity of hub genes controlling distinct functional pathways. For instance, the authors identified the gene encoding for *DUSP6* in females and *EMX1* in males as drivers of stress susceptibility in a sex-specific fashion. The downregulation of *DUSP6* in the medial prefrontal cortex (mPFC) increased stress susceptibility while its overexpression rescued stress-induced depressive and anxiety-like behavioral deficits in females but not males (Labonté et al., 2017). This was associated with changes in the activity of the ERK intracellular signaling cascade and in the activity of pyramidal neurons in the mPFC of females but not males. Alternatively, the overexpression of *EMX1* in the mPFC increased depressive and anxiety-like behavioral responses in males but not females. This was also consistently associated with a potentiation of pyramidal neuron activity in a sex-specific fashion (Labonté et al., 2017). It should be emphasized that *DUSP6* was consistently downregulated in the mPFC of both women with MDD and stressed female mice after CVS. Additionally, an increased phosphorylation of ERK was found in females from both species in pyramidal neurons but not GABAergic interneurons. *DUSP6* downregulation in the mPFC, while increasing stress susceptibility, also reproduced a large proportion of the transcriptional changes observed in depressed and stressed females. Together, these findings highlight the contribution of *DUSP6* in the mPFC as a female-specific driver of stress susceptibility, and strongly supports the capacity of CVS to reproduce specific behavioral and molecular aspects of MDD in a sex-specific fashion.

Similar analyses with human cohorts also revealed a major sex difference in the expression of long non-coding RNAs (lncRNAs; Issler et al., 2020). Issler and colleagues recently revealed regulation of lncRNAs associated with depression in brain region and in a sex-specific fashion. Roughly 3% of differentially expressed lncRNA were commonly affected in men and women with MDD, similar to the levels reported above for protein coding genes (5%–10%; Labonté et al., 2017). The authors identified the primate-specific lncRNA *LINC00473* as a potential sex-specific mediator of depression in females specifically. The analyses revealed that this lncRNA was consistently downregulated across brain regions in women but not men with MDD, and its expression was strongly correlated with protein coding genes previously associated with MDD including *DUSP6*, *ARC*, *NR4A1*, *EGR1*, and *EGR2* (Orsetti et al., 2008; Covington et al., 2010; Li et al., 2015; Labonté et al., 2017). Interestingly, the downregulation of *LINC00473* in the mPFC was sufficient to rescue the social withdrawal induced by CSDS, and anxiety- and compulsive-like behaviors induced by CVS in females but not males (Issler et al., 2020). The authors further provided functional data suggesting that the pro-resilient effects induced by the downregulation of this lncRNA are associated with a reduction of the activity of pyramidal neurons (Issler et al., 2020). Interestingly, these effects are similar to what was reported with the downregulation of *DUP6* in females' mPFC (Labonté et al., 2017), by impacting the activity of the CREB pathway known for its involvement in MDD (Carlezon et al., 2005). Whether these effects may be mediated by similar intracellular cascades or not, these results suggest that lncRNAs, while interacting with protein coding genes, are involved in the control of depressive- and anxiety-like behaviors in humans and mice.

More recently, insights into the transcriptional signatures associated with depressive traits and states have been made (Shukla et al., 2021). Using RNAseq from the anterior cingulate gyrus, Shukla and colleagues investigated transcriptional signatures from four different cohorts during: a first depressive episode; remission after the first episode; recurrent episodes, or remission after recurrent episodes. Interestingly, these analyses highlighted several patterns of differentially expressed genes, some of which showed consistent changes across every

phase, but also robust patterns oscillating between episodes and remission phases. Importantly, only minimal overlap was found between genes found in the episode and remission phases. Further deconvolution analyses suggested that a cluster of genes co-expressing GABAergic markers such as SST, VIP (vasoactive intestinal peptide), and CRH (corticotrophin-releasing hormone) displayed phasic changes according to the disease states. This suggests that changes in interneuron function in the mPFC may be involved in the transition from state to trait phases in men's MDD. Unfortunately, this study included only a limited number of samples from women, that prevented the authors to perform sex-specific analyses. It would be wrong to assume these findings are applicable to women with MDD, as transcriptional signatures from men and women with MDD differ. More work will be required to identify the transcriptional signatures defining state and trait MDD in the female brain. Nevertheless, these findings are consistent with previous studies performed in humans and mouse models of stress and support the alteration of the GABAergic signaling as a potential driver of depressive-like behaviors (Tripp et al., 2012; Soumier and Sibille, 2014; Hodes et al., 2015; Lin and Sibille, 2015; Shepard et al., 2016; Fee et al., 2017; Fuchs et al., 2017; Czéh et al., 2018; Shepard and Coutellier, 2018; Todorović et al., 2019; Girgenti et al., 2021). By dissociating transcriptional changes identified with depressive state and trait, these findings represent a significant step forward in the understanding of the molecular mechanisms underlying the expression and the consolidation of the disease.

Transcriptional Studies in Mouse Models

It is interesting to note that a number of studies confirmed the capacity of different types of stress to reproduce a significant proportion of the molecular alterations associated with MDD in both sexes. For instance, consistent low transcriptional overlap has been reported in the mPFC and nucleus accumbens (NAc) of males and females after CVS (Hodes et al., 2015; Labonté et al., 2017). Several functional pathways have also been shown to be enriched with differentially expressed genes (DEG) in both human MDD and stressed males and females (Labonté et al., 2017; Scarpa et al., 2020). These changes result from alterations in the epigenetic regulation of gene expression that include modifications at the DNA methylation level. Indeed, the overexpression of the DNA methyltransferase 3 alpha (Dnmt3a) in the NAc was shown to increase stress susceptibility in both sexes while its downregulation made female mice resilient to 6 days of variable stress with no effect in males (Hodes et al., 2015). Interestingly, these behavioral effects were associated with significant transcriptional alterations distinctly affecting males and females. CVS was also shown to alter the regulation of microRNA (miRNA) expression discernably in males and females (Pfau et al., 2016). Previous analyses using RNAseq to screen miRNA profiles in males and females that underwent CVS revealed highly sex-specific signatures proposing that susceptibility and resilience to sCVS exhibited by males and females may result from a complex remodeling of miRNA signatures affecting coding genes. This was suggested for lncRNAs in human brains as well (Issler et al., 2020).

A similar reorganization of transcriptional structures was observed following CSDS (Bagot et al., 2016, 2017; Lorsch et al., 2018, 2019; Scarpa et al., 2020). In addition to what extent stress changes transcriptional profiles in the brain, these studies confirmed that resilience is a mechanism involving the activation of specific transcriptional programs required to elaborate and consolidate appropriate behavioral strategies to cope with stress. This was reported both at the differential expression and the gene network levels (Bagot et al., 2016, 2017; Lorsch et al., 2018, 2019; Scarpa et al., 2020), similar to what was observed in human MDD, but also after CVS and SI (Labonté et al., 2017; Seney et al., 2018; Scarpa et al., 2020). However, none of these studies included females, limiting their interpretation to males only. At the differential expression level, the number and identity of genes differentially expressed across brain regions were drastically different between males susceptible and resilient to CSDS (Bagot et al., 2016, 2017; Scarpa et al., 2020). The transcriptional organization of gene networks was also different between both phenotypes, with distinct gene networks being associated with the expression of stress susceptibility and resilience in males after CSDS. Importantly, the behavioral contribution of these gene networks was confirmed by a series of behavioral and functional studies. The susceptible-specific hub genes encoding for the Dickkopf Like Acrosomal Protein 1 (*Dkkl1*) and the neurogenic differentiation transcription factor 2 (*NeuroD2*), increased susceptibility to social stress, and induced behavioral despair and anxiety-like behaviors when overexpressed in the ventral hippocampus (vHPC) but not in the mPFC of male mice (Bagot et al., 2016). Overexpression of the gene sidekick cell adhesion molecule 1 (*Sdk1*), in the vHPC also promoted depressive and anxiety-like behavioral features to social stress. However, its overexpression in the mPFC induced pro-resilient effects in male mice (Bagot et al., 2016). Interestingly, the behavioral effects observed after the overexpression of these genes were associated with changes in neuronal activity in the vHPC. The overexpression of both *Dkk1l* and *Sdk1* increased spontaneous excitatory postsynaptic current frequency with no effect on amplitude (Bagot et al., 2016). Furthermore, the overexpression of these two hub genes induced a significant reorganization of the transcriptional structure of their respective gene networks in the vHPC (Bagot et al., 2016). Overall, this suggests that the regulation of specific hub genes promotes the expression of stress susceptibility by imposing functional changes in the activity of specific neuronal populations *via* a reorganization of its own network transcriptional structure. As these findings apply to males only, more work is needed to define the transcriptional profiles underlying the expression of susceptibility and resilience to social stress in female mice.

Further research on the transcriptional organization of gene networks in susceptible and resilient mice identified the *Esr1* gene, encoding for the estrogen receptor 1, as an upstream regulator that drives resilience to social stress in the NAc. The overexpression of *Esr1* in the NAc generated a robust pro-resilient phenotype in males exposed to CSDS and in females that experienced sCVS (Abelaira et al., 2013). These behavioral changes coincided with a consistent reorganization of transcriptional signatures. The authors noted a major

overlap between transcriptional signatures from males after *Esr1* overexpression and resilient but not susceptible males after CSDS. In contrast, no significant overlap was observed between transcriptional signatures from males and females after *Esr1* overexpression, suggesting that the molecular mechanisms underlying the expression of resilience induced by *Esr1* may differ in males and females. It is also important to consider that *Esr1* may indeed be a driver of stress resilience but only in CSDS; classically, CVS in males and females does not induce the expression of a resilient phenotype. Further work is needed to address these important questions.

Lorsch et al. (2019) identified the transcription factor Zinc finger protein 189 (*Zfp189*) as an additional driver of resilience to social stress in the mPFC. The analyses revealed that *Zfp189* is one of the most connected key drivers within a resilient-specific gene network, and significantly upregulated in the mPFC of resilient mice after CSDS. Consistently, the human homolog *ZNF189* was significantly downregulated in the mPFC from MDD post-mortem tissue. Interestingly, the overexpression of *Zfp189* in the mPFC was shown to trigger pro-resilient responses when administered before stress exposure and rescued the susceptible phenotype when injected after exposure to CSDS consistent with a pro-resilient and antidepressant-like role for this key-driver. Further analyses confirmed that the pro-resilient effect of *Zfp189* was mediated by a specific reorganization of its own gene network, which is associated with resilience in the mPFC. More importantly, the authors showed that this effect was driven through direct interactions with CREB. Despite that *Zfp189* and its gene network have been identified in males, CREB knockdown (KO) induced the expression of a depressive-like phenotype to social stress in males and sCVS in females. The expression of *Zfp189* in CREB KO mice rescued these effects in both sexes (Lorsch et al., 2019). These results strongly support the role of Zfp189 as a driver of resilience to stress in both sexes, regardless of the type of stress used. Finally, the direct relationship of both proteins was shown through an elegant set of experiments that combined CRISPR gene editing with behavioral assessment. The authors used a specific strategy to specifically target CREB and *Zfp189* to either associate or segregate them in order to induce or prevent their physical interactions. Interestingly, targeting CREB to *Zfp189 via* this approach increased resistance to social stress while creating a repressive environment around *Zfp189* gene loci. This decreased its expression in the mPFC and induced a pro-susceptible phenotype in male mice. Together these analyses provide substantial evidence for the role of *Zfp189* in mediating pro-resilient effects *via* a complex molecular cascade that involves direct interactions with CREB in the mPFC.

ELS has also been recently shown to induce different transcriptional changes across brain regions of males and females. This series of analyses was based on the two-hit stress model in mice: postnatal stress that occurs during postnatal days 10–20 increases susceptibility to social stress later in life (Peña et al., 2017, 2019). These behavioral effects have been associated with a series of transcriptional changes affecting several brain regions differently, including the ventral tegmental area (VTA), NAc, and mPFC in males and females, depending on the history of previous ELS. These analyses suggest that ELS primes molecular programs in different brain regions to be in a depressive-like state, thus being more plastic to a significant reorganization when challenged by additional stress during adulthood (Peña et al., 2017, 2019), or even drug abuse in a sex-specific fashion (Walker et al., 2022a). These findings led to the identification of specific genes as upstream regulators of transcriptional structures in these brain regions driving stress responses in a sex-specific fashion. While the genes encoding for alpha-synuclein (SNCA) and beta catenin (CTNNB1) were both predicted upstream regulators in female VTA and NAc, the orthodenticle homeobox 2 encoding gene, *Otx2*, was the highest-ranked upstream regulator of the pro-depressive transcriptional signature in males' VTA (Peña et al., 2017, 2019). The functional and behavioral implication of *Otx2* as an upstream regulator of pro-depressive transcriptional signatures was further assessed by a series of behavioral experiments following its viral modification directly in the VTA. Transient *Otx2* overexpression in the VTA of juvenile male mice blocked susceptibility to adult social defeat and rescued the downregulation of several *Otx2* targets in this brain region (Peña et al., 2017). The transient juvenile suppression of *Otx2* expression in the VTA recapitulated the effects of postnatal stress on the expression of susceptibility to social stress during adulthood, which is associated with significant changes in the expression of its downstream target genes. It is important to note that these effects were specifically associated with the juvenile developmental period, as the overexpression of *Otx2* during adulthood only partially rescued behavioral and transcriptional effects, while its downregulation failed to induce behavioral susceptibility and changes in *Otx2* target gene expression (Peña et al., 2017).

Further analyses suggest that these effects may be mediated at least in part by epigenetic changes. Indeed, several targets of *Otx2* in the VTA were predicted to be enriched with the presence of the open chromatin mark H3K4me3 (Peña et al., 2017). Similar observations have been made concerning the epigenetic mechanisms mediating the effects of ELS in the NAc of males and females (Kronman et al., 2021). Kronman and colleagues showed that ELS induces a significant suppression of the repressive histone mark H3K79 specifically in males (Kronman et al., 2021). These effects were accompanied by cell-type-specific changes in the expression of the H3K79 writer and eraser, *Dot1l* and *Kdm2b*, respectively, in the NAc following a developmental trajectory. The expression of both genes was significantly increased in D2-expressing medium spiny neurons (MSN) of both males and females, an effect that was not seen early postnatally (PND21) but that became significant at a later developmental stage (PND35). This was maintained until adulthood, suggesting an incubation effect of ELS across developmental stages. Interestingly, *Dot1l* downregulation in D2-MSNs reversed the behavioral consequences of ELS-mediated behavioral susceptibility, while its overexpression in the same neuronal population replicated the behavioral phenotype induced by ELS in males, and to a lower extent in females. Conversely, the overexpression of *Kdm2b* in D2 expressing MSNs reversed ELS-induced

behavioral phenotypes, whereas its downregulation increased stress susceptibility in males exclusively. As shown before with other key drivers and upstream regulators, the transcriptional profiles initiated by ELS were strikingly similar to those induced by *Dot1l* overexpression and inversed to *Dot1l* downregulation in D2 MSNs. Interestingly, further analyses were done to address the discrepancy between the upregulated expression of *Dot1l* and the downregulation of H3K79me2 in whole NAc after ELS. The results showed that the upregulation of *Dot1l* is associated with increased deposits of H3K79me2 at more genomic sites, but the loss of H3K79me2 found at a subset of sites is more important. This loss could be due to the coordinated induction of *Kdm2b* in the NAc.

