Journal of Visualized Experiments

Using a murine model of psychosocial stress in pregnancy as a translationally relevant paradigm for psychiatric disorders in mothers and infants

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62464R1
Full Title:	Using a murine model of psychosocial stress in pregnancy as a translationally relevant paradigm for psychiatric disorders in mothers and infants
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TITLE:

Using a Murine Model of Psychosocial Stress in Pregnancy as a Translationally Relevant Paradigm for Psychiatric Disorders in Mothers and Infants

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KEYWORDS:

chronic psychosocial stress, pregnancy, behavior, neuroendocrine function, perinatal mood, and anxiety disorders

SUMMARY:

The chronic psychosocial stress (CGS) paradigm employs clinically relevant stressors during pregnancy in mice to model psychiatric disorders of mothers and infants. Here, we provide a step-by-step procedure of applying the CGS paradigm and downstream assessments to validate this model.

ABSTRACT:

The peripartum period is considered a sensitive period where adverse maternal exposures can result in long-term negative consequences for both mother and offspring, including the development of neuropsychiatric disorders. Risk factors linked to the emergence of affective dysregulation in the maternal-infant dyad have been extensively studied. Exposure to psychosocial stress during pregnancy has consistently emerged as one of the strongest predictors. Several rodent models have been created to explore this association; however, these models rely on the use of physical stressors or a limited number of psychosocial stressors presented in a repetitive fashion, which do not accurately capture the type, intensity, and frequency of stressors experienced by women. To overcome these limitations, a chronic psychosocial stress (CGS) paradigm was generated that employs various psychosocial insults of

different intensity presented in an unpredictable fashion. The manuscript describes this novel CGS paradigm where pregnant female mice, from gestational day 6.5 to 17.5, are exposed to various stressors during the day and overnight. Day stressors, two per day separated by a 2 h break, range from exposure to foreign objects or predator odor to frequent changes in bedding, removal of bedding, and cage tilting. Overnight stressors include continuous light exposure, changing cage mates, or wetting bedding. We have previously shown that exposure to CGS results in the development of maternal neuroendocrine and behavioral abnormalities, including increased stress reactivity, the emergence of fragmented maternal care patterns, anhedonia, and anxiety-related behaviors, core features of women suffering from perinatal mood and anxiety disorders. This CGS model, therefore, becomes a unique tool that can be used to elucidate molecular defects underlying maternal affective dysregulation, as well as trans-placental mechanisms that impact fetal neurodevelopment and result in negative long-term behavioral consequences in the offspring.

INTRODUCTION:

The mechanisms underlying increased susceptibility to neuropsychiatric disorders in mothers and infants following adverse maternal exposures in the peripartum period remain largely unknown. Substantial maternal physiological alterations occur during pregnancy and the transition to the postpartum period, including several neuroendocrine adaptations that are hypothesized to be critical not only for healthy offspring neurodevelopment but also for preserving maternal mental health[1,2]. At the level of the maternal hypothalamic pituitary adrenal (HPA) axis, adaptions in both circadian and stress-induced levels of glucocorticoid release are observed, including a more flattened rhythm of diurnal HPA axis activity and dampened HPA axis response to acute stressors[3–5]. Given that enhanced HPA axis activity is reported in a subset of women with postpartum affective dysregulation, including increased levels of circulating glucocorticoids and inhibited negative feedback[6–8], exposure to stressors that result in increased postpartum stress reactivity and prevent maternal HPA axis adaptions are thought to increase susceptibility to neuropsychiatric disorders.

To elucidate the effects of stress on affective dysregulation in mothers and infants, several rodent models of stress in the peripartum period have been generated. A majority of these models are characterized by the application of physical stressors that result in homeostatic challenges and alterations in dam physiological status[9], such as chronic restraint stress[10] and swim stress during gestation[11], or postpartum shock exposure[12]. Although these paradigms have been shown to result in the emergence of postpartum depressive-like behaviors and alterations in maternal care[10–12], they have been limited by their inability to accurately capture the psychosocial nature of stressors commonly experienced by human mothers. This becomes particularly important when attempting to reveal the neuroendocrine consequences of chronic stress in the peripartum period, given that processing of different types of stressors is thought to be mediated by varying neural networks orchestrating HPA axis activation[9].

In order to overcome this limitation, several groups have designed stress paradigms employing psychosocial insults or a combination of physical and psychosocial stressors. The maternal separation model, where dams are separated from her pups for several hours per day during the

postpartum period[13,14], and the chronic social stress model, where the dams are exposed to a male intruder in the presence of their litters[15,16], have been able to reproduce the emergence of abnormalities in maternal care and depressive-like phenotypes associated with physical stress paradigms. The chronic ultramild stress paradigm, where pregnant female mice are exposed to a variety of psychosocial insults, including cage tilt and overnight illumination, as well as substantial physiological insults, such as restraint stress and food restriction, has further revealed exposure to a mixed nature of stressors results in abnormalities in maternal behavior, including impairments in maternal aggression, as well as dysregulation in the circadian activity of the HPA axis[17,18]. Consistent with these results, an alternating restraint stress and overcrowding model during gestation results in elevations in postpartum maternal circadian corticosterone levels as well as alterations in maternal care, although no differences are observed in HPA axis re-activity following postpartum exposure to novel acute insults[1].

An expansion of this work, generating a gestational stress paradigm that employs multiple psychosocial insults presented in an unpredictable fashion and minimizes the use of physiological stressors. Studies have previously shown this chronic psychosocial stress paradigm (CGS) results in the development of maternal HPA axis dysfunction, including enhanced stress reactivity in the early postpartum period[19]. These changes are associated with abnormalities in maternal behavior, including alterations in the quality of maternal care received by pups, and the emergence of anhedonic and anxiety-like behaviors[19], features consistent with perinatal mood and anxiety disorders[20,21]. Furthermore, offspring weight gain reduces during the postnatal period following in-utero exposure to CGS[19], suggesting CGS may have persistent negative programming effects in future generations.

The goal in developing the CGS paradigm was to primarily utilize clinically relevant stressors, which accurately capture the type, intensity, and frequency of insults often associated with neuroendocrine dysregulation and the development of perinatal mood and anxiety disorders. Here, the study provides a detailed protocol of how to subject pregnant female mice to CGS, as well as downstream assessments that can be used to test the validity of the model.

PROTOCOL:

All animal experiments described were approved by the Animal Care and Use Committee at Cincinnati Children's Medical Center and were in accordance with the National Institutes of Health guidelines. *Ad libitum* access to standard rodent chow and water was provided at all times to mice, including during the CGS paradigm. Mice were housed on a 14 h/10 h light-dark cycle (lights on 06:00 h) unless otherwise specified (i.e., exposure to lights overnight).

