Immune receptor toll-like receptor 4 contributes to stress-induced affective responses in a sex-specific manner

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Abstract

Stress activates innate immune Toll-like receptors (TLRs) and enhances susceptibility to depression, a condition that is more prevalent in females. The TLR4 receptor type is involved in inflammatory responses and its expression levels associate with depressive symptoms and their successful treatment. Yet, little preclinical research has examined the role of TLR4 in stress-induced affective responses to determine if these are sex-specific. One group per genotype of male and female Tlr4 knockout (KO) and wild type (WT) rats were exposed to predator odor in a place conditioning apparatus with others exposed to saline. Affective behaviors evaluated included distance traveled and center time in an open-field apparatus, sucrose preference and fluid intake in a two-bottle test, and conditioned place aversion to the odor-paired compartment. Predator odor exposed rats showed conditioned place aversion to the odor-paired compartment, demonstrating predator odor was aversive. Such exposure led to anhedonia (decreased sucrose preference) across genotypes and sex. Predator odor exposure decreased distance traveled, an effect that was greater in KO rats, especially in females. Tlr4 deletion also resulted in sex-specific effects on anxiety-like behavior. Compared to WTs, female KO rats showed lower center time after predator odor exposure whereas genotype did not affect this response in male rats. Across litters, fewer male KO and heterozygous rats and more WT rats were born whereas female rats showed the typical genotype distribution. Results suggest predator odor alters affective behaviors, consistent with the preclinical literature, and deletion of Tlr4 enhances some stress-induced affective responses, often in a sex-specific manner.

Keywords:
- TLR4
- Predator odor
- Sex differences
- Depression
- Anhedonia
- Innate immune system
- Rat

1. Introduction

Major depressive disorder (MDD) is a debilitating and often recurring disease (Mueller et al., 1999) with a lifetime prevalence rate of over 20% among adults in the United States (Hasin et al., 2018). Women are about twice as likely as men to have MDD (Kessler et al., 1993) and more often show the classic signs of anhedonia, sadness, and feelings of worthlessness as well as altered sleep and feeding patterns (Frank et al., 1988; Kornstein et al., 2000; Zagni et al., 2001; Marcus et al., 2008). In contrast, depression in men is often accompanied by anger or addiction-like behaviors. Although men are less likely than women to seek treatment (Martin et al., 2013) and evidence suggests the sex gap is closing (Platt et al., 2020), the rate disparity in MDD may reflect, in part, the many sex differences seen in neurobiological systems that contribute to depression (Rubinow and Schmidt, 2019).

Historically, understanding the neurobiology of depression has focused on neural and hormonal systems activated by stress because depressive episodes are often precipitated by stressful life events. More recently, attention has turned to the role of the innate immune system in MDD (Maes, 1995; Frank et al., 2016; Liu et al., 2019) that communicates bi-directionally with neural and hormonal systems (Maier, 2003). Stress activates the innate immune system leading to the production of pro-inflammatory cytokines (Dhabhar and McEwen, 1997; Padgett and Glaser, 2003). Inflammatory cytokines affect brain systems known to contribute to MDD (Miller et al., 2008) and peripheral levels are increased in depressed patients (Levine et al., 1999; Dantzer et al., 2008; Rizavi et al., 2016; Zhang et al., 2018).

Toll-like receptors (TLRs) play a major role in the innate immune system and are considered pattern recognition receptors (PRRs) that are expressed by immune cells in addition to neural and glial cells (Vaure and Liu, 2014). TLRs promote the production of inflammatory factors such as cytokines (Akira et al., 2006). Toll-like receptor 4 (TLR4) is part of the mammalian family of TLRs that is activated by lipopolysaccharide (LPS) and is a particular focus of research in depression (Cheng et al., 2016).
TLRs are also activated by damage- or danger-associated molecular proteins (DAMPs) that occur endogenously after psychological or physical stress (Franklin et al., 2018).

Whether there are sex differences in TLR4 function in humans (Jiang and Gilkeson, 2014) or rodents (Santos-Galindo et al., 2011) is not clear. In response to an immune challenge, men show higher TLR4 activation and greater pro-inflammatory cytokine production, whereas women have increased macrophage activation, phagocytic capacity, and IL-10 production (Klein and Flanagan, 2016). Yet, several innate and adaptive immune responses differ between the sexes (Marriott and Huet-Hudson, 2006) and are linked to depression (Bekkhat and Neigh, 2018; Rainville et al., 2018). Although few animal studies investigating the role of the innate immune system in depressive-like effects include females, greater behavioral and immune response to inflammatory challenges are seen in female rats compared to males (Tonelli et al., 2008).

