Severe, multimodal stress exposure induces PTSD-like characteristics in a mouse model of single prolonged stress

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Abstract

Appropriate animal models of posttraumatic stress disorder (PTSD) are needed because human studies remain limited in their ability to probe the underlying neurobiology of PTSD. Although the single prolonged stress (SPS) model is an established rat model of PTSD, the development of a similarly-validated mouse model emphasizes the benefits and cross-species utility of rodent PTSD models and offers unique methodological advantages to that of the rat. Therefore, the aims of this study were to develop and describe a SPS model for mice and to provide data that support current mechanisms relevant to PTSD. The mouse single prolonged stress (mSPS) paradigm, involves exposing C57Bl/6 mice to a series of severe, multimodal stressors, including 2 h restraint, 10 min group forced swim, exposure to soiled rat bedding scent, and exposure to ether until unconsciousness. Following a 7-day undisturbed period, mice were tested for cue-induced fear behavior, effects of paroxetine on cue-induced fear behavior, extinction retention of a previously extinguished fear memory, dexamethasone suppression of corticosterone (CORT) response, dorsal hippocampal glucocorticoid receptor protein and mRNA expression, and prefrontal cortex glutamate levels. Exposure to mSPS enhanced cue-induced fear, which was attenuated by oral paroxetine treatment. mSPS also disrupted extinction retention, enhanced suppression of stress-induced CORT response, increased mRNA expression of dorsal hippocampal glucocorticoid receptors and decreased prefrontal cortex glutamate levels. These data suggest that the mSPS model is a translationally-relevant model for future PTSD research with strong face, construct, and predictive validity. In summary, mSPS models characteristics relevant to PTSD and this severe, multimodal stress modifies fear learning in mice that coincides with changes in the hypothalamo-pituitary-adrenal (HPA) axis, brain glucocorticoid systems, and glutamatergic signaling in the prefrontal cortex.

1 **Abbreviations:** PTSD = posttraumatic stress disorder, mSPS = mouse single prolonged stress, CON
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3 = control, OF = open field, PRX = paroxetine, Dex = dexamethasone, HPA = hypothalamo-pituitary-
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5 adrenal, PFC = prefrontal cortex, GR = glucocorticoid receptor, CORT = corticosterone
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1 Introduction

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4 Posttraumatic stress disorder (PTSD) can develop after exposure to a psychologically-
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6 traumatic event and manifests as clusters of symptoms including re-experiencing of trauma,
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8 behavioral avoidance, emotional numbing, hyper-arousal, as well as additional affective and cognitive
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10 abnormalities (Association, 2013). Persistence of aberrant conditioned fear responses and deficits in
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12 retention (recall) of fear extinction have been frequently reported in individuals with PTSD (Jovanovic
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14 et al., 2009; Milad et al., 2008; Norrholm et al., 2011), which may underlie the chronic re-experiencing
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16 of traumatic memories. The neurocircuitry alterations linked to conditioned fear learning deficits
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18 include decreased ventromedial prefrontal cortex (PFC) activity, which might lead to insufficient
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20 inhibition of amygdalar activity (Hayes et al., 2012), resulting in impaired extinction retention and
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22 emotional dysregulation (Elzinga and Bremner, 2002; Pitman et al., 2012; Shin et al., 2006).
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24 Physiologically, PTSD is characterized by a dysfunction in the normal stress response mediated by
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26 the hypothalamo-pituitary-adrenal (HPA) axis, particularly enhanced negative feedback of the HPA
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28 axis (Belda et al., 2008; Kanter et al., 2001; Liberzon et al., 1997; Yehuda, 2005; Yehuda et al., 1993)
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30 and increased numbers of glucocorticoid receptors (GRs) in the lymphocytes of PTSD subjects
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32 (Yehuda et al., 1991). Effective pharmacotherapies for PTSD are quite limited. They include mainly
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34 selective serotonin reuptake inhibitors (SSRIs) , which have been shown to reduce aspects of all
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36 three clusters of behavioral symptoms (Ipser et al., 2006), with the only two FDA approved agents
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38 being paroxetine (Paxil; PRX) and sertraline (Zoloft).
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49 While studies of PTSD patients have become increasingly sophisticated, clinical studies
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51 remain limited in their ability to probe the underlying neurobiology of PTSD, underscoring the utility of
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53 appropriate PTSD animal models. The rat single prolonged stress (SPS) model has emerged as a
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55 model with strong face, predictive and, most importantly, construct validity in modeling certain
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57 behavioral and biological aspects of PTSD (Pitman et al., 2012; Yamamoto et al., 2009). In this model
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59 rats are exposed sequentially to restraint, forced group swim and ether followed by an undisturbed
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period that is necessary for the development of PTSD-like characteristics. The exposure to severe, multimodal stress, including psychological, physical, and pharmacological stressors, demonstrates strong construct validity by producing similar HPA-axis alterations, such as enhanced negative feedback of the HPA axis (Liberzon et al., 1997; Liberzon et al., 1999), increased GR expression in the dorsal hippocampus (Eagle et al., 2013b; George et al., 2014; Knox et al., 2012b; Kohda et al., 2007; Liberzon et al., 1999; Wang et al., 2009), enhanced contextual fear, extinction, and extinction retention deficits (George et al., 2014; Iwamoto et al., 2007; Knox et al., 2012a; Knox et al., 2012b; Matsumoto et al., 2013; Takahashi et al., 2006; Takei et al., 2011; Yamamoto et al., 2008), hyperarousal (increased sensitivity) (George et al., 2013a; Khan and Liberzon, 2004), increased avoidance behavior (Brand et al., 2008; Eagle et al., 2013a), and emotional/cognitive deficits (Eagle et al., 2013a; Peng et al., 2010; Wang et al., 2010). The SPS model also demonstrates predictive validity in that PRX can block enhanced fear learning (Takahashi et al., 2006). Use of the rat SPS model has likewise provided additional insight into mechanisms that underlie the cortical hypoactivity observed in PTSD subjects. For example, we have found that SPS-exposed rats demonstrate reduced glutamate (Glu) in the PFC compared to controls, indicative of decreased PFC excitatory tone and hypoactivity (George et al., 2013b; Knox et al., 2010). Conversely, few reports of mouse models of PTSD have been documented. One such report by Wang and colleagues describes a mouse SPS model that incorporates conditioned fear stress (Wang et al., 2012) in addition to the established multimodal stress paradigm that is akin to the rat SPS model. Use of this mouse model demonstrates efficacy of PRX to reduce fear behaviors, hippocampal apoptosis and structural damage associated with fear stress/SPS exposure. However, additional PTSD-related outcomes were not examined, including HPA axis alterations and GR expression levels, and extinction/extinction retention of learned fear behaviors, which may be difficult to interpret considering the inclusion of fear conditioning in the SPS exposure paradigm.

