PNAS

Supporting Information for

Transcriptional signatures of early-life stress and antidepressant treatment efficacy

Sero Toriano Parel, Shannon N. Bennett, Cindy J. Cheng, Olivia C. Timmermans, Laura M. Fiori, Gustavo Turecki, Catherine Jensen Peña*

*Corresponding author: Catherine Jensen Peña
Email: cpena@princeton.edu

This PDF file includes:

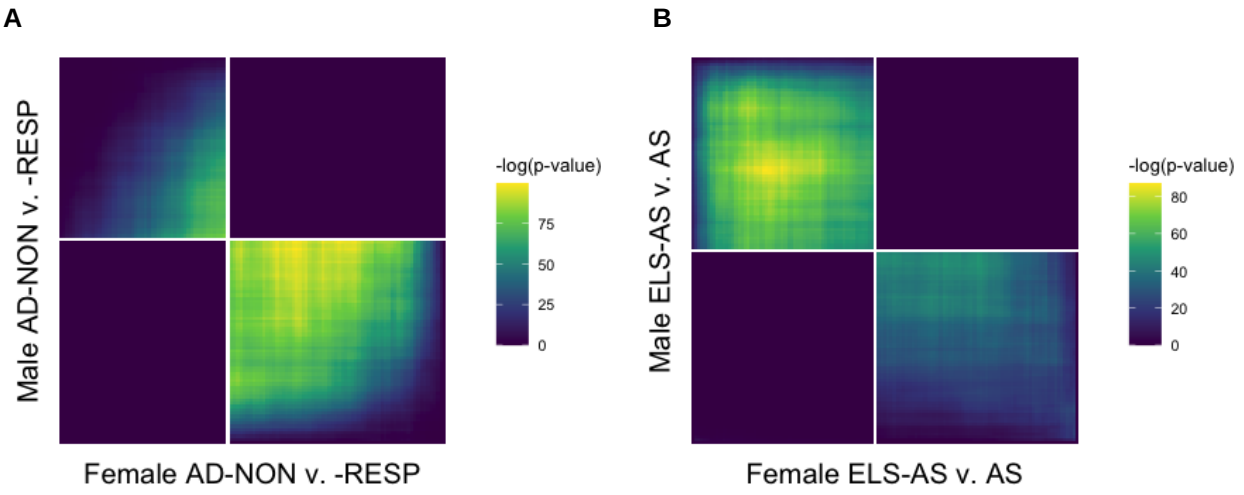

Fig. S1. Sex differences in transcriptomic response to antidepressant treatment or stress.

Threshold-free RRHO analysis compares gene expression changes across two comparisons. Lower left quadrant includes co-upregulated genes; upper right quadrant includes co-downregulated genes, and upper left and lower right quadrants include oppositely regulated genes (up-down and down-up, respectively). Genes along each axis are sorted from most to least significantly regulated from the middle to outer corners. Pixels represent the overlap between DEGs in each comparison, with the significance of overlap (-log10(p-value)) of a hypergeometric test color-coded as indicated by the key for each RRHO map. (*A*) Gene expression patterns from white blood cells of MDD patients who were non-responsive vs responsive to antidepressant treatment are opposite between female-identifying and male-identifying patients. (*B*) Gene expression patterns from NAc of mice exposed to ELS+AS vs only-AS are opposite between assigned-females and assigned-males. It is important to note, however, that mouse NAc RNA-seq data used in *B* was generated from different cohorts, and males and females experienced different adult stressors, making it impossible to distinguish between sex and cohort effects.

