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microRNA-19b predicts widespread pain and posttraumatic stress symptom risk in a sex-dependent manner following trauma exposure

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Introduction

Motor vehicle collision (MVC) and sexual assault (SA) are common traumatic stress exposures that occur worldwide.[10] For every 10 individuals experiencing MVC or SA, evidence suggests that two will develop persistent posttraumatic widespread pain (PTWP) and three to seven will develop posttraumatic stress symptoms (PTSS).[9; 43; 89] These morbid sequelae of traumatic stress exposures frequently co-occur[63], suggesting shared pathogenic mechanisms, and are more common in women[6; 47], suggesting that such mechanisms may differ in women and men. However, biologic mechanisms responsible for PTWP and PTSS and contributing to sex differences in these adverse outcomes remain poorly understood, despite increasing evidence indicating that pain and PTSS processing are sex-dependent[41; 85].

microRNA (miRNA) are small non-coding RNA molecules that primarily regulate gene expression by binding to target mRNA. Because a single miRNA can regulate many different gene transcripts, each miRNA can act as a gene regulatory hub. Previous studies have shown that the study of miRNA can be a valuable tool for gaining insight into PTWP and PTSS pathogenesis.[24; 58] However, many of these previous studies used predominately male cohorts[8; 61; 105] or focused on a single sex in animal model studies[7; 42; 92; 95]. Therefore, whether miRNA expression levels predict PTWP and PTSS in a sex-dependent manner has not been addressed, despite evidence indicating that miRNA expression might be differentially regulated in men and women[66].

The current study used an *in silico* approach to first identify pain and PTSS miRNA regulatory hubs; that is, miRNA that are predicted to regulate more pain and PTSS-associated transcripts than expected by chance[90]. One of the top candidate miRNA regulatory hubs, miR-19, was then assessed for differential expression in women and men who developed PTWP and/or PTSS vs those who recovered following MVC and in women who developed these outcomes following SA. Secondary analyses identified whether sex-dependent expression extended to nervous system tissues relevant to PTWP and PTSS and identified relevant pathways/transcripts regulated by miR-19.

Materials/Subjects and Methods

Motor vehicle collision cohort study (Discovery cohort)

This prospective longitudinal study enrolled African American individuals ≥ 18 and ≤ 65 years of age who presented within 24 hours of MVC to one of eleven emergency departments (EDs) in six states/districts (Michigan, Pennsylvania, Florida, Alabama, Massachusetts, and Washington D.C.) between July 2012 and July 2015. This study has been described in detail previously.[53]

In brief, individuals who did not have a fracture or other injury requiring hospital admission were screened for eligibility. Patients who were not alert and oriented were excluded, as were patients who did not self-identify as African American, were pregnant, prisoners, unable to read and understand English, or taking opioids above a total daily dose of 30 mg of oral morphine or equivalent. The study was approved by the institutional review boards of

all participating hospitals. Each participant provided written informed consent before enrollment.

Sexual assault cohort study (Replication cohort)

This prospective longitudinal study is similar in design to its pilot study described previously[64] and to the MVC study described above. Multiethnic women ≥ 18 and ≤ 65 years of age presenting to one of thirteen sexual assault nurse examiner (SANE) programs within 72 hours of sexual assault trauma were enrolled. Women unable to give informed consent (e.g., due to intoxication) were excluded, as were women who were hospitalized after sexual assault, lived with their assailant, were prisoners, were pregnant, did not have a telephone, and/or did not live within driving distance for follow-up interviews. Institutional Review Board (IRB) approval was obtained at all study sites, and all study participants provided written informed consent.

Pain and PTSS assessments and outcome definitions in humans

MVC- and SA- related pain intensity and distribution in the past week was assessed six months following trauma exposure using the modified Regional Pain Scale.[99] Pain intensity was evaluated in each of 19 body regions[98] via a 0 (no pain) to 10 (maximum possible pain) numeric rating scale (NRS).[28] PTWP was defined according to American College of Rheumatology 1990 criteria (i.e., axial pain, left and right sided pain, and upper and lower segment pain).[100]

MVC-related PTSS were assessed six months following MVC using the Impact of Event Scale: Revised (IESR).[96] This 22-item questionnaire includes avoidance, intrusion and hyperarousal subscales. Scores range from 0–88; a validated cut-off of 33 was used to define individuals with substantial PTSS vs individuals with low PTSS scores.[23]

SA-related PTSS was assessed six months following SA using the civilian version of the posttraumatic stress disorder checklist (PCL)[12]. This validated 17-item questionnaire assesses posttraumatic distress symptoms in relation to a stressful experience. A total symptom severity score (range = 17–85) can be obtained by summing the scores from each of the 17 items that have response options ranging from 1 “Not at all” to 5 “Extremely”.

Distress in the ED following MVC was measured using the Peritraumatic Distress Inventory, a 13-item questionnaire assessing the level of distress experienced immediately after a traumatic event.[15] Each item on the questionnaire was evaluated using a 0 (no distress) to 4 (high distress) numeric rating scale. A validated cut-off of 23 was used to identify those with substantial distress[69]; this was also the mean and median level of distress in the cohort.

Statistical analyses

MVC and SA cohort sociodemographic characteristics were summarized using standard descriptive statistics. Logistic regression analyses adjusted for participant age and ED or SANE study site were used to assess the relationship between miR-19b expression levels and PTWP or PTSS outcomes six months following trauma exposure, and to derive β

coefficients and p-value significance. Sex-dependent effects were evaluated using an interaction variable (miR-19b*sex) because of evidence that such interactions are frequently present and important (e.g.[65]). Differences in the predicted probability of developing PTWP and PTSS based on peritraumatic miR-19b expression levels was evaluated by using β coefficient estimates from the following logistic regression equation and the predict function in R (predict() function, R statistical computing software).

$$\text{Estimate of } P(\text{develop the outcome}) = \frac{1}{1+\exp[-(\beta_0+\beta_1 mir19+\beta_2 Sex+\beta_3 Age+\beta_4 mir19\times Sex+\sum\beta_k newSite_k)]}$$

Bivariate correlations were used to determine Pearson correlation coefficients and p-values for the relationship between miR-19b and potential miR-19b regulated transcripts. Statistical analyses were carried out using SPSS software v24.0, SAS software v9.4, or R statistical computing.

Bioinformatics

For *in silico* analyses aimed at identifying potential miRNA regulatory hubs for PTWP and PTSS, we first generated a list of previously identified pain and PTSS associated genes. Few studies evaluating genes associated specifically with post-traumatic widespread pain (PTWP) have been performed; genes associated with pain outcomes more generally were identified via the following pain gene databases: PainNetworks,[72] Algynomics Pain Research Panel v2.0,[60] and the PainGenes Database[49]. Similarly, genes associated with PTSS were identified via the PTSDgene database[102], and genes that overlapped between the four total databases were included only once. A full list of genes used for these analyses are presented in Supplementary Table 1 (n=604). miRNAs predicted to bind to the 3'UTR of these identified pain or PTSS genes were identified using TargetScan 7.0.[1] Monte Carlo simulations (x10,000) consisting of randomly selected sets of 604 genes were then used to generate a distribution of the number of predicted pain and PTSS gene targets for each known human mature miRNA (miR-Base v.22).[90] This distribution was used to determine an empirical p value and thus identify miRNA with the greatest preferential binding to genes previously associated with pain and PTSS phenotypes. (Of note, this *in silico* approach does not account for extracellular miRNA that have been shown previously to influence pain by directly activating nociceptive neurons[71]).

The ontology relationship between pain or PTSS genes predicted to be targeted by miR-19b was assessed using the DAVID v6.7 online algorithm (https://david.ncifcrf.gov/home.jsp).[40] All miR-19b predicted target genes were input into DAVID and the 'Gene Ontology Biological Process 1' annotation was selected.

Predicted miRNA/mRNA binding duplex/hybrids were determined using the RNA Hybrid online algorithm (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/).[75]

Animals

Experiments were performed on adult female and male Sprague Dawley rats (180–300 g; Charles River, Hollister, CA, Raleigh, NC, and Kingston, NY). Rats were housed under a 12-hour light/dark cycle in the Laboratory Animal Resource Center of the University of California, San Francisco, in the Division of Laboratory Animal Medicine at The University of North Carolina at Chapel Hill, or in the Veterinary Medical Unit at The University of Michigan. In all facilities, experiments were performed on independent female and male cohorts. Each of these cohorts underwent the same conditions, were tested either consecutively in the same day or at the same time of day in sequential days, and independent cohorts at each site were performed by the same experimenter. Additionally, each set of experiments that directly compared male and female behavior or data collected from the animals were performed at a single facility/site. Animal care and use conformed to NIH guidelines. Experimental protocols were approved by the Institutional Animal Care and Use Committee at each university.

Animal stress exposures

Unpredictable Sound Stress—This set of behavioral experiments and related tissue isolation was performed at the University of California, San Francisco. The details of the unpredictable sound stress (USS) protocol have been reported previously.[34; 44–46] Exposure to sound stress occurred over 4 days, on days 1, 3, and 4. Animals were placed in a soundproof chamber in sets of three individual cages that were positioned 25 cm from a speaker that emitted a 105-dB tone of mixed frequencies (11 to 19 kHz). Over a period of 30 min, rats were exposed to 5- or 10-s sound epochs each minute at random intervals during the minute. Following each stress session, animals were returned to the animal facility and pair-housed. Two weeks following the final stressor, animals were tested for pain hypersensitivity using von Frey monofilaments on the left hind paw following injection of the algogen prostaglandin E2 (PGE_2). Stock solutions of PGE_2 (Sigma, St. Louis, MO) were prepared with 4 mg/ml in 10% ethanol, with subsequent dilutions made in 0.9% saline. Mechanical nociceptive thresholds were determined following intraplantar injection of linearly increasing doses of PGE_2 (0.1–1000 ng), administered cumulatively at 25-minute intervals in a volume of 2.5 μl. Paw withdrawal thresholds were determined using the methods developed by Chaplan et al[19]. The results of these experiments have been published previously[44–46] and thus the pain behavioral data were not included in the current manuscript. However, we include these methods because, in order to ensure the development of USS-induced hyperalgesia as defined previously, paw withdrawal thresholds in response to PGE_2 were assessed before tissue isolation and measurement of miR-19b levels. Control animals were pair-housed and left undisturbed in the animal facility during stress tests, but were tested for hypersensitivity similarly to stressed animals.

Single Prolonged Stress—This set of experiments were performed at the University of Michigan. However, to ensure that SPS resulted in hyperalgesia, an additional cohort of rats that underwent SPS (in a manner identical to rats used for tissue expression studies) were tested at UNC. The details of the single prolonged stress (SPS) protocol have been reported previously.[48] Briefly, rats were exposed to serial stressors on one day as follows: restraint for 2 h, forced swim for 20 min, and exposure to ether until general anesthesia was induced

(generally < 5 minutes). Rats were pair-housed until the time of SPS, after which time they were single-housed and left undisturbed in the animal facility for 7 days, a period crucial for the development of PTSS symptomatology.[48] Control animals were left undisturbed in the animal facility during the SPS procedure and were single-housed during the same period as the SPS animals.

