microRNA -19b is a sex-dependent regulator of posttraumatic stress symptoms and widespread pain

Abstract

Posttraumatic widespread pain (PTWP) and posttraumatic stress symptoms (PTSS) are frequent forms of trauma that occur at different rates in women and men. Genetic approaches to study pathways using model organisms and mutants have identified hundreds of genes correlated with PTWP/PTSS. Our lab sought to identify microRNAs (miRNAs) that contribute to sex-dependent differences in vulnerability to these outcomes. In the current study, we first identified miRNA that are predicted to regulate PTWP/PTSS genes using Monte Carlo simulations. We found that the most significant miRNA predicted to target PTWP/PTSS genes was miR-19b, a microRNA that has been shown previously to be regulated in response to estrogen and stress exposure. Next, we assessed whether miR-19b expression predicts PTWP/PTSS in a cohort of individuals experiencing motor vehicle collision, one of the most common forms of trauma currently experienced by Americans. Logistic regression demonstrated a sex dependent relationship between initial miR-19b levels following motor vehicle collision and later development of PTWP/ PTSS. The sex-dependent expression of miR-19b was also observed in a rat model of single prolonged stress, which is thought to be analogous to PTSS. We found miR-19b to be regulated by 17-β-estradiol in rat dorsal root ganglion neurons and amygdala, which are neural tissues commonly implicated in PTSS. The potential importance of miR-19b to PTWP/PTSS pathogenesis is highlighted by results showing that miR-19b can directly bind a number of pain and PTSS associated transcripts including circadian rhythm pathway genes. Together, our results suggest that miR-19b plays a regulatory role in PTWP and PTSS development following trauma/stress exposure. Thus, the level of miR-19b expression following motor vehicle collision may predict PTWP/PTSS and enable preventative treatment.

Introduction

Motor vehicle collision (MVC) is one of the most common traumatic stress exposures worldwide.[1] For every 10 individuals experiencing MVC, evidence suggests that two will develop persistent posttraumatic widespread pain (PTWP) and three will develop posttraumatic stress symptoms (PTSS).[2,3] These morbid sequelae of traumatic stress exposures such as MVC frequently co-occur,[4-9] suggesting shared pathogenic mechanisms, and are more common in women,[10-13] 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 PTSS and pain processing are sex-dependent[14-20].

microRNA (miRNA) are small non-coding RNA molecules that 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 PTSS and PTWP pathogenesis (e.g.[21-30]). However, many of these previous studies used predominately male cohorts[24,25,31,32] or focused on a single sex in animal model studies[23,33-36]. Therefore, whether miRNA expression levels predict PTSS and PTWP in a sex-dependent manner has not been addressed, despite evidence indicating that miRNA expression might be differentially regulated in men and women[21,37-40].

The current study used an unbiased *in silico* approach to first identify PTSS and pain miRNA regulatory hubs; that is, miRNA that are predicted to regulate more PTSS and pain- associated transcripts than expected by chance[41]. The top candidate miRNA regulatory hub, miR-19, was then assessed for differential expression in males and females who developed PTSS and/or PTWP vs those who recovered following MVC. Secondary analyses identified whether sex-dependent expression extended to nervous system tissues relevant to PTSS and PTWP and identified relevant pathways/transcripts regulated by miR-19.

Materials/Subjects and Methods

Bioinformatics

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,[42] Algynomics Pain Research Panel v2.0,[43] and the PainGenes Database[44] (n = 560 unique genes, **Supplementary Table 1**). A structured literature review of peer-reviewed original research and review articles written in English was performed in May 2015 to identify genes previously associated with PTSS related phenotypes (n = 154 genes, **Supplementary Table 2**) using the following search terms: post-traumatic stress disorder, anxiety, attention deficit hyperactivity disorder, bipolar disorder, memory, panic disorder, depression, neuroticism, seasonal affective disorder, substance abuse, schizophrenia, obsessive compulsive disorder, OR social behavior AND gene, genetic, GWAS, OR transcript. miRNAs predicted to bind to the 3'UTR of these identified pain or PTSS genes (n = 629) were then identified using TargetScan 7.0.[45]

Monte Carlo simulations (x10,000) consisting of randomly selected sets of genes, normalized to 3'UTR length, were used to generate a distribution of the number of predicted pain and PTSS gene targets for each known human mature miRNA (miR-Base v.21).[41] This distribution was used to identify miRNA with the greatest preferential binding to genes previously associated with pain, PTSS, and pain and PTSS phenotypes.