1 In order to take advantage of techniques that will allow for additional lines of investigation of
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3 the neuroendocrine, neurobiological and behavioral abnormalities observed in PTSD, for example
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5 using optogenetic and transgenic technologies amenable to use in mice, a valid model is required in
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7 this species. Likewise, development of a mouse model of PTSD that parallels the rat SPS model
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9 emphasizes the validity of each model independently, supporting their use in cross-species
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11 examination of not only the neurobiology of PTSD, but also PTSD-related fields. The ability to
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13 investigate the neurobiology of PTSD using both rats and mice with cross-species consistency will
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15 advance our knowledge in the field more rapidly, underscoring the importance of the current study.
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17 The aims of this study were to develop a SPS model for use in mice that allows exploration of
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19 mechanisms relevant to PTSD. Modification of the rat SPS paradigm has yielded a mouse SPS
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21 (mSPS) model that replicates key behavioral and physiological characteristics of PTSD, including
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23 enhancement of cue-induced fear, deficits in conditioned fear extinction retention, enhanced negative
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25 feedback of the HPA axis, increased hippocampal GR mRNA expression and blunted Glu levels in
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27 the PFC. Furthermore, enhancement of cued fear behaviors observed in mSPS mice were
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29 attenuated with oral PRX treatment, providing a translationally-relevant model for future PTSD
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31 research.
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Materials and Methods

The Guide for the Care and Use of Laboratory Animals 8th edition (Institute of Laboratory Animal Resources (U.S.), 2011) was followed and all experimental procedures were approved by the Institutional Animal Care and Use Committee at Wayne State University and in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care.

Animals and the mSPS paradigm

Naïve C57Bl/6 male mice (N = 152) were bred at Wayne State University facilities and group-housed in standard microisolator polycarbonate (home) cages with bedding in a temperature- (~24°C) and humidity- (35-40%) controlled environment. Animals were allowed food and water *ad libitum* in their home cages and housed on a 12 h light-dark cycle with lights on at 7AM. All animals were acclimated to the laboratory for 5-7 days before experimentation and were handled throughout the study. All mice weighed approximately 25-30 g and were 12-14 weeks of age at the start of experimentation. A separate cohort of naïve mice were used for each set of experiments. Group sizes are stated in the figure legends.

A portion of the mice were exposed to the mSPS procedure, which is a modified version of a previously described SPS procedure for rats (Eagle et al., 2013a; Eagle et al., 2013b; Eagle and Perrine, 2013; George et al., 2014; Knox et al., 2010; Liberzon et al., 1997). Mice underwent a series of short, multimodal stressors in the following order. 1) Animals were restrained in BD Falcon® 50 ml conical tubes with a screw-on top (with air holes located approximately ½ cm apart) for 2 h. 2) Restraint was immediately followed by a 10 min group swim (n=4-8 per swim) in a 4 L beaker (diameter = 18.4 cm) with a water depth of ~25 cm (3.5 L) at room temperature (~23°C). 3) Mice were then towel dried and placed back into their original cages where they were exposed to a beaker of soiled bedding taken from cages of sentinel rats, which was placed inside the home cage during recuperation to serve as a predator scent (Papes et al., 2010). 4) After 15 min of rat bedding scent exposure, mice were exposed to anhydrous diethyl ether until loss of consciousness, which took ~3

min. Ether exposure was conducted by gradually adding diethyl ether-soaked cotton balls at 1 min intervals to a standard microisolator polycarbonate cage. This protocol was chosen to minimize rapid onset of loss of consciousness and possible asphyxiation. Control animals were handled briefly and weighed instead of being exposed to mSPS treatment. Following the mSPS procedure (or control treatment), all animals were housed in new cages with fresh bedding and returned to the vivarium, where they were left undisturbed for seven days.

Measurement of cue-induced freezing behavior

Mice (mSPS, $n = 7$; control, $n = 7$) were tested for cued fear behavior using a tone paired with the mSPS or control procedure to examine behavioral responses to trauma cue. Here, the cue was composed of a series of 5 brief bursts of tone (1 kHz at 70-80 dB, 1 s), separated by 0.5 s intervals with a 1 s period between each series. The tone originated from speakers located approximately 1 m away from the animals. The tone was present during the entire mSPS procedure. Control mice were separately exposed to the tone while in their home cages for the same duration as mSPS mice. After the undisturbed period, mice were tested for cue-induced freezing behavior in an open field arena as a measure of cued fear. The open field consisted of a large testing chamber made of clear Plexiglas material with no lid (62.2 cm x 62.2 cm x 35.6 cm; Formtech Plastics, Oak Park, MI). The outside of the walls and floor were surrounded with white paper. Mice were placed individually into the center of the open field for two 3 min trials and activity was continuously recorded for both trials. First, activity was recorded for a 3 min 'No Tone' trial. Immediately after the 'No Tone' trial, activity was recorded while the tone, originally paired with mSPS and control treatment, was played. Activity was recorded with a digital CCD camera located above the arena that was connected to a PC computer for data storage and subsequent analysis. Freezing behavior was measured as time spent immobile, scored by two independent observers that were unaware (blinded) of experimental conditions. Freezing was defined as lack of skeletal movement not including normal breathing, sniffing and/or grooming behaviors. Inter-rater reliability between raters was $r^2 = 0.81$.

