Sex differences in inflammation in the hippocampus and amygdala across the lifespan associations with cognitive bias

Travis E. Hodges  
University of British Columbia

Stephanie E. Lieblich  
University of British Columbia

Rebecca K. Rechlin  
University of British Columbia

Liisa A.M. Galea (liisa.galea@ubc.ca)  
University of British Columbia

Research Article

**Keywords:** adolescence, young adult, middle-age, TNF-α, IL-1β, cognitive bias, doublecortin, dorsal hippocampus, basolateral amygdala, ventral hippocampus

**Posted Date:** July 8th, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-1746768/v1

**License:** This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Background: Cognitive symptoms of major depressive disorder, such as negative cognitive bias, are more prevalent in women than in men. Cognitive bias involves pattern separation which requires hippocampal neurogenesis and is modulated by inflammation in the brain. Previously, we found sex differences in the activation of the amygdala and the hippocampus in response to negative cognitive bias that varied with age. Given the association of cognitive bias to neurogenesis and inflammation, we examined associations between cognitive bias, neurogenesis in the hippocampus, and cytokine and chemokine levels in the ventral hippocampus (HPC) and basolateral amygdala (BLA) of males and females across the lifespan.

Results: After cognitive bias testing, males had more IFN-γ, IL-1β, IL-4, IL-5, and IL-10 in the ventral HPC than females in adolescence. In young adulthood, females had more IFN-γ, IL-1β, IL-6, and IL-10 in the BLA than males. Middle-aged rats had more IL-13, TNF-α, and CXCL1 in both regions than younger groups. Neurogenesis in the dorsal hippocampus was negatively associated with negative cognitive bias in young adult males.

Conclusions: Overall, the association between negative cognitive bias, hippocampal neurogenesis, and inflammation in the brain differs by age and sex. Hippocampal neurogenesis and inflammation may play greater role in the cognitive bias of young males compared to a greater role of BLA inflammation in adult females. These findings lay the groundwork for the discovery of sex-specific novel therapeutics that target region-specific inflammation in the brain and hippocampal neurogenesis.

Highlights

- Adolescent males had more hippocampal inflammation than females after cognitive bias testing
- Adult females had more basolateral amygdalar inflammation than males after cognitive bias testing
- HPC neurogenesis was negatively associated to cognitive bias in young adult males

Background

Major depressive disorder (MDD) affects 20% of the population and is characterized by an array of behavioral, emotional, and cognitive symptoms [1]. Cognitive symptoms of MDD, such as negative cognitive bias, persist in individuals in remission from MDD and are associated with increased relapse rates in these individuals [2–4]. Current treatments are not effective in reducing negative cognitive bias in MDD [5, 6] and the presence of negative cognitive bias can predict the efficacy of antidepressants in MDD [7, 8]. Thus, there is a need to develop novel therapeutics to treat MDD and attenuate negative cognitive bias in MDD. Human females are more likely to present with MDD and display cognitive symptoms of MDD compared to human males [9, 10]. Discovering the underlying mechanisms of negative cognitive bias with a focus on sex will aid in the discovery of precision treatments for negative cognitive bias in MDD.
Pattern separation, the ability to distinguish between highly similar inputs, is impaired in MDD [11–13], is involved in cognitive bias [14, 15], and relies on hippocampal neurogenesis [16–21]. Neurogenesis in the hippocampus declines with MDD and age in humans and in rodent models [22–30]. Further, treatment with antidepressants, such as selective serotonin reuptake inhibitors (SSRIs), is linked to increased neurogenesis in MDD and in rodent models with some suggestion of sex differences [23, 31, 32]. Intriguingly, there are sex differences in pattern separation and neurogenesis in response to pattern separation [33, 34]. However, the association between hippocampal neurogenesis and cognitive bias has not been examined.

Meta-analyses indicate that peripheral cytokines (including interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF)-α) and hippocampal inflammation are increased in individuals with MDD [35–38], indicating inflammation as a biomarker of MDD. Indeed, levels of cytokines are associated with poor treatment response in individuals with MDD, indicating they may play a role in remission [39]. Moreover, there are sex and age differences in proinflammatory cytokine production with higher levels in young and middle-aged women compared to men at baseline and in response to a challenge [40–42]. However, sex differences are seldom examined in studies of inflammation in MDD, even though females may be more susceptible than males to the effects of inflammation on depressed mood [40].

Both inflammation and neurogenesis in the hippocampus influences cognition, including pattern separation [16–21, 43], indicating that both may be involved in cognitive bias. The basolateral amygdala, which is associated with mood regulation, modulates negative affect and depressive-like behavior after immune challenge [44–47] and interacts with the hippocampus to regulate neurogenesis [48]. Further, projections between the ventral hippocampus and the basolateral amygdala are required for fear memory, anxiety, and pattern separation [49–51], but sex differences have not been analyzed. Previously we found greater neural activity in dorsal and ventral hippocampal subregions (CA1, CA3, dentate gyrus) and amygdala subregions (basolateral, lateral, central amygdala) of young adult females compared to young adult males in response to a similar cognitive bias, indicating a sex difference in the role of these regions to negative cognitive bias. Sex differences in the association between inflammation in the hippocampus and amygdala, neurogenesis in the hippocampus, and negative cognitive bias have yet to be examined.

