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The Single-Prolonged Stress Model Fails to Produce Behavioral or Corticosterone Alterations in Rats

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Abstract

There is a critical need for robust and reliable preclinical models for posttraumatic stress disorder (PTSD) to better understand pathophysiological mechanisms and support the development of novel treatments. The single prolonged stress (SPS) model has been previously utilized to investigate various acute behavioral effects and stress hormone changes in rodents. This study paired anxiety-like and social behavioral evaluations with corticosterone assessment as a complementary physiological biomarker to determine the presence of robust and intervenable phenotypes following SPS. Sprague Dawley rats ($N = 36$, 30 male and 6 female) received SPS model induction (e.g., restraint with odorant, forced-swim, diethyl ether exposure, and isolation) or control handling. Serum corticosterone and behavioral assessments, including the open field test (OFT) and a social motivation test (SMT), were investigated at 1 and 2 weeks following SPS induction. This SPS model did not induce anxiety-like or locomotive differences assessed in the OFT (p's $>$ 0.05). Similarly, SPS did not appear to alter social preference or avoidance in the SMT (p's > 0.05), as groups had similar novel social and novel object interaction levels. SPS-paired cue re-exposure did not unmask group differences in these behaviors. Corticosterone levels were also unaltered between groups in the weeks following SPS ($p = 0.178$). In the absence of other stressors or modifications, the null behavioral and corticosterone findings in the weeks following SPS suggest that this SPS protocol may not reliably produce adequately robust or intervenable phenotypes.

Key words: anxiety-like behavior; corticosterone; HPA axis; rat; single prolonged stress model; social behavior

Significance Statement

Having robust and intervenable preclinical models for PTSD is critical, and outcomes that resolve acutely may not relate to the long-term consequences of trauma. Further, sufficiently aversive models can be paired with a cue to enhance behavioral phenotypes or test interventions during cue re-exposure. This study evaluated the robustness and reproducibility of phenotypes in rats 1 and 2 weeks following SPS and with cue re-exposure. Anxiety-like and social behavioral changes were not recapitulated following SPS, and cue re-exposure did not enhance any phenotypes. Corticosterone was not altered 1 or 2 weeks following SPS. These null findings suggest that the SPS model, without modification for enhanced trauma induction, does not induce adequate psychological or physiological stress to reliably produce these phenotypes.

Introduction

Preclinical, rodent models have been utilized in attempts to better understand posttraumatic stress disorder (PTSD) development, develop new therapies, and define treatment targets. PTSD, a complex and severe psychiatric disorder, significantly impacts individuals

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following exposure to trauma (Goldstein et al., 2016; Wisco et al., 2022). However, susceptibility and resistance factors related to PTSD remain poorly understood (Benjet et al., 2016; Skórzewska et al., 2020). Moreover, current psychotherapeutic or pharmacologic approaches may offer limited relief to symptoms experienced, including intrusive thoughts, avoidance, negative alterations to cognition and mood, arousal, and reactivity (Brady et al., 2000; Stein et al., 2003). Despite the clear need for robust preclinical models, developing or choosing a single, accepted model for translational PTSD studies remains a challenge.

One commonly reported preclinical model for PTSD is the Single Prolonged Stress (SPS) model. In the classic induction protocol for SPS, rats are exposed to a series of multi-modal stressors (i.e., restraint, forced-swim, and diethyl ether exposure for loss of consciousness) followed by a 7 d isolation window (Liberzon et al., 1997). Variations in the SPS model range from the described classic SPS protocol, SPS with a paired cue, or SPS with a modified or additional stressor (Knox et al., 2012a; Canto-de-Souza et al., 2021). Odorants have been previously paired with the restraint period of SPS induction to create a conditioned stimulus (CS; Toledano and Gisquet-Verrier, 2014), which may reinvigorate or unmask behavioral phenotypes during re-exposure. Past findings have suggested that the classic SPS model in rats produces both behavioral (i.e., anxiety-like, hyperarousal, social interaction changes) and physiological changes (Yamamoto et al., 2009; Souza et al., 2017; Lisieski et al., 2018; Ferland-Beckham et al., 2021).

However, SPS outcomes may be temporally limited and sensitive to procedural differences, leading to variable findings. The reported timing of behaviors following SPS varies, with most studies focusing on acute effects in the days following model induction and some reports 2–4 weeks later (Wu et al., 2016; Mancini et al., 2021). Key behavioral phenotypes that have been evaluated following SPS are anxiety and depressive-like outcomes, social behaviors, and cognitive impairments (Lisieski et al., 2018). The open field test (OFT) is a common assay of anxiety-like and exploratory behaviors in rodents (Walsh and Cummins, 1976). Some groups have reported anxiety-like behavioral phenotypes (Wu et al., 2016; Iqbal et al., 2024), while others failed to recapitulate the phenotypes (Harvey et al., 2006; Eagle et al., 2013b; Lisieski and Perrine, 2017; Wu et al., 2017). Individuals with PTSD have also been reported to have changes in sociability, including increased avoidance (Charuvastra and Cloitre, 2008; Janssen et al., 2022). Some studies have shown altered social interactions, avoidance, and contextual fear following SPS model induction (Kohda et al., 2007; Eagle et al., 2013a), indicating potential social and motivational dysregulation.

In humans, the glucocorticoid cortisol has been shown to play a critical role in the body's stress response, similar to corticosterone (CORT) in rodents (Souza et al., 2017). The hypothalamic-pituitary-adrenal (HPA) axis release of glucocorticoids has been reported to be dysregulated in individuals with PTSD (Speer et al., 2019; Danan et al., 2021). Rodent studies have shown that SPS may lead to dysregulated CORT (Liberzon et al., 1997; Knox et al., 2012a,b; Toledano et al., 2013; Verbitsky et al., 2020), although timing and effects differ between studies. Physiological changes noted in individuals with PTSD should be reflected in reliable preclinical models and provide a complementary biomarker to behavioral or therapeutic evaluation.

This study aimed to provide a multilevel readout of stress-related behavioral and corticosterone changes to evaluate the robustness and reproducibility of the SPS model in rats. We hypothesized that SPS model induction would result in increased anxiety-like and social avoidant behaviors as previously reported and that trauma cue re-exposure would enhance behavioral phenotypes between groups. We further hypothesized that serum CORT levels would be dysregulated when sampled 1 week following SPS.

Materials and Methods

Animal use. Sprague Dawley rats ($N = 36$, 30M, 6F) were purchased from Charles River Laboratories at 60 d of age and were individually housed on a reverse 12 h light cycle with *ad libitum* food and water unless otherwise specified. Experiments began at 10 weeks of age (~70 d). All experiments for this study were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Dartmouth College.

Experimental timeline. Male and female rats ($N = 36$), received in three cohorts, were acclimated (pair-housed) for 1 week after arrival prior to collection of a baseline serum sample via lateral tail vein blood collection. Cohorts were counterbalanced for SPS or control group sample sizes. Twenty-four hours following the baseline blood collection, rats were then exposed to the SPS model (restraint + odorant, forced swim, and diethyl ether exposure) or control handling with odor exposure (Fig. 1). Further behavioral testing and blood sampling occurred 1 and 2 weeks following induction of the SPS model. Following the experimental conclusion, rats were humanely killed.

