SUPPLEMENTARY INFORMATION

Gains and Losses: Resilience to Social Defeat Stress in Adolescent Female Mice

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Supplementary Methods and Materials

Animals

All mice were housed in a temperature-controlled and humidity- controlled (21–22 °C; ~60%) colony room of the Neurophenotyping center of the Douglas Mental Health University Institute, on a 12/12 h light/dark cycle (light on at 8 A.M.). All mice had *ad libitum* access to food and water throughout the experiments (except during food restriction for Go/No-Go experiments). Female C57BL/6J wildtype mice arrived at postnatal day (PND) 21 and were assigned randomly to each experimental condition at PND25 (Jacksons Laboratories; *n* = 171). Mice were housed in groups of four or five animals per cage before exposure to AcSD and single-housed after AcSD. Male CD-1 retired breeder mice (more than three months of age) were obtained from Charles-River Canada and used as aggressors in the AcSD paradigm. CD-1 mice were used for a maximum of three consecutive experiments and for no longer than three months. CD-1 mice were single housed throughout the study. Adult male C57BL/6 wild-type mice (>PND65) and adolescent female C57BL/6 wild-type mice (three weeks old) used for screening and priming, were obtained from Charles-River Canada. Only female C57BL/6J mice were used in the experiments except for a cohort of

adolescent C57BL/6J male mice used in the experiments aimed at reproducing "female" pattern of attacks.

Accelerated social defeat stress paradigm for adolescent female mice

To study early adolescent social stress in female mice, we used the social defeat stress paradigm (Fig. 1 A) that we employed in our previous studies in male mice (1,2), but made modifications for early adolescent females. Table 1 shows how we operationally defined an "attack" in AcSD.

CD-1 mice priming and screening for aggressive behavior: Before the AcSD sessions, CD-1 mice were primed for aggressive behavior in two phases. In phase 1, an adult (>PND65) male C57BL/6 mouse was introduced into their home cage for 3 min or until 10 consecutive attacks, whichever came first on day one. Latency to attack and the number of attacks made by CD-1 mice were recorded, as well as the wounds received by the C57BL/6 mice. Adult male C57BL/6 mice that were severely injured were excluded from priming until they recovered. In the second phase (day 2), an adult (>PND65) male C57BL/6 mouse was introduced to a CD-1 mouse's home cage for one min or until it was attacked 10 times, and immediately after it was replaced by an adolescent (PND23–PND28) C57BL/6 female mouse for 5 min. This was done twice a day (9:00 and 14:00) for a total of five days or until the CD-1 mice became consistently aggressive toward the adolescent C57BL/6 female mice. Only CD-1 mice that attacked the adolescent C57BL/6 female mice at least five times on at least 2 consecutive days were selected as aggressors for the AcSD procedure. Adolescent female mice used during the priming phase are not used in the rest of the experiment.

Social defeat sessions: The AcSD apparatus used is the same as the one described previously (1,2). Briefly, mice were housed in a transparent rat cage with two mouse housing compartments separated by a

transparent and perforated central divider that allowed sensory but not physical contact between mice. The CD-1 mice that were selected for aggressive behavior in the screening phase were housed in one of the two compartments for at least 48 h before the beginning of AcSD to enhance territorial behavior. One day before the experiment, we repeated the priming procedure to verify that CD-1 mice were still aggressive toward adolescent C57BL/6J female mice. The AcSD protocol consisted of two sessions per day, one 9:00 and the second one at 14:00, for a total of four days. In each session, an adult C57BL/6 mouse was introduced into the CD-1 compartment for a period of 30 seconds (to prime the CD-1 for aggressive behavior) and was immediately replaced by an experimental adolescent (PND25) C57BL/6J female mouse, which was left with the CD-1 for 10 min or until 10 attacks occurred, whichever came first. At the completion of each session, the female adolescent mouse was housed in the neighboring compartment to provide psychological stress. Adolescent female mice were exposed to a new aggressor every session. There were 8 sessions of social defeat in total, animals that received 0 attacks in four or more social defeat sessions were excluded from all analyses (9/171 mice). The latency and the number of attacks made by CD-1 mice were recorded as well as the wounds received by the C57BL/6 mice. Adult C57BL/6 mice that were used for priming and that had severe wounds were retired from priming and replaced with a new mouse. Control C57BL/6J adolescent females were housed in similar two-compartment rat cages with a conspecific every day and no physical interaction occurred between conspecific mice. After the final AcSD session, all mice were single housed in standard mice cages for the remainder of the experiments.

