MicroRNA-mediated protective effects of peritraumatic 17β-estradiol on posttraumatic chronic pain in women

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INTRODUCTION

- Chronic posttraumatic musculoskeletal pain (CPMP) is a common outcome of traumatic stress exposure and is highly morbid and costly (1,2).
- Women are disproportionately burdened by CPMP (3,4).
- Few peritraumatic biomarkers and mediators of CPMP vulnerability have been identified (3).
- In a previous study, we showed across three independent longitudinal cohorts of trauma survivors that peritraumatic circulating levels of 17β-estradiol (E2) are a strong predictor of CPMP in women (but not men), such that higher circulating levels of E2 in the early aftermath of traumatic stress exposure is associated with clinically and statistically significant lower levels of CPMP over the course of a year following trauma.(5)
- In the current study, we aimed to further validate these findings using samples and data from the AURORA study (6). Further, we aimed to assess potential mechanisms through which E2 influences CPMP using available microRNA data from the same human cohort and complimentary *in vitro* cell-based studies.

HYPOTHESIS

Higher levels of E2 in the early aftermath of traumatic stress exposure is associated with lower levels of CPMP. microRNA mediate the relationship between E2 and CPMP development, and these mediators are involved in neuronal processes important to the pathogenesis of CPMP.

METHODS

The AURORA study is a longitudinal cohort designed to improve the prevention, diagnosis, and treatment of adverse posttraumatic neuropsychiatric sequelae, including CPMP, following traumatic stress exposure. Participants aged 18-75 years who presented to the ED within 72 hours of trauma exposure at 23 participating ED sites were enrolled. Overall musculoskeletal pain severity was assessed in the immediate aftermath of trauma and at three follow-up timepoints (eight weeks, three months, and six months) via a 0-10 numeric rating scale (NRS: 0 (no pain) to 10 (maximum possible pain)). Blood samples were collected in the early aftermath of trauma exposure into EDTA and PAXgeneRNA tubes. 17β-estradiol levels were measured in duplicates using the Ultrasensitive Estradiol Enzyme-linked Immunosorbent Assay (ALPCO, Salem, NH, Catalog #20-ESTHUU-E01) according to manufacturer's specifications. Sample absorbance (450nm) was measured using a Synergy HTX Multi-mode Microplate Reader (BioTek Instruments). Hormone concentrations were calculated from a standard curve generated using samples with known concentration; these standards were included on each 96-well plate containing study samples, along with two controls of known concentration and two samples in duplicate that was measured across all plates to control for cross-plate variability. For RNA analysis, template libraries for miRNA next-generation sequencing were produced from 1.0 ug total RNA. Samples were prepped using PAXgene Blood miRNA library prep kits according to manufacturer's instructions (Qiagen, Germantown, MD). Raw sequence reads were processed using a custom bioinformatics pipeline and were normalized using upper quartile normalization. We examined the direct transcriptional impact of 17β-estradiol on dorsal root ganglia (DRG), known centers for nociception-related gene regulation by estrogen receptor signaling (7). Lumber DRG (L4-L6) were dissected and prepared from naive, female Sprague-Dawley rats (4-5 weeks old) and prepared cells were stimulated with 100-nM 17β-estradiol (Sigma, E2758). RNA was isolated after 3 hours using TRIzol Reagent (Invitrogen, Carlsbad, CA, 15596026) and miRNA expression changes were measured using the GeneChip™ miRNA 4.0 Array (Thermo Fisher Scientific, MA, 902411). ***Statistical Analyses.*** E2 levels were determined to be non-normally distributed via the Shapiro-Wilk test; therefore, E2 levels were transformed by taking the square root of raw E2 values. Linear mixed models were used to assess the relationship between peritraumatic E2 levels and CPMP using the lmer function from the lme4 package (v. 1.1.23)(8) in R (v. 3.6.2)(9). Repeated measures mixed modeling was used, including participant age, ethnicity, BMI, education level, baseline pain levels, ELISA array, and time since trauma exposure as covariates. To explore specific RNA transcripts that might mediate the relationship between E2 and CPMP, mediation model analyses were performed independently for each miRNA transcript detected via RNA sequencing, adjusting for age, BMI, education level and baseline pain. Ingenuity Pathway Analysis (IPA) software (Qiagen, Germantown, MD) was used to identify enriched pathway relationships amongst miRNA transcript mediators in the context of known biological networks and canonical pathways. Significantly upregulated or downregulated miRNA from rat DRG neurons were identified using the R/Bioconductor package. Differential expression statistics were conducted using Linear Models for Microarray Data (LIMMA) and the eBayes (an empirical Bayes moderated t-statistic) method while controlling for false discovery rate (FDR). A p-value threshold of 0.05 was used.