DRG isolation and stimulation with 17β-estradiol

Lumbar dorsal root ganglia (DRG) (L4-L6) were dissected from naïve, female Sprague Dawley rats (4–5 weeks old) and digested with 2 mg/ml collagenase (Sigma, St. Louis, MO, C5138) and 5 mg/ml dispase II (Sigma, D4693) in 1x HBSS at 37°C for 30 min. Cells were triturated with flame-polished Pasteur pipets, and plated at 2×10^5 cells/ml onto 96-well plates pre-coated with poly-D-lysine (Sigma, P7886) and Laminin (Sigma, L2020). Twenty-four hours after plating, 1 μM cytosine β-D-arabinofuranoside (Sigma, C1768) was added. DRG neurons were deprived of hormones by growing them in neurobasal A (ThermoFisher, 12349015) lacking phenol red, supplemented with 10% dextran-coated charcoal treated FCS, 50 μg/mL gentamicin, and murine nerve growth factor 2.5S (ThermoFisher, 13257019) for 72 hours. Stimulation with 100 nM 17β-estradiol (Sigma, E2758) lasted for 3 hours. RNA was isolated after 3 hours using TRIzol Reagent (Invitrogen, Carlsbad, CA, 15596026).

RNA collection and isolation

MVC cohort blood samples were collected in the ED at the time of enrollment using PAXgene RNA tubes. Total RNA (including miRNA) was isolated using the PAXgene blood miRNA kit (Qiagen, Germantown, MD) and RNA was stored at −80 ˚C until use.

For both stressed and non-stressed rats, blood and tissue RNA were collected immediately following pain or fear learning protocols. RNA was collected from animals in the single prolonged stress protocol via tail bleed immediately following CO_2 euthanasia into RNAprotect Animal Blood Tubes (Qiagen). Total RNA was isolated using RNeasy Protect Animal Blood Kits (Qiagen) and stored at −80 ˚C until use. Plasma was collected from animals in the sound stress protocol by collecting trunk blood immediately following live decapitation. (While CO_2 euthanasia was used on our first set of animals, above, we used live decapitation for all other animals to address the possibility that CO_2 might influence miR-19b expression levels.) RNA was isolated from plasma using miRNeasy Serum/Plasma kits (Qiagen). Tissue RNA (aside from DRG for 17β-estradiol study, above) was collected by isolating the specific tissue and immediately storing in RNA later (ThermoFisher) according to manufacturer's instructions. Animals used for tissue isolation were sacrificed via live decapitation without anesthesia, after which tissue samples (amygdala, hippocampus, hypothalamus, adrenal glands, DRG and peripheral nerve) were isolated within 30 min. Tissue was homogenized using Bashing Beads (Zymo Research, Irvine, CA) or a motorized homogenizer, RNA isolated using DirectZol (Zymo Research), and total RNA stored at −80˚C until use.

For all RNA samples, RNA concentration and purity were measured using a NanoDrop One (Nanodrop Technologies, Wilmington, DE), and RNA integrity was measured using an

Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only RNA meeting an RNA integrity score of 7.0 or greater were used in this study.

Next Generation Sequencing

Small RNAs—Template libraries for miRNA Next Generation Sequencing were produced from 1.0 μg total RNA. For the MVC cohort an initial set of 69 samples (randomly selected from the full MVC cohort) were prepped using an adaptation of published protocols as described previously.[54; 73] A second set of 89 samples (also randomly selected) were prepped using TruSeq Small RNA library prep kits according to manufacturer's specifications (Illumina, San Diego, CA). Six samples were library prepped using both methods. For the SA cohort, all samples were prepped using TruSeq Small RNA library prep kits. Twelve barcoded libraries were combined per lane and sequenced on a HiSeq 2000 (Illumina). Raw sequence reads were processed using a custom bioinformatics pipeline as described previously[54], and were normalized using upper quartile normalization. In order to normalize potential technical biases between the two methods of library preparation, seq reads were adjusted for batch effects using the ComBat package in R.[51]

Total RNA (excluding miRNA)—Template libraries for total RNA sequencing were produced from 600ng total RNA using Ovation Human Blood RNA-Seq Library Systems kit (NuGen, San Carlos, CA) according to manufacturer's specifications. Libraries were multiplexed in groups of six and sequenced on a HiSeq 2000 at the University of North Carolina at Chapel Hill High Throughput Sequencing Facility. Raw sequencing reads were aligned to the human hg19 genome assembly using STAR (version 2.4.2a).[26] Expression levels of each transcript were estimated via RSEM[52] using University of California Santa Cruz (UCSC) known gene transcript and gene definitions. Raw RSEM read counts for all samples were normalized to the overall upper quartile[16] before comparison and visualization.

Reverse transcription-quantitative PCR

miR-19b expression levels were measured in 26 samples from the MVC cohort using reverse transcription – quantitative PCR (RT-qPCR) according to the methods of Chen et al[20]. These samples were not part of the cohort that was used for RNA sequencing, thus they expanded the final cohort size to 179 participants. The samples were chosen based on case (PTSS and PTWP) control status at 6 months and were matched on age (within 5 years) and sex. Stem-loop RT primers and TaqMan probes for miR-19b (Assay ID: 000396) and endogenous control RNU48 (Assay ID: 001006) detection were obtained from ThermoFisher Scientific (Waltham, MA).

miR-19b expression levels from animal model samples were measured via RT-qPCR using the same miR-19b RT primers and TaqMan probes as from human studies but used U87 (Assay ID: 001712) as the endogenous control RNA. For detection of *CGRP* in animal experiments assessing effect of 17β-estradiol on RNA expression, TaqMan probes for *CGRP* (Assay ID: Rn00569199_m1) and *HPRT* (Assay ID: Rn01527838_g1) were obtained from ThermoFisher.

Luciferase assays

Cloning—The 3'UTRs of human genes *RORA, CLOCK,* and *NPAS2* were amplified from human 293T cell line genomic DNA using primers as indicated in Supplementary Table 2. The amplified 3'UTRs consisted of either the entire 3'UTR or a portion thereof, which preserved the relative location of the miR-19b binding site in the context of the full length 3'UTR. The resulting PCR products were cloned downstream of the firefly luciferase gene in pL-SV40-GL3 using XhoI and NotI or EcoR1 restriction enzyme sites. These newly created constructs were then mutated at the predicted miR-19b binding sites such that 2–3 mismatches were incorporated into the seed binding region (primers in Supplementary Table 2).

Transfections/luciferase assays—Nine fmol pL-SV40-GL3 + 3'UTR and 36 fmol pL-SV40-Rluc were transfected into 293T cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). Additionally, 136 fmol of an empty plasmid containing GFP was transfected into cells to monitor transfection efficiency. Six hours later, the media was replaced and the cells were transfected a second time with either 10 μM miR-19b mimic (Ambion, MC10629, Waltham, MA) or 10 μM negative control (Ambion, 4464058) using Lipofectamine RNAiMAX (Invitrogen). Seventy-two hours later, cells were lysed and assayed for Luciferin protein levels using the Dual Luciferase Reporter Assay System (Promega, Madison, WI). Firefly and Renilla luciferase levels were quantified using a Synergy HTX multi-mode Reader (BioTek, Winooski, VT). The measured firefly luciferase luminescence was normalized by dividing by the corresponding sample's Renilla luciferase luminescence. The normalized luminescence values from each triplicate were averaged. Change was measured by dividing the average luminescence of samples containing miR-19b mimic by the level from the negative control.

Results

In silico analyses identify miR-19 as a regulatory hub for the expression of genes involved in the pathogenesis of PTWP and PTSS

In order to gain insight into candidate miRNA that predict and are potentially involved in the pathogenesis of PTWP and PTSS development, while circumventing potential type I and type II error via miRNome analyses (due to limited cohort sample sizes (as cautioned previously[24]), we first performed *in silico* analyses to define potential miRNA regulatory hubs. Using previously validated methods[90], we found that among all known human mature miRNAs (n=2,654, miRBase v22.0), 185 (6.9%) miRNA were predicted to target one or more gene transcripts previously associated with pain and PTSS outcomes (n=604) (Supplementary Figure 1, range 1– 108). However, this analysis does not account for the probability that each miRNA might be predicted to target the same number of gene transcripts given any list of n=604 genes. Therefore, Monte Carlo simulations (x10,000) were used to determine the degree of preferentially predicted binding of each miRNA to mRNA transcripts previously shown to be associated with pain and PTSS, while accounting for the distribution of predicted targeting for each of 10,000 random lists of gene transcripts. Based on these *in silico* analyses, seventeen miRNA were defined as potential candidate regulatory hubs (Figure 1, $p \leq 0.05$) including miRNA previously shown to be associated with

pain, PTSS, neuronal processes, and stress, such as miR-19, miR-103, miR-25, miR-18, miR-34 and miR-15 [7; 14; 25; 27; 29; 56; 78; 88; 91–94; 105; 106]. Top amongst the miRNA with the highest probability for preferentially targeting pain and PTSS genes was the miR-19 family of transcripts (adjusted p=0.017, Figure 1). The miR-19 family includes miR-19a and miR-19b (Supplementary Figure 2); these two miRNA have identical seed sequences, and thus similar predicted targeting, but originate from different genomic loci (miR-19a: 13q31.3, miR-19b: 13q31.3 and Xq26.2). Because a secondary aim of the study was to identify miRNA that predict PTWP and PTSS in a sex-dependent manner and miR-19b has been shown previously to be estrogen regulated[18], subsequent analyses focused on this miR-19 family member.

miR-19b predicts posttraumatic widespread pain (PTWP) and/or posttraumatic stress symptoms (PTSS) differently in men and women following motor vehicle collision (MVC)

To assess for evidence that miRNA-19b regulates the expression of genes involved in the pathogenesis of PTWP and PTSS, we evaluated whether miRNA-19b blood levels in the immediate aftermath of motor vehicle collision (MVC, i.e. the Discovery cohort) predict PTWP and PTSS outcomes. Participants (n = 179) were drawn from a prospective cohort study of individuals presenting to the emergency department (ED) after MVC. Blood samples were obtained in the ED, and PTWP and PTSS outcomes were assessed at six months (Figure 2a). Most study participants were women less than 40 years of age who presented to the ED within an hour of MVC (Table 1). In this cohort, 28.1% of participants reported PTWP and 31.7% reported PTSS at six months. miRNA blood levels were assayed using either RNAseq (n = 153) or RT-qPCR (n = 26).