Interspecies Transcriptional Studies

Each of these studies provides valuable evidence that distinct mouse models are useful in testing the contribution of specific genes and transcriptional programs on behavioral responses to chronic stress. However, they still do not directly compare the extent of how they can accurately reproduce the transcriptional signatures relevant to MDD in the brain. This precise question was recently addressed by comparing the RNAseq transcriptional profiles generated from human post-mortem brain samples and three models of chronic stress including CVS, SI, and CSDS (Labonté et al., 2017; Scarpa et al., 2020). These analyses revealed a significant overlap between transcriptional alterations in the mPFC and NAc from human MDD and stressed mice, with each of the chronic stress paradigms capturing distinct aspects of MDD abnormalities. At the differential expression level, CVS and SI were shown to better reproduce the human conditions in the NAc and mPFC (Scarpa et al., 2020). It should be mentioned that these analyses have been done by controlling for the effect of sex. Indeed, not every dataset included females, and sex-specific analyses were not possible which limits the interpretation of these results. Nevertheless, these findings are consistent with previous comparative studies showing that both males and females that experienced CVS reproduce a significant proportion of the differential expression profiles observed in men and women with MDD (Labonté et al., 2017). These analyses also revealed a significant number of functional pathways that are enriched for DEGs in humans with MDD, and each of the different mouse models of stress. This suggests that the behavioral consequences of stress may be mediated by similar functional pathways in both species (Scarpa et al., 2020).

Importantly, network-based approaches provided similar conclusions. Consistent with previous studies (Tsaparas et al., 2006; Monaco et al., 2015; Eidsaa et al., 2017), all three mouse models were shown to share a significant level of co-expression structure in the mPFC and the NAc, although it is accepted that the human transcriptome acquired a certain complexity throughout evolution that is not shared in mouse (Pembroke et al., 2021). This approach identified gene networks sharing common co-expression structures associated with MDD and stress and enriched with genes differentially expressed in human and mouse models. For instance, the authors reported a gene network associated with the function and structure of oligodendrocytes (Scarpa et al., 2020). Interestingly, impaired myelin-related gene expression, along with reduced myelin thickness, have been reported in the cortex from suicide completers with a history of child abuse (Lutz et al., 2017; Tanti et al., 2018, 2021). Similarly, prolonged social isolation and social stress in mice have been shown to change oligodendrocyte gene expression that interferes with myelin integrity in the mPFC (Liu et al., 2012; Zhang et al., 2016). Amongst all the genes in this network, *Gab1* was identified as a hub gene preserved in humans with MDD and each of the three mouse models of chronic stress. *Gab1* is also known to enhance PI3K/AKT activation and to extend the duration of Ras/MAPK signaling (Kiyatkin et al., 2006). Additionally, it was shown to indirectly trigger myelination by increasing the expression of *Egr2* when activated by the protein kinase A (PKA; Ghidinelli et al., 2017). Altered oligodendrocyte function in MDD has also been supported by a recent study using single nuclei RNA sequencing to probe changes in gene expression across every cell type found in the mPFC of men with MDD (Nagy et al., 2020). Amongst all genes found differentially expressed, the majority were found in oligodendrocytes and a subpopulation of deep layer excitatory cells in the mPFC. Based on their predictions, the authors concluded that the relationship between these two clusters of cells could be explained in part by impairments in fibroblast growth factor signaling, steroid hormone receptor cycling, immune function, and cytoskeletal regulation, which could underly changes in mPFCsynaptic plasticity (Nagy et al., 2020). These results are also consistent with previous results showing metabolic, functional, and morphological changes in the mPFC with depression and chronic stress (Hare and Duman, 2020).

Overall, these studies suggest that each mouse model can reproduce common but also unique molecular features relevant to the expression of the disease in humans with no unique model better than the others (**Table 1**; **Figure 2**). In other words, the decision for an appropriate model should be based not only on its capacity to reproduce certain behavioral aspects, but also its capacity to reproduce the transcriptional alterations relevant to the human condition. However, as female transcriptional data are not consistently available for each model, it is impossible to predict whether this capacity applies to both males and females. This cannot be simply addressed by directly overlapping human and mouse profiles, as important considerations such as gene orthology, correlation structures, and connectivity need to be taken when comparing the transcriptional structures of two different species. Additional clinical variables such as age, hormonal status, and pathological comorbidities that are difficult to account for in human post-mortem studies are also important considerations when performing interspecies sex-specific studies. Nevertheless, based on previous findings from human and mouse studies (Labonté et al., 2017; Lorsch et al., 2018, 2019; Scarpa et al., 2020), it is tempting to speculate that both males and females would reproduce specific aspects of the human condition, but most likely not the same. More work will be required to address this important question and consolidate the benefits of using mouse models to study specific molecular mechanisms underlying the expression of MDD in both sexes.

TABLE 1 | Summary of recent transcriptomic analyses done by RNA-sequencing characterizing transcriptional profiles in human MDD post-mortem brains and different animal models of depressive-like behaviors.

Transcriptional studies in humans from post-mortem tissues:

Human sex samples (M/W)	Sample size	Region of Interest	Main Findings	Studies
M & W	26 MDD (13 M & 13 W) and 22 Ctrl (13 M & 9 W); A cohort of 32 M (15 MDD & 17 Ctrl); A cohort of 18 W (6 MDD & 12 Ctrl)	vmPFC, OFC, dlPFC, aINS, NAc, vSUB	Low transcriptional overlap and divergent gene network structures between males and females across brain regions	Labonté et al. (2017)
M & W	50 MDD (26 M & 26 controls, 24 W & 24 Ctrl)	dlPFC, sgACC, BLA	Low transcriptional overlap between males and females across brain regions	Seney et al. (2018)
M & W	143 samples from 46 Ctrl (26 M & 20 W), 52 PTSD (26 M & 26 W), and 45 MDD (27 M & 18 W)	PFC, AMY, HIPP, dlPFC	Divergent transcriptomic signatures between PTSD and MDD. Low transcriptional overlap between males and females	Girgenti et al. (2021)
M & W; adolescents & children (M & W)	Cohort 1: 289 samples from 93 W (48 MDD & 45 Ctrl) and 1960 M (81 MDD & 115 Ctrl); Cohort 2: 584 children and adolescents with 350 MDD & 234 Ctrl; Cohort 3: 1774 samples from 879 MDD & 756 Ctrl	Blood samples analysis associated with six brains regions of interest. Only a significant result with BA25/ACC is presented	High overlap of the GR transcripts between sexes with only an enrichment of the eQTL in females	Moore et al. (2021)
M & W	Cohort of 50 MDD & Ctrl	OFC, dlPFC, vmPFC, NAc, aINS, vSUB	*LINC00473* is a sex-specific mediator of depression in females specifically	Issler et al. (2020)
M & W with few proportions of W	90 samples (20 Ctrl, 20 MDD, 15 in remission after one episode, 20 in recurrent episodes & 15 remissions after recurrent episodes)	dlPFC/ACC	Changes in interneurons function in the mPFCare involved in the transition from state to trait in MDD	Shukla et al. (2021)
M & W	78 samples (27 MDD suicided with CA, 25 without CA & 26 Ctrl)	ACC	CA induces epigenetic reprogramming of myelin in adults	Lutz et al. (2017)
M & W	36 samples (18 MDD with CA & 18 MDD without CA)	vmPFC	Long-term changes in connectivity related to imbalance of oligodendrocytes and myelin remodeling in MDD patients with CA	Tanti et al. (2018)

(Continued)

TABLE 1 | Continued

Transcriptional studies in humans from post-mortem tissues:

Human sex samples (M/W)	Sample size	Region of Interest	Main Findings	Studies
M & W	11 Ctrl from 9 M & 2 W, 26 MDD without CA from 14 M & 12 W, 12 MDD with CA from 9 M & 3 W	vmPFC/BA11-12	Decreased neuroplasticity of cortical circuits through the enhancement of developmental OPC-mediated PNN formation in MDD patients with CA	Tanti et al. (2021)
M	34 samples (17 MDD & 17 Ctrl)	dlPFC	Significant differential expression of oligodendrocytes associated with dysregulation of excitatory neurons in MDD	Nagy et al. (2020)

Transcriptional studies in mouse models:

Animals	Models	Sample size	Age	Region of Interest	Main Findings	Studies
C57BL/6J M & F mice	CVS	40 mice (10 M/groups, & 10 F/groups)	8 weeks	vmPFC and NAc	*DUSP6* and *EMX1* are drivers of stress susceptibility in a sex-specific manner	Labonté et al. (2017)
C57BL/6J M & F mice	CVS	3-5 mice/groups	8 weeks	OFC, dlPFC, vmPFC, NAc, aINS, vSUB	LncRNA *LINC00473* is a sex-specific mediator of depression in females specifically	Issler et al. (2020)
C57BL/6J M & F mice	sCVS	48 (4 mice/library & 3 libraries/sex/stress condition)	8-12 weeks	NAc	Low overlap between transcriptional profiles in the NAc and PFC in stressed males and females	Hodes et al. (2015)
C57BL/6J M & F mice	sCVS	60 (5 mice/library & 3 libraries/sex/stress condition)	8 weeks	NAc	Little overlap of the transcriptional and post-transcriptional profiles between sexes	Pfau et al. (2016)
C57BL/6J M mice	CSDS	12 (4 mice/library & 3 libraries/sex/stress condition)	8 weeks	vHIP, PFC, NAc, AMY	Overexpression of two specific hub genes induced a significant reorganization of the transcriptional structure of their respective gene networks in the vHIP	Bagot et al. (2016)

(Continued)

TABLE 1 | Continued

Transcriptional studies in mouse models:

Animals	Models	Sample size	Age	Region of Interest	Main Findings	Studies
C57BL/6J mice	CSDS	10 Ctrl, 8 resilient, 14 non-responders to treatments (8 + 6), 6 susceptible, 12 responders to treatments (6 + 6)		PFC, NAc, HIP, AMY	Transition from susceptible to resilient transcriptional profiles following pharmacological treatments	Bagot et al. (2017)
C57BL/6J mice	CSDS (M), CVS (F)	27 mice (6-8M mice/groups & 6-7 F mice/groups)	8 weeks	NAc, PFC	Estrogen receptor 1 is an upstream regulator that drives resilience to social stress	Lorsch et al. (2018)
C57BL/6J M & F mice	CSDS (M) & sCVS (F)	10 mice (5/groups)	8 weeks	PFC, vHIP, BLA, NAc	*Zfp189* is a hub gene driving resilience to social stress	Lorsch et al. (2019)
C57BL/6J M & F mice	ELS (MS and limited nesting) alone or followed by STVS or CSDS	4-6 mice/groups 5-6 mice/groups	Adult mice	VTA, NAc, PFC	ELS primes molecular programs toward a reorganization when challenged by stress during adulthood	Peña et al., 2019
C57BL/6J M mice	2-hit stress model, CSDS	3 mice/groups/sex	Adult and adolescent mice	VTA	*Otx2* overexpression rescued depressive-like behaviors and reversed *Otx2*-targets gene expression	Peña et al. (2017)
C57BL/6J M & F mice	CSDS, ELS	2 mice/groups/sex	10-12 weeks for CSDS	NAc	ELS induces a sex and cell type specific reorganization of H3K79 profiles	Kronman et al. (2021)

Interspecies transcriptional studies:

Subjects	Models	Age	Sample size	Region of Interest	Main Findings	Studies
C57BL/6J mice; M & F humans	CSDS, SI, CVS	MDD: 45+/-17 years old & Ctrl: 48+/-17	26 MDD (13 M & 13 W), 22 Ctrl (13 M & 9 W); 10 CVS mice/sex; 30 SI M & 15 M Ctrl; 11 M CSDS/phenotypes	PFC & NAc	CVS, SI and CSDS reproduce common but also unique transcriptional changes relevant to the expression of MDD	Scarpa et al. (2020)

Abbreviations: M, men/males; W, women; F, females; PTSD, post-traumatic stress disorder; MDD, major depressive disorder; Ctrl, controls; CA, child abuse; eQTL, cis-expression quantitative trait loci; LncRNA, long non-coding RNA; sCVS, subchronic variable stress; CVS, chronic variable stress; CSDS, chronic social defeat stress; ELS, early life stress; MS, maternal separation; STVS, subthreshold variable stress; SI, social isolation; OPC, oligodendrocytes progenitor cells; PNN, perineuronal nets; vmPFC, ventromedial prefrontal cortex; OFC, orbitofrontal cortex; dlPFC, dorsolateral prefrontal cortex; aINS, anterior insula; NAc, nucleus accumbens; vSUB, ventral subiculum; sgACC, subgenual anterior cingulate cortex; AMY, amygdala; HIPP, hippocampus; mPFC, medial prefrontal cortex; vmPFC, ventromedial prefrontal cortex; vHIP, ventral hippocampus; PFC, prefrontal cortex; BLA, basolateral amygdala; VTA, ventral tegmental area; BA, Brodmann area; ACC, anterior cingulate cortex.