Single prolonged stress model. A total of $N = 36$ rats were either exposed to the components of the SPS model or were handled as controls. SPS induction is a multistressor sequence involving 2 h of restraint via decapiCone paired with odorant exposure of 20% lemon oil in mineral oil, individual forced-swim (15 min), and diethyl ether exposure until loss of consciousness (10 ml, <5–10 min). The restraint portion of SPS was paired with odor exposure, and control animals were exposed to the odorant for 2 h in their home cage after handling. All stressors were done individually in SPS animals; no stressors were conducted in a group context. All animals were closely monitored for health concerns during SPS

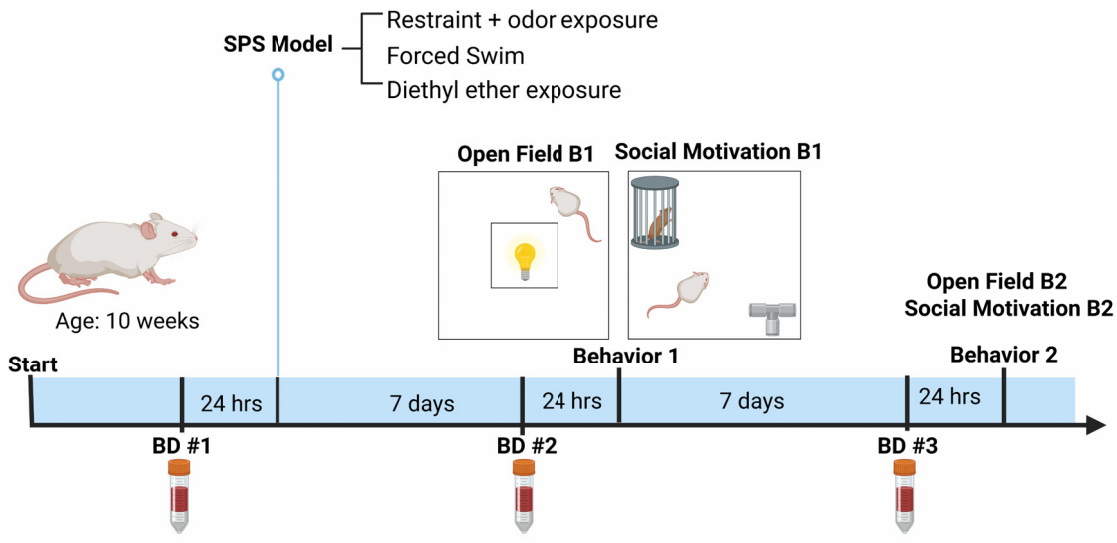


Figure 1. Timeline of experimental methodology for the single prolonged stress model and assessments. *N* = 36 rats received either SPS induction or control handling, where SPS induction consisted of the multimodal stressor sequence including 2 h. restraint and odorant exposure followed by forced swim (15 min) and diethyl ether exposure. In addition, three lateral tail vein blood draws were collected (BD1, BD2, BD3) and two behavioral timepoints at 1 (B1) and 2 weeks (B2) post-SPS induction were assessed. Behaviors investigated at these timepoints were the open field test and social motivation test.

and were allowed to recover for 5–10 min between stressors. After diethyl ether recovery, rats were returned to clean single-housed cages, and rats were isolated for a week (no handling or housekeeping from lab or animal care staff). At each stressor step, control animals were handled to simulate the operator transfer and handling.

Serum corticosterone collection and analysis. To collect serum for corticosterone assessment, lateral tail vein blood samples were collected at experimental timepoints BD1, BD2, and BD3 (pre-SPS, 1 week, and 2 weeks post-SPS). Baseline (BD1) was collected 24 h prior to the SPS model, as a preinduction comparison of CORT levels in both groups and to assess the variation in groups. The second and third samplings (BD2, BD3) were chosen to capture the CORT levels immediately following the week of isolation and 2 weeks following SPS induction. BD2 and BD3 serum samplings were performed 24 h before behavioral testing for temporal comparison of behavioral and CORT changes. To achieve this, rats were briefly anesthetized using 2–3% isoflurane, and tails were warmed via a heating pad before utilizing a heparinized butterfly catheter to gather 200–300 µl whole blood. Brief isoflurane exposure allowed rapid sampling without pain or distress and uniformity of sampling across subjects and repeated collections. Samples were collected starting ∼1 h before the end of their light phase, and each sampling was 5–7 min from start to finish. After sampling, animals were returned to their home cage. Whole blood samples were transferred to a collection tube and clotted at room temperature for 45 min before being held on ice until further processing. Samples were centrifuged for 15 min at 1,960 × *g* at 4°C. Supernatant was collected, aliquoted, and stored at −80°C until ready for use. To provide quantification of corticosterone in each sample, ELISA kits (Bio-Techne, R&D Systems, KGE009) were run with duplicate samples and appropriate standards. To control batch differences among kits and between cohorts, sample results were normalized to the standard curve of their kit before being aggregated.

Open field test. OFT was used to assess general locomotion, exploration, and anxiety-like behaviors following SPS induction. The apparatus for this task was a 60 cm × 60 cm × 40 cm arena, with walls and floors painted black for contrast and lighting. An overhead light was concentrated on the center one-third of the arena, providing center lighting (350 lumens). Light around the arena edges was <200 lumens, providing an anxiogenic center field. At timepoints B1 and B2 (i.e., 1 and 2 weeks following SPS), rats were placed individually in the arena for 15 min by the operator. Behavior recognition software (Noldus EthoVision XT) was used for tracking and locomotion of animals, as well as scoring various center-based metrics. Behavioral metrics from the OFT [i.e., velocity (cm/s), percent time in the center zone] were calculated for statistical comparison across time bins in B1 and B2. The sessions were sectioned into five 3 min time bins during analysis for comparison of behavioral outputs between groups and behavioral changes throughout the duration of the session.

Social motivation test. Social motivation test (SMT) was employed to test motivation or avoidance toward novel social and novel object interactions. The apparatus for this task was a 60 cm × 60 cm × 40 cm arena, with walls and floors painted black for contrast and lighting. The lighting condition for this test was low lighting, with only minimal diffuse light

(<100 lumens). The novel social rat was a sex and age-matched conspecific, and different novel animals were used for behavior tests 1 and 2 weeks post-SPS. Two visually and texturally different novel objects were used for the two SMT timepoints, B1 and B2 (1, 2 weeks post-SPS). The object for B1 (blue PVC pipe fitting, 4 in length, three-way pipe) and the object for B2 (1.0 in white PVC ball valve, 3.8 in length) differed by behavioral timepoint, but the location in the arena was consistent. Novel social rats and novel objects were placed on opposing corners of the arena, and rats were placed in the center of the arena before freely behaving for 15 min. Behavioral metrics from this task were again determined through video analysis. These metrics from the SMT (i.e., percent time in novel object zone, percent time in novel social zone) were calculated across time bins in B1 and B2 (five 3 min time bins).

