Fighting Females: Neural and Behavioral Consequences of Social Defeat Stress in Female Mice

Supplement

Notes on stress and age in C57BL/6J female mice

We would like to emphasize the importance of using ***twelve-week-old*** C57BL/6J (B6) females for chronic defeat studies, as we were unable to generate a consistent defeat phenotype in 6-8-week-old mice. This may be due to irregular estrous cycling in virgin female mice until they are 12-20 weeks old at which point cycling remains regular for 7-10 months (1).

Notes on effective methods for culling non-aggressive females

In our hands, the most intensely aggressive pregnant females were the most likely to later engage in rival agonistic behavior whereas females that were non-aggressive during pregnancy did not exhibit rival aggression. From a methodological standpoint, this suggests that housing females briefly with intact males prior to rehousing them with castrated mice may be an efficient technique for culling non-aggressive female residents prior to social defeat experiments.

Vivarium

Mice were housed in a temperature-regulated vivarium maintained on a 12-hour reverse light/dark photocycle (lights off: 0730-1930h) with unrestricted access to water and rodent chow (Purina LabDiet 5001) through wire mesh cage lids.

Surgeries

Ovariectomies on Swiss Webster (CFW) females were performed as described below. Castrated males were either purchased directly from Charles River Laboratories (Wilmington, MA, USA), or intact CFW males were purchased and castrated in-house as described below.

Ovariectomy: After anesthesia was induced with an injection of 10 mg/mL ketamine/1 mg/mL xylazine, the fur on the flank skin over each ovary was shaved and cleaned with 70% ethanol and povidone-iodine x3 and the rest of the animal was covered with a clean drape. A 2-cm incision was made in the skin and the underlying connective tissue was blunt dissected. A small incision was made through the dorsal muscle wall over the ovary and the ovary and distal uterine horn were identified. A ligature was made with sterile 4-0 absorbable suture near the junction between the oviduct and ovary; the oviduct and blood vessels were severed distal to the ligature and the ovary was removed. After the tissue proximal to the cut had been inspected to make certain there was no bleeding, the uterine horn was replaced inside the abdominal cavity. The muscle wall incision was closed with absorbable 4-0 suture and the skin incision closed with a sterile wound clip. This procedure was repeated for the remaining ovary. The mouse was returned to the vivarium after it recovered from anesthesia and received a subcutaneous injection of carprofen (0.5 mg/mL). Experiments involving ovariectomized females commenced after wound clips were removed 7-10 days after the surgery.

Castration: Once anesthesia was induced with an injection of 10 mg/mL ketamine/1 mg/mL xylazine, the scrotal skin was shaved and cleaned with 70% ethanol and povidone-iodine x3 and the rest of the animal covered with a clean drape. A 15-mm incision was made through the scrotal skin unilaterally and the cremasteric muscle was exposed with blunt dissection. A 10-mm incision was made through the muscle and the testis and attached epididymis and fat pad were gently pulled through the incision. The connective tissue between the testis and epididymis was cut to isolate the testis and a ligature of sterile 4-0 absorbable suture was placed around the spermatic cord, close to the testis to clamp off the spermatic artery and vein; the spermatic cord was then cut distal to the ligature and the testis was removed. After inspection for bleeding, the remaining spermatic cord and epididymis were replaced inside the scrotum. The muscle incision was closed with sterile absorbable 4-0 sutures, and 4-0 monofilament non-absorbable suture was used to close the skin incision. This procedure was then repeated for the remaining testis. The

animal was returned to the animal holding room after it recovered from anesthesia and received a subcutaneous injection of carprofen (0.5 mg/mL). Castrated males were housed with intact females after sutures were removed, 7-10 days following surgery.

Estrous cycle monitoring

Once dry, cell samples were stained with 0.5% cresyl violet acetate (Sigma Aldrich, C5042) in dH_2O and inspected under light microscopy at 10x magnification with a Nikon Optishot microscope. Photomicrographs were taken with a Spot Insight 2.0 CCD digital camera affixed to the microscope, and estrous phase (metestrus/diestrus, proestrus, estrus) was determined for each day of the defeat protocol based on the proportion of cornified epithelial cells, nucleated epithelial cells, and leukocytes within each sample.

Tissue collection for c-Fos immunohistochemistry

On the tenth day of the chronic stress protocol, brains were collected from a subset of animals after either no defeat (*n*=5 controls) or a five-minute social defeat stress episode (*n*=6 acute defeat; *n*=4 chronic defeat) followed by an hour-long threat period. Mice were deeply anesthetized with an injection of 10 mg/mL ketamine/1 mg/mL xylazine prior to transcardial perfusion with 5 mL of ice-cold 1xphosphate-buffered saline (PBS; Thermo Fisher Scientific, BP39920) diluted from 10xPBS with dH_2O, then 50 mL of 4% paraformaldehyde in PBS (PFA; Thermo Fisher Scientific, J19943K2) at a rate of 5 mL/min using a Masterflex C/L peristaltic pump (Cole-Parmer, EW-77120-62). Brains were post-fixed overnight in 4% PFA at 4^{o}C, cryoprotected in ascending concentrations of sucrose (10%, 15%, then 30% sucrose in PBS) at 4^{o}C, frozen in histology molds filled with optimal cutting temperature compound (OCT; Tissue Tek, 4583) on crushed dry ice for one hour, and stored at -20^{o}C until whole brains were sliced on a Leica CM1850 cryostat. Fifty-micron slices containing the anteromedial bed nucleus of the stria terminalis (amBNST), ventral lateral septum (LSv), hypothalamic paraventricular nucleus (hPVN),

