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European Journal of Neuroscience – Special Issue: Stress, brain and behavior

Differential chronic social stress models in male and female mice

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Running Title: Sex difference in social stress

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ejn.15481

Total number of pages: 42

Total number of figures: 5

Total number of supplemental figures: 3

Total number of supplemental tables: 1

Total number of words in the whole manuscript: 6,277

Total number of words in the Abstract: 247

Keywords: depression, animal model, affective neuroscience, sex difference, behavior

Abstract

Chronic stress creates an allostatic overload that may lead to mood disorders such as anxiety and depression. Modern causes of chronic stress in humans are mostly social in nature, relating to work and relationship stress. Research into neural and molecular mechanisms of vulnerability and resilience following chronic social stress (CSS) is ongoing and uses animal models to discover efficient prevention strategies and treatments. To date, most CSS studies have neglected the female sex and used male-focused aggression-based animal models such as chronic social defeat stress (CSDS). Accumulating evidence on sex differences suggests differences in the stress response, the prevalence of stress-related illness and the treatment response, indicating that researchers should expand CSS investigation to include female-focused protocols alongside the popular CSDS protocols. Here, we describe a novel female mouse model of CSS and a parallel modified male mouse model of CSDS in C57BL/6 mice. These new models enable the investigation of vulnerability, coping and downstream effectors mediating short-term and long-term consequences of CSS in both sexes. Our data demonstrate differential effects on male and female mice during, soon after, and many weeks after CSS. Female mice are more prone to body weight loss during CSS and hyperactive anxious behavior following CSS. Both sexes show reduced social interaction, but only stressed male mice show long-term changes in emotional memory and neuroendocrine function. We further discuss future avenues of research using these models to investigate mechanisms pertaining to sensitivity to CSS and treatment response profiles, in a sex-appropriate manner.

Introduction

Long-term exposure to stressful conditions is associated with the development of a manifold of pathophysiological conditions, including those affecting behavior, immune physiology, neuronal signaling, and cardiovascular function as well as chronic mood disorders such as anxiety and depression (McEwen 1998; Popoli et al. 2011; Yu 2016; Glaser and Kiecolt-Glaser 2005; Segerstrom and Miller 2004; Slavich and Irwin 2014; Boscarino and Chang 1999). In humans, chronic stress in modern life is usually social in nature, relating to socioeconomic status, work stress, relationship stress or conflicts with others (Kessler 1997; 1995; Björkqvist 2001; Slavich et al. 2010). Research in humans has established sex-differences in the biology of the hypothalamic-pituitary-adrenal (HPA) axis and stress response (Kudielka and Kirschbaum 2005; Bale 2006), the vulnerability to chronic stress (Hostetler and Ryabinin 2013; Bebbington 1996; Kessler and Mcleod 1984), the ensuing pathological conditions such as depression and auto-immune diseases (Whitacre 2001; Nolen-Hoeksema 1987); and finally, the response to drug treatments (Franconi et al. 2007; Kornstein et al. 2000). These data highlight the need for pre-clinical research that investigates mechanisms of pathology and resilience following CSS in both males and females.

Currently the majority of animal models of social stress, both acute and chronic, use male animals only (Wood and Bhatnagar 2015). Models of psychosocial stress in rodents include manipulations of living conditions at different ages, such as maternal separation (Schmidt, Wang, and Meijer 2011), isolation stress (Hatch et al. 1963), crowding stress (Haller et al. 1999), as well as aggression-based manipulations like social instability (Hostetler and Ryabinin 2013), resident intruder protocols, social defeat (Koolhaas et al. 1997) and maternal aggression (Lonstein and Gammie 2002).

As many effects of psychosocial stress are sex dependent (Goel and Bale 2009), the impact of stress on behavior and physiology should be compared between sexes. Indeed, a few noteworthy research projects that used less common rodent species with unique ethological characteristics were able to describe stress-related social behaviors in both males and females, including aggression (in golden hamsters), pair-bonding (in voles) and social defeat stress (in Californian mice). In nature, Californian mice, both male and female, aggressively defend territories (Ribble and Salvioni 1990), allowing investigators to assess effects of social defeat stress in females. This sex-suitable research project revealed that the long-term effects of social defeat stress are sex-specific (Trainor et al. 2011; 2013), and has resulted in follow-up studies on cognitive flexibility following stress in mice and humans (Shields et al. 2016; Laredo et al. 2015).

The use of laboratory-reared mice allows many advantages when looking for cellular and molecular mechanisms underlying pathologies. The CSS protocols described here employ a common mouse strain (C57BL/6), and allow manipulating conditions before and after stress while minimizing physical injuries in both males and females. Furthermore, it enables the differential assessment in males and females of short- and long-term effects of individual differences and effects of social environment on the stress response.

In the present study, we investigated the effects of two CSS ethologically-minded sex-specific protocols on several measures of anxiety, depression, learning and memory, social behavior and endocrine response profile. These two sex-specific protocols were developed in order to allow the comparative study of short-term and long-term effects of CSS.

Materials and Methods

Animals and Housing

Male and female (8-week old) C57BL/6 black mice (Harlan) were maintained in a pathogen-free, temperature-controlled (22ºC±1ºC) mouse facility on a reverse 12/12 hr light/dark cycle, with lights switched on at 8 p.m. Male white ICR (CD-1) outbred mice (Harlan) were used as residents for the social defeat paradigm. Female white ICR (CD-1) outbred mice (in-house breeding) were used for the social crowding stress paradigm. All experimental animals were pair-housed with same-sex partners for the duration of the experiment. Home-cage control animals were not housed with stressed animals but with other control animals. We did not monitor females' estrous cycle and used freely cycling females, as several studies suggest behavioral effects of exposure to stress may overshadow effects of different estrus cycle stages on behavior (Prendergast, Onishi, and Zucker 2014; Dalla et al. 2008; Horovitz et al. 2014). All experimental animals were weighed weekly to monitor general health. The study contains data from several batches that served as internal control. The Institutional Animal Care and Use Committee (IACUC) of The Weizmann Institute of Science approved all procedures. Animals were given ad libitum access to food and water.

Female CSS paradigm

Combining elements from several social stress protocols, including resident-intruder, crowding stress and social instability, we generated a novel female mouse model of CSS. Our model employed 8-week old female C57BL/6, socially housed in groups and then in pairs in order to create highly familiar low aggression pairs.

During the 15 days of the social stress protocol, each experimental C57BL/6 female mouse was daily transported to a testing room where it was exposed to crowding by a variable number (5-9) of unfamiliar ICR female mice in a novel cage (see below description of maintaining the ICR females) for 1 h. For each stress session, the experimental mouse was allowed to interact

directly with the unfamiliar ICR female mice for 30 mins; mice behavior included agonistic behavior such as sniffing and grooming as well as antagonistic behavior such as chasing, physical attacks and related vocalizations. For the remaining 30 min, the experimental mouse was placed inside a metal mesh within the same cage, enabling visual, olfactory and auditory interaction but no physical contact. In order to increase instability and unpredictability, stress sessions were conducted without habituation, each day at different times during the active dark phase and in different testing rooms. After each 1 h stress session, the experimental mice were returned to the homecage containing the same highly-familiar partner. In this manuscript, we describe behavioral and physiological response of the experimental mice to social stress, while the behavior of the partner mice is reported in a separate manuscript.

Control female C57BL/6 mice were housed in pairs and handled daily for 15 days.

The ICR female mice were housed in conditions of social instability for the duration of the social stress protocol: at the end of each stress session ICR mice were randomly divided into groups of 4 until the following day. At the beginning of each stress session, ICR mice were dispersed in novel cages containing 5-9 animals per cage. This procedure created high levels of arousal and exploration of conspecifics among ICR female mice, and rendered female ICR mice more aggressive towards the C57BL/6 female experimental mice.