SPS-paired cue re-exposure during behavior. All cohorts were characterized for behavioral metrics at B1 and B2 ($N = 12$ SPS, $N = 12$ Ctrl) as described. Cohort 3 [$N = 6$ SPS (3M, 3F), $N = 6$ Ctrl (3M, 3F)] experienced re-exposure of the SPS-paired cue during OFT and SMT as a CS associated with model induction stress. Five milliliters of the odorant were placed on a cotton pad attached to the arena wall for each animal after arena cleaning and was left for the duration of each OFT and SMT behavioral session as an SPS trauma cue.

Statistical analysis. The analysis plan for these measures includes the statistical approach for behavioral and physiological outcomes, differential analysis of cohorts based on condition, and assessment of variation within groups. Following ELISA quantification of absorbance values (450 nm), the concentration of CORT (ng/mL) was determined through interpolation of absorbance values determined by within-kit standard curve, using a sigmoidal four-parameter fit. Statistical analysis of normalized CORT values for group differences at 1 and 2 weeks post-SPS between groups was performed using a one-way ANOVA and Bonferroni's multiple-comparison correction. Additionally, the percent change in CORT from baseline to 1 and 2 weeks post-SPS was statistically assessed via multiple unpaired *t* tests. Following video analysis using behavioral recognition software, time-binned values for behavioral metrics from OFT and SMT were statistically compared with separate two-way ANOVAs for behavioral timepoints B1 and B2. A combined analysis was performed for cohorts 1 and 2 (i.e., $N = 12$ per group, males), and a separate analysis was performed for cohort 3 (i.e., $N = 6$ (3 M, 3F) per group) to differentiate the effects of behavioral outcomes of cue re-exposure. There was not a high enough sample size of females to perform a sex-specific analysis or to include sex as a cofactor (i.e., in a mixed effects analysis), so males and females of cohort 3 were combined within SPS and Ctrl conditions. To address individual variation within groups across assessments, *Z*-scores from the Ctrl group mean were determined for each animal (e.g., OFT percent time in center zone, SMT percent time in social zone, % change from BD1-BD2, BD1-BD3). For a more comprehensive review of statistical analysis approaches and outcomes in this study, see Table 1. For statistical analysis and figure creation, we use Prism (v. 10.2.1, GraphPad) and BioRender (BioRender.com).

Results

$N = 18$ rats were exposed to the SPS model, and $N = 18$ were tested as paired controls through 3 cohorts. Blood sampling was not possible for one animal, resulting in an $N = 17$ sample size for SPS CORT metrics. Results for cohorts that were not re-exposed to the cue during behavior [cohorts 1 and 2, $N = 12$ (males) per group] and the cohort with cue re-exposure [cohort 3, $N = 6$ (3M, 3F) per group] are separated for behavioral comparison.

Effects of the SPS model on anxiety-like and social behavior in rats

OFT outcomes

The main metrics of OFT are velocity for locomotion and arena center-based metrics to evaluate anxiety-like behaviors. These metrics were assessed per 3 min time bins for group-based comparisons and for potential behavioral changes through time in the task. Velocity (cm/s) during the OFT had a significant effect of time across bins revealed via two-way ANOVA in B1 (Fig. 2*A*; $F_{(3.147,69.24)} = 12.02$, $p = 0.0001$) and B2 (Fig. 2*B*; $F_{(3.240,71.29)} = 7.895$, $p = 0.0001$), as velocity decreased through the five time bins. No significant group effect ($F_{(1,22)} = 4.286$, $p = 0.0504$) or group–time interaction effect ($F_{(4,88)} = 0.9203$, $p = 0.4559$) was revealed in B1, similarly no group ($F_{(1,22)} = 0.899$, $p = 0.3533$) or group × time effect ($F_{(4,88)} = 0.8290$, $p = 0.5102$) was revealed in B2. SPS and control rats were evaluated for percent time spent in the center zone during the OFT, and no group differences ($F_{(1,22)} = 0.4213$, $p = 0.5230$), effect of time ($F_{(2.738,60.23)} = 2.347$, $p = 0.0870$), or group × time effect ($F_{(4,88)} = 0.4490$, $p = 0.7729$) were found in B1 (Fig. 2*C*). This was also true when tested at timepoint B2, with no effect of group ($F_{(1,22)} = 0.5910$, $p = 0.4502$), time ($F_{(2.136,46.99)} = 2.243$, $p = 0.1141$), or their interaction ($F_{(4,88)} = 0.9808$, $p = 0.4223$) revealed (Fig. 2*D*). No significant differences in distance traveled, center zone frequency, or rearing were revealed by two-way ANOVA (data not shown; p's > 0.05).

SMT outcomes

The main metrics of this task are the percent of time per 3 min bin spent in the novel object or novel social zones to assess motivation or avoidance of these interactions. When comparing the percent of time spent in the novel object zone at timepoint B1 (Fig. 2*E*), no group differences were revealed including no effect of group ($F_{(1,22)} = 0.4412$, $p = 0.5134$), effect of time through bins ($F_{(3,65.99)} = 1.720$, $p = 0.1713$), or the group × time interaction effect

Table 1. Detailed statistical table

Fig	Comparison	Type of test	Statistic	95% CI
2a	Velocity (cm/s), B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,22)} = 4.286$	$p = 0.0504$
2a	Velocity (cm/s), B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(3.147,69.24)} = 12.02$	$p = 0.0001$
2a	Velocity (cm/s), B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,88)} = 0.9203$	$p = 0.4559$
2b	Velocity (cm/s), B2, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,22)} = 0.899$	$p = 0.3533$
2b	Velocity (cm/s), B2, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(3.240,71.29)} = 7.895$	$p = 0.0001$
2b	Velocity (cm/s), B2, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,88)} = 0.8290$	$p = 0.5102$
2c	Time (%) in center zone, B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,22)} = 0.4213$	$p = 0.5230$
2c	Time (%) in center zone, B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(2.738,60.23)} = 2.347$	$p = 0.0870$
2c	Time (%) in center zone, B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,88)} = 0.4490$	$p = 0.7729$
2d	Time (%) in center zone, B2, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,22)} = 0.5910$	$p = 0.4502$
2d	Time (%) in center zone, B2, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(2.136,46.99)} = 2.243$	$p = 0.1141$
2d	Time (%) in center zone, B2, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,88)} = 0.9808$	$p = 0.4223$
2e	Time (%) in object zone, B1, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,22)} = 0.4412$	$p = 0.5134$
2e	Time (%) in object zone, B1, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(3,65.99)} = 1.720$	$p = 0.1713$
2e	Time (%) in object zone, B1, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,88)} = 0.8620$	$p = 0.4901$
2f	Time (%) in object zone, B2, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,22)} = 0.4028$	$p = 0.5322$
2f	Time (%) in object zone, B2, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(2.916,64.15)} = 3.167$	$p = 0.0315$
2f	Time (%) in object zone, B2, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,88)} = 0.4716$	$p = 0.7564$
2g	Time (%) in social zone, B1, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,22)} = 0.1374$	$p = 0.7144$
2g	Time (%) in social zone, B1, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(3.190,70.18)} = 3.957$	$p = 0.0101$
2g	Time (%) in social zone, B1, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,88)} = 0.5001$	$p = 0.7357$
2h	Time (%) in social zone, B2, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,22)} = 1.754$	$p = 0.1990$
2h	Time (%) in social zone, B2, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(3.236,71.18)} = 1.533$	$p = 0.2106$
2h	Time (%) in social zone, B2, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,88)} = 1.096$	$p = 0.3637$
3a	Velocity (cm/s) with cue re-exposure, B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,10)} = 0.2608$	$p = 0.6206$
3a	Velocity (cm/s) with cue re-exposure, B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(2.215,22.15)} = 15.21$	$p = 0.0001$
3a	Velocity (cm/s) with cue re-exposure, B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,40)} = 0.6494$	$p = 0.6307$
3b	Velocity (cm/s) with cue re-exposure, B2, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,10)} = 0.05611$	$p = 0.8175$
3b	Velocity (cm/s) with cue re-exposure, B2, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(2.031,\ 20.31)} = 20.89$	$p = 0.0001$
3b	Velocity (cm/s) with cue re-exposure, B2, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,40)} = 1.377$	$p = 0.2591$
3c	Time (%) in center zone (cue re-exposure), B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,10)} = 0.0730$	$p = 0.7924$
3c	Time (%) in center zone (cue re-exposure), B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(2.9,29)} = 1.629$	$p = 0.2052$
3c	Time (%) in center zone (cue re-exposure), B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,40)} = 1.023$	$p = 0.4070$
3d	Time (%) in center zone (cue re-exposure), B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,10)} = 0.086$	$p = 0.7749$
3d	Time (%) in center zone (cue re-exposure), B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(2.059,20.59)} = 2.019$	$p = 0.1574$

