Adolescent environmental enrichment induces social resilience and alters neural gene expression in a selectively bred rodent model with anxious phenotype.

***Angela M. O'Connor**, ***Megan H. Hagenauer,** Liam Cannon Thew Forrester, Pamela M. Maras, Keiko Arakawa, Elaine K. Hebda-Bauer, Huzefa Khalil, Evelyn R. Richardson, Farizah I. Rob, Yusra Sannah, Stanley J. Watson, Jr., Huda Akil

Univ. of Michigan, Ann Arbor, MI, USA

*Co-first authorship

Corresponding Author:
Angela O'Connor
oconnam@med.umich.edu

Abstract

Stress is a major influence on mental health status; the ways that individuals respond to or copes with stressors determine whether they are negatively affected in the future. Stress responses are established by an interplay between genetics, environment, and life experiences. Psychosocial stress is particularly impactful during adolescence, a critical period for the development of mood disorders. In this study we compared two established, selectively-bred Sprague Dawley rat lines, the "internalizing" bred Low Responder (bLR) line versus the "externalizing" bred High Responder (bHR) line, to investigate how genetic temperament and adolescent environment impact future responses to social interactions and psychosocial stress, and how these determinants of stress response interact. Animals were exposed to social and environmental enrichment in adolescence prior to experiencing social defeat and were then assessed for social interaction and anxiety-like behavior. Adolescent enrichment caused bLR rats to display less social avoidance, more social interaction, less submission during defeat, and resilience to the prolonged effects of social stress on corticosterone, while enrichment caused bHR animals to show greater aggression during defeat and during a neutral social encounter, and decreased anxiety-like behavior. To gain insight into the development of social resilience in the anxious phenotype bLRs, RNA-seq was conducted on the hippocampus and nucleus accumbens, two brain regions that mediate stress regulation and social behavior. Gene sets previously associated with stress, social behavior, aggression and exploratory activity were enriched with differential expression in both regions, with a particularly large effect on gene sets that regulate social behaviors. These findings provide further evidence that adolescent enrichment can serve as an inoculating experience against future stressors. The ability to induce social resilience in a usually anxious line of animals by manipulating their environment has translational implications, as it underscores the feasibility of intervention strategies targeted at genetically vulnerable adolescent populations.

Keywords: environmental enrichment; social stress; adolescence; genetic environment interactions

1. Introduction

Social stress is a major predictor for the development of future mood disorders, changing both short-term behavioral responses and longer-term developmental trajectories and coping mechanisms [1, 2].Resilience or susceptibility to social stress shapes how individuals respond to these experiences and the likelihood of impact on future behavior and brain function [3, 4]. Social stress resilience is determined by an interplay of genetics and environment prior to encountering a stressor [1, 5], and may be modifiable by these same factors [3]. The impact of developmental and environmental factors on resilience and susceptibility to social stress has been studied in mice using behavioral screening [6-9]. Less is known about how genetic predisposition and social temperament influence the experience of social stress, as well as the response to interventions designed to increase social resilience, including adolescent social experience.

The bred High Responder (bHR) and bred Low Responder (bLR) rat lines robustly model heritable extremes in temperament [10]. Bred based on locomotor reactivity to a novel environment, bHRs exhibit an “externalizing-like” temperament, with disinhibited, hyperactive, and sensation-seeking behavior, while bLRs exhibit an “internalizing-like” temperament, with inhibited, hypoactive, anxious- and depressive-like behavior [10-13]. These bred lines also differ in their response to stressors, including social stress [13-16], enabling insight into the genetic, molecular and circuit mechanisms that underlie variability in stress responses [13, 17, 18]. Notably, social interaction styles reliably differ between the two lines; bHRs display more aggressive, bold social behaviors and bLRs exhibit more defensive, submissive social behaviors [18, 19].

The divergent temperament in bHR and bLR rats is reflected in differences in brain gene expression [20-24]. Recent work has uncovered genetic differences that underlie these distinct phenotypes [25, 26]. The consistency, stability and predictability of these bred phenotypes across generations [11, 27], along with their innate differences in stress response [13] and social behavior [18, 19] make bLR and bHR animals an excellent model for assessing the interaction of genetics, social stress, and environmental influences, including social experience during adolescence.

Environmental enrichment, where animals are housed in complex caging with increased opportunity for sensory stimulation, motor activity and social interaction, can effectively decrease the incidence of anxiety-like behaviors within various animal models [28-32]. Enrichment has long been considered a “eustressor”, the experience of which inoculates against subsequent larger stressors [29, 33]; it is unknown how much genetic vulnerability or stress resilience determines the efficacy of this stress buffering effect. Two previous studies have tackled this question using the bHR/bLR lines; exposure to enrichment during adulthood reduced anxiety-like behavior in the bLR line [34]. Adult enrichment also shifted social behavior,

decreasing aggression in bHR animals and increasing positive-affect ultrasonic vocalizations produced by bLR animals [19].

These previous studies focused on enrichment during adulthood, but adolescence is a critical period for the development of mood disorders, with most mood disorder diagnoses occurring between the ages of 12-18 years [35]. During adolescence, emotional, social, and cognitive circuits undergo a critical period [36-38], allowing social stress reactivity and resilience to be molded by social and environmental conditions [39].

The current study focuses on the impact of adolescent social and environmental enrichment on social interaction, anxiety-like behavior, and social stress resilience in our bred lines to provide insight into how genetics, environment and stress interact. To characterize the hormonal changes accompanying shifts in social behavior, we measured circulating levels of corticosterone, testosterone, oxytocin, and interleukin-6. To characterize alterations in affective circuitry, we used RNA-seq to measure gene expression within the Nucleus Accumbens (NAcc) and Hippocampus (HC). These brain regions are both impacted by stress [40, 41] and implicated in mediating stress resilience [42, 43] and social behavior [44, 45]. Thus, the current work aims to elucidate the role of these two important brain regions in mediating the interplay between genetic, developmental and environmental factors in shaping social vulnerability or resilience.

2. Methods

All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals and comply with ARRIVE guidelines. All efforts were made to minimise animal suffering and the number of animals used in our experiments.

2.1 Experimental Animals:

We used male rats from generations F49, F53 and F56 of our in-house selectively bred bHR and bLR lines [10] (*n* for each experiment: **Fig S1**). Litters were culled on postnatal day 1 (P1) to even sex ratios (minimum litter size: 3M/3F, maximum: 6M/6F) (**Fig 1A**). Litters were weaned at P21 and males were pair- or triple-housed with littermates. Animals were housed on a 12:12 hour light:dark cycle (lights on: 7am) with *ad libitum* access to water and food.

