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TRAUMA AND CUE-ASSOCIATED WORKING MEMORY DEFICITS IN A RAT MODEL OF POSTTRAUMATIC STRESS DISORDER

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ABSTRACT

Posttraumatic stress disorder (PTSD) is associated with a variety of neural and behavioral alterations in response to trauma exposure, including working memory impairments. Rodent models of PTSD have not fully investigated chronic or reactive working memory deficits, despite clinical relevance. The present study utilizes footshock trauma to induce a posttraumatic stress state in rats and evaluates the effect of trauma and trauma-paired odor cues on working memory performance in the odor span task. Results demonstrate the emergence of chronic deficits in working memory among traumatized animals by three weeks post-trauma. The presentation of a trauma-paired odor cue was associated with further decrement in working memory performance. Further, anxiety-like behaviors modeling PTSD symptoms can be predicted by the degree of working memory impairment in response to the trauma-paired odor cue. This study enhances existing animal models by establishing face validity of rodent PTSD models through replication of the clinical observations of working memory deficits associated with PTSD. This will pave the way for future work to probe underlying mechanistic dysregulation of working memory following trauma exposure and for future development of novel treatment strategies.

KEYWORDS: working memory, trauma/stress, posttraumatic stress, odor span test, cue reactivity

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INTRODUCTION

Posttraumatic stress disorder (PTSD) is a psychological condition characterized by a pervasive maladapted stress state, elicited by a significant trauma. PTSD is defined by in four primary symptom clusters: intrusive thoughts and memories, avoidance, negative alterations in mood and cognition, and hyperarousal and reactivity (American Psychiatric Association, 2013). Intrusions and changes in reactivity are linked with adverse reaction to internal or external trauma reminders and demand substantial attentional and cognitive resources (Cox & Olatunji, 2017). Working memory also is dependent upon these attentional and cognitive resources and is commonly impaired in PTSD patients (Honzel et al., 2014). This relationship has brought about successful potential treatments in clinical applications where working memory training has been demonstrated to improve symptoms of posttraumatic stress disorder, suggesting an intersection between working memory and PTSD mechanisms (Larsen et al., 2019; McDermott et al., 2016; Saunders et al., 2015). Despite these successes in clinical applications, working memory has not been probed in animal models of PTSD, which will be necessary to provide a mechanistic understanding of the relationship.

Posttraumatic Stress Disorder

PTSD affects approximately 8% of the population, although this only represents a small percentage of the population that has experienced trauma (Kilpatrick et al., 2013). Little is known about the mechanisms underlying susceptibility to PTSD, but it is clear that not everyone exposed to significant trauma will develop PTSD. Susceptibility also is accompanied by significant sex differences. Despite being less likely to experience trauma in their lifetime, women are nearly twice as likely as men to develop PTSD (Kessler et al., 1995, 2005).

Animal models have traditionally evaluated all traumatized animals as a single, uniform group, limiting the nuanced exploration of susceptibility factors (Richter-Levin et al., 2019). A series of approaches have attempted to mimic clinical diagnostic procedures by employing a

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battery of behavioral tests selected to approximate symptom clusters to determine whether an animal is presenting with a PTSD-like state (Cohen et al., 2003; Richter-Levin et al., 2019). Multivariate approaches such as this accommodate individual differences in symptom presentation and may better capture sex differences associated with symptom profiles (Ardi et al., 2016; Murphy et al., 2019). Additionally, animal models of PTSD utilize varied trauma procedures, which may further variability in symptom presentation.

Fear conditioning has been a dominant tool for modeling PTSD and generates a phenotype of lasting reactivity toward cues and context associated with the stressor, most often footshock (Bienvenu et al. 2021). Footshock trauma has strong face and predictive validity for PTSD, resulting in behavioral alterations akin to the primary symptom clusters of clinical PTSD and responding to pharmaceutical interventions for PTSD (e.g. Paroxetine (Paxil) and Fluoxetine (Prozac)) (Bali & Jaggi 2015). Footshock trauma meets another important criterion of a valid PTSD model in that the behavioral responses persist and/or progress with the passage of time (Mikics et al 2008; Yehuda & Antelman, 1993). Footshock generally is not administered at painful levels, but its success as a trauma model relies upon the ability for surprising, unpleasant stimuli to induce long-lasting behavioral alterations (Wellman et al., 2014; van Dijken et al., 1992).

Methods of modeling PTSD-like trauma have largely focused on replicating physiological and reactivity phenotypes of PTSD (Liberzon et al., 1997; Cohen et al., 2012). However, additional assessment is needed of the trauma memories at the root of these physiological and reactivity profiles. PTSD has a multifaceted relationship with memory, defined by overactive and inappropriate memories of the trauma, but also commonly resulting in generalized memory impairments, including working memory (Nejati et al., 2018).

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Working Memory

Working memory is a limited-capacity mechanism for temporarily retaining and manipulating information for use in goal-directed tasks (Barch et al., 2009). Stress and trauma create chronic energetic and attentional demands that reduce available resources for normal functioning. This diversion of attentional resources may provide a mechanism to understand working memory deficits in PTSD (Block & Liberzon, 2016; Balderston et al., 2017; Peters et al., 2017). PTSD has been associated with elevated levels of attentional bias to aversive stimuli and it is theorized that the persistence of trauma-related memories serves as a low-level distractor that is omnipresent for the affected individual (Woodward et al., 2017). Clinical studies have demonstrated working memory deficits following acute stress, as well as in persistent states of stress such as PTSD (e.g. Moran, 2016; Shields et al., 2016; Scott et al., 2015). Rodent models have replicated the effects of acute stress on working memory performance but have not been utilized to study the effect of long-lasting stress responses that characterize PTSD on working memory (Davies et al., 2013). Span tasks, such as the odor span task, are the primary method for testing working memory capacity in rodents and therefore optimal to determine the effects of stress on this behavior (Davies et al., 2013; Dudchenko et al., 2013; Dudchenko, 2004).

The present study utilized the odor span task to assess working memory performance in a rodent model of PTSD. Rats underwent footshock trauma in the presence of a trauma-paired odor (TPO), and subsequent working memory performance was evaluated in the presence that TPO. Two hypotheses were tested: that working memory performance would be impaired acutely and/or chronically; and that working memory performance would deteriorate when presented with the TPO in the odor span task.

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RESULTS

Rats were assessed for the impact of trauma on both anxiety-like and working memory behaviors, as illustrated in Figure 1A. To examine working memory, an odor span task was utilized, in which rats identified novel odors in an incrementing delayed non-match-to-sample (DNMS) task (Figure 1B), as described in Materials & methods. Prior to beginning odor span training, animals went through a pre-test elevated zero maze test to evaluate baseline anxiety-like behavior (Fig. 1C). Following two weeks of initial training, DNMS performance was evaluated to assess baseline task performance (Fig. 1D). Animals were counterbalanced into control and trauma groups on these scores. Anxiety-like behavior, as assessed by time spent in the open arms of the zero maze, did not differ by condition [$F(1,28) = 0.0050$, $p = 0.94$], but there was a main effect of sex [$F(1,28) = 5.33$, $p=0.024$]. Females spent more time exploring the open arms of the maze, which is typical in locomotor-based tasks. There were no significant differences in DNMS performance between the control and trauma conditions [$F(1,28) = 0.27$, $p = 0.61$] or sexes [$F(1,28) = 2.07$, $p = 0.16$].