(*Table continues.*)

Table 1. Continued

Fig	Comparison	Type of test	Statistic	95% CI
3*d*	Time (%) in center zone (cue re-exposure), B1, OFT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,40)} = 1.301$	$p = 0.2861$
3*e*	Time (%) in object zone (cue re-exposure), B1, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,10)} = 0.5165$	$p = 0.4888$
3*e*	Time (%) in object zone (cue re-exposure), B1, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(2.274,22.74)} = 3.319$	$p = 0.0490$
3*e*	Time (%) in object zone (cue re-exposure), B1, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,40)} = 0.4447$	$p = 0.7756$
3*f*	Time (%) in object zone (cue re-exposures), B2, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,10)} = 0.4575$	$p = 0.5141$
3*f*	Time (%) in object zone (cue re-exposures), B2, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(3.073,30.73)} = 0.5013$	$p = 0.6885$
3*f*	Time (%) in object zone (cue re-exposures), B2, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,40)} = 0.9342$	$p = 0.4539$
3*g*	Time (%) in social zone (cue re-exposures), B1, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,10)} = 0.6011$	$p = 0.4561$
3*g*	Time (%) in social zone (cue re-exposures), B1, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(1.821,18.21)} = 0.7636$	$p = 0.4689$
3*g*	Time (%) in social zone (cue re-exposures), B1, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,40)} = 0.7680$	$p = 0.5524$
3*h*	Time (%) in social zone (cue re-exposures), B2, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group: $F_{(1,10)} = 2.669$	$p = 0.1334$
3*h*	Time (%) in social zone (cue re-exposures), B2, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Time: $F_{(2.626,26.2)} = 3.412$	$p = 0.0371$
3*h*	Time (%) in social zone (cue re-exposures), B2, SMT time bins in SPS and Ctrl rats	Two-way ANOVA	Group × time: $F_{(4,40)} = 1.352$	$p = 0.2679$
4*a*	Normalized CORT levels (ng/ml), BD1-BD3, SPS, and Ctrl rats across cohorts	One-way ANOVA	Group: $F_{(5,101)} = 1.56$	$p = 0.1778$

Comprehensive report of statistical analyses performed.

($F_{(4,88)} = 0.8620$, $p = 0.4901$). This lack of group differences remained true in B2 (Fig. 2*F*) with no effect of group ($F_{(1,22)} = 0.4028$, $p = 0.5322$) or the interaction of group and time ($F_{(4,88)} = 0.4716$, $p = 0.7564$), although a significant effect of time across was revealed ($F_{(2.916,64.15)} = 3.167$, $p = 0.0315$) showing groups spent less time in the novel object zone across time bins. Time spent in the novel social rat zone was also compared between groups; in B1 (Fig. 2*G*) no significant differences in this metric were found between SPS and control groups with two-way ANOVA revealing no effect of group ($F_{(1,22)} = 0.1374$, $p = 0.7144$) or interaction of group × time ($F_{(4,88)} = 0.5001$, $p = 0.7357$) but a significant effect of time ($F_{(3.190,70.18)} = 3.957$, $p = 0.0101$) as both groups spent less time in the social zone through time bins. In the second behavioral timepoint tested, B2 (Fig. 2*H*), there were again no group differences in time spent with in the novel social zone, with two-way ANOVA revealing no effect of group ($F_{(1,22)} = 1.754$, $p = 0.1990$), time ($F_{(3.236,71.18)} = 1.533$, $p = 0.2106$), or group × time through bins evaluated ($F_{(4,88)} = 1.096$, $p = 0.3637$). Rats of both groups spent more time on average in the novel social zone than in the novel object zone during both timepoints. No significant difference in distance traveled, object or social zone frequencies, or zone alterations were revealed by two-way ANOVA (data not shown; p's > 0.05).

Behavioral results during SPS-paired cue re-exposure

The effect of re-exposure to the SPS-paired cue during OFT and SMT was determined for SPS and control rats in cohort 3 at behavioral timepoints B1 and B2 across 3 min time bins (Fig. 3).

OFT outcomes with cue re-exposures

Evaluation of locomotion and center-based metrics during cue re-exposure in OFT. When assessing velocity differences in B1 (Fig. 3*A*), a two-way ANOVA did not reveal a group effect ($F_{(1,10)} = 0.2608$, $p = 0.6206$), but an effect of time was discovered ($F_{(2.215,22.15)} = 15.21$, $p = 0.0001$). Similarly, in the second OFT timepoint, B2 (Fig. 3*B*), velocity did not differ between groups ($F_{(1,10)} = 0.05611$, $p = 0.8175$) but a significant effect of time was revealed ($F_{(2.031,\ 20.31)} = 20.89$, $p = 0.0001$). No group × time effects were revealed during B1 ($F_{(4,40)} = 0.6494$, $p = 0.6307$) or B2 ($F_{(4,40)} = 1.377$, $p = 0.2591$). Groups were additionally assessed for percent time in the center zone of OFT with no significant differences found during B1 (Fig. 3*C*), including effect of group ($F_{(1,10)} = 0.0730$, $p = 0.7924$), time through bins ($F_{(2.9,29)} = 1.629$, $p = 0.2052$), or group × time ($F_{(4,40)} = 1.023$, $p = 0.407$). Similarly, in B2 (Fig. 3*D*), no significant effect was revealed for group ($F_{(1,10)} = 0.086$, $p = 0.7749$), time ($F_{(2.059,20.59)} = 2.019$, $p = 0.1574$), or group × time ($F_{(4,40)} = 1.301$, $p = 0.2861$) via two-way ANOVA. SPS and control performances were similar across timepoints and to individuals who did not experience cue