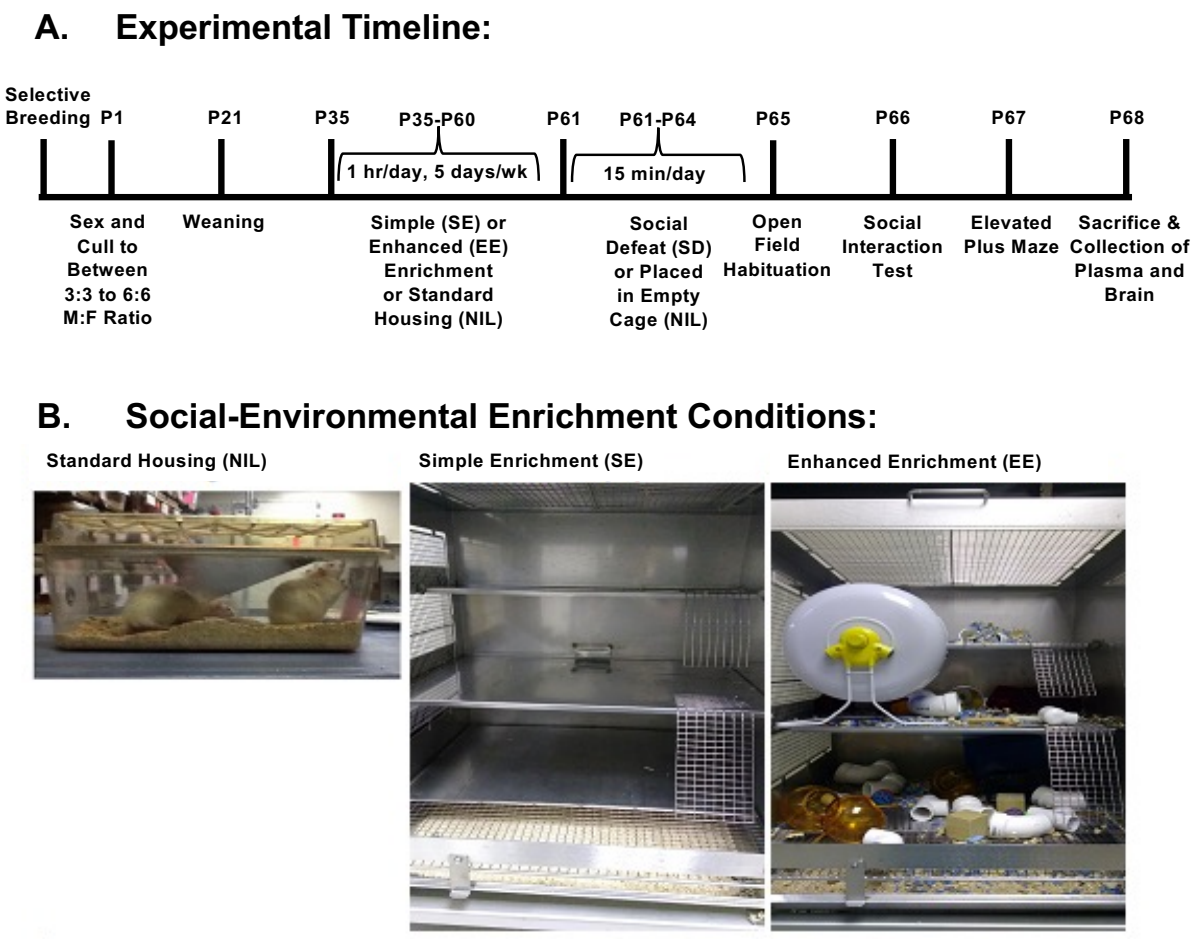


Fig 1: Behavioral Methodology

A. Experimental timeline outlining the timing of behavioral interventions and testing. The day of birth is considered postnatal day 0 (P0). **B.** Examples of the standard ("NIL"), simple enrichment ("SE") and enhanced enrichment ("EE") cages. Enrichment cages consisted of a large (50x40x50cm) cage with three separate levels connected by mesh ramps. The EE condition also contained various objects that were added to the cage and moved around over the duration of enrichment period, including running wheels, plastic and cardboard tunnels, plastic igloo houses, Nylabones and dog chew toys. Different starting combinations of objects were used each week. All animals from one litter (n=3-6) were placed into the same enrichment cage.

2.2 Adolescent Social-Environmental Enrichment

At P35, animals were assigned to standard housing (NIL), simple enrichment (SE) or enhanced enrichment (EE) (**Fig 1A**). SE and EE groups spent an hour a day in large enrichment cages (09:30–10:30 hrs, **Fig 1B**), 5 days/week from P35-P60. All animals from one litter (n=3-6) were placed into the same enrichment cage. The EE condition also contained various objects (**Fig 1B**)

that were added to the cage over each 5-day period and moved daily. All objects were cleaned between weeks (bleach+detergent).

2.3 Repeated Social Stress:

The repeated social stress paradigm consisted of a four-day training phase and four-day repeated stressor phase as previously described ([46], see Supplement). All training and social stress took place during the dark phase of the light cycle (between 19:00-00:00 hours).

During the training phase, male Long-Evans were trained to attack non-experimental outbred Sprague-Dawley intruders. During training they needed to reach aggression scores of at least 4 (scores: 1: non-aggressive social interaction; 2: lateral threat and rearing; 3: boxing/scuffling; 4: pinning; 5: pinning and attempted biting) to be used as an aggressor during the stressor phase. Long-Evans that were not included in the aggressor group were used as novel targets in the social interaction test.

From P61-64, the experimental bHR/bLR rats were each introduced individually to a Long-Evans aggressor's cage for a daily 15-minute stressful social encounter. The bHR/bLR intruder could move freely throughout the cage until an aggressive interaction (score>3). Intruders were then placed into a protective wire mesh container (10x10x15cm) within the Long-Evans cage for the remainder of the trial.

Each day, each bHR/bLR intruder was exposed to a different Long-Evans aggressor. Any rat that sustained physical wounds during social defeat was excluded from the study. Experimental bHR/bLR rats that were in the no defeat group ("NIL") were placed in a clean, empty novel cage within the same testing room and allowed to move freely for the 15-minute period.

Video recordings of behavior during the social stress sessions were hand-scored by a blind observer using The Observer XT software (Noldus Information Technology), with bHR/bLR intruder behaviors classified as submissive or aggressive according to [47, 48], and normalized as a percent of the total trial time prior to physical separation from the Long-Evans aggressors (maximum: 15 min).

2.4 Social Interaction Test:

On P65-66 during the light period (07:00-11:00 hours), experimental animals underwent a social interaction test consisting of two 5-minute trials on consecutive days (Day 1: habituation, Day 2: testing). The testing arena was a white Plexiglass open field (100x100cm, dim lighting: 40 lux), which was cleaned (70% ethanol) after every animal. The bHR/bLR rats were placed into the center of the testing field on both days. On Day 2, there was a caged novel stimulus male Long-Evans rat ("target") present, with zones of the open field predefined as target, interaction, and social avoidance zones. A video tracking system (Ethovision XT 11.5, Noldus Information Technology) calculated the percent time the bHR/bLR rats spent in each zone. Precise location

and social behavior were observed by hand-scoring videos (The Observer XT software, Noldus Information Technology).

2.5 Elevated Plus Maze (EPM) Test:

On P67 during the light period (between 07:00-11:00 hours), animals underwent the EPM. The EPM consists of four intersecting black Plexiglass arms (45cmx12cm) shaped like a cross, elevated 70cm from the floor. Two opposite arms are enclosed (45cm walls) and two remain open; at the intersection a square platform (12x12cm) allows access to all arms. During the five-minute test, the room was dimly lit (40 lux) and animals' behavior monitored using a video tracking system (Ethovision XT 11.5, Noldus Information Technology) that recorded latency to enter the open arms, the amount of time spent in the open arms and centre square. The testing apparatus was cleaned (30% ethanol) after every animal.

2.6 Tissue and Blood Collection

On P68 during the light period (between 14:00-17:00 hours), animals habituated to a new room (>30 minutes), then were removed to the sacrifice room and immediately decapitated without anaesthesia. Trunk blood was collected in EDTA tubes and placed onto ice before centrifugation (3000 rpm, 4°C, 10 minutes). Plasma supernatant was stored at -80°C. Brains were dissected within 2 minutes of death, flash-frozen (-30°C), and stored at -80°C.

2.7 Corticosterone ELISA

Plasma corticosterone levels were measured using an enzyme immunoassay (EIA) kit (Arbor Assays catalogue# K014 https://www.arborassays.com/). Immediately prior to EIA, 5µl of plasma from each sample underwent dissociation following kit protocols. Samples and freshly prepared corticosterone dilution standards were pipetted into well plates in duplicate and kit protocols followed. The optical density (450nm) of each well was determined in a plate reader. Plasma corticosterone concentrations were calculated using Arbor Assays' calculator (https://www.myassays.com/). See supplement for other hormone assays (testosterone, oxytocin, interleukin-6).

2.8 RNA-seq tissue extraction and data processing

All brains were hemi-sected (groups counter-balanced by side), and one half dissected within a cryostat (-20°C). The NAcc (+1.5mm to +1mm Anterior-Posterior [49]) was extracted by 2mm hole-punch, and whole dorsal HC (-3mm to -4mm Anterior-Posterior [49]) extracted using dissection tools.

Nucleotides were extracted from HC and NAcc tissue from a subset of bLR animals (NIL+NIL, NIL+SD, EE+NIL, EE+SD, sample sizes: **Fig S1**), using Qiagen AllPrep DNA RNA miRNA Universal Kit 50. Extracted RNA was evaluated for total concentration and quality using a Nanodrop spectrophotometer (concentration range: 285-432 ng/ul, 260/280 ratio range: 1.61-1.80). Samples were then processed by the University of Michigan DNA Sequencing Core (https://brcf.medicine.umich.edu/cores/dna-sequencing/). RNA integrity (RIN) was assessed using the TapeStation automated sample processing system (Agilent, Santa Clara, CA). Samples with RINs<8 were excluded. A DNA library targeting polyadenylated transcripts was constructed for each sample (100ng total RNA) with the KAPA hyper mRNA stranded library prep kit (Roche, catalogue# KK8581) in a 12-cycle PCR. Final cDNA libraries were checked for quality by TapeStation (Agilent) and qPCR using Kapa's library quantification kit for Illumina Sequencing platforms (catalogue# KK4835, Kapa Biosystems, Wilmington MA). Samples were clustered and sequenced on a NovaSeq S2 Run (Illumina) with 80 samples/flow cell and 50 base paired end reads (targeted sequencing depth=45 million reads/sample) using NovaSeq S2 reagents.