Male CSS paradigm

Based on chronic social defeat stress (CSDS) protocols in male mice (Berton, Mcclung, et al. 2006), we developed a protocol of CSDS that is comparable to the female protocol described above. Briefly, our model employed 8-week old male C57BL/6, housed in groups and then in pairs until 12 weeks old in order to create low aggression levels, as is known to occur in groups of males cohabiting for weeks (Haller et al. 1999). During the 15-day social stress protocol, each experimental C57BL/6 male mouse was transported daily to a testing room and spent 1 h

in the home-cage of an aggressive and unfamiliar ICR outbred mouse (Harlan); see below the description of maintaining the ICR males. During the first 5 min of that 1 h exposure the mice were allowed to physically interact. During this time, the ICR mouse attacked the intruder mouse and the intruder displayed subordinate posturing. For the remaining 55 mins, the C57BL/6 experimental mouse was placed in a cage with a metal mesh, which enabled visual, olfactory and auditory interaction but no direct physical contact. After each 1 h stress exposure, the experimental mice were returned to their homecage, containing the same highly familiar mouse partner. In this manuscript, we describe behavioral and physiological response of the experimental mice to social stress, while the behavior of the partner mice is reported in a separate manuscript.

Control male C57BL/6 mice were housed in pairs and handled daily for 15 days.

ICR male mice were housed alone in cages for one week prior to CSDS in order to establish territoriality.

Behavioral assessments

All behavioral assessments were performed during the dark phase following a 2 h habituation to the test room before each test. Behavioral tests were conducted in the following order, from the least stressful procedure to the most stressful: social interaction test (SIT), dark-light transfer (DLT), forced swim test (FST), fear conditioning (FC) and acoustic startle response test (ASR).

SIT

A SIT was conducted based on prior protocols (Berton, McClung, et al. 2006; Krishnan et al. 2007b). Briefly, 24 h following the last social stress session, each experimental mouse (either male or female) was placed in a dimly illuminated open field arena (white plastic box, 50 x 50

x 22 cm, 3-5 lux) and its trajectory was tracked for two consecutive sessions of 3 min each. Video recordings were performed as previously described (Regev et al. 2011). During the first session ("No Target") the open field contained an empty wire mesh cage (circular, 10 cm diameter) located at one end of the field. During the second session ("Target"), the conditions were identical except that a social target animal (an unfamiliar same-sex ICR mouse) was introduced. Between the two sessions, the experimental mouse was removed from the arena, and was placed in a temporary novel cage for approximately 1 min. An automated video-tracking software (Ethovision 9, Noldus) was used to determine the time spent by experimental mice in a defined "interaction zone" (a semi-circle surrounding the mesh) during the "No Target" and "Target" conditions. An interaction ratio was calculated as follows:

$$interaction\ ratio = \frac{time\ spent\ in\ the\ interaction\ zone\ during\ the\ \text{Target}\ session}{time\ spent\ in\ the\ interaction\ zone\ during\ the\ \text{No Target}\ session}$$

DLT test

The DLT test takes advantage of the natural conflict of a rodent between the exploration of a novel environment and the aversive properties of a large, brightly lit open-field (Regev et al. 2011). The DLT test apparatus consists of a polyvinyl chloride box divided into a black dark compartment (14 x 27 x 26 cm) connected to a larger white 1200 lux illuminated light compartment (30 x 27 x 26 cm). Mice were placed in the dark chamber, and behavior was quantified during the 5 min test starting when the door connecting the dark and light chambers was opened. A video tracking system (VideoMot2; TSE Systems) was used to quantify time spent in the light compartment, the distance traveled in light area and number of dark-light transitions.

FST

When forced to swim in a narrow space from which there is no escape, mice adopt an immobile posture after an initial period of vigorous activity, which is considered a behavioral despair reaction. The FST is considered an index of "depressive-like symptoms" and was performed as previously described (Issler et al. 2014). Briefly, mice were placed in Plexiglas cylinders (13 cm diameter × 24 cm high) containing water (22◦C ± 2◦C) to a depth of 15 cm, their activity during 6 minutes was recorded and immobility duration was quantified off-line with an automated video-tracking system (Ethovision 9, Noldus).

FC

FC is a behavioral learning paradigm that allows organisms to learn to predict aversive events, by pairing an aversive event to a neutral stimulus (a foot shock paired with a neutral sounds). Previous studies reported effects of chronic stress on memory formation and retrieval using different conditioning protocols (e.g., Rodrigues et al., 2009; Roozendaal et al., 2009), as well as sex differences in fear memory learning and extinction that were related to anxiety disorders (McDermott et al. 2015). We therefore employed a long-term protocol of FC (Karpova et al. 2011) that allowed us to examine male and female behavior during conditioning, extinction training, and long term fear memory retrieval. A computer-controlled fear-conditioning system (TSE Systems, Bad Homburg, Germany) monitored the procedure while measuring freezing behavior (defined as lack of movement except for respiration for at least 3 s); the measure was expressed as a percentage of time spent freezing. FC and extinction took place in two different contexts: FC context (A) was a transparent Plexiglas chamber cage (21 cm × 20 cm × 36 cm) with metal grids on floor and constant illumination (250 lux) whereas extinction context (B) was a black nontransparent Plexiglas chamber (same size) with planar floor and no illumination except dim red light. Both context A and context B were cleaned before each session with 70

% ethanol and 1 % acetic acid, respectively. On day 1, mice were conditioned using 5 pairings of the conditioned stimulus, CS (CS duration 30 s, 1 Hz, white noise, 80 dB) with the unconditioned stimulus, US (1 s foot-shock 0.6 mA, co-terminated with the CS); inter-trial interval (ITI): randomly varying 20-120 sec. On day 2, conditioned mice were submitted to extinction training in context B during which they received 12 presentations of the CS alone (ITI: randomly varying 30-60 s). On day 10 mice were tested first for spontaneous recovery of fear (Cue test) in context B, and two hours later were tested for context dependent fear renewal (Context test) in context A. Both tests included four presentations of the CS (ITI: randomly varying: 20-60 s).

ASR test

The ASR test reflects a transient motor response to a sudden unexpected stimulus, such as a loud noise. Animals suffering from anxiety and hyperarousal are found to express an enhanced startle response; the startle reaction is potentiated by previous stressful experiences such as footshocks and is attenuated by prepulse inhibition (PPI: Koch 1999). Startle response and PPI protocol were assessed in a Startle Response System (TSE Systems) using a protocol adapted from Neufeld-Cohen et al. (Neufeld-Cohen et al. 2010). Briefly, mice were placed in a small Plexiglas and wire mesh cage on top of a vibration-sensitive platform in a sound-attenuated, ventilated chamber. A high-precision sensor, integrated into the measuring platform, detected movement. Two high-frequency loudspeakers inside the chamber produced all the audio stimuli. The session began with 5 min acclimation to white background noise [65 db] maintained through the whole session. Thirty-two startle stimuli [120 db, 40 ms in duration with a randomly varying ITI of 12–30 ms] were presented interspersed with an additional 40

ms startle stimuli randomly preceded by 40 ms pre-pulses of either 74 db, 78 db, or 82 dB. Latency to peak startle response and response amplitude were measured both in response to startle stimuli and in response to startle stimuli preceded by pre-pulses.