In initial logistic regression modeling (n = 153), a sex*miR-19b interaction was observed for both PTWP outcomes ($\beta = -2.41$, p = 0.034, Table 2) and PTSS ($\beta = -3.01$, p = 0.008, Table 2). (These associations persisted after adjusting for prior trauma exposure.) Subsequent analyses were therefore stratified by sex. In such stratified analyses, miR-19b expression levels were lower in women who developed PTWP and substantial PTSS than in those who recovered (1.40–1.45 fold lower, p = 0.026 for PTWP, p = 0.044 for PTSS, Supplementary Table 3) and expression levels of miR-19b were negatively correlated with the probability of developing PTWP and PTSS (Figure 2b, c). In contrast, miR-19b expression levels were higher in men who developed PTWP and substantial PTSS than in those who recovered (1.42–1.45 fold higher, p = 0.113 for PTWP, p = 0.039 for PTSS, Supplementary Table 3) and expression levels of miR-19b were positively correlated with the probability of developing PTWP and PTSS (Figure 2b, c). These observations were further expanded by assessing miR-19b expression levels via RT-qPCR in twenty-six additional women and men (69% women) from the MVC study. These participants were selected based on whether they met the criteria for co-morbid PTWP/PTSS or did not report either outcome (“recover”). In men, peritraumatic miR-19b expression levels were significantly higher in those individuals who developed PTWP/PTSS vs those who recovered (Mean difference = 3.41, p = 0.024; Figure 2d). In contrast, in women, peritraumatic miR-19b expression levels were lower in those individuals who developed PTWP/PTSS vs those who recovered, though this comparison was not statistically significant (Mean difference = −2.79, p = 0.336; Figure 2d). In secondary analyses using

small RNA-seq data, we found that circulating blood miR-19a levels also predicted PTWP and substantial PTSS in a sex-dependent manner (Supplementary Table 4).

The relationship between miR-19b and PTSS replicates in women in a second cohort of trauma survivors

In order to assess whether early peritraumatic expression levels of miR-19b were also associated with PTWP and PTSS development following a different type of single trauma exposure, we performed the same analyses described above but in a cohort of women sexual assault survivors (SA, the Replication cohort). Of note, this cohort is comprised of only women due to the disproportionate number of women who are sexually assaulted each year[67] and the pressing need to understand the trauma recovery process in these individuals[17]. We used this SA cohort for replication because the key novel finding from MVC survivors was the association between miR-19b and PTWP and PTSS in women (men and male animals had been shown previously to express higher levels of miR-19b in high pain and stress states[7; 61; 78; 92; 95]). Participants (n=74) were drawn from a larger prospective study of SA survivors (Table 1). 21.6% of the participants reported PTWP six months following trauma exposure and 74.3% of the participants reported PTSS. Consistent with results from the MVC dataset, in logistic regression modeling adjusted for age and study site, miR-19b expression levels significantly predicted PTSS outcomes ($\beta = -0.91$, $p = 0.013$) and were negatively correlated with the probability of developing PTSS (Figure 2e). In contrast, miR-19b expression levels were not significantly predictive of PTWP outcomes in this cohort of SA survivors ($\beta = 0.265$, $p = 0.439$).

miR-19b is expressed differently in male and female animals following stress exposure

We next evaluated whether the association between miR-19b and PTWP/PTSS observed in our human cohort studies translates to relevant expression levels in male and female animals exposed to psychological and physiological stressors, unpredictable sound stress (USS) [45] and single prolonged stress (SPS)[48] (Figure 3a). Previous studies have shown that these stressors cause enduring stress induced hyperalgesia[39; 44–46; 86; 103; 104] and PTSD-like phenotypes (reviewed in [55]). We also observe such behaviors following stress exposure in rats in our laboratory (Supplementary Figure 3). Because our human studies focused on blood expression levels of miR-19b, we first examined circulating levels of miR-19b in animals exposed to USS and SPS. In these samples, we found results paralleling our human data: male animals exposed to SPS demonstrated higher levels of circulating miR-19b as compared to unstressed control male animals, whereas female animals exposed to USS demonstrated lower levels of miR-19b compared to unstressed control female animals (Figure 3b, 3c).

We next evaluated for male and female differences in miR-19b expression levels following stress exposure in animal tissue known to be relevant to PTWP and PTSS pathogenesis. miR-19b was expressed in all tissues examined (Cycle threshold values <30), with highest expression in the blood (Supplementary Figure 4). When comparing expression levels between male and female unstressed control rats, males expressed higher levels of miR-19b in blood and dorsal root ganglia than females (blood, $t=4.03$, $df=6$, $p=0.001$; DRG, $t=2.544$, $df=5$, $p=0.035$) and similar levels of miR-19b between sexes in amygdala, hippocampus,

hypothalamus, and adrenal gland (Supplementary Figure 4). In male animals exposed to USS, miR-19b expression levels were higher in the amygdala and hypothalamus relative to male animals not exposed to USS (Figure 3d, 3e). This result was consistent with previous reports demonstrating increased miR-19b expression levels in the amygdala of male animals following stress exposure.[7; 92] However, in female animals exposed to USS, miR-19b expression levels in the amygdala and hypothalamus were similar to miR-19b expression in female animals not exposed to USS (Figure 3d, 3e).

Estrogen stimulation of primary neurons results in a decrease in miR-19b expression

The above data demonstrate that miR-19b predicts PTWP and substantial PTSS differently in men and women following trauma exposure, and is expressed at lower levels in females than males in blood and DRG at baseline in animal tissues. These data, together with previous data showing that miR-19b is under the transcriptional control of the main female sex hormone 17β- estradiol (albeit in non-relevant cell lines to PTWP and PTSS),[18] led us to hypothesize that 17β-estradiol regulates miR-19b expression in the peripheral tissues examined in this study. We therefore tested whether 17β-estradiol stimulation alters miR-19b expression in female primary cultures of dorsal root ganglion neurons. miR-19b expression decreased 3 hours following stimulation with 100 nM 17β-estradiol (Mean difference =0.547, p =0.009, Supplementary Figure 5). As a positive control, we also assessed expression of *CGRP* mRNA expression following stimulation with 17β-estradiol. Consistent with previous reports (e.g.[31]), *CGRP* expression increased 3 hours after stimulation with 100 nM 17β-estradiol (Mean difference = 1.408, $p < 0.051$, Supplementary Figure 5).

Rhythmic processes are predicted to be over-represented in targeting by miR-19b

The primary mechanism through which miRNA influence disease onset/outcomes is by regulating the expression of transcripts in biological pathways influencing pathogenic processes. A single miRNA can act as a gene regulatory hub by regulating many such transcripts. To identify potential biologic pathways regulated by miR-19b, we evaluated gene ontology (GO) relationships within miR-19b predicted pain and PTSS associated targets using the DAVID online algorithm. The GO group with the highest fold enrichment was “rhythmic processes” (GO:0048511) (9.867 fold enrichment, $p = 1.5\times10^{-5}$, Supplementary Table 5); similar results were obtained using the reactome online algorithm (high enrichment for the “circadian clock” pathway, $p=1.56\times10^{-5}$). This ontology group and pathway included well-known circadian rhythm pathway genes such as *CLOCK, NPAS2* and *RORA*. Overall, the results of GO bioinformatics predictions suggest that miR-19b may influence PTWP and PTSS outcomes by regulating circadian rhythm pathway genes.

Circadian rhythm gene transcripts are differentially associated with miR-19b expression levels in men and women who develop PTWP and/or PTSS following MVC and are directly regulated by miR-19b in vitro

If miR-19b regulates circadian rhythm pathway genes *in vivo*, then individuals with high levels of miR-19b would be expected to have low levels of miR-19b targets (due to repression of the transcript by the miRNA). We therefore examined the relationship between miR-19b and key circadian rhythm transcripts *RORA, CLOCK* and *NPAS2* using mRNA

sequencing data from MVC cohort participants (n=90). We found that, in general, these relationships were stronger in individuals who developed PTWP/PTSS following MVC vs those who recovered (Figure 4a – 4c). Further, we observed a consistent inverse relationship (either at the trend or statistically significant level) between miR-19b and all three transcripts in women who developed PTWP/PTSS (*RORA*, R=−0.36, p=0.09; *CLOCK*, R=−0.47, p=0.04; *NPAS2*, R=−0.25, p=0.25 Figure 4a – 4c). In contrast, in men, we observed a statistically significant inverse relationship between miR-19b and *RORA* (R=−0.51, p=0.04 Figure 4a) but no relationship or a trend-level positive relationship for *CLOCK* and *NPAS2* respectively. Using *in vitro* dual luciferase reporter assays, we also found that, consistent with bioinformatics predictions, miR-19b could directly bind and repress the 3'UTRs of these genes (Figure 4d – 4e). Together these data suggest that the influence of miR-19b on PTWP/PTSS development could be due, at least in part, to regulation of key circadian rhythm pathway transcripts by miR-19b.

Discussion

We evaluated the potential role of miRNA in the development of PTWP and PTSS using *in silico*, *in vitro*, animal, and human data. Initial *in silico* analyses implicated members of the miR-19 family in PTWP and PTSS pathogenesis based on their highest predicted preferential binding to PTWP and PTSS-associated transcripts. Circulating miR-19b levels in the immediate aftermath of MVC indeed predicted both PTWP and substantial PTSS six months after trauma exposure. Interestingly, associations between miR-19b expression levels and PTWP and PTSS outcomes were different in women and men; in women, we observed an inverse relationship between miR-19b expression levels and the probability of developing PTWP and PTSS and in men, we observed a positive relationship between miR-19b expression levels and the probability of developing these outcomes. The inverse relationship between miR-19b expression levels and PTSS in women was also replicated in an independent cohort of sexual assault survivors. Male and female differences in miR-19b expression were also observed in animal models of PTWP and PTSS following stress exposure, both in blood and in tissues relevant to PTWP and PTSS pathogenesis. Our study further shows that miR-19b expression in peripheral nervous tissue may be mediated at least in part by 17β-estradiol.

Bioinformatics analyses indicated that gene pathways most likely to be targeted by miR-19b were those associated with circadian processes. Such processes have been implicated in the development of adverse posttraumatic neuropsychiatric sequelae such as PTWP and PTSS. Consistent with these data, in *in vitro* analyses, miR-19b was found to directly bind and repress the 3'UTRs of key circadian rhythm transcripts. In concert, an inverse relationship was observed between miR-19b and these transcripts in women experiencing MVC trauma, particularly those developing PTWP or PTSS. However, negative relationships were not consistently observed for men who experienced MVC trauma.

The molecular mechanisms accounting for the above associations between miR-19b and the development of PTWP or substantial PTSS, and sex differences in the direction of these associations, remain unknown. It is possible that miR-19b expression levels predict vulnerability to PTWP or PTSS but is not involved in the pathogenesis of these outcomes.

However, if miR-19b not only marks vulnerability to PTWP or PTSS, but also influences their development, then it is likely that miR-19b does so by altering the levels of key gene transcripts, such as those involved in circadian rhythm homeostasis. Current evidence suggests that disruption in circadian rhythm activation in either direction (i.e. increased or decreased activation) can have negative downstream effects on rhythmic gene expression across multiple tissues and bodily systems.[62; 101] Thus it is possible that miR-19b expression levels that push biological systems too far from the mean in men and women in either direction are pathogenic. However, further studies are needed to understand possible mechanisms accounting for the relationship between miR-19b and PTWP or PTSS, and sex differences in the direction of these associations.