In the present study, we examined sex and age differences in hippocampal neurogenesis and inflammatory cytokines (interferon gamma (IFN-γ), IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, TNF-α) and chemokine (C-X-C motif ligand 1; CXCL1) levels in the basolateral amygdala and ventral hippocampus after cognitive bias testing. We hypothesized that there would be sex differences in the associations of inflammation and neurogenesis with cognitive bias. As cognitive bias changes with age, we examined adolescent, young adult, and middle-aged rodents, and hypothesized that the association between cognitive bias, inflammation, and neurogenesis would differ by age.

**Results**
Males had higher inflammatory cytokines in the ventral hippocampus than females in adolescence. Adolescent males had higher IFN-γ, IL-1β, IL-4, IL-5, and IL-10 levels in the ventral hippocampus compared to adolescent females (p's < 0.006; IFN-γ: sex by age interaction: F(2,48) = 5.865, p = 0.005, $\eta_p^2 = 0.196$; IL-1β: sex by age interaction: F(2,47) = 5.557, p = 0.007, $\eta_p^2 = 0.191$; IL-4: sex by age interaction: F(2,46) = 5.683, p = 0.006, $\eta_p^2 = 0.198$; IL-5: sex by age interaction: F(2,47) = 4.352, p = 0.019, $\eta_p^2 = 0.156$). Adolescent males also had higher IFN-γ, IL-1β, IL-4, IL-5, IL-10, and IL-6 cytokine levels compared to young adult and middle-aged males (p's < 0.011; IL-6: sex by age interaction: F(2,48) = 3.500, p = 0.038, $\eta_p^2 = 0.127$) and adolescent and young adult females had higher IL-1β, IL-4, and IL-10 levels compared to middle-aged females (p's < 0.046). See Fig. 1.

Middle-aged rats had higher CXCL1, IL-13, and TNF-α levels in the ventral hippocampus compared to young adults.

Regardless of sex, middle-aged rats had higher TNF-α levels in the ventral hippocampus compared to young adults (p = 0.004; main effect of age: F(2,48) = 5.96, p = 0.005, $\eta_p^2 = 0.199$). There was a trend for middle-aged rats to have higher CXCL1 levels compared to adolescents (p = 0.059; main effect of age: F(2,49) = 3.12, p = 0.053, $\eta_p^2 = 0.113$), regardless of sex. Both adolescents and middle-aged adults had higher IL-13 levels compared to young adults (p's < 0.003; main effect of age: F(2,49) = 8.06, p = 0.001, $\eta_p^2 = 0.247$). See Fig. 1.

Females had higher inflammatory cytokines in the basolateral amygdala than males in adulthood. Middle-aged rats had higher levels of TNF-α compared to other ages, regardless of sex. In contrast to the ventral hippocampus, females had higher levels of inflammation in the basolateral amygdala (BLA) in adulthood to middle-age than males, depending on the cytokine. Young adult females had higher levels of IFN-γ, IL-1β, IL-6, and IL-10 than young adult males [IFN-γ (p = 0.002; sex by age interaction: F(2,49) = 5.8003, p = 0.006, $\eta_p^2 = 0.191$), IL-1β (p = 0.005; sex by age interaction: F(2,47) = 3.622, p = 0.034, $\eta_p^2 = 0.134$), IL-6 (p = 0.001; sex by age interaction: F(2,46) = 8.44, p = 0.0008, $\eta_p^2 = 0.268$), and IL-10 (p = 0.024; sex by age interaction approached significance: F(2,49) = 2.618, p = 0.08, $\eta_p^2 = 0.097$), IL-13 (p = 0.002; sex by age interaction: F(2,49) = 5.482, p = 0.007, $\eta_p^2 = 0.183$)]. There were no sex differences in these cytokines in adolescent or middle-aged rats (p's > 0.424). Furthermore, young adult females had higher IFN-γ, IL-6, and IL-10 levels compared to all other groups (p's < 0.048, although p = 0.09 compared to middle-aged females for IL-10). Middle-aged females had higher levels of IL-5 and IL-13 than middle-aged males and all other groups (p's < 0.0002). Middle-aged males had higher CXCL1 levels in the BLA compared to middle-aged females and all other groups (p's < 0.0002; sex by age interaction: F(2,44) = 10.03, p = 0.0003, $\eta_p^2 = 0.313$). Regardless of sex, middle-aged rats had higher TNF-α levels in the BLA compared to adolescents and young adults (p's < 0.0002; main effect of age: F(2,48) = 14.81, p = 0.00001, $\eta_p^2 = 0.382$). See Fig. 2.
Negative correlations between basolateral amygdala and ventral hippocampal cytokines in young adulthood, sex difference in correlations between basolateral amygdala IL-6 and ventral hippocampus cytokines in adolescence. Correlations of cytokine and CXCL1 levels within the BLA and the ventral hippocampus were largely positive in all age groups, although correlations between regions were more negative in young adults, regardless of sex, compared to the other age groups. In adolescence, there was a sex difference in the correlations between BLA IL-6 and cytokines in the ventral hippocampus, with positive correlations in adolescent females compared to negative correlations in adolescent males (sex difference in BLA IL-6 and ventral hippocampal IFN-γ (z = 2.768, p = 0.003), IL-1β (z = 3.335, p < 0.001), IL-4 (z=-3.464, p < 0.001), IL-5 (z = 1.979, p = 0.024), IL-10 (z=-2.694, p = 0.004), IL-13 (z = 2.767, p = 0.003), TNF-α (z=-2.727, p = 0.003)). See supplementary Fig. S1.