1. Preparing for timed matings

1.1. At least 2 weeks prior to setting up timed matings, house the adult female mice together in a standard mouse cage (18.4 cm x 29.2 cm x 12.7 cm), four mice per cage. Label each female mouse with a specific ID number via an ear tag.

NOTE: C57BL6 female mice with no prior pregnancy and between 3 to 6 months of age were used for this protocol.

1.2. At least 1 week prior to setting up timed matings, individually house the adult male mice to be used for mating.

2. Setting up timed matings

2.1. Set up the timed matings at 18:00 h. Take two female mice and place them inside a cage that holds an individually housed male mouse. Separate the timed matings the following morning by 08:00 h.

3. Checking the copulatory plug, designated as gestational day 0.5 (G0.5)

3.1. Immediately after separating timed matings, check for the presence of a copulatory plug in the female mice. The presence of a copulatory plug will mark G0.5. Allow the mouse to hold the wire grid inside the cage and gently lift it by the tail to visualize the vaginal opening.

NOTE: The presence of a copulatory plug indicates sexual activity has occurred but does not guarantee a pregnancy. When attempting to calculate the number of experimental mice needed, expect 50% of mice to be plugged from timed matings and a pregnancy to plug incidence of 60%–70%.

3.2. Use simple visual examination to identify the presence of a copulatory plug (an opaque whitish hardened mass within or slightly protruding from the vaginal opening). If the copulatory plug is not easily identified by simple visual examination, gently insert a blunt end probe into the vaginal opening. Identify the plugs located further back in the vagina by the resistance of probe insertion.

3.3. Separate the female mice with copulatory plugs and group house in standard mouse cages, 3 to 4 mice per cage.

4. Preparing for CGS paradigm

4.1. Randomly assign cages housing female mice with copulatory plugs into two groups on G5.5: Control and CGS group. Attempt to randomize cages to have an approximately equal number of mice per group. Transfer the mice to clean standard mouse cages and label with a “do not disturb” sign. Designate these cages as “home cages” for mice to place them at the end of each stressor.

4.2. Designate a separate room in the mouse facility to perform the CGS paradigm. Design a 11-day stressor regimen, which runs from G6.5 to G17.5, to utilize each of the 7 day stressors [exposure to foreign objects (marbles or legos), predator odor exposure (dirty rat bedding), 30° cage tilt, frequent changes of bedding, bedding removal, movement on shaker] twice per day,

and to utilize each of the 3 night stressors (overnight lights on, cage mate change, exposure to wet bedding) overnight in a random fashion. For a possible sample schedule and schematic of experiments described below, see **Figure 1**.

NOTE: Each day stressor should fall within the light cycle of mice (lights on 06:00 h–20:00 h), and last 2 h, with at least a 2 h break in between stressors. Each night stressor should be set up at the beginning of the dark cycle (lights off 20:00 h) and separated at the start of light cycle (lights on 06:00 h).

5. Performing the CGS paradigm

5.1. Set up specific stressors on a standard static cage with filtered top and water bottle in the room designated for the CGS paradigm. Prepare the number of static cages needed for the experiment depending on the number of mouse cages designated to undergo CGS during randomization. Before starting each stressor, transfer the mouse cages of the CGS group from the housing room to the CGS room.

NOTE: Perform the handling/transfer of mice from home cage to experimental cage and back in laminar flow hoods.

5.2. Apply the following stressors according to the pre-designed regimen (refer to step 4.2).

5.2.1. Exposure to foreign objects (marbles or legos): Place six marbles (14 mm in diameter) or six legos (different shapes, not to exceed 4 cm in height) randomly distributed into a clean static cage with mouse bedding, without including the mouse nestlets. Place the mice together with their home cage counterparts into the static cage with foreign objects for 2 h. Return the mice to their home cage with the same counterparts at the conclusion of the stressor.

NOTE: Clean the foreign objects after use.

5.2.2. Predator odor exposure (dirty rat bedding): Place 1 cm in depth of fresh dirty rat bedding from female rats into a clean static cage with no mouse bedding, without including the mouse nestlets. Place the mice together with their home cage counterparts into the static cage with dirty rat bedding for 2 h. Return the mice to their home cage with the same counterparts at the conclusion of the stressor.

5.2.3. 30° cage tilt: Place the mice with their home cage counterparts into a clean static cage with mouse bedding, without including the mouse nestlets. Tilt the cage at 30° against the wall for 2 h. Return the mice to their home cage with the same counterparts at the conclusion of the stressor.

5.2.4. Frequent changes of bedding: Place the mice with their home cage counterparts into a clean static cage with mouse bedding, without including the mouse nestlets. Substitute the mouse bedding with clean mouse bedding every 10 min for 2 h. During mouse bedding changes,

gently place the mice in a different clean cage to avoid direct contact with the mice. Return the mice to their home cage with the same counterparts at the conclusion of the stressor.

5.2.5. Bedding removal: Place the mice together with their home cage counterparts into an empty clean static cage (with no mouse bedding or nestlets) for 2 h. Return the mice to their home cage with the same counterparts at the conclusion of the stressor.

5.2.6. Movement on shaker: Place the mice with their home cage counterparts into a clean static cage with mouse bedding, without including the mouse nestlets. Place the static cage atop a reciprocal lab shaker set to 140 strokes per min for 2 h. Return the mice to their home cage with the same counterparts at the conclusion of the stressor.

5.2.7. Overnight exposure to lights: Place the mice with their home cage counterparts into a clean static cage with mouse bedding, without including the mouse nestlets. Keep the lights on overnight (20:00 h–06:00 h) to interfere with dark cycle. Return the mice to their home cage with the same counterparts at the conclusion of the stressor.

5.2.8. Cage mate change: Transfer the mouse into a clean static cage with mouse bedding which is being housed by a different group of two female mice (intact females not part of treatment or control group). Keep the mouse in the static cage with unfamiliar cage mates overnight. Return the mouse to its home cage with its specific home cage counterparts at the conclusion of the stressor.

5.2.9. Exposure to wet bedding: Fill the static cage with mouse bedding with clean water kept at 24 °C until bedding is saturated with water. Place the mice together with their home cage counterparts into the static cage with wet bedding overnight. Return the mice to their home cage with the same counterparts at the conclusion of the stressor.

5.3. During CGS paradigm, keep the control mice undisturbed in their home cages inside the housing room.

5.4. Replace the used home cages with new home cages on G10.5. On G17.5, at the conclusion of the overnight stressor, single-house all the experimental mice to prepare for parturition and downstream functional assessments.

6. Monitoring the experimental mice during the CGS paradigm

6.1. Monitor the mice every 1 h during stressor application, except during overnight stressors.

6.2. Exclude the mice displaying distress signs, including wounds, lethargy, or any physical abnormality from the experiment. Contact the veterinary staff as needed.