AcSD with "limited attacks" in adolescent male mice: The number of attacks received by female adolescent mice in the AcSD were found to be fewer than those previously recorded in adolescent males (as defined by our operational definition of an attack (1,2)). We therefore performed an experiment where we exposed male adolescent mice (n = 10) to a "female" pattern of attacks, by matching the number of

attacks and the length of time with the CD-1 to those recorded in females. The number of attacks were controlled using a ruler to separate the mice.

Social Interaction Test (SIT)

Twenty-four hours after the last AcSD session, C57BL/6J adolescent female mice were assessed in the SIT to measure approach and/or avoidance behavior toward a social target (3,4). The SIT consisted of two sessions of 2.5 min each, in which defeated, and control mice were allowed to explore a squared arena (42 × 42 cm). In the first session, an empty wire mesh enclosure was located against one of the walls of the arena to determine baseline exploration. In the second session, an unfamiliar CD-1 mouse was placed inside the wire mesh enclosure. The area that surrounded the enclosure was designated as the social interaction zone (14 × 9 cm), and the corners of the wall opposite to the enclosure were designated as corners (9 × 9 cm) and represented the farthest point from the social interaction zone. The time spent (in seconds) in the interaction zone and the corners was estimated during both sessions of the test. The time in between session 1 and session 2 is approximately 1 min. The social interaction ratio was calculated as the time spent in the interaction zone with the CD-1 aggressor present divided by the time spent in the interaction zone with the CD-1 aggressor absent. Defeated mice with a ratio < 1.00 were classified as susceptible and with a ratio >1.00 were classified as resilient. To ensure that high social interaction ratios reflected interest in the social target, mice that did not spend at least 60 seconds inside the interaction zone during the habituation phase were excluded from all analyses (3/171 mice). Mice with outlier interaction ratio were excluded from the analysis (2/171 mice). The SIT was performed under red light conditions between 11:00 and 16:00 and mice were tested in a counterbalanced order. Mouse behavior was recorded with an overhead video camera for offline analysis using the software TopScan^{TM} 3.0 (Clever Systems Inc.)

SIT with an awake versus an anesthetized CD-1 target: A separate cohort of female mice exposed to AcSD (n=17) or control conditions (n=5) in early adolescence, were tested in the SIT 24h later using an awake CD-1 as target. Twenty-four hours later, the same mice were tested in the SIT, but using an anesthetized CD-1 target during the second phase of the test. This novel CD-1 mouse was anesthetized using a mixed solution containing ketamine 50 mg/kg, xylazine 5 mg/kg, acepromazine 1 mg/kg and administered i.p.

Elevated Plus Maze (EPM)

On day 6 (24 h after the SIT), female mice were tested in the EPM to assess risk-like taking behavior. The maze consists of four arms in the form of a cross, two with high walls and two with open arms that are elevated 50 cm from the floor. Adolescent C57BL/6J female mice (*n* = 58) were placed in the intersection of the four arms and were left to explore the maze for five minutes. The amount of time spent in the closed arms, open arms and center was be measured with the software Topscan^{TM} 3.0 (Clever Systems).

Go/No-Go task

To assess cognitive function in adulthood, we tested mice in the Go/No-Go task as previously described (1,2,5–7). Adult (PND 75 ± 10) female mice (*n* = 49) that underwent AcSD in adolescence were food restricted and started training for the behavioral task. Sessions took place in operant behavioral boxes (Med Associates, Inc., St Albans, VT, USA) that were equipped with a house light, an adjustable Sonalert module that produces a tone, two nose-poke holes (Right and Left) with a cue light within each hole, and a reward dispenser. Chocolate-flavored dustless precision pellets (BioServ) were used as a reward. The experimental procedure consisted of three stages: Stage 1-Discrimination training, Stage 2-Reaction Time training, and Stage 3-the Go/No-Go task. Animals were subject to one training or testing session per day.