Animal models of depression use stressors to induce depressive-like behaviors, such as anhedonia assessed by decreased sucrose preference (Papp et al., 1991), immobility in the forced swim test (Porsolt et al., 1977), and decreased activity in the open field test (Hall, 1936; Weiss et al., 1980). Stressors include chronic mild stress, inescapable shock, or learned helplessness models (Weiss et al., 1980; Willner, 1997; Maier and Watkins, 2005), all of which engage the innate immune system and associate with depressive-like effects (De La Garza, 2005; Kubera et al., 2011; Cheng et al., 2016). Predator odor exposure is considered an ethologically valid stressor devoid of physical stress that induces indices of anxiety, depression, and post-traumatic stress disorder (Cohen and Zohar, 2004; Whitaker et al., 2014; Wu et al., 2019). Predator odor exposure leads to enhanced affective responses that persist for weeks after a single exposure (Cohen et al., 2004; Edwards et al., 2013; Roltsch et al., 2014; Whitaker and Gilpin, 2015) and alters cytokine levels in the brains of rats and mice (Wilson et al., 2013b; Deslauriers et al., 2017).

Yet, studies of predator odor exposure typically use male rodents only with some exceptions (Adamec et al., 2006; Mazor et al., 2009; Cozzoli et al., 2014). Interestingly, male and female rodents appear differentially vulnerable to the effects of predator odor stress, although this is task-dependent (Adamec et al., 2006; Albrechet-Souza et al., 2020).

Rodent studies that examined the role of TLR4 in the ability of stress to induce depressive-like effects employed male subjects only and show discrepant findings (Garate et al., 2011; Biesmans et al., 2016; Cheng et al., 2016; Couch et al., 2016; Deslauriers et al., 2017; Fu et al., 2019). One study that used predator odor stress showed that immune deficient and T-cell deficient male mice exhibited greater unconditioned fear and anxiety-like behaviors but depressive-like behaviors were not assessed (Cohen et al., 2006). Thus, the current study was designed to investigate if deletion of the Tlr4 gene in rats alters the ability of predator odor stress to induce depressive-like behavioral responses and whether such effects are sex-specific. We predicted that Tlr4 knockout (KO) rats would show decreased stress-induced depressive-like behaviors compared to wild type (WT) rats and that these effects would be greater in female vs. male rats. It is worth noting that this relatively new Tlr4 knockout rat model (Ferguson et al., 2013) does not result in genotype differences in restraint stress-induced CRF modulation of GABAergic transmission in the amygdala (Varodayan et al., 2018) or in alcohol consumption (Harris et al., 2017).

2. Methods

2.1. Animals

Male and female Wistar rats (Rattus norvegicus) heterozygously expressing an inactive variant of the Tlr4 gene were generated by and acquired from Gregg E. Homanics, PhD (University of Pittsburgh, Department of Anesthesiology) (Ferguson et al., 2013). All animals were obtained through breeding. The 91 rats employed in this study were derived from 19 litters comprised of 216 pups total, although only 193 (17 litters) were genotyped. There were two additional male KO’s in the study that were born prior to our use of mLIMS transgenic mouse colony management system (Bioinformatics Madison, WI) used to keep track of breeding data. Litter information on these two rats is missing but their data are included in the behavioral analyses. No more than two pups per sex per genotype per litter were assigned to one of the two experimental groups. The group sizes used in the behavioral studies by sex, genotype, and odor-exposure group are seen in Table 1.

Rats were caged with their dam and littersmates until weaning on postnatal day 21. Afterwards, they were group-housed by sex and genotype (2–4 to a cage) in amber polysulfone cages in a humidity- and temperature-controlled environment with ad libitum access to food and water except where noted differently. A 12-h light-dark cycle (lights on at 07:00) was maintained throughout the study. Animals were at least 65 days of age at the start of testing. Procedures were approved by the University of Houston Institutional Animal Care and Use Committee in accordance with the NIH’s Guide for the Care and Use of Laboratory Animals.