Oral PRX treatment and cue-induced freezing behavior

A separate group of animals was assessed for the effects of oral PRX on mSPS-dependent cued-induced freezing forming the following groups: control+vehicle, n = 7; control+PRX, n = 7; mSPS+vehicle, n = 7; mSPS+PRX, n = 7. Mice were exposed to the mSPS paradigm or control treatment, including the presentation of the tone, as described. During the 7-d undisturbed period following mSPS or control treatment, half of the mice from each group were given oral PRX HCl (LTK Laboratories, Inc., St. Paul, MN) dissolved in their drinking water. During acclimation, the amount of total daily water consumption was determined for each cage of mice and used to provide an estimate of daily drinking for oral dosing calculation. Mice consumed approximately 10 mg/kg/day for 7 days. Only one drinking bottle with PRX in water or water alone (vehicle control) was provided to the mice (i.e. a forced choice) and solutions were replaced daily. At the end of the 7-d undisturbed period, freezing behavior was assessed in the presence and absence of the trauma cue, as described.

Measurements of fear conditioning, extinction, and extinction retention

Using methods similar to those previously described in PTSD patients (Milad et al., 2008) and in rats exposed to SPS (George et al., 2014; Knox et al., 2012a; Knox et al., 2012b), we tested fear conditioning, extinction, and extinction retention in a separate cohort of mice following mSPS (n = 9) or control (n = 9) treatment. Fear conditioning was conducted in standard conditioning chambers (Coulbourn Instruments, Allentown, PA), each positioned within a sound-attenuating cubicle. A camera was mounted atop each chamber to assess freezing during trials by frame-to-frame pixel comparison using FreezeFrame v4 software (Actimetrics, Willmette, IL). A small house light (28 V, 0.04 A) was continuously on within the cubicle to provide dim illumination. Two unique contexts were used: context A – two black and two clear walls, ventilation fan on to provide background noise (~65 dB), shock grid floor; and context B – two white and two walls with striped and polka-dotted patterns, white textured Plexiglas solid panel for flooring, insertion of a white wall to create a simple convex polygon rather than a square chamber (thereby changing chamber geometry) and introduction of a

new smell of vanilla poundcake oil (Hosley Candle Co., Lynwood, IL). The apparatus was cleaned with 70% ethanol after each use in contexts A and B and with deionized water after ethanol in context B.

Beginning on day 7 of the undisturbed period, mice were given a 5 min habituation session in which they were exposed to context A for 5 min with no tones or shocks. Later that day, mice were placed back in context A, and after a 180 s pretrial period, presented with 3 tone-shock pairings. Each trial consisted of a 10 s, ~70 dB, 4 kHz tone (conditioned stimulus, CS) co-terminating with a 1 s, 0.4 mA footshock (unconditioned stimulus, UCS), with a 60 s intertrial interval (ITI). Freezing behavior (conditioned response, CR) was measured. On day 8, mice were placed in a new context, context B. There was no habituation to context B. After a 180 s pretrial period, mice were exposed to twenty 10 s, ~70 dB, 4 kHz tones with a 60 s ITI to evaluate extinction of freezing behavior. On day 9, mice were returned to context B and the same procedure as the previous day (day 8) was used to assess extinction retention behavior. For data analysis, movement thresholds were set for each animal individually, based on their histogram of activity during the retention trial. Bouts of freezing were defined as time periods of greater than 1.75 seconds spent below the set movement threshold. Data were exported from FreezeFrame in "bins" and as a percentage (%) of the time spent freezing. Data were binned as follows: trials 1-3 for acquisition, blocks 1-10 (2 trials per block) for extinction, and blocks 1-2 (5 trials per block) for extinction retention.

HPA negative feedback testing

Mice were tested for mSPS effects on dexamethasone (Dex) suppression of plasma corticosterone (CORT) in response to restraint stress forming the following groups: control+vehicle, n = 9; control+Dex, n = 10; mSPS+vehicle, n = 9; mSPS+Dex, n = 12. Control and mSPS mice received subcutaneous (s.c.) injection of either Dex (0.1 mg/kg; Sigma-Aldrich Corp., St. Louis, MO) or normal sterile saline. Immediately after injections, mice were placed into restraint tubes for 30 min.

Mice were then euthanized and trunk blood was collected for measurement of CORT. Trunk blood was centrifuged at 6000 x g for 5 min to collect plasma.

Plasma CORT levels were measured using a commercially available ELISA kit (Neogen Corp., Lexington, KY) following the manufacturer methods and as previously described (Cox et al., 2011; Perrine et al., 2014). CORT was extracted from plasma by adding ethyl ether (in excess) to the sample and then vortexing the mixture, allowing it to separate into organic and inorganic fractions, and exposing the sample to a stream of N₂ to evaporate the organic phase. Extraction buffer provided with the kit was used to reconstitute the remaining inorganic residue. CORT-horseradish peroxidase conjugate and the reconstituted samples were added to the antibody-coated 96-well plate. After a 1-h incubation period at room temperature on an orbital shaker at low speed, the wells of the plate were thoroughly rinsed with wash buffer to leave only bound material. Tetramethylbenzidine in H₂O₂ was added to the wells of the plate and bound conjugate was detected by absorbance at 650 nm. Quantification of the CORT concentration in each sample was determined by comparison to external corticosteroid standards run in parallel. CORT data are shown as ng/ml, and were determined in control and mSPS groups in the presence and absence of Dex.