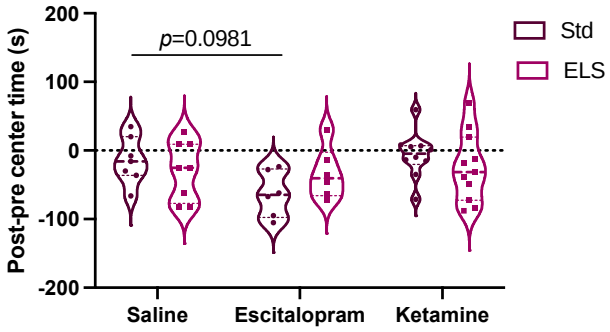

Fig. S2. Impact of antidepressant treatment on female open field center time.
There was a trend for an effect of escitalopram relative to saline [$F(1,23)=2.945$, $p=0.098$] to reduce time in the center of the open field, but no effect of ketamine, nor an interaction with ELS.

Table S1

ASSIGNED-FEMALES

		Pre-treatment			Post-treatment			
Fig.	**Behavior**	**ELS**	**Defeat**	**Interaction**	**ELS**	**Escitalopram**	**Ketamine**	**Interaction**
3*B*	OFT: Time spent in arena center	–	▼	–	–	(▼)	–	(ELS × Escitalopram)
3*C*	OFT: Distance traveled	–	–	–	–	–	–	–
3*D*	Social interaction: Ratio	▲	–	–	N/A	N/A	N/A	N/A
3*E-K*	NSF: Latency to eat	–	▲	Std-Defeat v. ELS-Defeat (Std-Ctrl v. ELS-Ctrl)	▲[1]	▼	▼	ELS × Escitalopram (▼ ELS × Ketamine)

ASSIGNED-MALES

		Pre-treatment			Post-treatment			
Fig.	**Behavior**	**ELS**	**Defeat**	**Interaction**	**ELS**	**Escitalopram**	**Ketamine**	**Interaction**
4*A,C*	OFT: Time spent in arena center	–	–	–	–	–	–	ELS × Ketamine
4*B,D-F*	Social interaction: Ratio	▼	▼	ELS × Defeat	–	–	–	ELS × Ketamine

Table S1. Summary of behavioral pharmacology. Direction of effects are stratified by assigned sex, early experience, adult experience, and treatments. For main effects and interactions that are statistically significant ($p < 0.05$) or trending ($0.10 > p > 0.05$; indicated by parentheses), ▲ indicates an increase, and ▼ indicates a decrease in the behavior. N/A indicates behavior was not measured. [1]Change over time in latency was significant when subtracting post-treatment latency from pre-treatment (corresponding to Fig. 3G), however this effect was not significant in standalone post-treatment measurement.