2.2. Genotyping

Prior to weaning, each rat was anesthetized with isoflurane to remove a 3-mm section from the tip of the tail. Tail snips were sent to TransnetFX Inc. (Cordova, TN) for genotyping.

2.3. Experimental procedure

Adult, wild type (WT) and knockout (KO), male and female rats were randomly assigned to either Odor Exposed or Non-Exposed groups as seen in Table 1. The experimental procedure timeline is shown in Fig. 1. First, baseline behavioral measures were obtained on two tests of anxiety- and depressive-like behaviors: 1) locomotor activity (distance traveled in cm) and center time (in sec) in the open field test (OFT); and 2) sucrose preference and total fluid intake in the two-bottle sucrose preference test (SPT). After baseline tests were conducted, some rats were subjected to a predator-associated odorant stressor (Exposed group) whereas the other rats were exposed to saline (Control group) in a place conditioning apparatus. A random number generator (random.org) was used to determine apparatus compartment pairing. Next, all rats were re-tested on OFT and SPT over the following 48 h. Finally, rats were tested for conditioned place aversion (CPA) by examining the change in time (sec) spent in the predator odor-paired compartment in the place conditioning apparatus at both 24-h and 10-days after predator odor (or saline) exposure. All tests were carried out during the animals’ light hours under low red-light conditions in designated testing rooms except for SPT that was performed over 48-h in the animals’ home cages. For tests occurring in designated testing rooms, animals were allowed to acclimate to the room in their home cages for at least 20-m. Behavioral apparatuses were cleaned thoroughly with 70% ethanol solution after each use.

2.4. Predator odor-exposure

Apparatus. Testing took place in one of eight Med Associates three-compartment place preference apparatuses (MED-CPP-013AT, Med Associates Inc., St Albans, VT), each consisting of: 1) a black compartment

| Table 1 | Group sizes by Experimental Group, Genotype, and Sex. |
|---------|-----------------------------------------------------|
| Experimental Group | Genotype and Sex | Wild Type | Knockout |
| | Male | Female | Male | Female |
| Odor Exposed | 11 | 6 | 8 | 12 |
| Non-Exposed | 21 | 9 | 10 | 14 |
with metal bar flooring, 2) a white compartment with metal grid flooring, and 3) a gray center compartment with solid flooring connecting the black and white compartments (main compartments). Each of the main compartments measured 11.75” L x 8.25” W x 8” H (29.85 × 20.96 × 20.32 cm) and the center compartment measured 4.75” L x 8.25” W x 8” H (12.06 × 20.95 × 20.32 cm). The three compartments were divided by vertical guillotine doors. Infrared sensors in each compartment tracked animal movement and recorded times in each compartment using the corresponding software (MED Test, v. 4.2.0.0, MED Associates, Fairfax, VA). Individual incandescent lights in each compartment had been manually adjusted to reduce inherent side preferences.

Procedure. Animals were individually exposed to the apparatus and baseline times (sec) in the two main compartments of the apparatus were recorded over the 15-m session. The following morning, animals were randomly assigned and confined to one distinct compartment by closing the vertical guillotine doors and exposed to saline-soaked filter paper contained in a plastic weigh boat placed beneath the floor of the apparatus for 15-m. That evening, each animal was placed in the opposite compartment and exposed to either bobcat urine- (Pmart, LLC, Sandy Point, ME) or saline-soaked filter paper for 15-m. Testing for conditioned place aversion (CPA) or avoidance of the odor-paired compartment was performed at 24-h post-exposure and again at 10-days post-exposure. In these tests, the vertical guillotine doors were lifted so that the rats had access to the entire apparatus. Time spent (s) on the odor-paired side over the 15-m test was tabulated. The measure of CPA was the time spent in the odor-paired compartment on the test day minus the time recorded on the baseline day.

2.5. Open field testing (OFT)

Animals were placed in a 43.2 cm × 43.2 cm (width) × 30.5 cm (height) open field box (Med Associates Inc., St. Albans, VT). The box consisted of white Plexiglas floors and clear Plexiglas walls, along the base of which were positioned arrays of infrared detectors. Animal movement was tracked using Med Associates Activity Monitor software (version 6.02, Med Associates Inc., St Albans, VT). Distance traveled (cm) and time spent (s) in the center zone (as determined by the software) were recorded over a 30-m testing period.