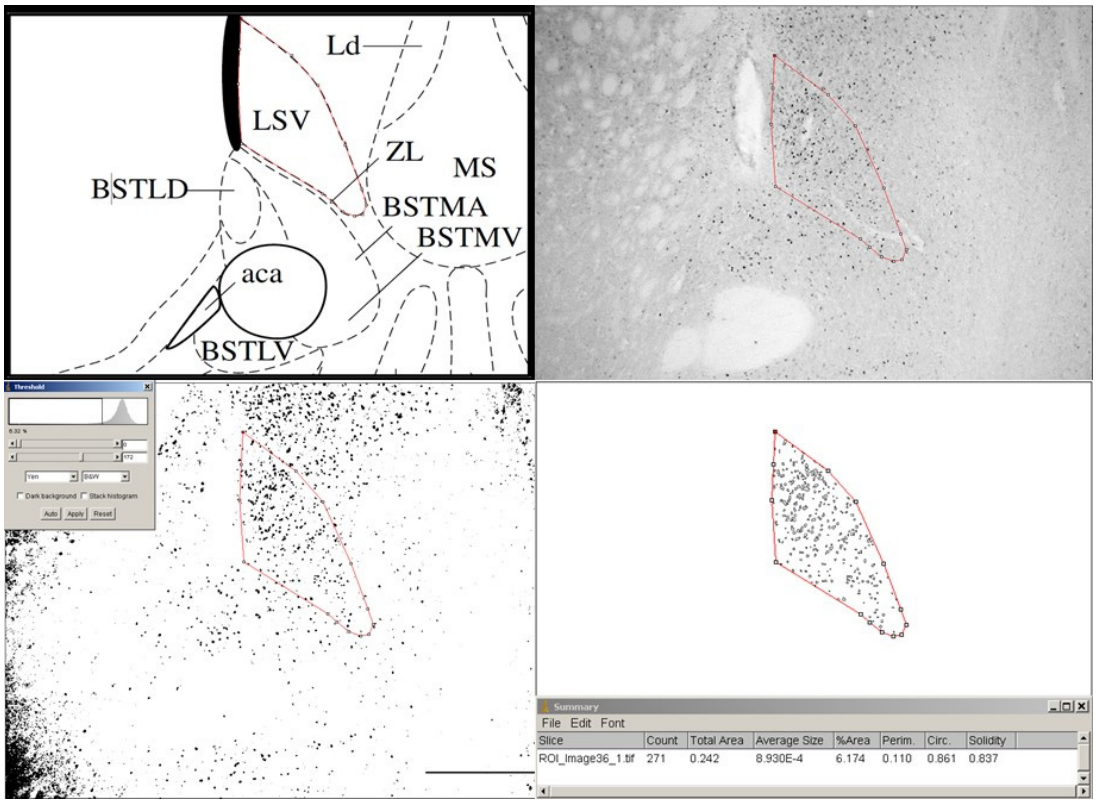
periventricular nucleus (PeN), ventromedial hypothalamus (VMH), medial amygdala (MeA), and dentate gyrus (DG) were selected for immunohistochemistry. Slices containing amBNST and LSv were identified as AP 0.50 to 0.38; slices including hPVN and PeN were AP -0.70 to -0.82; and slices containing VMH, MeA, and DG were located AP -1.58 to -1.82 (2).

Sections were incubated in 3% H_2O_2 diluted from 30% (Sigma-Aldrich, H1009) in PBS for 30 min, then in 50% ethyl alcohol diluted from 100% (Pharmco-AAPER, 111000200) in PBS for 30 min, rinsed three times in PBS, and blocked in 10% goat serum (Sigma-Aldrich, G9023) in 0.1% Triton X-100 (Sigma-Aldrich, X100) PBS for one hour. Sections were subsequently incubated with the primary antibody for c-Fos (1:1000; rabbit anti-c-Fos, Proteintech Group, Inc., 26192-1-AP) in PBS with 0.1% Triton X-100 for 40 hours at 4°C on a plate rocker. Following three PBS rinses, brain slices were incubated with biotinylated secondary antibody (1:500; biotinylated goat anti-rabbit, Vector Laboratories, BA-1000) for one hour, then transferred for one hour to Vectastain avidin-biotin complex (ABC) solution containing100 µL of reagents A (avidin DH) and B (biotinylated horseradish peroxidase H) per 5 mL PBS (Vector Laboratories, PK-4000). After three washes in PBS, sections were exposed to a solution of 0.7 mg/mL 3,3'-diaminobenzidine (DAB) in dH_2O (Sigma-Aldrich, D4418) for ten minutes, and rinsed thoroughly in PBS. Slices were incubated with solutions on a plate rocker at room temperature unless otherwise specified; all PBS washes were 5 min.

Slices were mounted on Superfrost Plus microscope slides (Fisher Scientific, 12-550-15), allowed to dry completely, cleared (1 minute in each: 50, 70, 95, 100% ethyl alcohol, then xylene), and cover slipped with Cytoseal 60 mounting medium (Richard-Allan Scientific, 8310-4). A Spot Insight 2.0 CCD digital camera affixed to a Nikon Optishot microscope was used to capture images at 4x and 10x magnification. All analyzed images were taken on the same day and camera settings remained consistent between animals and slices.

Predetermined unilateral regions of interest were selected in ImageJ from stereotaxic reference figures (2) to ensure the same area in each ROI between animals (e.g. for the ventral lateral septum illustrated below**, top left**). Images taken at 10x were changed to 8-bit grayscale images in ImageJ (**top right**). The Yen threshold setting was applied (**bottom left**) before cells were counted using the Analyze Particles function (**bottom right**). Particles with 3-to-infinity pixels were counted in the amBNST, MeApd, LSv, VMHvl, and PeN while particles with 20-to-infinity pixels were counted in the DG and hPVN. Images collected from pilot c-Fos IHC assays were subjected to manual counting using the ImageJ multi-point function and the described automated counting technique. A correlational analysis revealed a significant relationship between the number of c-Fos+ cells identified using the manual vs. automated method (r=0.9872).

ROI	Area (mm^2)
MeA	0.235
LSv	0.158
hPVN	0.086
VMHvl	0.087
PeN	0.017
amBNST	0.129
DG	0.623



Sex-specific patterns of aggressive behavior

The frequency of aggressive behaviors including sideways threats, attack bites, tail rattles, and pursuits and the duration of non-aggressive behaviors including walking, rearing, and grooming were quantified and lag sequential analyses were conducted to identify sex-specific behavioral patterns during aggressive encounters (Observer XT v. 12).