Real-time quantitative PCR (RT-PCR) analysis of dorsal hippocampal GR mRNA expression

Dorsal hippocampal tissues were dissected 7 d after mSPS (n = 10) or control (n = 9) exposure, flash frozen in liquid nitrogen and stored at -80°C until use. Tissues were prepared using the GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, MO), by homogenizing the cells with an electric pestle in the provided Lysis Solution with 2-mercaptoethanol. The homogenized tissue was filtered using the provided columns and centrifuged at 12,000 x g. Next, 500 µL of 70% EtOH was mixed with the lysate and this mixture was added to the GenElute Binding Column and centrifuged at 12,000 x g for 15 s. The column was washed with 500 µL of Wash Solution 1 and centrifuged at 12,000 x g for 15 s. For the second wash, 500 µL of Wash Solution 2, diluted with EtOH, was added to the column and centrifuged at 12,000 x g for 15 s. An additional

500µL of Wash Solution 2 was added to the column, and it was centrifuged at 12,000 x g for 2 min. RNA was eluted by adding 50 µL of Elution Solution into the binding column and centrifuging for 1 min at 12,000 x g. Eluted RNA was stored at -80°C until use. Quantification of RNA was determined by measuring the absorbance of 2 µL of RNA in 98 µL of water at 260 nm and 280 nm in quartz cuvettes.

Following reconstitution of the RNA, first strand cDNA was produced using the SuperScript III First-Strand Synthesis System (Life Technologies, Grand Island, NY). In brief, 2.5 µM oligoDTs, 1 µl sample RNA, and 0.5 µM dNTPs were incubated at 65°C for 5 min to allow annealing. cDNA synthesis mix was prepared as per the manufacturer's instructions and was added to the RNA, which was then incubated at 50°C for 50 min, followed by incubation at 85°C to terminate the reaction.

This cDNA was used for RT-PCR using SYBR green (Life Technologies, Grand Island, NY) with primers for either GR [forward sequence 5'- AGCTCCCCCTGGTAGAGAC-3' and reverse sequence 5'-GGTGAAGACGCAGAAACCTTG-3'] or β-actin [forward sequence 5'-AGAGGGAAATCGTGCGTGAC-3' and reverse sequence 5'-CAATAGTGATGACCTGGCCGT---3'] (Wynne et al., 2012; Yu et al., 2013). The PCR was performed in an ABI7700 Prism quantitative real-time PCR cycler, which yielded optimal conditions of: 1 cycle at 95°C for 10 min followed by 40 cycles of 95°C for 15s then 60°C for 1min. Primer concentrations were optimized and used at the following concentrations: (0.4 µM GR Forward, 0.2 µM GR Reverse; 0.2 µM β-actin Forward, 0.9 µM β-actin Reverse). A dissociation curve was generated and GR mRNA expression was normalized to β-actin mRNA for each sample run in duplicate. Data were analyzed using the comparative Ct method, as previously described (Livak and Schmittgen, 2001).

For each replicate, Ct, defined as the fractional cycle number at which the fluorescence passes a fixed threshold (based on the baseline data collected in the first 15 cycles), was calculated. Therefore, for example, for each sample an average Ct for GR (Ct_{GR}) and β actin ($Ct_{\beta-actin}$) was obtained. Samples were normalized against β-actin using the following equation:

1 $Ct_{\beta \text{ actin-GR}} = \text{mean}(Ct_{\beta \text{-actin}}) - \text{mean}(Ct_{GR}).$

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3 $\Delta Ct_{\beta \text{-actin-GR}}$ was calculated for each experimental group and differences between groups were
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6 calculated using the following equation as an example:

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8 $Ct_{GR} = \text{control}(Ct_{\beta \text{ actin-GR}}) - mSPS(Ct_{\beta \text{-actin-GR}}),$

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10 where $\Delta \Delta Ct_{GR}$ represents the difference in GR expression between experimental groups compared.

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12 The magnitude of difference in GR gene expression is equal to $2^{\Delta \Delta Ct_{GR}}$. Relative GR expression levels
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14 were normalized to β -actin and plotted relative to expression in control mice, which equals 1.
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17 ***Immunoblot analysis of dorsal hippocampal GR protein levels***