Figure 1. Study Design. (A) Blood plasma was collected from AURORA participants and assayed for circulating 17β-estradiol (E2) (high sensitivity ELISA) and miRNA levels (small RNA sequencing). (B) Dorsal root ganglion (DRG) neurons (L4-L6) were extracted from pre-pubescent female rats, cultured, and treated with E2 or vehicle for 3 hours. RNA was isolated using Trizol and assayed for microRNA expression using ThermoFisher's GeneChip MiRNA 4.0 Microarray.

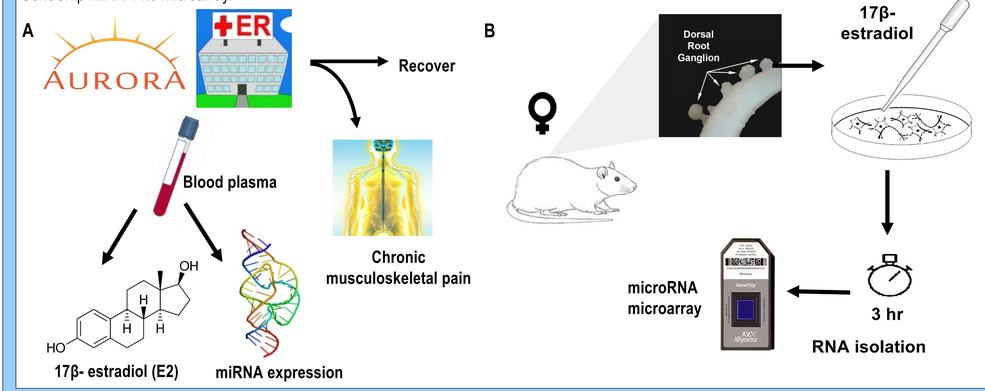
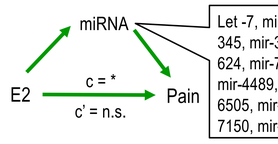


TABLE 2. Repeated Measures Regression Analyses. The relationship between circulating 17β-estradiol levels at the time of trauma exposure and chronic posttraumatic pain severity over the course of six months (WK8, M3, M6) following trauma exposure in multiethnic women and men (n=227).

	AURORA Women			AURORA Men		
	β	S.E.	p value	β	S.E.	p value
Intercept	0.990	1.904	0.603	-10.455	6.694	0.121
Time	-0.260	0.071	<0.001	-0.280	0.122	0.023
17β-estradiol	**-0.137**	**0.064**	**0.033**	0.015	0.118	0.902
Age	0.019	0.017	0.283	0.054	0.028	0.056
BMI	0.022	0.029	0.459	0.047	0.067	0.481
Baseline pain	0.343	0.091	0.002	0.444	0.167	0.009