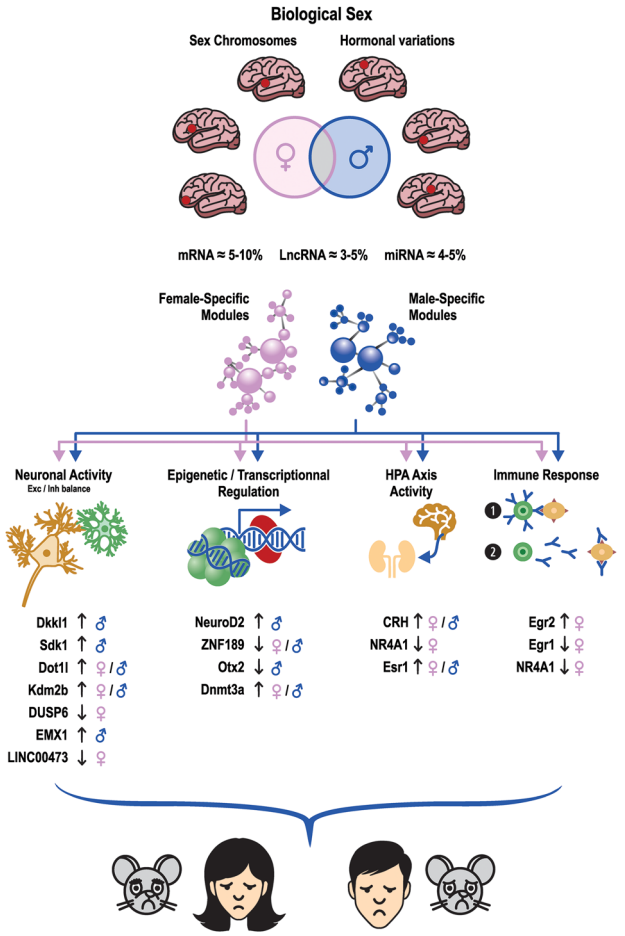

FIGURE 2 | Males and females with MDD or stress share minimal transcriptional overlap across brain regions. These sex differences may be driven by biological factors such as sex chromosomes and hormonal variations. Similar differences are also observed at the gene network level where sex-specific transcriptional networks are associated with the expression of MDD in either males or females in humans but also with the expression of stress susceptibility or resilience in stressed male and female mice. These transcriptional changes interfere with the activity of several molecular, biological, and cellular processes such as neuronal activity, epigenetic and transcriptional regulation, the function of the HPA axis, and immune response. Ultimately, this leads to the expression of converging depressive-like behaviors in males and females sharing similar symptomatic and behavioral features. The orientation of the arrows next to listed genes indicates whether gene expression is upregulated or downregulated in the depressed/stressed conditions. ♂: male symbol, ♀: female symbol.

CONCLUSION

Fundamental research using animals is an absolute necessity to improve our understanding of complex human conditions. Here, we have reviewed the strengths and weaknesses of some of the most widely used models to study the molecular and functional impact of chronic stress on the expression of depressive and anxiety-like behaviors. Amongst the multiple conclusions that can be drawn, no unique model can fully reproduce the human condition. Indeed, the clinical manifestation of the disease varies between individuals either qualitatively or quantitatively (Soderlund and Lindskog, 2018) which cannot be accounted for in animals. Several complex behavioral features and traits related to the disease cannot be evaluated without falling into anthropomorphic considerations. Furthermore, the clinical representation of the disease keeps evolving throughout the pathological process (Zahn-Waxler et al., 2000). Thus, rather than mimicking MDD and anxiety in mice as a whole, one should consider modeling specific aspects of the disease that can be accurately reproduced and quantified in mice and more importantly differently in each mouse model.

Nevertheless, data strongly support the use of animal models to study the molecular mechanisms underlying the expression of stress susceptibility and resilience in both males and females, although only a few studies properly integrated females in their analysis. As of now, studies investigating the transcriptional programs underlying the expression of MDD and anxiety in humans have revealed drastic differences between men and women. This should be considered carefully since the lack of overlap in DEG between stressed males and females should not always be interpreted as a sign of sex differences (Mukamel, 2022). With the development of novel approaches combining the assessment of differential expression profiles with transcriptional overlap, gene ontology and gene network-based approaches integrating correlation structures and connectivity measures, the sum of converging evidence is strongly supporting the existence of true sex differences in the transcriptional organization of gene networks across the brain that may drive the expression of behavioral alterations in a sex-specific fashion (Labonté et al., 2017; Lorsch et al., 2018, 2019; Seney et al., 2018; Walker et al., 2022a,b).

Most importantly, the transcriptional signatures associated with each type of stress share common core features but also unique aspects relevant to the human condition. In this sense, types of stress with psychosocial constructions affect the brain transcriptome differently than other stress types relying on physical paradigms. In perspective, this is in line with our understanding of how environmental challenges are impacting brain activity through epigenetic mechanisms (Fatma and Labonté, 2019) and adds to the importance of considering not only the behavioral features but also the molecular systems affected by different types of stress when choosing an appropriate mouse model. Ultimately, this choice may have a crucial impact on behavioral, morphological, functional, and molecular findings. For instance, transcriptional alterations that increase the activity of mPFC neurons have been shown to promote stress susceptibility in animals undergoing CVS (Labonté et al., 2017; Issler et al., 2020) while changes that induce similar functional impacts on mPFC activity have been associated with resilience and anti-depressant properties in the CSDS model (Bagot et al., 2016). Similarly, certain transcriptional changes triggering stress susceptibility in females induce no effect in males and the opposite has also been shown (Labonté et al., 2017).

Probably the most important remaining question is what are the mechanisms underlying these differences either at the behavioral or transcriptional levels. Amongst the different potential players, sex chromosomes and gonadal hormones come

to mind. Both X and Y chromosomes contain genes encoding for different chromatin writers and erasers as well as several transcription factors (Sene et al., 2013; Seney et al., 2013; Dossat et al., 2017). These genes are crucially involved in various developmental processes and are likely to be impacted differently by environmental factors and ultimately by hormonal influences (Puralewski et al., 2016; Jaric et al., 2019). Similarly, molecular processes and emotional responses are also importantly regulated by gonadal hormones which broaden the contribution of sex-specific biological correlates underlying stress responses in males and females (Bangasser and Cuarenta, 2021; Bhargava et al., 2021; Rainville et al., 2022). More recently, FCG mice were used to dissect the behavioral and transcriptional impact of gonadal hormones and sex chromosomes over stress responses in males and females (Paden et al., 2020). Interestingly, results show that XX male carriers recapitulate XX females' behavioral profiles. Similar findings were also reported for XY female carriers and XY males. At the transcriptional level, 25% of the differences between males and females were related to sex chromosomal influences while 23%–31% of these differences were associated with gonadal hormones (Paden et al., 2020). Interestingly, despite the extent of the transcriptional differences, the authors reported that a large proportion of the transcriptional changes in males and females were in fact clustered on similar functional pathways (Paden et al., 2020). This is very similar to the findings reported in human post-mortem tissue (Labonté et al., 2017; Seney et al., 2018; Girgenti et al., 2021) and supports the idea that common functional pathways may be impacted in males and females with MDD but *via* different genes. However, the contribution that sex chromosomes and gonadal hormones have, especially during crucial developmental phases, remains unknown and more work will be required to fully understand the complex interplay between sex chromosomes, gonadal hormones, and transcriptional programs in controlling the development of emotional responses in stressed males and females (Paden et al., 2020; Seney and Logan, 2021).

Overall, this suggests that several transcriptional programs are in place to control neuronal activity and brain function and these programs are affected distinctly by different types of stress in males and females. As of now, only the tip of the iceberg has been revealed and much more work is needed to provide a better understanding of the molecular mechanisms underlying stress susceptibility and resilience in males and females. While work in human populations is crucial to drive this initiative, animal models remain one of the best strategies to provide mechanistic insights into the effects. With this in mind, future work should consider using these approaches to reveal the transcriptional signatures underlying specific symptomatic profiles in humans. With the knowledge that each of the models can accurately reproduce specific behavioral and molecular aspects of MDD and anxiety in males and females, such initiatives should provide interesting insights into the systems to target more precisely in order to treat specific symptoms, rather than the complex syndrome.

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Sex Differences in Blood–Brain Barrier Transport of Psychotropic Drugs

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Treatment of neuropsychiatric disorders relies on the effective delivery of therapeutic molecules to the target organ, the brain. The blood–brain barrier (BBB) hinders such delivery and proteins acting as transporters actively regulate the influx and importantly the efflux of both endo- and xeno-biotics (including medicines). Neuropsychiatric disorders are also characterized by important sex differences, and accumulating evidence supports sex differences in the pharmacokinetics and pharmacodynamics of many drugs that act on the brain. In this minireview we gather preclinical and clinical findings on how sex and sex hormones can influence the activity of those BBB transporter systems and affect the brain pharmacokinetics of psychotropic medicines. It emerges that it is not well understood which psychotropics are substrates for each of the many and not well-studied brain transporters. Indeed, most evidence originates from studies performed in peripheral tissues, such as the liver and the kidneys. None withstanding, accumulated evidence supports the existence of several sex differences in expression and activity of transport proteins, and a further modulating role of gonadal hormones. It is proposed that a closer study of sex differences in the active influx and efflux of psychotropics from the brain may provide a better understanding of sex-dependent brain pharmacokinetics and pharmacodynamics of psychotropic medicines.

Keywords: sex differences, blood–brain barrier, psychotropics drugs, transporters and channels, brain, females, transporters, mental disorders

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INTRODUCTION

Neuropsychiatric disorders carry a significant burden and disproportionally affect more women than men (Wittchen et al., 2011). Their treatment relies on effective drug delivery to the brain. However, such drug delivery is challenging, as the blood–brain barrier (BBB) allows only endo- and xeno-biotics (including medicines) with specific physicochemical characteristics (lipophilicity, molecular weight, and charge) to enter. This barrier is achieved as brain capillary endothelial cells (BCECs), in very close proximity between them, form complex and tight junctions (**Figure 1**). The BBB functions within the context of the neurovascular unit (McConnell et al., 2017), a structure consisting of neurons, interneurons, astrocytes, pericytes, basal lamina covered with smooth muscular cells, microglia as well as endothelial cells and extracellular matrix, and regulates the cerebral blood flow (Muoio et al., 2014). Although some substances may diffuse passively

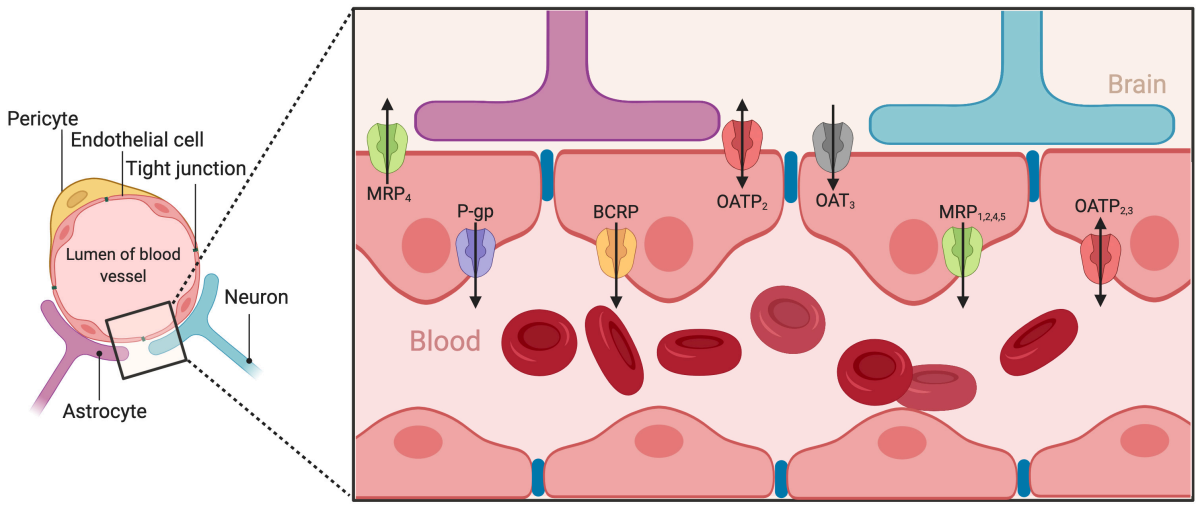

FIGURE 1 | Graphical representation of the blood–brain barrier and its transporters.

though the BBB, the influx and efflux of most substances is actively regulated by a complex system of transporters expressed on the BBB. Emerging evidence suggests that brain pharmacokinetics, and thus psychotropic pharmacodynamics is greatly influenced by these transport systems (O'Brien et al., 2012). However, such knowledge is relatively new and now unfolding for many of those systems, especially with the help of evidence gathered from the presence of those transporters in peripheral barriers, such as in the gastrointestinal tract, the liver, and the kidneys. On the other hand, there is strong evidence that many neuropsychiatric disorders present significant sex differences (Balta et al., 2019) and preclinical research is progressing into incorporating sex as an important biological variable (Butlen-Ducuing et al., 2021). Moreover, psychotropic medication present noteworthy pharmacodynamic and interestingly, pharmacokinetic sex differences (Kokras et al., 2011; Seeman, 2021). Given that psychotropic medication must reach the brain to exert their therapeutic action, it emerges that potential sex differences in the brain's transport systems might be involved in the action of psychotropic medicines in men and women. Therefore, in this minireview, we gather preclinical and clinical findings on how sex and sex hormones can influence the activity of BBB transporters and, discuss the current state of the art.

P-GLYCOPROTEIN

The ABCB1 gene expresses P-glycoprotein (P-gp) (or multi-drug resistance protein 1) in humans and two homologs in rodents, the abcb1a and abcb1b (O'Brien et al., 2012). P-gp has a broad binding site for a wide range of substances, as it is not restricted stereochemically and currently is the most studied transport protein. Regarding psychiatric disorders, P-gp plays an important role in CNS drugs bioavailability (De Klerk et al., 2011). Several antidepressants, like citalopram/escitalopram, paroxetine, imipramine, and venlafaxine are substrates of P-gp (Uhr and Grauer, 2003; Karlsson et al., 2010; O'Brien et al., 2013a,b). Thus, their brain pharmacokinetics are altered by P-gp and response to treatment is affected (Lin et al., 2011). However, other drugs appear not affected by P-gp, like fluoxetine and mirtazapine (Uhr et al., 2000, 2003). Interestingly, some psychotropic medications show a complex interaction with P-gp. For example, sertraline displays a biphasic and time-dependent interaction, fluctuating between inhibition and stimulation of P-gp (Kapoor et al., 2013). Another example is that high doses of nortriptyline saturate the P-gp-dependent transport and thus decrease its clearing effectiveness (Ejsing and Linnet, 2005). Abundant evidence indicates sex differences in the P-gp transport (Baris et al., 2006; Lifschitz et al., 2006; Ueno and Sato, 2012; Tornatore et al., 2013). However, there are also reports showing no significant sex differences (Dagenais et al., 2001; Gottschalk et al., 2011; Long et al., 2016). Such discrepancies, as discussed later, are probably explained by several factors, such as differences in species, the studied substrate, the tissue sampled, etc. Moreover, many P-gp polymorphisms affecting therapeutic drug efficacy are reported (Dizdarevic et al., 2014; Peng et al., 2015; Skalski et al., 2017; Rahikainen et al., 2018). Some are linked with sex-differentiated drug responses and development of specific side effects (Alzoubi et al., 2013; Rahikainen et al., 2018). This highlights the importance of sex segregation in pharmacogenetic research. Lastly, there is evidence that gonadal hormones, such as estrogens, testosterone and progesterone affect the activity of P-gp, and its activity may vary across the menstrual cycle (Axiotis et al., 1991; Peng et al., 2015; Kanado et al., 2019).