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19 Dorsal hippocampal tissue samples from mSPS (n = 8) and control (n = 8) mice were obtained
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21 by dissection (7 d after mSPS or control exposure) and prepared for immunoblot analysis of GR and
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23 β -actin levels. Frozen tissue was placed into homogenization buffer [10% 1 M NaCl, 10% glycerol,
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25 1% 1M Tris, 1% Triton X-100, protease inhibitor EDTA-free cocktail and phosphatase inhibitor
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27 cocktail (Thermo Fisher Scientific, Rockford, IL) in dH₂O] and then sonically disrupted (Misonix,
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29 Newton, CT). Homogenates were centrifuged at 10,000 x g for 20 min at 4°C and supernatant were
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31 extracted and frozen at -80°C until measurement of protein concentration. Protein concentrations
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33 were measured using the Folin-Lowry assay as previously described (Eagle et al., 2013b; Lowry et al.,
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35 1951; Perrine et al., 2008).
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39 Equal amounts (30 μ g) of protein were analyzed using sodium dodecyl sulfate-polyacrylamide
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41 electrophoresis, as previously described (Eagle et al., 2013b), which began by loading them into 10%
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43 sodium dodecyl sulfate-polyacrylamide Tris-HCl custom-poured gels. After gel electrophoresis,
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45 proteins were transferred to polyvinylidene fluoride membranes for 90 min at 100 V. Transferred
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47 proteins were visualized by Ponceau S Red to ensure efficient transfer and equal protein loading.
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49 Proteins of interest on the PVDF membrane were visualized as follows: PVDF blots were incubated
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51 for 2 h in blocking solution [Tris-buffered saline with 0.1% Tween-20 (TBS-T) with 5% non-fat dry
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53 milk] and then in primary antibodies overnight at 4°C. Blots were washed in TBS-T and then
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incubated for 1 h in goat anti-rabbit (1:10,000; Bio-Rad, Hercules, CA) secondary antibody conjugated to horseradish peroxidase. Protein bands were visualized using enhanced chemiluminescence substrate (West Pico; Thermo Fisher Scientific, Rockford, IL) on a Chemic-Doc XRS+ imaging system (Bio-Rad, Hercules, CA). Membranes were stripped between antibodies with RestorePlus Western Blot stripping buffer (Thermo Fisher Scientific, Rockford, IL) using manufacturer instructions. All membranes were separately probed for GR (GR-20, 1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and β -actin (1:5000, Sigma-Aldrich, St. Louis, MO). Bands of interest were confirmed by comparison to molecular weight standards (Protein Plus Western C Standards, BioRad, Hercules, CA).

Proton magnetic resonance spectroscopy analysis of PFC Glu levels

Tissue samples of the PFC, including prelimbic and anterior cingulate cortex, from mSPS (n = 6) and control (n = 10) mice were obtained by dissection (7 d after mSPS or control exposure) for analysis of whole-tissue levels of Glu by high resolution magic angle spinning ^1H -magnetic resonance spectroscopy (^1H -MRS) at 11.7 T *ex vivo*, as previously described (Knox et al., 2010; Perrine et al., 2011). Frozen intact tissue samples were weighed then analyzed with a vertical wide-bore (8.9 cm) Bruker 11.7 T magnet using a Bruker magic angle spinning probe maintained at 4°C and controlled with an Avance DRX-500 console (Bruker Biospin Corp., Billerica, MA). Spectra were acquired with a 1-D Carr-Purcell-Meiboom-Gill pulse sequence and Bruker-XWINNMR version 3.6 software using the same settings as previously described (Knox et al., 2010; Perrine et al., 2014; Perrine et al., 2011). Spectra were analyzed with a custom LCModel version 6.1-4 (Provencher, 1993), and Cramér-Rao bounds for Glu were less than 10%. Spectral data were normalized to tissue weight and are presented as a percent of control.

Statistical Analyses

All statistical analyses were conducted in Graphpad Prism 5 (Graphpad Software, Inc., La Jolla, CA) or SPSS 17.0 (IBM, Armonk, NY). For cue-induced behavior assessed in the open field and Dex

1 suppression testing, separate two-way ANOVAs were conducted. Planned pairwise comparisons
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3 were conducted using Sidak tests unless otherwise stated. Planned LSD pairwise comparisons were
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5 performed for analysis of PRX on cued freezing. Freezing behavior was examined across fear
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7 conditioning phases. Separate 2-way repeated-measures ANOVAs were conducted to determine the
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9 effects of group on % freezing across blocks. Planned Sidak-corrected comparisons were conducted
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11 to analyze group differences at each block during the retention phase. One-tailed *t*-tests were
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13 conducted for analysis of hippocampal mRNA expression and two-tailed *t*-tests were used to analyze
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15 hippocampal protein levels and PFC Glu levels.
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Results

mSPS induces cue-induced freezing

mSPS (n = 8) and control (n = 7) mice were tested for cue-induced freezing behavior in the open field (Fig. 1). A two-way mixed-design ANOVA was conducted for the effects of group (mSPS vs. control) and trial (tone vs. no tone) on freezing (Fig. 1). There was a significant main effect of trial [$F(1, 13) = 13.17, p = 0.003$] as well as a significant interaction of trial and group [$F(1, 13) = 5.04, p = 0.043$]. mSPS increased freezing in response to the tone, when compared to controls, $p = 0.048$, and to the no tone trial, $p < 0.001$. Controls did not exhibit more freezing in response to the tone. In the absence of tone, mSPS did not increase freezing compared to control treatment. Therefore, mSPS mice exhibited more cue-induced fear behavior in response to a tone previously paired with stressors than did controls.

Oral PRX reduces mSPS-mediated cue-induced freezing

A separate cohort of mice was assessed for the effects of PRX on trauma cue-specific fear. A subgroup of mice exposed to either mSPS or control treatment received water alone, as a control (n = 7 CON; n = 7 mSPS), or PRX (n = 7 PRX; n = 7 mSPS+PRX) in their drinking water during the undisturbed period of mSPS (Fig. 2). A two-way ANOVA was conducted for the effects of group (CON, mSPS, PRX, mSPS+PRX) and trial condition (tone vs. no tone) on freezing (Fig. 2B). A significant effect of trial was observed [$F(1, 48) = 13.08, p = 0.001$] reflecting expected trial-to-trial habituation, no significant effects for group or the group $\times$ trial interaction were found.