Reads were aligned to genome assembly Rnor6 (STAR) and summarized into counts per transcript (featureCounts: Ensembl 96 annotation). Following quality control (*see Supplement*), differential expression for the variables of interest (Social Defeat, Enrichment) was calculated using the limma/voom method (*limma* v.3.32.5 [50, 51]) with observed precision weights in a weighted least squares linear regression using models that included region-specific technical co-variates (RNA concentration, RNA extraction batch, dissection batches – model selection procedure in Supplement):

NACC:

Model 1 ("M1: Main Effects Model"):

$$y \sim \beta_0 + \beta_1 SocialDefeat + \beta_2 Enrichment + \beta_3 RNAconc + \beta_4 RNAextractBatch + \beta_{5-6} DissectionBatches + \varepsilon$$

Model 2 ("M2: Interactive Effects Model"):

$$y \sim \beta_0 + \beta_1 SocialDefeat + \beta_2 Enrichment + \beta_3 (SocialDefeat * Enrichment) + \beta_4 RNAconc + \beta_5 RNAextractBatch + \beta_{6-7} DissectionBatches + \varepsilon$$

HC:

Model 1 ("M1: Main Effects Model"):

$$y \sim \beta_0 + \beta_1 SocialDefeat + \beta_2 Enrichment + \beta_{3-4} DissectionBatches + \varepsilon$$

Model 2 ("M2: Interactive Effects Model"):

$$y \sim \beta_0 + \beta_1 SocialDefeat + \beta_2 Enrichment + \beta_3 (SocialDefeat * Enrichment) + \beta_{4-5} DissectionBatches + \varepsilon$$

Contrasts were defined by treatment, with the intercept for Enrichment and Social Defeat set as "NIL". Standard error was moderated towards a global value using an empirical Bayes distribution (function *eBayes()).* Multiple comparison correction was performed to calculate false discovery rate (FDR or q-value [52]).

2.8.1 Functional Ontology:

We evaluated whether our differential expression results (pre-ranked by t-statistic) were enriched for genes representing particular functional, anatomical, and cell-type categories using fGSEA [53] (v.1.2.1, nperm=10000, minSize=10, maxSize=1000) and a custom gene set file (Brain.GMT v.2: 15,545 gene sets).

2.8.2 Analysis Code Availability:

Initial RNA-Seq data pre-processing was performed using a standard pipeline (MBNI Analysis Hub: https://ahub.mbni.org). All downstream analyses were performed in Rstudio (v.1.0.153, R v. 3.4.1) (code: https://github.com/hagenaue/bHRbLR_Enrichment_Stress_BehaviorAndHormoneData, https://github.com/hagenaue/bHRbLR_Enrichment_Stress_RNASeqData). Full statistical methods are in the Supplement.

3. Results

We used selectively-bred animals that show an internalizing (bLRs) or externalizing (bHRs) temperament to examine how genetic background and adolescent social and environmental enrichment interact to shape social behavior, anxiety, and endocrine responses to repeated social stress. We then used RNA-Seq to examine the effects of these interventions on gene expression in the anxious phenotype (bLR) animals in brain regions related to social and emotional behavior and stress regulation (NACC, HC).

3.1 Behavior during Social Defeat Depends on Genetic Temperament and Adolescent Experience

bHR and bLR rats in the social defeat (SD) group experienced 15 minutes of social defeat stress daily for four days by being placed as intruders into the cage of a larger, territorial Long Evans male. Behavior during these defeat sessions indicated that social defeat stress is not a uniform experience, but instead experienced through the lens of both genetic predisposition and previous social and environmental experience (**Fig 2, Fig S6,** *details in supplement*). Compared to bHRs, bLRs responded with greater submissive behavior during social defeat (**Fig 2,** Line: (X^2(1, N=70)=88.81, p=2.20e-16), and bLR submissive behavior increased with each daily defeat session (Day: X^2(1, N=70)=39.35, p=3.545e-10, Day*Line: X^2(1, N=70)=9.91, p=0.00165). Notably, the increase in submissive behavior in bLRs was reduced by adolescent exposure to enrichment (Enrichment: X^2(1, N=70)=8.21, p=0.0165, Line*Enrichment: (X^2(1, N=70)=3.42, p=0.0405). Conversely, bHRs showed more aggressive behavior than bLRs (**Fig S6**; Line: X^2(1, N=70)=60.99, p=5.735e-15), and bHR aggression increased with each daily defeat session (Day: X^2((1, N=70)=9.01, p<1e-16, Day*Line: X^2(2, N=70)=1.84, p=0.00525) in a manner that was enhanced by adolescent enrichment (Enrichment: X^2(2, N=70)=8.03, p=0.0180, Day*Line*Enrichment: X^2(2, N=70)=7.22, p=0.0270).

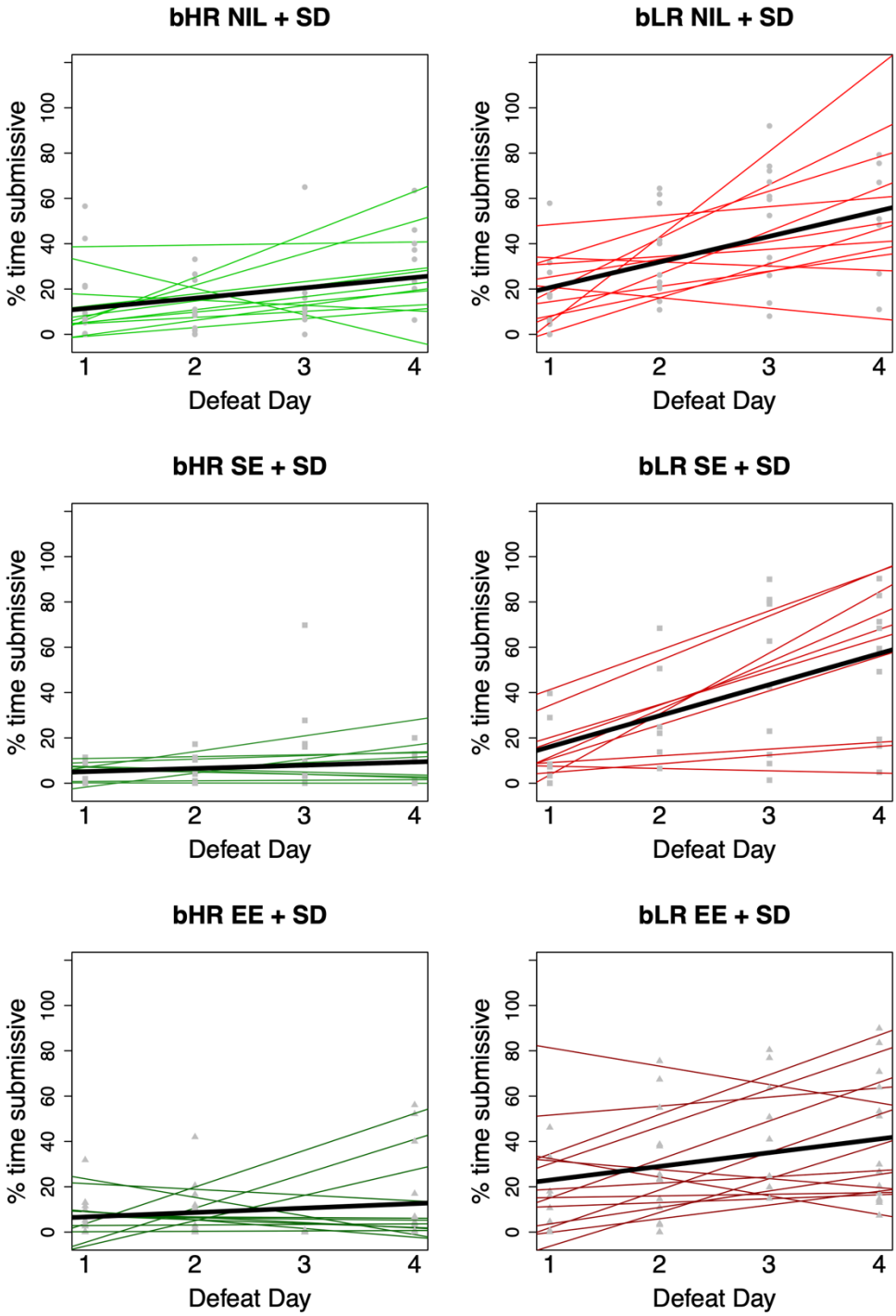


Fig 2. bLR rats responded submissively to social defeat and this increased with each social defeat session, but was moderated by enrichment during adolescence. *Grey dots=individual data points, thin red or green lines: best fit line for each rat across the four days of defeat, thick black line: best fit line for the experimental subgroup across the four days of defeat. Bred line is illustrated with color (bHR=green, bLR=red), and adolescent enrichment by datapoint shape*

(circle=standard housing (NIL), square=simple enrichment (SE), triangle= enhanced enrichment (EE)).

