Disrupted Estrogen Homeostasis Drives Female-Specific Depressive-Like and Anxiety-Like Behaviors via Convergent ERα Downregulation in the Ventral Hippocampus

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This article contains Supplementary Tables S1–S2, Supplementary Figures S1-S5, Supplementary Data S1-S2, Supplementary Methods S1-S3, and Supplementary Original Western Blots

Supplementary Table S1. Primer sequences for quantitative real-time PCR

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Gene	Forward	Reverse
GAPDH	CAATGACCCCTTCATTGACC	TGGACTCCACGACGTACTCA
TNF	GCCTATGTCTCAGCCTCTTCTC	GCCATTTGGGAACTTCTCATCC
IL-1β	TCTTGGGACTGATGCTGGTG	CAGAATTGCCATTGCACAACTC
IL-6	GCCTTCTTGGGACTGATGCT	GCCATTGCACAACTCTTTTCTC
ERα	CACCAGATCCAAGGGAA	CGGCGTTGAACTCGTAG
ERβ	GACTGTAGAACGGTGTGGTCATCAA	CTGTGAGGTAGGAATGCGAAAC

Supplementary Table S2. Baseline characteristics of included participants in NHANES 2013-2016 and 2021-2023

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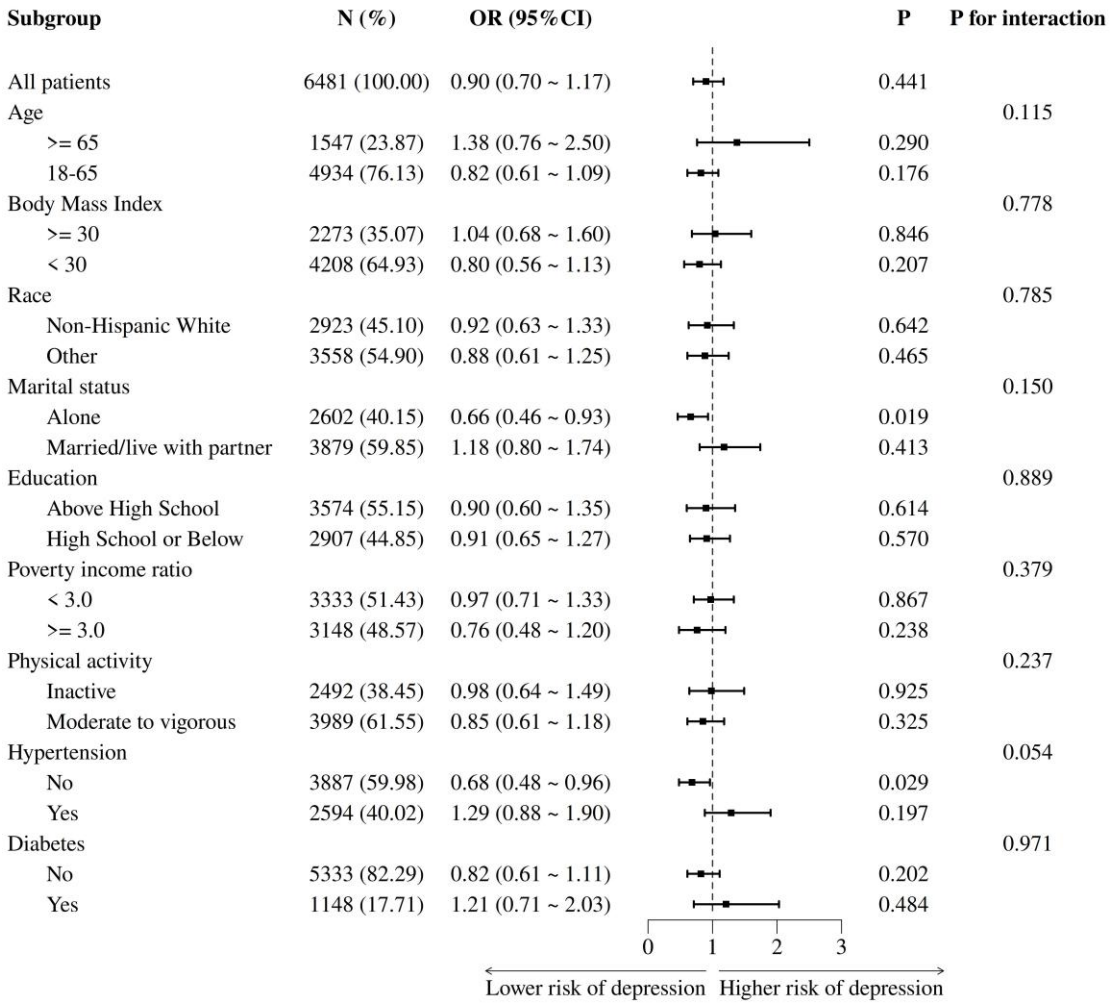
Characteristics	Overall	Serum Estradiol, pg/mL				*P*
		Q1 (< 18.6)	Q2 (18.6-24.0)	Q3 (24.0-30.1)	Q4 (≥ 30.1)	
Participants, n	6481	1606	1628	1616	1631	
Age, years	48.47 ± 18.47	49.24 ± 18.05	47.74 ± 18.28	47.94 ± 18.31	48.95 ± 19.20	0.052
Body Mass Index, kg/m^2	27.80 (24.50, 31.80)	27.15 (24.20,30.40)	27.40 (24.40,31.10)	28.20 (24.80,32.10)	28.60 (24.80,33.60)	<.001
Race						0.866
Non-Hispanic White	2923 (45.10)	734 (45.70)	740 (45.45)	726 (44.93)	723 (44.33)	
Other	3558 (54.90)	872 (54.30)	888 (54.55)	890 (55.07)	908 (55.67)	
Smoking						<.001
Current	1308 (20.18)	371 (23.10)	323 (19.84)	313 (19.37)	301 (18.45)	
Former	1783 (27.51)	491 (30.57)	459 (28.19)	418 (25.87)	415 (25.44)	
Never	3390 (52.31)	744 (46.33)	846 (51.97)	885 (54.76)	915 (56.10)	
Drinking						<.001
No	1828 (28.21)	435 (27.09)	436 (26.78)	433 (26.79)	524 (32.13)	
Current	4653 (71.79)	1171 (72.91)	1192 (73.22)	1183 (73.21)	1107 (67.87)	
Physical activity						0.383
Inactive	2492 (38.45)	589 (36.67)	629 (38.64)	631 (39.05)	643 (39.42)	