Corticosterone measurement

Plasma was extracted from blood samples that were collected by tail bleed under basal conditions at two time points: 1 h before onset of the active phase (7 AM) and 1 h after offset of the active phase (9 PM). Blood samples were centrifuged immediately (3500 rpm for 25 min at 4°C) and extracted plasma was stored at -80°C until assayed for corticosterone (CORT) using a radioimmunoassay kit (ImmuChemTM Double Antibody Corticosterone ^{125}I RIA KIT, MP biomedicals, NY, USA).

Metabolic assessments

Manual measurement of food intake

CSS and homecage female mice were isolated for 8 days, and chow was weighed once a day in order to assess food intake.

Metabolic cages

Calorimetry, food and water intake, and locomotor activity were measured using the Labmaster system (TSE Systems). The LabMaster instrument consists of a combination of sensitive feeding and drinking sensors for automated online measurement. The calorimetry system is an open-circuit system that determines O2 consumption, CO2 production, and respiratory exchange rate (RER). A photobeam-based activity monitoring system detects and records ambulatory movements, including rearing and climbing, in every cage. All the parameters are measured continuously and simultaneously. Data were collected after 48 h of adaptation in acclimated singly housed mice.

Blood glucose level measurements

Glucose-tolerance tests (GTT) were performed as previously described (Kuperman et al. 2010). Briefly, following 4 h of fasting, glucose (2g/kg of body weight) was injected i.p., and whole venous blood obtained from the tail vein at 0, 15, 30, 60, 90, and 120 min after injection, and was measured for glucose by using an automatic glucometer (One Touch; Lifescan). Glucose levels in fed animals were measured using the same glucometer.

Body composition

Body composition was assessed using Echo-MRI (Echo Medical Systems).

Statistical Methods

Data are expressed as mean ± SEM. As stress protocols were comparable but not identical for male and female mice, we did not employ statistical models directly comparing male and female mice, but compared each stressed group to its control group. All dataset distributions were assessed for normality using the Kolmogorov-Smirnov test to determine which statistical tests should be employed. When comparing normally distributed data, the independent Student's t-test was used; analysis of weight changes, reflecting within-animal comparisons necessary to address individual variability, employed Repeated Measures Analysis of Variance (RM-ANOVA), followed by post-hoc comparisons corrected by Tukey test. When data departed from normality, the Mann-Whitney U test was applied. An alpha level of 0.05 was determined significant.

Results

Changes in body weight

As expected, both CSS paradigms affected body weight (BW) gain. However, female mice were affected for a longer period of time than male mice (Fig. 2 a,b). Stressed female mice (n=27) gained significantly less weight than homecage female mice (n=23) during weeks 2-4 of monitoring (including stress protocol and one week post-stress). RM-ANOVA revealed significant main effects for Time [weeks 2-9] [$F_{(7,336)} = 150.6$; $P < 0.0001$] and for Group [$F_{(1,48)} = 12.47$; $P< 0.001$]; the interaction Time × Group was also significant [$F_{(7,336)} = 5.3$; $p<0.0001$]. Follow-up comparisons were significant for the 3-5th weeks of weight monitoring [week 3: $P< 0.0001$; week 4: $P<0.0001$; week 5: $P<0.0005$; Fig. 2a]. Male stressed mice (n=13) significantly differed from homecage control mice (n=12) during week 3. RM-ANOVA indicated significant main effects for Time [weeks 2-9] [$F_{(7,161)} = 147.6$; $P < 0.0001$] but not of Group [$F_{(1,23)} = 1.96$; $P=0.17$]; the interaction Time × Group was significant [$F_{(7,161)} = 7.54$; $p<0.0001$] and follow-up comparisons indicated a difference only during the 3rd week of weight monitoring [week 3: $P<0.005$, Fig. 2 b].

At the end of the experiment (after ASR, some 2 months after CSS), we conducted explorative metabolic analysis of female mice, described in Supplementary Fig. 3. At this time point there was no difference in BW between CSS and homecage female mice (Fig S3F), which limits the ability of this dataset to provide evidence on mechanisms at work. Briefly, In order to test food intake, we isolated CSS and homecage female mice for 8 days and we weighed once a day the chow in order to assess food intake. We found no differences between groups (Fig. S3A). At a later timepoint, female CSS and homecage mice were tested in metabolic cages using the Labmaster system (TSE Systems). Data were collected after 48 h of adaptation in acclimated singly housed mice (Kuperman et al. 2010) . We found no differences between groups in food intake (Fig. S3B) or water intake (Fig. S3c); no difference in energy expenditure (Fig. S3d,e), RER (Fig. S3f), body weight (Fig. S3g), or mobility (Fig. S3h). Next we measured body composition, but no difference was found in lean mass (Fig. S3i) or fat mass (Fig. S3j). Finally,

we measure blood glucose in fed animals, where we found significantly reduced glucose level in CSS animals (unpaired two-tailed t- test, $t(23)=2.39$, $p<0.05$, Fig. S3K). Follow up analysis using the Glucose Tolerance Test did not reveal differences between groups (Fig. S3L)

SIT

It was previously shown that CSDS causes long-term reduction in social interaction, measured 24 hr after the last stress session (Tsankova et al. 2006; Berton, McClung, et al. 2006). During SIT, most control mice spent more time interacting with a social target than with an empty mesh. Therefore, we compared the interaction ratio between mice undergoing CSS (StressF, n=26; StressM, n=22) and the control group (HomeF, n=22; HomeM, n=10) and found significantly reduced interaction ratio 24 h after the last stress session in stressed mice of both sexes (Mann-Whitney U test, females $U=161$; $P<0.01$, males $U=13$; $P<0.0001$, Fig. 2c,d). An additional Chi-square test was performed on the proportion of mice categorized as "susceptible" to the stress protocol (mice with interaction scores <1), or "unsusceptible" to stress (mice with interaction scores >1). In male mice, the proportion of susceptible mice was significantly higher in the stressed vs. the control group ($X^2(1,N=32)=12.37$, $p<0.0005$) and the vast majority of stressed mice (21 out of 22) were categorized as susceptible, as opposed to 50% in standard CSDS protocols (Krishnan et al. 2007b). In female mice, there was no difference between ratio of susceptible stressed vs. control mice ($X^2(1,N=48)=0.01$, N.S.) and most stressed mice (25 out of 26) had an interaction ratio higher than 1 (Fig. 2c).

DLT test

The DLT test took place one week after the last social stress session, in order to measure long term effects of CSS on anxiety and neophobia. We were surprised to find that stressed female mice spent more time in the light [$t_{(49)} = 2.16$; $P<0.05$, Fig. 3a], traveled greater distance in the

light [$t_{(49)}$ = 4.26; P<0.0001, Fig. 3c], made more dark-light crossings [$t_{(49)}$ = 3.53; P<0.001, Fig. 3e] and entered the light more readily [Mann-Whitney U = 182.5; P<0.01, Fig. 3g], than female control mice. There were no significant differences between CSS male mice and their respective control group [time in light: $t_{(33)}$ = 0.75; P=0.45, Fig. 3b; distance in light: $t_{(33)}$ = 0.8; P=0.42, Fig. 3d; dark-light crossing: $t_{(32)}$ = 1.05; P=0.29, Fig. 3f; time to enter light: Mann-Whitney U = 90; P=0.24, Fig. 3h]. However, stressed females (Fig.3i), but not males (Fig.3j), exhibited increased average velocity during the test relative to control group mice (females: $t_{(49)}$ = 4.9; P<0.0001; males: $t_{(33)}$ = 1.35; P=0.18).