Planned LSD pairwise comparisons revealed that mSPS enhanced freezing to a tone, compared to controls [CON; $t(48) = 2.27, p = 0.027$] and compared to the no tone trial freezing [$t(48) = 2.81, p = 0.007$] thus replicating the previous findings. Furthermore, the mSPS-induced freezing in response to the tone presentation was attenuated in the mSPS+PRX group, which had decreased freezing during the tone compared to mSPS [$t(48) = 2.11, p = 0.040$]. Thus these data demonstrate that PRX can reduce mSPS-mediated, cue-induced freezing behavior.

mSPS disrupts extinction behavior

Control (n = 9) and mSPS (n = 9) mice were conditioned to associate a tone (CS) with a footshock (UCS) and freezing (CR) was assessed. During acquisition, mice significantly increased the percentage of time spent freezing (% freezing) [$F(3, 42) = 58.17, p < 0.001$], with no significant mSPS effect, nor significant mSPS x time interaction (Fig. 3A, left). Next, mice underwent extinction training, where the tone was no longer paired with a footshock. Mice significantly decreased % freezing across extinction [$F(10, 140) = 5.13, p < 0.001$]; however, freezing was not completely abolished by the end of the session. As with the fear acquisition, there was no significant effect of mSPS or any interaction of mSPS x time (Fig. 3A, right). Finally, mice were tested for extinction retention by conducting a second extinction session (Fig. 3B). There was no significant main effect of mSPS, however there was a significant effect of testing block across extinction retention testing [$F(2, 28) = 15.49, p < 0.001$], with no significant interaction of mSPS x testing block. Sidak-corrected *t*-tests revealed that the main effect of block was largely caused by a significant difference between mSPS and CON mice in % freezing in the 2nd extinction retention testing block [$t(42) = 2.41, p = 0.023$], suggesting that mSPS-exposed mice had a decrement in extinction learning or failed to retain extinction memory at late phase retention.

mSPS enhances dexamethasone suppression of plasma CORT levels

mSPS and CON mice were tested for Dex suppression of plasma CORT in response to restraint stress (Fig. 4). Either saline (n = 9 mSPS; n = 9 control) or Dex (0.1 mg/kg; n = 12 mSPS; n = 10 control) was administered s.c. immediately prior to 30 min restraint. Plasma was assayed for CORT levels using ELISA. A two way ANOVA did not reveal significant main effects, however the interaction trended towards significance [$F(1, 36) = 3.38, p = 0.074$] (Fig 4). Planned LSD pairwise comparisons revealed that mSPS enhanced Dex suppression of plasma CORT levels relative to controls that received Dex [$t(36) = 2.74, p = 0.010$] and compared to the mSPS mice that received only saline [$t(36) = 2.65, p = 0.012$].

mSPS increases dorsal hippocampal GR mRNA expression with no effect on GR protein levels

Homogenized tissue punches from dorsal hippocampus (represented in Fig. 5A) were separately assayed for GR protein levels via immunoblotting or mRNA levels using quantitative RT-PCR 7 d after mSPS or control exposure. Analysis by *t*-test revealed that mSPS (n=10) significantly [$t(17)=1.78$, $p = 0.047$] upregulated GR mRNA in the dorsal hippocampus compared to controls (n=9) (Fig. 5B). When normalized to β -actin mRNA expression, mSPS mice demonstrated an approximate 1.26-fold increase in GR mRNA compared to controls. No differences were observed between mSPS (n=8) and control (n=8) mice in GR protein levels normalized to β -actin (Fig. 5C).

mSPS decreases PFC Glu levels

Whole tissue punches from PFC (represented in Fig. 6A) were separately assayed for Glu levels via ^1H -MRS 7 days after mSPS or control exposure. Analysis by *t*-test revealed that mSPS (n=6) significantly [$t(14) = 2.96$, $p = 0.010$] decreased Glu levels in the PFC compared to controls (n=10) (Fig. 6B).

Discussion

Severe, multimodal stress exposure in mice modifies fear-related behaviors similar to those observed in PTSD patients. These behavioral responses were accompanied by neurohormonal changes to the HPA axis, hippocampal GR mRNA expression and Glu levels in the PFC. Together these findings establish mSPS as a model of PTSD with substantial face, predictive, and construct validity.

mSPS causes abnormal fear learning

In the present study, mice that had a unique tone paired with mSPS exposure had significantly greater amounts of freezing in an open field only when the tone was replayed (i.e. cue-induced), but not in the open field alone (Figure 1), which models the increased fear response to trauma cues observed in clinical PTSD (Jovanovic et al., 2010; Shin and Handwerger, 2009). This finding is also congruent with a recent study using rat SPS and a trauma cue to measure heightened freezing (Toledano and Gisquet-Verrier, 2014). In a separate cohort of mice, we replicated our findings and also found that the tone-associated freezing behavior was attenuated by treatment with PRX (Figure 2). This models pharmacotherapeutic treatment in humans with PTSD, in that PRX reduces aspects of all three clusters of behavioral symptoms (Ipser et al., 2006), and is congruent with findings in the rat SPS paradigm, in which PRX dose-dependently suppresses the enhancement of contextual freezing after SPS (Takahashi et al., 2006).