Social defeat sessions are complex dyadic interactions, and behavior of resident aggressors may be influenced by the phenotype of intruder rats. Indeed, territorial aggressive behavior displayed by the Long-Evans aggressors varied depending on whether they encountered a bHR or bLR intruder (**Fig S7**), with the most aggressive intruders receiving more severe defeat (% time aggressive vs. social defeat score: n=242 scored encounters, β=6.305, p=2.00e-16). Therefore, the more submissive bLRs received less severe defeat (lower social defeat scores) than the aggressive bHRs (main effect of Line: $X^2(1, N=75)=11.17$, p=0.000830, main effect of day: $X^2(1, N=75)=4.65$, p=0.0311, generation co-variate: $X^2(1, N=71)=90.32$, p<2.20e-16). Following full defeat (pinned: social defeat score 4 or 5), the experimenter intervened with a divider to prevent further direct interaction and potential injury. Since aggressive intruders elicited more intense territorial aggression, they tended to spend less time directly interacting with the resident Long-Evans (% time aggressive vs. time caged: n=242 scored encounters, β= -0.881, p<2e-16). Thus, the more-submissive bLRs spent slightly more time caged with the aggressor than the bHRs (main effect of Line: $X^2(1, N=71)=5.69$, p=0.0170, main effect of Day: $X^2(1, N=71)=8.06$, p=0.00453, generation co-variate: $X^2(1, N=71)=128.16$, p<2.20e-16).

Altogether, we conclude that the experience of social defeat stress differs dramatically for animals that are innately more submissive (bLRs) than animals that are more aggressive (bHRs) and can be modulated by adolescent social-environmental experience.

3.2 Exposure to adolescent enrichment altered bLR and bHR social behavior

The impact of our manipulations on social interactions in each of the two lines was assessed using a caged novel (neutral) Long-Evans stimulus animal in an open-field arena (**Fig 3A**). Overall, bLRs showed more socially avoidant behavior (reflected by time spent in the avoidance zones away from the social stimulus animal) than bHRs (**Fig 3B**; main effect of Line: F(1, 128)=22.37, p=6.667e-05; Generation co-variate: F(2,128)=4.575, p=1.233e-02). Previous experience with adolescent enrichment decreased social avoidance in both bHRs and bLRs (**Fig 3B;** main effect of Enrichment: F(2, 128)=4.57, p=1.247e-02)).

This decrease in social avoidance following enrichment did not necessarily translate into pro-social behavior, depending on bred line. Adolescent enrichment increased the percent time spent engaging in social interaction, especially for bLR rats, in a manner that seemed to scale with the complexity of the enrichment intervention (NIL<SE<EE, **Fig 3C**). This was evident in a detailed analysis of social approach behavior performed on a subset of the videos (generations F53, F56) by a scientist blinded to group status (main effect of Line: F(1,67)=3.501, p=0.0665*trend; main effect of Enrichment: F(2, 67)=2.965, p=0.0563*trend, interactive effect of Line*Enrichment: F(2, 67)=4.917, p=0.00940). This was also observed within an automated Ethovision analysis of the time spent in the zone nearby the stimulus animal's cage using videos

from all subjects (**Fig S8A**, main effect of Line: F(1, 128)=3.52, p=0.0625**trend*, main effect of Enrichment: F(2, 128)=10.51, p=0.0002; Generation Co-variate: F(2,128)=5.378, p=0.00633).

Adolescent enrichment also increased the percent time spent on top of the stimulus animal's cage, especially for bHR animals, in a manner that scaled with the complexity of the enrichment intervention (NIL<SE<EE, **Fig 3D**). This was evident in a detailed analysis performed on a subset of the videos (generations F53, F56) by a scientist blinded to group status (main effect of Line: F(1, 67)=52.88, p=6.667e-05; main effect of Enrichment: F(2,67)=41.15, p=6.667e-05; interactive effect of Line*Enrichment: F(2, 67)=21.18, p=6.667e-05; Generation co-variate: F(1,67)=18.120, p=1.333e-04). This was also shown with an automated Ethovision analysis quantifying the time spent within the zone on top of the stimulus animal's cage using the videos from all subjects (**Fig S8B,** main effect of Line: F(1, 128)=31.26, p=6.667e-05; main effect of Enrichment: F(2,128)=18.14, p=6.667e-05; interactive effect of Line*Enrichment: F(2, 128)=7.723, p=4.000e-04; Generation Co-variate: F(2,128)=8.168, p=5.333e-04). This behavior appeared aggressive, and was often accompanied by loud vocalizations, urinating on the Long-Evans rat, and biting the bars of the Long-Evans' cage.

Overall, adolescent enrichment made bLRs more interactive than avoidant, so that their behavior came to more closely resemble that typical of bHRs, who also displayed increased social aggression following adolescent enrichment. In contrast, social defeat did not have any residual effects on social behavior within this task in either line (p>0.09 for all effects of SD, SD*Line, SD*Enrichment, SD*Enrichment*Line).