Timing for behavioral testing and blood collection for corticosterone measurements

To control for circadian fluctuations in behavioral and physiological measures, procedures were conducted between 1200 and 1500h. Blood was collected at two time points: three days prior to any defeat experience (i.e., baseline) and on the tenth day of the chronic social defeat stress protocol after a 20-min threat period preceded by no defeat for controls or by a 5-min social defeat experience for acutely and chronically defeated females (**Fig. S1A**). Samples were taken between 1300-1500h on both blood collection days; animal sequence was determined by randomizing subjects within stress condition, then alternating between stressed and control mice. This sequence was used on both collection days so that blood was taken from the same animal at approximately the same time (±10 min) during baseline and post-defeat or post-threat collection.

Light-dark box

Females were initially placed in the dark chamber (29x29x36 cm) for a 5-minute habituation period, after which a door was raised, allowing mice to freely explore both the light (>300 lux; 29x29x36 cm) and the dark (<5 lux) chambers for an additional five minutes. Ethovision XT v.14 tracked animal behavior, and the latency to enter the light chamber as well as the duration and distance travelled within the light chamber were measured. The apparatus was cleaned with tap water and wiped dry between each test.

Novel object investigation

Trials were videotaped and behaviors including the duration of novel object investigation, burrowing, walking and rearing, the latency to investigate and burrow, and the frequency of flinching/jumping were quantified (Observer XT v.12). Novel objects (i.e., rubber stoppers, 14-135G/14-130G; Fisher Scientific, Agawam, MA) were removed after each test and cleaned in a cage washer.

Home cage social interaction

The duration of experimental female-initiated social contact was quantified manually during social interaction testing. Trials were also videotaped to determine baseline differences in the behavioral composition of social interactions in control, acutely, and chronically defeated females. For videotaped trials, behaviors scored using Observer XT v. 12 included: duration of experimental female-initiated contact (nasal and flank/anogenital), burrowing, time spent following the stimulus animal, frequency of defensive kicks/flinches, and the number of escapes. Lag sequential analyses were used to determine differences in patterns of behavior in control and chronically defeated females during social interaction tests.

Statistics

Behavioral data collected from intact aggressive male and female residents were analyzed with two-tailed unpaired t-tests. Daily attack bite frequencies and attack latencies during the 10-day social defeat stress protocol were analyzed with one-way analyses of variance (ANOVA). Likewise, body weights of non-defeated and chronically defeated females were analyzed over the course of the 10-day stress protocol using a repeated-measures one-way ANOVA.

Plasma corticosterone concentrations were analyzed with a two-way repeated measures ANOVA with factors of time (levels: baseline, post-social defeat protocol) and social defeat condition (levels: control, acute defeat, chronic defeat). For each brain region of interest, one-way

ANOVA was used to reveal differences in the number of c-Fos+ cells in control and acutely or chronically defeated females. Correlational analyses were also conducted to explore interregional patterns of c-Fos activation according to stress condition; corresponding Pearson's *r* values are reported in **Table S1**.

In resident females (*n*=23), attack bites were evaluated according to estrous cycle phase (levels: proestrus, estrus, metestrus/diestrus) using a between-subjects ANOVA (**Fig. S3**). Estrous cycling data were also collected from control and chronically defeated females during the 10-day chronic social defeat protocol and analyzed using chi-square tests to detect differences between the ratio of days that controls spent in metestrus/diestrus: proestrus: estrus and the ratio of days that defeated females spent in these phases. Analyses were conducted on defeated samples from the first five days and the last five days of the 10-day stress protocol (i.e., approximately the first and second cycles). Estrous cycle data were also analyzed based on regularity (1, 3), defined as two days with leukocytic cells (metestrus or diestrus), one day with nucleated cells (proestrus), and one or two days with cornified cells (estrus or metestrus). The proportion of regular and irregular cycles in defeated females was compared for the first and second estrous cycles with chi square analyses; control data served as the expected outcome (**Fig. S5**).

Repeated-measures two-way analyses of variance identified significant main effects and interactions between stimulus female condition (levels: non-anesthetized, anesthetized) and defeat condition (levels: control, acute defeat, chronic defeat) in behaviors exhibited during home cage social interactions. In females treated with saline or ketamine, baseline home cage social contact durations were analyzed with an unpaired t-test comparing controls to chronically defeated females. These baseline values were used to calculate the change in social contact time (s) for each animal that subsequently received either saline or ketamine: Change in social contact time = (Post-injection contact time - Baseline contact time). Change in social contact time and the number of behavioral transitions during post-treatment social interaction testing were analyzed

with two-way analyses of variance with factors of drug dose (levels: saline, ketamine) and defeat condition (levels: control, chronic defeat).

Two-way analyses of variance were used to identify potential interactions between body temperatures collected prior to and following social or novel object investigation (levels: pre-test, post-test) and defeat condition (levels: control, chronic defeat). Measures of nesting, open field social interactions, and novel object investigation were analyzed in control vs. chronically defeated females with two-tailed unpaired t-tests or with a Mann-Whitney U test in the case of nest scores. For open field social interaction tests, social interaction scores were calculated as the ratio of time spent in the social interaction zone in the presence vs. the absence (i.e., habituation trial) of a social stimulus animal: SI score = SI zone time with social stimulus present/social stimulus absent. SI scores were analyzed with a two-tailed unpaired t-test (**Fig. S7A**). Correlational analyses were conducted between behavioral measures according to stress condition, and the corresponding Pearson's *r* values are given in **Table S2**. In addition, average daily number of attack bites received by chronically defeated females served as the independent variable in regression analyses with behavioral measures as dependent variables; significant findings are displayed in **Fig. S9**. All statistical analyses were conducted in GraphPad Prism 7 with the alpha level set to 0.05 for parametric tests; when appropriate, Sidak's multiple comparisons tests were used to identify significant differences between levels, post-hoc.