Moderate to vigorous	3989 (61.55)	1017 (63.33)	999 (61.36)	985 (60.95)	988 (60.58)	
Marital status						<.001
Alone	2602 (40.15)	536 (33.37)	609 (37.41)	671 (41.52)	786 (48.19)	
Married/live with partner	3879 (59.85)	1070 (66.63)	1019 (62.59)	945 (58.48)	845 (51.81)	
Education						0.339
Above High School	3574 (55.15)	858 (53.42)	897 (55.10)	896 (55.45)	923 (56.59)	
High School or Below	2907 (44.85)	748 (46.58)	731 (44.90)	720 (44.55)	708 (43.41)	
Diabetes						0.309
No	5333 (82.29)	1309 (81.51)	1363 (83.72)	1331 (82.36)	1330 (81.55)	
Yes	1148 (17.71)	297 (18.49)	265 (16.28)	285 (17.64)	301 (18.45)	
Hypertension						<.001
No	3887 (59.98)	980 (61.02)	1030 (63.27)	987 (61.08)	890 (54.57)	
Yes	2594 (40.02)	626 (38.98)	598 (36.73)	629 (38.92)	741 (45.43)	
Poverty income ratio						0.012
< 3.0	3333 (51.43)	848 (52.80)	797 (48.96)	807 (49.94)	881 (54.02)	
≥ 3.0	3148 (48.57)	758 (47.20)	831 (51.04)	809 (50.06)	750 (45.98)	
Self-reported health						0.439
Very good to excellent	2900 (44.75)	694 (43.21)	737 (45.27)	725 (44.86)	744 (45.62)	
Good	2361 (36.43)	582 (36.24)	598 (36.73)	602 (37.25)	579 (35.50)	