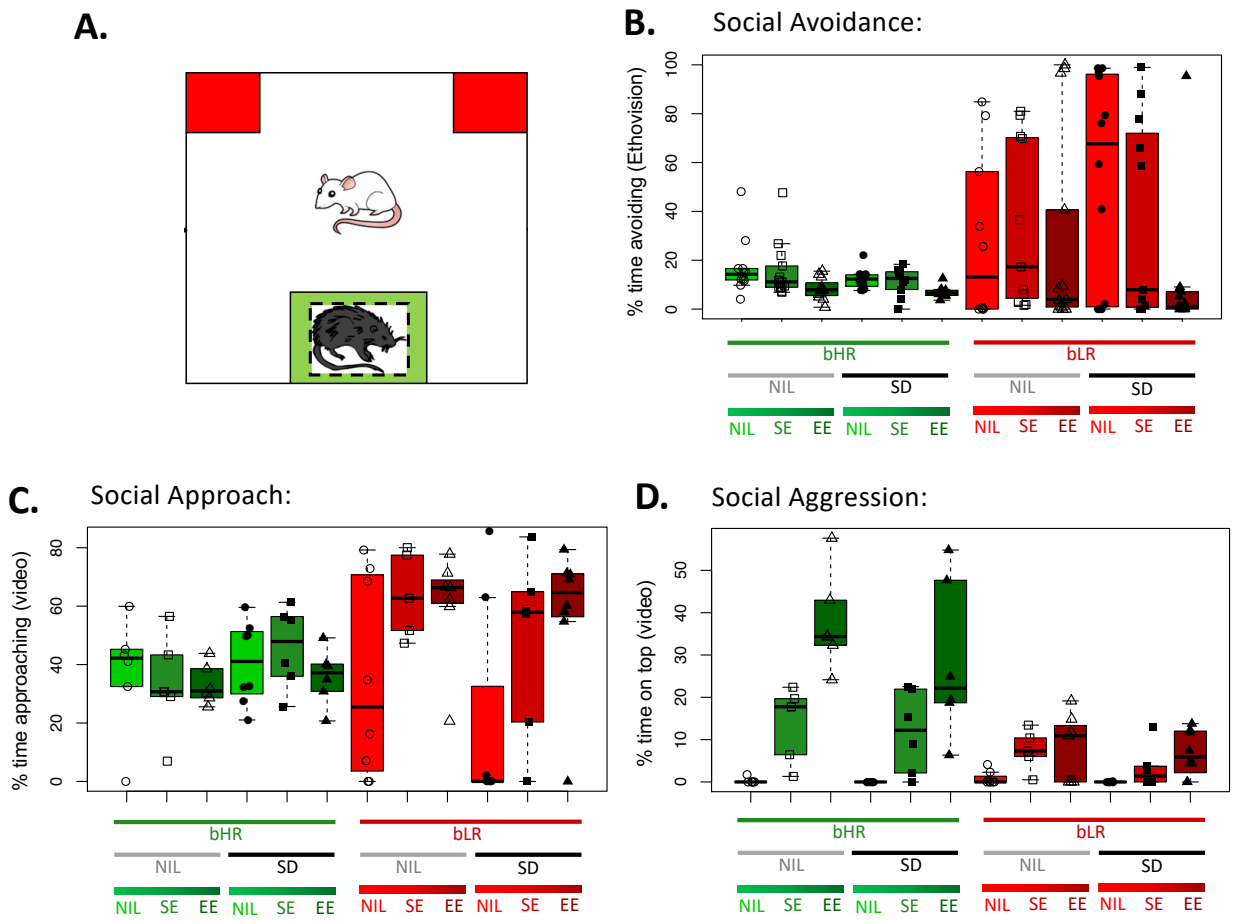


Fig 3. Adolescent social and environmental enrichment decreased social avoidance, leading to increased approach behavior in bLRs and increased aggression in bHRs. A. *An illustration of the social interaction task for the bHR/bLR animals (white rat), with each zone delineated: caged novel target Long Evans rat (black rat), avoidance zone (red), and interaction zone (green).* ***B-D.*** *Boxplots illustrate the median and interquartile range for each treatment group (+/- whiskers illustrating the range and/or 1.5x interquartile range). Bred line is illustrated with box fill color (bHR=green, bLR=red), adolescent enrichment by datapoint shape (circle=standard housing (NIL), square=simple enrichment (SE), triangle= enhanced enrichment (EE)), and social defeat is indicated by datapoint color (open=no defeat (NIL), black filled=defeated (SD)).* ***B.*** *Adolescent enrichment decreased the percent time spent in the socially avoidant zone (as determined by an automated Ethovision analysis). As expected, bLR rats were generally more avoidant than bHR rats.* ***C.*** *Adolescent enrichment increased the percent time spent approaching the stimulus animal, especially for bLR rats, as measured by detailed video analysis by a blinded experimenter.* ***D.*** *Adolescent enrichment increased the percent time on top of the stimulus animal's cage, especially for more aggressive bHRs, as measured by detailed video analysis by a blinded experimenter.*

3.3 Adolescent Enrichment Decreased Anxiety-like Behavior in bHR Rats

On the elevated plus maze (EPM), bHRs and bLRs exhibited expected phenotypical differences [10], with bHRs spending a greater percent time exploring the open arms than bLRs, indicating decreased anxiety-like behavior (**Fig 4A**, main effect of Line: $F(1, 128)=134.01$, $p=6.667e-05$, Generation co-variate: $F(2,128)=7.488$, $p=7.333e-04$). Percent time exploring the open arms was also increased following adolescent exposure to enrichment (main effect of Enrichment: $F(2, 128)=7.73$, $p=9.333e-04$), especially in bHRs (interactive effect of Line*Enrichment: $F(2,128)=9.668$, $p=2.000e-04$), in a manner that seemed to scale with the complexity of the enrichment intervention (NIL<SE<EE). Social defeat did not have any residual effects on anxiety-like behavior within this task in either line ($p>0.18$ for effects of SD, SD*Line, SD*Enrichment, SD*Enrichment*Line).

Phenotypical differences in exploratory behavior were also observed when examining the distance travelled during habituation to the open field used for social interaction testing (**Fig S9,** main effect of Line: $F(1,128)=512.26$, $p=6.667e-05$, Generation co-variate: $F(2, 128)=5.08$, $p=7.333e-03$). There was also a small decrease in distance travelled following social defeat (main effect of social defeat (SD): $F(2, 128)=6.19$, $p=1.293e-02$), that might depend on line (interactive effect of SD*Line: $F(1,128)=3.64$, $p=5.780e-02$ ***trend***), but no residual effects or interactive effects of enrichment (all $p>0.26$).

Taken together, these results affirm our selectively-bred model, and provide further evidence that positive effects of adolescent enrichment on behavior depend on genetic predisposition.

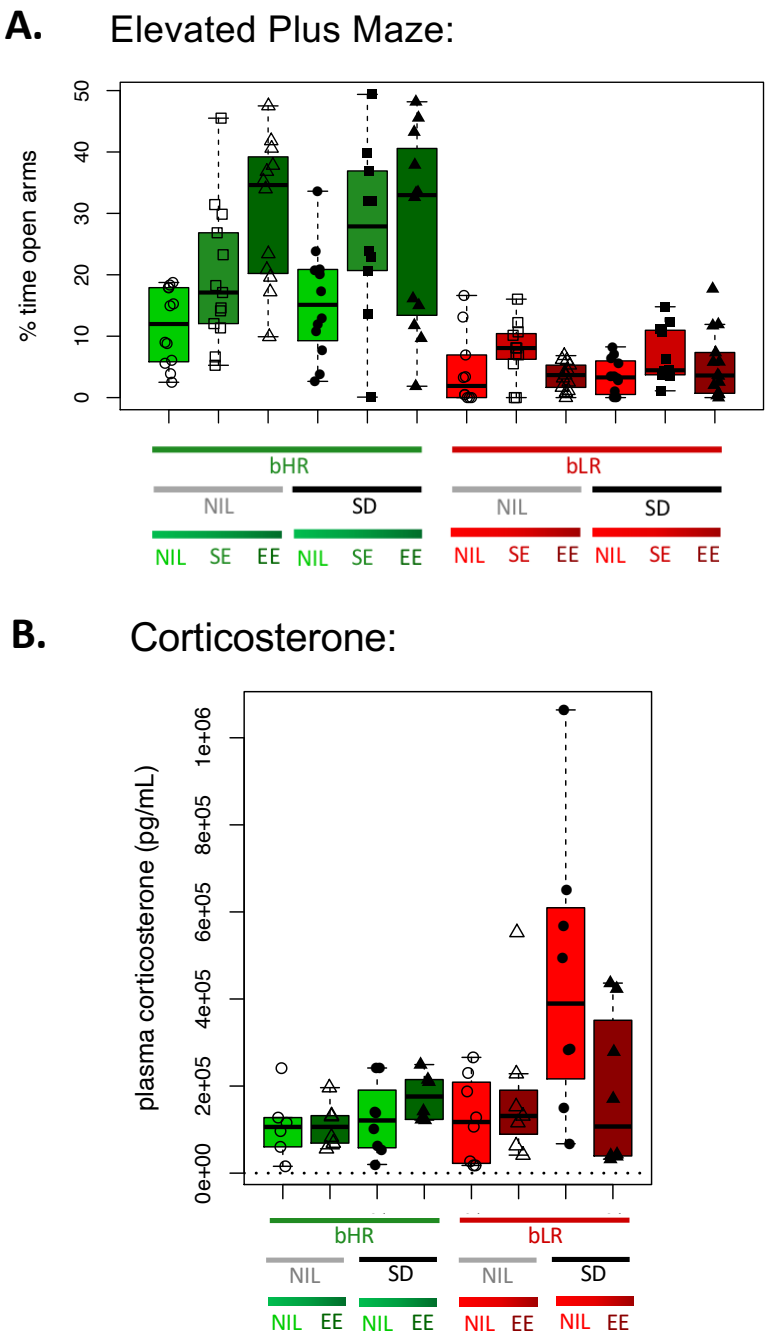


Fig 4. Anxiety and Stress Response: Adolescent enrichment decreased anxiety-like behavior in bHR rats and reduced the prolonged elevation of corticosterone following social defeat in vulnerable bLR rats. A. *The elevated plus maze (EPM) revealed expected phenotypical differences in anxiety and exploratory activity due to selective breeding, with bLRs showing less exploratory activity and elevated anxiety-like behavior, as illustrated by decreased percent time spent in the open arms. bHR rats also showed large increases in the percent time exploring the open arms of the EPM following adolescent enrichment, whereas neither bHR nor bLR rats showed a clear change in anxiety-like behavior following social defeat. Boxplots illustrate the*

median and interquartile range for each treatment group (+/- whiskers illustrating the range and/or 1.5x interquartile range). Bred line is illustrated with box fill color (bHR=green, bLR=red), adolescent enrichment by datapoint shape (circle=standard housing (NIL), square=simple enrichment (SE), triangle=enhanced enrichment (EE)), and social defeat is indicated by datapoint fill (open=no defeat (NIL), black filled=defeated (SD)). ***B.*** *Plasma corticosterone was found to be elevated several days following social defeat, especially in bLRs kept in standard housing conditions. Only plasma from a subset of animals was used for this assay (generations F53 and F56: NIL+NIL, NIL+SD, EE+NIL, and EE+SD). The dotted line represents the limit of detection for the assay.*

3.4 Social Stress Produced a Prolonged Increase in Corticosterone in bLR Rats that Was Reduced by Adolescent Enrichment

To determine whether circulating hormones might contribute to the observed behavioral profiles, trunk blood was collected at sacrifice from a representative subset of animals at baseline the day after behavioral testing concluded (generations F53 and F56: NIL+NIL, NIL+SD, EE+NIL, and EE+SD, sample sizes: **Fig S1**). Plasma was assessed for baseline circulating levels of corticosterone, testosterone, oxytocin and IL-6.