Phase 1- Discrimination training: At the beginning of each session, the house light comes on and remains on for the entire 20-min session. During each session, a cue light in a nose-poke hole is presented for 9 seconds. If a mouse responds by nose-poking on the illuminated hole during this period, a chocolate food pellet is dispensed, and the trial is counted as a "reward" trial. If the mouse does not respond by nose-poking on the illuminated hole, the cue light is extinguished for inter-trial interval (ITI) of 10 sec. The location of the cued (active) nose-poke hole (either left or right) is counterbalanced within groups and remains the same for each mouse for the entire experiment. Responses to the active nose-poke hole when the cue light is off and responses to the non-active nose-poke hole – which is never illuminated – do not result in a reward but are recorded. Mice advanced to the second phase of training after reaching a criterion of >70% responses to cued trials.

Phase 2- *Reaction time training*: To receive a food reward, mice need to nose-poke only following the illumination of the cued light, and within 5 seconds of the cue illumination. Each 30-min session starts with a pretrial period during which the house light is illuminated for a variable amount of time (3, 6, or 9 seconds) *without* the cue light. If a mouse nose-pokes during this pretrial period, the house light is extinguished, and a 10 s inter-trial interval (ITI) begins. This is recorded as a "premature response" and is considered a measure of waiting impulsivity (8–10). If the mouse does not respond during the pretrial period, the cue light is then illuminated for 5 seconds. A response within this timeframe results in the delivery of the food reward, but failure to respond results in the extinguishing of the house lights and the beginning of ITI of 10 seconds. Mice advance to the next task when reaching <50 % of the pretrial periods with a premature response.

Phase 3- Go/No-Go task: Mice are tested in 30-min daily sessions for a total of 14 days. To receive the food reward, mice are required to respond to a 'Go' cue (an illuminated nose-poke hole) within a 3-second

timeframe, or to inhibit their response when this cue is presented simultaneously with an auditory 'No-Go' cue (a 80 dB acoustic tone lasting 3-seconds). If mice respond during the 'No-Go' trial, an ITI begins, and no reward is delivered. Responses to the Go cue are recorded as "Hits" and responses to the No-Go cue are counted as "Commission Errors" and are considered a measure of action impulsivity (8–10). A randomized variable pretrial period of 3-9 seconds precedes each trial. Within each session, the number of 'Go' and 'No-Go' trials are given in an approximately 1:1 ratio and are presented in a randomized order. Each session consists of ~30-50 'Go' and 30-50 'No-Go' trials.

Western blot and quantitative PCR (qPCR)

Tissue dissection: Adolescent female mice from the control, susceptible and resilient groups (n = 23) were euthanized by decapitation 7 d after the SIT. Brains were removed and flash frozen with 2-methylbutane chilled in dry ice. Bilateral punches of the ventral tegmental area (VTA) and the nucleus accumbens (NAcc) were taken from 1 mm-thick coronal sections, starting from plate 55 for VTA and plate 14 for NAcc, of the mouse brain atlas (11).

RNA extraction and qPCR: As previously described (1), total RNA fractions were isolated from the frozen brain tissue with the miRNeasy Micro kit protocol (QIAGEN). All RNA samples were determined to have 260/280 and 260/230 values ≥ 1.8, using the Nanodrop 1000 system (Thermo Scientific). Reverse transcription for *Dcc* mRNA was performed using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time qPCR was performed in technical triplicates using TaqMan assay (Applied Biosystems) using an Applied Biosystems QuantStudio 6 Flex Real-Time PCR system. Data for *Dcc* mRNA expression were analyzed using the relative quantification method with *Gapdh* as reference gene.