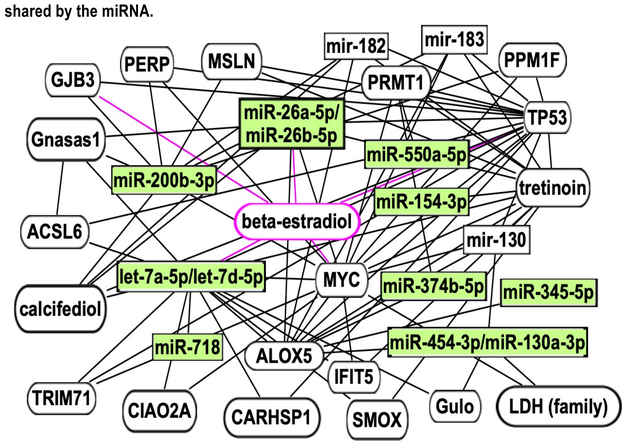
Chronic posttraumatic pain severity was measured via a 0-10 numeric rating scale (NRS). Participants' education level, race/ethnicity, and the plate that the 17β-estradiol was run on were also included as a categorical variable in the model, but for simplicity (due to multiple categories), was not included in the table.

FIGURE 3. Secondary analyses assessing potential miRNA that mediate the relationship between E2 and chronic pain severity identified 25 full mediators.



Let -7, mir-10, mir-26, mir-154, mir-200, mir-345, mir-374, mir-454, mir-510, mir-550, mir-624, mir-718, mir-4257, mir-4323, mir-4485, mir-4489, mir-4638, mir-5582, mir-6731, mir-6505, mir-5692, mir-6516, mir-6501, mir-7150, mir-7846

FIGURE 4. Pathway analyses using the 25 miRNA mediators shown in Figure 3 identified "Neurological Disease, Organismal Injury, and Psychological Disorders" as the top network shared by the miRNA.



Top network: "Neurological Disease, Organismal Injury, and Psychological Disorders"

FIGURE 2. Women with high circulating levels of 17β-estradiol at the time of trauma exposure report lower pain severity in the subsequent weeks and months (n=164).

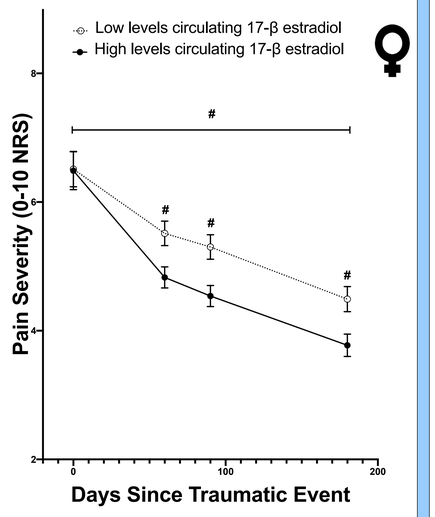


FIGURE 5. Of the miRNA identified as mediators of the relationship between E2 and CPMP in human AURORA participants, both miR-454 (*p = 0.0183*) and miR-374b (*p = 0.0207*) were significantly downregulated in female rat DRG following treatment with E2. This was the same direction of effect as seen in AURORA women.

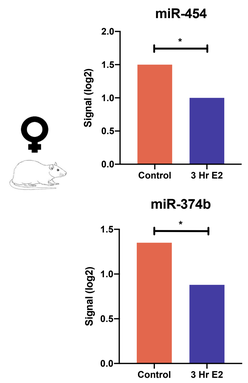


TABLE 1. Cohort characteristics. Women (n=164) and men (n=63) from the AURORA cohort were included. All participants were enrolled in the immediate aftermath of trauma.