Supporting Information Text

MATERIALS AND METHODS

Transcriptomic Datasets

Three independent RNA-seq datasets were used for bioinformatic analyses for predicting the effects of a history of ELS on future antidepressant response. The first dataset (GEO GSE89692) includes the NAc of a mouse model for ELS wherein assigned female and male mice experienced ELS and/or adult stress (AS) in a 2×2 design (6, 13). Adult stress in these datasets consisted of chronic social defeat stress for assigned-male mice and sub-threshold variable stress for assigned-female mice. In these datasets, ELS sensitized mice to experience of AS to produce greater depression-like behaviors. Whole-tissue RNA-seq was performed on 4-6 subjects per group as previously described (6, 13). Differential gene expression analyses were computed via the R package `DESeq2` (63). The following comparisons were used, separately for assigned-female and -male mice: ELS-Control vs Std-Control; Std-AS vs Std-Control; ELS-AS vs Std-AS. The second dataset (GEO GSE81672) includes the NAc of adult assigned-male mice that underwent chronic social defeat stress and were categorized as stress-naive, resilient to adult stress, susceptible to adult stress and responsive to either antidepressant treatment with the tricyclic imipramine or sub-anesthetic ketamine, or susceptible and non-responsive to one of the treatments (12). The primary comparisons used were Imipramine Non-Responders vs Imipramine Responders and Ketamine Non-Responders vs Ketamine Responders. Whole-tissue RNA-seq was included on 6-10 subjects per group, as previously described (70). Differential expression analyses were computed via the R package limma::voom (71, 72). The third RNA-seq dataset (see Fig. 1A) includes peripheral white blood cell samples from control subjects and patients with major depressive disorder (MDD) at two time points, before and 8 weeks after antidepressant treatment with either escitalopram (a selective serotonin reuptake inhibitor) or desvenlafaxine (a serotonin and norepinephrine reuptake inhibitor) and assessment for response or non-response to treatment on two depression rating scales: Montgomery-Åsberg Depression Rating Scale (MADRS) and Hamilton Depression Rating Scale (HAMD). The patient cohort, blood sample collection, processing, and sequencing as previously described (23). This sample included 31 healthy control subjects (22 self-identified female and 9 self-identified male, with sex additionally matched by genotyping) and 186 MDD subjects (111 female, 75 male). Of the MDD patients, 91 received escitalopram and 95 received desvenlafaxine. MDD patients were classified as responders or non-responders based on a ≥ 50% reduction in scores from baseline, and only patients where response to antidepressant treatment was consistent on both rating scales were included, resulting in 69 RESP (n=41 female and 28 male) and 70 NRES (n=47 female and 23 male) subjects. The primary comparisons used were antidepressant (escitalopram + desvenlafaxine) Non-Responders vs Responders, separately for female- and male-identifying subjects. RNA-seq normalization for these subjects was conducted using `DESeq2` and were log2-transformed and corrected for age, gender, and RIN using the removeBatchEffect function of limma. For comparison between mouse and human datasets, only homologous genes with baseMean expression >2 in both datasets were included.

Bioinformatic comparisons

Bioinformatic analyses were performed using R and Python. Genes included in differential analyses had the following parameters for each comparison: base mean > 2, significance as uncorrected $p < 0.05$, and threshold for fold-change > 30% (log2 FC, LFC > |0.3875|). DEGs included in Venn diagrams were limited to protein-coding genes. Gene lists for heatmaps included a union of all DEGs from any one comparison with matched LFC values regardless of significance in other comparisons.

Bioinformatic analyses were performed using R and Python. Rank-rank hypergeometric overlap (RRHO) analysis was implemented to compare pairs of independent gene expression datasets in a threshold-free manner as previously described (64, 65). A two-sided version of RRHO analysis was used to evaluate coincident and opposite enrichment (65). In each pairwise comparison of datasets, genes are ranked by the -log10(p-value) multiplied by the sign of the fold change from the differential expression analysis (63). For each set of comparisons, gene lists were filtered for genes expressed (>2 base mean) in both lists, such that human-mouse comparisons only included orthologous genes. RRHO difference maps were produced for each pair of match comparisons by calculating for each pixel the normal approximation of difference in log odds ratio and standard error of overlap; this Z-score was then converted to a *P*-value and corrected for multiple comparisons across pixels. We then performed gene ontology analysis for genes with high coincidence among comparisons using the R packages `clusterProfiler` enrichment tool for interpreting omics data, `org.Hs.eg.db` for human genome-wide annotations, and `org.Mm.eg.db` for mouse genome-wide annotations (66, 67).

Animals

All experiments with animals were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at Princeton University and of the Society for Neuroscience. All mating occurred in-house to prevent shipping stress during gestation. Mice were maintained on a 12-h light/dark cycle with *ad libitum* access to food and water. Two nulliparous C57BL/6J female mice (Jackson) were mated with one male in the animal facility. Males were removed after 5 days and females were separated into individual cages 1-3 days prior to parturition. Litters were weighed and counted and cages cleaned on the day of birth (postnatal day or P0) but otherwise undisturbed. C57BL/6J pups were assessed for sex at birth and assigned "female" or "male" at weaning on postnatal day P21 by their gonadal anatomy, or external genitalia. Assigned-females and -males were weaned separately into cages of 3-5 mice, keeping littermates together and only combining pups from different litters and of the same experimental condition to maintain at least three mice per cage. Up to only two mice of the same assigned-sex were used per litter per initial control/defeat random assignment, and up to one mouse per sex per litter was used for each treatment group.