The ontology relationship between PTWP/PTSS genes predicted to be targeted by miR-19b was assessed using the DAVID v6.7 online algorithm (https://david.ncifcrf.gov/home.jsp).[46] 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/).[47]

MVC Cohort Study

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. The study only enrolled African Americans because of the pressing need for pain studies that focus on understudied, high risk groups.[48-50] This study has been described in detail previously.[51] 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.

Pain and PTSS assessments and outcome definitions in humans

MVC-related pain intensity and distribution in the past week was assessed six months following MVC using the modified Regional Pain Scale.[52] Pain intensity was evaluated in each of 19 body regions[53] via a 0 (no pain) to 10 (maximum possible pain) numeric rating scale (NRS).[54] 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).[55]

MVC-related PTSS were assessed six months following MVC using the Impact of Event Scale: Revised (IESR).[56] 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.[57]

Distress in the ED was measured using the Peritraumatic Distress Inventory, a 13-item questionnaire assessing the level of distress experienced immediately after a traumatic event.[58] 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[59]; this was also the mean and median level of distress in the cohort.

Statistical analyses

MVC cohort sociodemographic characteristics were summarized using standard descriptive statistics. Logistic regression analyses adjusted for participant age and ED study site were used to assess the relationship between miR-19b expression levels and PTSS or PTWP outcomes 6 months following MVC, and to derive odds ratios (ORs) and p-value significance. miR-19b is expressed across almost all cell types[115] and at high levels in whole blood; therefore miR-19b sequencing reads were divided by 1000 and ORs can be interpreted as

increased odds per 1000 sequencing reads of miR-19b. Sex- and stress-dependent effects were evaluated using interaction variables (miR-19b*stress and miR-19b*sex) because of evidence that such interactions are frequently present and important (e.g.[60-65]). Differences in ED miR-19b expression levels between women and men who did and did not subsequently develop PTSS or PTWP at 6 months were evaluated by comparing mean expression levels in the two groups. 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 or SAS software v9.4.

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. 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: The details of the unpredictable sound stress (USS) protocol have been reported previously.[66-69] 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. 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. Control animals were left undisturbed in the animal facility during stress tests, but were tested for hypersensitivity similarly to stressed animals.

Single Prolonged Stress: The details of the single prolonged stress (SPS) protocol have been reported previously.[70] 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 under 5 minutes). Rats were single housed and left undisturbed in the animal facility for 7 days, a period crucial for the development of PTSS symptomatology.[70] Following this period, fear conditioning, fear extinction, and extinction retention testing was conducted, as described previously.[71] Control animals were also single housed and left undisturbed in the animal facility during the SPS procedure but experienced fear conditioning, fear extinction, and extinction retention testing similarly to stressed 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) and 5 mg/ml dispase II (Sigma, St. Louis, MO) in 1x HBSS at 37°C for 30 min. Cells were triturated with flame-polished Pasteur pipets, and plated at $2x10^5$ cells/ml onto 96-well plates pre-coated with poly-D-lysine (Sigma, P7886) and Laminin (Sigma, St. Louis, MO). Twenty-four hours after plating, 1 µM cytosine β-D-arabinofuranoside was added. DRG neurons were deprived of hormones by growing them in neurobasal A (Gibco, Waltham, MA) lacking phenol red, supplemented with 10% dextran-coated charcoal treated FCS, 50 µg/mL gentamicin, and murine nerve growth factor 2.5S (Gibco, Waltham, MA) for 72 hours. Stimulation with 100 nM 17β-estradiol (Sigma, St. Louis, MO) lasted for 3 or 6 hours. RNA was isolated after each time point using TRIzol (Invitrogen, Carlsbad, CA).