7. Measuring the percentage body weight gain during gestation in the experimental mice (optional)

7.1. On G6.5, weigh the mice individually before the exposure to stressors. On G17.5, at the conclusion of the overnight stressor, weigh the mice individually. Weigh the control mice at the equivalent gestational time points.

7.2. Measure the percentage body weight gain during gestation by setting the weight of the first day of the CGS paradigm (G6.5) as 100%.

8. Measuring the postpartum relative adrenal gland weights in experimental mice (optional)

8.1. On postpartum day 2 (PP2), weigh the control and the CGS dams individually. Sacrifice the dams by carbon dioxide inhalation followed by cervical dislocation in a fume hood.

8.2. Place the mice on a dissection plate, sterilize the abdominal area with 70% ethanol, and open the abdominal cavity using scissors to make a vertical cut. Isolate the adrenal glands located adjacent to the anterior pole of the kidneys with forceps, bilaterally. Carefully dissect the fat tissue surrounding the adrenal glands underneath a dissecting microscope.

8.3. Weigh the bilateral adrenal glands individually. Calculate the relative adrenal gland weights in milligrams per gram (total weight of the right and the left adrenal glands/body weight).

9. Measuring the postpartum hypothalamic pituitary adrenal (HPA) axis activity in the experimental mice (optional)

9.1. In preparation for HPA axis measurements, cull litters to 6 pups per litter on postpartum day 0 (PP0).

9.2. On postpartum day 2 (PP2), individually restrain the control and the CGS dams inside a well-ventilated 50 mL polypropylene conical tube for 20 min. Immediately after restraint stress, remove the mouse from the conical tube and restrain the mouse with the non-dominant hand by holding the loose skin over the shoulders and posterior to the ears to have the skin over the mandible taut.

9.3. Puncture the submandibular vein with a lancet slightly behind the mandible but anterior to the ear canal. Collect up to 100 µL of maternal blood in a serum separator tube. After sample collection, apply gentle pressure with gauze to the puncture site to stop the bleeding. Return the dams to the home cage once the bleeding stops.

9.4. Centrifuge the serum separator tube at 21,130 x *g* for 6 min and carefully remove the serum. Store the serum at -20 °C for later use. Measure the serum corticosterone concentration by an ELISA kit following the manufacturer's protocol.

10. Measuring the postpartum behavioral changes in the experimental mice (optional)

10.1. To prepare for the behavioral analysis, cull litters to 6 pups per litter on PP0.

10.2. Perform analysis of the maternal care fragmentation from PP2 to PP5. On each day, during the light cycle, expose the dams to the testing room for a 5 min habituation period before videotaping the maternal behavior for a 30 min period.

10.2.1. Assess the maternal care fragmentation by measuring the average length of an individual licking/grooming bout and the total number of bouts performed by dams[19].

NOTE: Licking/grooming behavior is defined as a behavior where the dam is making contact with the pup's body with her tongue, or the pup is being handled by the dam with her forepaws. A bout is defined as an uninterrupted period of time where the dam is engaged in licking/grooming of her pups.

10.3. Perform analysis of anhedonia via sucrose preference test (SPT) from PP0 to PP6. Expose the dams to one 100 mL bottle of clean water and one 100 mL bottle of 4% sucrose solution in their home cage. Measure the amount of water and sucrose consumed (in mL) daily. Interchange the bottle placement in the home cage. Calculate the sucrose preference using the averages from the last 4 days: preference % = [(sucrose consumption / sucrose + water consumption) x 100].

10.4. Perform analysis of anxiety-like behavior via elevated zero maze (EZM) on PP8. Place the dams individually on the EZM apparatus consisting of two closed quadrants and two open quadrants elevated from the floor. Allow the dams to explore the maze undisturbed for 5 min. Quantify the time spent in the open quadrant and the number of entries into the open quadrants.

11. Measuring the postnatal offspring weight changes (optional)

11.1. To prepare for the offspring weight analysis, cull litters to 6 pups per litter on the day of birth (postnatal day 0, PN0).

11.2. Record the weight of pups on PN0 and at different time points during the postnatal period (PN2, 7, 15, 21).

REPRESENTATIVE RESULTS:

Exposing the pregnant female mice to CGS results in changes in chronic stress-relevant parameters, including a reduction in body weight gain during pregnancy (**Figure 2A**) and increased adrenal gland weights in the early postpartum period (**Figure 2B**)[19]. Importantly, exposure to CGS results in postpartum abnormalities in maternal neuroendocrine function. CGS dams exhibit a hyperactive HPA axis as evidenced by the increased serum corticosterone levels following the application of a novel acute insult (**Figure 3**)[19].

Exposing the pregnant female mice to CGS further results in behavioral abnormalities in the early postpartum period that appear to reflect the emergence of a depressive-like phenotype. CGS

dams display alterations in maternal care as reflected by an increase in the degree of fragmentation of maternal signals received by the pups. The average duration of licking/grooming bouts is reduced and associated with an increase in the mean number of bouts following CGS, indicating numerous short episodes of nurturing behavior (**Figure 4A,B**)[19]. Sucrose preference is also depressed in CGS dams when compared to control dams, suggesting the presence of anhedonia (**Figure 4C**)[19]. Lastly, the CGS dams also display increased anxiety-related behaviors as measured by a reduction in the time spent in the open quadrants of the EZM when compared to control dams (**Figure 4D**)[19].

In the offspring, exposure to CGS in-utero results in decreased weight gain during the postnatal period, from postnatal day 7 to 21, although no changes are observed at birth. This reduction in body weight gain is present in offspring of both sexes (**Figure 5**)[19]. Of note, the CGS paradigm did not have any effect on gestational length, litter size, or sex ratio per litter (data not shown)[19].

FIGURE LEGENDS:

Figure 1: Schematic of CGS paradigm and functional assessments for validation. This figure has been modified from Zoubovsky, S.P. et al.[19].

Figure 2: Changes in the chronic stress-related parameters in the dams following CGS exposure. (**A**) Body weight changes from G6.5–G17.5, Control = 17, CGS = 17. (**B**) Relative maternal adrenal gland weights at PP2, Control = 20, CGS = 15. Data presented as mean + SEM. *$p < 0.05$, ****$p < 0.0001$. This figure has been modified from Zoubovsky, S.P. et al.[19].

Figure 3: Maternal HPA axis measurements following CGS exposure. Maternal serum corticosterone levels measured after 20 min of restraint stress on PP2, Control = 8, CGS = 5. Data presented as mean + SEM. *$p < 0.05$. This figure has been modified from Zoubovsky, S.P. et al.[19].