Working memory performance in the DNMS training phase was assessed immediately following the footshock trauma session. Data are expressed as percent change in correct responses compared to the previous DNMS session (Fig. 2). There were no group differences between male and female animals [$F(1,28) = 0.64$, $p = 0.43$], control and trauma animals [$F(1,28) = 0.18$, $p = 0.68$], or interaction between sex and condition [$F(1,28) = 0.043$, $p = 0.84$]. No change in DNMS performance was observed following acute stress (male control [$t(7) = 1.96$, $p = 0.090$]; male trauma [$t(7) = 1.32$, $p = 0.23$]; female control [$t(7) = 1.11$, $p = 0.30$]; female trauma [$t(7) = 1.33$,$p = 0.23$].

Working memory performance was assessed during the final week of training as an average measure of baseline performance (Fig. 3A). There was a main effect of sex [$F(1,28) = 4.87$, $p = 0.036$], demonstrating that female working memory performance was, on average,

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higher than male performance. There was a main effect of condition [$F(1,28) = 4.76$, $p = 0.038$], revealing that animals with a trauma history obtained lower maximum spans, on average, than control animals. There was no interaction between sex and condition [$F(1,28) = 0.19$, $p = 0.66$]. Omissions also were summed from the final week of training to examine whether the trauma-history spans could be explained by animals prematurely "quitting" trials through omissions (Fig. 3B). Two-way repeated measures ANOVA revealed no main effect of condition [$F(1,28) = 2.42$, $p = 0.13$], supporting the fact that trauma-history animals did not omit responses significantly more often than control animals. There was no main effect of sex [$F(1,28) = 0.097$, $p = 0.76$], suggesting that the sex difference in working memory performance was not driven by an increase in trial omissions by males.

All animals received two days of behavioral testing, elevated zero maze and marble burying, discussed below, and then entered the testing phase of the odor span task. In this phase, animals were given a normal testing day to serve as baseline and a TPO testing day. On the TPO testing day, the TPO, coriander, was presented as the target cup at span 4. Maximum span performance was compared between baseline and TPO testing days (Fig. 4A). A three-way ANOVA revealed a main effect of day [$F(1,28) = 5.46$, $p = 0.027$], which reflected that span performance on average decreased on the TPO sessions, but no main effect of condition [$F(1,28) = 3.41$, $p = 0.075$] or sex [$F(1,28) = 1.25$, $p = 0.27$]. Percent change in span length on the TPO session was calculated as a metric of how each animal's working memory performance was affected by the presence of the TPO (Fig. 4B). One-sample t-tests revealed that male control [$t(7) = 0.72$, $p = 0.49$] and female trauma [$t(7) = 0.69$, $p = 0.51$] animals did not have significantly altered spans on the TPO test session. On the other hand, male trauma [$t(7) = 2.82$, $p = 0.026$] and female control [$t(7) = 3.30$, $p = 0.017$] animals demonstrated significant decreases in maximum span on the TPO session.

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Latency to dig was compared between non-TPO trials and the TPO trial, calculated as the total latency to dig divided by the number of cups sampled (Fig. 4C). A three-way mixed models ANOVA was run because one female was omitted from this analysis due to a time-out omission on the TPO trial. There was a main effect of trial type on latency to dig [$F(1,28) = 16.86$, $p = 0.0003$], where latency to dig was longer on TPO trials, and a main effect of sex [$F(1,28) = 7.86$, $p = 0.0093$], with males having longer latencies to dig than females. There was no main effect of condition [$F(1,28) = 2.73$, $p = 0.11$] or interactions. Again, a change score was calculated to directly compare the change in latency to respond within each group (Fig. 4D). Female control animals did not significantly increase latency to dig on the TPO trial [$t(7) = 1.68$, $p = 0.14$], but male control [$t(7) = 2.50$, $p = 0.041$], male trauma [$t(7) = 2.50$, $p = 0.041$], and female trauma [$t(7) = 2.71$, $p = 0.030$] animals all significantly increased latency to dig on the TPO trial. Deficits in span were hypothesized to result from cue reactivity, which is approximated by latency to dig on the TPO trial. Interestingly, the latency to dig on the TPO trial was only significantly correlated with the decrement in span performance for male trauma animals (Pearson's $r = -0.80$, $p = 0.0017$). All other groups demonstrated non-significant relationships between latency and span change [male control ($r = 0.099$, $p = 0.82$); female control ($r = 0.10$, $p = 0.83$); female trauma ($r = -0.35$, $p = 0.40$)].

Susceptibility tests were run before and after the odor span testing days to protect against the possibility that re-exposure to the trauma-paired cue substantially affected the behavior of the trauma-history animals. Elevated zero maze behavior was measured by time spent in the open arms of the maze (Figure 5A). Two-way repeated measures ANOVA revealed no main effect of condition [$F(1,28) = 0.11$, $p = 0.74$] or sex [$F(1,28) = 0.070$, $p = 0.79$] on open-arm time. General locomotor activity was evaluated via the number of entries into the open arms during the elevated zero maze test (Fig. 5B). There was no main effect of condition [$F(1,28) = 0.0049$, $p = 0.94$], but there was a main effect of sex [$F(1,28) = 19.13$, $p = 0.0002$],

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demonstrating that females displayed greater general locomotion during the zero maze test even though their total time in the open arm did not differ from males. Marble burying results are not shown because only 3 trauma-history and 1 control out of the 32 total animals engaged in marble-burying behavior. Open field behavior was scored as the total amount of time that the animal spent in the center of the arena (Fig. 5C). There were no main effects of condition [$F(1,28) = 0.025$, $p = 0.88$] or sex [$F(1,28) = 0.48$, $p = 0.49$] on center time. Total distance traveled, scored with ANY-Maze as number of zone crossings multiplied by the size of each zone in the maze (15 cm), was analyzed for locomotor behavior (Fig. 5D). There was no main effect of condition [$F(1,28) = 0.078$, $p = 0.78$], but a trend toward a main effect of sex [$F(1,28) = 3.99$, $p = 0.056$], again supporting that females tend to have higher levels of general locomotion than males during anxiety-like behavior tests. Behavior in the social interaction test was evaluated by the distribution of time spent engaged in non-social, pro-social, and antisocial encounters (Fig. 5E). A priori hypotheses were that a PTSD-like phenotype would be associated with less total social interaction and greater engagement in antisocial behavior. Two-way ANOVAs were performed on each of these metrics. Total social interaction did not differ by condition [$F(1,28) = 0.21$, $p = 0.65$], but there was a main effect of sex [$F(1,28) = 8.74$, $p = 0.0063$], demonstrating that females spent less total time engaged in social interaction. There were no main effects of condition [$F(1,28) = 0.045$, $p = 0.83$] or sex [$F(1,28) = 2.43$, $p = 0.13$] in time spent engaged in antisocial behavior. Data were converted to z-scores within each group (male control, male trauma, female control, and female trauma) to allow for combination of male and female data by condition, while taking into account differences between the sexes. When normalized within condition, the change in span on TPO trials is significantly correlated with antisocial behavior for trauma-history animals only (Fig. 5F; Pearson's $r = 0.64$, $p = 0.007$). Control animals did not display a relationship between antisocial interaction and change in span ($r = -0.38$, $p = 0.16$).