We next correlated negative cognitive bias score with inflammatory markers in both the BLA or hippocampus but no correlations survived Bonferroni correction. See supplemental Table S1.

Neurogenesis decreased with age, regardless of sex. As expected, adolescents had higher DCX expression in the dorsal hippocampus compared to the ventral hippocampus (p = 0.0001) and higher DCX expression compared to the adult groups, regardless of sex (p's < 0.00014). Moreover, young adults had higher DCX expression compared to middle-aged adults, regardless of sex (in dorsal, p = 0.0009, in ventral p = 0.03; region by age interaction: F(2,45) = 14.789, p = 0.00001, $R_p^2 = 0.397$). See Fig. 3.

Negative cognitive bias was negatively associated with dorsal neurogenesis in young adult males. Dorsal DCX expression was negatively correlated with freezing ($r = -0.787, p = 0.036$) and negative cognitive bias score ($r = -0.7643, p = 0.045$) in young adult males only as there were no significant correlations with freezing or negative cognitive bias in any other group (p's > 0.241). However, these correlations do not survive Bonferroni corrections. See supplemental Table S2.

Inflammation was positively associated ventral hippocampal neurogenesis in males but with dorsal hippocampal neurogenesis in females. In males, ventral hippocampal DCX was positively associated with ventral hippocampal IL-13 and TNF-α, but dorsal hippocampal DCX was negatively associated with BLA IL-1β (p's < 0.043). However, only the correlation between ventral hippocampal DCX and IL-13 survived Bonferroni (p = 0.001). In females, dorsal hippocampal DCX was positively associated with ventral hippocampal IFN-γ, IL-5, IL-13, and CXCL1 in young adulthood (p's < 0.03) but changed to positive correlations between the dorsal hippocampal DCX and BLA IL-4, IL-5, IL-10, and IL-13 in middle-age (p's < 0.043). See supplemental Table S3.

Associations between cytokine/chemokine levels differ by age and sex after cognitive bias testing. Principal component analysis was used to identify clusters/pathways of interest or components [52–54]. The first two principal components accounted for 60.43% of the variance of all cytokine/chemokine and neurogenesis data. Component 1 accounts for 38.66% of the variance and is associated with cytokine/chemokine levels in the ventral HPC and hippocampal neurogenesis compared to cytokine/chemokine levels in the BLA. Component 2 accounts for 21.78% of the variance and is
associated with all cytokines/chemokine levels in both regions. The loadings for PC1 and PC2 are shown in Table 1. An ANOVA on Principal Component 1 found that hippocampal inflammation and neurogenesis were higher in adolescents compared to the adult age groups (p's < 0.00013), and IL-4, L-5, IL-13, TNF-α, and CXCL1 levels in the BLA were higher in middle-aged rats compared to the younger age groups (p's < 0.00013) after cognitive bias testing (main effect of age: F(2,48) = 100.5, p < 0.000001, $\eta_p^2 = 0.807$). A priori we expected sex differences and hippocampal inflammation and neurogenesis were elevated in adolescent males compared to adolescent females (p < 0.006; sex by age interaction: F(2,48) = 2.851, p < 0.068, $\eta_p^2 = 0.106$) with no other sex differences seen. There was no significant main effect of sex (p > 0.102; see Fig. 4A). An ANOVA on Principal Component 2 found higher cytokine associations in young adult females compared to males (p = 0.005) after cognitive bias testing (sex by age interaction: F(2,48) = 8.578, p = 0.0007, $\eta_p^2 = 0.263$). There were no other significant main or interaction effects (all p's > 0.09). See Fig. 4B.