Poor to fair	1220 (18.82)	330 (20.55)	293 (18.00)	289 (17.88)	308 (18.88)	
Testosterone, total (ng/dL)	413.00 (306.00, 539.00)	323.00 (237.19,427.00)	393.00 (313.00,502.00)	438.00 (334.00,563.00)	503.00 (379.00,652.50)	<.001
Depression						0.016
No	6026 (92.98)	1480 (92.15)	1542 (94.72)	1494 (92.45)	1510 (92.58)	
Yes	455 (7.02)	126 (7.85)	86 (5.28)	122 (7.55)	121 (7.42)	

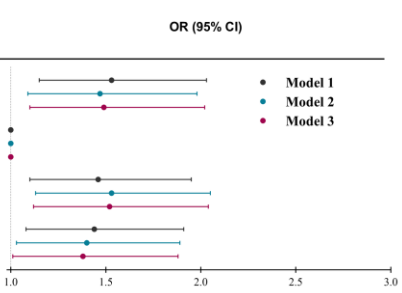
Supplementary Figure S1. Subgroup analyses of the association between serum estradiol levels and depression in NHANES 2013-2016 and 2021-2023



The plot shows the odds ratios (ORs) and 95% confidence intervals (CIs) per unit increment in log-transformed serum estradiol concentrations. Models were fully adjusted for age, race, body mass index, poverty income ratio, marital status, education level, smoking, drinking, physical activity, hypertension, diabetes, self-reported health, and log-transformed testosterone. Stratification variables were excluded from the corresponding models. The solid vertical line represents the reference value of 1.0. The dashed vertical line indicates the overall OR for the entire cohort.

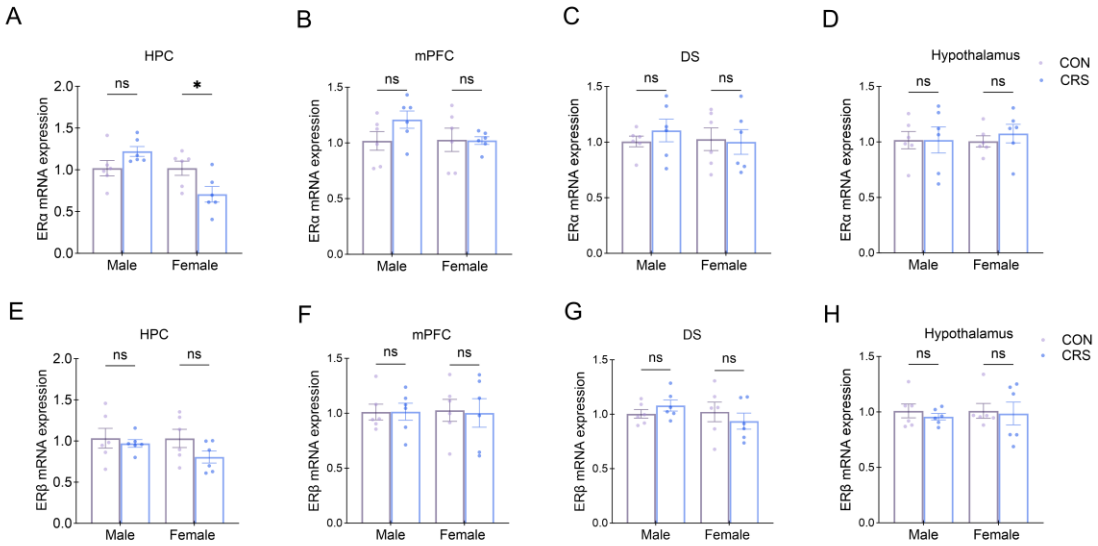
Supplementary Figure S2. Sensitivity analysis of the association between serum estradiol levels and depression in NHANES 2013-2016 and 2021-2023

Estradiol, pg/mL	Model 1		Model 2		Model 3	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Q1 (< 18.6)	1.53 (1.15 ~ 2.03)	0.003	1.47 (1.09 ~ 1.98)	0.011	1.49 (1.10 ~ 2.02)	0.010
Q2 (18.6-24.0)	1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
Q3 (24.0-30.1)	1.46 (1.10 ~ 1.95)	0.009	1.53 (1.13 ~ 2.05)	0.006	1.52 (1.12 ~ 2.04)	0.006
Q4 (≥ 30.1)	1.44 (1.08 ~ 1.91)	0.013	1.40 (1.03 ~ 1.89)	0.030	1.38 (1.01 ~ 1.88)	0.043