Volk et al. and Balakathiresan et al. previously identified higher levels of miR-19b expression (in the amygdala and serum, respectively) in male animals exposed to chronic stress,[7; 92] and Wang et al. and Sakai et al. found that miR-19a was associated with chronic neuropathic pain in male rats.[78; 95] In humans, Martin et al. identified miR-19a as the most differentially up-regulated miRNA in male combat veterans who developed PTSD.[61] Our findings in male animals and humans are consistent with these data, but extend previous findings by placing them in sex-specific context, as miR-19b consistently predicts PTWP or substantial PTSS in females, but in a direction opposite to males. These data highlight the importance of evaluating potential sex-specific effects on stress-related disorder pathogenesis, as studies examining both sexes would likely not identify an association between miR-19a/b and these outcomes unless specifically stratifying for sex.

Our results indicate that 17-β-estradiol may influence miR-19b expression levels in females (males were not assessed). While it is important to validate this finding in larger sample sizes, it is also important to test the effects of 17-β-estradiol on miR-19b in additional peripheral and in central nervous system tissues. This is because 17-β-estradiol has been shown to act as both pro- and anti- nociceptive mediators[3; 22], potentially based on its differing roles in peripheral and central nervous system tissues[77; 85]. In addition, 17-β-estradiol has been shown to modulate fear extinction via multiple mechanisms within the brain network consisting of the amygdala, hippocampus, and the prefrontal cortex[21]. Therefore, even though we have observed effects of 17-β-estradiol on miR-19b in peripheral tissue (i.e. dorsal root ganglion neurons), additional or differing transcriptional regulators might influence miR-19b both peripherally and centrally. For instance, X chromosome-specific regulatory events, such as X chromosome inactivation, might also influence miR-19b levels, as this miRNA originates from two evolutionary paralogous genomic regions, one on chromosome 13 and the other on the X chromosome.

While our analyses focused on miR-19b, our *in silico* analyses identified a number of other miRNA that may also influence posttraumatic pain and PTSS pathogenesis and/or maintenance. Many of these miRNA, including miR-34, miR-18, miR-15, and miR-103, have already been shown to be associated with pain, PTSS, or stress-related disorders.[25; 29; 78; 88; 91; 94; 106] It should be noted, however, that while our *in silico* selection criteria prioritized miRNA that bind many gene targets, other miRNA not selected as top candidates using this method may also have an important influence on posttraumatic pain or PTSS if

they affect the expression of only one or a few highly influential genes. Examples of such miRNA include miR-135[13; 42; 54], miR-30[76; 80; 87], and miR-320[11; 70; 74].

The fact that miR-19b regulates key transcripts involved in the circadian rhythm pathway is interesting given the wealth of (albeit conflicting) literature linking this pathway to PTSS vulnerability. For instance, RAR-related orphan receptor alpha (*RORA*), which we showed to be regulated by miR-19b in both men and women in this study, has been shown to be both genetically associated[5; 57; 59] and not associated[36] with PTSD. Additional studies have also shown physiological relationships between the circadian rhythm and PTSD pathogenesis,[32] and literature from the pain field also implicates sleep and circadian abnormalities in pain vulnerability[2; 4; 30; 37; 50; 68; 79; 81–83]. However, very few studies have examined whether there are sex-dependent differences in the contribution of the circadian rhythm pathway to PTWP/PTSS pathogenesis. This study adds evidence to this growing body of literature implicating the circadian rhythm pathway and core circadian rhythm genes in the pathogenesis of pain and PTSS development.

The many strengths of this study include being one of the largest human studies of miRNA predictors of PTWP or PTSS development in male and female trauma survivors to date, and that it used coordinated translational studies to guide and strengthen the validity of the findings. However, several limitations should be considered when interpreting this work. First, the miR-19b association with PTWP has not been replicated in a second cohort of post-trauma survivors. This is an essential next step for future studies. However, this study did replicate the miR-19b association with PTSS in women sexual assault survivors, and internally replicated the RNA sequencing findings from the MVC cohort by examining miR-19b expression via RT-qPCR in an expanded cohort of 26 age-matched cases and controls. Second, this study did not assess the influence of 17-β-estradiol levels on miR-19b association with PTWP/PTSS in female participants following MVC or SA. This would have been an interesting experiment given the cell culture results demonstrating that 17-β-estradiol can negatively regulate miR-19b in peripheral nervous tissue. Third, this study focused on the experimental validation of only a subset of the many predicted targets of miR-19b, those of the circadian rhythm pathway. This decision is supported by bioinformatics pathway analyses, however, it is possible that other transcripts predicted to be regulated by miR-19b are also important to the pathogenesis of PTWP/PTSS, such as *DICER1*[97], *ESR1*[22; 33], or *YY1*[84]. Additionally, pathogenic mechanisms related to PTWP/PTSS that have been previously defined for miR-19 but were not assessed in the current study, such as mechanisms related to hippocampal neuron migration[38], are additional candidates for miR-19b-associated roles in the trauma recovery process. Fourth, consistent with a previous report[104], we did not detect sex differences in the development of enduring stress induced hyperalgesia following SPS. However, as described previously, underlying mechanisms can still differ in males and females despite similar phenotypes observed across sexes [35]. Finally, this work was performed in relatively small epidemiological cohorts and in one strain of rats. Therefore, whether sex differences in the relationship between peritraumatic miR-19b expression levels and PTWP/PTSS outcomes replicates across larger sample sizes that include additional trauma types, ethnicities, and animal species/strains remains to be determined.

In summary, we report the first set of evidence indicating that expression levels of miR-19b in the early aftermath of trauma/stress exposure is associated with PTWP or PTSS development six months following trauma exposure. Further, our data suggests that miR-19b may affect the development of these stress-related disorders by altering the levels of key circadian rhythm gene transcripts. Future studies are needed to delineate the sex-dependent effects of miR-19b on circadian rhythm signaling and on adverse posttraumatic outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

[1]. Agarwal V, Bell GW, Nam J-W, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. Elife 2015;4:e05005.

[2]. Alexandre C, Latremoliere A, Ferreira A, Miracca G, Yamamoto M, Scammell TE, Woolf CJ. Decreased alertness due to sleep loss increases pain sensitivity in mice. Nature Medicine 2017.

[3]. Amandusson Å, Blomqvist A. Estrogenic influences in pain processing. Frontiers in neuroendocrinology 2013;34(4):329–349. [PubMed: 23817054]

[4]. Ambriz-Tututi M, Rocha-González HI, Cruz SL, Granados-Soto V. Melatonin: a hormone that modulates pain. Life sciences 2009;84(15):489–498. [PubMed: 19223003]

[5]. Amstadter A, Sumner J, Acierno R, Ruggiero K, Koenen K, Kilpatrick D, Galea S, Gelernter J. Support for association of RORA variant and post traumatic stress symptoms in a population-based study of hurricane exposed adults. Molecular psychiatry 2013;18(11):1148. [PubMed: 23319003]

[6]. Andrews P, Steultjens M, Riskowski J. Chronic widespread pain prevalence in the general population: A systematic review. European Journal of Pain 2018;22(1):5–18. [PubMed: 28815801]

[7]. Balakathiresan NS, Chandran R, Bhomia M, Jia M, Li H, Maheshwari RK. Serum and amygdala microRNA signatures of posttraumatic stress: Fear correlation and biomarker potential. Journal of psychiatric research 2014;57:65–73. [PubMed: 24998397]

[8]. Bam M, Yang X, Zhou J, Ginsberg JP, Leyden Q, Nagarkatti PS, Nagarkatti M. Evidence for epigenetic regulation of pro-inflammatory cytokines, interleukin-12 and interferon gamma, in peripheral blood mononuclear cells from PTSD patients. Journal of Neuroimmune Pharmacology 2016;11(1):168–181. [PubMed: 26589234]

[9]. Beck JG, Coffey SF. Assessment and treatment of posttraumatic stress disorder after a motor vehicle collision: Empirical findings and clinical observations. Professional Psychology: Research and Practice 2007;38(6):629.

[10]. Benjet C, Bromet E, Karam E, Kessler R, McLaughlin K, Ruscio A, Shahly V, Stein D, Petukhova M, Hill E. The epidemiology of traumatic event exposure worldwide: results from the

World Mental Health Survey Consortium. Psychological medicine 2016;46(2):327–343. [PubMed: 26511595]

[11]. Bjersing JL, Bokarewa MI, Mannerkorpi K. Profile of circulating microRNAs in fibromyalgia and their relation to symptom severity: an exploratory study. Rheumatology international 2015;35(4):635–642. [PubMed: 25261961]

[12]. Blanchard EB, Jones-Alexander J, Buckley TC, Forneris CA. Psychometric properties of the PTSD Checklist (PCL). Behaviour research and therapy 1996;34(8):669–673. [PubMed: 8870294]

[13]. Bray N Psychiatric disorders: miR-135: a marker of mood. Nature Reviews Neuroscience 2014;15(8):495–495.

[14]. Brett JO, Renault VM, Rafalski VA, Webb AE, Brunet A. The microRNA cluster miR-106b~ 25 regulates adult neural stem/progenitor cell proliferation and neuronal differentiation. Aging (Albany NY) 2011;3(2):108. [PubMed: 21386132]

[15]. Brunet A, Weiss DS, Metzler TJ, Best SR, Neylan TC, Rogers C, Fagan J, Marmar CR. The Peritraumatic Distress Inventory: a proposed measure of PTSD criterion A2. The American journal of psychiatry 2001;158(9):1480–1485. [PubMed: 11532735]

[16]. Bullard JH, Purdom E, Hansen KD, Dudoit S. Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. BMC bioinformatics 2010;11(1):94. [PubMed: 20167110]

[17]. Campbell R, Dworkin E, Cabral G. An ecological model of the impact of sexual assault on women's mental health. Trauma, Violence, & Abuse 2009;10(3):225–246.

[18]. Castellano L, Giamas G, Jacob J, Coombes RC, Lucchesi W, Thiruchelvam P, Barton G, Jiao LR, Wait R, Waxman J. The estrogen receptor-α-induced microRNA signature regulates itself and its transcriptional response. Proceedings of the National Academy of Sciences 2009;106(37):15732–15737.

[19]. Chaplan SR, Bach F, Pogrel J, Chung J, Yaksh T. Quantitative assessment of tactile allodynia in the rat paw. Journal of neuroscience methods 1994;53(1):55–63. [PubMed: 7990513]

[20]. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR. Real-time quantification of microRNAs by stem–loop RT–PCR. Nucleic acids research 2005;33(20):e179–e179. [PubMed: 16314309]

[21]. Cover K, Maeng L, Lebrón-Milad K, Milad M. Mechanisms of estradiol in fear circuitry: implications for sex differences in psychopathology. Translational psychiatry 2014;4(8):e422. [PubMed: 25093600]

[22]. Craft RM. Modulation of pain by estrogens. Pain 2007;132:S3–S12. [PubMed: 17951003]

[23]. Creamer M, Bell R, Failla S. Psychometric properties of the impact of event scale—revised. Behaviour research and therapy 2003;41(12):1489–1496. [PubMed: 14705607]

[24]. Daskalakis NP, Provost AC, Hunter RG, Guffanti G. Noncoding RNAs: stress, glucocorticoids and PTSD. Biological psychiatry 2018.