Video legends

Video S1. Resident CFW female engaging in species-typical rival aggression toward an unfamiliar B6 female. Interfemale aggression is characterized by a greater number of rapid bite bouts and pursuits leading to bites compared to intermale aggression. The frequencies of aggressive behaviors are similar between males and females (**Fig. 1C; Video S2**).

Video S2. Resident CFW male exhibiting species-typical aggression toward an unfamiliar B6 male. Attack bites are often preceded by sideways threats (**Fig. 1C**).

Video S3. Resident CFW female aggression toward an intruder B6 female on the fifth day of the chronic social defeat stress protocol.

Video S4. Typical home cage social interaction between a chronically defeated female (no tail marking) that received saline and a non-aggressive stimulus female (black tail marking; **Fig. 3**).

Video S5. Typical home cage social interaction between a saline-treated control female (no tail marking) and a non-aggressive stimulus female (black tail marking; **Fig. 3**).

Video S6. Home cage social interaction behavior in a chronically defeated female (no tail marking), 24 hrs after receiving a systemic ketamine (20.0 mg/kg) injection. The non-aggressive social stimulus female has a black tail marking. Ketamine increased social interactions in defeated females (**Fig. 3**).

Video S7. Social interactions in the home cage of a control female that received ketamine (20.0 mg/kg) on the previous day. The control female has no tail marking while the social stimulus has a black tail marking. Ketamine had no effect on social interactions in control females (**Fig. 3**).

Video S8. Vigilance-like behavior in a chronically defeated female during the open field social interaction test. The stimulus cage holds an unfamiliar, aggressive CFW female resident (**Fig. 5**).

Figure S1.

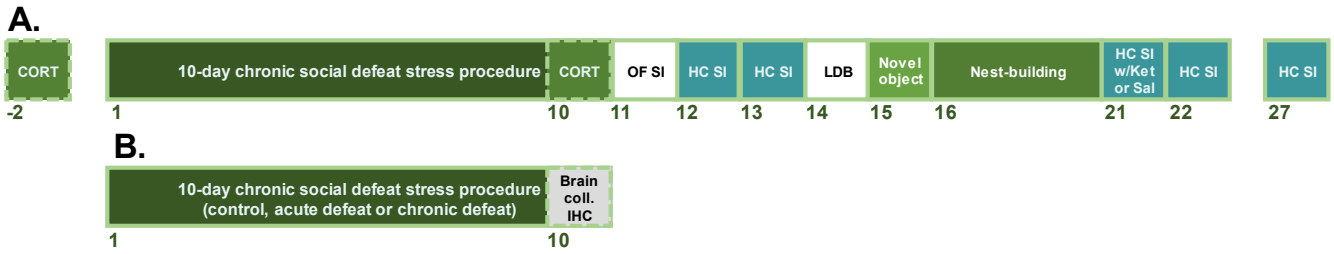


Figure S1. Twelve-week-old C57BL/6J females were either non-defeated controls, defeated once (i.e., acute defeat) or chronically defeated by aggressive females for ten consecutive days. Control and defeated females were used for (**A**) corticosterone measurements and/or behavioral phenotyping or for (**B**) c-Fos immunohistochemistry. Experimental days are indicated below each timeline. (**A**) HC SI tests with awake and anesthetized stimulus animals occurred on days 12 and 13, respectively; all subsequent HC SI tests were conducted with awake stimulus females. blood collection for corticosterone assay, CORT; open field social interaction test, OF SI; home cage social interaction test, HC SI; light dark box test, LDB; novel object investigation test, Novel object; nest-building test, Nest-building; home cage social interaction test 30-min after systemic ketamine (20 mg/kg) or saline administration, HC SI w/Ket or Sal; brains collected 60 minutes after the final defeat for c-Fos immunohistochemistry, Brain coll. IHC.

Figure S2.