Figure 4: Behavioral changes in the early postpartum period in dams following CGS exposure. (**A**) Mean duration and (**B**) number of licking/grooming bouts recorded from PP2–PP5, Control = 17, CGS = 17. (**C**) Percent sucrose preference in SPT, Control = 17, CGS = 19. (**D**) Total amount of time spent in open quadrant of EZM during the 5 min period, Control = 17, CGS = 19. Data presented as mean + SEM *$p < 0.05$, **$p < 0.01$. This figure has been modified from Zoubovsky, S.P. et al.[19].

Figure 5: Changes in the offspring body weight during postnatal development following in-utero exposure to CGS. The offspring body weight measured from PN0 to PN21, Control = 17 litters, CGS = 17 litters. Data presented as mean + SEM. *$p < 0.05$, ****$p < 0.0001$. This figure has been modified from Zoubovsky, S.P. et al.[19].

DISCUSSION:

Exposing the pregnant mice to CGS perturbs postpartum maternal neuroendocrine function, including HPA axis response to novel stressors, and is associated with various behavioral abnormalities relevant to perinatal mood and anxiety disorders. Given that the model employs utilization of an environmental risk factor, higher phenotypic variation is expected than

otherwise observed in genetic models[22]. Nevertheless, results obtained from application of the CGS paradigm can be consistent across research laboratories if care is taken to minimize variables that may confound results.

Critical steps in the protocol include steps related to general husbandry practices, including housing control mice separate from CGS mice, and timed-mating steps. Co-housing control and CGS mice could in itself be a stressful stimulus for the control group and therefore confound neuroendocrine or behavioral results[23,24]. Likewise, initiating experiments with pregnant mice shipped from the supplier is not recommended. To maximize the efficiency of timed matings, it is recommended to house adult female mice together at least 2 weeks prior to setting up timed matings to synchronize their estrus cycles. Likewise, using sexually experienced adult male mice and preventing males from mating at least 1 week prior to setting up timed matings will maximize their fertility and increase the potential for successful pregnancies. The timeline of the CGS paradigm must also be carefully followed. Applying these stressors of varying intensity too early in gestation could affect uterine decidualization and inhibit embryo implantation[25]. Stress exposure during different gestational time windows has also been found to carry varying sex-specific neurodevelopmental disease risk for offspring, where male offspring are significantly more vulnerable than female offspring to stressors during early gestation[26,27]. CGS schedule must be designed in such a way that it ensures unpredictability to prevent the development of adaptation mechanisms and acclimation often associated with repeated exposure to predictable stressors[28]. Lastly, litters should be culled to six pups on the day of birth to ensure comparable conditions across all dams and prevent litter size variabilities from confounding maternal hormone or behavior analysis. Likewise, different cohorts should be employed for neuroendocrine and behavioral assessments to minimize confounding effects of restraint stress and submandibular bleeds on behavior. Different cohorts should also be used for maternal care assessment and analysis of other behavioral parameters to minimize disruption of maternal interaction with the pups.

There are several limitations to the current protocol. The inability to accurately predict the number of pregnant mice prior to the start of the CGS paradigm can pose a considerable financial and animal use burden. Modifications could be made to the protocol to achieve more predictable success with timed matings, including evaluating vaginal cytology to identify mice in estrus stage, where both mating and ovulation typically occur[29]. Ultrasonographic examination of mice could also be incorporated into the CGS paradigm as an alternative non-invasive technique to accurately identify pregnancies from very early stages of gestation[30]. The use of special breeding chow, with increased fat content, has also been employed by other groups to improve mating success[31]. However, caution must be taken when instituting changes in diet, given that this could affect maternal stress reactivity and behavior[32]. In addition, the current protocol has been shown to be effective in wild-type C57BL/6 mice, but modifications of the protocol may be needed for different strains or genetic backgrounds as well as species, for they may have large variations in stress sensitivity, maternal care, and emotional regulation.

Compared to currently existing peripartum stress models, the CGS paradigm proves to be more translationally relevant given the resulting disease-relevant endophenotypes observed, including

enhanced maternal stress reactivity and postpartum abnormalities in maternal care, anhedonia, and anxiety. These alterations seem to recapitulate clinical findings associated with perinatal mood and anxiety disorders. Future applications of this model include utilizing the CGS paradigm to identify sex-specific effects of maternal psychosocial stress on offspring brain development and disease susceptibility. Studying the effects of CGS on placental function should be considered, given that dysfunction in key placental functions have been shown to impact fetal brain development[33]. Incorporating cross-fostering experiments with the CGS paradigm would further help understand the individual contributions in-utero CGS exposure and associated maternal hormonal milieu changes versus postpartum abnormalities in nurturing behavior play in shaping offspring emotional development.

ACKNOWLEDGEMENTS:

The authors wish to acknowledge support from the National Institute of General Medical Sciences T32 GM063483-14 grant and Cincinnati Children's Research Foundation. For data adapted from Zoubovsky et al., 2019, Creative Common License can be found at the following location: http://creativecommons.org/licenses/by/4.0/.

DISCLOSURE:

The authors have no conflicts of interest to disclose.

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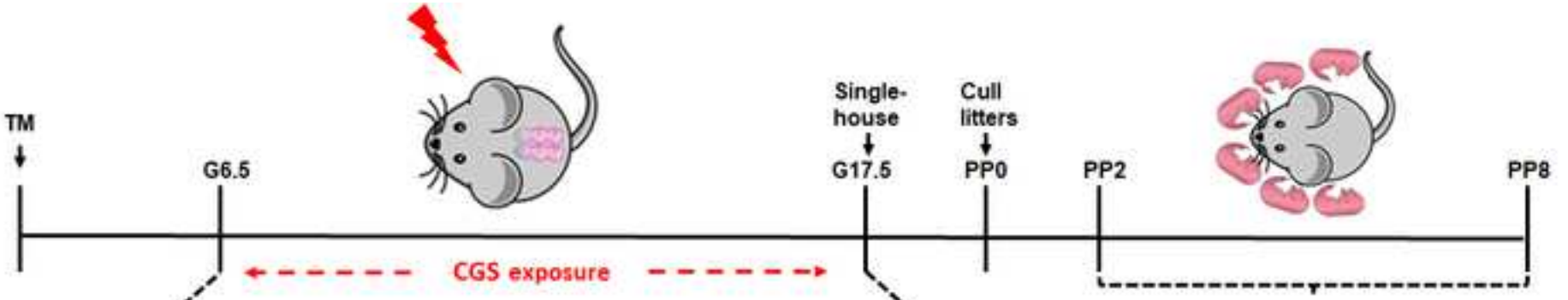
Figure 1