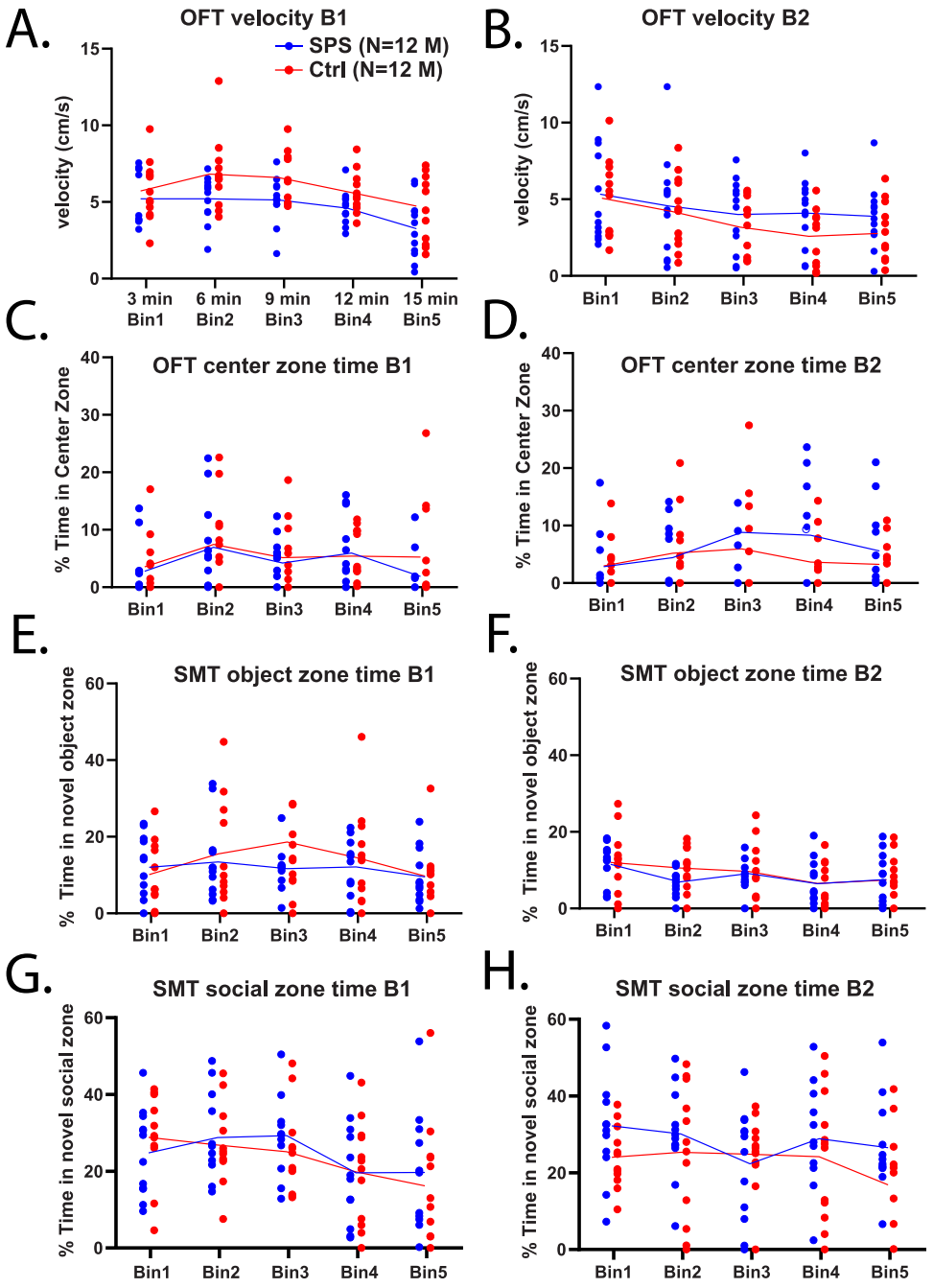


Figure 2. Assessment of behavioral changes following SPS in the OFT and SMT. $N = 12$ SPS (blue) and $N = 12$ control (red) rats had 15 min OFT and SMT sessions. OFT and SMT were tested at 1 week post-SPS (B1) and 2 weeks post-SPS (B2). Results for each behavior is displayed per 3 min bin. Each bin is the average value over 3 min per animal. General locomotion, exploratory and anxiety-like behaviors were explored in the OFT. Average velocity of groups during the OFT at timepoints B1 (***A***) and B2 (***B***). The percent time spent in the center zone of OFT while freely exploring the arena was determined for B1 (***C***) and B2 (***D***) timepoints per group. SMT was utilized to assess motivation or avoidance toward novel object or social experiences, and rats had free access to both over 15 min. Metrics for interaction in the SMT were determined, % time in the novel object zone is shown per bin at B1 (***E***) and B2 (***F***). Interactions in the novel social zone, shown as % time for B1 (***G***) and B2 (***H***).

re-exposure. No significant differences in distance traveled, center zone frequency, or rearing were revealed by two-way ANOVA (data not shown; p's > 0.05) during OFT with cue re-exposure.

SMT outcomes with cue re-exposures

Novel object and novel social interactions were not altered by cue re-exposure in SPS rats. While assessing percent time in the novel object zone during cue re-exposure, no effects of group ($F_{(1,10)} = 0.5165$, $p = 0.4888$) or group × time effect