BREAST CANCER RESISTANT PROTEIN

Breast cancer resistant protein (BCRP) is an ABC transporter expressed in different tissues, including the brain epithelial cells, and may be responsible for the low bioavailability of

several psychotropics. A recent study showed that sertraline is a BCRP substrate along with its P-gp inhibiting properties (Feng et al., 2019). Venlafaxine dose-dependently induces the BCRP expression (Bachmeier et al., 2011). Moreover, BCRP is known to work in synergy with P-gp, cooperatively eliminating xenobiotics from the brain and thus impeding treatment (Kodaira et al., 2010; Agarwal et al., 2011). Several preclinical studies highlight sex differences in BCRP, whose regulation is testosterone-induced and estradiol-inhibited, and point to a higher expression of BCRP in males (Teo et al., 1993; Fu et al., 2012). Hormonal manipulations, such as gonadectomy or hormonal treatment significantly affected its expression, and in general lower BCRP expression in females led to higher drug exposure (Merino et al., 2005; Gulilat et al., 2020). However, most results are obtained from tissues other than the brain. Interestingly a single study showed that specifically in the brain, BCRP expression is higher in female than male mice (Tanaka et al., 2005).

MULTIDRUG RESISTANCE-ASSOCIATED PROTEINS

Multidrug resistance-associated protein (MRP) is a family of ABC transporters comprising of currently seven known members which are located at luminal membranes, and also found at the BBB (Ueno et al., 2010). Although considered to be an important drug transport mechanism, there is limited information regarding most psychotropics. One study showed that phenytoin and carbamazepine brain levels were lower following upregulation of MRP1 (Chen et al., 2013). Interestingly, no sex differences were identified regarding Mrp1 and Mrp2 mRNA expression in the choroid plexus. However, after its removal, BBB expression levels of Mrp1, Mrp2, and Mrp4 were twice as higher in female mice than in males (Flores et al., 2017). Studies in tissues such as the liver and the kidneys generally corroborate that females have higher MRP expression (Maher et al., 2005; Lu and Klaassen, 2008) and some evidence points to a progesterone and/or dehydroepiandrosterone regulation of this sex difference (Rost et al., 2005; Evseenko et al., 2007).

ORGANIC ANION TRANSPORTERS

Organic anion transporter (OAT) is an heterogenous family of negatively charged proteins, mainly located in kidneys and the liver, but OAT1/OAT3 are also found in the brain and are responsible for transporting hydrophobic organic anions. Evidence suggests that valproate, used as a mood stabilizer, is a substrate of OAT1 and homovanillic acid, a metabolite of dopamine, is a substrate of OAT3 (Sekine et al., 2000; Mori et al., 2003). In the kidneys and the liver, OAT expression is affected by androgens, and perhaps different OAT isoforms are stronger expressed in males and females in these tissues. Overall, renal Oat1 expression is androgen-regulated, renal Oat2 expression is modulated by female GH secretion pattern, and hepatic Oat3 expression is influenced by both androgens and female GH secretion pattern (Buist et al., 2003). Although OAT sex differences have been demonstrated in rodents, the direction of sex difference is not consistent and are not confirmed in other species, such as in rabbits (Groves et al., 2006) and in human cells (Breljak et al., 2016). Moreover, regarding specifically the brain, an *in vivo* BBB preclinical study did not identify a sex difference in OAT3 (Ohtsuki et al., 2005).

ORGANIC ANION TRANSPORTING POLYPEPTIDES

These transporters form a superfamily of membrane-solute carriers characterized by significant functional diversity and a widespread role in the transport of endo/xenobiotics (Hagenbuch and Meier, 2004). There is scarce data on whether they are involved in the brain transport of psychotropics, but we know that transport of DHEA-S and opioids occurs *via* OATP1A2 and a small sex difference favoring women was recently reported (Asaba et al., 2000; Gao et al., 2000; Taniguchi et al., 2020). However, DHEA administration led to a gender-neutral Oatp1a1 and Oatp1b2 decrease and a further decrease in Oatp1a4 expression only in males (Rost et al., 2005). Evidence on sex differences is convoluted because there are many organic anion transporting polypeptide (OATP) transporters with a broad tissue distribution. Most preclinical evidence converges that activity of Oatp1a4, which is also expressed in the BBB, is higher in females, with testosterone probably suppressing it (Zhang et al., 2013; Brzica et al., 2018). However, several preclinical studies showed a tissue-specific variability in the direction or even absence of sex differences regarding various members of the OATP family (Cheng et al., 2005, 2006; Fu et al., 2012; Muzzio et al., 2014; Prasad et al., 2014; Badee et al., 2015).

ORGANIC CATION TRANSPORTERS

Organic cation transporter (OCT) are responsible for transporting cationic substances, like monoamine neurotransmitters, nicotine, the opioid agonist oxycodone, and antipsychotics like amisulpride and haloperidol (Bostrom et al., 2006; Okura et al., 2008; Sekhar et al., 2019). Interestingly, OCT2 and rOCT are found in the brain, and regulate the concentration of neurotransmitters in the neurons rather than the BBB (Busch et al., 1998). Very few data exist on potential sex differences, mostly on renal OCT2, which is expressed more strongly in males than females and it is upregulated by androgens (Alnouti et al., 2006; Groves et al., 2006; Basit et al., 2019). Plasma membrane monoamine transporter (PMAT/SLC29A4), a known transporter for cationic substances, is implicated in the efflux of amisulpride and haloperidol from the brain and is inhibited by nicotine (Tega et al., 2018; Sekhar et al., 2019). Some evidence on sex differences exist for PMAT, as behavioral changes were noted only in female, but not male, PMAT knockout mice (Gilman et al., 2018).

MONOCARBOXYLATE TRANSPORTERS

Monocarboxylate transporter (MCT) mediate the transport of short chain monocarboxylates such as lactate and pyruvate, indicating their involvement in regulating brain energy substrates. Of 14 MCT members identified, MCT1, MCT2, MCT4, and the sodium-coupled SMCT1 have been described in the brain (Pierre and Pellerin, 2005). They are implicated in the brain transport of several drugs, including notably statins, salicylates and in relation to psychotropics, valproic acid, and γ-hydroxybutyrate (GHB) (Vijay and Morris, 2014). Sex differences have been identified, and are attributed in a tissue-specific regulation by both male and female sex hormones (Felmlee et al., 2020). Hepatic MCT1 and MCT4 regulation appears dependent on both estrogens and androgens (Cao et al., 2017). In muscles testosterone increases MCT1/4 expression but decreases testicular MCT2/4. However, there is a paucity of data regarding sex-dependent patterns of brain MCT regulation, which is important given the tissue-specific profile that emerges.

MULTIDRUG AND TOXIN EXTRUSION PROTEINS

Multidrug and toxin extrusion protein (MATE) family transporters function in concert with OCT, are mostly expressed in the liver and the kidneys, but they are also found in the brain, and are involved in the transport of cationic drugs (Lickteig et al., 2008). Amisulpride and haloperidol, both antipsychotics, as well as nicotine, have been identified as possible substrates of MATE1 (Tsuda et al., 2007; Sekhar et al., 2019). This family of transporters is very recently discovered, and few data exist on potential sex differences. No data is available for the brain, but it appears that hepatic mRNA of MATE1 was notably increased in females in relation to males, but on the contrary, renal mRNA expression was found notably lower in females compared to males (Lickteig et al., 2008; Fu et al., 2012).

OTHER TRANSPORTERS

Several other transporters, of which relatively little is known, are located at the BBB. Alanine/serine/cysteine transporter 2 (ASCT2) is located at the abluminal membrane of BACEs and is the only transporter of the Solute Carrier 1A (SLC1A) family to transport glutamine (Albrecht and Zielinska, 2019). BBB also expresses Betaine/GABA transporter-1, which in mice can be found as GAT2 transporter, regulating the efflux of GABA, and is different from GABA transporters, GAT1/3, that mediate transport across neurons and astrocytes (Takanaga et al., 2001). Enkephalins and AVP are effluxed by Peptide Transport System 1 and 2, respectively (Banks, 2006; Ueno et al., 2010). Several sodium-coupled transporters (NHE1, NHES, NBCn1, and NKCC1) are implicated in the active transport of lithium, a mood stabilizer across the BBB (Luo et al., 2018). System A and System L are transport systems of small and large neutral amino acids, respectively. Several drugs are carried by system L into the brain, and there is a strategy to design drugs that resemble the amino acids L-histidine and L-tryptophan for enhanced CNS delivery through LAT1 transporter (Hall et al., 2019). However, for all those transport systems little is known about their potential sex differences.

DISCUSSION

In this minireview we summarized findings about sex differences in brain transport systems. These may affect pharmacokinetics of psychotropic medications in a sex-dependent manner and are important for precision medicine and treatment. In summary, for many transporter systems little is known about their function and the role of sex and gonadal hormones. Some protein transporters are indeed recently discovered, but for many other, evidence accumulates at a slow pace. Moreover, data are more abundant for the peripheral expression and function of these transporters, and less is known about the BBB, with the exception perhaps of the P-gp. This is surprising, as brain-transport systems regulate the influx and massively the efflux (clearance) of psychotropics. Moreover, BBB dysfunction has been implicated in many neuropsychiatric disorders and other diseases which are sex-differentiated (Greene et al., 2020; Profaci et al., 2020; Dion-Albert et al., 2022b). Admittedly, studies on peripheral transporters are methodologically easier, especially in humans where access to the BBB is significantly hindered. However, preclinical studies are also lacking, and more research is needed on which psychotropics are substrates of which BBB transporter system and whether this is sex-differentiated. This research could lead to clinical important findings regarding the treatment of psychiatric disorders in a more precise way.

Despite the paucity of evidence, preclinical studies collectively support the notion of male and female predominant transporters mainly in the periphery (Maher et al., 2005; Klaassen and Aleksunes, 2010; Zhu et al., 2017; Basit et al., 2019). The existence of protein transporter systems in the periphery also adds another layer of complexity in understanding their impact on pharmacokinetics. Most, if not all, of those transporters are heavily expressed in peripheral tissues (intestine, liver, and kidneys) that are crucially implicated in absorption, distribution, and metabolism of drugs. Peripheral transporters play as much an important role in psychotropic pharmacokinetics as do the BBB transporters in delivering to and clearing psychotropics from the brain. Therefore, a psychotropic that is a substrate for a specific transporter may be more extensively absorbed, more broadly distributed and at the same time more readily cleared from the brain and then metabolized and excreted. It remains unknown whether these effects cancel themselves out and, in the context of this review, whether male or female sex affects those transporters equally, in all of their expression sites (brain and periphery) (Cummins et al., 2002; Gottschalk et al., 2011). It is possible that their function is also influenced locally by estrogens – or other steroid – receptors in the BBB. These local interactions represent an interesting new research pathway that could promote our understanding of the BBB and its transporter proteins in the healthy and diseased brain in a sex-dependent way.

Indeed, transporter function, and thus potential sex differences, are not necessarily identical in peripheral tissues (such as in liver, kidneys, and intestine) and the brain. Although transporters present significant, but not absolute conservation across species, some sex differences observed in one species are not confirmed in another. Therefore, future research should focus on whether findings from one species to another are translatable, regarding both substrates for each transporter, as well as on the significance of potential sex differences of transporters in relation to human disease and treatment. A recent study on P-gp comparing gastro-intestinal tissue from Wistar rats and humans confirmed the translatability of experimental findings on discovered sex differences (Mai et al., 2021). P-gp activity is altered in patients with depression and recent evidence, in post mortem brain, suggest that vascular alterations in the BBB are present in women with depression (de Klerk et al., 2009; Dion-Albert et al., 2022b). Interestingly, BBB dysfunction has been associated with many other diseases, such as dementia, autoimmune disorders, epilepsy, and stroke, that also present sex differences and often co-exist with depression (Greene et al., 2020; Profaci et al., 2020). Therefore, future studies should investigate sex differences in specific transport proteins of the BBB in relation to its dysfunction during depression and other comorbidities. Moreover, transporter activity may be affected by factors such as stress, disease, exercise, or diet in a brain-region specific manner. Indeed, chronic variable stress altered BBB integrity in female, but not in the male mouse prefrontal cortex and this could have contributed to stress vulnerability (Dion-Albert et al., 2022b).

This mini-review focused on sex differences in psychotropic transport across the BBB. As the purpose of such sex differences remains unclear, it is postulated that the mammalian reproductive process exerted a selection pressure that explains those sexual dimorphisms (Gilks et al., 2014; Della Torre and Maggi, 2017). As elegantly reviewed elsewhere, this is reflected to several sex differences at the BBB in health and disease, regarding, but not limited to BBB strength, metabolism, response to stressors and involvement of several pathways, classic and non-classic genomic, as well as non-genomic, involving NO signaling, matrix metalloproteinases, the RhoA/Rho-kinase-2 pathway and other estrogens-mediated pathways (Weber and Clyne, 2021; Dion-Albert et al., 2022a).

In conclusion, accumulated evidence supports the existence of several sex differences in expression and activity of BBB transporters, and a further modulating role of gonadal hormones. A closer study of sex differences in the active influx and efflux of psychotropics from the brain may provide a better understanding of sex-dependent brain pharmacokinetics and pharmacodynamics of psychotropics. This would have a significant impact in precision medicine and treatment. Furthermore, in combination with BBB permeability studies, research on sex differences in BBB transporters will contribute to our understanding of the neurobiology and treatment of psychiatric diseases and their relationship with other disorders, such as autoimmune and neurological.

AUTHOR CONTRIBUTIONS

D-GS and TG searched the literature and gathered relevant evidence. PP compiled the first draft. NK and CD conceived and coordinated the study, mastered the final and revised manuscripts, and provided guidance. All authors contributed and approved the manuscript.