FST

In order to measure long term effects of CSS on depressive like symptoms, we tested the mice in the FST 3-4 weeks after the end of the stress protocol. No differences in total immobility time were observed between the CSS and control groups in either female or male mice (females: Mann-Whitney U = 232; P=0.26, Fig. S1c; males: $t_{(23)}$ = 0.44; P=0.44, Fig. S1d). Similarly, no differences were found in latency to first immobility session in chronically stressed groups relative to their controls (females: Mann-Whitney U = 270; P=0.74, Fig. S1a; males: $t_{(23)}$ = 0.88; P=0.38, Fig S1b).

Fear conditioning

No differences in freezing duration were observed between stress and control groups during fear conditioning and extinction (Supp. Table1). However, in the cue test, that assessed spontaneous recovery of fear, stressed males, but not stressed females, exhibited increased freezing relative to their control group (male: $t_{(21)}$=2.09; P<0.05; female: $t_{(48)}$=0.53; NS; Fig. 5b,c). Specifically, stressed male mice showed more freezing than controls upon initial entry into the experimental chamber, before hearing any tones, and also during later presentations of tones (Fig. 5c right panel). There were no differences in the context test that took place 2 h

after the cue test, and similar freezing levels were observed between stressed and control groups, both female and male (Fig. 5 d,e, Supp. Table 1).

Acoustic startle response test

We used the ASR test as a measure of long-term anxiety following CSS. We did not find significant differences between stressed and control groups, in neither male nor female mice, in any of the examined indices (see supplementary information and Fig. S2).

Corticosterone

HPA axis activity was assessed by measuring basal CORT levels two months after the last stress session. Only male mice showed a significant alteration in HPA activity following CSS: RM-ANOVA indicated significant main effect of group [$F_{(1,22)}$ = 5.18; P<0.05], significant main effect of sample time [$F_{(1,22)}$ = 46.03; P<0.0001] but non-significant interaction [$F_{(1,22)}$ = 1.37; P=0.25]. CSS mice Female mice showed a significant main effect of sample time [$F_{(1,22)}$ = 48.79; p<0.0001] but there was no significant group effect [$F_{(1,22)}$ = 0.67; P=0.41] or interaction [$F_{(1,22)}$ = 0.4; P=0.52].

Discussion

In this study, we investigated the long-term effects of CSS in female and male mice exposed to a sex-specific ethologically valid stress protocol. A battery of behavioral tests were administered over a relatively long-time period, eight weeks following CSS, examining social interaction, anxiety and depression, learning and memory as well as the neuroendocrine profile. We found that female mice are more prone to BW loss during CSS and to hyperactive anxious behavior following CSS, both sexes show disturbances in social interaction, but only male mice

show long-term changes in neuroendocrine function and in memory performance after fear conditioning.

We avoided using an identical stressor for both sexes for several reasons: I) ~~as~~ previous studies demonstrate sex-specific vulnerability to different social stressors (Haller et al. 1999; Kessler and Mcleod 1984), II) there is an emerging lack of consensus regarding the ecological validity of CSDS in female lab-reared rodents (e.g., males are chemo-genetically manipulated to display aggressive behavior towards females (Takahashi et al. 2017), III) Many researchers avoid exploring sex differences related to social stress due to a lack of protocols for female rodents (Palanza 2001; Schmidt et al. 2010; Beery and Zucker 2011; Zilkh ethologically-minded a et al. 2016).

Our aim was to augment existing CSS research protocols in female mice, in order to promote exploration of sex differences in causes, effects and treatment of stress-related disorders. We therefore developed a protocol that exposed female mice to a modified crowding /social instability stress that created mild aggression between females without necessitating manipulations such as brain lesions (Haller et al. 1999), which make comparisons to males problematic. Furthermore, we modified the CSDS protocol for males accordingly, to control for experimental parameters such as age, housing conditions, social nature of stressor, stress duration, and physical characteristics during stress exposure.

We maintained males and females under stable-paired housing conditions using a highly familiar same-sex partner throughout the experiment, as previous research has shown that social isolation is a potent stressor in rats (Weiss et al. 2004; Hatch et al. 1963); that group housing increases trait variability in mice (Prendergast, Onishi, and Zucker 2014); and that social instability is a potent social stressor in mice (Schmidt et al. 2007). On the other hand, social support and attachment are a major individual resilience factor ~~for~~ against development

of anxiety in rats (Stranahan, Khalil, and Gould 2006; Isovich et al. 2001). We therefore designed housing conditions to preclude additional stress and to promote optimal coping with CSS. We did not monitor estrous cycle in female mice as it is an additional source of stress. While it may be argued that variability in female behavioral and physiological data (e.g., in CORT measurement, Fig. 5) might be explained by estrous cycle stages, recent studies and meta-analyses of mice and rats suggest males and females show similar variability (Smarr et al. 2017; Prendergast, Onishi, and Zucker 2014).

A known consequence of chronic stress is dysregulation of feeding habits and metabolism (Harris 2015; Andrews and Abizaid 2014). When single-sex CSDS studies were conducted, male mice and rats showed a reduction in BW gain (Krishnan et al. 2007a; Becker et al. 2008), as did female rats intruding on lactating female rats (Shimamoto et al. 2011) and female mice subjected to male mice aggression produced by brain manipulation (Takahashi et al. 2017). Two studies comparing male and female rats undergoing CSDS found BW reduction only in males (Page, Opp, and Kozachik 2016; Haller et al. 1999). We found BW reduction in both male and female mice (Fig. 2 a,b), with more pronounced and enduring effects in females and only short-term effects in males. These findings validate the stressful nature of the current CSS protocols in both sexes, even when stress exposure was limited to one hour per day. Preliminary profiling of metabolic function in female CSS and control mice at the end of study (after ASR) found no difference in food intake, activity or energy expenditure (Sup Fig. 3). Elucidation of molecular mechanisms underlying CSS effects on BW and possible sex differences awaits further investigation.

The emotional response to CSS is variable and may range between resilience and susceptibility, depending on a complex interaction between genetic and environmental elements (Nestler et al. 2002). CSDS in male mice was used successfully to describe molecular and circuit-level differences between resilient and susceptible mice, based on a behavioral phenotype of reduced

social exploratory behavior, assessed by SIT (Elliott et al. 2010; Krishnan et al. 2007a). In order to assess the effects of CSS on social behavior, we employed the SIT one day after the end of CSS in both female and male mice and found a significant reduction in social interaction in both sexes (Fig. 2 c,d). When applying the published cutoff between susceptible and resilient mice at an interaction ratio of 1, we find that proportion of susceptible male mice in our modified CSDS protocol was much higher than the published 50% (Krishnan et al. 2007a). This would suggest that even 1-hour long exposure to CSDS, as opposed to 24-hour exposure in other protocols, is an extremely efficient stressor for male mice. It seems that interaction ratio analysis that employs a cutoff at 1 is not appropriate to differentiate susceptible vs. resilient females, emphasizing the need to base analyses of female behaviors on empirical data obtained from female and not male subjects. Research into factors affecting resilience to stress would benefit from inclusion of females (Russo et al. 2012) as response profiles to chronic stress vary between the sexes (Beck and Luine 2002).

In order to test anxiety, we used the DLT test, a test calibrated on male rodents, where decreased activity in the light chamber is considered a marker of anxiety. We found that in several parameters, chronically stressed female mice exhibited significantly *increased* activity in the lit section, including an *increase* in velocity. Findings of increased locomotor activity and increased exploration by female rodents in anxiety tests like DLT, elevated plus maze (EPM) and open field (OF) were reported, either at baseline without previous stress exposure (Fernandes et al. 1999; An et al. 2011; Archer 1975; Johnston and File 1991), after non-social stress like restraint (R. E. Bowman et al. 2009) or inescapable shocks (Steenbergen et al. 1990). Adrenalectomy and CORT replacement did not change these findings (Kokras et al. 2012), suggesting that sex differences go beyond the baseline differences in physiology of the stress response and HPA axis function.