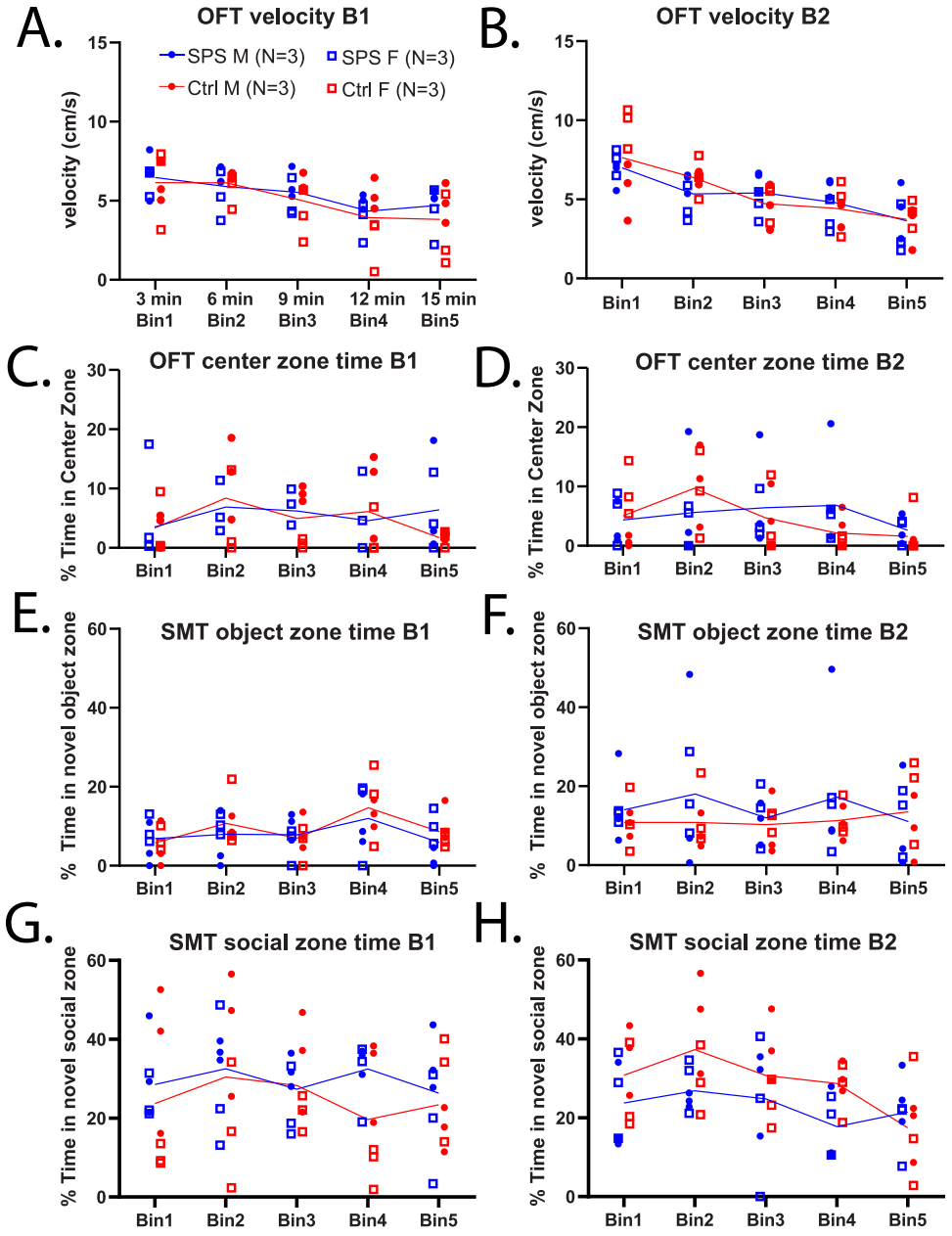


Figure 3. Assessment of behavioral changes following SPS in the OFT and SMT with paired cue re-exposure (cohort 3). SPS (blue) and control (red) rats were in the OFT and SMT each for 15 min, and the cue (odor) was placed in the arena for both tasks. Results for each behavior are displayed per 3 min bin. Each bin is the average value over 3 min per animal. OFT and SMT were tested at 1 week post-SPS (B1) and 2 weeks post-SPS (B2) as in Figure 2 results, but with the addition of cue re-exposure during behavioral testing. General locomotion, exploratory, and anxiety-like behaviors were explored in the OFT. Average velocity of groups during the OFT at timepoints B1 (***A***) and B2 (***B***). The percent time spent in the center zone of OFT while freely exploring the arena was determined for B1 (***C***) and B2 (***D***) timepoints per group. SMT was utilized to assess motivation or avoidance toward novel object or social experiences, and rats had free access to both over 15 min. Metrics for interaction in the SMT were determined, % time in the novel object zone is shown per bin at B1 (***E***) and B2 (***F***). Interactions in the novel social zone, shown as % time for B1 (***G***) and B2 (***H***).

($F_{(4,40)} = 0.4447$, $p = 0.7756$) but a significant effect of time ($F_{(2.274,22.74)} = 3.319$, $p = 0.0490$) in B1 was found (Fig. 3*E*). No significant differences in percent time in the novel object zone, including group effect ($F_{(1,10)} = 0.4575$, $p = 0.5141$), effect of time ($F_{(3.073,30.73)} = 0.5013$, $p = 0.6885$), or group × time effect ($F_{(4,40)} = 0.9342$, $p = 0.4539$) occurred in B2 (Fig. 3*F*). Finally, cue re-exposure did not produce group differences in percent time in the novel social rat zone, with no effect of group ($F_{(1,10)} = 0.6011$, $p = 0.4561$), time through bins ($F_{(1.821,18.21)} = 0.7636$, $p = 0.4689$), or group × time effect ($F_{(4,40)} = 0.7680$, $p = 0.5524$) in B1 (Fig. 3*G*). In B2 (Fig. 3*H*), only a significant effect of time ($F_{(2.626,26.2)} = 3.412$, $p = 0.0371$) was revealed, with no group ($F_{(1,10)} = 2.669$, $p = 0.1334$) or group × time effects ($F_{(4,40)} = 1.352$, $p = 0.2679$). During cue re-exposure, both groups of rats spent a greater amount of time in the social zone than the object zone,

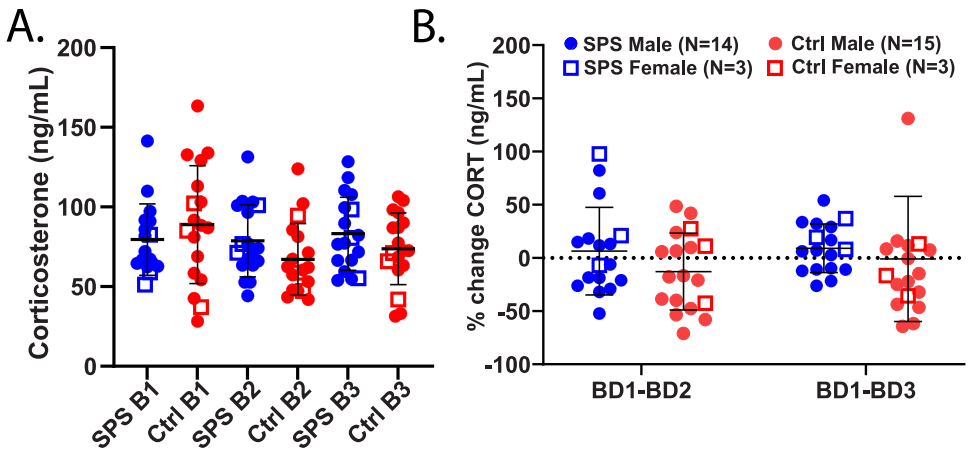


Figure 4. Assessment of corticosterone following SPS induction. ***A***, Normalized corticosterone values for SPS and control rats, assessed via ELISA kit in three cohorts. Sample sizes were $N = 18$ for all groups, in the exception of $N = 17$ SPS at timepoint BD1. CORT (ng/ml) is shown for both groups across timepoints from baseline (BD1) to 1 week (BD2) and 2 weeks (BD3) following SPS induction. ***B***, Within animal percent change in CORT from baseline to BD2 and BD3, where individual percentage changes are shown for both SPS (red) and control (blue) rats.

with similar preferences to rat behavior without cues (Fig. 2). No significant differences in other metrics including distance traveled, object or social zone frequencies, or zone alterations were revealed by two-way ANOVA (data not shown; p's > 0.05) during the SMT with cue re-exposure.