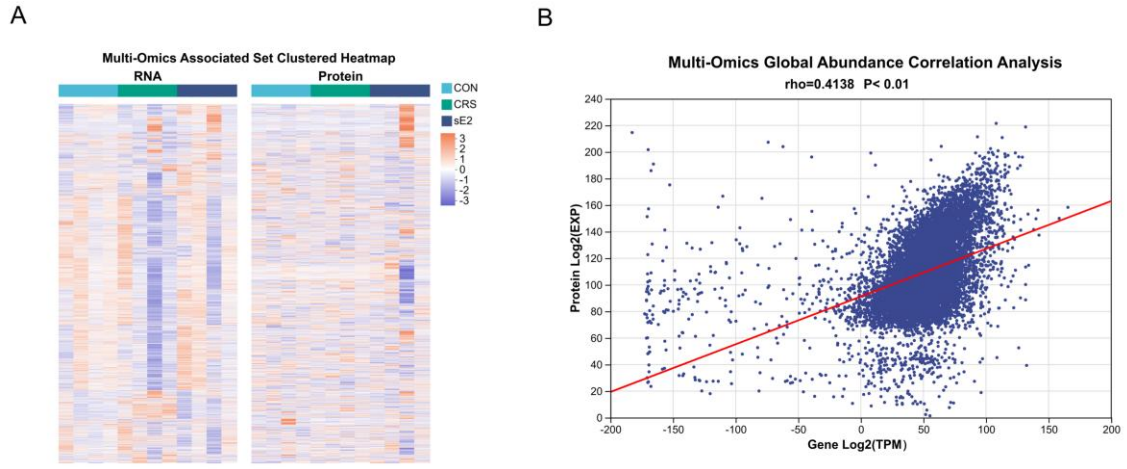
Analyses were performed using the original dataset without missing value imputation. Multivariable-adjusted ORs (95% CI) for depression across quartiles of estradiol concentrations. Model 1: unadjusted; Model 2: adjusted for age, race, body mass index, poverty income ratio, marital status, education level, smoking, drinking, physical activity, hypertension, diabetes, self-reported health; Model 3: additionally adjusted for log-transformed testosterone. Significant associations were observed in Q1, Q3, and Q4 compared to the reference group Q2 (all $p < 0.05$). OR, odds ratio; CI, confidence interval.

Supplementary Figure S3. CRS selectively decreases ERα but not ERβ mRNA expression in the hippocampus of female mice