Early-life and adult stress paradigms

The two-hit ELS and adult stress paradigm in a mouse model implemented in the current study were as previously described (6, 13). Briefly, ELS involved limited nesting from P10-17 (73–75). Litters were randomly assigned to standard-rearing (Std) or ELS groups. Pups were separated together as a litter to clean cages with distinct bedding for 3-4 h/day at random times each day during the light cycle to minimize predictability and habituation. Nesting material was depleted to one-third of that provided to standard-reared cages during the days of separations and then restored on the final day. For adult stress, we adapted a social defeat paradigm that can be used for both female and male mice (28, 69). Adult mice were assigned randomly to either stress or control (Ctrl) conditions, and littermates were assigned to different groups for within-litter controls. For stress in adulthood, mice experienced chronic non-discriminatory social defeat stress, an improved model for depression and social stress-related disorders. In this paradigm, female and male C57BL/6J pairs were simultaneously placed into the home cage of a novel Swiss Webster aggressor mouse (28, 69). An experimental male mouse was introduced to an aggressor's cage first for ~3 minutes, followed by an experimental female mouse for an additional 5 minutes. Male mice were then moved across a perforated plexiglass barrier within the aggressor cage for the remainder of the day, for sensory stress without physical interaction. Female mice were removed to individual cages with aggressor bedding for the remainder of the day. Experimental mice were introduced to a new aggressor each day for 10 days, and male and female mice rotated in different directions so that trios were unique each day. While aggressors were observed to attack female mice, attacks were predominantly directed at male mice. No copulations were observed. Control mice were housed in a standard mouse cage in pairs of the same sex, separated by a perforated plexiglass barrier. At the conclusion of social defeat, all mice were rehoused in clean cages and underwent three consecutive days of behavioral testing before tissue collection.

Behavioral testing

Overview: Mice underwent behavioral testing twice in order to assess initial susceptibility or resilience to adult social stress and the effect of antidepressant treatment: first, one day after the 10 days of social defeat and, second, one day after the conclusion of antidepressant or saline treatment (Fig. 3A). For each round of testing, one behavioral test was conducted per day. Due to the large number of animals (n=237 in total)], testing was done in two cohorts. All mice were tested in all three tests described below after social defeat for the first cohort. However, there was a strong main effect of sex on social interaction behavior such that defeat decreased social investigation among males but not females. Pilot studies in our lab also showed a stronger effect of social defeat on novelty-suppressed feeding behavior among females than males. We thereafter tested male mice in the social interaction test and female mice in the novelty-suppressed feeding test. All mice were tested on the open field test.
Arenas used for behavioral testing measured 44 × 44 × 20 cm. Video tracking software (Ethovision, Noldus) was used to record and quantify mouse behavior in each test. To mitigate habituation in repeated testing, the patterns on the walls of the chambers were altered for each testing session.

Social interaction testing: We assessed social avoidance behavior using a two-stage social interaction test conducted. Testing was conducted in the light cycle but under red lighting, described previously (6, 12). Social avoidance has been shown to be related to depression-like behavior and altered response to antidepressant treatment (26, 27). In the first stage with no target present, the experimental mouse explored an arena containing a novel plexiglass and wire mesh enclosure (novel object; 10×6 cm) centered against one wall of the arena for 2.5 minutes. In the second stage with the target present, the experimental mouse was immediately returned to the arena with a novel Swiss Webster mouse (aggressor strain) within the enclosure for another 2.5 minutes. A social interaction ratio (SI ratio) was calculated as time that the experimental mouse spent in an "interaction zone" (14×26 cm) surrounding the enclosure in stage two with the aggressor present divided by time in the zone during stage one with the empty novel enclosure. Mice with an SI ratio <1

were categorized as susceptible to social defeat stress, while mice with an SI ratio >1 were categorized as resilient to stress.