2.6. Two-bottle choice sucrose preference testing (SPT)

Animals were housed singly in their home cages and the lixit water system removed. For baseline measurement, access to two bottles with leak-proof OptiRat Plus lids (Animal Care Systems, Inc., Centennial, CO) containing water was given for four consecutive days. One water bottle was then replaced with a bottle containing a 1% sucrose solution and animals were given free choice between water and sucrose solution for a period of 48 h. Bottles were removed and weighed at 24- and 48-h. Bottle positions were switched at the 24-h weighing time to eliminate possible position preferences. After the second weighing, the standard lixit water access was restored. Sucrose preference was recorded by dividing the weight of the sucrose solution bottle by the weight of both bottles together (i.e., total fluid intake water). During sucrose preference testing, animals were housed singly and returned to ad libitum drinking and their original group housing conditions after testing was completed.

2.7. Statistical analysis

For CPA data, a 2 × 2 × 2 Analysis of Variance (ANOVA) was employed to determine between groups interactions of sex (male or female), exposure condition (saline or predator odor), and genotype (WT or KO), respectively. For OFT and SPT, a series of 2 × 2 × 2 Analysis of Covariance (ANCOVAs) was employed to determine between groups interactions of sex, exposure condition, and genotype, respectively. Baseline measures in the OFT and SPT were used as a covariate in these statistical analyses. All data were analyzed using PROC GLM in SAS software version 9.4 (SAS Institute Inc., Cary, NC). Correlations across the various behavioral measures were made but none were significant so they will not be reported here. Graphical visualization of data was completed with GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA). Birth and litter data (sex ratio; genotype ratio) were analyzed by chi-square with Statistica software version 13 (Palo Alto, CA) using theoretical results as the expected values (i.e., 50%/sex; 25%/25%/50% for WT/KO/HET genotypes).

3. Results

3.1. Birth and litter data

Of the 193 pups born in the 17 litters bred and genotyped, 100 were male and 93 were female resulting in a male/female sex ratio of 51.8%, p

| Genotype          | Sex   | Wild Type | Knockout | Heterozygous | Total |
|-------------------|-------|-----------|----------|--------------|-------|
| Male*             | 45    | 16        | 39       |              | 100   |
| Female            | 22    | 25        | 46       |              | 93    |
| Total             | 67    | 41        | 85       |              | 193   |

* p < 0.00002.
> 0.10. The proportions of pups per sex per genotype are shown in Table 2. The proportions of WT, KO, and heterozygous (HET) female rats born were what would be expected from breeding HET dams with HET sires, p > 0.10. On the other hand, male pups born to these litters showed a disproportionately higher number of WTs and lower numbers of KOs and HETs, chi-square = 21.66; df = 2; p < 0.00002.

3.2. Baseline behaviors

There are several sex differences in baseline (i.e., pre-predator odor exposure) behaviors as seen in Table 3. Female rats show greater distance traveled and spend less time in the center in the open field test (OFT). These statements are supported by significant main effects of Sex for both distance traveled, F(1, 87) = 10.31, p = 0.002, and time spent in the center of the open field, F (1,87) = 7.38, p = 0.005. There are no significant main effects of Genotype (p = 0.809; p = 0.0947) nor a Sex X Genotype interaction for distance traveled (p = 0.774) or time in the center of the open field (p = 0.846). Female rats also ingest more total fluid and show greater sucrose preference compared to male rats in the two-bottle sucrose preference test (SPT). These statements are supported by main effects of Sex on total fluid intake, F(1, 92) = 17.48, p < 0.0001, and sucrose preference, F(1, 92) = 10.22, p = 0.002. There are no significant main effects of Genotype (p = 0.467; p = 0.149) nor Sex X Genotype interactions (p = 0.925; p = 0.777) for total fluid intake or sucrose preference, respectively. To compensate for these baseline differences in OPT and SPT, these measures were used as co-variates in the ANCOVA.