[25]. Dias BG, Goodman JV, Ahluwalia R, Easton AE, Andero R, Ressler KJ. Amygdala-dependent fear memory consolidation via miR-34a and notch signaling. Neuron 2014;83(4):906–918. [PubMed: 25123309]

[26]. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29(1):15–21. [PubMed: 23104886]

[27]. Dwivedi Y, Roy B, Lugli G, Rizavi H, Zhang H, Smalheiser N. Chronic corticosterone-mediated dysregulation of microRNA network in prefrontal cortex of rats: relevance to depression pathophysiology. Translational psychiatry 2015;5(11):e682. [PubMed: 26575223]

[28]. Farrar JT, Young JP Jr., LaMoreaux L, Werth JL, Poole RM. Clinical importance of changes in chronic pain intensity measured on an 11-point numerical pain rating scale. Pain 2001;94(2): 149–158. [PubMed: 11690728]

[29]. Favereaux A, Thoumine O, Bouali-Benazzouz R, Roques V, Papon MA, Salam SA, Drutel G, Léger C, Calas A, Nagy F. Bidirectional integrative regulation of Cav1. 2 calcium channel by microRNA miR-103: role in pain. The EMBO journal 2011;30(18):3830–3841. [PubMed: 21804529]

[30]. Finan PH, Goodin BR, Smith MT. The association of sleep and pain: an update and a path forward. The Journal of Pain 2013;14(12):1539–1552. [PubMed: 24290442]

[31]. Gangula P, Lanlua P, Wimalawansa S, Supowit S, DiPette D, Yallampalli C. Regulation of Calcitonin Gene-Related Peptide Expression in Dorsal Root Ganglia of Rats by Female Sex Steroid Hormones 1. Biology of reproduction 2000;62(4):1033–1039. [PubMed: 10727274]

[32]. Germain A Sleep disturbances as the hallmark of PTSD: where are we now? American Journal of Psychiatry 2013;170(4):372–382. [PubMed: 23223954]

[33]. Glover EM, Jovanovic T, Norrholm SD. Estrogen and extinction of fear memories: implications for posttraumatic stress disorder treatment. Biological psychiatry 2015;78(3):178–185. [PubMed: 25796471]

[34]. Green PG, Alvarez P, Gear RW, Mendoza D, Levine JD. Further validation of a model of fibromyalgia syndrome in the rat. The Journal of Pain 2011;12(7):811–818. [PubMed: 21481648]

[35]. Greenspan JD, Craft RM, LeResche L, Arendt-Nielsen L, Berkley KJ, Fillingim RB, Gold MS, Holdcroft A, Lautenbacher S, Mayer EA. Studying sex and gender differences in pain and analgesia: a consensus report. Pain 2007;132:S26–S45. [PubMed: 17964077]

[36]. Guffanti G, Ashley-Koch A, Roberts AL, Garrett M, Solovieff N, Ratanatharathorn A, De Vivo I, Dennis M, Ranu H, Smoller J. No association between RORA polymorphisms and PTSD in two independent samples. Molecular psychiatry 2014;19(10):1056–1057. [PubMed: 25048002]

[37]. Haack M, Lee E, Cohen DA, Mullington JM. Activation of the prostaglandin system in response to sleep loss in healthy humans: potential mediator of increased spontaneous pain. PAIN® 2009;145(1–2):136–141. [PubMed: 19560866]

[38]. Han J, Kim HJ, Schafer ST, Paquola A, Clemenson GD, Toda T, Oh J, Pankonin AR, Lee BS, Johnston ST. Functional implications of miR-19 in the migration of newborn neurons in the adult brain. Neuron 2016;91(1):79–89. [PubMed: 27387650]

[39]. He Y-Q, Chen Q, Ji L, Wang Z-G, Bai Z-H, Stephens RL, Yang M. PKCγ receptor mediates visceral nociception and hyperalgesia following exposure to PTSD-like stress in the spinal cord of rats. Molecular pain 2013;9(1):35. [PubMed: 23837410]

[40]. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols 2009;4(1):44–57. [PubMed: 19131956]

[41]. Inslicht SS, Metzler TJ, Garcia NM, Pineles SL, Milad MR, Orr SP, Marmar CR, Neylan TC. Sex differences in fear conditioning in posttraumatic stress disorder. Journal of psychiatric research 2013;47(1):64–71. [PubMed: 23107307]

[42]. Issler O, Haramati S, Paul ED, Maeno H, Navon I, Zwang R, Gil S, Mayberg HS, Dunlop BW, Menke A. MicroRNA 135 is essential for chronic stress resiliency, antidepressant efficacy, and intact serotonergic activity. Neuron 2014;83(2):344–360. [PubMed: 24952960]

[43]. JunMei H, Bortsov Andrey V, Lauren B, Orrey Danielle C, Swor Robert A, David P, Jeffrey J, Niels R, Lee David C, Robert D. Chronic Widespread Pain after Motor Vehicle Collision Typically Occurs via Immediate Development and Non-Recovery: Results of an Emergency Department-Based Cohort Study. Pain 2016;157(2):438. [PubMed: 26808013]

[44]. Khasar SG, Burkham J, Dina OA, Brown AS, Bogen O, Alessandri-Haber N, Green PG, Reichling DB, Levine JD. Stress induces a switch of intracellular signaling in sensory neurons in a model of generalized pain. The Journal of Neuroscience 2008;28(22):5721–5730. [PubMed: 18509033]

[45]. Khasar SG, Dina OA, Green PG, Levine JD. Sound Stress–Induced Long-Term Enhancement of Mechanical Hyperalgesia in Rats Is Maintained by Sympathoadrenal Catecholamines. The Journal of Pain 2009;10(10):1073–1077. [PubMed: 19576859]

[46]. Khasar SG, Green PG, Levine JD. Repeated sound stress enhances inflammatory pain in the rat. Pain 2005;116(1):79–86. [PubMed: 15936144]

[47]. Kilpatrick DG, Resnick HS, Milanak ME, Miller MW, Keyes KM, Friedman MJ. National estimates of exposure to traumatic events and PTSD prevalence using DSM-IV and DSM-5 criteria. Journal of traumatic stress 2013;26(5):537–547. [PubMed: 24151000]

[48]. Knox D, George SA, Fitzpatrick CJ, Rabinak CA, Maren S, Liberzon I. Single prolonged stress disrupts retention of extinguished fear in rats. Learning & Memory 2012;19(2):43–49. [PubMed: 22240323]

[49]. LaCroix-Fralish ML, Ledoux JB, Mogil JS. The Pain Genes Database: An interactive web browser of pain-related transgenic knockout studies. Pain 2007;131(1):3. e1–3. e4. [PubMed: 17574758]

[50]. Lautenbacher S, Kundermann B, Krieg J-C. Sleep deprivation and pain perception. Sleep medicine reviews 2006;10(5):357–369. [PubMed: 16386930]

[51]. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. Bioinformatics 2012;28(6): 882–883. [PubMed: 22257669]

[52]. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC bioinformatics 2011;12(1):323. [PubMed: 21816040]

[53]. Linnstaedt SD, Hu J, Liu AY, Soward AC, Bollen KA, Wang HE, Hendry PL, Zimny E, Lewandowski C, Velilla M-A. Methodology of AA CRASH: a prospective observational study evaluating the incidence and pathogenesis of adverse post-traumatic sequelae in African-Americans experiencing motor vehicle collision. BMJ open 2016;6(9):e012222.

[54]. Linnstaedt SD, Walker MG, Parker JS, Yeh E, Sons RL, Zimny E, Lewandowski C, Hendry PL, Damiron K, Pearson C, Velilla MA, O'Neil BJ, Jones J, Swor R, Domeier R, Hammond S, McLean SA. MicroRNA circulating in the early aftermath of motor vehicle collision predict persistent pain development and suggest a role for microRNA in sex-specific pain differences. Mol Pain 2015;11:66. [PubMed: 26498117]

[55]. Lisieski MJ, Eagle AL, Conti AC, Liberzon I, Perrine SA. Single-Prolonged Stress: A Review of Two Decades of Progress in a Rodent Model of Post-traumatic Stress Disorder. Frontiers in psychiatry 2018;9.

[56]. Liu Y, Wang L, Lao J, Zhao X. Changes in microRNA expression in the brachial plexus avulsion model of neuropathic pain. International journal of molecular medicine 2018;41(3):1509–1517. [PubMed: 29286067]

[57]. Logue MW, Baldwin C, Guffanti G, Melista E, Wolf EJ, Reardon AF, Uddin M, Wildman D, Galea S, Koenen KC. A genome-wide association study of post-traumatic stress disorder identifies the retinoid-related orphan receptor alpha (RORA) gene as a significant risk locus. Molecular psychiatry 2013;18(8):937–942. [PubMed: 22869035]

[58]. López-González MJ, Landry M, Favereaux A. MicroRNA and chronic pain: From mechanisms to therapeutic potential. Pharmacology & therapeutics 2017.

[59]. Lowe SR, Meyers JL, Galea S, Aiello AE, Uddin M, Wildman DE, Koenen KC. RORA and posttraumatic stress trajectories: main effects and interactions with childhood physical abuse history. Brain and behavior 2015;5(4).

[60]. Luda Maixner WD; Hamilton Michael; Fillingim Roger; Gracely Richard; Slade Gary; Mogil Jeffrey; Sleptsov Alex. Algynomics Pain Research Panel v2.0. In: Luda WD Maixner; Hamilton Michael; Fillingim Roger; Gracely Richard; Slade Gary; Mogil Jeffrey; Sleptsov Alex editor, 2016.

[61]. Martin CG, Kim H, Yun S, Livingston W, Fetta J, Mysliwiec V, Baxter T, Gill JM. Circulating miRNA associated with posttraumatic stress disorder in a cohort of military combat veterans. Psychiatry Research 2017;251:261–265. [PubMed: 28222310]

[62]. McClung CA. Circadian genes, rhythms and the biology of mood disorders. Pharmacology & therapeutics 2007;114(2):222–232. [PubMed: 17395264]

[63]. McLean SA, Clauw DJ, Abelson JL, Liberzon I. The development of persistent pain and psychological morbidity after motor vehicle collision: integrating the potential role of stress response systems into a biopsychosocial model. Psychosomatic medicine 2005;67(5):783–790. [PubMed: 16204439]

[64]. McLean SA, Soward AC, Ballina LE, Rossi C, Rotolo S, Wheeler R, Foley KA, Batts J, Casto T, Collette R. Acute severe pain is a common consequence of sexual assault. The Journal of Pain 2012;13(8):736–741. [PubMed: 22698980]

[65]. Meloto CB, Bortsov A, Bair E, Helgeson E, Ostrom C, Smith S, Dubner R, Slade GD, Fillingim RB, Greenspan JD, Ohrbach R, Maixner W, McLean S, Diatchenko L. Modification of COMT-dependent pain sensitivity by psychological stress and sex. Pain 2015.

[66]. Morgan CP, Bale TL. Sex differences in microRNA regulation of gene expression: no smoke, just miRs. Biology of sex differences 2012;3(1):22. [PubMed: 23009289]

[67]. Morgan REaK, Grace. Criminal Victimization, 2016: Revised In: USDo Justice editor. Office of Justice Programs: Bureau of Justice Statistics, 2018.