Plasma corticosterone was elevated in bLRs in comparison to bHRs (**Fig 4B,** main effect of Line: $F(1, 48)=5.35$, $p=0.0249$). Corticosterone was also elevated in the SD animals (main effect of Social Defeat: $F(1, 48)=5.77$, $p=0.0186$), especially for the bLRs kept in standard housing conditions (interactive effect of Line*Social Defeat*Enrichment: $F(1, 48)=4.90$, $p=0.0314$). Interestingly, these effects of social defeat were present in the bLRs even though the plasma was collected four days after the final defeat session, without evidence of residual social avoidance or anxiety-like behavior. Therefore, exposure to adolescent enrichment appeared to be protective and prevented a persistent elevation of plasma corticosterone following defeat for bLRs.

Testosterone, oxytocin, and IL-6 did not show any effects of treatment group (**Fig S10**-**S11**, *details in supplement*).

3.5 RNA-seq: Adolescent Enrichment and Social Defeat Alter Social-Emotional Circuitry

To better understand the impact of both adolescent enhanced enrichment (EE) and social defeat stress (SD) on the more vulnerable bLR rats, we used RNA-Seq to characterize gene expression in two brain regions known for their involvement in social-emotional processing: the nucleus accumbens (NACC) and hippocampus (HC).

The NACC and HC RNA-Seq studies were partially independent but revealed surprisingly similar results. Following quality control, the NACC dataset included reads aligned to 17,765 Ensembl-annotated transcripts (median library size=30 million) from 46 subjects (sample sizes: **Fig S1**).

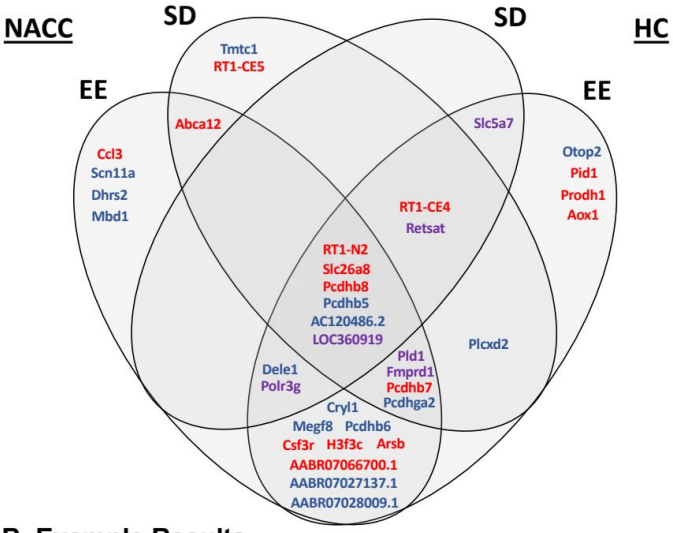
Within a model targeting only the main effects of enrichment and social defeat (M1), twelve genes were differentially expressed with enrichment (FDR<0.10, **Fig S12, Table S1**), many of which were from the protocadherin family (Pcdhb6, Pcdhga2, Pcdhb5, Pcdhb8, **Fig 5, Fig S12**). Five genes were differentially expressed with social defeat (FDR<0.10, **Fig S13**), with many representing the Major Histocompatibility Complex (RT1-CE4, RT1-CE5, RT1-N2**, Fig 5, Fig S13**). Within a model containing both the main effects of EE and social defeat as well as their interactive effects on gene expression (EE*SD, "M2"), a similar set of genes was identified for enrichment (7 genes, FDR<0.10) and SD (6 genes, FDR<0.10), with no significant interactive effects between the two variables (EE*SD, all FDR>0.10, **Figs S12-S13, Table S1**).

The HC RNA-Seq dataset was smaller and had a shallower read depth but showed a surprising degree of overlap with the NACC results. Following quality control, the HC dataset included reads aligned to 17,629 Ensembl-annotated transcripts (median library size=20 million) from 25 subjects (sample sizes: **Fig S1**). Within both models (M1 and M2), there was a similar set of genes differentially expressed with EE (FDR<0.10, M1: 12 genes, M2: 2 genes, **Fig S14, Table S2**) and no significant differential expression with social defeat or interactive effects of social defeat and EE (all FDR>0.10**, Table S2**).

Notably, there was substantial overlap between the genes that were identified as differentially expressed in response to the two interventions within both brain regions (**Fig 5).** Most genes that showed differential expression with either EE or social defeat in one region (FDR<0.10) showed at least nominal effects (p<0.05) with the other intervention in the same brain region, or with either intervention in the other brain region. These overlapping effects of EE and social defeat were often surprisingly in the same direction in both brain regions (**Fig 5, Figs S12-S14).**

RNA-Seq: bLR Rats

A. Venn Diagram



B. Example Results

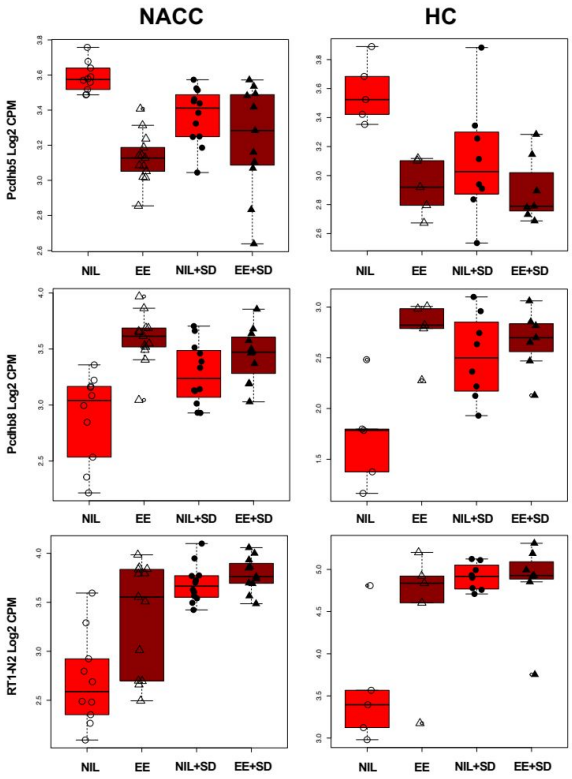


Fig 5. bLR RNA-Seq: A similar set of genes was differentially expressed in the Nucleus Accumbens (NACC) and Hippocampus (HC) in response to adolescent enrichment and social defeat*. **A.** A Venn Diagram illustrating the overlap of the bLR RNA-Seq results from the two brain regions (NACC, HC) and treatment groups (adolescent enrichment: standard housing (NIL) vs. enhanced enrichment (EE); social defeat: no defeat (NIL) vs. social defeat (SD)). To be included in the Venn Diagram, a gene needed to be differentially expressed in association with either EE or SD in at least one region (FDR<0.10). Then, to be considered overlapping, there needed to be at least nominal (p<0.05) differential expression with the other intervention in the same brain region, or in association with either intervention in the other brain region. Surprisingly, the overlapping effects of EE and SD were often in the same direction in both brain regions: Red=upregulation, Blue=down-regulation, Purple=differential expression in opposing directions under different conditions/regions. For the full table of top differentially expressed genes (FDR<0.10) see **Figs S12-14**. For the full results for all genes see **Tables S1-S2**. **B.** Example boxplots illustrating the relationship between gene expression (log2 CPM) and treatment group. Adolescent enrichment is illustrated by datapoint shape (circle=standard housing (NIL), triangle=enhanced enrichment (EE)) and social defeat is indicated by datapoint fill (open=no defeat (NIL), black filled=defeated (SD)).*

To explore the brain functions associated with these genes, we compared our differential expression results to a custom gene set file (Brain.GMT v.2) that included not only traditional gene ontology categories, but also previously-published gene sets related to brain cell type, co-expression networks, stress, internalizing behavior, and neuropsychiatric illness. Using gene set enrichment analysis, we found that our differential expression results showed enrichment within a disproportionately large number of gene sets in categories paralleling our behavioral results: stress, fear conditioning, social behavior, aggression, and activity level (**Fig 6**, full results: **Table S3-S4)**. Within these gene sets, typically adolescent EE and social defeat had opposing effects within the NACC and similar effects within the HC (**Fig S15-20).** This is interesting, because although environmental enrichment and social defeat are often considered opposing interventions in terms of their effects on stress susceptibility, both interventions involve a novel environment, social stimuli, increased activity, and some amount of stress.