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Limbic Responses Following Shock Wave Exposure in Male and Female Mice

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Blast traumatic brain injury (bTBI) presents a serious threat to military personnel and often results in psychiatric conditions related to limbic system dysfunction. In this study, the functional outcomes for anxiety- and depressive-like behaviors and neuronal activation were evaluated in male and female mice after exposure to an Advanced Blast Simulator (ABS) shock wave. Mice were placed in a ventrally exposed orientation inside of the ABS test section and received primary and tertiary shock wave insults of approximately 15 psi peak pressure. Evans blue staining indicated cases of blood-brain barrier breach in the superficial cerebral cortex four, but not 24 h after blast, but the severity was variable. Behavioral testing with the elevated plus maze (EPM) or elevated zero maze (EZM), sucrose preference test (SPT), and tail suspension test (TST) or forced swim test (FST) were conducted 8 days–3.5 weeks after shock wave exposure. There was a sex difference, but no injury effect, for distance travelled in the EZM where female mice travelled significantly farther than males. The SPT and FST did not indicate group differences; however, injured mice were less immobile than sham mice during the TST; possibly indicating more agitated behavior. In a separate cohort of animals, the expression of the immediate early gene, c-Fos, was detected 4 h after undergoing bTBI or sham procedures. No differences in c-Fos expression were found in the cerebral cortex, but female mice in general displayed enhanced c-Fos activation in the paraventricular nucleus of the thalamus (PVT) compared to male mice. In the amygdala, more c-Fos-positive cells were observed in injured animals compared to sham mice. The observed sex differences in the PVT and c-Fos activation in the amygdala may correlate with the reported hyperactivity of females post-injury. This study demonstrates, albeit with mild effects, behavioral and neuronal activation correlates in female rodents after blast injury that could be relevant to the incidence of increased post-traumatic stress disorder in women.

Keywords: anxiety, blood-brain barrier, c-Fos, depression, limbic, post-traumatic stress disorder, blast traumatic brain injury (bTBI), righting reflex

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INTRODUCTION

Blast exposure is the leading cause of traumatic brain injury (TBI) in military personnel and a serious threat to civilian populations in proximity to regional conflicts and civil unrest. Blast TBI (bTBI) is considered the "invisible wound" in modern day combat zones, such as Iraq and Afghanistan, and milder injury with ∼80% prevalence is the most common form. Blast exposure has been associated with a variety of psychiatric conditions, including post-traumatic stress disorder (PTSD), depression, and anxiety (Walker et al., 2015). Post-mortem examination of chronic bTBI cases also found histories of enduring neuropsychiatric symptoms (Rosenfeld and Ford, 2010; Shively et al., 2016; Mac Donald et al., 2017; Ryan-Gonzalez et al., 2019).

Preclinical reports have shown central nervous system (CNS) limbic system areas are particularly vulnerable to bTBI, and are often associated with neurobehavioral changes related to anxiety, depression, and PTSD (Elder et al., 2012; Blaze et al., 2020; Kostelnik et al., 2021). Alterations in the basolateral amygdala (BLA) have been reported following bTBI with changes to neuronal cytostructure, gene expression, and neuroimmune responses with associated anxiety-like behavior (Sajja et al., 2015; Ratliff et al., 2019; Blaze et al., 2020). Likewise, blast injury in rats altered a marker associated with PTSD, stathmin 1, in the amygdala, but not in the hippocampus (Elder et al., 2012). However, ultrastructural rat hippocampal changes have been reported after blast (Cernak et al., 2001). The paraventricular nucleus of the thalamus (PVT) is another important area for emotion-based responses, especially fear. The PVT is primarily involved in stress, arousal, and motivated behaviors with projections to the amygdala and limbic cortex (Kirouac, 2015; Azevedo et al., 2020; Rowson and Pleil, 2021), and it was recently found to mediate PVT-central amygdala freezing responses (Ma et al., 2021). The PVT's connection to depressive-like behavior is less understood, but reduced tail suspension test immobility with PVT inhibition has been reported (Kato et al., 2019; Barson et al., 2020). Other cortical changes after bTBI include a decrease in Thy-1 stained cortical neuronal afferents, possibly from the medial prefrontal cortex, which terminate in the BLA (Heldt et al., 2014). These limbic regions are also involved in fear conditioning; a common preclinical model for evaluating PTSD. Blast exposure increased responses in the acoustic startle reflex and anxiety-related behaviors in the elevated plus and zero mazes (Xie et al., 2013; Awwad et al., 2015). Preclinical and clinical imaging data following blast exposure correspondingly indicate greater amygdala activation and long-term anxiety post-bTBI, including region-specific imaging differences in brain metabolism in the amygdala and blood-brain barrier (Matthews et al., 2011; Rubovitch et al., 2011; Jaiswal et al., 2019).

Acute immediate early gene (IEG) responses in the cerebrum are observed after a broad range of stimuli including cell insults, immune activation, apoptosis, neuronal depolarization, and learning and memory experiences (Curran and Morgan, 1995; Raghupathi et al., 1995; Chaudhuri, 1997; Gallo et al., 2018). Alterations in c-Fos expression may play a role in encoding transient stimuli to long-term genetic changes. A marker of neuronal activation, c-Fos expression becomes elevated in the PVT after stressors such as the forced swim test and elevated plus maze (Curran and Morgan, 1995; Gallo et al., 2018; Barson et al., 2020). Changes in c-Fos have been reported as early as 1–3 h after blast exposure in the hippocampus and amygdala (Säljö et al., 2002; Du et al., 2013; Rex et al., 2013; Ou et al., 2022), and elevated levels can persist (Säljö et al., 2002; Russell et al., 2018b). As described earlier, the PVT and central amygdala are involved in fear conditioning, and increased c-Fos activation in these regions was reported in a single prolonged stress mouse model of PTSD (Penzo et al., 2015; Park and Chung, 2019; Azevedo et al., 2020). Only one study has examined IEG response 7 days after a restraint stressor and bTBI, and found sex differences in c-Fos response in the paraventricular nucleus of the hypothalamus (Russell et al., 2018b). Sex differences in functional outcome are also observed with more activity displayed by females compared to males after TBI (Tucker et al., 2016, 2017). One group described increased risk behavior in males in response to negative outcomes, lack of reward during a gambling task with the choice to select a more secure option, compared to female rats (Ishii et al., 2018).

The objective of this exploratory study was to determine how a shock wave exposure alters physiological and behavioral outcomes. To assess the effects of primary and tertiary shock wave injury, the Advanced Blast Simulator (ABS) was utilized as a reliable state-of-the-art model of bTBI (Sawyer et al., 2016). The elevated plus and zero maze (EPM, EZM), sucrose preference test (SPT), tail suspension test (TST), and forced swim test (FST) were performed to assess anxiety- and depressive-like behaviors after ABS, while blood-brain barrier (BBB) and c-Fos neuronal activation were studied for acute pathological changes.

MATERIALS AND METHODS

Animals

Eight-week old male and cycling female C57BL/6J mice (00664) were obtained from the Jackson Laboratory (Bar Harbor, ME, United States) and housed in an AAALAC-accredited animal facility for at least 3 days of acclimation before the experiments were started. All procedures were approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee. Until the SPT was conducted, all animals were group-housed (five per cage), had access to food (Harlan Teklad Global Diets 2018, 18% protein) and water *ad libitum*, and were maintained on a standard 12 h: 12 h light-dark cycle. Animals that underwent the SPT were divided into separate cages with standard enrichment (cotton nestlets and huts) for the duration of the study, but singly housed mice were still able to view neighboring cages. All experimental procedures were performed by female investigators.

Advanced Blast Simulator

Male and female mice were randomly assigned to injured or sham conditions. Injured groups were exposed to a single blast overpressure ∼ 15 psi peak pressure using the USUHS Advanced Blast Simulator (ABS) as previously described (Vu et al., 2018). Briefly, the ABS contains a driver and driven chamber with

pressure transducers placed within the inner wall to monitor incident pressure and shock wave velocity. A pencil gauge probe immediately adjacent to the mouse holder measured incident pressure (Quartz, free-field, ICP blast pressure pencil probe, 50 psi, 104.2 mV/psi, 137B23A, PCB Piezotronics). A membrane consisting of two or three 0.254-mm thick clear acetate sheets (Grafix Plastics, Cleveland, OH, United States) and two layers of vinyl-coated polyester mesh (Pet Screen, Hanover/New York Wire, Cat. No. 70589, mesh size: 14.5 × 10 grids/in^2, wire diameter: 0.635 mm) separated the driver and the driven chambers. Sham-treated mice were anesthetized and placed near the blast chamber, but were not exposed to the blast wave. All mice were first placed in an isoflurane induction chamber (3% isoflurane in 100% oxygen for 4–6 min) and once anesthetized, head and body wraps (a modified tongue depressor and Vet Wrap) were used to minimize movement before the mouse was placed inside the simulator. A hatch in the driven chamber allowed the ABS mouse to be secured in a mesh holder (same material as the membrane, 14cm × 15cm with a cross-sectional areal occlusion of ∼5.6% for the shock wave) supported by metal posts (12.7 mm diameter, about 2.9 m distal to the driver membrane) in a vertical orientation, exposing the ventral surface of the mouse to the oncoming blast wave. Once the hatch to the driven chamber was tightly sealed with the mouse inside the ABS, compressed air was allowed to accumulate in the driver end, until the pressure (150–160 psi) was great enough to rupture the membrane and the shock wave travelled down the ABS to where the animal was placed (about 2.9 m away from the membrane). After the blast wave was delivered, the mouse was removed from the mesh pocket and assessed for the occurrence of apnea. Sham-treated mice were placed in a clean cage in a supine position outside of the ABS, and the latency to recover the righting reflex was recorded for all mice. Once both ABS and sham animals regained consciousness, they were returned to their home cages and provided with acetaminophen (Tylenol) in their drinking water (1 mg/ml; ∼200 mg/kg b.w. for 24 h). Animals were weighed both immediately before and one day after ABS exposure.

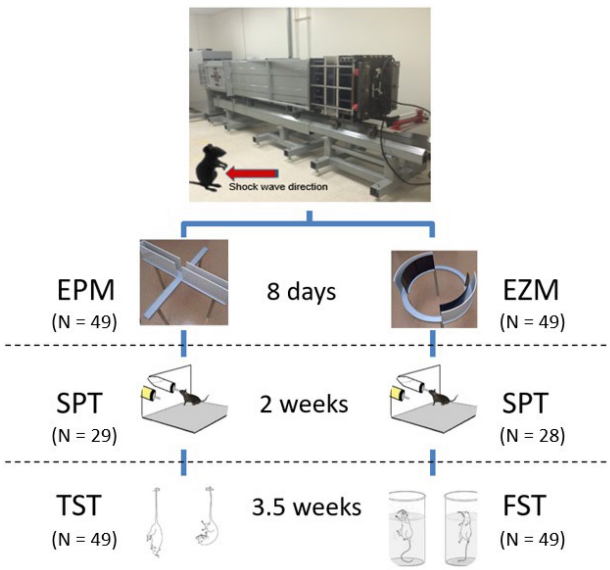

FIGURE 1 | Advanced Blast Simulator and behavioral testing paradigms. A single shock wave (∼15 psi) was delivered with the mouse in an upright position with the ventral surface of the mouse exposed to the oncoming shock wave (see Vu et al., 2018 for a detailed description of the ABS). Eight days post-injury, animals underwent either EPM or EZM testing followed by the SPT 2 weeks post-injury or sham treatment, and lastly either TST or FST 3.5 weeks following ABS. Images reproduced from Abelaira et al. (2013), with permission from the Brazilian Journal of Psychiatry. Images reproduced from Brigman et al. (2010), Tucker and McCabe (2017).

Behavioral Testing

Animals were randomly assigned to different behavioral task cohorts. The five cohorts consisted of n = 18–20 mice evenly distributed between males and females as well as injured and sham conditions (**Figure 1**). Following ABS as described above, each cohort underwent one of two testing paradigms: Elevated Plus Maze (EPM, 8 days post-injury) then Sucrose Preference Test (SPT, 2 weeks post-injury) and Tail Suspension Test (TST, 3.5 weeks post-injury), or Elevated Zero Maze (EZM, 8 days post-injury) then Sucrose Preference Test (SPT, 2 weeks post-injury) and Forced Swim Test (FST, 3.5 weeks post-injury).

Elevated Plus Maze

The EPM (Stoelting, Wood Dale, IL, United States) was conducted 8 days following ABS to assess anxiety-like behavior (Pellow et al., 1985). The EPM is a cross-shaped platform with equal 35 cm length and 5 cm width arms raised 50 cm above the floor. Two opposite side arms are "open," with 1 cm high edges and the remaining arms are "closed" with opaque, dark 16 cm high walls (**Figure 1**). Animals were allowed to acclimate to the room for 30 min before testing began. Overhead fluorescent lights illuminated the maze during testing with 1600 lux illuminance for the open arms and 200 lux for the closed arms. To start the test, individual mice were placed at the 5 cm center square region and allowed to explore the maze for 5 min. A ceiling camera and Any-Maze software (Stoelting) tracked animal movement throughout the test and were used to calculate the time spent in the open and closed arms, distance travelled, and number of entrances to the open arms during the session.

Elevated Zero Maze

The Stoelting EZM (Shepherd et al., 1994) as previously described by Tucker et al. (2017) was performed 8 days post-injury to examine anxiety-like behavior. Briefly, the EZM is a 60 cm diameter ring platform raised 50 cm above the floor. The ring is divided into four equally sized areas with two opposite side "open" quadrants and two remaining "closed" quadrants (**Figure 1**). The open quadrants have 1 cm high edges and are exposed to 1600 lux light from overhead fluorescent lamps whereas the closed quadrants have 16 cm high dark, opaque walls and have a brightness of 200 lux. All quadrants have 5 cm width lanes. Animals spent 30 min acclimating to the room before starting the test. At the beginning of the test, mice were placed individually at an arbitrary boundary between an open and closed quadrant facing the closed quadrant. Animals were allowed to freely explore the maze for 5 min and a ceiling camera tracked the animal movement

throughout the test. Any-Maze software was used to calculate the time spent in the open and closed quadrants, distance travelled, and the number of entrances to the open quadrants during the test.