Open field test (OFT): To assess general locomotor and exploratory behavior, experimental mice were allowed to freely explore a brightly lit, empty arena for 10 min as previously described (6). Time spent in the center (20 × 20 cm) of the open field arena, distance traveled in the arena, and locomotor activity were measured using Ethovision.

Novelty-suppressed feeding (NSF): Depression- and anxiety-like behavior among female mice was assessed by NSF testing, as in (76). Mice were deprived of food in a clean home cage for 18 hours before testing. Testing was conducted in the dark phase of the day when mice typically eat most. On the day of testing, mice were placed in a corner of a brightly-lit arena covered with corncob bedding on its floor. A single yogurt chip was attached to a white circular platform (10 cm diameter) located at the center of the arena. The test session lasted until mice took their first bite of the chip, which was defined as the moment the mouse sat on its haunches, grasped the chip with its forepaws, and bit into the chip; or for a maximum of 10 min. Mice that failed to consume the food within 5 min were assigned a latency score of 600 seconds. Immediately after the first bite or at 10 min if the mouse failed to eat, the mouse and the yogurt chip in its arena were transferred to its home cage with *ad libitum* access to water, and latency to eat in the home cage was then recorded by hand. An NSF ratio was then calculated as the latency to eat in the novel arena, divided by the latency to eat in its home cage. Mice with an NSF ratio > mean + 0.25*SD of the Std-Ctl mice for that cohort were categorized as susceptible to social defeat stress.

Antidepressant treatment

Mice categorized as susceptible to social defeat stress were randomly assigned to treatment with either saline, escitalopram, or ketamine, beginning the day after the first round of behavioral testing ended. Saline-treated mice received 0.1 mL sterile normal saline (i.p.) daily for 21 days. Escitalopram-treated received escitalopram (10 mg/kg, i.p.; Henry Schein 1263605, dissolved in sterile saline) for 21 days, wherein dosage was demonstrated in previous literature (32-34). Mice assigned to the ketamine group were given 0.1 mL sterile normal saline (i.p.) daily for 20 days, followed by a single dose of ketamine (10 mg/kg, i.p.) on day 21 of treatment, wherein dosage was demonstrated in previous literature (12). The second and final round of behavioral testing began the day following the last day of treatment.

Statistical analyses of behavior

Behavioral and gene expression data were analyzed with R, SPSS (IBM, version 28), or Prism (GraphPad, version 9). General linear model (two-way ANOVA) was used to determine main effects and interactions, as described. To account for within-animal change in behavior with treatment, a behavioral difference score was calculated (post-treatment minus pre-treatment). Male mice were categorized as responsive to treatment if their SI-Ratio difference score was positive; female mice were categorized as responsive to treatment if their NSF-latency difference score was negative. Differences in latency to eat in the NSF test were calculated by Log-rank Mantel-Cox survival analysis. Effects of escitalopram and ketamine were compared to saline separately. Post-hoc analyses used Tukey's HSD multiple comparisons correction. Including cohort as a covariate did not alter any statistical outcomes. Outliers were defined as > 2 standard deviations away from the mean and were removed. Fixed effects models were generated to examine the effects of ELS, treatment, and their interactions on latency to eat in the NSF test (females) and SI ratio (males). All significance thresholds were set at $p < 0.05$.