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Multiple regression analysis using the PTSD-like behavioral profile significantly predicts the degree to which an animal's span was altered by presentation of the trauma-paired cue (span change) for trauma-history animals. For this analysis, data from each predictor variable (training span, TPO latency, elevated zero maze open zone time, open field center time) and the outcome variable (percent change in span) were converted to z-scores to standardize data scaling across groups and collapsed across sex since few effects of sex were present in the data. Sex was factored into the regression analysis, but because it was not a significant predictor variable, it was excluded as a predictor of interest. The regression model was built with training span, TPO latency, elevated zero maze, open field, and antisocial interaction scores to predict the change in span in the presence of the TPO. This model was significant only for trauma-history animals [Table 2; $F(5,10) = 18.12$, $p < 0.001$, $R^2 = 0.90$, all variables contributed significantly to the prediction, $p < 0.05$], but not for control animals [Table 1; $F(5,9) = 1.023$, $p = 0.46$, $R^2 = 0.36$; no variables contributed significantly to the prediction, $p > 0.05$]. Covariance matrices are presented in Figure 6.

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DISCUSSION

The present study is among the first to examine working memory in an animal model of PTSD. A footshock trauma procedure and the odor span task were used to evaluate the effect of trauma history and trauma-paired cue reactivity on working memory performance. The data demonstrated that while footshock trauma did not acutely affect working memory performance in DNMS (Figure 2), trauma history was associated with persistent deficits in working memory performance (Figure 3), and exposure to a trauma-paired cue further impaired working memory (Figure 4). Additionally, multivariate analysis of PTSD phenotypic behaviors significantly predicts the degree to which an individual's working memory is impaired by the presentation of trauma-associated cues further validating the change in span as an index that models certain features of PTSD. Collectively, these data move the PTSD field toward an animal modeling approach that will allow for investigation of the mechanisms and implications for working memory deficits in PTSD.

Lack of acute effects of footshock trauma

Footshock sessions were delivered one animal at a time and acute effects of the trauma were assessed immediately. Animals were transported directly from the footshock trauma session to the behavioral testing room, where they completed 8 DNMS trials. There was no effect of acute trauma on DNMS performance, which appears to contrast with evidence that acute stress impairs working memory capacity in the odor span task (Davies et al., 2013). It is possible that the lack of acute stress effects is driven by differences in mechanisms (or resources required) for working memory capacity versus two-sample discrimination. It may not be surprising to find such conflicting results, as acute stress has been found to both impair and enhance working memory performance in different working memory tasks, and whether performance is impaired or enhanced may be partially dependent on the sex of subject (Khayyer et al., 2021; Schoofs et al., 2013). Further, in the present study, animals were still in a

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training phase and the lack of established stable responding may have obscured the true effect of acute footshock trauma on working memory performance.

Past trauma triggered chronic working memory deficits

Following footshock trauma, animals were given three additional weeks of odor span training, which also served as a stress-incubation period to allow for the emergence of persistent stress phenotypes (e.g. Harvey et al., 2003). Maximum odor span was averaged across the final week of training, and trauma history resulted in lower overall working memory performance at three weeks post-trauma compared to the control group. Both sexes with trauma history had lowered maximum spans compared to controls, although females had higher spans regardless of condition. Clinical research indicates that females may have slightly better working memory performance than males, but these results may be impacted by factors such as general motivation and attention or olfactory sensitivity (Voyer et al., 2021; Sanchez-Andrade et al., 2005). It should be noted that span performance in this study was high compared to previous odor span studies (Dudchenko et al., 2000; Davies et al., 2013; de Falco et al., 2019). The most apparent methodological differences between past odor span work and the current study was our use of Wistar rats and inclusion of both sexes. Previous studies exclusively used Long Evans and Sprague Dawley strains, and primarily focused on males. It is possible that working memory, stress response, olfactory sensitivity, or general cognitive performance may be confounded by strain and gender (Andrews, 1996; Martis et al., 2018). Probe trial performance indicated that animals were not marking cups or selecting based on the smell of the buried food reward, but that does not rule out possible engagement in strategies like chunking in a manner that promoted attainment of higher spans (Guida et al., 2012).

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Trauma-paired cue reduced span performance for trauma-exposed rats

Following the three weeks of OST training and stress incubation, animals performed a baseline odor span test, which was identical to training days. On the next day, they were given a TPO test, in which the TPO was presented at span 4. Male trauma-history animals had significantly lower span performance when the TPO was present. Female trauma-history animals did not demonstrate a significant deficit in span in the presence of the TPO, although this may have been driven by greater variability among trauma-history females. The apparent working memory deficit in female control animals in the presence of the TPO is likely driven by the fact that many of these females were regularly attaining the maximum span possible in the test, and so a small variation from this ceiling was significant. Average latency to dig was significantly higher on the TPO trial in all groups but female control, likely indicating deliberation or hesitancy to interact with the TPO. Male trauma history animals are the only ones for whom latency to dig on the TPO trial was significantly correlated with the deficit in span performance in the presence of the TPO. This correlation was not driven by animals who were unable to continue the span following the TPO trial. Rather, animals who successfully completed the TPO trial subsequently achieved a lower span than at baseline.

The effect of the TPO on working memory demonstrates cue reactivity in our model, which is an important aspect of the PTSD-like phenotype. Cue reactivity is associated with two of the primary PTSD symptom clusters: intrusions and hyperarousal/reactivity. The disruption of task performance in the presence of the TPO may indicate that the animals were distracted by its presence. Attention plays a central role in the working memory mechanism, and distractors generally impair working memory performance (Cowan, 2008). A chronic stress response to trauma may involve frequent allocation of attentional resources to trauma-associated information (Peters et al., 2017). Therefore, the working memory deficits following exposure to trauma cues may be related to impairments in the ability to maintain attention on the task when

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the trauma-paired odor is present. Indeed, targeting attention maintenance and working memory deficits in individuals diagnosed with PTSD reduced their symptoms (Badura-Brack et al., 2015; McDermott et al., 2016).