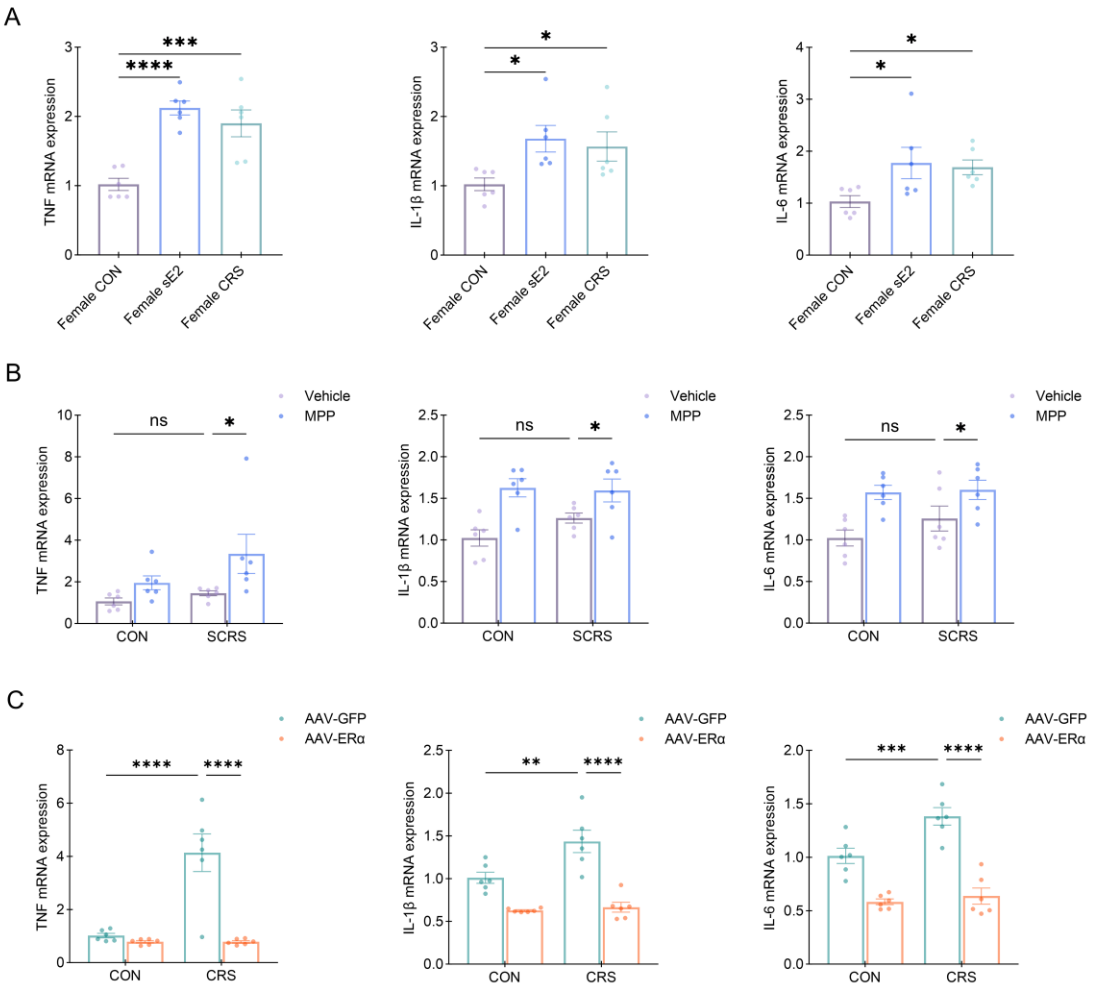
(A–D) ERα mRNA expression levels in the hippocampus (HPC), medial prefrontal cortex (mPFC), dorsal striatum (DS), and hypothalamus. Note the selective downregulation in the HPC. **(E–H)** ERβ mRNA expression levels in the respective brain regions. Data are presented as mean ± standard error of mean, $*p < 0.05$.

Supplementary Figure S4. Integrated analysis of the transcriptome and proteome



(A) Clustered heatmap displaying the relative expression levels (Z-score normalized) of RNA (left) and proteins (right) across Female CON, Female CRS, and Female sE2 groups. **(B)** Global abundance correlation analysis between the transcriptome and proteome (Spearman's rho = 0.4138, $p < 0.01$).

Supplementary Figure S5. Evaluation of neuroinflammatory factors



Relative mRNA expression levels of pro-inflammatory cytokines (TNF, IL-1β, IL-6) in the ventral hippocampus of female mouse models. Data are presented as mean ± standard error of mean, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Supplementary Data S1. Human MDD GWAS-identified candidate genes

Data were adapted from Als TD, et al. "Depression pathophysiology, risk prediction of recurrence and comorbid psychiatric disorders using genome-wide analyses," Nat Med. 2023;29(7):1832-1844.

Supplementary Data S2. Catalog of genes associated with mental disorders from the PsyGeNET database

Data were adapted from Ma G, et al., "Single-cell transcriptomics reveals Sox6 positive interneurons enriched in the prefrontal cortex of female mice vulnerable to chronic social stress," Mol Psychiatry. 2025;30(11):5058-5071.