Several authors have suggested the motivation of male and female rodents in behavioral tests of anxiety is different, with males being driven by anxiety and females driven by activity (An et al. 2011; Fernandes et al. 1999). Archer (1975) has argued that behavioral sex differences in anxiety tests showcase the different form that fearful reaction takes in different sexes, namely that males tend to become immobile and females tend to actively attempt to escape (Archer 1975). Extending these suggestions to the current DLT findings implies that CSS female mice are more anxious than control female mice, despite presenting a behavioral pattern similar to stress-resilient male mice. CSS male mice did not differ from control male mice in this test.

We did not detect CSS related depression-like behaviors in the FST or hyperarousal and anxiety-related behaviors in the ASR or PPI. One possible reason is that FST and ASR provide behavioral readout that is not sensitive enough to detect long-term changes in behavior following CSS. It is likely that subtle long-term effects may be revealed only when readout extends to physiological measures such as heart rate (Page, Opp, and Kozachik 2016). Alternatively, it may be that detection of long-term effects following CSS requires anxiety-provoking social circumstances, while these tests were of a non-social nature. It is less likely that the lack of effects stems from "recovering from the CSS" over time since CSS-related long-term changes were found in females (metabolic profile, as discussed above) and males (emotional memory and neuroendocrine function, as discussed below).

Learning and memory processes are affected by stress in complex ways, depending on stressor duration and intensity (acute stressors differ from chronic ones), the learning material (emotional vs. non-emotional items), the learning stage (consolidation vs. retrieval of memory), and the subjects' age and sex (Sandi and Pinelo-Nava 2007). For example, non-social chronic stress in rats (6 h restraint stress a day, over 21 days) was found to affect several types of memory in a sex-specific manner, with males showing impairments in object recognition and spatial memory while females reveal enhanced performance or no impairment (Luine 2002;

Bowman, Beck, and Luine 2003; Andreano and Cahill 2009; Bowman et al. 2009). Given this complexity, we focused on the effects of chronic social stress on emotional learning.

We investigated the effects of CSS on emotional memory in male and female mice using a protocol assessing long-term fear conditioning (FC) memory (Tsankova et al. 2006). Emotional memory in general, and FC memory in particular, rely on functioning and plasticity of the amygdala, hippocampus and prefrontal cortex – brain structures known to be affected by stress, both acute and chronic (Roozendaal, McEwen, and Chattarji 2009). In addition, retrieval of FC memory relies on different brain structures depending on the type of retrieval: amygdala activity underlies cue memory while hippocampal activity underlies context memory (Phillips and LeDoux 1992). Furthermore, studies employing different methodologies in humans and rodents reported sex-dependent activity in the amygdala and hippocampus during different FC memory stages (Stark et al. 2006; Maren, De Oca, and Fanselow 1994; Andreano and Cahill 2009). Sex-hormone dependent differences were reported in emotional memory, with males exhibiting enhanced FC memory acquisition and context-triggered retrieval, while females exhibited enhanced extinction, (Milad et al. 2010; Chang et al. 2009; McDermott et al. 2015). Finally, social isolation stress was reported to affect FC learning and memory male mice and rats (Pibiri et al. 2008; Pugh et al. 1999). Corresponding sex-dependent effects of social stress on emotional memory were noted among healthy human subjects (Jackson et al. 2006).

Surprisingly, we did not find stress- or sex-dependent differences during FC memory acquisition or extinction (Table S1). However, male, but not female, CSS mice, exhibited enhanced freezing during cue-triggered memory retrieval (hearing CS in a context different from the conditioning context) but not during context-triggered retrieval (Fig. 4). This finding suggests that future studies using the CSS protocols should focus on neurocircuitry and neurochemistry of the amygdala. Relevant research questions should pertain to long-term CSS effects on long-term emotional memory, why females seem to be resilient to enhanced retrieval

compared to males, with special emphasis in assessing social factors (e.g., housing conditions, social parameters during memory retrieval) and their effects at different times after emotional memory formation.

One of the explanations for sex differences in susceptibility to stress-induced affective disorders such as depression and anxiety is that females show heightened sensitivity to stress at multiple levels of HPA axis function (Bangasser and Valentino 2014). Sex-related differences were also noted in response to manipulations of social factors; isolated female rats have higher CORT levels than crowded or group housed female rats whereas male rats show an opposite pattern (Brown and Grunberg 1995). In Californian mice, a unique monogamous species that undergoes CSDS, females, but not males, show higher CORT levels than controls immediately after defeat (Trainor et al. 2010). However, 4 weeks after the defeat, males show higher CORT levels than controls but females do not (Trainor et al. 2011). These sex-dependent effects of CSDS on HPA axis activity were related to function of sex hormones, as gonadectomy performed before CSDS had opposite effects on the sexes (Trainor et al. 2013).

When we assessed baseline CORT levels eight weeks after CSS, we found a decrease in CORT levels in males but not females (Fig. 5). This finding corresponds with the long-term male-specific CORT differences after CSDS in Californian mice (Trainor et al. 2011). However, we found decreased, and not increased, CORT levels in CSS males. The difference may stem from variation in stress resilience that were not assessed in the Californian mice. When examining male mice 8 weeks after social defeat, Krishnan et al. found a decrease in AM CORT levels in susceptible mice but an increase in unsusceptible mice (Krishnan et al. 2007a). Our SIT revealed that almost all CSS male mice were susceptible to the protocol (interaction ratio < 1, Fig. 2d). Resilience assessment in female mice likely requires a different cutoff point and future studies are encouraged to strengthen this finding and further examine sex-specific differences in stress-related CORT levels, e.g., after FST or restraint challenges.

To conclude, this study reported the short- and long-term effects of differential chronic social stress protocols in male and female mice. We report a complex array of effects on social, anxiety-like and depressive-like behaviors, emotional memories and endocrine stress-response mechanisms. Collectively, the data suggest a need for a more nuanced application of sex-relevant manipulations and assessments in preclinical studies, just as they are applied in contemporary clinical trials.

Finally, an individuals' genetic and epigenetic background has an important role in determining their reaction to stressful environmental conditions (Schmidt, Sterlemann, and Müller 2008), and as our data indicate, there is wide phenotypic variability even among genetically identical lab-reared mice (e.g., Fig.4, Fig. S2). The CSS protocols described here could aid researchers in investigating molecular and cellular mechanisms of resilience and vulnerability to CSS in a sex-appropriate manner. Our protocols can be combined with established early-life perturbations such as maternal separation or trauma exposure during adolescence. Hopefully, such combined methods will enable a better understanding of the mechanisms underlying the sex-specific susceptibility to stress-related psychopathologies in adulthood. Combining physiological readout methods as suggested above with pharmacological therapeutic interventions, such as SSRI administration after CSS, would allow investigation of sex-appropriate treatment of psychopathologies. This could create empirical support for sex-specific, and perhaps individual-specific treatment regiments, that will promote the wellbeing of patients that are currently suffering as non-responders to existing treatment.