Serum corticosterone evaluation following SPS in rats

Serum samples collected at three timepoints (BD1, BD2, BD3) were processed, and quantification of corticosterone (ng/ml) was determined for duplicates of each sample. Normalized CORT values (ng/ml) were compared between SPS and Ctrl groups across timepoints (Fig. 4*A*). No group differences in CORT were revealed at 1 or 2 week timepoints following SPS via one-way ANOVA ($F_{(5,\ 101)} = 1.56$, $p = 0.1778$), and no significant differences were revealed after Bonferroni's multiple comparisons. Corticosterone value averages were similar between cohorts. The percent change in CORT from baseline to 1 and 2 weeks post-SPS in both groups was also similar, with high variation in within-animal changes over time (Fig. 4*B*). Multiple unpaired t tests of % changes did not reveal any group differences in the BD1-BD2 ($p = 0.1505$) or BD1-BD3 ($p = 0.5149$) comparisons.

Assessment of individual rat behavioral and corticosterone variation

To determine the level of variation across groups, individual performances across behavioral metrics and CORT measurements were Z-scored to the control group performance for Ctrl (Fig. 5*A*) and SPS rats (Fig. 5*B*). A low number of comparisons in either group were significantly different from the control group mean ($|Z\text{-score} > 2|$), with 7 in SPS and 5 in controls. Approximately 5% of z-scores reach significance, which is expected by chance given the number of comparisons. No subgroups (i.e., susceptible, resilient) in behavioral or physiological outcomes were revealed.

Discussion

The expected behavioral and physiological changes following the SPS model in rats were not recapitulated in this study. SPS and control rats displayed similar behavior in the OFT and preferences for the novel object and novel social interaction in the SMT. These metrics were not modulated by re-exposure to the odorant previously paired with restraint during SPS. Serum CORT levels, assessed as a physiological biomarker of stress in the model, were not altered at either the 1 or 2 week timepoint. The null behavioral findings, coupled with the lack of CORT changes, suggest that published SPS models may fail to produce reliable phenotypes. Without a robust and repeatable effect in established behavioral and physiological outcomes, the usefulness of this SPS protocol is diminished. Many groups have already moved to alternate or modified SPS protocols, while still reporting them as "SPS," highlighting the lack of robust or replicable outcomes across research groups.

No behavioral deficits in OFT or SMT 1 or 2 weeks following SPS

Under the conditions tested, the SPS model did not produce the anxiety-like phenotype at the chosen timepoints in OFT. This differs from reported findings at 1 week post-SPS (Souza et al., 2017; Sarapultsev et al., 2024) but aligns with groups that failed to recapitulate anxiety-like phenotypes (Harvey et al., 2006; Eagle et al., 2013b; Lisieski and Perrine, 2017; Wu et al., 2017). Other studies have employed additional or alternative anxiety-related tasks (Walf and

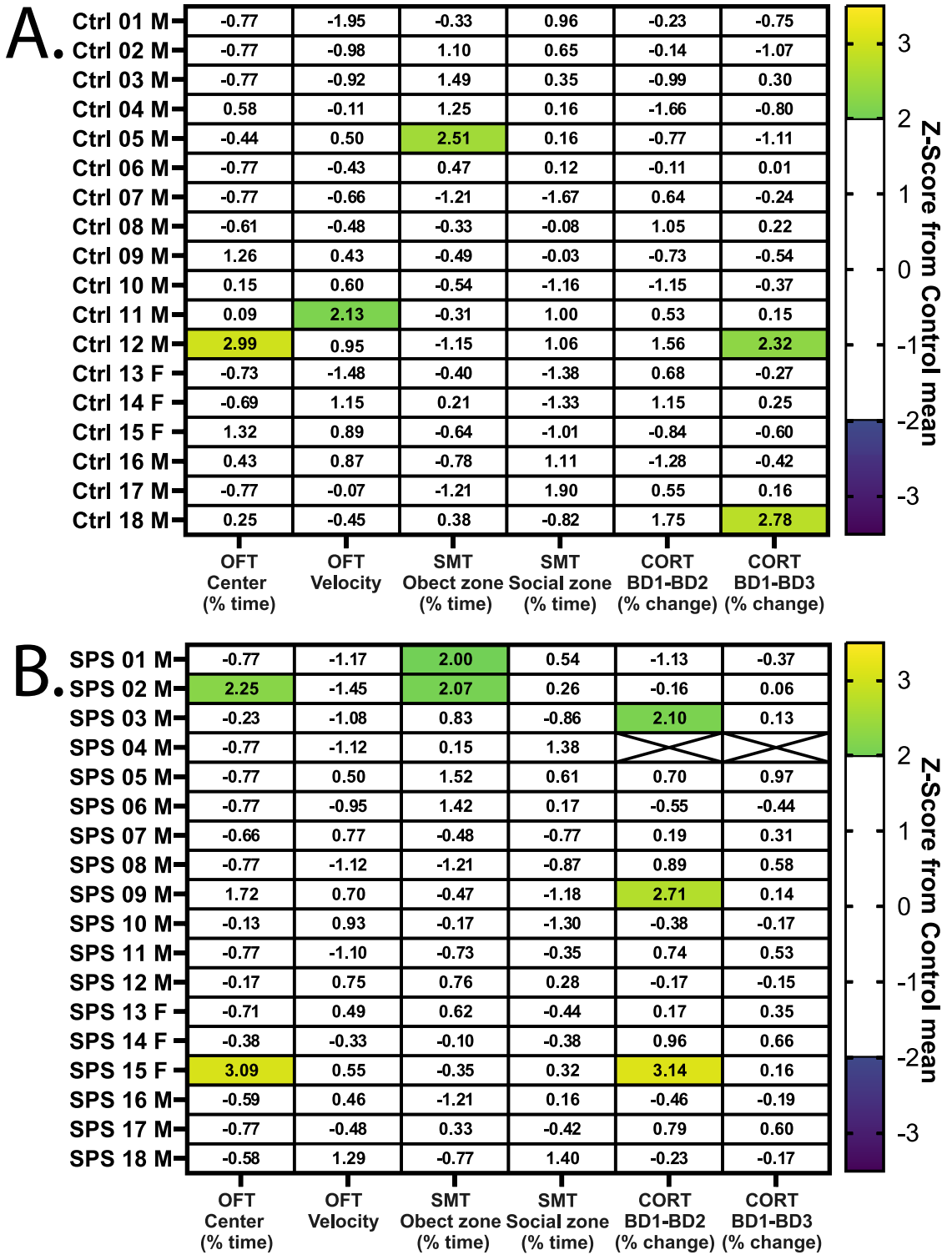


Figure 5. Individual and group variation across OFT and SMT behavioral and CORT phenotypes. Behavior comparison includes metrics from timepoint B1, tested 1 week following SPS. Comparisons for all metrics include the individual rat performance *Z*-scored to the mean of the control group to statistically test if the metric is significantly different than the control population (|*Z*-score >2|), to provide a multimetric comparison per rat and identify potential subgroups. ***A***, Control ($N = 18$) rat individual variation across behavioral and CORT-related metrics, in *Z*-score to control mean. ***B***, SPS ($N = 18$) rat individual and group variation across metrics, shown as *Z*-score to the control mean. $N = 1$ rat was excluded for CORT metrics due to missing sample at the pre-SPS blood draw (BD1).