Supplementary Methods

Supplementary method S1. Preliminary cross-sectional study

Study Population and Cohort Selection

Our preliminary cross-sectional analysis utilized data from the National Health and Nutrition Examination Survey (NHANES) across three cycles: 2013 – 2016 and 2021 – 2023 [1]. To ensure analytical rigor, participants were selected according to pre-specified inclusion and exclusion criteria. As illustrated in the flowchart (Figure 1a), the initial pool comprised 32,079 participants. After excluding individuals aged < 18 years (n = 11,821), those using exogenous hormones (n = 1,603) or antidepressants (n = 1,003), pregnant or lactating women (n = 263), and participants with incomplete patient health questionnaire-9 items (PHQ-9) or sex hormones data (n = 4,940), 12,449 eligible participants remained [2-4]. To minimize physiological hormonal heterogeneity (menstrual cycle fluctuations and reproductive status), 5,968 female participants were further excluded, resulting in a final core analytical cohort of 6,481 male participants, focusing on the association between serum E2 and depression risk [5-6].

The original NHANES study was approved by the National Center for Health Statistics (NCHS) Research Ethics Review Board, and all participants provided written informed consent. This secondary analysis of de-identified public data was conducted in accordance with the Declaration of Helsinki.

Measurement of Sex Hormones

Serum concentrations of E2 and testosterone were quantified simultaneously using isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC–MS/MS). As the international gold standard for hormone measurement, ID-LC–MS/MS provides superior sensitivity and specificity compared to traditional immunoassays, effectively eliminating matrix interference and ensuring high precision. The lower limits of detection (LLOD) for serum E2 and testosterone were 2.994 pg/mL and 0.750 ng/mL, respectively. For values below the LLOD, concentrations were substituted with LLOD divided by the square root of 2 (LLOD/$\sqrt{2}$) to maintain data integrity for statistical analysis [7-8].

Assessment of Depressive Symptoms

Depressive symptoms were quantified using the PHQ-9, a validated 9-item self-report screening tool based on the DSM diagnostic criteria. Each item was scored from 0 ("not at all") to 3 ("nearly every day") based on symptoms experienced over the past two weeks, with a total score range of 0–27. According to widely accepted clinical standards, a total score $\geq$ 10 was utilized as the threshold for

clinically significant depression. To handle missing PHQ-9 data, we followed the NHANES standardized protocol [29]: if at least seven of the nine items were answered (missing ≤ 2 items), the total score was calculated by imputing the missing values with the mean score of the responded items. If more than two items were missing, the participant was excluded from the depression-related analysis to ensure the reliability of the clinical assessment [9-10].

Definition of Covariates

A comprehensive set of covariates was extracted and adjusted to control for potential confounding factors: (1) Demographics: Age, race, education, and marital status; (2) Socioeconomic status: family income to poverty (PIR); (3) Lifestyle factors: smoking, drinking, physical activity, and body mass index (BMI); (4) Clinical variables: Medical history of hypertension and diabetes, and self-reported health [11-12].

Statistical Analysis

Statistical analyses were performed using R software (version 4.4.1), with $p < 0.05$ considered statistically significant.

Missing values were imputed via multiple imputation by chained equations (mice) using a random forest algorithm [13]. Baseline characteristics across serum E2 quartiles were compared using one-way ANOVA, Kruskal-Wallis, or Chi-square tests as appropriate. The dose-response relationship between log-transformed serum E2 and depression was modeled using restricted cubic splines (RCS) with three knots (10th, 50th, and 90th percentiles); non-linearity was tested via the likelihood ratio test. Multivariable logistic regression estimated odds ratios (ORs) and 95% confidence intervals (CIs) across three models: Model 1: unadjusted; Model 2: adjusted for age, race, BMI, PIR, marital status, education, smoking, drinking, physical activity, self-reported health, hypertension, and diabetes; Model 3: additionally adjusted for log-transformed testosterone to account for androgen-independent effects. Subgroup analyses were performed to assess the consistency of the observed associations across different population characteristics, including age (18–65 vs. ≥ 65 years), BMI (< 30 vs. ≥ 30 kg/m²), race (Non-Hispanic White vs. Other), marital status (Married/Cohabiting vs. Alone), education (High school or less vs. Above high school), PIR (≥ 3.0 vs. < 3.0), physical activity (Moderate-to-vigorous vs. Inactive), and chronic disease status (Hypertension/Diabetes: No vs. Yes). Sensitivity analyses using the non-imputed dataset were conducted to evaluate the robustness of the findings [14-16].