Acknowledgments

We thank Yael Kuperman, Mariana Schroeder, Ilana Adler and Maya Lebow for their contribution to this work via insightful discussions and technical expertise. A.C. is the incumbent of the Vera and John Schwartz Family Professorial Chair in Neurobiology at the

Weizmann Institute of Science and the head of the Max Planck Society–Weizmann Institute of Science Laboratory for Experimental Neuropsychiatry and Behavioral Neurogenetics. M.T. is the incumbent of the Carolito Stiftung Research Fellow Chair in Neurodegenerative Diseases. This work is supported by: an FP7 Grant from the European Research Council (260463, A.C.); a research grant from the Israel Science Foundation (1565/15, A.C.); the ERANET Program, supported by the Chief Scientist Office of the Israeli Ministry of Health (3-11389, A.C.); the project was funded by the Federal Ministry of Education and Research under the funding code 01KU1501A (A.C.); I-CORE Program of the Planning and Budgeting Committee and The Israel Science Foundation (grant no. 1916/12 to A.C.); Ruhman Family Laboratory for Research in the Neurobiology of Stress (A.C.); research support from Bruno and Simone Licht; the Perlman Family Foundation, founded by Louis L. and Anita M. Perlman (A.C.); the Adelis Foundation (A.C.); and Sonia T. Marschak (A.C.).

Abbreviations

ASR – acoustic startle response

BW – body weight

CORT - corticosterone

CS – conditioned stimulus

CSDS – chronic social defeat stress

CSS – chronic social stress

DLT – dark light transfer test

EPM – elevated plus maze

FC – fear conditioning

FST – forced swim test

GTT – glucose tolerance test

HPA – hypothalamic pituitary adrenal

ITI – inter trial interval

OF – open field

PPI – pre pulse inhibition

RER – respiratory exchange rate

RM-ANOVA – repeated measured analysis of variance

SIT –social interaction test

Competing Interests

The authors declare no conflict of interests.

Author Contributions

O.F. conceived and designed the experiments, collected the data, performed data interpretation and analysis, drafted the paper and approved the final version of the paper to be published. M.T. assisted in collecting the data, data interpretation and analysis, provided critical revision of the paper and approved the final version of the paper to be published. A.C. provided guidance in conceiving and designing the experiments, provided critical revision of the paper and approved the final version of the paper to be published.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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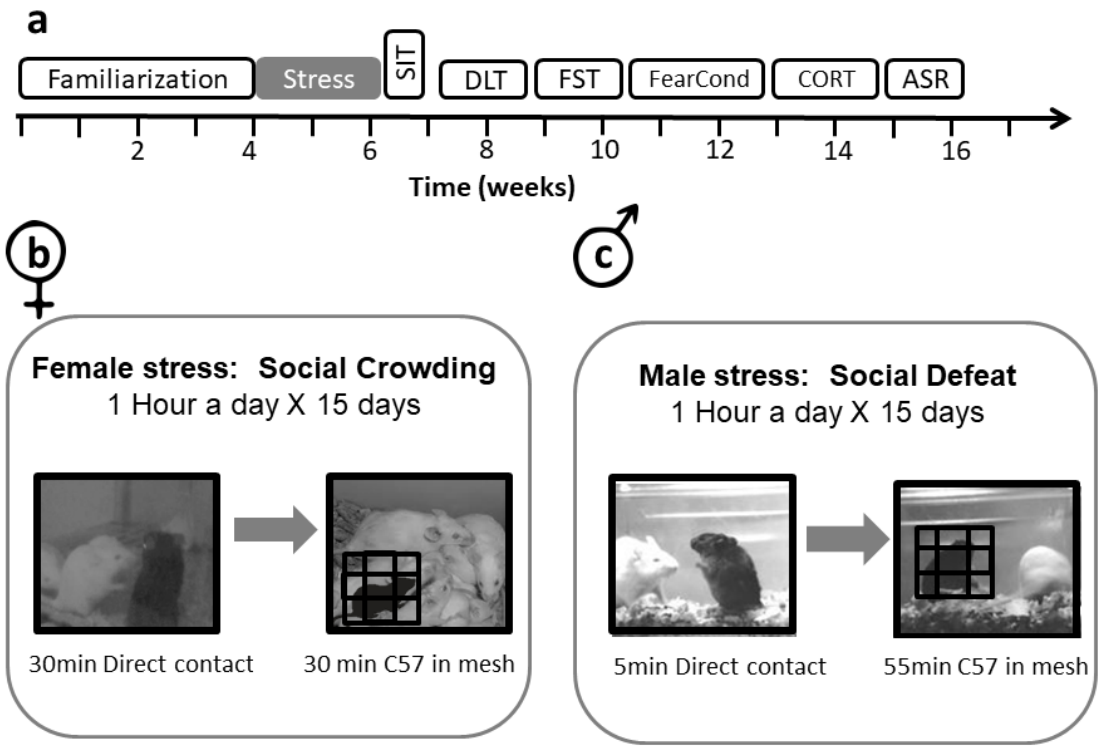

Fig. 1. Chronic Social Stress protocols for male and female mice. (a) Timeline of the experimental design. **(b)** Female C57/BL black mice were exposed to social crowding stress for one hour a day over 15 days, with groups of different female white ICR mice. **(c)** Male C57/BL black mice were exposed to social defeat stress for one hour a day over 15 days, with different male white ICR mice (see *Methods* for more details). SIT – Social interaction test; DLT – Dark light transfer test; FST – Forced swim test; FearCond – Fear conditioning; CORT – Corticosterone; ASR – Acoustic startle response.