Odor span is considered to capture working memory capacity, which is theoretically distinct from the virtually limitless recognition memory (Turchi & Sartor, 2000; Dudchenko, 2000). The distinction between working and recognition memory lies in whether the animal is tasked with remembering the order of stimulus presentation, as is the case for span tasks. It has been shown that working memory capacity frequently falls into the range of 7 +/- 2 items in humans and animals, although smaller limits have been proposed (Miller, 1956; Cowan, 2001; Hahn et al., 2021). Performance within the odor span task remains high when more than 70 odors are to be recognized if the maintenance of presentation order is not required. Unique patterns of neural activity have been observed to support recognition of “novel” versus “familiar” odors, suggesting that the odor span task may not be accomplished simply by recognition and memory of each individual odor (De Falco, 2019). This may indicate the use of chunking strategies or suggest that the odor span task captures a more general feature of cognition. Attentional deficits and biases have been associated with impaired working and recognition memory, indicating that both forms of memory are dependent upon attentional resources (Spataro et al., 2022; Fosco et a., 2020). Thus, future work must expand on the current findings, particularly by examining the mechanisms underlying attentional allocation toward trauma cues and manners through which the resulting attentional bias may be corrected in animal models.

Multivariate prediction of cue reactivity

Animals were tested on elevated zero maze, marble burying, open field, and social interaction to evaluate PTSD-like phenotypes. Distinct differences by group and by sex were observed across these tests, except marble burying, as well as throughout the training and testing phases of the odor span. PTSD is a multifactorial disorder characterized by clusters of

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symptoms; therefore, a multiple linear regression model was built to determine whether this array of behavioral indices could predict how trauma cues impact working memory performance (change in span). Significant predictors included working memory span performance during training, latency to dig in the TPO trial, elevated zero maze open arm time, open field center time, and antisocial behavior. Only antisocial behavior independently correlated significantly with change in span, but when combined in the regression model, all behavioral metrics of the PTSD-like phenotypes significantly related to change in span. This predictive relationship was only present for animals with a trauma history. No behaviors were significant predictors of span performance in control animals. These results suggest that trauma-paired cue reactivity is associated with working memory deficits and relates to a wider profile of PTSD-like symptoms.

Previous studies have employed strategies with fewer measures (generally two tests) of anxiety-like behavior to determine extent of the PTSD-like phenotype in animals. Clinical diagnosis of PTSD requires symptoms across four primary clusters, and male and female symptoms may differ, so the utilization of multiple tests is important to capture individual variation (Carmassi et al., 2014; Carragher et al., 2016; Gruene et al., 2015). Future work will be needed to validate the use of a multiple linear or logistic regression approach to detect susceptible and resilient phenotypes in animal models of PTSD. Additionally, the current study relied upon multiple behavioral endpoints, which are inherently variable. Larger sample sizes would provide additional power for detecting significant effects, particularly with respect to sex differences and susceptible phenotypes. Future studies would benefit from using a higher throughput model, such as an automated odor span task (Galizio et al., 2020). Additionally, because male and female rodents exhibit differing anxiety-like behavioral profiles, future work should incorporate anxiety-like tests designed to capture more female-typic behaviors (Gruene et al., 2015; Meyerson et al., 2006).

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Conclusion

PTSD models lack standardization, resulting in variations in models associated with differing behavioral outcomes (Bienvenu et al., 2021; Knox et al., 2012). The current study presents a model for PTSD-like impairment of working memory performance, with different impacts in the absence versus presence of a trauma cue. Additionally, the regression model produced a quantitative assessment of the impact of the trauma-paired cue on span performance. The sample size used in the present study was quite small for use in multiple linear regression. Bootstrapping and k-fold cross validation methods were used to provide assurance of model robustness, but the risk remains that these models built on small samples will not generalize well. This method should be validated using a larger sample size to provide further confidence about the ability for a holistic PTSD-like phenotype to predict reactive impairments in working memory. Future studies with this and other PTSD models must validate working memory phenotypes to identify molecular underpinnings of these persistent effects of trauma and to further the development of treatments that may have increased efficacy in individuals suffering PTSD-related working memory deficits.

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MATERIALS AND METHODS

Subjects

Three separate cohorts of adult male and female Wistar rats (initial weights M: 253-306 g; F: 146-206 g Charles River, NC) were obtained at 8 weeks of age. Upon arrival, the rats were pair-housed with lights on a 12-hr light/dark cycle (lights on at 0600) in a humidity- and temperature-controlled vivarium with ad libitum access to food and water. Subsequently, subjects were individually housed, mildly food restricted to maintain 90% of free feeding weight, and handled 5 minutes each day for 5 days prior to the first experimental task. All experimental procedures were conducted during the light phase of the light cycle. All procedures were approved by the IUPUI School of Science Animal Care and Use Committee.

Experimental Overview

The experiment consisted of the odor span task, footshock trauma, and susceptibility tests (Fig. 1A). All behavioral apparati were cleaned with water between same-sex subjects and with 70% ethanol between sexes to minimize odor distractions. Prior to each anxiety-related behavioral test, animals were given an acclimation period in the antechamber of the testing room. White noise (approximately 54 dB) was played during all behavioral tests and tests were run under normal house lights unless otherwise specified. Behavioral tests were recorded via an overhead camera and scored by an observer blind to experimental condition in BORIS (Friard & Gamba, 2016).

Odor Span Task

Methods for the Odor Span Test followed Davies et al. (2013). The apparatus featured a grey textured plastic platform (36” round, 12” border, 31” above the ground). Odors (0.5 g of dried spice; allspice, anise seed, basil, caraway, celery seed, cinnamon, cloves [0.1 g], cocoa, coffee, cumin, dill, fennel seed, garlic, ginger, lemon, marjoram, nutmeg, onion powder, orange,

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oregano, paprika, rosemary, sage, and thyme) were mixed in Premium Play Sand (100 g; Quikrete Cement Products) in 3.25-oz plastic cups (2" tall; 3" wide). Twenty-four Velcro attachment points were equally spaced along the perimeter of the platform and cups were randomly attached to one of these points in each trial to prevent the use of spatial strategies. Animals were trained five days each week in either a morning group or an afternoon group, with sexes and conditions represented equally across groups.

Initial Training

Initial training involved rats learning to dig for a cereal reward (1/4 of a Kellogg's Froot Loops®) in unscented sand (3.25-oz cups containing 100 g of sand). For each trial in this phase, a single cup of unscented sand was placed in a random position on the platform. After the subject retrieved the reward or following a maximum response time of 2 minutes, it was removed from the platform and placed in a separate intertrial box for a 40-second period. The experimenter then moved the cup to a new location before the next trial. The reward was first placed on the surface of the sand, then half buried once animals reliably retrieved the unburied reward. After animals were consistently obtaining the half-buried reward, the reward was fully buried under the surface of the sand and animals were trained until they would consistently dig for and retrieve the reward from the unscented sand. Initial training took approximately 3-4 days.

Delayed Non-Match-to-Sample

Rats were next trained on a DNMS task. In this phase, rats were first presented with a sample trial where they were presented with a cup of scented sand randomly placed on the platform. After retrieving the reward, rats were moved to the intertrial interval box for a 40-s delay period, during which time the experimenter moved the sample cup to a new location and randomly placed a second bowl containing a different odor and a reward on the platform. The animals were then returned to the platform for their choice trial and allowed to freely sample

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(sniff) the cups. A choice was determined by the animal contacting the sand with their nose or paws. An error was scored if the rats selected the sample odor, rather than the novel odor. The animals were given 8 DNMS choice trials each day until they selected the novel odor on 6 out of 8 choice trials for three consecutive days. DNMS training took approximately 1.5 weeks.