Frye, 2007), which may produce different results. Both groups spent a low percentage of time in the center zone, which may suggest a possible floor effect in exploratory behavior.

The SPS model did not significantly alter social or object-related interaction or induce avoidance in the SMT at 1 or 2 weeks. It was believed that a decrease in social interactions post-SPS would relate to socially avoidant behavior, while increased preference for social versus object novelty may lean toward a compensatory increase in social motivation. However, this lack of change or difference between groups suggests that SPS exposure did not alter social phenotypes in these metrics. It is also possible that the timepoints or the SMT are not sensitive to potential social or motivational changes.

No effect of SPS cue re-exposure in behavioral assessments

Previous studies have found that trauma-exposed animals had greater avoidance of an odorant CS in behaviors (Toledano and Gisquet-Verrier, 2014). However, the addition of SPS cue re-exposure during OFT and SMT did not enhance or unmask changes in this study. Cue re-exposure during the OFT did not induce group differences in anxiety-like behaviors in SPS rats, suggesting re-exposure was not sufficient to trigger expected phenotypes. This was also noted in the SMT, as cue re-exposure did not alter motivation for novel social and novel object interactions. The lack of cue-driven behavioral changes in the SPS group was unexpected in this study and suggests that the SPS model (i.e., US) was not sufficiently aversive to create a robust fear memory associated with the paired neutral odor (i.e., CS).

Although only male rats were initially assessed for changes without cue re-exposure, both females and males were included in the investigation of SPS cue re-exposure during OFT and SMT. This limitation prevented direct comparison of males and females outside of this group, and sample sizes were not adequately powered for sex-based analyses in the cue re-exposure group. The overall lack of behavioral phenotypes in either condition following SPS further limits the evaluation of any sex differences that may exist when outcomes are more robust. A much larger sample size may be required to detect subtle effects in OFT or SMT in either males or females. Although these tasks were chosen to assess general locomotion, anxiety-like behavior, and social motivation or avoidance, alternative behavioral tasks may provide more sensitive readouts of stress-induced phenotypes.

CORT levels were unaltered at 1 and 2 weeks following SPS

There were no significant group changes in serum CORT levels 1 or 2 weeks following SPS in rats. Additionally, there was no evidence of CORT changes (i.e., pre to post SPS) that aligned with behavioral phenotypes. A reduced baseline cortisol level (i.e., hypocortisolism) has been commonly related to PTSD, demonstrating HPA axis dysregulation even without restress (von Majewski et al., 2023). Decreased baseline CORT has been reported following SPS in rats, although not until 28 d post-SPS (Zhang et al., 2012). Our lack of CORT signal, in the absence of a restress event, may suggest an inadequate physiological stress from SPS that aligns with the null behavioral findings. The timepoints of CORT sampling were chosen to put potential HPA axis changes, and their timing, in the context of behavioral assessments. However, the lack of an acute sampling (e.g., within hours or days of SPS) limits interpretability and leaves open the possibility that our SPS model did not elicit the acute stress response that has been reported following the individual components of SPS (Abel, 1993; Zardooz et al., 2010; Bekhbat et al., 2018) and contributed to our negative results. It is also possible that a dexamethasone suppression test would have provided an alternate approach to assess HPA axis functionality. Methodological choices, such as the use of isoflurane during serum collection, were made to reduce variation and maximize potential group comparisons. It is unlikely the brief use of isoflurane would have significantly altered CORT levels. Previous studies have shown little to no effect following brief exposure in males, although some evidence suggests more female sensitivity (Zardooz et al., 2010; Bekhbat et al., 2016).

High individual and group variation across metrics

Individual behavioral and CORT variation was assessed relative to the mean control group performance to determine the extent of variation in the groups and to identify potential deficits across metrics per animal. Further, this assessment was performed to identify potential subgroups of SPS rats or if susceptibility and deficits only occurred in some individuals. No evidence of latent SPS subgroups emerged from this question, as both groups had very few individual animal *Z*-scores that were significantly different from controls. Approximately 5% of comparisons were significant, as would be expected by chance. Additionally, individual SPS rats did not have significant performance differences from controls in multiple metrics, whether behavioral or CORT-related, and individual variation was not altered by the presence cue re-exposure during behavior. In sum, this confirms that despite SPS model induction, there were no substantial or consistent changes in behavioral performance or CORT levels.

Considerations for the future of the SPS model

The SPS model has been widely used and published to study PTSD related behavioral outcomes in rodents, but the consistency and validity of this model are still subject to debate. SPS is intended to produce phenotypes relevant to individuals with PTSD, but many diagnostic criteria are subjective or overlooked (e.g., criteria of duration in PTSD) and limit translatability when designing experiments. Small procedural changes can also strongly influence the presence or absence of group differences, so comparison across studies remains difficult (Ferland-Beckham et al., 2021).

Additionally, there are limited reports addressing the SPS model in female rats, with mixed results depending on behavior and timeline (Keller et al., 2015; Pooley et al., 2018a,b; Nahvi et al., 2019; Mancini et al., 2021). Evidence that SPS in females may not reliably induce deficits related to PTSD continues to emerge, which limits translational value.

Factors that divide studies into "produced phenotype" versus "no phenotype" may come down to minor procedural differences, such as individual versus group exposure to stressors (Vanderheyden et al., 2015) or degree of isolation during quiescence week. This suggests that the model lacks robustness to produce consistent behavioral changes across research groups. Some studies chose to combine SPS with additional stress-related paradigms like contextual fear conditioning to demonstrate enhanced fear memory (Kohda et al., 2007) or have switched to alternative models to enhance the robustness of phenotypes in certain assays (Skórzewska et al., 2020; Pitcairn et al., 2024). Future research should focus on maximizing reliability and consistency of outcomes, particularly in producing robust trauma-induced phenotypes, which may require modifications to the SPS model, testing alternate behaviors, or consideration of timepoints.

Conclusions

In this study, SPS did not produce behavioral phenotypes in anxiety-like or social domains, or evidence of dysregulated HPA axis function at 1 or 2 weeks in rats. Cue re-exposure did not enhance weak behavioral outcomes, showing SPS also failed to elicit trauma-cue avoidance. The protocol used for this study did not appear to induce sufficient stress to produce robust, expected phenotypes at a group level. These null findings differ from reported literature, where SPS has largely been upheld as a common model for studying behavioral and physiological changes relevant to PTSD. The lack of a robust behavioral effect limits the ability to assess temporal changes or potential novel therapies. We chose not to employ a modified SPS protocol, as this paradigm has been reported as sufficient to produce behavioral and CORT changes 1 week post-SPS. This SPS model may produce some acute deficits (i.e., <1 week), but acute or temporary changes may not relate to symptoms and dysregulation in individuals with PTSD. It is well understood that all preclinical models have inherent limitations, but findings suggest that this induction protocol for SPS, without modification or enhancement, lacks robustness and replicability for continued use and consideration as a good model for translational PTSD studies.

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