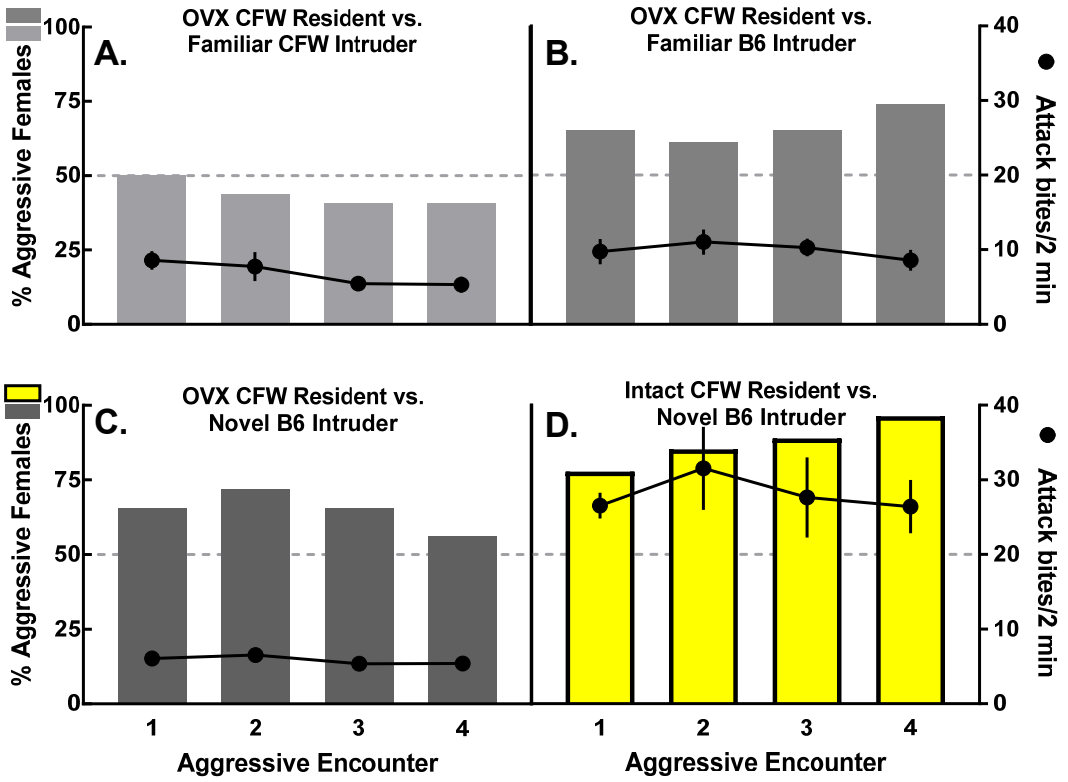


Figure S2. (**A-C**) Ovariectomized (OVX) or (**D**) intact resident Swiss-Webster (CFW) female mice were housed with intact CFW males and tested for aggression toward intact (**A**) familiar CFW, (**B**) familiar C57BL/6J (B6), or (**C, D**) novel B6 intruder females. Reliably high levels of aggressive behavior were observed in intact CFW females that confronted novel B6 intruders, as indicated by both the percentage of CFW females that were aggressive (left axes; data portrayed as the percentage of all CFW female residents that expressed aggression during that encounter) and by their attack bite frequencies during 2-minute resident-intruder encounters (right axes; data portrayed as the Mean ± SEM, calculated from females that fought).

Figure S3.

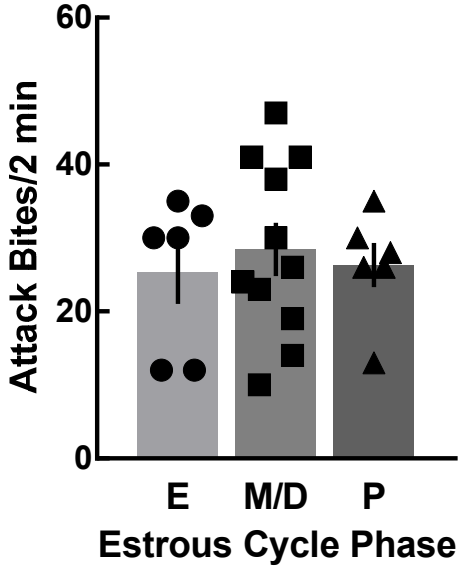


Figure S3. Swiss-Webster (CFW) females (*n*=23) housed with castrated males were evaluated for aggression toward unfamiliar C57BL/6J intruder females during different phases of the estrous cycle: estrus, E; metestrus/diestrus, M/D; proestrus, P. Each point corresponds to an individual female and bars depict the data as the group Mean ± SEM.

Figure S4.

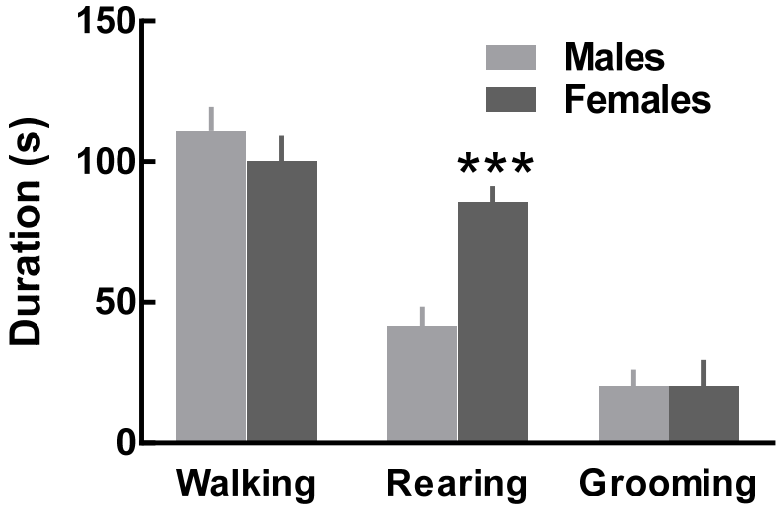


Figure S4. Non-aggressive behaviors were quantified during intermale and interfemale 5-min resident-intruder confrontations with novel C57BL/6J conspecifics. Resident females engaged in more rearing behavior (i.e., front paws off the ground and balancing on the hind legs) than males ($t(18)=4.834$, ***$p=0.0001$), but the durations of walking and self-grooming were comparable between males and females. Data are shown as Mean ± SEM.

Figure S5.