Western blot: As previously described (1), protein samples (20 mg, *n* = 24) were separated on a 10% SDS-PAGE and transferred to a PVDF membrane which was incubated overnight at 4 °C with antibodies against Netrin-1 (Abcam, catalog #126729) and alpha-tubulin (Cell Signaling, catalog #2144S) for the loading control. Since Netrin-1 is a secreted protein which can be produced remotely (12), we routinely quantify protein rather than mRNA levels (1,13).

Neuroanatomical experiments and stereology

Brain samples were obtained from a separate cohort of adult (PND70±15) female mice (*n* = 24) that were exposed to AcSD in adolescence or served as controls. They were anesthetized i.p. with a rodent cocktail containing ketamine 50 mg/kg, xylazine 5 mg/kg, acepromazine 1 mg/kg and were perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). Coronal sections of the pregenual PFC were obtained at 35 µm using a vibratome. Immunostaining was visualized with Alexa Fluor 594-conjugated secondary antibody raised in rabbit (Alexa Jackson).

As described previously (5,14,15), the density of tyrosine hydroxylase (TH)+ fibers and varicosities in the inner layers of the cingulate (Cg), prelimbic (PrL) and infralimbic (IL) regions of the pregenual medial PFC were evaluated using a stereological fractionator sampling design (16), with the optical fractionator probe of the Stereoinvestigator software (MicroBrightField). In the PFC, the TH antibody labels predominantly DA axons and rarely labels norepinephrine axons (14,17). Regions of interest were delineated according to the mouse brain atlas (11), and contours of the TH+ projection within these regions were traced at 5x magnification with a Leica DM4000B microscope. Stereoinvestigator calculates, for each brain region, a volume (in cubic micrometers) measure from the contour area, a section thickness, and a section periodicity (MicroBrightField). Sections spanning plates 14– 18 of the mouse brain atlas (11) were studied. Stereoinvestigator calculates the total number of TH+ varicosities based on the experimenter's random sampling of a known fraction of the region. Counting frame and grid size were chosen to consistently sample 33 sites per region.

Experimental design and statistical analyses

The data derived from the EPM, stereological and Go/No-Go experiments were gathered from separate cohorts of female mice. All mice underwent the SIT in adolescence, 24 h after the last defeat or housing control session. The data from these tests were pooled together from all cohorts and can be found in Supplemental figure S1. Mean values are presented as mean ± SEM. The significance threshold was set at $\alpha = 0.05$. Data that were normally distributed and with similar variance across groups were analyzed with one-way or two-way ANOVAs. Significant main effects and/or interaction effects were followed by Holm–Sidak *post hoc* tests. Data that were not normally distributed or with heterogeneous variances were analyzed with a Kruskal–Wallis non-parametric test or a Brown–Forsythe's ANOVA, respectively, followed by Dunn's or Dunnett's T3 *post hoc* tests. Outliers were identified using the ROUT method with a Q = 1%. Correlations were calculated using the Pearson's correlation coefficient with 2-tailed analysis. The binomial and chi-squared tests were used to analyze categorical data. Additionally, hierarchical cluster analysis was conducted on the proportion of commission error data obtained from the Go/No-Go task using the Ward method and squared Euclidean distance as a measure of similarity (18,19). The statistical tests were conducted using GraphPad Prism (GraphPad Software) and SPSS software.