[68]. Morioka N, Saeki M, Sugimoto T, Higuchi T, Zhang FF, Nakamura Y, Hisaoka-Nakashima K, Nakata Y. Downregulation of the spinal dorsal horn clock gene Per1 expression leads to mechanical hypersensitivity via c-jun N-terminal kinase and CCL2 production in mice. Molecular and Cellular Neuroscience 2016.

[69]. Nishi D, Matsuoka Y, Yonemoto N, Noguchi H, Kim Y, Kanba S. Peritraumatic Distress Inventory as a predictor of post-traumatic stress disorder after a severe motor vehicle accident. Psychiatry and clinical neurosciences 2010;64(2):149–156. [PubMed: 20447011]

[70]. Orlova IA, Alexander GM, Qureshi RA, Sacan A, Graziano A, Barrett JE, Schwartzman RJ, Ajit SK. MicroRNA modulation in complex regional pain syndrome. Journal of translational medicine 2011;9(1):195. [PubMed: 22074333]

[71]. Park C-K, Xu Z-Z, Berta T, Han Q, Chen G, Liu X-J, Ji R-R. Extracellular microRNAs activate nociceptor neurons to elicit pain via TLR7 and TRPA1. Neuron 2014;82(1):47–54. [PubMed: 24698267]

[72]. Perkins JR, Lees J, Antunes-Martins A, Diboun I, McMahon SB, Bennett DL, Orengo C. PainNetworks: A web-based resource for the visualisation of pain-related genes in the context of their network associations. PAIN® 2013;154(12):2586. e2581–2586. e2512. [PubMed: 24036287]

[73]. Pfeffer S, Sewer A, Lagos-Quintana M, Sheridan R, Sander C, Grasser FA, van Dyk LF, Ho CK, Shuman S, Chien M, Russo JJ, Ju J, Randall G, Lindenbach BD, Rice CM, Simon V, Ho DD, Zavolan M, Tuschl T. Identification of microRNAs of the herpesvirus family. Nature methods 2005;2(4):269–276. [PubMed: 15782219]

[74]. Qureshi RA, Tian Y, McDonald MK, Capasso KE, Douglas SR, Gao R, Orlova IA, Barrett JE, Ajit SK, Sacan A. Circulating microRNA Signatures in Rodent Models of Pain. Molecular neurobiology 2015:1–12.

[75]. Rehmsmeier M, Steffen P, Höchsmann M, Giegerich R. Fast and effective prediction of microRNA/target duplexes. Rna 2004;10(10):1507–1517. [PubMed: 15383676]

[76]. Rodgers AB, Morgan CP, Bronson SL, Revello S, Bale TL. Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation. Journal of Neuroscience 2013;33(21):9003–9012. [PubMed: 23699511]

[77]. Rosen S, Ham B, Mogil JS. Sex differences in neuroimmunity and pain. Journal of neuroscience research 2017;95(1–2):500–508. [PubMed: 27870397]

[78]. Sakai A, Saitow F, Maruyama M, Miyake N, Miyake K, Shimada T, Okada T, Suzuki H. MicroRNA cluster miR-17–92 regulates multiple functionally related voltage-gated potassium channels in chronic neuropathic pain. Nat Commun 2017;8:16079. [PubMed: 28677679]

[79]. Schuh-Hofer S, Wodarski R, Pfau DB, Caspani O, Magerl W, Kennedy JD, Treede R-D. One night of total sleep deprivation promotes a state of generalized hyperalgesia: a surrogate pain model to study the relationship of insomnia and pain. PAIN® 2013;154(9):1613–1621. [PubMed: 23707287]

[80]. Shao J, Cao J, Wang J, Ren X, Su S, Li M, Li Z, Zhao Q, Zang W. MicroRNA-30b regulates expression of the sodium channel Nav1. 7 in nerve injury-induced neuropathic pain in the rat. Molecular pain 2016;12:1744806916671523. [PubMed: 27765894]

[81]. Simpson NS, Scott-Sutherland J, Gautam S, Sethna N, Haack M. Chronic exposure to insufficient sleep alters processes of pain habituation and sensitization. Pain 2018;159(1):33–40. [PubMed: 28891869]

[82]. Smith MT, Klick B, Kozachik S, Edwards RE, Holavanahalli R, Wiechman S, Blakeney P, Lezotte D, Fauerbach JA. Sleep onset insomnia symptoms during hospitalization for major burn injury predict chronic pain. Pain 2008;138(3):497–506. [PubMed: 18362052]

[83]. Smith MT, Remeniuk B, Finan PH, Speed TJ, Tompkins DA, Robinson M, Gonzalez K, Bjurstrom MF, Irwin MR. Sex differences in measures of central sensitization and pain sensitivity to experimental sleep disruption: Implications for sex differences in chronic pain. Sleep 2018.

[84]. Sorge R, LaCroix-Fralish M, Tuttle A, Khoutorsky A, Sotocinal S, Austin JS, Melmed K, Labialle S, Schmidt J, Wood J. The Yin and Yang of pain: variability in formalin test nociception and morphine analgesia produced by the Yin Yang 1 transcription factor gene. Genes, Brain and Behavior 2013;12(4):405–413.

[85]. Sorge RE, Totsch SK. Sex differences in pain. Journal of neuroscience research 2017;95(6): 1271–1281. [PubMed: 27452349]

[86]. Sun R, Zhang Z, Lei Y, Liu Y, Lu Ce, Rong H, Sun Ye, Zhang W, Ma Z, Gu X. Hippocampal activation of microglia may underlie the shared neurobiology of comorbid posttraumatic stress disorder and chronic pain. Molecular pain 2016;12:1744806916679166. [PubMed: 27852966]

[87]. Tramullas M, Francés R, de la Fuente R, Velategui S, Carcelén M, García R, Llorca J, Hurlé MA. MicroRNA-30c-5p modulates neuropathic pain in rodents. Science Translational Medicine 2018;10(453).

[88]. Uchida S, Nishida A, Hara K, Kamemoto T, Suetsugi M, Fujimoto M, Watanuki T, Wakabayashi Y, Otsuki K, McEwen BS. Characterization of the vulnerability to repeated stress in Fischer 344 rats: possible involvement of microRNA-mediated down-regulation of the glucocorticoid receptor. European Journal of Neuroscience 2008;27(9):2250–2261. [PubMed: 18445216]

[89]. Ulirsch J, Ballina L, Soward A, Rossi C, Hauda W, Holbrook D, Wheeler R, Foley K, Batts J, Collette R. Pain and somatic symptoms are sequelae of sexual assault: results of a prospective longitudinal study. European Journal of Pain 2014;18(4):559–566. [PubMed: 24019249]

[90]. Vickers KC, Shoucri BM, Levin MG, Wu H, Pearson DS, Osei-Hwedieh D, Collins FS, Remaley AT, Sethupathy P. MicroRNA-27b is a regulatory hub in lipid metabolism and is altered in dyslipidemia. Hepatology 2013;57(2):533–542. [PubMed: 22777896]

[91]. Volk N, Pape JC, Engel M, Zannas AS, Cattane N, Cattaneo A, Binder EB, Chen A. Amygdalar MicroRNA-15a Is Essential for Coping with Chronic Stress. Cell Reports 2016;17(7):1882–1891. [PubMed: 27829158]

[92]. Volk N, Paul ED, Haramati S, Eitan C, Fields BK, Zwang R, Gil S, Lowry CA, Chen A. MicroRNA-19b associates with Ago2 in the amygdala following chronic stress and regulates the adrenergic receptor beta 1. The Journal of Neuroscience 2014;34(45):15070–15082. [PubMed: 25378171]

[93]. Von Schack D, Agostino MJ, Murray BS, Li Y, Reddy PS, Chen J, Choe SE, Strassle BW, Li C, Bates B. Dynamic changes in the microRNA expression profile reveal multiple regulatory mechanisms in the spinal nerve ligation model of neuropathic pain. PloS one 2011;6(3):e17670. [PubMed: 21423802]

[94]. Vreugdenhil E, Verissimo CS, Mariman R, Kamphorst JT, Barbosa JS, Zweers T, Champagne DL, Schouten T, Meijer OC, Ron de Kloet E. MicroRNA 18 and 124a down-regulate the glucocorticoid receptor: implications for glucocorticoid responsiveness in the brain. Endocrinology 2009;150(5):2220–2228. [PubMed: 19131573]

[95]. Wang C, Jiang Q, Wang M, Li D. MiR-19a targets suppressor of cytokine signaling 1 to modulate the progression of neuropathic pain. International journal of clinical and experimental pathology 2015;8(9):10901. [PubMed: 26617805]

[96]. Weiss DS. The impact of event scale: revised. Cross-cultural assessment of psychological trauma and PTSD: Springer, 2007 pp. 219–238.

[97]. Wingo AP, Almli LM, Stevens JJ, Klengel T, Uddin M, Li Y, Bustamante AC, Lori A, Koen N, Stein DJ. DICER1 and microRNA regulation in post-traumatic stress disorder with comorbid depression. Nature communications 2015;6.

[98]. Wolfe F Pain extent and diagnosis: development and validation of the regional pain scale in 12,799 patients with rheumatic disease. The Journal of rheumatology 2003;30(2):369–378. [PubMed: 12563698]

[99]. Wolfe F Pain extent and diagnosis: development and validation of the regional pain scale in 12,799 patients with rheumatic disease. J Rheumatol 2003;30(2):369–378. [PubMed: 12563698]

[100]. Wolfe F, Smythe HA, Yunus MB, Bennett RM, Bombardier C, Goldenberg DL, Tugwell P, Campbell SM, Abeles M, Clark P, et al. The American College of Rheumatology 1990 Criteria for the Classification of Fibromyalgia. Report of the Multicenter Criteria Committee. Arthritis and rheumatism 1990;33(2):160–172. [PubMed: 2306288]

[101]. Wulff K, Gatti S, Wettstein JG, Foster RG. Sleep and circadian rhythm disruption in psychiatric and neurodegenerative disease. Nature Reviews Neuroscience 2010;11(8):589–599. [PubMed: 20631712]

[102]. Zhang K, Qu S, Chang S, Li G, Cao C, Fang K, Olff M, Wang L, Wang J. An overview of posttraumatic stress disorder genetic studies by analyzing and integrating genetic data into genetic database PTSDgene. Neuroscience & Biobehavioral Reviews 2017.

[103]. Zhang Y, Gandhi PR, Standifer KM. Increased nociceptive sensitivity and nociceptin/orphanin FQ levels in a rat model of PTSD. Molecular pain 2012;8(1):76. [PubMed: 23082795]

[104]. Zhang Y, Schalo I, Durand C, Standifer KM. Sex Differences in Nociceptin/Orphanin FQ Peptide Receptor-mediated pain and anxiety symptoms in a preclinical model of post-traumatic stress disorder. Frontiers in psychiatry 2018;9.