Day	Morning - 2 hours	Afternoon - 2 hours	Overnight Stressor
G6.5	Exposure to foreign object (marble)	No bedding	Lights on O/N
G7.5	Dirty rat bedding exposure	Frequent changes of bedding	Cage mate change
G8.5	Cage tilt – 30°	No bedding	Wet bedding
G9.5	Dirty rat bedding exposure	Exposure to foreign object (lego)	Cage mate change
G10.5	Exposure to foreign object (marble)	Movement on shaker	Lights on O/N
G11.5	Frequent bedding changes	No bedding	Wet bedding
G12.5	Cage tilt – 30°	Movement on shaker	Lights on O/N
G13.5	No bedding	Exposure to foreign object (lego)	Cage mate change
G14.5	Dirty rat bedding exposure	Frequent changes of bedding	Wet bedding
G15.5	Movement on shaker	Cage tilt – 30°	Lights on O/N
G16.5	Dirty rat bedding exposure	No bedding	Wet bedding

Dams should be weighed on G6.5 and G17.5.
Control dams are left undisturbed.
TM, timed matings; G, gestational day; PP, Postpartum day

Functional assessment:

HPA axis measurements including adrenal gland weights

Maternal care assessments

Sucrose preference test

Elevated zero maze

***Offspring weight can be measured throughout postnatal development**

Figure 2

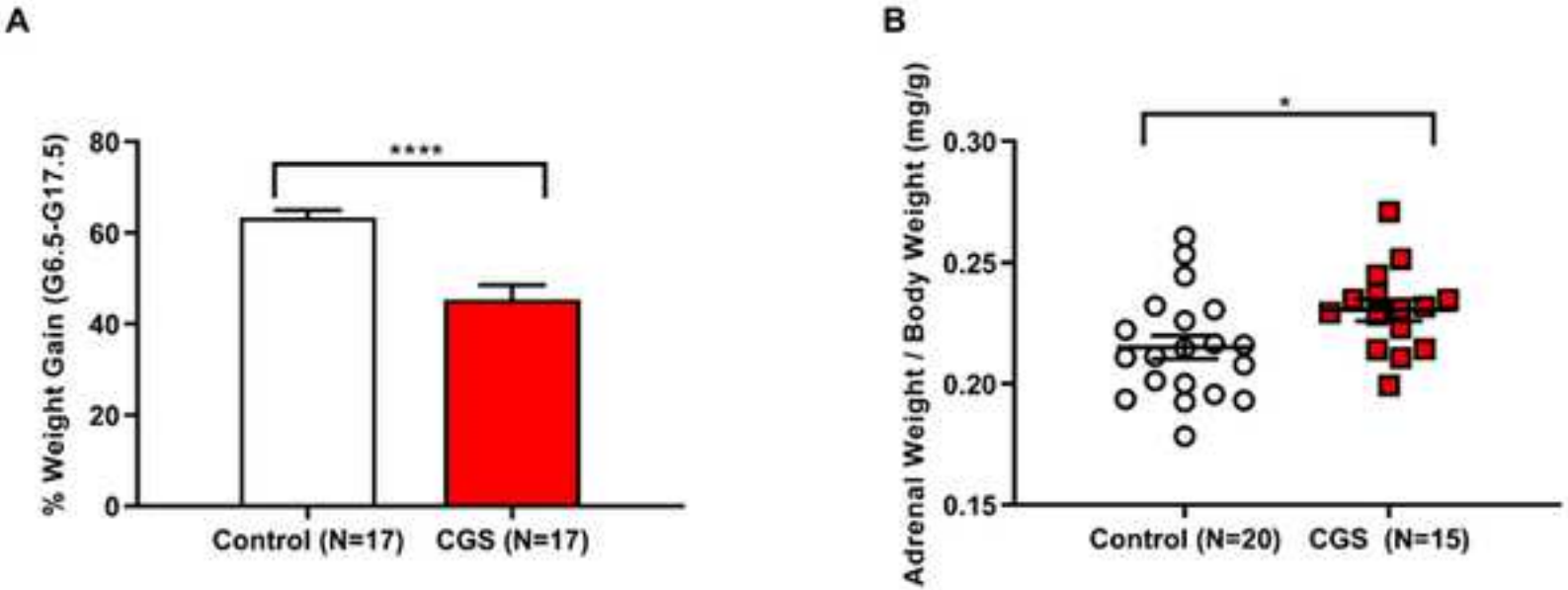


Figure 3

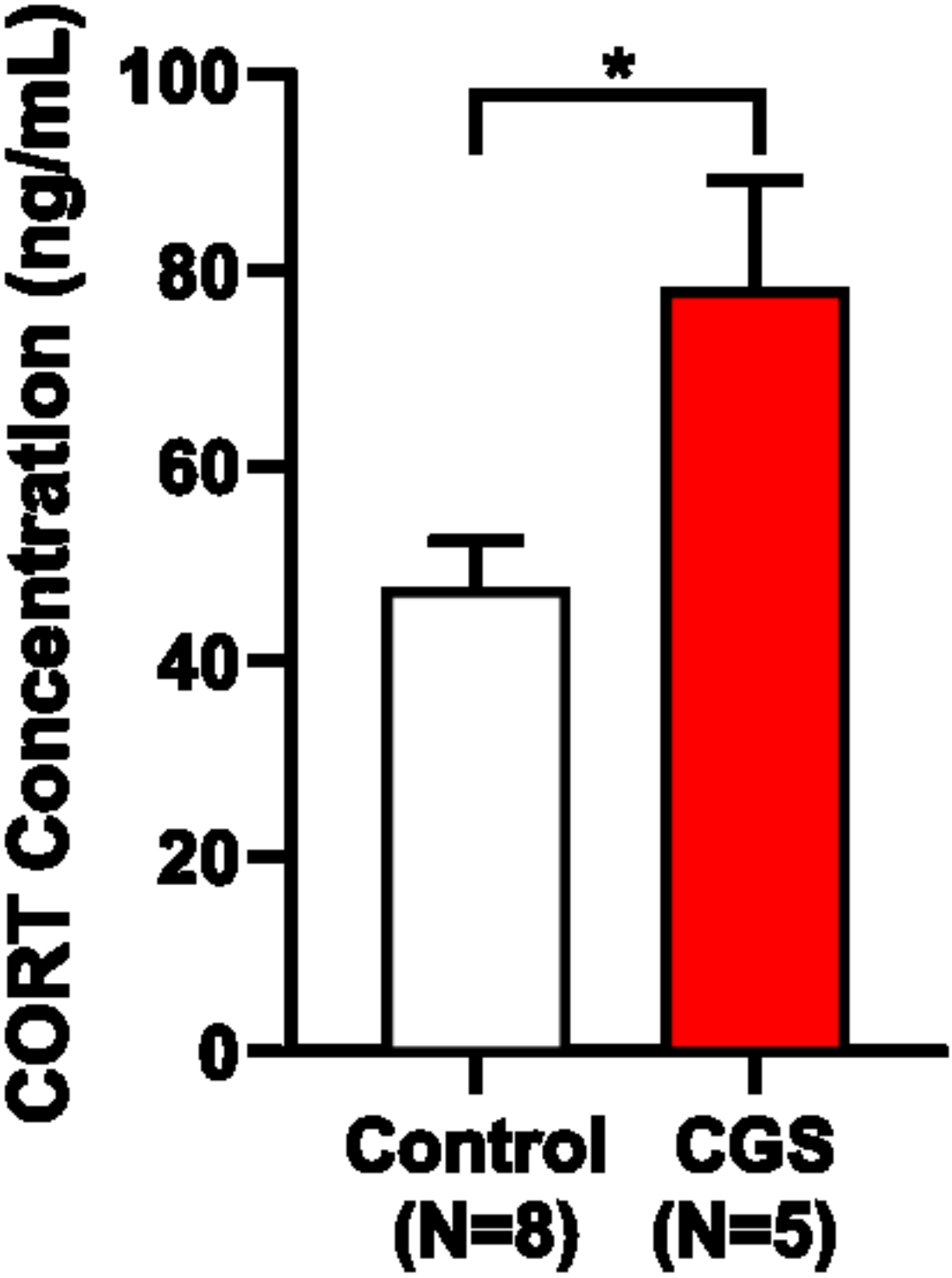


Figure 4

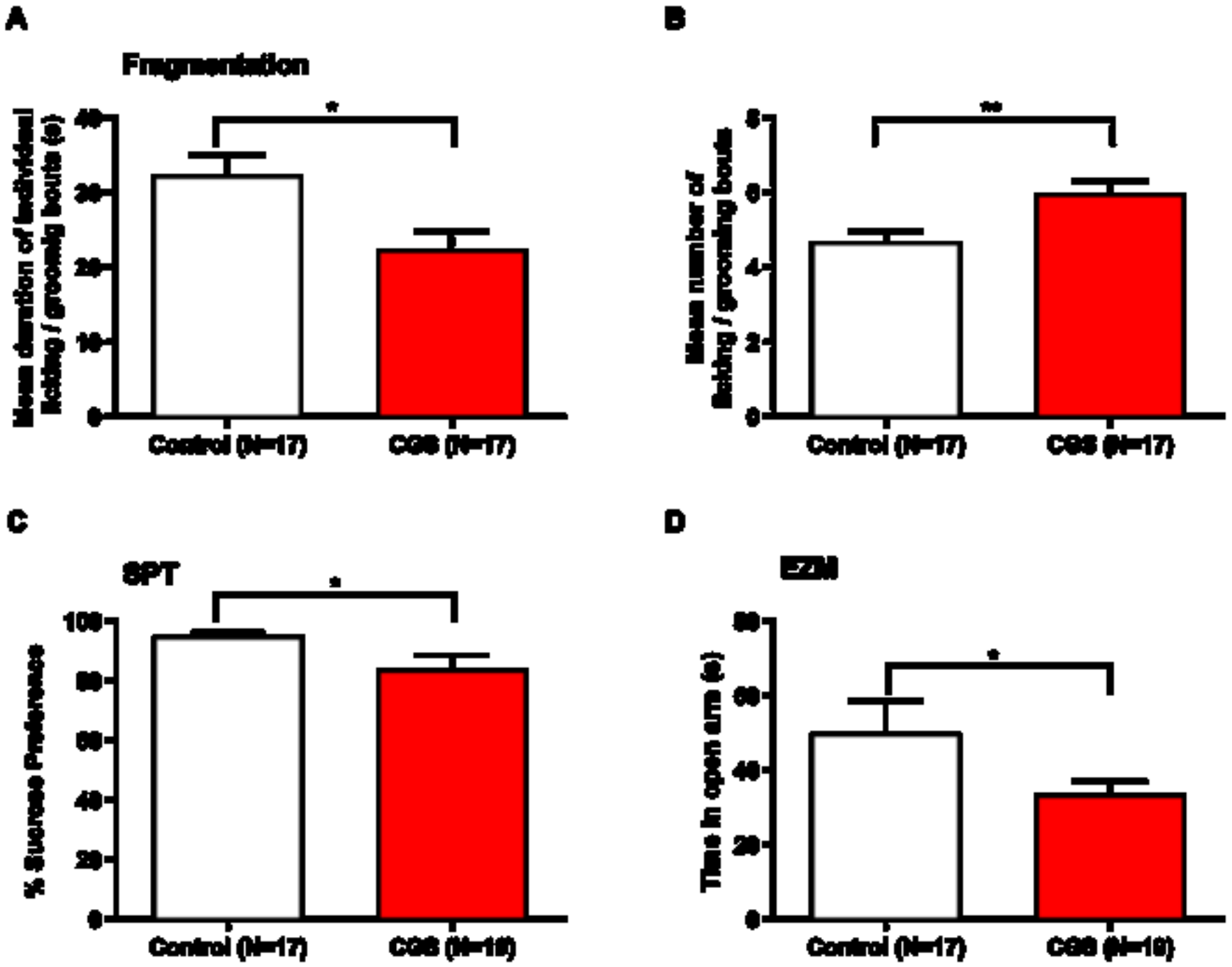


Figure 5

Click here to access/download;Figure;JOVE_FIGURE 5R.tif

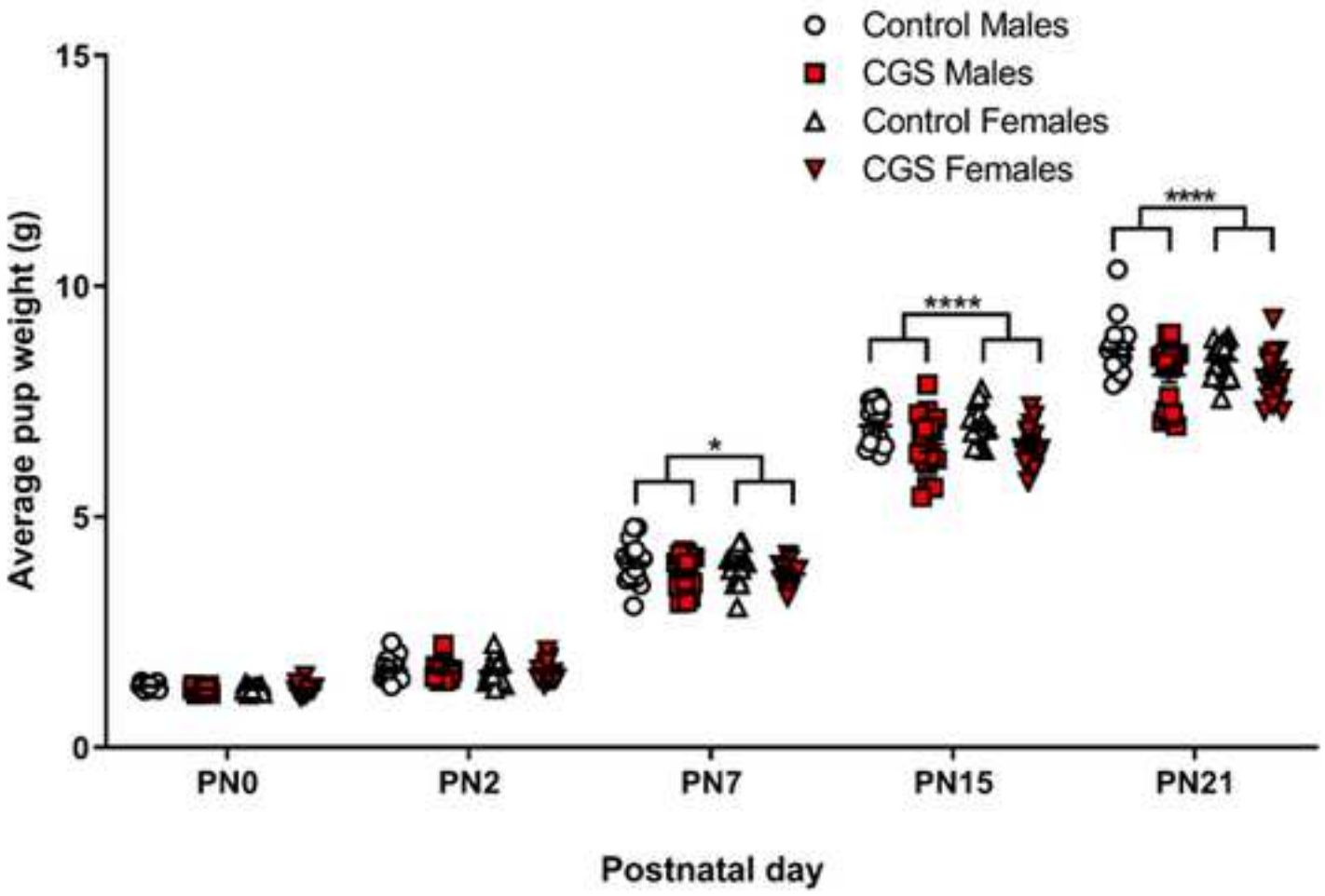


Table of Materials

Name of Material/Equipment	Company	Catalog Number
Animal lancet	Braintree Scientific Inc.	GR4MM
Blunt end probe	Fine Science Tools	10088-15
Bottles for SPT	Braintree Scientific Inc.	WTRBTL S-BL
Conical tubes (50 mL)	Corning Inc.	352098
Legos	Amazon	-
Marbles	Amazon	-
Mouse Corticosterone ELISA kit	Biovendor	RTC002R
Mouse EZM	TSE Systems	-
Reciprocal laboratory shaker	Labnet international	S2030-RC-B
Serum separator tubes	Becton Dickinson	365967
Static cage- bottom	Alternative Design Manufacturing and Supply Inc.	RC71D-PC
Static cage - filtered ventilated tops	Alternative Design Manufacturing and Supply Inc.	FT71H-PC

Comments/Description
Used to check for copulatory plugs
100 mL glass water bottle with stopper and sipper ball point tube, graduted by 1 mL.
Used for restraining mice to measure HPA axis response to acute stress. Make sure conical tube has small opening at the end for ventilation.

RE: *JoVE* 62464