Supplementary Figures

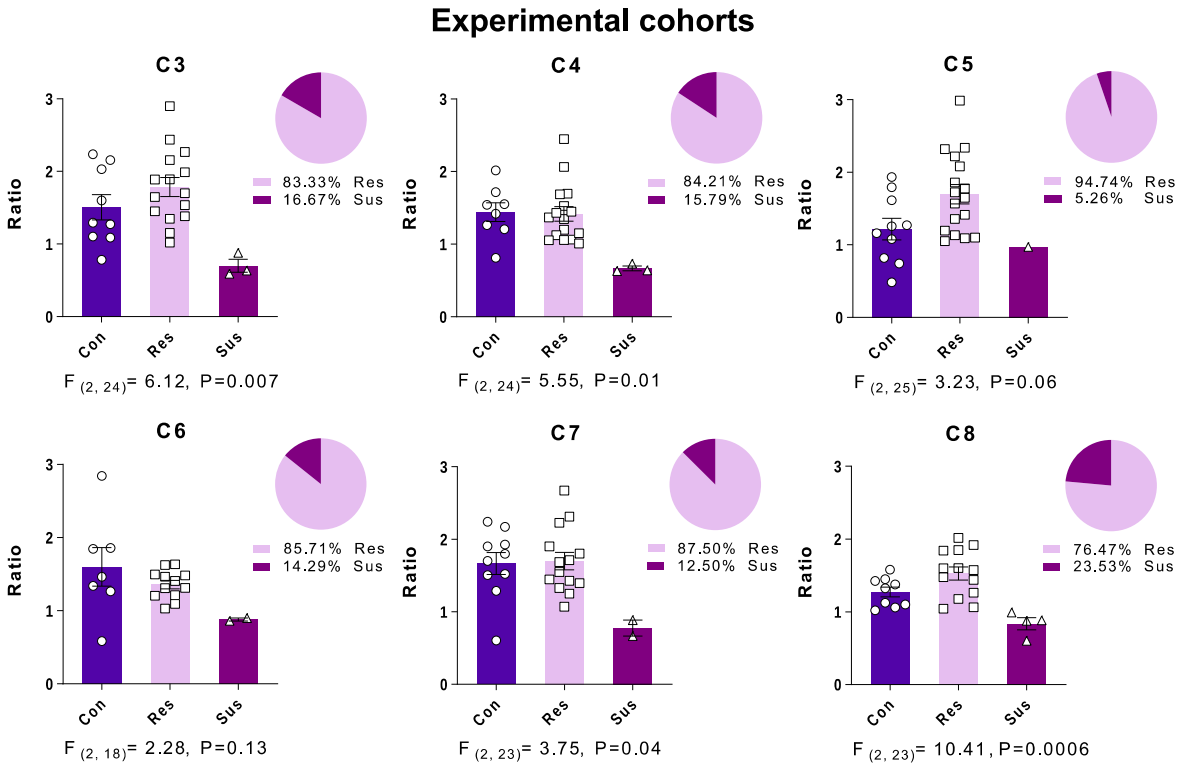

Figure S1. **Individual SIT of each experimental cohort and proportion of res/sus mice.** Cohorts C3 and C8 were used for molecular experiments (*Dcc* mRNA and Netrin-1 protein). Cohorts C4 and C6 were used for behavioral experiments. Cohorts C5 and C7 were used for neuroanatomy and stereological quantification. Data were analyzed with one-way ANOVA. All data are shown as mean ± SEM.