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degrades estradiol and is linked to depression in premenopausal females. Cell Metab. 2023;35(4):685-694.e5.

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Supplementary method S2. Sample Size and Exclusion Criteria

All sample sizes were determined by power analysis. All experiments were independently repeated at least 3 times. A random subset of behaviorally validated mice was used for molecular analyses. Female mice in estrus were excluded except for the sE2-treated group. Mice that died, showed abnormal behavior, or had incorrect viral injections were also excluded. Final effective sample sizes:

1. Male CON (11), Male CRS (11), Female CON (12), Female CRS (11);
2. Female CON (12), Female sE2 (20), Female CRS (18);
3. CON+Vehicle (9), CON+MPP (8), SCRS+Vehicle (9), SCRS+MPP (10);
4. CON+GFP (16), CON+ERα (18), CRS+GFP (15), CRS+ERα (16).

Supplementary method S3. Integrative Multi-Omics Pipeline and Translational Bioinformatics Analyses

Rank-Rank Hypergeometric Overlap

Transcriptomic data and corresponding phenotypic information (accession number GSE102556) were retrieved from the NCBI Gene Expression Omnibus (GEO) database using the GEOquery package in the R programming environment [1]. Based on brain region annotations, samples derived from the ventral subiculum were selected and subsequently stratified by sex. Within each cohort, the log2 fold change and p-value for each gene were calculated using a standardized differential expression analysis pipeline [2]. Subsequently, the Rank-Rank Hypergeometric Overlap (RRHO) method was employed to compare the differential expression profiles across the human sex cohorts without relying on predefined significance thresholds, followed by a cross-species comparative analysis against mouse ventral hippocampal transcriptomic data [3]. First, genes were ranked based on the metric −log10(p-value) × sign(log2 fold change), ensuring that the most significantly upregulated and downregulated genes were positioned at opposite ends of the ranked list. Next, the analysis was conducted using the RRHO package with a sliding window step size set to 200. Hypergeometric tests were applied to evaluate the statistical significance of gene overlap between the two ranked lists at various cutoffs. Finally, RRHO heatmaps were generated to visualize the comparative results, where regions of significant enrichment indicate either concordant or discordant regulatory trends between the gene sets [4].

From the RRHO2 output, we extracted the gene sets corresponding to the maximally significant overlap (MSO) within the downregulated-downregulated (DD) quadrants for each pairwise comparison (sE2 vs. Human MDD and sE2 vs. CRS). To identify a highly conserved transcriptional signature, a consensus core was defined by intersecting these two pairwise DD gene lists. Functional enrichment analysis was subsequently performed on the consensus genes using the R package clusterProfiler. Gene Ontology (GO) terms were further identified, with *p*-values adjusted using the Benjamini-Hochberg (BH) method.

Gene Set Enrichment Analysis

To further elucidate the biological functions and signaling pathways associated with depression, Gene Set Enrichment Analysis (GSEA) was performed using the clusterProfiler R package [5]. Instead of focusing solely on individual differentially expressed genes, GSEA was employed to evaluate whether

predefined sets of genes exhibited statistically significant, cumulative differences between groups. We conducted a comprehensive functional enrichment analysis across four primary libraries: the MSigDB Hallmark gene sets, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and the Reactome Pathway Database. All detected genes were pre-ranked based on the ranking metric −log10(p-value) × sign(log2 Fold Change) derived from the differential expression analysis. The Enrichment Score (ES) was calculated using a weighted Kolmogorov-Smirnov statistic, followed by 1,000 permutations to determine statistical significance. Pathways with a nominal *p*-value < 0.05, and a False Discovery Rate (FDR) q-value < 0.25 were considered significantly enriched [6].