Odor Span Task

Following DNMS, animals were trained on the odor span task (Fig. 1B). These trials were conducted identically to the DNMS task, except cups containing novel odors were added following each correct choice until the rat made an incorrect selection or omitted a response, resulting in an increasing number of odors present in the arena. Span for a given trial is defined as the number of choice trials completed successfully, which is equivalent to the total number of cups present minus one. Each rat performed a maximum of three consecutive spans per day, with no trials beginning more than 15 minutes into the session. Maximum span is reported for each day. Because training duration was time-locked to the three-week stress incubation period, animals who reached a stable level of high performance were reduced to training three times per week to reduce the likelihood of overtraining.

Following three weeks of OST training, the effect of trauma cue presentation on working memory performance was evaluated. Animals were given a baseline testing day, which was identical to the OST training days except that following an incorrect choice, they returned to their homecage for approximately 30 minutes between spans while other rats were tested. The following day, on span 4 (5 cups present on the table), the TPO (coriander) was presented for the first time as a target odor in the OST task. Interaction with the coriander as well as maximum span achieved while coriander was on the platform were recorded and evaluated. Several probe trials were conducted throughout training to confirm that subjects were using cup odor to solve the task and to ensure coriander performance could not be attributed to novelty. The first was a non-baited trial, in which the odor presented at span 4 (five cups on the table)

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did not contain a food reward. Upon selection (nose or paw contact with the sand) of the correct cup, a food reward was dropped onto the surface of the sand for the animals to consume. Successful completion of this trial was indicative of animals not selecting the correct cup because of pursuing the smell of the food reward. The second probe trial was a cup-change trial, in which the cups at span 1 (2 cups on the table) were swapped out with clean cups of the same scents. Successful completion of this trial was indicative that the animals were not marking the cups as a strategy to identify previously sampled cups (Davies et al., 2017). Swapping out to clean cups later in the span is not feasible while maintaining a 40-s intertrial interval. Non-baited and cup-changed trials were conducted once per week during the 3-week odor span training period. Lastly, a novel odor probe trial was run with two of the three cohorts to ensure that animals could successfully perform the task when presented with a completely novel odor, confirming that any observed reactions on the TPO test day were not confounded by strong reactivity to task-novel odors. The novel odor trial was conducted at the end of the final week of odor span training. For animals who did not successfully pass probe trials, a second probe was provided in the same session. Performance on the second probe is not reported here, but all animals did pass subsequent probe trials, suggesting that the initial failure was likely not directly related to the employment of strategies being probed. Performance across all animals on the non-baited trial was 90%, on the cup-change trial was 91%, and on the novel odor probe was 96%. These rates were on par with the expected rate of correct responses on a given non-probe trial.

Footshock Trauma

Two weeks into the training procedure, animals underwent footshock trauma. They were provided two days of brief acclimation to the footshock chambers prior to the trauma day (<20 min). Animals were counterbalanced into footshock trauma and control groups based on DNMS training performance and initial elevated zero maze performance to maximize chances of

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equalizing cognitive ability and baseline anxiety-like behavior between the groups. Acclimation and footshock sessions were run in illuminated operant boxes (MedPC), with no additional cues preceding footshock. On the day of the footshock trauma, coriander was placed in the operant boxes to allow scent dispersion. This coriander served as a trauma-paired odor cue, which subsequently was presented in the odor span test to assess the impact of trauma cues on working memory performance. The trauma group had five minutes in the box before footshocks began, followed by 20 uncued shocks at 0.8 mA (1-s duration, VI-40 schedule, resulting in average of 17-minute total footshock session length). Control animals were placed in the boxes with coriander scent present but did not experience any footshock. Similar parameters were utilized in fear conditioning procedures that produced substantial conditioned freezing responses three weeks post-stressor, indicating its ability to generate a chronic stress response that could be considered a model of PTSD (Wellman et al., 2014). Animals were shocked one at a time and then immediately brought to the odor span room for that day's training and assessment of acute stress effects on task performance. Footshock chambers were not used for more than one animal per cohort.

Elevated Zero Maze

Elevated zero maze performance was evaluated the day following the final odor span training day as the first of the battery of behavioral tests to assess PTSD-like phenotype. This test shows an animal's response to the conflict between exploration of a novel environment and exposure to an unsafe (open, unwalled) environment. Behavior approximates the avoidance cluster of PTSD-like symptoms (Shepherd et al., 1994; Verbitsky et al., 2020). The maze consists of a circular pathway (105 cm maze diameter, 10 cm track), elevated approximately 50 cm above the floor, with alternating walled and open zones (2 walled and 2 open zones) evenly dividing the total circumference of the track. This circular version of the maze shows fewer sex differences than the plus-shaped version of the maze because it lacks the ambiguous center

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zone and has been shown to be more repeatable than the elevated plus maze (Braun et al., 2012; Tucker & McCabe, 2017). Testing was conducted in dim lighting conditions (~0 lux in closed arms, 6 lux on open arms). Rats were placed on the maze facing the entrance of the closed arm and allowed to freely explore for five minutes. Overhead video of the session was captured and scored for time and entries into the open arm, defined by all four paws entering an arm.

Marble Burying

The marble burying test approximates the PTSD-like symptom cluster of alterations in arousal and reactivity and was conducted on the day following elevated zero maze testing (Kedia & Chattarji, 2014; Verbitsky et al., 2020). Marble burying is conducted most successfully in mice, but the tendency to express neophobia through burying behaviors is common among rodent species (Himanshu et al., 2020). Following homecage acclimation to the testing room (normal light, approximately 100 lux), each animal was moved into a standard shoebox cage with 5cm packed-down bedding and allowed to freely explore for 15 minutes. Then 20 marbles (2-cm diameter) were arranged in a 4 x 5 grid across one half of the testing box. The other half of the box contained no marbles, creating two zones that the animals could freely explore (de Brouwer & Wolmarans, 2018). The rats were placed on the marble-free side of the testing box and allowed 30 minutes to explore the testing box and interact with marbles, after which they were returned to their home cage and the number of marbles buried was counted. A marble was considered buried if more than ½ was under the bedding. Marbles were soaked in a 1-2% bleach solution for 15 minutes and then rinsed and dried thoroughly after each test.

Open Field

The open field test provides an index of anxiety-like behavior, as the center of the field is an exposed and unprotected region. Behavior in this task approximates PTSD-like avoidance

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and possibly captures phenotypes aligned with the cluster of negative alterations in mood (Gould et al., 2009; Katz et al., 1981; Verbitsky et al., 2020). This test was performed the day following TPO testing in the odor span task. The open field apparatus is a square arena surrounded by high walls (63 x 63 x 50 cm), and testing was performed under moderately dim (40 lux) illumination. The floor of the apparatus is divided into sections to quantify position relative to the walls versus center and movement via line crossings. At the beginning of the test, each rat is placed into the center of the open field arena and allowed to freely explore for 10 minutes. Overhead video of the session was captured and scored for time in the center of the open field and for overall locomotor activity, measured by number of zone crossings multiplied by the size of each zone.