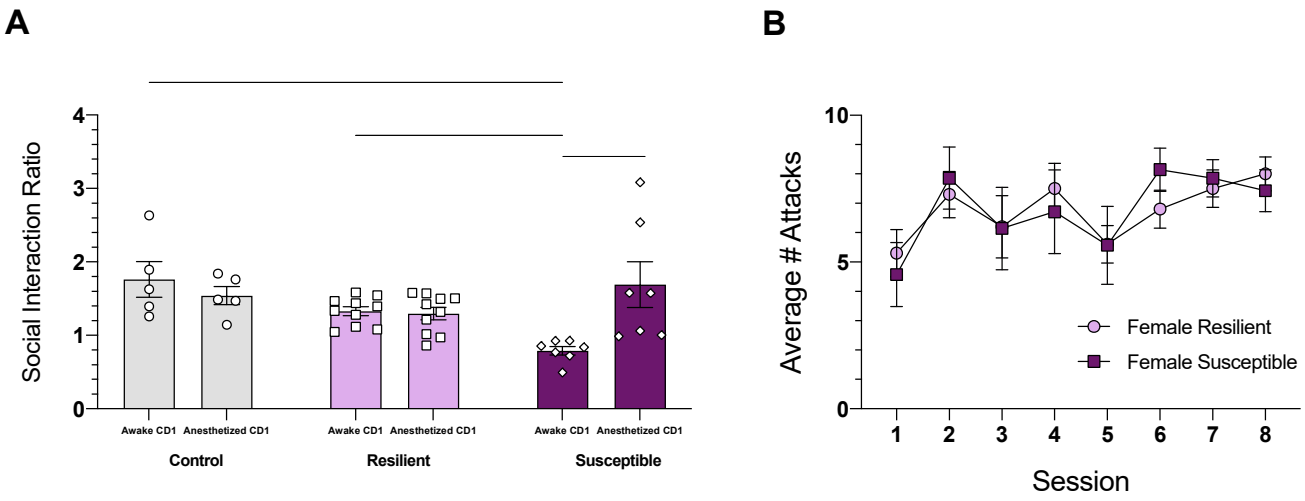

Figure S2: **An awake social target is necessary for susceptible female mice to show social avoidance in adolescence**. Twenty-four hours after undergoing AcSD (n=17) or control conditions (n=5) in early adolescence, female mice underwent a SIT, in which a subset group showed social avoidance to the CD-1 target. The following day, female mice underwent another SIT, but during the second phase of this test, an anesthetized CD1 mouse was placed in the mesh enclosure. **A)** Susceptible females show social avoidance in the presence of an awake, but not anesthetized CD-1 target. (Two-way repeated measures ANOVA, CD-1 state x phenotype interaction $F_{(2,19)}$= 7.411, *p*=0.004, Holm–Sidak *post hoc* tests: susceptible/awake vs susceptible/anesthetized, *p*=0.0016; control/awake vs susceptible/awake *p*=0.009; resilient/awake vs susceptible/awake *p*=0.0251) **B)** There is no difference in the number of received attacks between resilient and susceptible groups (Two way repeated measures ANOVA, session x phenotype interaction $F_{(7, 105)}$= 0.366, *p*=0.9201).

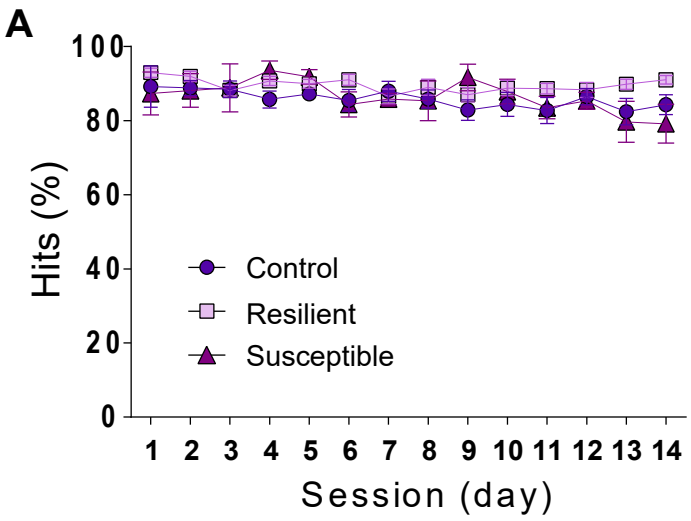

Figure S3. **No difference in female Go/No-Go Hits. A**, there are no significant differences in hits between groups following AcSD in females. Two-way repeated measures ANOVA, $F_{(26, 598)} = 1.34$, $p=0.12$. All data are shown as mean ± SEM.

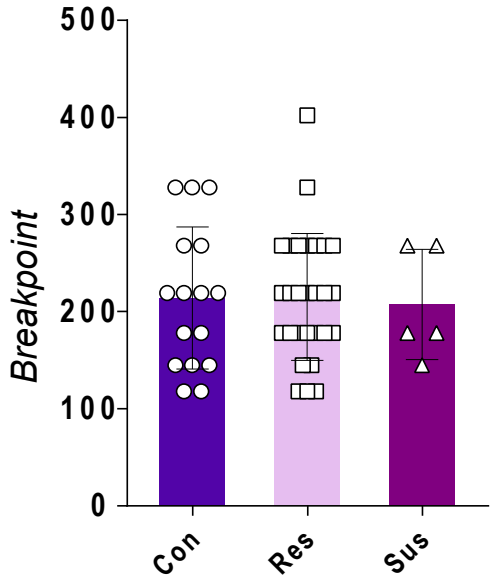

Figure S4. **No difference in breakpoint in female progressive ratio.** Progressive ratio following the Go/No-go task shows no differences across female groups. one-way ANOVA $F_{(2, 46)} = 0.03$, $p = 0.9$. All data are shown as mean ± SEM.