Sucrose Preference Test

The SPT was administered 2 weeks following injury as a measure of anhedonia. Due to laboratory spatial and time constraints, housed mice cages were randomly selected so that ∼57% of the mice were tested. Cages of mice not selected for testing remained group housed. Individually housed mice were evaluated over the course of 5 days. Mice were offered two 20 mL bottles of 1% sucrose diluted with water placed about 7.5 cm apart at equal heights in their home cages for the first 2 days of testing to acclimate them to the sweet taste and to the new bottles. The amount of sucrose consumed was measured by weighing each bottle daily. On the third day, one bottle was replaced with filtered tap water, so that each mouse was given a choice of drinking solutions of sucrose or tap water. On the fourth day, the positions of the bottles were switched to control for potential side bias. SPT bottles were again weighed and the sucrose preference ratio was calculated for the final 48 h of testing with the following equation: sucrose preference ratio =

$$\frac{consumed\ sucrose}{consumed\ water + consumed\ sucrose}\ x\ 100$$

Tail Suspension Test

The TST to measure depressive-like behavior was performed 3.5 weeks after ABS. As previously described by Can et al. (2012), mice were suspended from their tails from laboratory benches with tape (12 mm wide, 24 cm long). The tape was adhered about 1 cm from the tip of the tail and a 4 cm length hollow polycarbonate tube (1.3 cm inner diameter, McMaster-Carr, Santa Fe Springs, CA #8585K41) was placed around the base of the tail to prevent tail-climbing during the test. Padding was placed below the mice in case of falls and mice were monitored throughout the 6 min test. A standard video camera was used to record the sessions and videos were uploaded into Any-maze. The time spent immobile (defined as animals with minimal movement) during the last 5 min of the session to account for initial test acclimation was later scored using Any-Maze with manual key presses by an investigator blinded to injury condition where a computer key was held down for the duration of animal immobility.

Forced Swim Test

The Porsolt FST (Porsolt et al., 1977) to study learned helplessness was conducted 3.5 weeks post-injury. As described by Tucker et al. (2017), FST chambers (Stoelting) were clear 42 cm in height and 19 cm diameter Plexiglas cylinders. The chambers were filled to a depth of about 25 cm with water at 25°C. Mice were placed into the cylinders for 6 min and allowed to swim or float. Mice were closely monitored from a separate room. Once the test concluded, mice were gently dried with paper towels and placed in a clean cage under a heat lamp to dry. FST cylinders were rinsed and replaced with fresh water for each animal. A standard video camera was used to record sessions and videos were imported into Any-Maze with key presses to measure immobility (defined as animals floating on the water surface with minimal movements). The first minute of the test was not scored to account for initial acclimation to the FST, so an investigator blinded to injury condition only scored the last 5 min of the test.

Immunohistochemistry

A separate cohort of animals, which did not undergo behavioral testing, were exposed to a single ABS shock wave (about 15 psi) and examined for the presence of Evans blue as a marker of blood-brain barrier disruption. An animal restrainer was used to intravenously administer Evans blue (2% diluted in buffer, 0.1 mL per animal) via the tail veil immediately prior to ABS exposure. The mice were euthanized 4 h (n = 25, n = 6–7 per injury and sex condition) or 24 h (n = 15, with Evans blue injection after ABS) post-injury and tissue was collected for c-Fos immunohistochemistry from only the 4 h group. Briefly, mice were anesthetized with a mixture of ketamine and xylazine and then transcardially perfused with cold phosphate buffer solution (0.1 M) and 4% paraformaldehyde in phosphate buffer. Brains were dissected and further fixed in paraformaldehyde for an additional 24 h. They were then transferred to 20% sucrose solution in phosphate buffer for 72 h before freezing the tissue and storage (−80°C) until sectioning. A Leica microtome was used to cut 30 μm thick coronal sections. The tissue was initially washed in tris-buffered saline with 0.05% triton (TBS-T). Sections were then processed with 0.3% hydrogen peroxide for 30 min and afterwards washed with TBS-T again before blocking buffer (TBS-T with 0.20% triton, goat serum, and 10% bovine serum albumin; BSA) incubation for 1 h at room temperature. C-Fos (1:1000; Millipore Sigma Cat: ABE457, Lot: 3585299) antibody was applied to the sections before storage at 4°C overnight. The next day, sections were washed with TBS-T and secondary antibody goat-anti-rabbit IgG (1:1000 Jackson Immunoresearch Cat: 111-065-003, Lot: 117316) was applied in blocking buffer (TBS-T with 0.05% triton, goat serum, and 10% BSA) for 1 h at room temperature. Sections were again washed with TBS-T before incubation in ABC solution (Vectastain ABC HRP Kit, PK-4000, Vector Laboratories) for 45 min at room temperature. The tissue was washed a final time with TBS-T prior to DAB development with the DAB Substrate Kit, Peroxidase (HRP), with Nickel, 3,3′-diaminobenzidine (SK-4100, Vector Laboratories) for 1 min. The free-floating sections were mounted onto glass slides and left to dry overnight. Lastly, sections were dehydrated in ethanol gradients (75–100%), cleared in xylene, and cover slipped with Permount mounting media the next day for analysis. Positive, activated neural tissue from restrained ABS-exposed mice, and negative, tissue processed without primary antibody, controls were included in all immunohistochemistry procedures.

Three regions of interest (ROIs), bilateral cerebral cortex, bilateral amygdala, and the PVT, were analyzed for c-Fos staining. Six mice per group were selected for Carl Zeiss El-Einsatz model #451485 light microscope imaging. Images for the PVT were taken at 100× magnification, the cerebral cortex at 50×, and the amygdala was captured at 25× magnification. To quantify c-Fos in each ROI, the threshold and particle analysis functions

on Image J software (NIH) were used for cell counts on black/white c-Fos images. The particle count was employed as an estimate for the number of c-Fos positive cells. The values were averaged for three or more sections per animal. Cresyl violet (Chroma-Gesellschaft Schmid GmbH & Co 1 A 396) and H&E (haematoxylin Sigma-Aldrich Cat: GHS132-1L, Lot: SLCH6216 and eosin Sigma-Aldrich Cat: HT110332-1L, Lot: SLCJ2544) staining were performed to indicate anatomical consistency for the ROIs and for microbleed analysis, respectively.

Statistics

GraphPad Prism version 8.42 (GraphPad Software, San Diego, CA, United States) and SPSS version 27.0.1.0 (IBM, Armonk, NY, United States) were used for statistical analysis and figure generation. Body weights were measured on the day of injury and 24 h later. Since there was a noticeable difference in baseline (before injury) body weights, a two-way Injury × Sex analysis of variance (ANOVA) was performed for the mice as a percentage of body weight loss after sham or injury treatment. Mann-Whitney U tests were performed to analyze righting time data using within sex comparisons and between sex comparisons for male ABS and female ABS groups. Photographs of the brains from the cohort of mice that were euthanized after Evans blue infusion and ABS exposure were visually ranked for degree of staining. The rankings for Evans blue staining intensity were evaluated with respect to righting reflex to determine if there was an association between staining and the duration of the righting reflex. Due to smaller samples sizes and score ties, the SPSS program to determine Kendall's tau (τ) was employed and the SPSS bootstrap procedure for estimation of the 95% confidence interval for the correlation coefficient, based upon 1000 bootstrapped samples, was performed.

Two-way ANOVAs (injury × sex) were performed for all behavioral tasks (EPM, EZM, SPT, FST, and TST). Behavior graphs depict the scores for individual animals and the embedded horizontal bar shows the arithmetic means for each group. Histology for c-Fos was analyzed using two-factor (injury × sex) ANOVAs. $p < 0.05$ was considered significant.

RESULTS

Shock Wave Characteristics, and Mouse Body Weight, Righting Times, Apnea, Morbidity

The ABS produced a characteristic Friedlander-like curve with a consistent peak pressure across the study (mean 15.56 psi, coefficient of variation = 4.5%) (**Figure 2**). The shock wave velocity was approximately 469.73 m/s and the positive and negative phases were 5.62 and 8.40 ms, respectively, with an average impulse (pressure × time) of 0.0358 psi × s.

Figure 3A summarizes the body weights of individual female and male mice before and after sham or ABS exposure. Since the baseline (preinjury) body weights for the mice in the ABS and sham groups were not equivalent, an Injury × Sex ANOVA was computed using the percentage change in body weights after

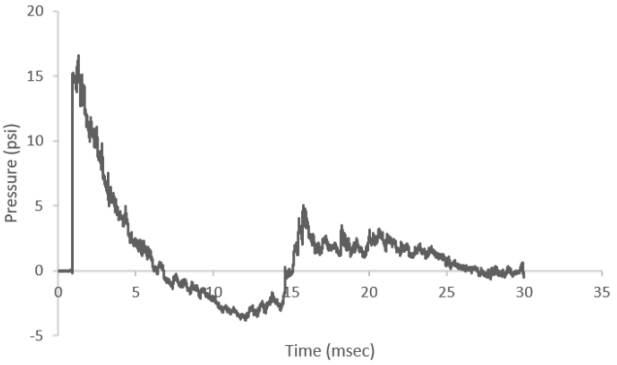

FIGURE 2 | Representative Friedlander-like ABS waveform. The ABS device produced a waveform with an average of 15.56 psi peak incident pressure and 469.74 m/s shock wave velocity. The shock wave positive phase duration was 5.62 ms and negative phase duration was 8.40 ms.

ABS injury or sham treatment compared to pre-injury body weights. The average percent change in body weight was −2.23 and 0.574% for the female and male mice, respectively, on the day after sham treatment, while the females lost −8.22 and the males −5.79% of their preinjury body weight after ABS exposure. The ANOVA indicated there was a significant overall main effect between the females and males in percentage of body weight loss before to after sham or ABS treatment (−2.619% change, $F_{1,76} = 24.214$, $p < 0.001$) and a difference in the percentage of body weight loss after ABS injury compared to sham treatment (−7.002%, $F_{1,76} = 134.584$, $p < 0.001$), but no interaction effect to suggest the injuries had a different effect on female and male mice ($F_{1,76} = 0.123$, $p = 0.727$).

Behavior and Evans blue-treated cohorts were combined for righting reflex analysis. Mann-Whitney U analyses showed injured animals required a longer time to regain consciousness compared to sham mice. Female shock wave exposed mice (462.5 s median) had increased righting times compared to female sham mice (46.0 s median; $U = 3$, $p < 0.0001$) and male shock wave exposed mice (316.0 s median) displayed longer righting reflexes than male sham animals (43.0 s median; $U = 1.5$, $p < 0.0001$). A third Mann-Whitney test showed injured male and female mice were significantly different, with male animals exhibiting overall shorter reflex durations than female mice ($U = 370.5$, $p = 0.0026$, **Figure 3B**). Five cases of apnea, ranging from about five to thirty seconds, were observed in two male and three female injured animals. One case with about 5 s of apnea corresponded to the longest female ABS righting time. Six animals died following ABS. Five female mice died immediately after injury and one male mouse died 4 days after ABS exposure.

Anxiety-Like Behavior

For the EPM and EZM, injured mice overall spent less time in the open regions, but the two-way ANOVAs were not statistically significant (**Figures 4A,B**). No statistical differences were detected for either EPM or EZM test in terms of the amount of time spent in or the number of entries into the open areas (**Figures 4C,D**). No difference in the total distance travelled

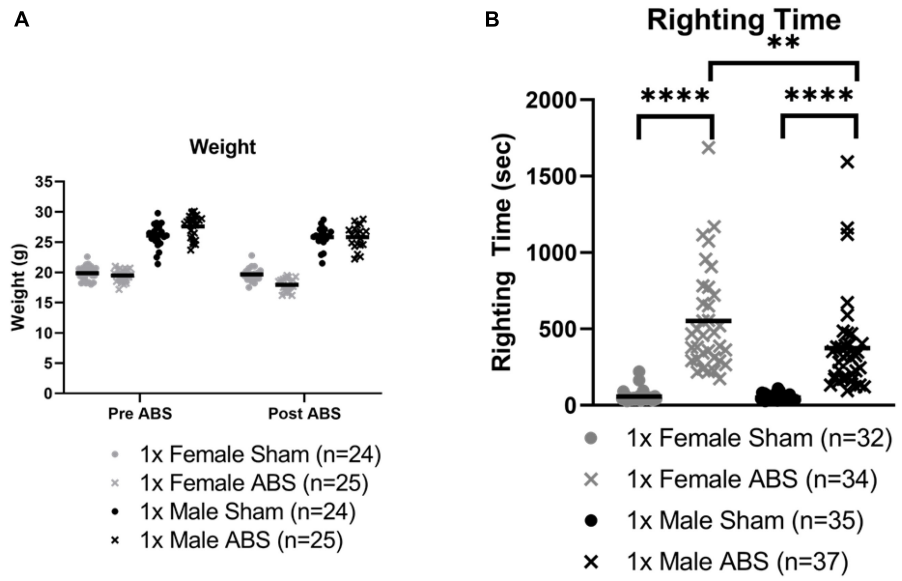

FIGURE 3 | Weight and righting time changes after bTBI. **(A)** Animal weight before and one day after ABS exposure for the behavior cohort of mice. Due to differences in baseline body weights, the percentage change in body weight for each mouse was computed for pre- and post-injury sham or ABS treatment (data not shown; see text). ANOVA indicated there was a significant greater difference in females compared to males overall and a significantly greater percentage of body weight loss in ABS animals, but no sex differences due to injury. **(B)** Righting times of all cohorts, behavior and Evans blue, immediately after a single blast. The Mann Whitney U test ($U = 3$, $p < 0.0001$) demonstrated that the duration of the righting reflex was longer for shock wave exposed females compared to female sham mice. Male injured mice also demonstrated a longer righting reflex compared to sham counterparts ($U = 1.5$, $p < 0.0001$). A comparison of righting reflex duration in injured mice indicated the responses after shock wave exposure in males and females were significantly different ($U = 370.5$, $p = 0.0026$) with longer righting times for female mice. Bars indicate means. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$. No significant correlations were found between righting reflex and loss of body weight ($\tau = 0.000$, $p = 1.000$) or percentage loss of body weight ($\tau = 0.032$, $p = 0.846$) for females, or righting reflex and loss of body weight ($\tau = -0.167$, $p = 0.312$) or percentage loss of body weight ($\tau = -0.105$, $p = 0.516$) for males.

during EPM testing was evident, but for the EZM there was a main effect of sex in which female animals travelled a greater distance compared to male mice ($F_{1,45} = 6.999$, $p = 0.0112$, **Figures 4E,F**).

Depressive-Like Behavior

The two-way ANOVA for the SPT data did not detect statistical differences between the sham and injured or male and female mice for sucrose intake. However, the two-way ANOVA for the TST data showed a main effect of injury ($F_{1,45} = 6.763$, $p = 0.0125$) with shock wave exposed animals spending less time immobile than sham-treated mice. No sex differences were reported for the TST. The FST two-way ANOVA indicated no group differences for immobility (**Figure 5**).