3.3. Conditioned place aversion

The aversive effects of predator odor exposure (i.e., decreased time spent in the odor-paired compartment after exposure compared to baseline time) were assessed in the conditioned place aversion (CPA) procedure at both 24-h and 10-days post-exposure. Results are shown in Fig. 2. There are no main effects of Exposure (p = 0.225), Sex (p = 0.316), or Genotype (p = 0.876), or their interactions (p’s > 0.05) on CPA when tested 24-h after conditioning sessions as seen in Fig. 2A-B. When CPA was measured 10-days post-exposure, there is a significant main effect of Exposure, F(1, 79) = 8.91; p = 0.004. Exposed animals spend significantly less time in the odor-paired compartment on the test relative to baseline (i.e., show greater CPA) compared to non-exposed controls as seen in Fig. 2C-D. There are no other significant main effects or interaction effects (p’s > 0.10).

3.4. Open field test

The effects of predator odor exposure on distance traveled (cm) and center time (s) in the open field test (OFT) are shown in Fig. 3. For distance traveled, there are significant main effects of Exposure, F (1,87) = 6.76; p = 0.011, Sex, F (1,82) = 10.93; p = 0.001, and Genotype, F (1,87) = 7.48; p = 0.007, but not in their interactions (p’s > 0.05). Exposed animals travel less distance than non-exposed animals when measured post-odor exposure as seen in Fig. 3A-B. Females show greater distance traveled compared to males and KO animals travel less distance than WT animals. For time in the center of the open field, there are significant main effects of Exposure, F (1,82) = 14.89; p = 0.0002), Sex, F (1,82) = 8.67; p = 0.004), and a Sex X Genotype interaction, F (1,82) = 5.15; p = 0.026. All other main effects and interactions are not significant (p’s > 0.10). Exposed animals spend less time in the center zone compared to non-exposed animals and female rats spend less time in the center zone relative to male rats as seen in Fig. 3C-D. Female KO rats with predator odor exposure spend the least amount of time in the center compared to the three other groups (Fig. 3C). This statement is supported by Tukey post hoc tests (p’s = 0.014 and 0.001).

3.5. Sucrose preference test

The effects of predator odor exposure on total fluid intake (water + sucrose in mL) and on sucrose preference (% sucrose intake out of total fluid intake) in the sucrose preference test (SPT) are shown in Fig. 4. There is a significant main effect of Exposure, F (1,87) = 6.29; p = 0.014, on total fluid intake as seen in Fig. 4A and B. Exposed animals ingested lower total fluid relative to non-exposed controls. There are no significant main effects of Sex (p = 0.395), Genotype (p = 0.541), or their interactions (p’s > 0.05) on total fluid intake. There is a significant main effect of Exposure, F (1,87) = 6.83; p = 0.011, on sucrose preference as seen in Fig. 4C and D. Exposed animals show lower sucrose preference compared to non-exposed controls. There are no significant main effects of Sex (p = 0.817), Genotype (p = 0.557), or their interactions (p’s > 0.05) on sucrose preference.

4. Discussion

The results of the present study demonstrate that predator odor exposure is aversive and alters affective responses, some of which depend upon sex and genotype. Across genotypes and sex, this stress causes anhedonia as measured by decreased sucrose preference and total fluid intake. A reduction in distance traveled also occurs due to predator odor exposure, particularly in Tlr4 KOs and in females that may reflect a greater depressive-like effect. Female KO rats spend the least amount of time in the center of the open field after predator odor exposure compared to male rats. This demonstrates a sex-specific effect of predator odor stress on anxiety-like behavior in rats in which the absence of the Tlr4 gene leads to an increased anxiety-like response to stress in females. The lack of effect of predator odor exposure by Tlr4 gene deletion in males may reflect, in part, that significantly fewer male KO rats were born. Thus, we confirm that predator odor exposure is aversive and show that this stress differentially alters affective behaviors that, in some cases, depend on the presence of the Tlr4 gene and on sex.

Both Tlr4 KO and WT rats showed conditioned place aversion (CPA) to the odor-paired compartment when measured 10-days after exposure. This confirms prior work demonstrating that predator odor exposure is aversive when assessed several days after exposure (Edwards et al., 2013; Whitaker and Gilpin, 2015; Albrecht-Souza and Gilpin, 2019). Along with studies that show enhanced stress hormone reactivity in response to predator odor (Wilson et al., 2013a; Rolsch et al., 2014; Whitaker and Gilpin, 2015; Whitaker et al., 2016; Albrecht-Souza and Gilpin, 2019; Albrecht-Souza et al., 2020), our findings support the notion that predator odor is a stressor for rodents. Like other stressors, predator odor exposure should induce depressive-like effects. Indeed, we find evidence of anhedonia in the sucrose preference test (SPT), consistent with prior studies that employed chronic unpredictable stress (Papp et al., 1991). However, results from mouse studies with predator odor exposure are inconsistent; findings of no effect in SPT in male or female mice (Adamec et al., 2006) as well as a decrease (Calvo-Torrent et al., 1999) and an increase in sucrose intake in male mice (Burgado et al., 2014) have been reported. Several methodological or species differences, as have been noted by others (Pruett et al., 2008), may explain the discrepancies. Alternatively, these disparities may reflect that the link between stress and consummatory behavior is bi-directional, sometimes leading to decreases and other times leading to increases in consumption (Maniam and Morris, 2012).