Research suggests PTSD results from deficits in retention of fear extinction that might contribute to persistence of trauma-related memories (Milad et al., 2008; Quirk et al., 2006; Ressler et al., 2004; Rothbaum and Davis, 2003). The literature on acquisition of fear conditioning/extinction in humans is less consistent regarding acquisition of extinction (Norrholm et al., 2011; Peri et al., 2000) in PTSD patients. The rat SPS model indicates that severe, multimodal (traumatic) stress exposure disrupts both contextual and cued retention of extinction memories (George et al., 2014; Knox et al., 2012a; Knox et al., 2012b). The data presented here suggests that an extinction retention deficit, or

1 potentially a delay in extinction learning, also develops in mice following exposure to severe,
2
3 multimodal stress (Figure 3). The observation of stress-induced deficits in extinction learning or
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5 extinction retention in rodent models, that parallels clinical evidence, further demonstrates important
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7 construct validity and highlights the opportunity for future pre-clinical studies to explore mechanisms
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9 that underlie these behaviors.
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13 ***mSPS disrupts HPA axis control and alters brain neurochemistry***

15 Currently, PTSD is defined by behavioral signs and symptoms, without identification of a
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17 corresponding biomarker. Nevertheless, enhanced negative feedback of the HPA axis has emerged
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19 as one of the most consistent research findings in PTSD. Our findings show that mSPS-exposed
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21 mice exhibit heightened negative feedback of HPA axis inhibition, as predicted, to a dose of DEX that
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23 was ineffective in control mice, which indicates that mSPS-exposed mice are particularly sensitive to
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25 HPA axis manipulation using the Dex suppression test and that mSPS exposure alters HPA axis
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27 reactivity in a clinically-relevant manner. Enhance negative feedback of the HPA axis in PTSD is
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29 thought to reflect greater peripheral GR sensitivity (Yehuda et al., 2004; Yehuda et al., 1993).
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31 Elevations in the number of GRs in lymphocytes of PTSD subjects have been reported (Yehuda et
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33 al., 1991), but GR levels in the human brain and the action of glucocorticoids in PTSD are still
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35 unclear. Research indicates that stress-induced changes in mitochondria membrane potential are
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37 regulated by genomic and non-genomic GR signaling in the dorsal hippocampus and this could
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39 potentially contribute to hippocampal atrophy in some cases of PTSD (Han et al., 2013; Sapolsky,
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41 2000; Zhang et al., 2006). However, a second hypothesis, and one that follows the well-known
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43 effects of glucocorticoids on brain activity, is that GR signaling in the brain, specifically the dorsal
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45 hippocampus and frontal cortex, regulates learning and memory processes. For example, Dex had a
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47 relatively negative effect on declarative memory function in healthy subjects, but not in those with
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49 PTSD, which is thought to reflect an altered sensitivity to certain memories following trauma (Bremner
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51 et al., 2004). More research is needed to clarify the implications of enhanced negative glucocorticoid
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1 feedback to understand the effects of trauma on brain GR levels and to determine the role of
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3 hippocampal GRs on learning processes in PTSD.
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6 Findings from the rat SPS model have contributed to our understanding of the peripheral (HPA
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8 axis) and central (brain) actions of glucocorticoids and the role of hippocampal and cortical GR in fear
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10 learning behavior following severe, multimodal stress. For example, it has been shown that SPS
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12 enhances negative (fast) feedback of the HPA axis which is accompanied by increased hippocampal
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14 GR (and reduced mineralcorticoid receptor) levels (Liberzon et al., 1997; Liberzon et al., 1999).
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16 Enhancement of HPA axis activity (Ganon-Elazar and Akirav, 2012; Liberzon et al., 1999) and
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18 increases in hippocampal GR levels (Eagle et al., 2013b; George et al., 2014; Knox et al., 2012b;
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20 Liberzon et al., 1999; Wang et al., 2009) are some of the most consistent findings in the SPS model,
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22 which are also replicated in the present study using the mSPS model (Figures 4 and 5). These
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24 findings suggest that GR expression and function may contribute directly to fear learning
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26 abnormalities reported in PTSD. Pretreatment with a glucocorticoid receptor antagonist prevents
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28 SPS-induced changes in conditioned fear and hippocampal-dependent long term potentiation (Kohda
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30 et al., 2007). Recent evidence indicates that hippocampal and cortical GR are necessary for the
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32 extinction retention deficits observed following SPS (Knox et al., 2012b). In addition to implicating GR
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34 upregulation in the PFC and dorsal hippocampus in fear memory abnormalities associated with
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36 PTSD, we have also shown that SPS decreases PFC Glu levels (Knox et al., 2010), thereby
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38 implicating Glu as a key contributor to hypoactivity of excitatory neural transmission in the aberrant
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40 behavioral effects caused by severe, multimodal stress. We replicate this finding by showing mSPS
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42 decreases Glu levels in the PFC (Figure 6) in association with deficits in fear learning (Figures 1-3).
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44 While stress-induced changes in HPA axis activity, hippocampal GR levels, and cortical Glu levels
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46 are observed in rodents, these mechanisms require further exploration in humans with PTSD.
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57 ***A validated mouse model of PTSD***

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In addition to the present mouse paradigm demonstrating neurohormonal and neurochemical findings that support current mechanisms thought to underlie PTSD, as a model in its own right, generation of a mouse model of PTSD is particularly valuable to research areas such as alcohol use behaviors and genetics, which are relevant to PTSD. For example, the alcohol field depends heavily on use of mice for their unique tendency to consume large quantities of ethanol and the utility that mice have provided for elucidating the genetic underpinnings of alcohol behaviors. Furthermore, a secondary aim of this study was to develop a mouse model of PTSD with behavioral characteristics that reflected the clinical condition. Design of the mSPS model was based on the previously established rat SPS model of PTSD (Pitman et al., 2012; Yamamoto et al., 2009) that has been used extensively since its development and has yielded translational findings that have shaped our clinical views of the mechanisms of PTSD. Three important criteria – face, construct, and predictive validity – were chosen for evaluating animal models of PTSD (Daskalakis et al., 2013; Pitman et al., 2012; Yehuda and Antelman, 1993) and guided a series of experiments to test the validity of the mouse model for translational and biological studies of PTSD. Here, we demonstrated *face validity* with the use of experimental manipulations intended to be “life-threatening” to the animal and observation of behavioral effects modeling PTSD. The *construct validity* of the mSPS model was assessed by multiple experimental approaches to determine if our model reflects the current, underlying conceptual theory of PTSD. The described behavioral, pharmacological, neurohormonal, and neurochemical data reflect clinical findings relevant to altered fear learning (i.e. enhanced reactivity to trauma reminders and impaired extinction learning), glucocorticoid negative feedback of the HPA axis and support current mechanisms relevant to PTSD. Thus our model has strong construct validity for modeling the construct of PTSD. Finally, the *predictive validity* of our model was assessed on the clinical observations that SSRIs are effective in the treatment of PTSD. In the present study, repeated oral administration of PRX reduces cue-induced freezing to a tone previously paired with the severe, multimodal stressor. This suggests that the predictive validity of mSPS for modeling the