Behavioral Testing Sequence Effects

Post hoc analyses to determine whether or not prior behavioral tests affected later testing performance was assessed. Specifically, we evaluated whether or not the EZM or EPM may have affected performance on the SPT, and if subgroups of mice that were or were not evaluated on the SPT may have affected performance on the subsequent TST and FST. The performance ratio measures in mice that previously received testing on the EPM and EZM were compared using a three-factor (Injury × Sex × EPM/EZM Testing). ANOVA indicated there was no significant main effect of EPM vs. EZM on the subsequent SPT ($F_{1,49} = 1.131$, $p = 0.293$). Likewise, a three-factor ANOVA (Injury × Sex × SPT Testing/No SPT Testing) indicated there was no difference on either the TST or the FST as a function of mice having been tested on the SPT (and single housing) compared to mice that had not been used on this test (and remained in group housing). The main effect for Injury on the TST was significant, as expected ($F_{1,41} = 8.206$, $p = 0.007$), indicating the time immobile on the TST was less in injured mice compared to the sham animals. For the FST, there were no significant differences between groups on any factors.

Histology

Evans Blue Staining

Evans blue staining was evaluated in a separate cohort of sham and injured animals 4 h post-ABS. No staining appeared on the brain parenchyma of sham animals (**Figure 6**). Of note was the observed variability in Evans blue deposition in the cerebral cortex following injury, and there was a trend (albeit with a small sample size) for the appearance of a more intense uptake in some females. There appeared to be one case of subdural hemorrhage isolated in the left hemisphere of an injured female (top photograph for the Female ABS mice in **Figure 6**) and general staining near the superior sagittal sinus in several animals. A smaller cohort of mice was analyzed for the presence of Evans blue 24 h after shock wave exposure, but no staining was evident in either the injured or sham animals (**Figure 6**). Kendall's tau was computed to determine whether or not there was an

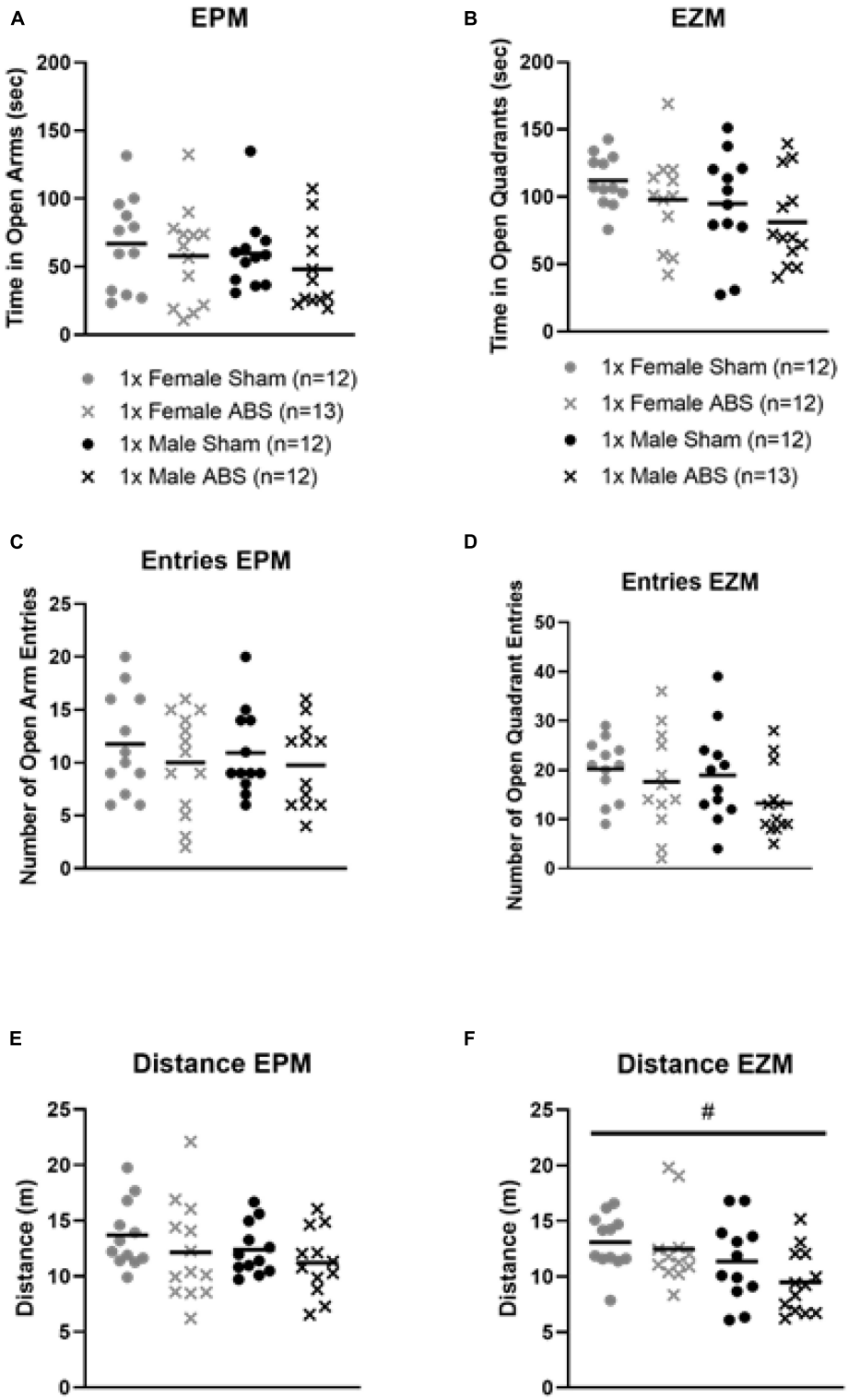

FIGURE 4 | No changes in anxiety-like behaviors on the EPM of EZM after injury, but a sex difference was displayed for EZM distance travelled. The results from the **(A)** EPM and **(B)** EZM testing show no difference in terms of time spent in the open areas for injury or sex. The number of open region entries also did not significantly differ for **(C)** EPM or **(D)** EZM. ABS group and sham animals for each behavioral task. **(E)** The total distance travelled was not statistically different between injured (ABS) or sham mice for EPM. **(F)** Results from the EZM testing indicated there was a main effect of sex where females travelled significantly farther than male mice ($p = 0.0112$). Bars indicate means. # indicates main effect of sex $p \leq 0.05$.

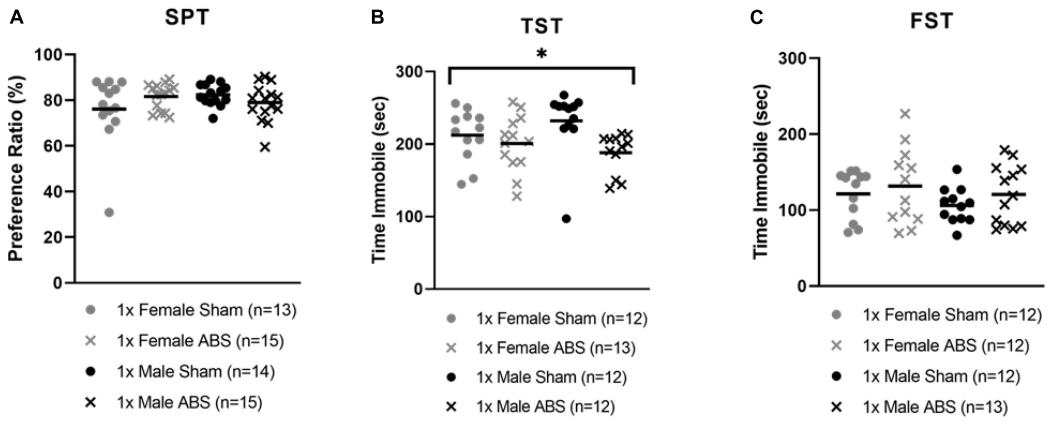

FIGURE 5 | Decreased time immobile on the TST following bTBI. **(A)** SPT did not show any significant difference in sucrose consumption as an effect of shock wave injury or sham treatment. **(B)** TST displayed a significant effect of injury with ABS mice spending less time immobile compared to sham mice. **(C)** The time immobile on the FST was not significant. Bars indicate means. $^{*}p \leq 0.05$ for main effect for the difference between uninjured and ABS exposed mice.

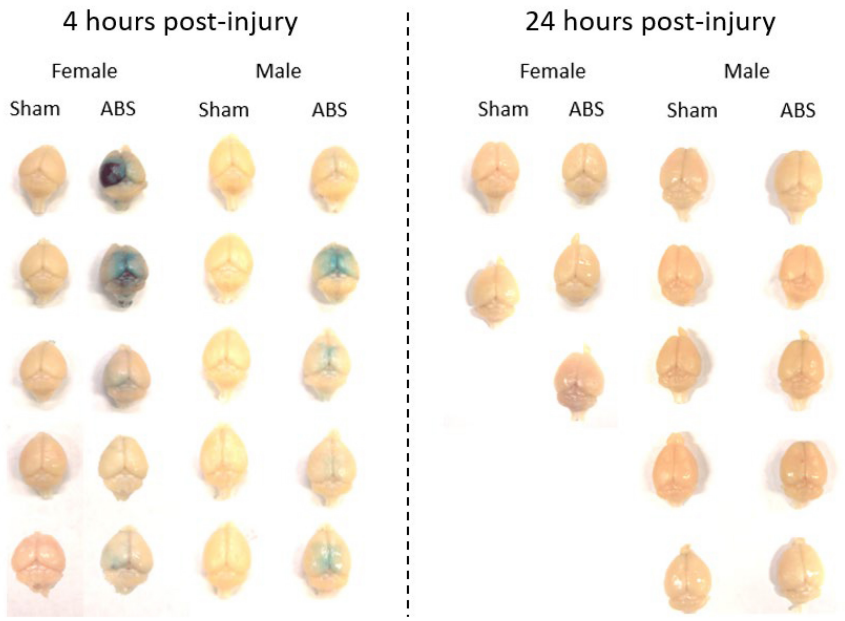

FIGURE 6 | Evans blue was evident 4 but not 24 h after shock wave exposure. Evans blue staining was present on the brain parenchyma of ABS-exposed male and female mice 4 h after injury, but was not apparent on the brains of sham animals. One day after shock wave exposure, Evans blue was not observed on the brain parenchyma of either ABS-injured male and female or sham animals.

association between the intensity of Evans blue staining 4 h after ABS injury and the duration of the righting reflex. There was a significant association, where longer righting reflex duration was associated with more intense Evans blue staining ($\tau = 0.580$, 95% confidence interval = 0.029–0.924, $p = 0.030$). A summary of the measures is presented in **Figure 7**.

H&E and Cresyl Violet

Qualitative analysis of H&E did not reveal any microbleeds in any brain regions in the samples from ABS or sham animals (data not shown). Adjacent sections immunolabeled for c-Fos were stained with Cresyl Violet to confirm consistent anatomical location of the amygdala (data not shown).

c-Fos

Data are presented collapsed across anatomical sides. No significant differences were observed in the cerebral cortex (**Figures 8A,D**). There was a main effect of sex for staining in the PVT ($F_{1,14.497} = 15.781$, $p = 0.001$) with females exhibiting more c-Fos staining compared to male mice (**Figures 8B,E**). There was a main effect of injury in the amygdala ($F_{1,44.567} = 16.036$ $p = 0.001$) with ABS-injured mice expressing more c-Fos positive cells compared to sham mice (**Figures 8C,F**).

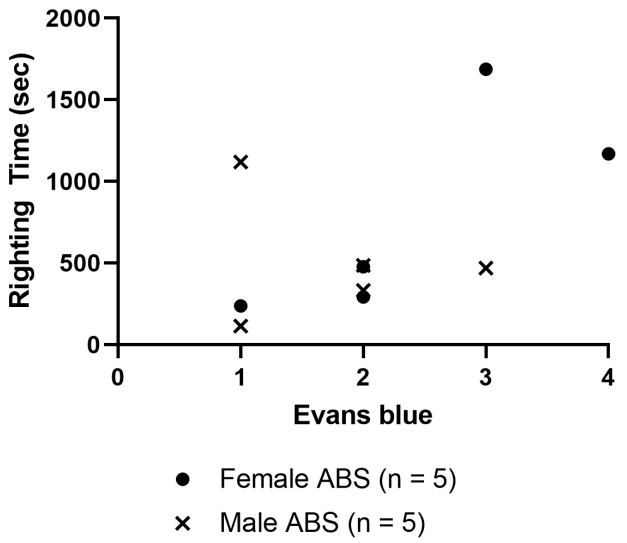

FIGURE 7 | Correlation of righting reflex time and Evans blue staining 4 h post-injury. The scatterplot displays the righting time of injured male and female mice 4 h after shock wave exposure with respect to the amount of Evans blue staining intensity ranked 1 (least) –4 (most), and Kendall tau analysis indicated a significant correlation between stain intensity and the duration of the righting reflex ($\tau = 0.508$, $p = -0.030$). No correlations (data not shown) were detected between Evans blue ranking and peak ABS pressure ($\tau = -0.025$, $p = 0.925$), Evans blue and body weight ($\tau = 0.090$, $p = 0.719$), righting time and peak pressure ($\tau = 0.244$, $p = 0.325$).

DISCUSSION

Physiological Changes After Blast Traumatic Brain Injury

The current study found that male and female mice experienced longer righting reflex times after shock wave exposure compared to sham animals, and female ABS-exposed mice expressed longer periods of unconsciousness than male injured mice. The sex differences observed for righting times may be correlated with variations in body weight. As expected, males were larger than females, which may affect injury-related biomechanics during the shock wave exposure, causing the smaller female mice to move more during the blast event. The decrease in body weight following shock wave, as well as the increase in righting reflex for animals in the injured condition, indicate that the shock wave produced physiological effects. The loss in body weight suggested that, acutely, injured animals were physically unable or uninterested in eating following ABS. The observed weight loss and longer righting reflexes after ABS are consistent with a recent ABS study in the laboratory with identical conditions (Nonaka et al., 2021), as well as other reports (Schindler et al., 2020). Previous experiments demonstrated only transient changes in water and food consumption post-blast with vertical orientation exposure, or no differences in male and female body weight after ABS when animals were placed in a prone position (Russell et al., 2018b; Vu et al., 2018). The sex differences in weight and righting time immediately following bTBI suggest that female mice sustained greater damage initially compared to male mice.