[105]. Zhou J, Nagarkatti P, Zhong Y, Ginsberg JP, Singh NP, Zhang J, Nagarkatti M. Dysregulation in microRNA expression is associated with alterations in immune functions in combat veterans with post-traumatic stress disorder. PloS one 2014;9(4):e94075. [PubMed: 24759737]

[106]. Zucchi FC, Yao Y, Ward ID, Ilnytskyy Y, Olson DM, Benzies K, Kovalchuk I, Kovalchuk O, Metz GA. Maternal stress induces epigenetic signatures of psychiatric and neurological diseases in the offspring. PloS one 2013;8(2):e56967. [PubMed: 23451123]

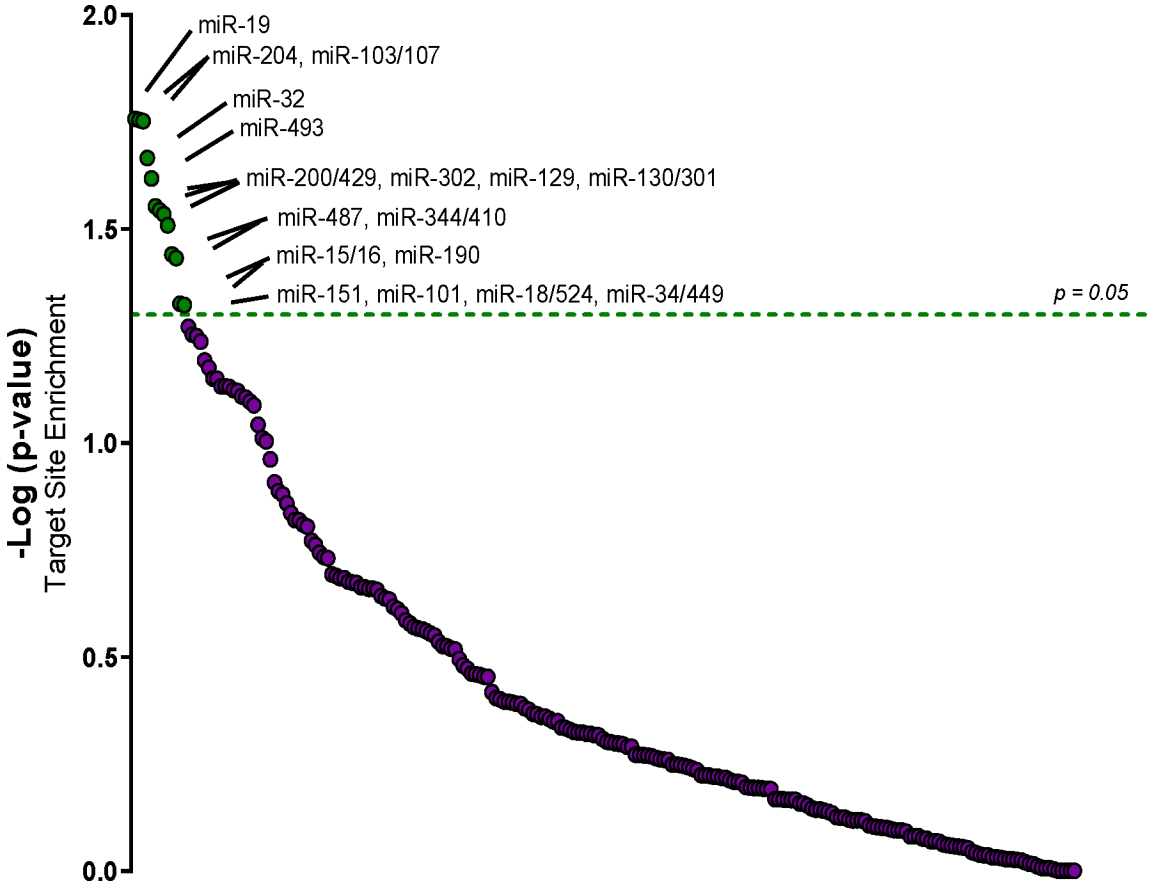

Fig 1.

miR-19 is a strong candidate regulatory hub for pain and PTSS. In silico analyses, using Monte Carlo cross validation approaches to estimate microRNA with the largest number of pain and PTSS associated mRNA targets, identified top candidate regulatory hubs for pain and PTSS. Individual microRNA (represented by circles) with the largest –Log p-value represent microRNA with the highest level of enrichment of predicted target sites in pain and PTSS related genes. A significance threshold corresponding to an empirical p value of p=0.05 is indicated by a green horizontal dotted line and microRNA with p values that are more significant than this threshold are shaded green. The most significant such microRNA and thus the strongest candidate regulatory hub for pain and PTSS is miR-19.

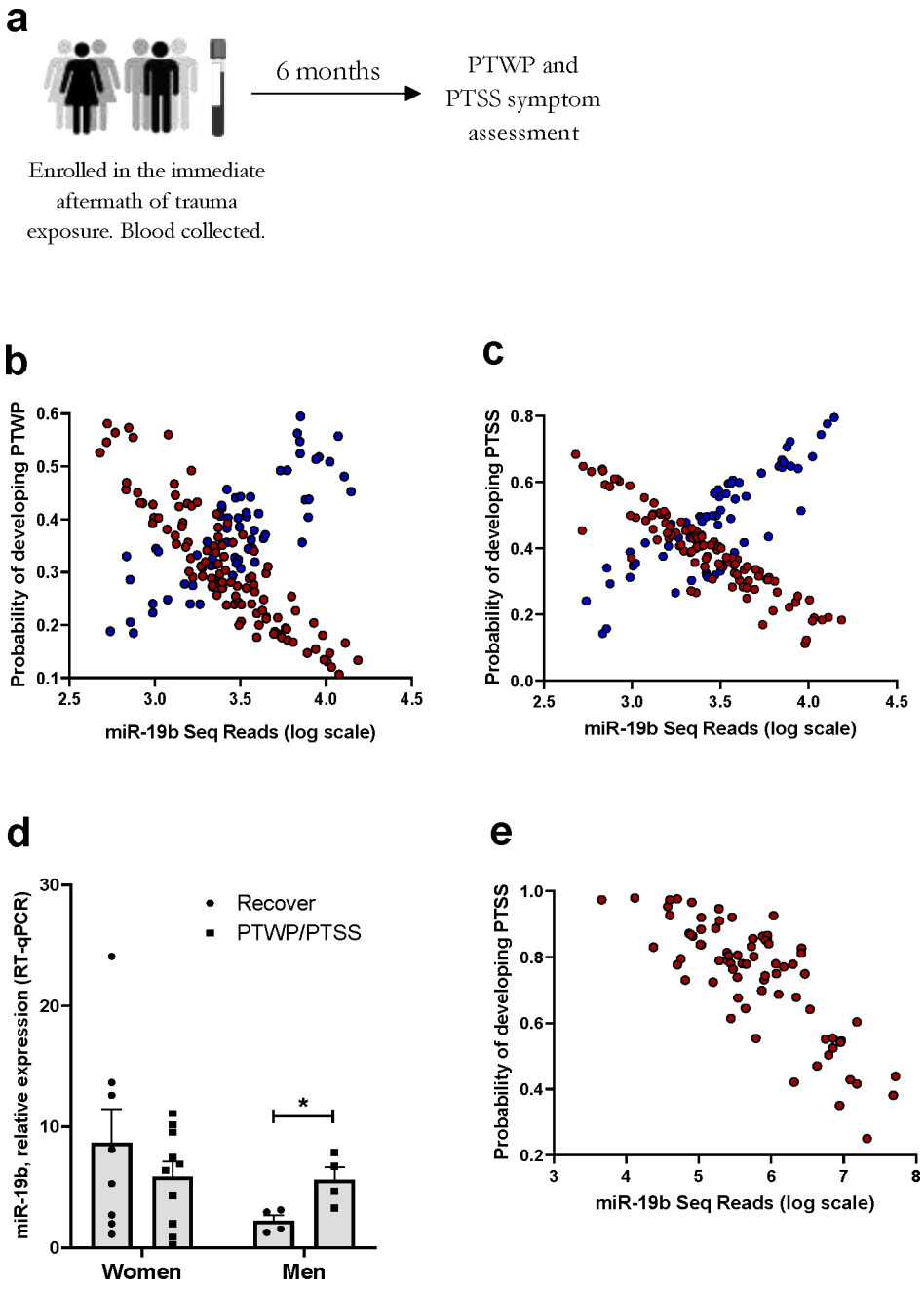

Fig 2.

miR-19b is differentially expressed in women and men who develop posttraumatic widespread pain (PTWP) and/or posttraumatic stress symptoms (PTSS) six months following trauma exposure. (a) Simplified study design schematic showing that women and men were enrolled in the emergency department following motor vehicle collision or at sexual assault nurse examiner sites following sexual assault, blood samples were obtained in the ED, and PTWP and PTSS outcomes were assessed six months following trauma exposure. (b and c) miR-19b expression levels (n=153) were negatively associated with the probability of developing PTWP and PTSS in women (n=95, 62.5%, red dots) and were

positively associated with the probability of developing PTWP and PTSS in men (n=57, 37.5%, blue dots) following motor vehicle collision. miR-19b expression levels were measured via small RNA-seq. (d) RT-qPCR data showing relative miR-19b expression levels in an additional subset of women and men (n=26, 69% women) who developed co-morbid PTWP/PTSS or recovered following motor vehicle collision trauma. Data are represented as mean ± SEM. *$p < 0.05$. (e) miR-19b expression levels (measured via small RNA-seq) from women sexual assault survivors plotted relative to their probability of developing PTSS six months following trauma exposure.