Table 3

| Measure                          | Females                      | Males                      |
|---------------------------------|------------------------------|----------------------------|
|                                 | Mean      | SEM      | Mean      | SEM      |
| Distance Traveled (cm; OFT)*    | 8993.10  | 506.31   | 7025.56  | 344.99   |
| Time in Center (sec; OFT)*      | 703.94   | 36.81    | 901.58   | 54.38    |
| Total Fluid Intake (ml; SPT)**  | 91.29    | 5.53     | 63.53    | 3.15     |
| Sucrose Preference (% of total intake; SPT)* | 95.16%  | 0.96%    | 88.97%   | 1.31%    |

*p < 0.01; **p < 0.0001.
Fig. 2. Predator odor exposure induces delayed and persistent avoidance behavior in the CPA test. Predator odor exposure animals did not display conditioned place aversion (CPA) at 24 h post-exposure ($p > 0.10$) (A, B). At 10 days post-exposure, predator odor-exposed animals showed CPA and spent significantly less time on the side of the apparatus previously paired with predator odor ($p = 0.004$) (C, D). There were no Sex or Genotype effects at either test time. M.E., main effect; **$p < 0.01$.

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A. Females

B. Males

C. Females

D. Males

Fig. 3. Predator odor exposure suppresses locomotion and decreases time spent in the center zone in the OFT. Predator odor-exposed rats showed decreased distance traveled during the open field test (OFT; $p = 0.011$). Overall, females traveled greater distances than males ($p = 0.001$) and WT animals traveled greater distances than KO animals ($p = 0.007$) (A, B). Time spent in center zone in the OFT was reduced in predator odor-exposed groups ($p = 0.0002$). Males spent more time in the center zone than females ($p = 0.004$). Of the predator odor exposed animals, female KOs spent the least amount of time in the center zone ($#, Sex X Genotype interaction; p = 0.026$) (C, D). Data presented in figure represent actual values; data were analyzed by ANCOVA to take into account significant sex differences in baseline values. M.E., main effect; $#, interaction effect, p < 0.05; *p < 0.05; **p < 0.01; ***p < 0.001$.

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A. Females

B. Males

C. Females

D. Males

Fig. 4. Predator odor exposure reduces total fluid consumption and sucrose preference in the SPT. Predator odor-exposed animals consumed less total fluid (water + sucrose solution) than non-exposed animals ($p = 0.014$) (A, B) and showed decreased preference for the sucrose solution in the sucrose preference test (SPT; $p = 0.011$) (C, D). Data presented in figure represent actual values; data were analyzed by ANCOVA to take into account significant sex differences in baseline values. M.E., main effect; *$p < 0.05$.  

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Predator odor stress decreases locomotor activity in the open-field test (OFT) in the present study consistent with a study in male mice (Deslauriers et al., 2017). Similarly, chronic predator odor stress blocks the increase in distance traveled upon the second exposure to an open field and decreases center zone distance in male mice (Burgado et al., 2014). Decreased locomotor activity can be interpreted as a depressive-like effect (Hall, 1936; Weiss et al., 1980). These results would therefore be consistent with our findings in the SPT. We also see that this stress affects center time in the OFT, a behavior thought to reflect anxiety. Other studies also show anxiety-like effects of predator odor exposure (Cohen et al., 2004; Wilson et al., 2013a; Whitaker and Gilpin, 2015; Deslauriers et al., 2017) although another study does not (Albrechet-Souza et al., 2020). Depression and anxiety are often co-morbid (Spijker et al., 2020) and are linked to alterations in the innate immune system in humans (Maes, 1995; Wu et al., 2015; Frank et al., 2016; Liu et al., 2019). Because predator odor affects cytokine levels in rats and mice (Wilson et al., 2013b; Deslauriers et al., 2017), such effects may promote these enhanced affective behavioral responses.