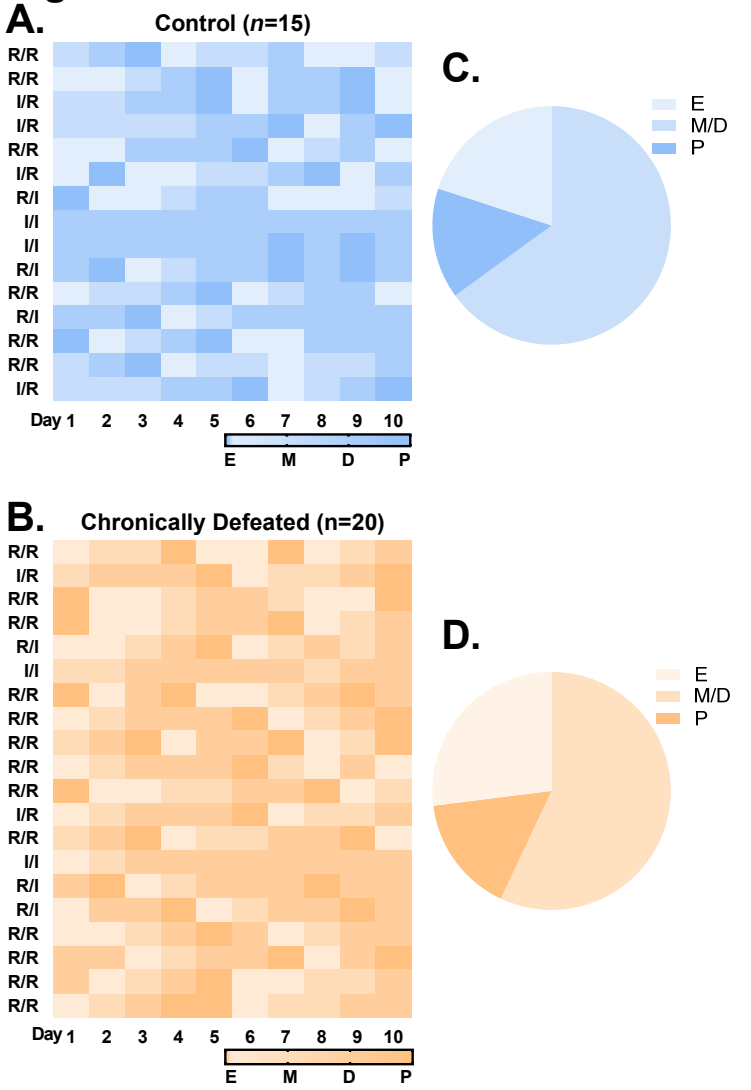


Figure S5. Chronic defeat stress did not affect estrous cycle regularity or body weight during the ten-day stress protocol. Heat maps illustrate estrous cycling in (**A**) control and (**B**) chronically defeated females with the lightest shades indicating estrus, E; medium shades indicating metestrus and diestrus, M and D; and the darkest indicating proestrus, P. Each column marks a day of the stress protocol and each row is an individual mouse. Left axis labels describe regularity of cycle 1 (days 1-5) and cycle 2 (days 6-10): regular/regular, R/R; irregular/regular, I/R; regular/irregular, R/I; irregular/irregular, I/I. Proportion of time in each estrous cycle phase is displayed for (**C**) controls and (**D**) chronically defeated females. (**E**) Control and chronically defeated female body weights were collected every other day and are depicted as the group Mean ± SEM.

Figure S6.

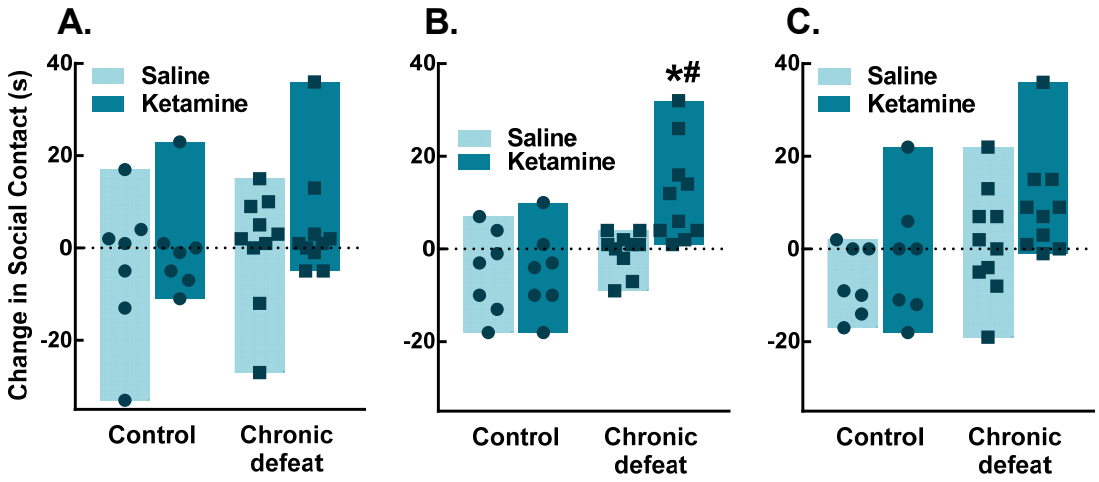


Figure S6. Ketamine increased social contact in chronically defeated females 24 hrs after treatment, but not 30 min or five days post-injection. Change in social contact is calculated within-subject as the post-injection social contact duration minus the baseline social contact time in seconds. (**A**) There was no difference social contact between non-defeated control and chronically defeated females thirty minutes after ketamine or saline injections. (**B**) Twenty-four hours post-injection, ketamine increased social contact in chronically defeated females (same as the data portrayed in **Fig. 3G**); *p<0.05, compared to ketamine-treated controls; #p<0.05, compared to saline-treated chronically defeated females. (**C**) This effect was no longer statistically significant five days after ketamine or saline administration. (**A-C**) Bars depict the max and min values. The dotted lines mark no change in social contact between baseline and post-injection tests; values above the dotted lines are increases from baseline while values below are decreases from baseline social contact time. Circles and squares indicate individual female mice.

Figure S7.