We thank the reviewers and the editor of *JoVE* for their very thoughtful reviews of our submitted manuscript, "Using a murine model of psychosocial stress in pregnancy as a translationally relevant paradigm for psychiatric disorders of the maternal-infant dyad". In addressing the concerns raised, we believe we have significantly improved the manuscript. We detail our individual responses to the editor and reviewers below. We hope with these modifications the manuscript will now be considered suitable for publication in *JoVE*. Thank you again for your consideration.

Sincerely,
Louis J Muglia MD, PhD

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the manuscript to correct spelling or grammar issues.

2. Please revise the following lines to avoid previously published work: 45-47, 289-290, 333-334.

We have revised the above mentioned lines.

3. Please specify the age and breed of the mice used.

Age and breed of mice have been specified: C57BL6 female mice between 3 to 6 months of age (lines 126-127 in track marked manuscript).

4. Line 146: Please revise "copulatory drug" to "copulatory plug".

"Copulatory drug" has been changed to "copulatory plug".

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

All text in the protocol section has been changed to the imperative tense. We have also added to our protocol that handling of mice and euthanasia/adrenal gland dissections should be performed in animal facility hoods.

6. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

The protocol has been edited so as to only contain action items. Non-action items that were more relevant to the discussion have now been moved to the discussion section (lines 449-453 in track marked manuscript).

7. For SI units, please use standard abbreviations when the unit is preceded by a numeral throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, etc.

For SI units, abbreviations have been edited throughout manuscript so as to have standard abbreviations only.

8. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step. Also, consider highlighting any other protocol sections for filming which could add more clarity/details to the protocol.

Shorter protocol steps have been combined so that steps now contain at least 2-3 actions and are a maximum of 4 sentences per step. This protocol describes the use of a novel psychosocial stress paradigm during pregnancy for rodents therefore the corresponding parts have been highlighted in the manuscript for filming.

9. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

One-line space has been included between each protocol step. As mentioned above, this protocol emphasizes the use of a psychosocial stress paradigm for rodents therefore the corresponding parts have been highlighted in the manuscript for filming.

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage –

LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names.

Reference list has been edited.

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12. Figure 2: Please consider adding labels in the figure to make it more informative.

Figure 2 has been deleted from the manuscript following suggestions from Reviewer # 1.

13. Figure 4: Please revise the y axis title to "ng/mL" instead of "ng/ml".

Figure 4 (which has now become Figure 3) y-axis title has been revised to "ng/mL".

14. Figure 5: Please revise the y-axis units in Figure 5A and 5D to "s" instead of "sec".

Figure 5 (which has now become Figure 4) y-axis units have been revised to "s".

15. Please sort the Materials Table alphabetically by the name of the material.

Materials Table has been sorted alphabetically by the name of the material.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript "Using a murine model of psychosocial stress in pregnancy as a translationally relevant paradigm for psychiatric disorders of the maternal-infant dyad" by Zoubovsky et al. provides a detailed protocol for subjecting pregnant female mice to chronic psychosocial stress. This manuscript is very well-written and clear. The authors provide a strong and compelling rationale in the Introduction, and then go on to provide detailed step-by-step instructions for executing the protocol, as well as data demonstrating that the chronic social stress protocol changes stress-relevant parameters. Overall this manuscript is excellent.

I have a few minor suggestions to improve clarity, detailed below:

Major Concerns:

None

Minor Concerns:

In the Predator Odor Exposure protocol (5.3.2), it would be helpful to specify whether this should be bedding from female or male rats, or if it does not matter. In addition, the authors write "Place 1 cm of fresh dirty rat bedding…" I assume this refers to the depth of the bedding on the cage floor, but this was not immediately clear.