The link between Tlr4 with depression and co-morbid anxiety is demonstrated by the finding that TLR4 expression levels correlate with severity of certain symptoms, specifically anxiety and weight loss, in patients with major depressive disorders (MDD) (Keri et al., 2014; Hung et al., 2015). In preclinical research, chronic mild stress increases Tlr4 expression in the prefrontal cortex of male rats (Garate et al., 2011) and deletion of Tlr4 decreases cytokine responses in hippocampus after stress in male mice (Cheng et al., 2016). Based on this literature, we predicted that deletion of the Tlr4 gene would lessen the ability of predator odor stress to induce depressive- and anxiety-like effects. However, our results in the OFT show the opposite: an enhanced depressive-like response seen as decreased distance traveled in KO rats of both sexes and an enhanced anxiety-like response in female KO rats. Male immune deficient and T-cell deficient mice exposed to predator odor exhibited greater unconditioned fear and anxiety behaviors (Cohen et al., 2006). Similarly, another study demonstrated that elimination of Tlr4 decreases novelty-associated exploratory behavior and social interaction in the absence of baseline anxiety-like differences in male mice (Li et al., 2016). Yet, a separate study of male and female Tlr4 deficient mice showed anxiety-like behavior and decreased social interaction in the absence of an experimental stressor, with a greater effect on anxiety in males (Femenia et al., 2018). Additionally, heightened anxiety behavior was observed in male mice with intracerebroventricular administration of a TLR4 antagonist (Okun et al., 2011). Other studies show that decreased Tlr4 activity associates with reduced stress-induced affective responses. Male mice with Tlr4 deletion exhibit decreased learned helplessness (Cheng et al., 2016) and inhibition of Tlr4 activity attenuates immobility in both forced swim and tail suspension tests after chronic mild stress (Fu et al., 2019). Finally, another study finds no relation between Tlr4 expression and behavior in the forced swim test in male rats (Garate et al., 2011). Despite correlational evidence in humans suggesting TLR4 mediates symptoms of anxiety and depression, the effects of TLR4 manipulation on anxiety- and depressive-like behaviors sometimes diverge in experimental animal studies that suggests a more nuanced role for TLR4 in affective responses that may vary depending on the type of stressor.

Tlr4 activity is increased by LPS administration and this reduces locomotor activity and center crossings in an open field in both male and female rats (Tonelli et al., 2008). Enhanced depressive-like effects in female mice are observed on administration of LPS in the absence of baseline anxiety-like differences in male mice when LPS is combined with chronic unpredictable stress (Couch et al., 2016). However, no effect of LPS combined with predator odor stress is seen in the OFT with male mice (Deslauriers et al., 2017). It is difficult to reconcile these discrepant findings in studies that examined responses to manipulations that either decreased or increased TLR4 activity, although they are likely due to procedural and species differences. Further, unlike our study that employed rats of both sexes wherein we found some sex-specific effects, much of the previous research was conducted with male rodents only. Although our results contradicted our original hypothesis, our findings stand in general agreement with most studies using knock-out, knock-down and antagonism strategies to target TLR4.

No genotype effect was seen in sucrose preference or in total fluid intake in the present study, consistent with a lack of effect of LPS administration in combination with chronic restraint stress in male rats (Biesmans et al., 2016). However, LPS administration combined with chronic mild stress did decrease sucrose preference in male mice (Couch et al., 2016). LPS alone attenuated saccharin preference in male rats, an effect that is reversed by chronic treatment with a tricyclic antidepressant drug (Yirmiya, 1996). Conversely, inhibition of Tlr4 activity attenuated sucrose preference after chronic mild stress in male mice (Pu et al., 2019). The sucrose concentration used in the present study may have been too high to uncover genotype or sex differences in this stress-induced affective response.