**Table 1.** Principal component loadings from DCX and cytokine/chemokine data.
<table>
<thead>
<tr>
<th>Region</th>
<th>PC1</th>
<th>PC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal hippocampus</td>
<td>DCX</td>
<td>0.792*</td>
</tr>
<tr>
<td>Ventral hippocampus</td>
<td>DCX</td>
<td>0.735*</td>
</tr>
<tr>
<td></td>
<td>IFN-γ</td>
<td>0.846*</td>
</tr>
<tr>
<td></td>
<td>IL-1β</td>
<td>0.910*</td>
</tr>
<tr>
<td></td>
<td>IL-4</td>
<td>0.904*</td>
</tr>
<tr>
<td></td>
<td>IL-5</td>
<td>0.593*</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>0.703*</td>
</tr>
<tr>
<td></td>
<td>CXCL1</td>
<td>-0.173</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>0.937*</td>
</tr>
<tr>
<td></td>
<td>IL-13</td>
<td>0.424*</td>
</tr>
<tr>
<td>Basolateral amygdala</td>
<td>IFN-γ</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>IL-1β</td>
<td>-0.064</td>
</tr>
<tr>
<td></td>
<td>IL-4</td>
<td>-0.761*</td>
</tr>
<tr>
<td></td>
<td>IL-5</td>
<td>-0.743*</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>-0.201</td>
</tr>
<tr>
<td></td>
<td>CXCL1</td>
<td>-0.567*</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>-0.099</td>
</tr>
<tr>
<td></td>
<td>IL-13</td>
<td>-0.678*</td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>-0.542*</td>
</tr>
</tbody>
</table>

Significant loadings are indicated in bold. *indicates significance at p < 0.022.

**Discussion**

Here, we report sex and age differences in cytokine and chemokine levels after cognitive bias testing that are dependent on region. Adolescent males had higher levels of cytokines in the ventral hippocampus than females, but adult (young and middle-age) females had higher levels of cytokines than adult males in the BLA. Furthermore, middle-aged rats had higher levels of TNF-α and the chemokine CXCL1 in both the hippocampus and amygdala than all other ages. Middle-aged rats also had higher levels of IL-13 compared to younger rats in the ventral hippocampus. Negative correlations between basolateral
Amygdala and ventral hippocampal cytokines were found in young adults, and whereas adolescent males had negative correlations, adolescent females had positive correlations between IL-6 levels in the basolateral amygdala and ventral hippocampal cytokines. Principal component analyses found high ventral hippocampus cytokine/chemokine levels in adolescents, high basolateral amygdala cytokine/chemokine levels in middle-aged rats, and that young adult females had higher levels of inflammation than young adult males after cognitive bias testing. These findings demonstrate sex and age differences in possible biomarkers (inflammation, neurogenesis) related to negative cognitive bias and regional differences in these results (BLA in females, HPC in males). Future studies should examine these biomarkers under stress conditions to determine their roll in rats displaying a depressive endophenotype.

Adolescent males had high ventral hippocampal inflammation after cognitive bias testing

There was a sex difference in ventral hippocampal inflammation, as adolescent males have higher IFN-γ, IL-1β, IL-4, IL-5, and IL-10 levels than adolescent females. Inflammation (IL-1β, IL-1α) and increased neural activation in the ventral hippocampus have been linked to an increased susceptibility to stressor exposure and increased depressive-like behaviors in male rats [55–57]. In the current study, negative cognitive bias was positively associated with IL-4 and IL-10 in the ventral hippocampus of adolescent males only, suggesting a greater role of hippocampal inflammation in the depressive-like behavior of adolescent males compared to all other groups. Overall, adolescents had higher cytokine levels in the ventral hippocampus than the adult groups, similar to past findings in plasma [58] and decreased hippocampal IL-4 with age in male rats (4 months to 22–23 months; [59, 60]). Put together, these data suggest a greater role of cytokine levels in the hippocampus in the behavior of adolescents and specifically in adolescent males compared to females. Future studies examining the role of inflammation in depressive-like behavior should take age and sex into account.

Young adult females had higher basolateral amygdala inflammation than young adult males

Young adult females had higher IFN-γ, IL-1β, IL-6, and IL-10 in the basolateral amygdala compared to young adult males. Higher IL-6 has been found in plasma in adult women compared to men at baseline [42] and there is an up-regulation of genes related to inflammation in the brain in women compared to men [61]. In mice, higher IL-6 was reported in the ventral hippocampus of aged female mice compared to aged male mice [62]. However, few papers have examined sex differences in the role of inflammation in depressive-like behavior or the role of inflammation in the basolateral amygdala in cognition. Our data suggests a greater role of cytokines in the basolateral amygdala of females than in males in cognitive bias.