Identification of Differentially Expressed Genes and Proteins

To identify molecular signatures associated with depression and the effects of estrogen signaling, differential expression analysis was performed. For transcriptomic analysis, two sets of criteria were applied: (1) In the initial experimental set (Female CON, Female CRS, and Female sE2), differentially expressed genes (DEGs) were identified using a threshold of absolute fold change (FC) > 1.3 with a raw *p*-value < 0.05, using the Female CON group as the reference. (2) In the intervention set (CRS+ERα vs. CRS+GFP), a more stringent statistical threshold was employed, where DEGs were identified using a threshold of absolute FC > 1.3 and an adjusted *p*-value (*p*-adj) < 0.05, with the CRS+GFP group serving as the control. For proteomic analysis, differentially expressed proteins (DEPs) were screened between the Female CON, CRS, and sE2 groups. Proteins with an FC > 1.2 or < 0.83 and a raw *p*-value < 0.05 were considered significantly altered, relative to the Female CON group. All fold changes and statistical significances were calculated via the DESeq2 and limma R packages for transcriptomics and proteomics, respectively [7-8].

Transcription Factor Enrichment and Upstream Regulator Identification

To investigate the potential upstream transcriptional regulators driving the observed molecular dysregulation, transcription factor (TF) enrichment analysis was performed using the ChEA3 (ChIP-Enrichment Analysis 3) platform. The 147 convergent DEGs and 94 convergent DEPs, representing the shared signatures between the CRS and sE2 models, were utilized as input gene sets. We specifically queried the Literature ChIP-seq library within ChEA3 to identify TFs with significantly overrepresented binding sites among these convergent targets. TFs were ranked based on their statistical significance, calculated as the -log10 (Fisher's Exact Test *p*-value). This integrative analysis aimed to identify core

regulatory nodes, such as ESR1, that may coordinate the transcriptomic and proteomic alterations in the vHPC across different experimental conditions [9].

Integrated Multi-Omics Analysis

To identify robust biological signatures shared between the transcriptome and proteome, an integrated meta-analysis was conducted. First, a global abundance correlation was performed by calculating the Spearman correlation coefficient (rho) between gene expression levels (Log2 TPM) and protein abundance levels (Log2 EXP). For the integrative functional analysis, we employed Stouffer's Z-score method to combine the results from the transcriptomic and proteomic datasets. Specifically, the p-values and the direction of enrichment (Normalized Enrichment Scores, NES) from the individual GSEA of each omics level were converted into standard Z-scores. These Z-scores were then integrated using a weighted approach to calculate a Meta-Z-score for each biological pathway. This meta-analysis allowed us to identify convergent multi-omics pathways that were consistently dysregulated at both the mRNA and protein levels across the CRS and sE2 models [10-11]. The resulting integrated scores were visualized using bar plots, and the expression patterns of the top-ranked convergent multi-omic genes were further validated and visualized using dot plots. Additionally, all abundance data for the multi-omics associated sets were Z-score normalized for clustered heatmap visualization.

Functional Enrichment Analysis (GO and KEGG)

To further characterize the functional impact of ERα (ESR1) overexpression in the vHPC, GO and KEGG enrichment analyses were performed on the DEGs identified from the CRS+ERα vs. CRS+GFP comparison. The GO analysis categorized the DEGs into three primary functional domains: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). The KEGG pathway analysis was employed to identify the biological signaling pathways and metabolic processes significantly enriched with these ESR1-regulated genes. Statistical significance for the enrichment was determined based on a raw p-value < 0.05 or a Benjamini-Hochberg adjusted p-value (p-adj) < 0.05 [12-13].

Clinical and Translational Relevance Analysis

To evaluate the clinical and translational significance of the ERα (ESR1)-regulated molecular signatures in the vHPC, three layers of integrative analyses were performed.

Firstly, after filtering for protein-coding transcripts and performing mouse-to-human ortholog mapping, a cross-model validation was conducted using a Venn diagram to identify the overlap between ESR1-regulated DEGs and the convergent DEGs previously identified from the CRS and sE2 models

[14]. Secondly, to assess the genetic relevance, these prioritized orthologs were overlapped with Genome-Wide Association Study (GWAS) hits linked to depression [15]. Thirdly, the refined ESR1-regulated gene set was queried against the PsyGeNET database to explore its association with a broad spectrum of psychiatric disorders [16].

Finally, to translate these molecular mechanisms into potential pharmacological interventions, a Connectivity Map (CMap) analysis was employed. The ERα-regulated gene signature was utilized to query the database to identify top small-molecule compounds (candidate therapy mimetics) capable of inducing transcriptomic profiles highly similar to the ERα-rescue effect [17].

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