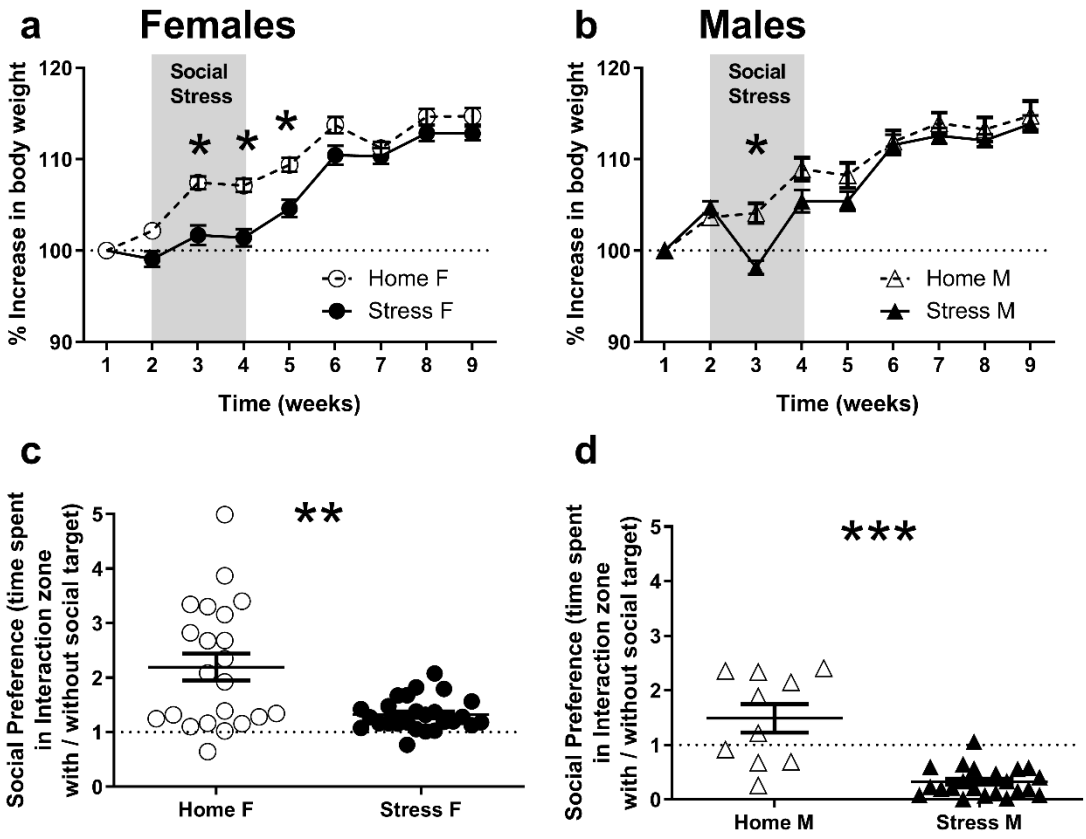

Fig. 2. Chronic social stress affected weight gain and social interaction with novel mice in both male and female mice. **(a)** Female chronically stressed mice (Stress F, n=27) gained less weight than homecage control mice (Home F, n=23) during the 3rd-5th weeks of weight monitoring [significant main effect for Time ($p < 0.0001$), Group ($p < 0.001$) and their interaction ($p < 0.0001$); week 3: $p < 0.0001$; week 4: $p < 0.0001$; week 5: $p < 0.0005$]. (b) Male chronically stressed mice (Stress M, n=13) gained less weight than homecage control mice (Home M, n=12) but only during stress [significant main effects for Time ($p < 0.0001$) but not for Group ($p = 0.17$); interaction was significant ($p < 0.0001$); week 3: $p < 0.005$]. **(c)** During the social interaction test (SIT), performed one day after end of the stress protocol, chronically stressed female mice (Stress F, n=26) show a significantly lower interaction ratio compared to female homecage control mice (Home F, n=22). Home F mice, but not Stress F mice, spend more time near the wire mesh when the other side contains a novel mouse vs. when it is empty (Mann-Whitney U test, $P<0.01$). Dotted line indicates equal preference. **(d)** Similarly, during SIT male chronically stressed mice (Stress M, n=22) show significantly reduced interaction ratio compared to male homecage control mice (Home M, n=10) (Mann-Whitney U test, $P<0.0001$. Error bars represent SEM.

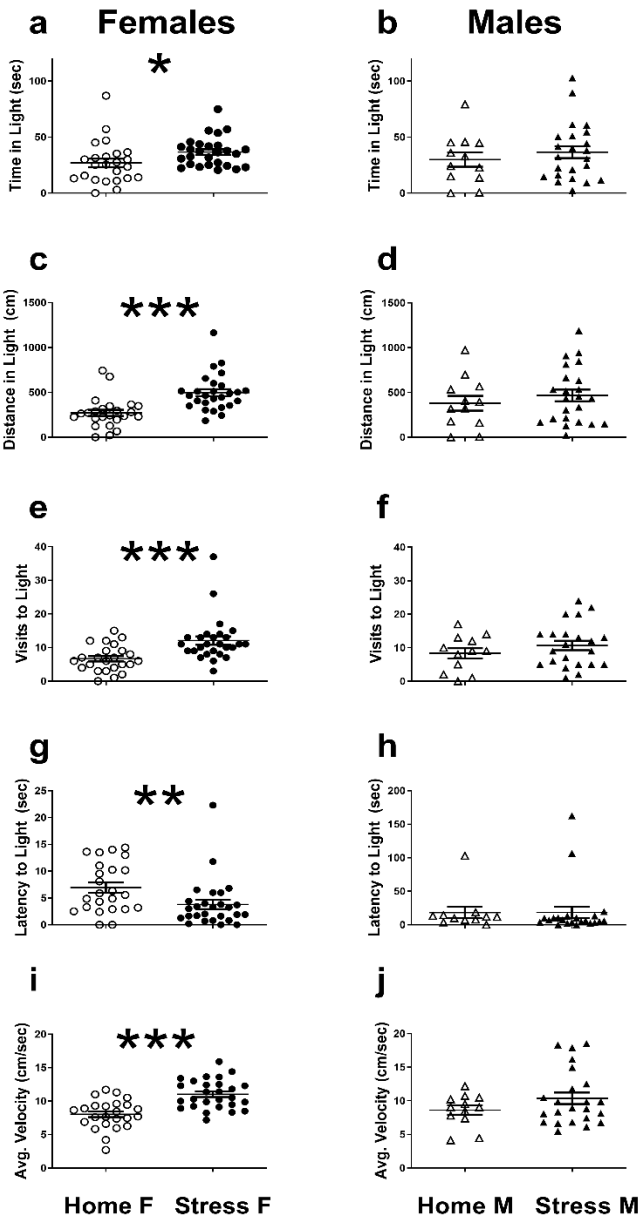

Fig. 3. Chronic social stress affected performance in Dark Light Transfer (DLT) test of anxiety in female but not male stressed mice. Chronically stressed female mice (Stress F, n=27) spent significantly more time in light area **(a)**, traveled longer distance **(c)**, made more visits to light area **(e)**, were quicker to enter the light area **(g)** and traveled faster **(i)** than homecage control female mice (HC F, n=24). There were no differences in any of these measures **(b,d,f,h,j)** between chronically stressed male mice (Stress M, n=23) and homecage control mice (HC M, n=12). Error bars represent SEM, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