Effects of Blast Traumatic Brain Injury on the Blood-Brain Barrier

Behavioral and neuropathological changes have been reported in blast studies in which the animal is placed in a vertical orientation (Koliatsos et al., 2011; Vu et al., 2018; Nonaka et al., 2021) and in a prone position (Bailey et al., 2016; Sawyer et al., 2016; Russell et al., 2018a; Arun et al., 2020). Body orientation may be particularly relevant to cases of tertiary blast effects. The BBB, for example, is an essential element in brain homeostasis and is one of the first sites to be altered following bTBI, and is a structure particularly vulnerable to impact compression (Gama Sosa et al., 2014; Huber et al., 2016). Acute cerebral vascular impairment and BBB protein dysregulation have been reported after single and multiple ABS exposure, respectively (Heyburn et al., 2019, 2021; Rodriguez et al., 2019), and our data support these findings with acute BBB permeability to Evans blue 4 h following ABS. Finite element modeling and shock tube experiments demonstrate that the prone orientation produces a lesser degree of damage compared to other body orientations (Hubbard et al., 2014; Heyburn et al., 2019; Unnikrishnan et al., 2021). In this study the vertical orientation was employed and Evans blue staining provided evidence of BBB disruption, but it was evident only on the dorsal surface of the brain. This suggests that the fixed vertical animal positioning enabled tertiary, acceleration artifacts from the shock wave-associated blast wind that pushed the mouse backwards, compressing the top of the head into the mesh holder. Interestingly, (Unnikrishnan et al., 2021) used almost identical peak pressure (100 kPa or 14.5 psi) modeling of vertical orientation inside of the ABS, and reported higher pressure on the ventral brain than the dorsal side.

Assessment of the degree of Evans blue staining on the dorsal surface of the neuraxis, 4 h after ABS injury, was associated with the duration of the righting reflex, suggesting longer righting reflex duration was reflected in greater severity of BBB compromise (**Figure 7**). However, although Evans blue staining was present in some injured animals 4 h after bTBI, H&E staining did not indicate the presence of microbleeds and no c-Fos changes were observed in the cerebral cortex, suggesting that the extent of the damage was limited to albumin extravasation to the superficial surface, but not deeper cortical layers (Weng et al., 2011). The damage, however, might be transient since only subtle changes in behavior were observed at later time points (Yang et al., 2016). Another study demonstrated the extravasation of dyes smaller than 70 kDa with barrier integrity restoration one day post-blast (Hue et al., 2015), which is consistent with the observed absence of Evans blue 24 h following shock wave exposure in the current study, indicating that the BBB may be repaired in rodents one day after bTBI. Evans blue has been used historically, but caution must be used with extravasation interpretation and its quantification continues to present challenges (Saunders et al., 2015). Taken together, many potential artifacts are present in preclinical bTBI designs and additional complications with Evans blue

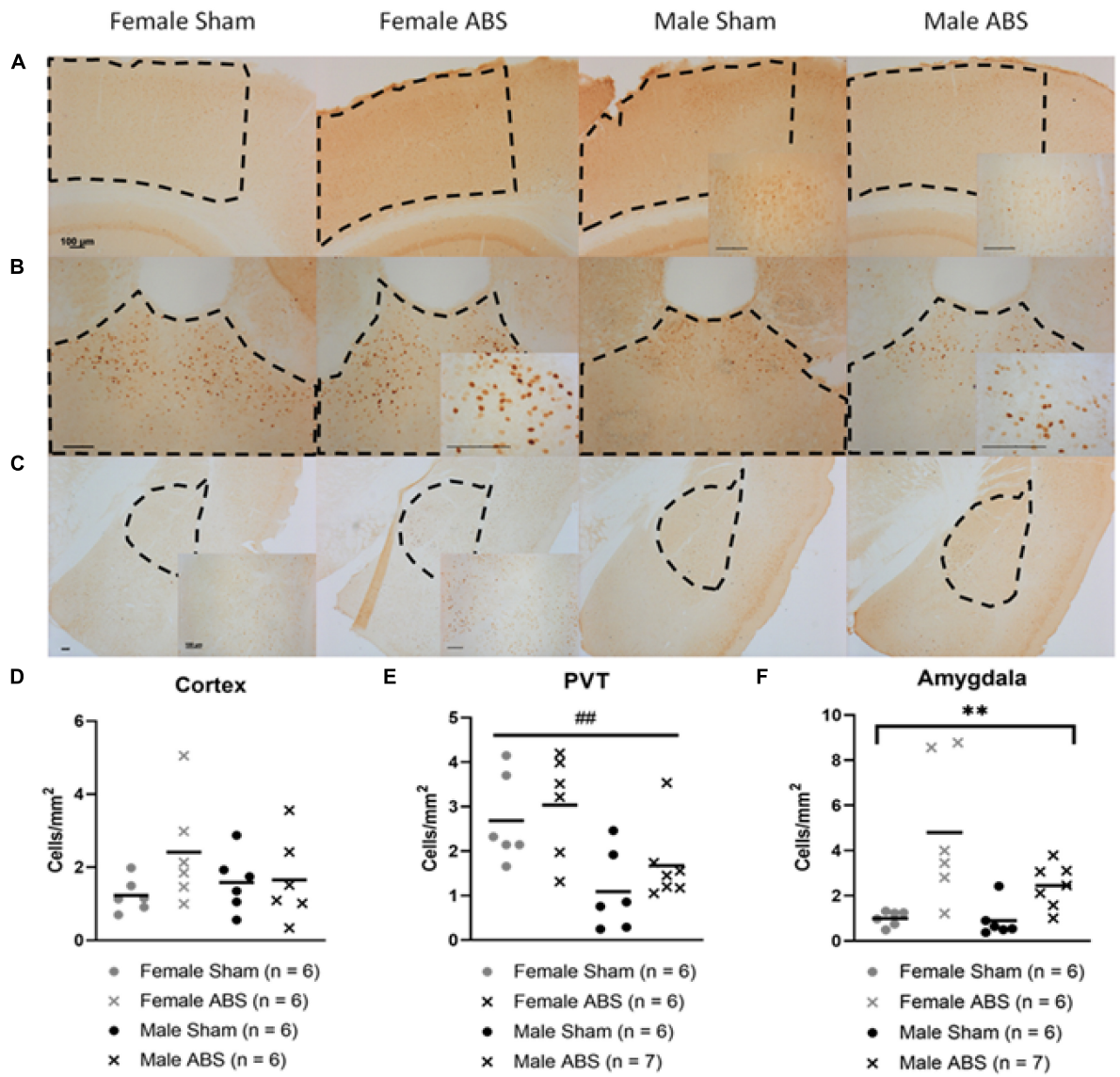

FIGURE 8 | c-Fos differences by sex and injury in the PVT and amygdala, respectively. Representative images of the **(A)** cerebral cortex, **(B)** PVT, and **(C)** amygdala (basolateral and central nucleus) with regions of interest outlined in dashed lines and magnified inserts of areas within the measured regions. All scale bars indicate 100 μm. **(D)** The cerebral cortex did not show any significant difference between injury or sex. **(E)** The PVT displayed an overall effect of sex in which females had more c-Fos positive cells compared to males. **(F)** There was a main effect of injury in the amygdala, where injured mice had greater c-Fos cell density than sham mice. Bars indicate means. ## indicates main effect of sex $p \leq 0.001$, ** indicates main effect of injury $p \leq 0.001$.

quantification, including observed variability in stain intensity, hinder definitive conclusions from being made regarding BBB disruption in this study.

Acute Changes in c-Fos Activation in the Amygdala and Paraventricular Nucleus of the Thalamus Following Blast Traumatic Brain Injury

In addition to BBB disruption, there were increases in c-Fos activation in the amygdala (males and females) following TBI and overall in the PVT (females only). The c-Fos activation reported in this study supports previous investigations of behavioral changes after blast injury through limbic activation. In previous studies, increases in c-Fos appeared in the central and basolateral amygdala and subsequent increases occurred in anxiety-like behavior and fear circuitry (Rowson and Pleil, 2021; Ou et al., 2022). Reports of changes in cytostructure after bTBI also may shed light on possible mechanisms of hyperactivity in the amygdala with increased dendritic branching and spine density after injury (Ratliff et al., 2019, 2020). The sex differences in c-Fos seen in the PVT may correspond to the higher hypothalamic-pituitary-adrenal axis activation in females and maladaptive behaviors in response to stress (Ishii et al., 2018; Rowson and Pleil, 2021). The acute changes in neuronal activation observed primarily in the amygdala after ABS and

overall c-Fos sex differences in the PVT were consistent with some of the behavioral alterations seen at later time points.

Effects of Blast Exposure on Depressive- and Anxiety-Like Behaviors

The mild and transient nature of the bTBI as assessed histologically was associated with only subtle changes in behavior. Previous bTBI studies described increased anxiety-like behavior in the EZM and EPM in injured rats and mice (Elder et al., 2012; Xie et al., 2013; Awwad et al., 2015; Russell et al., 2018a). However, the present study did not find significant differences for time in the open region, nor for the number of entrances into the open zones for EPM and EZM. Some of the differences in reported anxiety-like behavior could be due to the type of shock tube used. One group reporting anxiety-like behaviors following bTBI used a compact, focal blast tube with animal head-only exposure (Awwad et al., 2015), whereas another group used a full body shock tube, but with multiple blast injuries (Elder et al., 2012). A different design of repetitive, automated blast exposures was implemented in an air blast chamber with anxiety-like behavior performed 5 min after injuries (Xie et al., 2013). Russell et al. (2018a) employed an injury paradigm that closely resembled the current study, but animals were placed in a prone instead of vertical orientation. These differences in animal body orientation and blast tube design alter injury biomechanics and therefore functional outcomes (Needham et al., 2015; Unnikrishnan et al., 2021).

While there was no change in the total distance travelled in the EPM, females travelled farther than males in the EZM. Hyperactivity has been observed in females during open field tests following controlled cortical impact (Tucker et al., 2016, 2017). Likewise, a recent study with repetitive concussive injuries directed at the frontal region likewise observed increased distance travelled in the open field test, and that female mice travelled farther in an acute test 4 days after injury, but not at later time points (Vu et al., 2021). The higher levels of activity, particularly in females as observed in the EPM and in open field in other studies, could be related to activational effects of estrogen (Morgan and Pfaff, 2002; Ogawa et al., 2003), but further investigation is needed to understand the mechanism and duration of this increased activity in female mice after blast.

Behavioral tasks measuring depressive-like behaviors showed varying trends. No sex differences were found, but the injured group had reduced immobility in the TST compared to sham-treated controls, an unexpected finding. Reduced immobility after blast exposure may indicate injury-induced agitation or a panic-like reaction and has been reported after other concussive TBI models (Anyan and Amir, 2018; Tucker et al., 2019). Interestingly, a study of CRF neurons after bTBI and restraint-induced stress found sex differences with females showing increased amygdala CRF2 gene expression and hippocampal decreased expression, whereas males displayed the opposite (Russell et al., 2018a). Increased neuronal activation in the amygdala of injured females was consistent with the current study. Hyperarousal is an element of PTSD and as discussed previously, blast is able to induce PTSD-like traits where greater activity levels during the TST in injured animals may correspond to the hyperactivity observed in female mice in the EZM (Elder et al., 2012; Perez-Garcia et al., 2019). Activity level, however, has been criticized as an inaccurate measurement of anxiety (Lister, 1990; Tucker and McCabe, 2021). The TST and FST are arguably models of acute stress, which could be interpreted as changes in anxiety rather than depression (Lister, 1990; Nestler and Hyman, 2010; Tucker et al., 2017; Anyan and Amir, 2018). Further study is needed to define the relationship between bTBI and subsequent hyperactivity.

Study Limitations and Future Prospects

Results from behavioral tests can depend on the animal strain and sex (McCabe and Tucker, 2020). Overall, female rodents exhibit more activity compared to males during behavioral tasks and this inherent difference can make data interpretation challenging (Tucker et al., 2016, 2017). Females may also have subtle neuroprotective effects associated with estrogen and progesterone, but estrous cycle stage does not appear to have a predominant effect on behavioral outcome after TBI (Wagner et al., 2004). Likewise, housing conditions with respect to sex differences in behavior should also be considered. Animals in the current study were singly housed for the SPT and remained individually housed for subsequent testing with the TST and FST. In this study, single housing did not affect depressive-like behavior. Increased corticosterone and anxiety-like behavior have been reported in female rodents that are singly housed, whereas individually housed males display less anxiety-like behavior (Brown and Grunberg, 1995; Palanza et al., 2001). A potential confound of this study's histological data included additional animal restraint during intravenous Evans blue administration, but injured and sham-treated mice underwent the same injection procedure. Previous literature demonstrated that blast alone without a stressor could increase contextual fear conditioning (Elder et al., 2012; Perez-Garcia et al., 2019; Perez Garcia et al., 2021). TST and FST alone can cause changes in c-Fos activation (Yanagida et al., 2016; Hiraoka et al., 2017), so separate cohorts of animals were included for behavioral testing and pathology in this study, and no behavioral stress confounds occurred for c-Fos staining (Cullinan et al., 1995; Yanagida et al., 2016; Hiraoka et al., 2017). Since c-Fos activation has been reported in other areas after bTBI, such as the paraventricular nucleus of the hypothalamus and hippocampus, additional brain regions should be investigated (Du et al., 2013; Russell et al., 2018b; Ou et al., 2022) and given reports of different PVT cellular divisions, specific PVT circuitry should be identified (Kirouac, 2015; Gao et al., 2020). Future directions could also include investigation of multiple blast exposures and bTBI with chronic stress as more clinically relevant designs for military populations (Owens et al., 2008; Kontos et al., 2013). The evaluation of later time points after blast for behavioral tasks may indicate delayed or biphasic behavioral changes, such as slowed onset or immediate deficits followed by a recovery and then prolonged alterations (Stemper et al., 2016; Arun et al., 2020; Perez Garcia et al., 2021). This study further highlights the importance of investigating potential sex differences, and indicates overall variance in limbic

activation and functional outcome between male and female rodents following bTBI exposure.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and all procedures were approved by the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

EM, LT, and JM contributed to the experiment design and development and edited the manuscript. PV assisted with ABS training. EM, AF, and YK performed the ABS procedures. EM and LT conducted the behavioral testing. AF assisted with tail injection training and performed tail injections. EM and JL performed the histology. EM and JM performed the statistical analyses and wrote the manuscript and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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