Neurogenesis was related to negative cognitive bias in males but not in females
In the present study we found that reduced hippocampal neurogenesis was associated with increased negative cognitive bias in young adult males. These data are consistent with findings of increased hippocampal neurogenesis being associated reduced depressive-like behavior, normally found after antidepressant treatment or voluntary exercise in both males and females [24, 27, 31, 32, 63]. It is difficult to understand why we saw this association in males but not in females in the present study but pattern separation training increases neurogenesis in male but not female rats [33]. Thus, it is possible that the cognitive training was sufficient to boost neurogenesis in the males but not the females in the present study. These data suggest that strategies to increase neurogenesis might help reduce negative cognitive bias in males more so than in females. Future studies should examine possible sex differences in the role of increasing neurogenesis on negative cognitive bias.

**Age influences cytokine and neurogenesis**

Aging was associated with a decline in neurogenesis as well as an increase in certain inflammatory signals in the hippocampus and basolateral amygdala. Middle-aged rats had higher TNF-α in the ventral hippocampus and basolateral amygdala than younger groups after cognitive bias testing. These findings are similar to past findings of increased immune-related genes with age in the hippocampus and cortex of humans and mice [61, 64, 65]. TNF-α is elevated in depressed adult men and women [35] and TNF-α levels in plasma are positively associated with the number of depressive symptoms in aged men and women [66]. In addition, TNF-α inhibition (Infliximab) attenuates cognitive impairments in adult male rats exposed to chronic unpredictable stress [67]. Along with TNF-α, the chemokine CXCL1 was also increased with age, particularly in the BLA of males in the present study. Both TNF-α and CXCL1 in the cerebrospinal fluid are upregulated after chronic stress and associated with depressive-like behavior in adult male mice [68]. These data suggest a greater role of hippocampal TNF-α and CXCL1 in negative cognitive bias with age. In addition, increased CXCL1 in the BLA of middle-aged males compared to middle-aged females may play a role in greater negative cognitive bias in middle-aged males as we found in a previous study that negative bias was increased in males relative to females in middle age only [69] and warrants further investigation. Overall, these data stress the importance of examining age and sex when exploring the link between inflammation and depressive-like cognitive endophenotypes.

Our findings that neurogenesis decreased with age are consistent with data from several studies that demonstrate a decline in hippocampal neurogenesis across the lifespan in humans, rodents, and non-human primates [70–74]. Changes in hippocampal neurogenesis and plasticity across the lifespan is associated with poor performance in hippocampal-dependent cognitive tasks with aging in male and female rodents [75–77]. Our data suggest that hippocampal neurogenesis may play a greater role in adult negative cognitive bias, particularly in males.

**Implications for cognitive bias treatment**

Although meta-analyses find that immune challenges (increased IL-6, IL-10, or TNF-α in plasma) are associated with increased negative mood and negative cognitive bias in humans [78], studies have yet to examine the association between negative cognitive bias and inflammation in the brain. Here, the amount
of inflammation in the brain after cognitive bias testing shifts from higher in males than females in adolescence to higher in females than males in young adulthood. Fluoxetine injections or CXC receptor 2 inhibitor injections reduce CXCL1 and depressive-like behavior in adult male mice [68], and our data suggest that these treatments might have improved efficacy on the cognitive bias of middle-aged groups. Our data stress the importance of considering sex, age, and region when studying the effects of inflammation on depressive-like behaviors.

The effects of pharmacological antidepressants on cognitive bias are mixed [79–82]. Some studies find an increase in positive cognitive bias after SSRI treatment [79–81, 83] whereas others find an increase in negative cognitive bias after SSRI treatment or no effect in adult men and women [79, 80]. These equivocal findings may be due to not examining sex or age differences. Further, negative cognitive bias has a direct role in SSRI efficacy, as a reduced negative bias early in SSRI treatment is associated with clinically significant improvement in later treatment for MDD [84]. The generalizability of cognitive bias modification to different aspects of negative cognitive bias is also questionable and suggests that more treatments options are needed [85, 86]. Because negative cognitive bias is a key factor in an increased risk for MDD and relapse, the maintenance of depressive symptoms, the severity of other depressive symptoms, and remains after remission of MDD [87–91] it is crucial to discover novel therapeutic targets for this cognitive symptom of MDD. Our data stresses the fact that sex and age need to be considered when investigating novel therapeutics targets for negative cognitive bias and related mechanisms.

**Conclusion**

Overall, inflammation in the brain reverses from higher in males than females in the ventral hippocampus during adolescence to higher in females than males in the basolateral amygdala during adolescence, and this is related to cognitive bias testing. Moreover, hippocampal neurogenesis is higher in adolescents than adult groups and hippocampal neurogenesis is associated with reduced negative cognitive bias in young adult males. Future studies should examine whether antidepressants, exercise, or other methods of increasing hippocampal neurogenesis will play a greater role in reducing negative cognitive bias in adult males compared to females. Future studies should also focus on age and sex as some treatments might have differential effects across the lifespan based on sex. These data provide potential biomarker targets to reduce negative cognitive bias in MDD that vary by age and sex.