SI References

6. C. J. Peña, *et al.*, Early life stress confers lifelong stress susceptibility in mice via ventral tegmental area OTX2. *Science* **356**, 1185–1188 (2017).
12. R. C. Bagot, *et al.*, Ketamine and Imipramine Reverse Transcriptional Signatures of Susceptibility and Induce Resilience-Specific Gene Expression Profiles. *Biol. Psychiatry* **81**, 285–295 (2017).
13. C. J. Peña, *et al.*, Early life stress alters transcriptomic patterning across reward circuitry in male and female mice. *Nat. Commun.* **10**, 5098 (2019).
23. S.-C. Fuh, L. M. Fiori, G. Turecki, C. Nagy, Y. Li, Multi-omic modeling of antidepressant response implicates dynamic immune and inflammatory changes in individuals who respond to treatment. *PLoS ONE*, e0285123 (2023).
26. O. Berton, *et al.*, Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science* **311**, 864–868 (2006).
27. V. Krishnan, *et al.*, Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* **131**, 391–404 (2007).
28. C. N. Yohn, *et al.*, Chronic non-discriminatory social defeat is an effective chronic stress paradigm for both male and female mice. *Neuropsychopharmacology* **44**, 2220–2229 (2019).
32. C. Sánchez, *et al.*, Escitalopram, the S-(+)-enantiomer of citalopram, is a selective serotonin reuptake inhibitor with potent effects in animal models predictive of antidepressant and anxiolytic activities. *Psychopharmacology* **167**, 353–362 (2003).
33. P. J. Fitzgerald, J. Y. Yen, B. O. Watson, Stress-sensitive antidepressant-like effects of ketamine in the mouse forced swim test. *PLoS One* **14**, e0215554 (2019).
34. M. J. Anwar, K. K. Pillai, A. Samad, D. Vohora, Effect of escitalopram on cardiomyopathy-induced anxiety in mice. *Hum. Exp. Toxicol.* **32**, 632–639 (2013).
63. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
64. S. B. Plaisier, R. Taschereau, J. A. Wong, T. G. Graeber, Rank-rank hypergeometric overlap: identification of statistically significant overlap between gene-expression signatures. *Nucleic Acids Res.* **38**, e169 (2010).
65. K. M. Cahill, Z. Huo, G. C. Tseng, R. W. Logan, M. L. Seney, Improved identification of concordant and discordant gene expression signatures using an updated rank-rank hypergeometric overlap approach. *Sci. Rep.* **8**, 9588 (2018).
66. T. Wu, *et al.*, clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *Innov. J.* **2**, 100141 (2021).
67. G. Yu, L.-G. Wang, Y. Han, Q.-Y. He, clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* **16**, 284–287 (2012).
68. C. J. Peña, E. J. Nestler, R. C. Bagot, Environmental Programming of Susceptibility and Resilience to Stress in Adulthood in Male Mice. *Front. Behav. Neurosci.* **13**, 40 (2019).
69. A. Dieterich, T. Liu, B. A. Samuels, Chronic non-discriminatory social defeat stress reduces effort-related motivated behaviors in male and female mice. *Transl. Psychiatry* **11**, 125 (2021).
70. R. C. Bagot, *et al.*, Ventral hippocampal afferents to the nucleus accumbens regulate susceptibility to depression. *Nat. Commun.* **6**, 7062 (2015).
71. C. W. Law, Y. Chen, W. Shi, G. K. Smyth, voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol.* **15**, R29 (2014).
72. M. E. Ritchie, *et al.*, limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* **43**, e47 (2015).
73. J. Molet, P. M. Maras, S. Avishai-Eliner, T. Z. Baram, Naturalistic rodent models of chronic early-life stress. *Dev. Psychobiol.* **56**, 1675–1688 (2014).
74. E. E. Gilles, L. Schultz, T. Z. Baram, Abnormal corticosterone regulation in an immature rat model of continuous chronic stress. *Pediatr. Neurol.* **15**, 114–119 (1996).
75. C. J. Rice, C. A. Sandman, M. R. Lenjavi, T. Z. Baram, A novel mouse model for acute and long-lasting consequences of early life stress. *Endocrinology* **149**, 4892–4900 (2008).
76. S. R. Bodnoff, B. Suranyi-Cadotte, R. Quirion, M. J. Meaney, A comparison of the effects of diazepam versus several typical and atypical anti-depressant drugs in an animal model of anxiety. *Psychopharmacology* **97**, 277–279 (1989).