In the predator odor exposure section of protocol, we have changed the description to specify addition of 1cm in depth of fresh dirty rat bedding from female rats (lines 218-219 in tracked marked document).

In the Frequent Change of Bedding protocol (5.3.4), is there a reason that the bedding is removed/substituted from the same cage, or could the animals be placed into a new clean cage every 10 minutes? Removing/replacing the bedding by hand without contacting the mice seems tricky and a potential source of variability.

This section of our protocol has been edited so as to clarify that animals should be placed in a new clean cage while the bedding from the experimental cage is being replaced by clean bedding (lines 234-237 in track marked manuscript).

In the Cage Mate Change protocol (5.3.8), is it common for females to sustain injuries? If so, this should be stated.

In our cage mate change protocol, it is not common for female mice to sustain injuries, therefore this has not been added to the manuscript.

Figure 2 is probably not necessary. The descriptions in the text are likely sufficient, particularly if there will be a video component as well.

Figure 2 has been deleted from the protocol.

Reviewer #2:
Manuscript Summary:
This is a straightforward step-by-step protocol of the chronic psychosocial stress paradigm during pregnancy of mice. Similar protocols are already used in the literature, but I find a visual presentation of this complex paradigm useful. I have only a few remarks/recommendations.

Major Concerns:
1. The term "psychiatric disorders of the maternal-infant dyad" suggests that mother and offspring somehow have the same common disorder, and the term is therefore misleading. Stress during pregnancy may lead to long-term negative consequences in both mother and offspring (but the consequences may be very different!), and these consequences may partly be due to postpartum affective dysregulation between mothers and infants because of altered maternal care behavior. I suggest the replace this term with 'psychiatric disorders in mothers and infants' or similar.

We thank the reviewer for this suggestion. The term “psychiatric disorders of the maternal-infant dyad” has been changed to “psychiatric disorders in mothers and infants”.

2. The description of measuring maternal care (10.2.1) is very superficial and should be either much more detailed or references should be added. Measuring rodent maternal care behavior is complicated and requires specific expertise. In the description, licking/grooming behavior is not defined, nor is a 'bout' of licking/grooming defined. Readers are not advised about recording methods (video records/personal observation and notes?) and analysis of these behaviors.

The description of measuring maternal care fragmentation has been edited to include more detail. Briefly, licking and grooming behavior has been defined as a behavior where the dam is making contact with the pup’s body with her tongue, or the pup is being handled by the dam with her forepaws. A bout has been defined as an uninterrupted period of time where the dam is engaged in licking/grooming of her pups. In addition, we have specified that maternal behavior sessions should be videotaped. Our original publication has also been cited as reference in the manuscript (lines 356-367 in track marked manuscript).

Original publication: Zoubovsky, S.P., et al. Chronic psychosocial stress during pregnancy affects maternal behavior and neuroendocrine function and modulates hypothalamic CRH and nuclear steroid receptor expression. *Translational Psychiatry*. **10** (6), 1-13 (2020).

3. It is well known that stress during pregnancy may lead to premature birth, lower birth weight and associated physiological conditions in the offspring. Have the Authors seen such effects in the presented experiments (e.g. length of pregnancy, litter sizes, birth weights and sex ratio of pups) that could account for later possible adverse behavioral outcome?

The CGS paradigm did not result in any changes in gestational length, litter size, or sex ratio of pups per litter. This has been added to the results section (lines 405-407 in track marked manuscript) and the original publication which contains the data has been cited. In-utero exposure to CGS did result in a reduction in offspring weight during the postnatal period, from postnatal day 7 to 21, although birth weights were not altered. This has more clearly been explained in the results section (lines 403-404 in track marked manuscript).

Minor Concerns:

4. Different mouse strains show large variations in stress sensitivity, emotional behavior, maternal care etc., therefore, the use of different strains of mice should be discussed. If Authors have experience with only one mouse strain, it should be stated.

We have only employed our protocol in C57BL6 female mice. This has been mentioned as a limitation in the discussion section (lines 461-465 in track marked manuscript).

5. Lighting conditions and day/night cycles should be described.

Lighting conditions and day/night cycles have been added to the protocol (lines 118-119 in track marked manuscript). Briefly, mice are housed on a 14-h/10-h light–dark cycle (lights on 0600 h) unless otherwise specified (ie: exposure to lights overnight). A description of lighting conditions has also been added when describing specific stressors in protocol (lines 186-188 in track marked manuscript).

6. Is the use of special breeding chow recommended? Such food is applied in many labs during gestation and the postpartum period.

Our animal facility does not employ the use of special breeding chow, therefore this was not originally described in our protocol. However, use of special breeding chow has been added to our discussion as a potential way to improve breeding success with

caveats given that chow higher in fat content could affect maternal stress reactivity and behavior (lines 476-479 in track marked manuscript).

Froberg-Fejko, K., Lecker, J. Using environmental enrichment and nutritional supplementation to improve breeding success in rodents. *Lab Animal.* **45** (1), 406-407 (2016).

Perani, C.V., Neumann, I.D., Reber, S.O., Slattery D.A. High-fat diet prevents adaptive peripartum-associated adrenal gland plasticity and anxiolysis. *Scientific Reports*. **5**, 14821-14831 (2015).

7. Line 132: does "experienced adult male mice" mean sexually experienced?

“Experienced adult male mice” refers to sexually experienced. This has been added to the protocol and this section of the protocol has now been moved to the discussion as advised by the Editor’s comment (now found in line 451 in tracked marked manuscript).

8. Line 146: use 'plug' instead of 'drug'

Plug has been used instead of drug (line 146 in track marked manuscript).

9. Line 201: what is the recommended size of marbles or legos?

Marbles used for our protocol are 14 mm in diameter. Legos used were different shape and did not exceed 4 cm in height (this will be highlighted in video). These specifics have been added to the protocol section of our manuscript (lines 208-209 in track marked manuscript).

10. Line 242: are unfamiliar female mice from the same treatment or do you recommend using intact females for this purpose?

Intact females (that were not part of treatment or control group) were used for this purpose so as to ensure there would be enough unfamiliar female mice for this particular stressor. This has been specified in the protocol section (lines 252-253 in track marked manuscript).

11. Why 'CGS' is used as abbreviation of chronic psychosocial stress?

CGS was used as abbreviation in our original publication because this chronic psychosocial stress paradigm also represents a chronic gestational stress paradigm. For consistency purposes, CGS abbreviation is also being utilized in this manuscript.

Original publication: Zoubovsky, S. P., et al. Chronic psychosocial stress during pregnancy affects maternal behavior and neuroendocrine function and modulates

hypothalamic CRH and nuclear steroid receptor expression. *Translational Psychiatry*. **10** (6), 1-13 (2020).

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