1 treatment of PTSD is good. However, it should be noted that PRX may have reduced the acquisition
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3 (e.g. development), rather than the expression, of conditioned fear since PRX was given orally during
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5 the undisturbed period. While an additional mouse model of SPS has been documented (Wang et al.,
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7 2012), incorporation of conditioned fear into the stress exposure paradigm may confound
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9 interpretation of PTSD-related outcomes such as extinction/extinction retention deficits. As such,
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11 SPS exposure in the present study avoids the use of conditioned fear, allowing fear behavior
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13 outcomes to be examined without potential confound.
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17 **Conclusion**

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19 Overall, these data indicate that mSPS models characteristics relevant to PTSD. In addition,
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21 severe, multimodal stress modifies fear learning in mice that coincide with changes in HPA axis, brain
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23 glucocorticoid systems and glutamatergic signaling in the PFC. The development of the mSPS model
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25 will be critical for integrating cutting edge techniques that are readily available in mouse models (e.g.
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27 optogenetics and the use of transgenic strains) and will greatly facilitate further exploration of the
28
29 neuroendocrine, neurobiological, and behavioral abnormalities that are relevant to PTSD.
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3 The authors report no biomedical financial interests or potential conflicts of interest.
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Figure Legends

Figure 1: Mouse single prolonged stress (mSPS) increases cue-induced freezing. Freezing in response to a tone cue previously paired with trauma of mSPS ($n = 8$) or control treatment ($n = 7$). mSPS produced significantly greater freezing in response to the tone compared to controls and compared to the trial without the tone present. $*p < 0.05$.

Figure 2: Oral PRX reduces mouse single prolonged stress (mSPS)-mediated cued-induced freezing. Control (CON) or mSPS-exposed mice were divided into subgroups and received either water ($n = 7$ CON; $n = 7$ mSPS) or paroxetine (PRX, 10 mg/kg/day for 7 days; $n = 7$ PRX; $n = 7$ mSPS+PRX) in their drinking water during the 7-day post-mSPS incubation period. At the end of the 7-day undisturbed period, mice were tested for tone-induced freezing. mSPS enhanced freezing to a tone and this effect was attenuated in the mSPS+PRX group. $*p < 0.05$, $***p < 0.001$.

Figure 3: Mouse single prolonged stress (mSPS) disrupts extinction retention. (A) Mice exposed to mSPS ($n = 9$) or control (CON, $n = 9$) significantly increased the percentage of time spent freezing (% freezing) across conditioning acquisition blocks and significantly decreased % freezing across extinction blocks; however, there was no significant effect of mSPS. (B) Analyses of extinction retention revealed a significant difference between mSPS and CON mice in % freezing in the 2nd (late phase) extinction retention block. $*p < 0.05$ compared to CON.

Figure 4: Mouse single prolonged stress (mSPS) enhances dexamethasone suppression of plasma corticosterone. Mice were first exposed to mSPS or control (CON), and then dexamethasone (Dex; 0.1 mg/kg; s.c.) or saline (s.c.) was administered prior to a 30 min restraint. This created the following groups: CON+saline ($n = 9$), CON+Dex ($n = 10$), mSPS+saline ($n = 9$), and mSPS+Dex ($n = 12$). Mice were then killed to assess plasma corticosterone (CORT) response to restraint. mSPS enhanced the dexamethasone-induced suppression of plasma CORT (% of saline)

1 compared to CON and to mSPS mice that received saline. $*p < 0.05$ compared to mSPS+saline, $**p$
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3 < 0.01 compared to CON+Dex.
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6 **Figure 5: Mouse single prolonged stress (mSPS) enhances dorsal hippocampal glucocorticoid**
7 **receptor (GR) mRNA expression with no effect on GR protein levels.** (A) Schematic (modified
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9 from (Franklin and Paxinos, 2007)) representing hippocampal tissue punches that were assayed for
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11 hippocampal GR mRNA using RT-PCR and protein levels using immunoblotting. Both protein and
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13 mRNA were normalized to β -actin and are plotted relative to control (CON) mice. (B) mSPS (n = 10)
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15 exposure significantly increased hippocampal GR/actin mRNA levels by approximately 1.26 fold
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17 compared to CON (n = 9). (C) mSPS exposure (n = 8) did not alter GR/actin protein levels in the
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19 dorsal hippocampus compared to controls (n = 8). For the representative blots C = control and S =
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21 mSPS. $*p < 0.05$ compared to CON.
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30 **Figure 6. Mouse single prolonged stress (mSPS) decreases glutamate levels in the prefrontal**
31 **cortex.** (A) Schematic (modified from (Franklin and Paxinos, 2007)) representing prefrontal cortex
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33 (PFC) tissue punches that were assayed for glutamate (Glu) levels using proton magnetic resonance
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35 spectroscopy at 12 T *ex vivo*. (B) mSPS exposure (n = 6) significantly decreased PFC Glu levels by
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37 approximately 20% of control (CON) levels (n = 10). $*p < 0.05$ compared to CON.
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Figure 1

Figure 2

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Figure 4

Figure 5

Figure 6