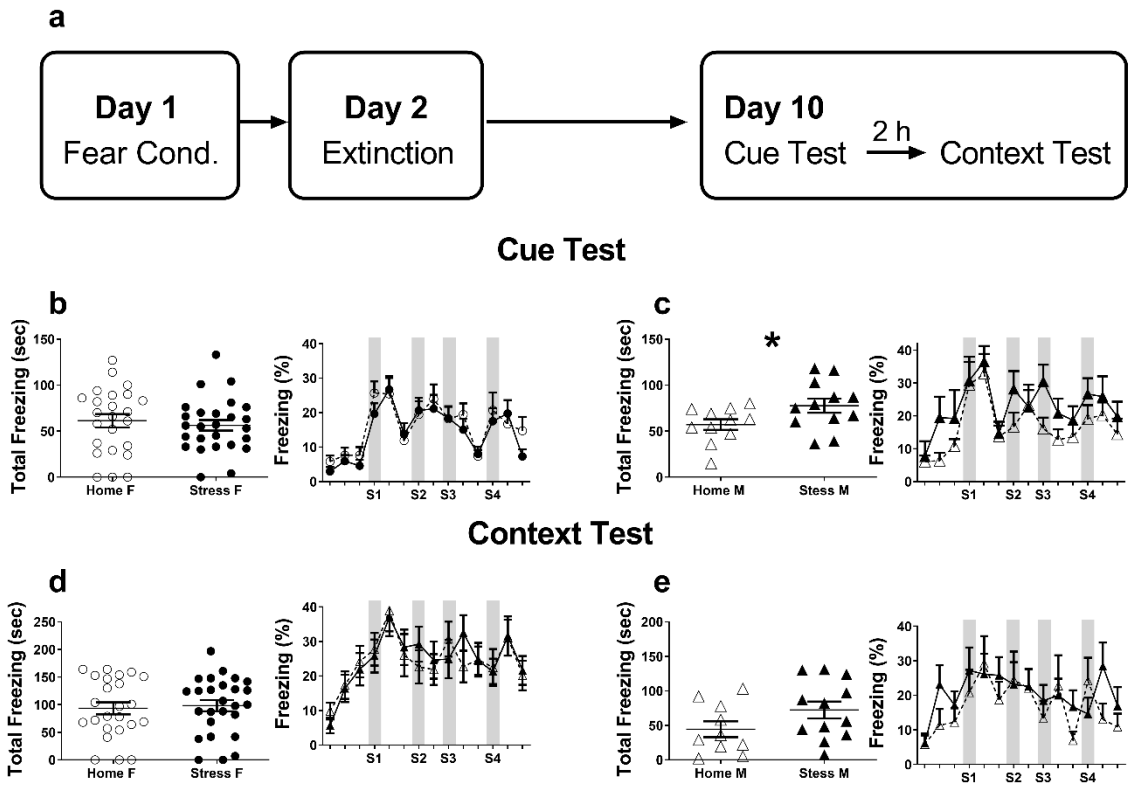

Fig. 4. Chronically stressed male, but not female, mice show enhanced spontaneous recovery of fear 10 days after conditioning. (a) Timeline of fear conditioning protocol. See *Methods* for details. Panels **b-e** present behavior during 5-min cue and context tests, both administered in succession, 10 days after fear conditioning. Behavior is presented as total freezing time (left panel) and as %freezing per 30-sec bins (right panel, gray squares indicate timing of sound reminders). During the cue test, male stressed mice (**c**, full triangles on left panel, full lines on right panel, Stress M; n=12) showed significantly more freezing than homecage control mice (**c**, empty triangles on left panel, dashed lines on right panel, Home M; n=11). There was no difference in freezing during the cue test between female stressed mice (**b**, full circles on left panel, full lines on right panel, Stress F; n=26) and homecage control mice (**b**, empty circles on left panel, dashed lines on right panel, Home F; n=24). During the context test there were no differences in freezing between chronically stressed and control female **(d)** or male **(e)** mice. Error bars represent SEM, * $p < 0.05$.

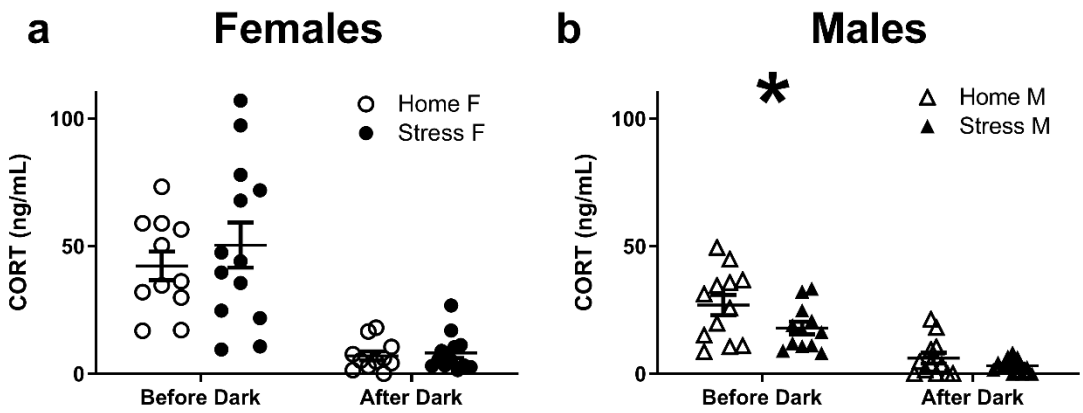

Fig. 5. Plasma corticosterone levels were lower in male but not female stressed mice 2 months after chronic stress. Samples from tail blood were collected under basal conditions, one hour before onset of active phase (Before Dark, 7am) and one hour after the end of the active phase (After Dark, 9pm) in male and female mice. **(a)** Female mice (Home F, n=11; Stress F, n=13) showed a trend for higher CORT levels Before Dark that did not reach significance [significant main effect of sample time ($p<0.0001$) but non-significant group effect or interaction]. **(b)** Chronically stressed male mice (Stress M, n=12) showed a significantly lower CORT levels than homecage controls (Home M, n=12) [significant main effect of group ($p < 0.05$), significant main effect of sample time ($p < 0.0001$) and non-significant interaction]. Error bars represent SEM.

Differential chronic social stress models in male and female mice

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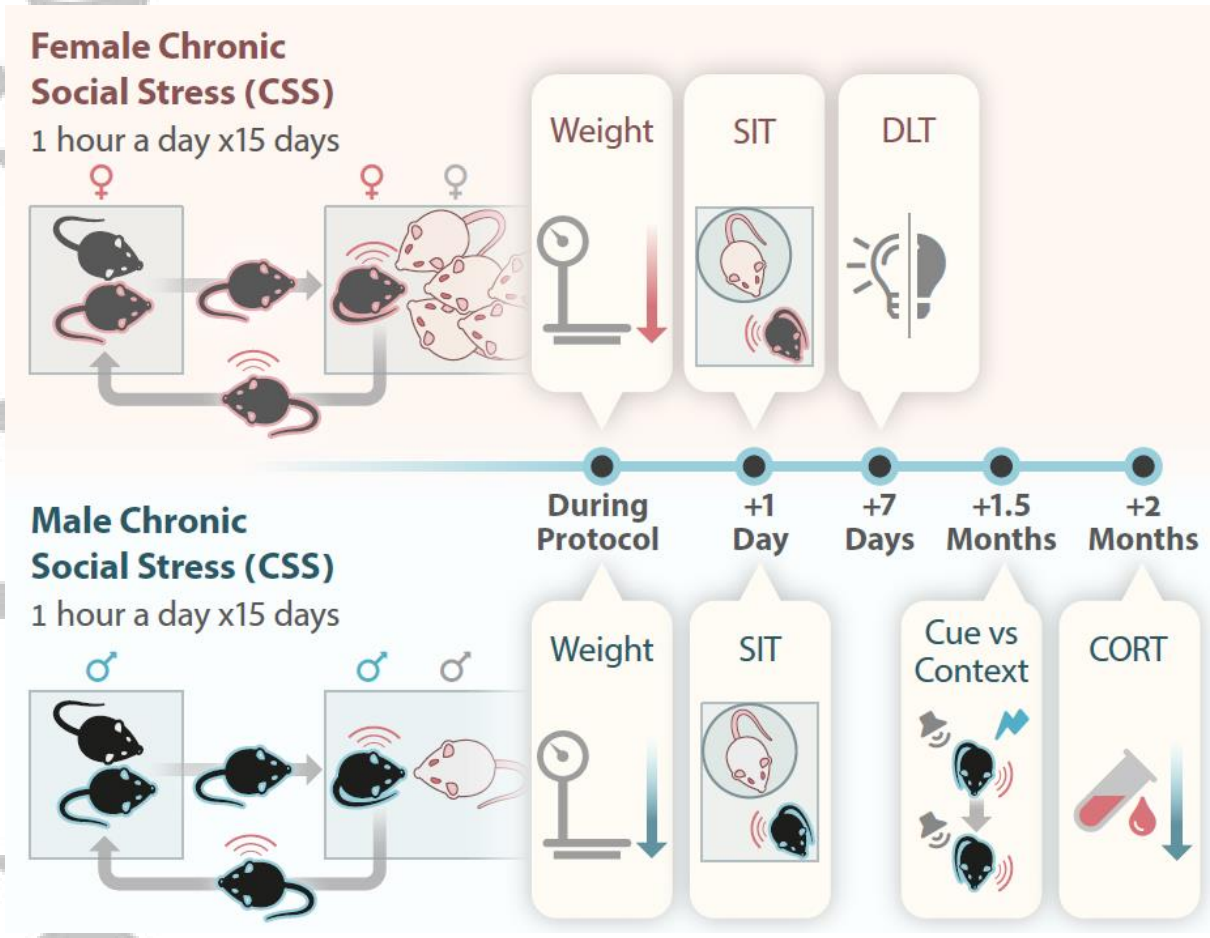

Furman and colleagues describe a novel female mouse model of chronic social stress (CSS) with a parallel male mouse model. These models reveal short- and long-term effects, demonstrating sex-related differential effects. Females present more prolonged body weight reduction during CSS and hyperactive anxious behavior following CSS. While both sexes manifest reduced social interactions, only stressed males exhibit long-term changes in emotional memories and neuroendocrine functions.