Social Interaction

This test examines whether rats show deficit in social interactions with an unknown, same-sex conspecific, and approximates negative alteration in mood symptom cluster (File & Seth, 2003; Verbitsky et al., 2020). Open field testing was performed the day prior to social interaction testing and served as the acclimation period for the test apparatus. Social interaction was tested under the same lighting conditions as the open field test (approximately 40 lux in the center of the arena). On the test day, rats were paired across condition (one control, one stress) with novel partners. Social interaction most commonly involves pairing animals of identical conditions or pairing with an experimentally naïve animal. However, due to hypothesized individual differences in trauma response and an attempt to limit animal use, animals were paired across condition and scored individually, which has successfully been implemented in previous studies (Hindley et al., 1985). One rat from each pair was randomly assigned to have a marked tail to allow for individual behavioral scoring during the test. At the beginning of the test, rats were placed in opposite corners of the arena and allowed to interact freely for 5 minutes. All behaviors initiated by the experimental rat are scored according to the following categories: pro-

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social behaviors towards the other rat (such as sniffing, contact, grooming), anti-social behaviors towards the other rat (attack, threat posture, mounting, fleeing, defensive posture, submission), and nonsocial behaviors (locomotion, rearing, grooming self).

The day following the social interaction test, animals were euthanized using isoflurane to induce rapid sedation, followed by rapid decapitation.

Statistical Analysis

Statistical significance was evaluated at $p < 0.05$ and graphed data are expressed as mean +/- standard error of the mean (SEM) unless otherwise noted. One-sample t-test was used to evaluate change scores from the null hypothesis of zero change for span and latency data. This was not run following an ANOVA and no corrections for multiple comparisons were performed because each group was treated as unrelated to one another Pearson correlation and multiple linear regression were used to assess the significance of bivariate and multivariate linear associations. Bootstrapping and 10-fold cross-validation techniques were used to affirm robustness of the multiple regression model. Three-way mixed measures ANOVA was used to evaluate baseline versus test day differences among condition and sex. Two-way repeated measures ANOVAs were used to compare differences among groups for remaining analyses. Z-score normalization was performed on each group (male control, male trauma, female control, and female trauma) to allow for collapse of antisocial interaction and change in span statistics across sex in a manner that preserved sex differences. Statistical testing and graphing were performed in GraphPad Prism version 9.3.1 (GraphPad Software, San Diego, California USA) and R (R Core Team, 2020). One control female was omitted from data analyses related to the trauma-paired odor latency because of a time-out response omission. All video behavioral scoring was conducted in BORIS (Friard & Gamba, 2016) by scorers blind to condition and sex of animals.

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Table 1. Multiple regression for control animals

Variable	Estimate	Standard error	P value
Intercept	0.02171	0.2666	0.9369
Training Span	0.3588	0.2487	0.1830
TPO latency	-0.2467	0.2985	0.4299
Elevated Zero Maze	0.02355	0.2986	0.9389
Open field	-0.3877	0.2966	0.2235
Antisocial interaction	-0.2150	0.2965	0.4868

Table 2. Multiple regression for trauma animals. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$.

Variable	Estimate	Standard error	P value
Intercept	4.764e-007	0.09327	>0.9999
Training Span	0.5549	0.1153	0.0007***
TPO latency	-0.4124	0.1050	0.0028**
Elevated Zero Maze	0.5439	0.1195	0.0011**
Open field	-0.3133	0.1194	0.0255*
Antisocial interaction	0.3929	0.1116	0.0055**

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FIGURE LEGENDS

Figure 1. Experimental procedures and counterbalancing measures. A. Experimental timeline. B. Depiction of sample odor span session. C. Group assignments show balanced pre-test elevated zero maze performance, as assessed by open arm time, although females spent greater time in the open arms of the maze than males. *p < 0.05, male vs female. D. Pre-stress delayed non-match-to-sample (DNMS) performance did not differ between the groups. Data are presented as mean +/- SEM, with individual data points overlaid. N = 8 per group.

Figure 2. Delayed non-match-to-sample change following acute stress. Acute stress did not significantly impact DNMS performance, expressed as a change score calculated as percent change from DNMS performance pre-stress. Data are expressed as mean +/- SEM, with individual data points overlaid. N = 8 per group.

Figure 3. Trauma history impacted training performance in the odor span test. A. Maximum span, calculated as the average maximum odor span across the final five days of training, was significantly lower in males (squares, left) and animals with a trauma history (dark bars, right in each pair). B. Summed trial omissions from the final five days of training did not differ between groups. Data are presented as mean +/- SEM with individual data points overlaid. N=8 per group. * indicates male vs female and control vs trauma ($p < 0.05$).

Figure 4. Trauma history impacted performance in the odor span test. A. Data show maximum span obtained on baseline test day (left bars) and trauma-paired odor (TPO) test day (right bars). B. Span change on the trauma-paired odor test day was significantly different from baseline for male trauma and female control animals. * $p < 0.05$, one-sample t-test compared to no change (0). C. Latency to respond on non-TPO trials, calculated as the average latency per sampled cup on choice trials 1-3, compared to TPO trial 4. D. Latency to dig on the TPO trial increased for all groups except female controls * $p < 0.05$, one-sample t-test compared to no change (0). E. Latency to dig on the TPO trial correlated with span impairment only for male

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trauma animals * indicates $p < 0.05$, male trauma. Data are presented as mean +/- SEM with individual data points overlaid (A-D) or as individual values with individual groups' correlation lines overlaid (E). N=8 per group.

Figure 5. Behavioral profiling to assess PTSD-like phenotype. A. Elevated zero maze open arm time did not differ between groups. B. Open arm entries in zero maze were more numerous for females, indicating greater locomotor activity. *$p < 0.05$, male vs. female. C. Open field time in the center zone did not differ between groups. D. Total distance traveled in the open field test did not differ between groups. E. Time spent engaged in non-social, pro-social, and antisocial behaviors in the social interaction test differed by sex. F. Correlation between normalized change in span and antisocial interaction was significant only for animals with trauma-history. *$p < 0.05$, control vs. trauma. Data are presented as mean +/- SEM with individual data points overlaid (A-E) or as individual values with correlation lines overlaid by trauma history grouping (F). N=8 per group.

Figure 6. Multiple linear regression covariance matrices. No parameters significantly covaried for control animals (A) or trauma-history animals (B). Blank cells indicate value < 0.01.