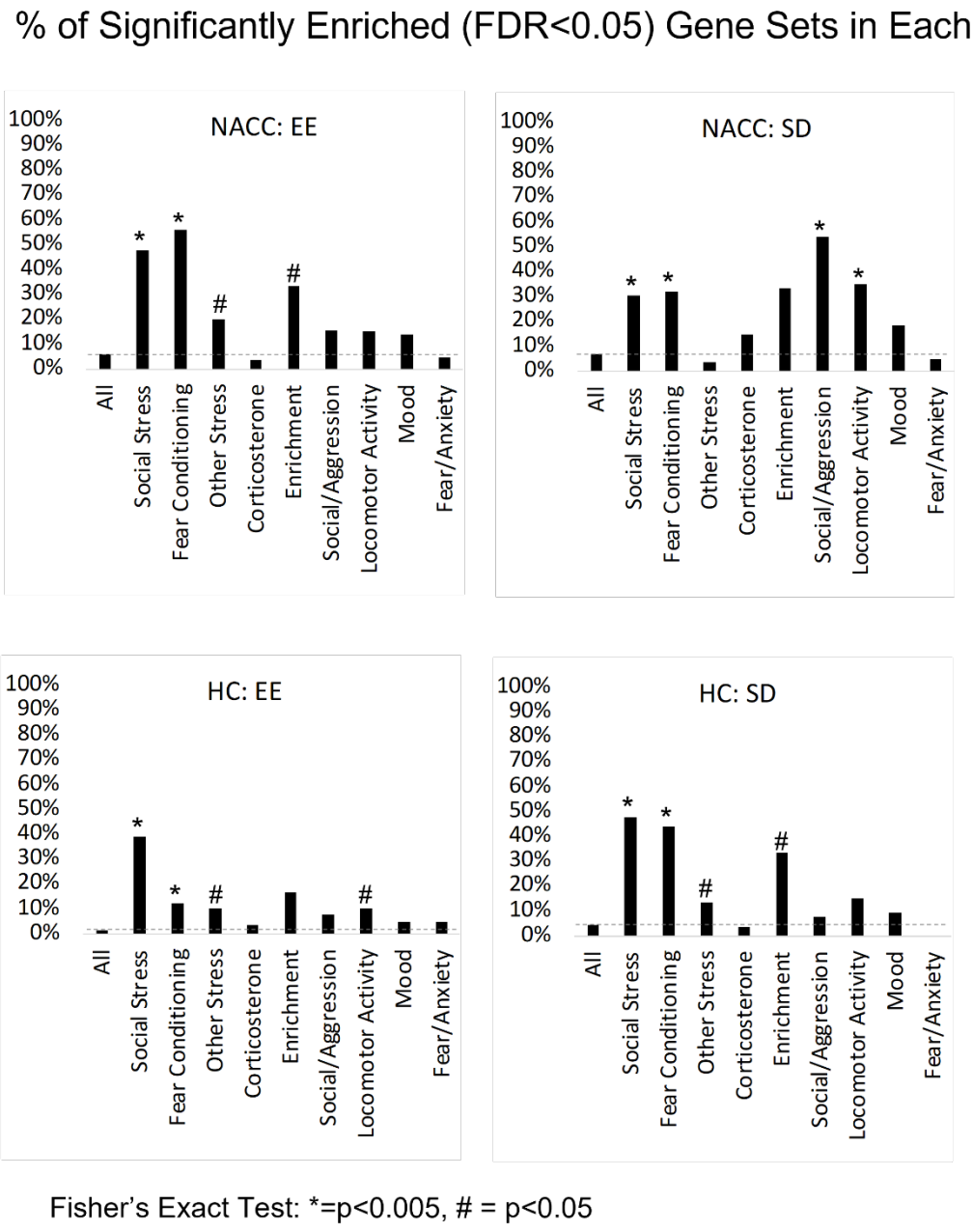


Fig 6. For each of our interventions, there was an enrichment of differential expression within gene sets related to social behavior, stress, aggression, enrichment, and activity level. *Using gene set enrichment analysis and a custom gene set file (Brain.gmt), we found that our differential expression results showed enrichment in gene sets in categories paralleling our behavioral results. Shown above are the percent of gene sets (y-axis) from each category (x-axis) that were found to be significantly enriched with differential expression (enrichment FDR<0.05 using output from the main effects model (M1) or interactive effects Model (M2)) for each intervention (enhanced enrichment (EE), social defeat (SD)) in each brain region (nucleus accumbens (NACC), hippocampus (HC)) (subpanels). Categories for which a disproportionately*

*large number of gene sets were enriched with differential expression were identified using fisher's exact test: *p<0.005, #p<0.05. The directionality for individual enriched gene sets can be seen in* ***Figs S15-S20.*** *For the full results for all gene sets, see* ***Tables S3-S4.***

Our differential expression results also showed disproportionate enrichment in gene sets related to cell type (**Figs S21-S22**). Gene sets related to oligodendrocytes were enriched with upregulation in both the NACC and HC following both social defeat and adolescent EE, perhaps implying an increase in connectivity. Other cell type related gene sets showed divergence across conditions: in the NACC, gene sets related to neurons were enriched with down-regulation following social defeat**,** and gene sets related to the brain vascular and ventricular systems (astrocytes, mural cells, endothelial cells, progenitor cells, neurogenesis-related cells, ependymal cells) were enriched with down-regulation following enrichment. In the HC, gene sets related to the choroid plexus were upregulated following social defeat and EE.

In conclusion, we found that adolescent EE and social defeat impact social and emotional neural circuitry and associated genes in a manner that is likely to reflect features shared across these social behavioral interventions and may be accompanied by structural as well as functional changes.

4. Discussion

The present results extend our understanding of how genetic predisposition influences individual responses to interventions intended to increase social resilience, on a behavioral, hormonal, and neural level. This is the first study to examine adolescent enrichment in combination with repeated social stress in a model with heritable differences in temperament. We found that a month of adolescent social and environmental enrichment produced robust long-term effects on social behavior, aggression, anxiety, and exploration that varied according to genetic background and temperament. In bHRs, adolescent enrichment increased aggressive behavior in response to both territorial aggression and more neutral social encounters. In bLRs, the impact of enrichment on social behavior was especially notable, decreasing submission in response to territorial aggression and increasing social approach during a neutral social encounter. Physiologically, social defeat produced a prolonged elevation in stress hormone in standard-housed bLRs. This elevation in corticosterone was reduced in bLRs that experienced adolescent enrichment, suggesting a lasting buffering effect of adolescent intervention. Therefore, following adolescent enrichment, the typically anxious bLRs developed greater resilience to social defeat and their social interactions came to resemble behavior more typical of bHRs. Thus, adolescent social and environmental complexity proved to be a powerful factor in selectively inducing social resilience in these vulnerable animals.

We therefore focused on the bLRs and characterized the impact of adolescent enrichment and social defeat stress on the brain. We used RNA-Seq to characterize gene expression in two brain regions involved in affect, stress regulation, social behavior, emotional memory, and behavioral inhibition: the NACC and HC. We found an enrichment of differential expression within many gene sets previously associated with stress, social behavior, aggression, and exploratory activity [40-45]. Additionally, we found that enrichment and social defeat often had similar effects on gene expression, emphasizing that although these interventions are considered to have opposite effects on stress susceptibility, they share many similarities, including novelty, social interaction, exploration, and stress.