Males

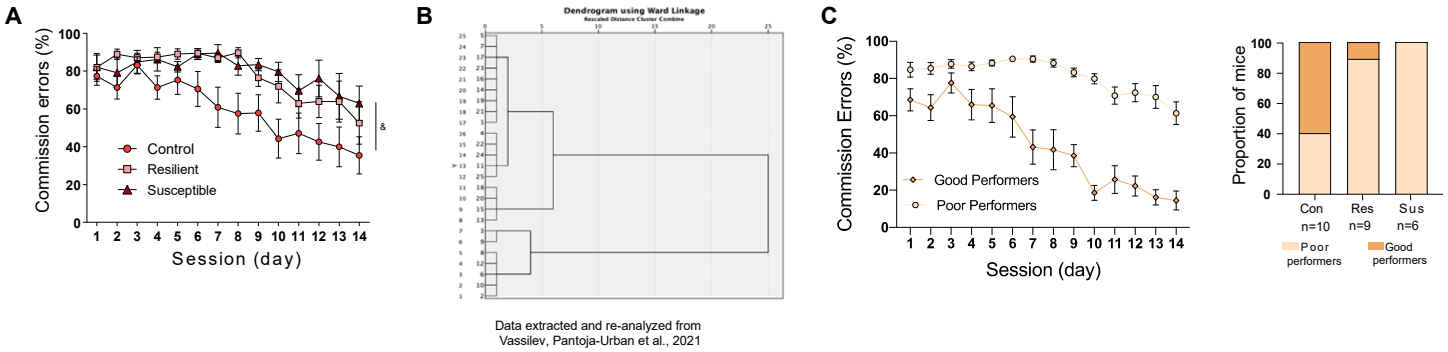

Figure S5. **Go/No-Go male data**. In male mice, AcSD in adolescence leads to deficits in inhibitory controls regardless of SIT phenotype**. A,** Data reproduced from (1) of the percentage of commission errors of adult male mice exposed to AcSD in adolescence and showing deficits in inhibitory control in all defeated mice, compared to controls, regardless of SIT in adolescence. **B,** dendrogram (k=2) showing hierarchical clustering of male subjects based on percentage of commission errors 14 days of the Go/No-Go task. **C,** Cluster analysis classified cases in two groups based on commission error scores: good and poor performers. Most male control mice (60%) were classified in the good performance cluster. However, almost all defeated mice were classified in the poor performance cluster (89% “resilient”; 100% “susceptible”; $\chi^2 = 8.7$, df = 2, $p = 0.01$).

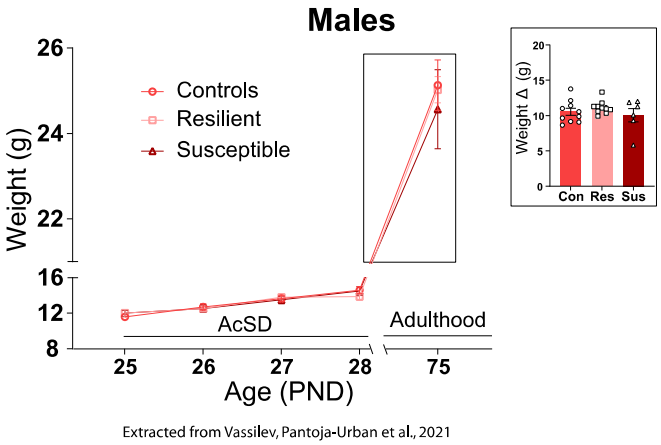

Figure S6. AcSD in adolescence does not alter body weight in male mice. Using data gathered from our published study in males (1), we analyzed body weight in adolescence and in adulthood and found no differences across groups (Data extracted from (1) see Fig. 6B, inset, Brown-Forsythe ANOVA $F_{(2,\ 10.37)} = 0.74$, $p = 0.5$).

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