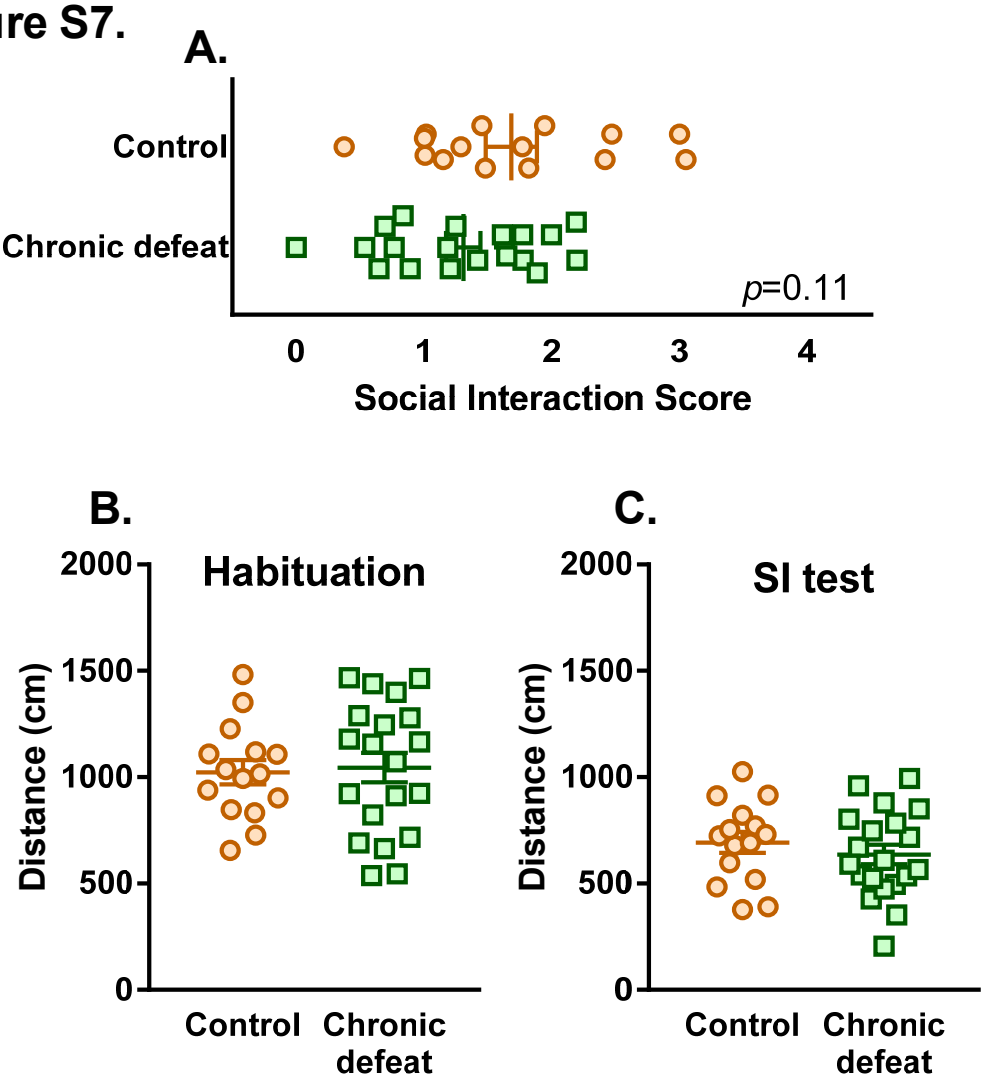


Figure S7. (**A**) Social interaction scores were similar between control and chronically defeated females. Distance travelled (cm) was also similar between control and defeated female mice during the (**B**) open field social interaction pre-test (i.e., habituation), and during the subsequent (**C**) open field social interaction (SI) test.

Figure S8.

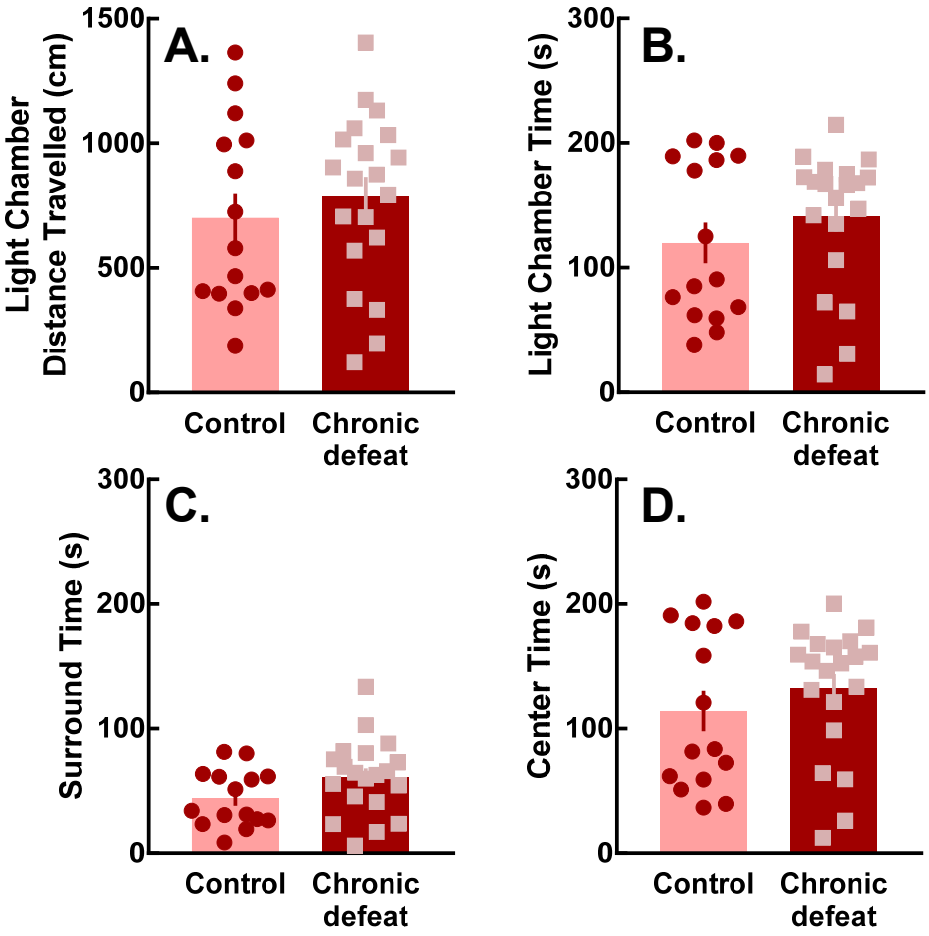


Figure S8. Chronically defeated and control females performed similarly during light-dark box testing on measures of (**A**) distance travelled, (**B**) duration in the light chamber, (**C**) duration in the perimeter (surround) of the light chamber, and (**D**) duration in the center of the light chamber.

Figure S9.