We predicted female rats would exhibit greater stress-induced affective behavioral responses than male rats. Indeed, this effect was seen in both measures of the OFT (distance traveled and center time) in the present study. This prediction is based on the fact that women with MDD are more likely than men to show signs of anhedonia and sadness (Frank et al., 1988; Kornstein et al., 2008; Zagni et al., 2001; Marcus et al., 2008). Our expectation that deletion of the Tlr4 gene would attenuate these stress-induced affective responses to a greater extent in females is based on findings of gender differences in the immune system and depression. Women show greater inflammatory and cellular immune responses, including cytokine production and T cell activation, in response to infections or antivirals compared to men (Standberry et al., 2002; Nalbandian and Kovats, 2005; Klein et al., 2010) that may reflect gonadal hormone differences (Gillgrass et al., 2005). An inflammatory challenge leads to greater depressive responses (Moieni et al., 2015) and antiviral treatments are more likely to cause depression (Udina et al., 2012) in women compared to men. Women also show higher rates of autoimmune diseases (Whitacre, 2001; Ortona et al., 2014) and the risk of developing a MDD is greater among those with autoimmune diseases (Siegert and Abernethy, 2005; Menard et al., 2017; Zhang et al., 2017). Interestingly, the pattern of sex differences in affective responses to stress seen in humans contrasts with that observed in rodents, where male animals often show greater stress sensitivity than females (Cohen and Yehuda, 2011). Further understanding of the biological mechanisms of stress reactivity in humans and rodents will likely provide insight into the occurrence of these sex differences both within and between species.

The lack of sex differences in the SPT induced by predator odor exposure in the present study contrasts with another report wherein female rats exhibited greater anhedonia in sucrose preference tests than males after a single prolonged stress, an effect that was not due to gonadal hormones (Pooley et al., 2018). We did not monitor estrous stage in the current study, but the anxiety- and depressive-like responses recorded at baseline prior to predator odor exposure confirm prior reports of sex differences (see Table 3). Like other research, we find sex differences in sweet solution intake (Scalfani et al., 1987; Marshall et al., 2017). This may reflect that the sensitivity to or the discrimination of the taste of sweet solutions is lower in female rats (Curtis et al., 2004). Sex differences in open field behaviors have also been documented (Gray and Buflery, 1971; Archer, 1975; Beatty and Fessler, 1976). Male rats show less ambulation and rearing in open field compared to female rats (Mastr et al., 1980) and spend more time in the center of the arena (Archer, 1975). Our findings are consistent with these prior reports.

Results of the present study are in line with our hypothesis that the role of the innate immune system, particularly the TLR4 receptor, in stress-induced depressive-like effects particularly in females. However, contrary to our prediction, deletion of the Tlr4 gene enhanced rather than attenuated stress-induced affective responses in some cases. This hypothesis was based on the evidence that depressive symptoms in both humans and rodents associate with activation of the innate immune system, specifically TLR4 activity. Thus, the lack of the Tlr4 gene would presumably attenuate the behavioral responses to the predator odor stress. Yet, the
literature on TLR4 activity and behavior is mixed; both activation and elimination of this receptor can lead to similar responses as discussed above. This may reflect that adaptive behavioral responses depend upon the integrity of the innate immune system as suggested by Cohen and colleagues (Cohen et al., 2006). This integrity may be disrupted by either increasing TLR4 activity via LPS administration or by deleting the gene or may relate to compensatory effects in other systems. That the anxiety-like effect of predator odor exposure is not altered by Tlr4 gene deletion in males may reflect, in part, the lower than expected number of male KO rats born, likely owing to unforeseen reproductive effects of Tlr4 deletion. TLR4s present in the female uterine epithelium mediate an immune response to seminal fluid and may affect successful fertilization (Schjenken et al., 2015; Ezz et al., 2019). While TLR4 is also present in sperm, the role of the receptor in reproductive processes remains uncharacterized (Sahnow et al., 2017; Fang et al., 2020). The presumed detrimental reproductive effects of TLR4 deletion in male rats may have led to obtaining KOs with overcompensation in other systems, perhaps in the expression of other DAMP-responsive TLRs (Park et al., 2005; Ivanov et al., 2007). Nonetheless, our findings suggest that the efficacy of pharmacological treatments for depression and co-morbid anxiety may vary by sex differentially by stress exposure.

Declaration of competing interest
None.

Acknowledgments
Support was provided by NIAAA grant, U01-013476 (TAK), by a Summer Undergraduate Research Fellowship from University of Houston, and an undergraduate grant from Psi Chi International Honor Society in Psychology (CBQ). We thank Dr. Gregg Homanics for providing the original breeding pairs of heterozygous TLR4 rats.

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