**Methods**

**Animals**

Male and female Sprague-Dawley rats (N = 55) were bred in house from animals obtained from Charles River (Québec, Canada). Only 1 male and 1 female rat per litter was assigned to each age group and each condition to avoid litter confounding effects. Males and females were housed (2–3 per cage) in separate colony rooms. Rats were maintained under a 12h light-dark cycle, with lights on at 07:00 h. Rats were housed in opaque polyurethane bins (48 x 27 x 20 cm) with aspen chip bedding and ad libitum access to
autoclaved tap water and rat chow (Jamieson’s Pet Food Distributors Ltd, Delta, BC, Canada). Rats were left undisturbed, apart from weekly cage changing, until they reached the correct age for testing. All experimental procedures were approved by the University of British Columbia Animal Care Committee and in accordance with the Canadian Council on Animal Care guidelines.

**Cognitive Bias Task Procedure**

Cognitive bias procedure and tissue collection methods are previously described in Hodges et al. ([69]). Briefly, male and female rats were randomly assigned to be tested in adolescence (postnatal day (PD) 40, \( n = 17 \)), young adulthood (PD 100, \( n = 18 \)), or middle-aged adulthood (PD 210, \( n = 24 \)) and then to one of the two groups - test rats (adolescents: \( n = 8 \), female \( n = 9 \); young adults: \( n = 9 \) per sex; middle-aged adults: \( n = 12 \) per sex). Rats were placed in a shock-paired context (Context A) and in a no-shock-paired context (Context B) for 5 min each daily for 16 consecutive days, one context in the morning (8:30 h – 11:00 h) and the other context in the afternoon (13:00 h – 15:30 h). After 16 days of training, rats were placed in an ambiguous context (Context C) on Test Day (Day 18). Context C partially resembled both Contexts A and B in terms of transport (duration and method), illumination (two lights), one lever out, and an intermediate pattern of lines on the walls (7 mm between lines). Testing in Context C lasted 5 min with no footshock. Time spent freezing (no head or body movement besides breathing; [92]) during the first 3 min of entering each context was measured on each day and percentage freezing was computed. Further, a difference score was created by subtracting percentage freezing in Context C on Day 18 from percentage freezing in Context B (no footshock-paired) on Day 16 and used to index negative cognitive bias scores (high freezing = negative cognitive bias; low freezing = neutral/positive cognitive bias; adapted from [93, 94]).

These behavioral data were published previously [69]. We found that negative cognitive bias was higher in the adult age groups compared to adolescents and middle-aged males had a higher negative cognitive bias than middle-aged females. Ninety min after exposure to Context C on day 18, test rats were euthanized by decapitation. Brains were removed from the skull and cut in equal halves along the sagittal plane. The left hemisphere was used for DCX immunohistochemistry and the right hemisphere was used for electrochemiluminescence (described below).

**DCX Immunohistochemistry**

We examined hippocampal neurogenesis using a marker of immature neurons and microtubule-associated protein, doublecortin (DCX; [95]). The left hemisphere was placed into a 4% paraformaldehyde solution for 24 h, and subsequently placed into a 30% sucrose in 0.1M phosphate buffered saline (PBS; pH 7.4) for another 24 h and then until sliced. Coronal sections (30µm) were sliced on a microtome and collected from approximately bregma 3.72mm to -6.96mm [96]. Sections were stored in an antifreeze solution (30% ethylene glycol, 20% glycerol in 0.1M phosphate buffer (PB; pH 7.4)) at -20°C until immunohistochemistry assays were conducted.

Coronal sections were successively washed 3x in PBS for 10 min per wash and incubated at room temperature in a 0.6% hydrogen peroxide (H2O2; H1009, Sigma-Alrich, St. Louis, MO, USA) in distilled
water (dH2O) for 30 min. Sections were then washed another 3x in 0.1M PBS for 10 min per wash, and then incubated at 4°C in DCX primary antibody (1:1000 goat Anti-DCX pAb; SC-8066; Santa Cruz Biotechnology, Dallas, TX, USA), 3% normal rabbit serum (VECTS5000, Vector Laboratories, Inc, Burlingame, CA), and 4% Triton-X in PBS for 24 hours. The next day, sections were washed 5x in 0.1M PBS for 10 min per wash and incubated overnight at 4°C in secondary antibody (biotinylated rabbit anti-goat IgG; 1:500; Vector Laboratories, Inc, Burlingame, CA). The last day, after another series of 5 washes in 0.1M PBS for 10 min per wash, sections were incubated in an avidin-biotin horseradish peroxidase solution (PK-4000, Vector Laboratories, Inc, Burlingame, CA) for 4 h at room temperature. Sections were washed 3x in 0.1M PBS for 10 min per wash and horseradish peroxidase was visualized using 3,3’ diaminobenzidine (DAB) in a 3 M sodium acetate buffer containing 2.5% nickel sulfate and 0.05% H2O2 (SK-4100, Vector Laboratories, Inc, Burlingame, CA) for 3 min. Sections were washed another 3x in 0.1M PBS for 10 min per wash and then mounted on Superfrost Plus slides (Fisher Scientific, Inc., Hampton, NH) and let dry. Sections were then dehydrated using increasing concentrations of ethanol (50%, 70%, 95%, 100% for 2, 2, 2, and 10 mins respectively), and then cleared with xylene for 10 min and coverslipped using Permount mounting medium (Fisher Scientific, Inc., Hampton, NH).