Characteristic	AURORA Women	AURORA Men
Participants, n	164	63
Age, years, mean (SD)	39.99 (14.2)	36.51 (14.5)
Trauma type, n (%)		
Motor vehicle collision	161 (98)	60 (95)
Physical assault	3 (2)	3 (5)
Sexual assault	0	0
Major thermal burn injury	0	0
Education, n (%)		
Less than high school graduate	16 (10)	8 (13)
High school graduate	31 (19)	20 (32)
Some college	73 (45)	25 (40)
College graduate	44 (27)	10 (16)
Ethnicity		
Non-Hispanic White	53 (32)	28 (44)
Non-Hispanic Black	105 (64)	34 (54)
Hispanic and Non-Hispanic other	6 (4)	1 (2)
BMI, mean (SD)	31.2 (7.9)	28.2 (6.4)
Pain sev in ED/ SANE, mean (SD)	6.5 (2.5)	6.2 (2.7)
Previous traumatic events, mean (SD)	3.3 (2.4)	3.1 (2.8)
E2 level (pg/ml), mean (SD)	100.4 (1107.9)	50.8 (58.8)

RESULTS

- Characteristics of the study sample are shown in Table 1. Most participants were less than 40 years old, had experienced motor vehicle collision, had some college education, and were overweight or obese.
- In women only, an inverse relationship between peritraumatic E2 and the development of CPMP was observed (β=-0.137, p=0.033) such that women with high E2 at the time of trauma had less CPMP over the following weeks and months.
- Secondary analyses identified 25 miRNA that mediated the relationship between E2 and CPMP; pathway analysis of the 25 miRNA identified "Neurological Disease, Organismal Injury, and Psychological Disorders" as the top network.
- Female rat lumbar DRG treated with E2 had decreased expression of miR-454 and miR-374b. In AURORA women, these miRNA were also downregulated in women with high E2.

CONCLUSIONS

We replicated the results from previous cohorts showing that increased peritraumatic E2 levels predict improved CPMP outcomes in women. This relationship might be mediated by E2 regulation of pain-associated miRNA transcripts. Further studies are needed to validate this finding.

REFERENCES

1. Herrera-Escobar JP, Apoj M, Weed C, Harlow AF, Al Rafai SS, Lilley E, et al. (2018): Association of pain after trauma with long-term functional and mental health outcomes. Journal of Trauma and Acute Care Surgery 85: 773–779.
2. Institute of Medicine (US) Committee on Advancing Pain Research, Care, and Education (2011): Relieving Pain in America: A Blueprint for Transforming Prevention, Care, Education, and Research. Washington (DC): National Academies Press (US). Retrieved November 16, 2021, from http://www.ncbi.nlm.nih.gov/books/NBK91497/
3. Linnstaedt SD, Zannas AS, McLean SA, Koenen KC, Ressler KJ (2020): Literature review and methodological considerations for understanding circulating risk biomarkers following trauma exposure. Mol Psychiatry 25: 1986–1999.
4. Tolin DF, Foa EB (2006): Sex differences in trauma and posttraumatic stress disorder: a quantitative review of 25 years of research. Psychol Bull. 132(6):959-92. doi: 10.1037/0033-2909.132.6.959.
5. Linnstaedt SD, Mauck MC, Son EY, Tungate AS, Pan Y, Ruekeis C, Yu S (2021): Peritraumatic 17β-estradiol levels influence chronic posttraumatic pain outcomes. Pain. 162(12):2909-2916. doi: 10.1097/j.pain.0000000000002282.
6. McLean SA, Ressler K, Koenen KC, Neylan T, Germine L, Jovanovic T, et al. (2020): The AURORA Study: a longitudinal, multimodal library of brain biology and function after traumatic stress exposure. Mol Psychiatry. 25(2):283-296. doi: 10.1038/s41380-019-0581-3
7. Chen Q, Zhang W, Sadana N, Chen X (2021): Estrogen receptors in pain modulation: cellular signaling. Biology of Sex Differences 12: 22.
8. Bates D, Mächler M, Bolker B, Walker S (2014): Fitting Linear Mixed-Effects Models using lme4. arXiv:14065823 [stat]. Retrieved December 6, 2021, from http://arxiv.org/abs/1406.5823
9. R: a language and environment for statistical computing (n.d.): Retrieved December 6, 2021, from https://www.gbif.org/tool/81287/r-a-language-and-environment-for-statistical-computing

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