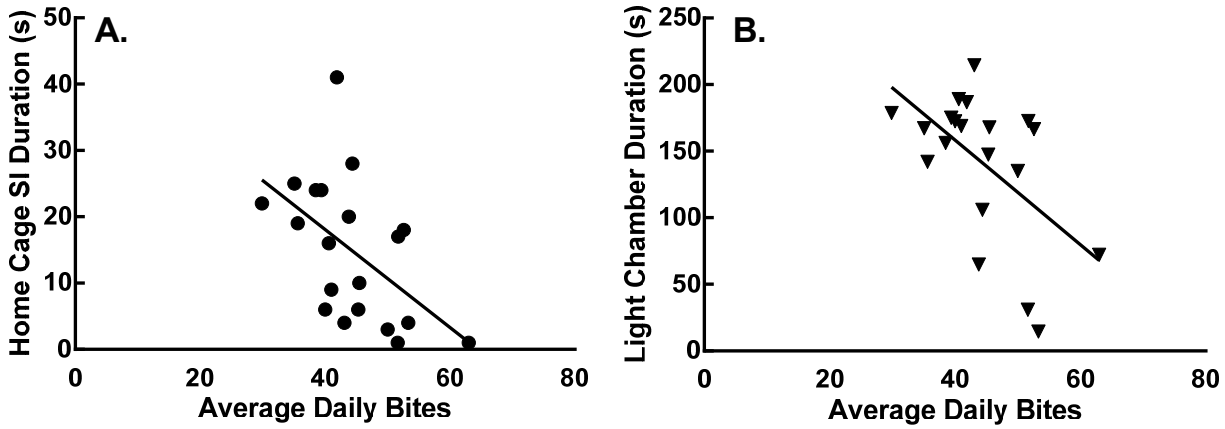


Figure S9. Attack bites received by chronically defeated females predicted the (**A**) duration of home cage social interactions ($F(1,18)=6.86$, $p=0.017$) and (**B**) time spent in the light chamber of the light dark box ($F(1,18)=7.74$, $p=0.012$). Lines generated from linear regression analyses; symbols represent individual mice.

Figure S10.

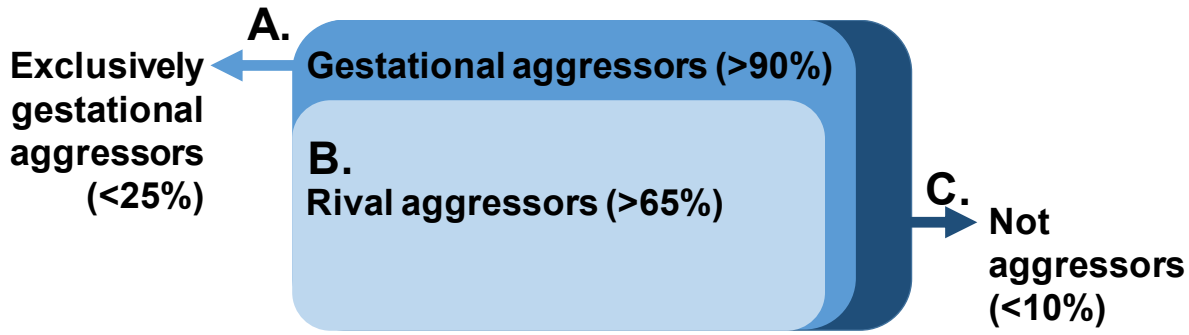


Figure S10. Schematic representation of the aggressive subgroups identified in a sample of intact, Swiss-Webster (CFW) females. Individuals can be categorized generally into one of three subgroups: (**A**) aggressive during gestation only (<25%), (**B**) aggressive both during gestation and outside of the gestational period (i.e., rival aggressors; >65%), and (**C**) non-aggressive even during pregnancy (<10%).

Table S1. Correlations in c-Fos activation between brain regions

				Control Females			
	MeA	**LSv**	**hPVN**	**VMHvl**	**PeN**	**amBNST**	**DG**
MeA	-						
LSv	0.48	-					
hPVN	0.85	0.68	-				
VMHvl	0.64	0.84	0.77	-			
PeN	-0.19	***-0.95***	-0.44	-0.75	-		
amBNST	***0.89***	0.06	0.68	0.27	0.26	-	
DG	0.29	-0.09	0.00	0.40	0.09	0.21	-
				Acutely Defeated Females			
	MeA	**LSv**	**hPVN**	**VMHvl**	**PeN**	**amBNST**	**DG**
MeA	-						
LSv	-0.26	-					
hPVN	-0.16	0.55	-				
VMHvl	-0.07	0.46	0.63	-			
PeN	-0.14	0.42	0.44	0.52	-		
amBNST	***-0.97***	0.20	0.19	0.20	0.04	-	
DG	-0.01	0.42	0.58	-0.17	-0.21	-0.03	-
				Chronically Defeated Females			
	MeA	**LSv**	**hPVN**	**VMHvl**	**PeN**	**amBNST**	**DG**
MeA	-						
LSv	0.53	-					
hPVN	0.25	0.60	-				
VMHvl	-0.47	0.33	-0.18	-			
PeN	0.47	-0.21	-0.74	-0.20	-		
amBNST	-0.28	0.40	0.85	0.18	***-0.98***	-	
DG	-0.18	0.35	0.90	-0.01	***-0.95***	***0.98***	-

Pearson's *r* values; bold italicized values indicate significant correlations with $p < 0.05$; medial amygdala, MeA; ventral lateral septum, LSv; hypothalamic paraventricular nucleus, hPVN; ventrolateral division of the ventromedial hypothalamus, VMHvl; periventricular nucleus, PeN; anteromedial bed nucleus of the stria terminalis, amBNST; dentate gyrus, DG

Table S2. Behavioral correlations in control and chronically defeated females

	Control Females				
	HC SI	**OF SI**	**OF SI Hyper**	**LDB**	**HC NO**
HC SI	-				
OF SI	0.44	-			
OF SI Hyper	-0.33	-0.40	-		
LDB	0.02	-0.42	0.33	-	
HC NO	0.14	-0.33	0.36	***0.73***	-
	Chronically Defeated Females				
	HC SI	**OF SI**	**OF SI Hyper**	**LDB**	**HC NO**
HC SI	-				
OF SI	0.40	-			
OF SI Hyper	-0.26	***-0.78***	-		
LDB	0.36	0.17	-0.04	-	
HC NO	0.43	0.28	-0.27	0.41	-

Pearson's r values; bold italicized values indicate significant correlations with $p < 0.05$; home cage social interaction time, HC SI; open field social interaction time, OF SI; time engaging in hypervigilance-like behavior during the open field social interaction test, OF SI Hyper; light dark box time in light chamber, LDB; home cage novel object investigation time, HC NO

Supplement References

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