DCX protein immunostained brain sections were analyzed using a Nikon Eclipse 80i microscope in the dorsal hippocampus (within bregma − 2.64 mm and − 4.56 mm) and ventral hippocampus (within bregma − 5.76 mm and − 6.36 mm). Photomicrographs were taken using a slidescanner (ZEISS Axioscan 7 Slide Scanner, Germany) and used to trace outline of each subregion of interest to calculate the area of each region using ImageJ software (Image J, 2020). Cell counts of DCX immunoreactive (ir) cells were conducted by experimenters’ blind to experimental condition and averaged across 2 sections per animal hippocampal region using a 40x objective. DCX-ir cell density for each subregion of interest was calculated by dividing the cell count by the corresponding area in mm

### Multiplex Cytokine Electrochemiluminescence

Electrochemiluminescence was done in accordance with previous protocols [97]. The right hemisphere of the brain was rapidly frozen and coronally sliced at 300 µm. The BLA (within bregma 1.92 mm and 0.96 mm) and the vHPC (within − 5.76 mm and − 6.36 mm) were identified and dissected out using tissue punching tools (0.75mm, 1.20mm, and 2mm in diameter; Harris Uni-Core, Sigma-Aldrich) and placed directly into tubes containing beads (1.4mm ceramic spheres, Lysing Matrix D, MP Biomedicals™, Santa Ana, CA, USA) on dry ice. Tissue was homogenized in complete lysis buffer using the Omni Bead Ruptor 24 (Omni International, Kennesaw, GA, USA). After homogenization, samples were centrifuged at 4°C at 1000g for 10 min and supernatant was collected and stored at -80°C until cytokine analysis.

Cytokine levels were quantified in samples using a multiplex electrochemiluminescence immunoassay kit (V-PLEX Proinflammatory Panel 2, Rat) from Meso Scale Discovery (Rockville, MD, USA). The following 8 cytokines and 1 chemokine were quantified in each sample: interferon gamma (IFN-γ), interleukin (IL)-1β, IL-4, IL-5, IL-6, IL-10, IL-13, tumor necrosis factor (TNF)-α, and the chemokine C-X-C motif ligand 1 (CXCL1). Samples were run in duplicates and plates were read using a Sector Imager 2400 (Meso Scale Discovery) and analyzed using the Discovery Workbench 4.0 software (Meso Scale Discovery). The lower
limits of detection (LLOD) were as follows for each individual plate (4 plates total) in pg/mL: IFN-γ: 0.674, 1.776, 1.62, 2.652; IL-1β: 1.995, 3.745, 3.616, 8.118; IL-4: 1.64, 4.613, 2.062, 5.75; IL-5: 0.552, 1.563, 0.541, 0.999; IL-6: 2.18, 4.09, 2.462, 3.718; IL-10: 0.789, 1.99, 1.744; 5.574; IL-13: 0.168, 0.698, 0.143, 0.252; TNF-α: 0.385, 0.97, 0.298, 0.399; and CXCL1: 0.99, 0.406, 0.967, 0.558. Inter-assay coefficient of variation was < 23% for all cytokines between plates.

**Data analyses**

General linear mixed model ANOVAs for levels of each cytokine/chemokine in the basolateral amygdala and ventral hippocampus were run with sex (male, female) and age (adolescence, young adulthood, middle-aged adulthood) as between-subjects factors. A repeated measures ANOVA using the same between-subjects factors as above was performed on the dorsal and ventral hippocampus DCX data. Pearson’s correlations were conducted between BLA or vHPC cytokine/chemokine levels, dorsal or ventral hippocampal DCX, and freezing in the ambiguous context or negative cognitive bias score. Principal component analyses were performed using DCX data and inflammation data in each brain region. Missing values, due to outliers (two standard deviations below or above the mean), which accounted for 1.65% of the data, were replaced by the mean for PCA analyses. One middle-aged male was completely removed from PCA analyses because they were missing 78% of cytokine/chemokine data in the ventral hippocampus due to cytokine levels two standard deviations above the mean. Post-hoc tests used Newman-Keuls comparisons. Any *a priori* comparisons examining sex differences were subjected to Bonferroni comparisons. Significance level of *p* < 0.05 was used. All statistical analyses were performed using Statistica software (v. 9, StatSoft, Inc., Tulsa, OK, USA).

Four animals were excluded from the following analyses due to their inability to distinguish between the shock- and no-shock-paired contexts on Day 16 of training (2 middle-aged males, 1 middle-aged female, 1 young adult male).