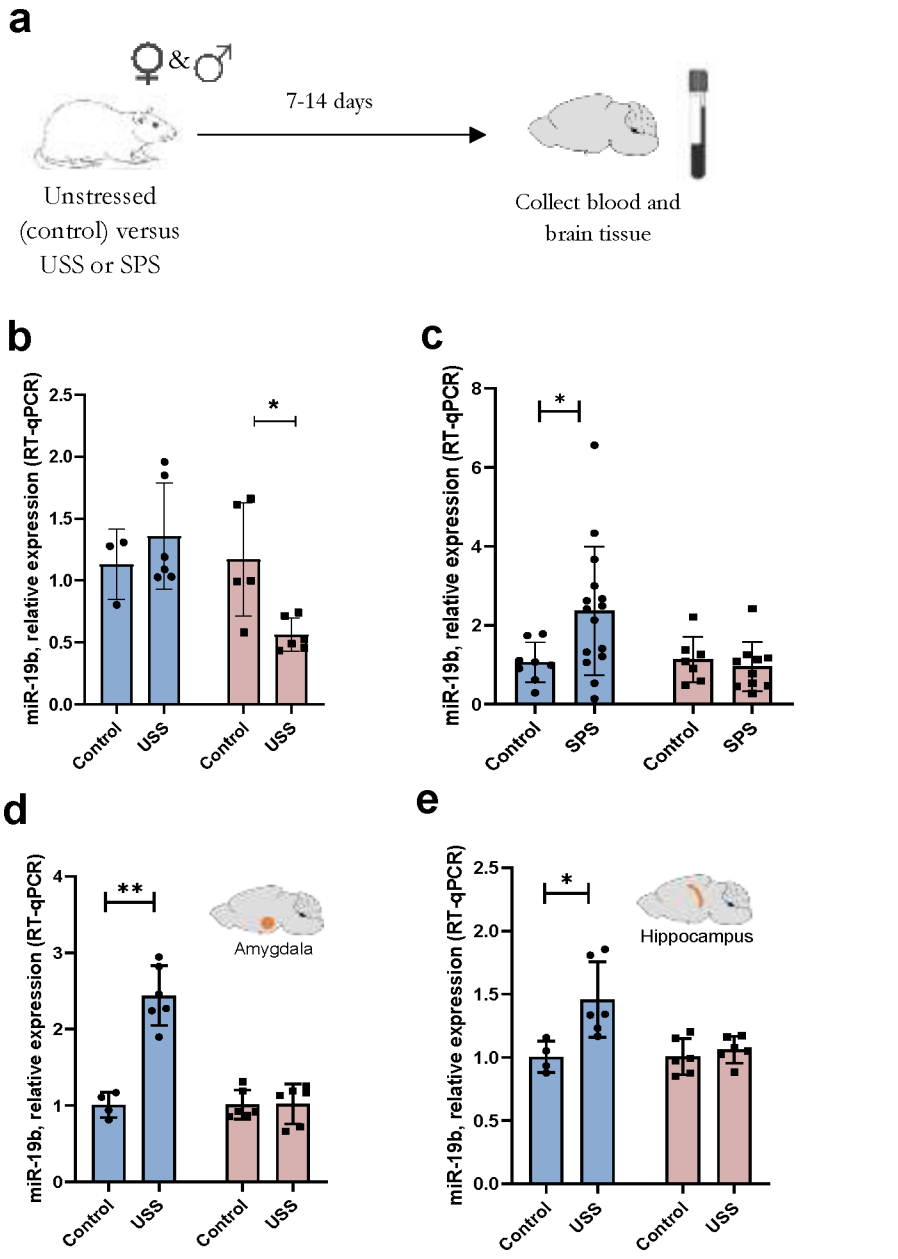

Fig 3.

miR-19b is expressed differently in male and female rats following stress exposure. (a) Schematic diagram of the animal protocol used to assess miR-19b expression levels in blood and brain of animal models of PTWP and PTSS. Male and female Sprague Dawley rats were either unstressed or exposed to unpredictable sound stress (USS) or single prolonged stress (SPS). Blood and brain tissue were isolated 7 (SPS) or 14 (USS) days following stress exposure, as these timepoints have been well-validated as the period in which stress-induced fear learning and PGE_2-responsive pain behaviors are observed (see text for details and references). (b) Circulating miR-19b expression levels in male (blue, n=10) and female (red, n=12) rats unstressed (Control) or exposed to USS. (c) Circulating miR-19b expression levels in male (blue, n=23) and female (red, n=17) rats unstressed (Control) or exposed to

SPS. (d) miR-19b expression levels in the amygdala of male (blue, n=10) and female (red, n=12) rats unstressed (Control) or exposed to USS. (e) miR-19b expression levels in the hippocampus of male (blue, n=10) and female (red, n=12) rats unstressed or exposed to USS. Data are represented as mean ± standard deviation and were analyzed by two-way ANOVA. Significant post-hoc differences between control and stress groups were determined with Tukey's multiple comparison test. *$p < 0.05$, **$p<0.001$

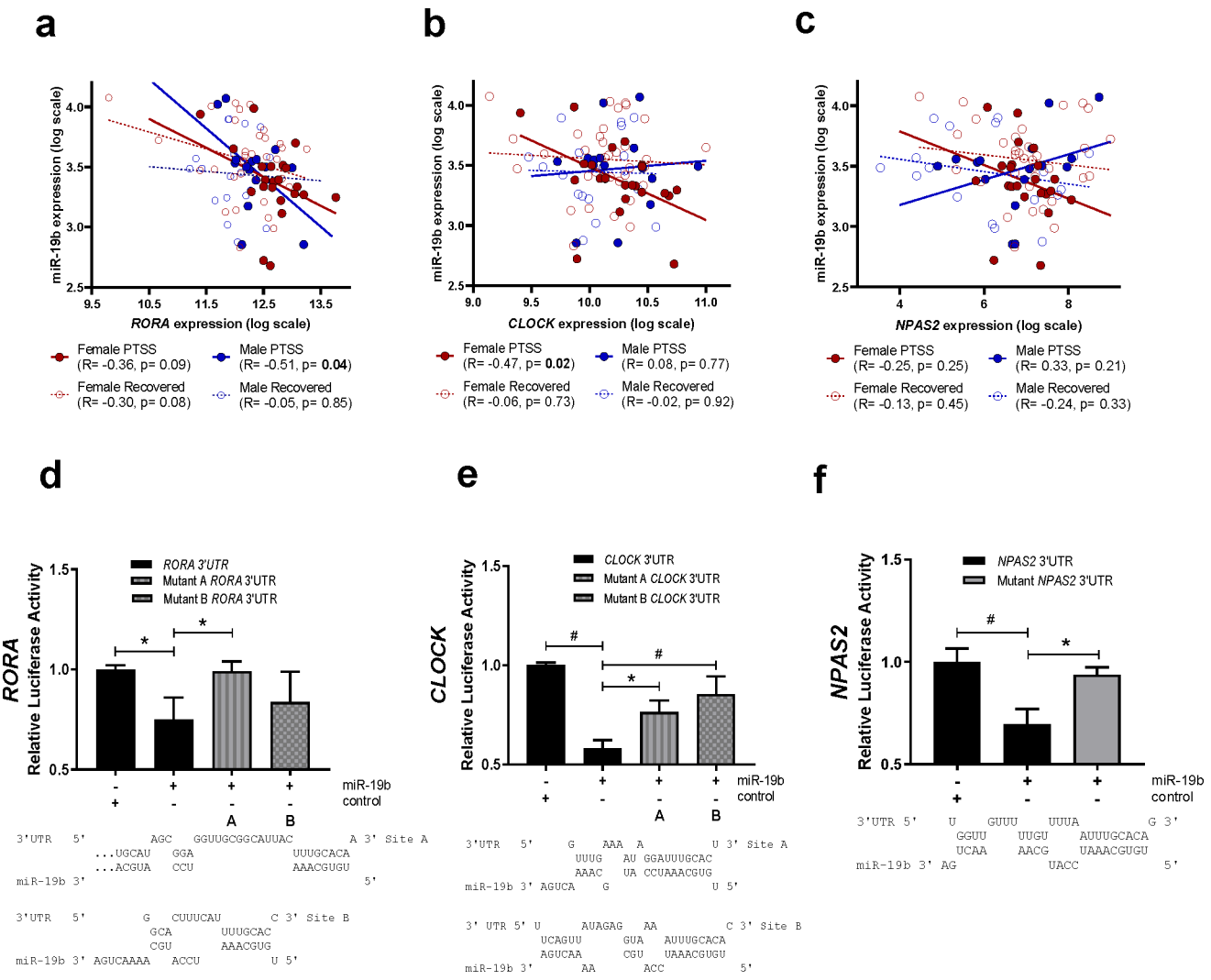

Fig 4.

Preliminary evidence suggesting that miR-19b regulates three genes involved in rhythmic processing. (a - c) Correlation between circulating miR-19b expression levels and circulating mRNA expression levels in men (blue circles, n=16 PTSS and n=18 Recovered) and women (red circles, n=22 PTSS and n=34 Recovered) in the early aftermath of MVC (n=90). The relationship between miR-19b and *RORA* (panel a), *CLOCK* (panel b), and *NPAS2* (panel c) mRNA is shown for men and women who developed PTSS six months following trauma exposure (filled circles) and in those who recovered (open circles). Expression levels of miR-19b and each mRNA transcript represent log transformed RNA seq reads. Pearson correlation coefficients (R) and p values are presented for each subgroup. (d – e) Dual luciferase reporter assays examining direct binding of miR-19b to the (d) *RORA* 3'UTR, (e) *CLOCK* 3'UTR, and (f) *NPAS2* 3'UTR. Black bars indicate binding of miR-19b mimic or control mimic to wild type 3'UTRs while gray bars represent binding of miR-19b or control mimic to 3'UTRs with miR-19b seed sites mutated in each of the predicted binding sites as indicated. Relative luciferase activity (y axis) refers to firefly luciferase activity/renilla luciferase activity (i.e. the firefly construct containing the 3'UTR is normalized to the

vector-only renilla construct). Predicted miR-19b – target hybrids are shown below each graph; *RORA* and *CLOCK* are predicted to have two miR-19b binding sites each and *NPAS2* is predicted to have one miR-19b binding site. p values were determined for panels (d - f) using the Mann-Whitney non-parametric test. *, $p<0.05$; #,$p<0.001$.

Table 1.

Baseline characteristics of study participants (Motor vehicle collision cohort, n = 179; Sexual assault cohort, n=74)

Characteristic	MVC (Discovery)	SA (Replication)
Enrolled, n	179	74
Age, years, mean (SD)	34 (12)	27 (8)
Females, n (%)	112 (63)	74 (100)
Ethnicity, n (%)		
Black or African American	179 (100)	9 (12)
White or European American	-	55 (74)
Multiethnic or Other	-	10 (14)
Education, n (%)		
HS or less	68 (39)	21 (29)
Some college	77 (43)	32 (43)
College	25 (14)	17 (23)
Post-college	7 (4)	4 (5)
Distress* in the early aftermath of trauma, mean (SD)	23 (11)	55 (18)
Overall pain in the ED or SANE (0–10 NRS), mean (SD)	7.2 (2)	5.0 (3)
Number of previous traumatic events**, mean (SD)	3.5 (3)	8.7 (8)

** Distress was measured with the peritraumatic distress inventory (scale of 0–52) in the MVC cohort and the posttraumatic stress disorder civilian checklist in the SA cohort (scale of 17–85)

*** Number of traumatic events in participants lifetime prior to MVC or SA (Life events checklist (assesses sixteen different types of trauma plus a question about 'other trauma'))

Abbreviations: MVC = motor vehicle collision trauma, SA = sexual assault trauma, SD = standard deviation, ED = emergency department, SANE = sexual assault nurse examiner, NRS = numeric rating scale

Table 2.

Logistic regression models examining the relationship between Emergency Department levels of miR-19b expression and Posttraumatic Stress (PTSS) or Posttraumatic Widespread Pain (PTWP) six months after Motor Vehicle Collision (n = 153).

	PTSS			PTWP		
Variable[a]	**β**	**S.E.**[b]	**p value**	**β**	**S.E.**[b]	**p value**
miR-19b	0.051	0.565	0.928	−0.259	0.566	0.647
x sex	−1.503	0.568	**0.008**	−1.205	0.569	**0.034**
Sex	4.889	1.973	0.013	4.024	1.983	0.043
Age	−0.006	0.015	0.654	0.019	0.015	0.216

[a] Site was also included in the model as a categorical variable.

[b] S.E. = standard error, CI = confidence interval.