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Figure 1

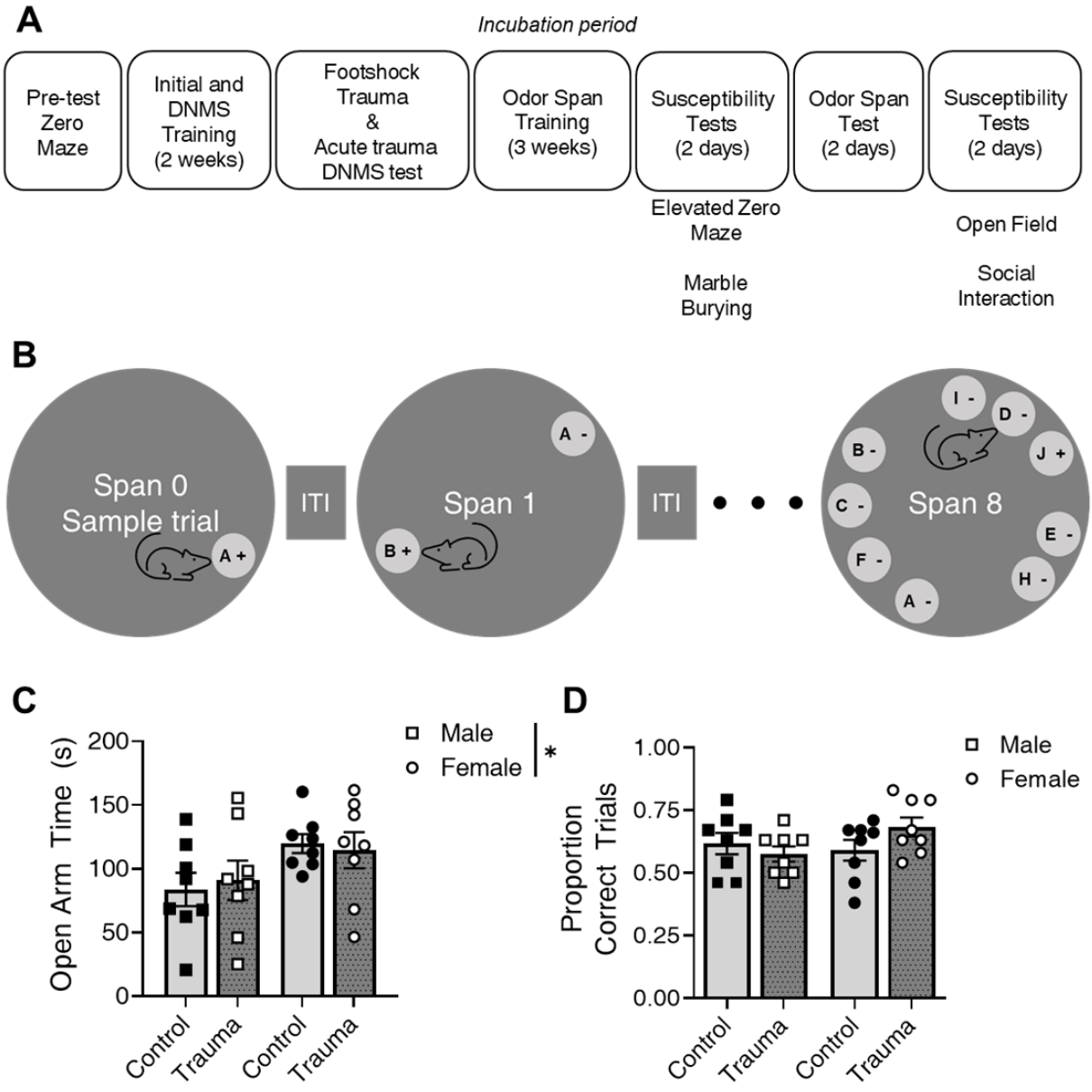

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Figure 2

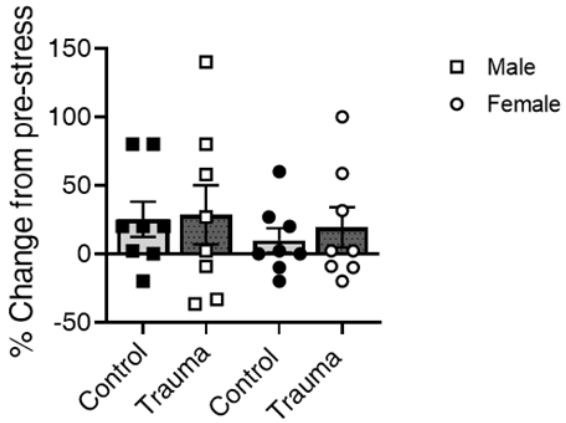

Figure 3

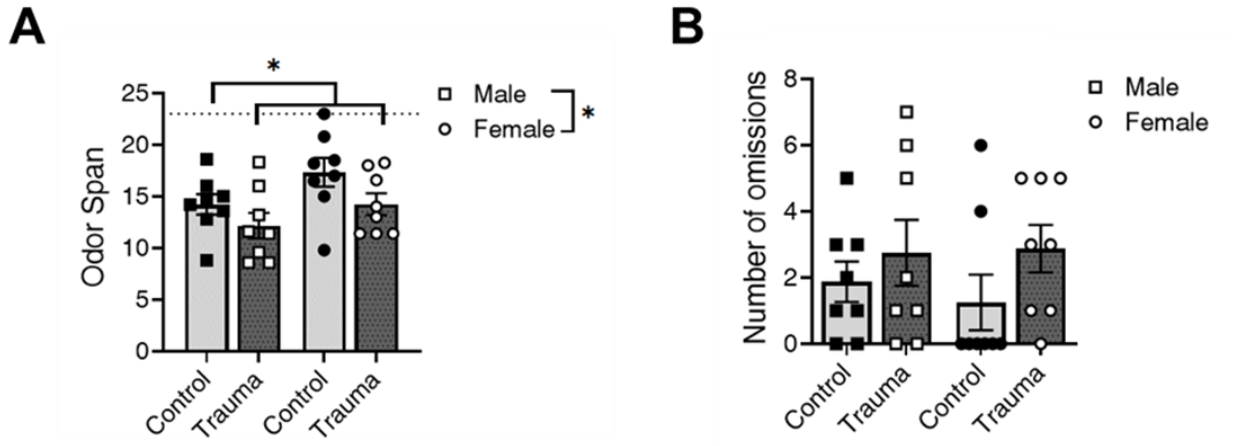