4.1 Behavior and Hormones

Studies in mice have shown that the primary impact of adolescent enrichment followed by social defeat stress is on social behavioral outcomes [54]. Similarly, adolescent enrichment increased social interaction in outbred Sprague-Dawley rats [55] and adult enrichment altered social behaviors in our bLR and bHR lines, increasing positive-affect ultrasonic vocalizations in both lines and decreasing bHR aggressive behaviors [19]. Previous work demonstrated that bHRs show higher levels of social interaction than bLRs [56]; our current findings show that bLRs exposed to adolescent enrichment have increased social approach and interaction. This increase may be due to motivation to exert social dominance over a restrained Long-Evans target, as both bLRs and bHRs spent more time on top of the stimulus animal's cage during social interaction testing. Studies in mice have shown that enrichment impacts resilience to social stressors depending on genetic background and baseline aggression [57-59], and that social enrichment alone has less impact on rodents than social-environmental enrichment [60]. In the current study, simpler adolescent enrichment conditions produced fewer changes in bLR social behavior compared to enhanced enrichment conditions, suggesting that the extra physical experiences available in the EE cage are important for producing long-term changes in social behavior in our usually anxious line.

In Sprague-Dawley outbred rats, adult enrichment increases stress resilience [61], and adolescent enrichment alters the neural circuitry underlying stress responsiveness [55]. Adult enrichment also decreased anxiety-like behaviors in outbred Sprague-Dawley rats [30], Roman Low Avoidance rats [32], bLR and bHR rats [34]. The current results using the EPM confirm that adolescent enrichment decreases bHR anxiety-like behavior, but does not appear to alter bLR anxiety-like behavior within our experimental timeline.

In contrast, adolescent enrichment decreased the systemic effects of social stress in bLRs. Elevated corticosterone could still be seen in standard-housed bLRs four days after social stress. This prolonged increase in corticosterone following social defeat was reduced by adolescent enrichment, parallelling previous findings that repeated social stress during adolescence increased corticosterone in outbred Sprague-Dawley rats [62], but contrasting with the increased corticosterone observed in bHR rats immediately after extended exposure to adult enrichment [19]. The response of corticosterone to stress was also enhanced in outbred rats

after adult enrichment [61]. Altogether, these findings suggest that enrichment can act as an eustressor [29, 33, 61], increasing stress responses acutely but decreasing them in the long-term.

These findings are important because inducing resilience in the anxious bLRs has proven difficult, and usually requires pharmacological intervention [27, 34]. bLRs that experienced adolescent enrichment showed decreased submission during social defeat, increased social interaction, decreased social avoidance, and a reduced elevation in corticosterone following social stress, suggesting that they had increased social resilience. Other studies have similarly observed large changes in baseline bLR behaviors following adolescent experiences [63, 64]. Recently, adolescent enrichment followed by repeated social stress was investigated in mice, showing similar positive effects [54], suggesting that adolescence is a time of both great vulnerability and great potential for positive intervention.

4.2 RNA-seq

RNA-seq was used to determine how adolescent enrichment and social defeat might impact the NACC and HC, two brain regions important for stress and social behavior [40-45]. Our findings suggest that the most impactful aspects of our two interventions on brain function may not be their assumed affective valence (positive vs. negative) but shared characteristics, such as repetitive exposure to social stimuli and a complex environment. The timing of our behavioral experiments may also have contributed to a similar impact of adolescent enrichment and social defeat stress on gene expression; previous work demonstrated that removing enrichment a week before behavioral tests induces a stress response [100, 101], which is similar timing to when we collected brain tissue.

Two groups of genes were particularly impacted by both interventions in both brain regions: the RT1 and protocadherin genes. The RT1 genes are part of the class III region within the rat major histocompatibility complex, and are involved in producing cytokines and complement components [65]. Changes in cytokine expression regulate both social behavior and neuronal connectivity [68], while social status and experience impact cytokine levels in multiple species [69-72]. Living in a social group is an immune challenge, and there is strong cross-talk between immune and social signalling pathways [73, 74]. The current data provide yet more evidence for this correlation between immune and social signalling and suggest that both positive and negative social experiences regulate immune signalling within the brain.

Protocadherins comprise a subset of the Cadherin family of transmembrane glycoproteins that regulate cell-to-cell contact through interactions via extracellular regions [75]. The protocadherins differentially expressed in the current study come from the class of clustered protocadherins [76], which regulate neurite formation, including dendritic self-avoidance, dendritic arborization, spine formation, axonal branching, and axonal pruning [66, 67, 77, 78]. Our results showed a downregulation of many protocadherins in both the HC and NACC following adolescent enrichment and sometimes social defeat. Previous research found that adult enrichment decreased other brain glycoproteins [79, 80] that regulate neuroplasticity [81-

83], reopening critical periods within the brain [80]. Protocadherins also regulate neuroplasticity and development [84, 85]; thus, it is possible that adolescent enrichment and social defeat might similarly extend or reopen plasticity gated by protocadherin expression.

Using gene set enrichment analysis, we found that our differential expression results showed disproportionate enrichment in categories paralleling our behavioral results: gene sets related to stress, social behavior, aggression, and activity level. Therefore, our current study not only provides evidence that adolescent enrichment and social stress impact social behavior, but also implicates gene expression networks that mediate these behaviors. We also observed robust enrichment within gene sets associated with fear conditioning and other stressors. While intuitive, these findings may be due to the prevalence of immediate early genes within these gene sets rather than to a "stress network" specifically.

Both adolescent enrichment and social defeat increased expression within oligodendrocyte-related gene sets within both brain regions. Previous studies found that enrichment increased myelination and oligodendrocyte markers and expression in mice, particularly during adolescence [86, 87]. The current results suggest that this effect of enrichment also occurs in rats. The impact of social stress on oligodendrocytes and brain myelination is less clear-cut, and may depend on brain region, stress susceptibility [88-91], and potentially species. In male adolescent Sprague Dawley rats, increased myelination within the hippocampus was similarly observed following juvenile stress [92].

There were opposing effects of adolescent enrichment and social defeat stress on the expression of ventricular and endothelial-related gene sets within the NACC. Consistent with the current results, social stress increased endothelial and ependymal markers in the brains of mice [93-95], suggesting an increased inflammatory state. The effects of enrichment on these supporting cells and systems within the brain are less straightforward, varying with the timing and duration of enrichment, rodent species/strain, and brain region [96-99]. We observed decreased expression of ventricular and endothelial-related genes within the NACC following adolescent enrichment in bLR rats.

Altogether, the gene expression profiling results underscore the multiple classes of mechanisms that participate in resilience induction in the bLRs, including cell type balance, immune signalling, and neuroplasticity. The overlap in affected genes and gene sets between both brain regions and behavioral interventions provides reassurance that our differential expression models properly controlled for dataset-specific confounding technical variability. A limitation of our study is that it only used male subjects, as the social defeat paradigm is best characterised in male rodents; thus any interactions between the effects of environmental enrichment and social stress in females may involve both shared and distinct mechanisms compared to males.

4.5 Conclusion

Repeated social stress is often used to model depression and anxiety; our current findings indicate that social defeat stress is not a uniform experience but should instead be considered through the lens of both genetic predisposition and previous social and environmental experience. Our findings support the concept that enrichment serves as a "eustressor", providing a mild inoculating dose of stress due to novelty that improves future coping with larger stressors. We find that adolescent enrichment influences future social interactions and anxiety-like behavior in a manner that depends on the genetic temperament. RNA sequencing of two brain regions in the vulnerable bLRs provides further evidence that social behavioral interventions such as adolescent enrichment and social defeat impact social-emotional circuitry and associated gene families. Our results suggest that these regions encode both the similar and divergent aspects of these two interventions. The ability to induce social resilience in a usually anxious line of animals by manipulating the adolescent environment provides an exciting avenue for the development of potential intervention strategies targeted at vulnerable human adolescent populations.

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Credit authorship contribution statement

Angela M. O'Connor: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. **Megan H. Hagenauer:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Software; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. **Liam Cannon Thew Forrester:** Data curation; Formal analysis; Methodology; Software; Writing - review & editing. **Pamela M. Maras:** Investigation; Methodology; Writing - review & editing. **Keiko Arakawa:** Investigation; Methodology; Writing - review & editing. **Elaine K. Hebda-Bauer:** Resources; Writing - review & editing. **Huzefa Khalil:** Data curation; Formal analysis; Software; Writing - review & editing. **Evelyn R. Richardson:** Data curation; Formal analysis; Software; Writing - review & editing. **Farizah I. Rob:** Data curation; Formal analysis; Software; Writing - review & editing. **Yusra Sannah:** Data curation; Formal analysis; Software; Writing - review & editing. **Stanley J. Watson, Jr.:** Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing - review & editing. **Huda Akil:** Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing - review & editing.

Declaration of competing interest

None

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Data Availability

All data are released on Figshare (*Link TBA- embargoed*) and GEO/SRA (*Link TBA - embargoed*).

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