**Declarations**

**Ethics declarations**

All experimental procedures were approved by the University of British Columbia Animal Care Committee (A20-0147) and in accordance with the Canadian Council on Animal Care guidelines.

**Availability of Supporting Data**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Consent for Publication**

Not applicable.

**Competing interests**
The authors disclose no conflicts.

**Funding**

This research was funded by an operating grant from the Natural Sciences and Engineering Research Council of Canada (NSERC; 2018-04301) to LAMG. TEH was supported for one year by the University of British Columbia, Canada (Institute of Mental Health Marshalls Scholars Program) and an NSERC (RGPAS-2018-522454) given to LAMG.

**Author Contributions**

THE conceptualized the project, acquired the behavioral and electrochemiluminescence data, performed doublecortin immunohistochemistry, analyzed data, drafted the manuscript, reviewed and edited the manuscript. SEL and RKR helped analyze doublecortin. LAMG conceptualized the project, supervised the studies, edited and revised the manuscript.

**Acknowledgements**

We thank Grace Y. Lee and Sophia H. Noh for helping with behavioral testing of the animals.

**References**


20. Fang J, Demic S, Cheng S. The reduction of adult neurogenesis in depression impairs the retrieval of new as well as remote episodic memory. PLOS ONE. Public Library of Science; 2018;13:e0198406.


24. Green AD, Galea LAM. Adult hippocampal cell proliferation is suppressed with estrogen withdrawal after a hormone-simulated pregnancy. Horm Behav. 2008;54:203–11.


70. He J, Crews FT. Neurogenesis decreases during brain maturation from adolescence to adulthood. Pharmacol Biochem Behav. 2007;86:327–33.


**Figures**

![Figure 1](image)

**Figure 1**

Mean (±SEM) IFN-γ (A), IL-1β (B), IL-4 (C), IL-5 (D), IL-6 (E), CXCL1 (F), IL-10 (G), IL-13 (H), TNF-α (I) levels in the ventral hippocampus, normalized by total protein concentrations. Adolescent males had higher IFN-γ, IL-1β, IL-4, IL-5, and IL-10 levels compared to adolescent females and adult groups and higher IL-6 compared to young adult and middle-aged males. Adolescent and young adult females had higher IL-1β, IL-4, and IL-10 levels compared to middle-aged females. Middle-aged rats had higher CXCL1 levels compared to adolescents and young adults and higher TNF-α levels compared to young adults. Both adolescents and middle-aged adults had higher IL-13 levels than young adult rats. * indicates p<0.05:
compared to all other groups. & indicates p<0.05: compared to middle-aged adults. # indicates p<0.05: compared to young adult and middle-aged adults. @ indicates p<0.05: main effects of age. n = 7-11.

Figure 2

Mean (±SEM) IFN-γ (A), IL-1β (B), IL-4 (C), IL-5 (D), IL-6 (E), CXCL1 (F), IL-10 (G), IL-13 (H), TNF-α (I) levels in the basolateral amygdala, normalized by total protein concentrations. Young adult females had higher IFN-γ, IL-1β, IL-6, and IL-10 levels compared young adult males and all other groups. Middle-aged females had higher levels of IL-5 and IL-13 compared to middle-aged males and all other groups, and middle-aged males had higher CXCL1 levels compared middle-aged females and to all other groups. Middle-aged adults had higher TNF-α levels compared to adolescents and young adults. * indicates p<0.05: compared to all other groups. @ indicates p=0.00001: main effect of age. n = 7-11.
Figure 3

Mean (±SEM) doublecortin (DCX) density in the dorsal hippocampus (A) and ventral hippocampus (B) or male and female adolescent, young adult, and middle-aged rats. Representative images of DCX+ cells in the granule cell layer of the dorsal and ventral hippocampus of adolescent, young adult, and middle-aged rats (C). DCX density is reduced with age and adolescents have higher DCX in the dorsal compared to the ventral hippocampus. *indicates p <0.00014.
Figure 4

Mean (±SEM) principal component (PC) scores for doublecortin (DCX) in the dorsal and ventral hippocampus, ventral hippocampus (VHPC) cytokine/chemokine levels, and basolateral amygdala (BLA) cytokine/chemokine levels. PC1 scores (A), associated with hippocampal DCX and VHPC cytokines compared to BLA cytokines, found higher hippocampal DCX and VHPC cytokine/chemokine levels in adolescent males compared to adolescent females and in adolescents compared to adult groups. Further
BLA cytokines/chemokines were higher in middle-aged rats compared to young rats. @indicates p<0.00014: comparison between each age group, *indicates p<0.006: compared to adolescent females. PC2 scores (B), associated with VHPC and BLA cytokine/chemokine levels, was more involved in young adult females compared to young adult males. *indicates p<0.005: compared to young adult males. n=8-11 per sex/age.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalJune82022.docx