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Figure 4

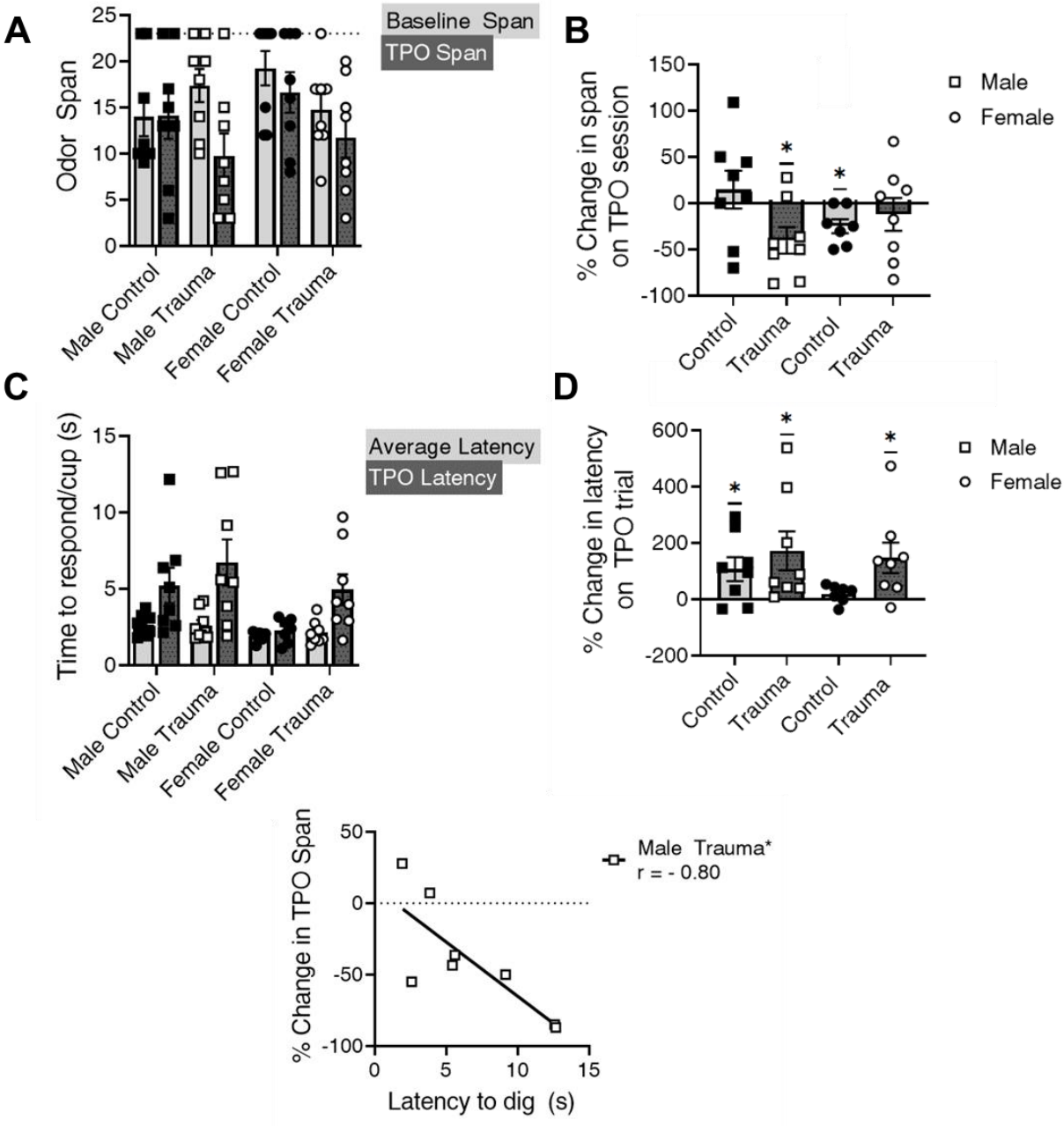

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Figure 5

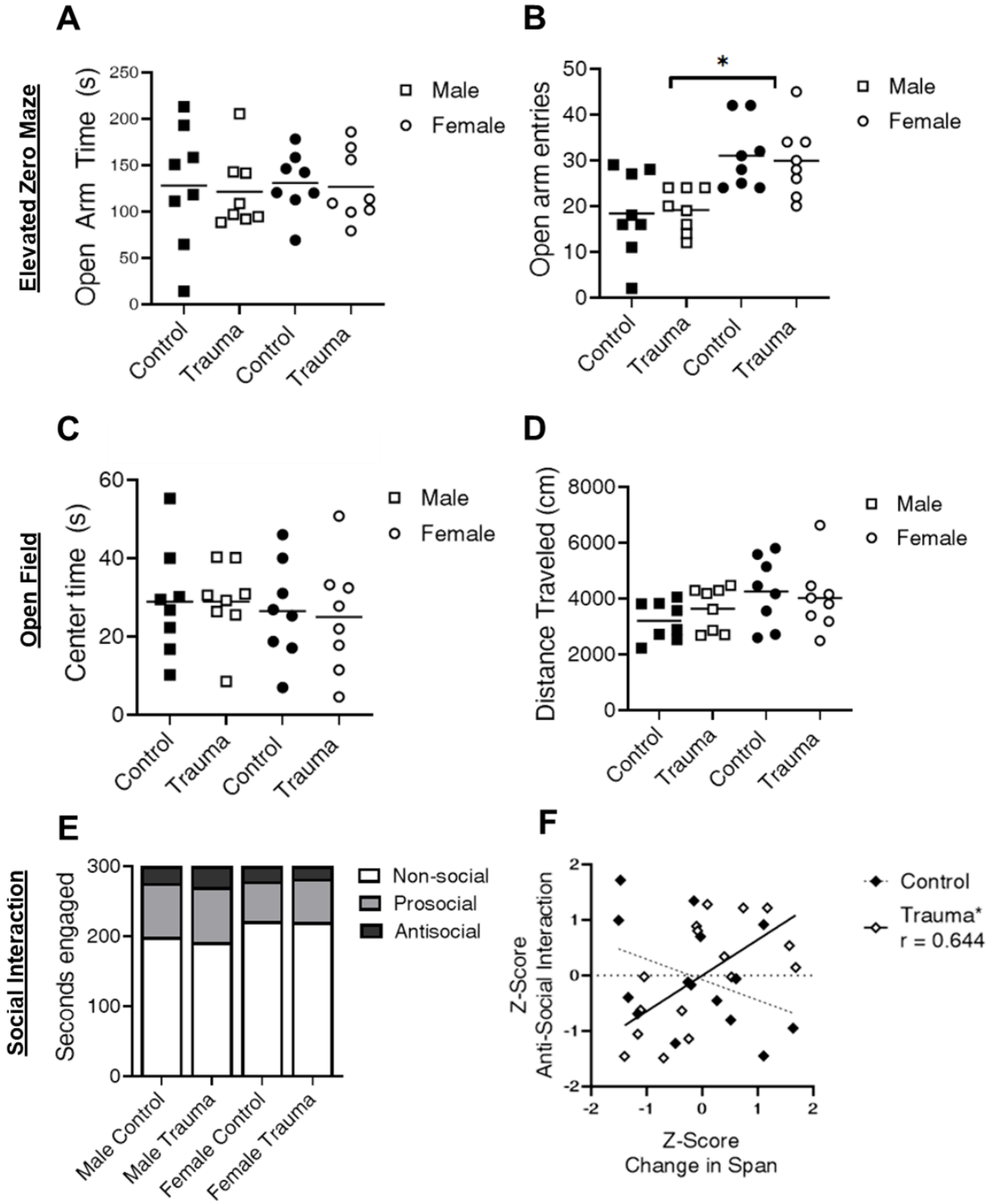

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Figure 6