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) 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, Germantown, MD). 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. 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 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 using two similar protocols. An initial set of 69 samples (randomly selected from the full MVC cohort) were prepped using an adaptation of published protocols as described previously.[21,72] 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. 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[21], and were normalized using upper quartile normalization. In order to normalize potential technical biases between the two methods of library preparation, sequencing reads were adjusted for batch effects using the ComBat package in R.[73]

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).[74] Expression level of each transcript were estimated via RSEM[75] 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[76] 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[77]. These samples were not part of the cohort that was used for RNA sequencing, thus they expanded the final cohort size to 178 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 and RNU48 detection were obtained from Life Technologies (Carlsbad, CA).

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 as the control RNA.

microRNA microarray

Total RNA isolated from rat DRG was end-labeled with biotin and hybridized onto Affymetrx miRNA 4.0 cartridge arrays. Total RNA was prepared using the Affymetrix FlashTag™ Biotin HSR RNA Labeling Kit and manual. Biotin-labeled RNA was then added to a hybridization cocktail (biotin-labeled total RNA, 50 pM control oligonucleotide B2, BioB, BioC, BioD and cre hybridization controls, 9.7 % DMSO, 4% formamide, 1x hybridization mix). Affymetrix miRNA 4.0 arrays (Affymetrix, Santa Clara, CA) were hybridized for 16 h at 48 °C in the GeneChip Hybridization Oven 645 (Affymetrix). The arrays were washed and stained with R-phycoerythrin streptavidin in the GeneChip Fluidics Station 450 (Affymetrix). The arrays were scanned with the GeneChip Scanner 3000 7G Plus with autoloader. GeneChip Command Console Software (AGCC) was used for washing, staining and scanning control of the instrumentation. Affymetrix Expression Console Software was used for basic data analysis and quality control.

Luciferase assays

Cloning. The 3'UTRs of human genes *RORA, NPAS2, and CLOCK* were amplified from human 293T cell line genomic DNA using primers as indicated in Supplementary Table 3. 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 3**).

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 h 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 form the negative control.

Results

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

Among all known human mature miRNAs (n=2,588, miRBase v21.0), 244 (9.4%) were predicted to target one or more gene transcripts previously associated with PTSS and pain outcomes (**Figure 1a**, range 1- 198). Monte Carlo simulations (x10,000) were used to determine the degree of preferentially predicted binding of each miRNA to transcripts associated with PTSS, pain, and PTSS and pain, while accounting for targeting distribution (**Figure 1b**). Based on these *in silico* analyses, the miR-19 family of transcripts was identified as most preferentially targeting PTSS and pain transcripts ($p < 0.05$, **Figure 1b**). The miR-19 family includes miR-19a and miR-19b (**Supplementary Figure 1**); 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). Subsequent analyses focused on miR-19b as a potential pain and PTSS-regulatory hub, because it has been shown to be associated with PTSS, pain, and stress previously.[21,23,24,35,78,79]

miR-19b predicts the development of PTSS and PTWP in humans following MVC

To assess for evidence that miRNA-19b regulates the expression of genes involved in the pathogenesis of PTSS and PTWP, we evaluated whether miRNA-19b blood levels in the immediate aftermath of motor vehicle collision (MVC) predict PTSS and PTWP outcomes. Participants (n = 178) were drawn from a prospective cohort study of individuals presenting to the ED after MVC. Blood samples were obtained in the ED, and PTSS and PTWP 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**). miRNA blood levels were assayed using either RNAseq (n = 152) or RT-qPCR (n = 26).

In initial general linear modeling (n = 152), a sex*miR-19b interaction was observed for both PTSS (OR = 1.41, p = 0.039, **Table 2**) and PTWP outcomes (OR = 1.46, p = 0.031, **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 = 0.044 for PTSS, p = 0.026 for PTWP, **Supplementary Table 4**, **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 = 0.039 for PTSS, p = 0.113 for PTWP, **Supplementary Table 4**, **Figure 2b, c**). These observations were replicated in the 26 individuals from the MVC study in whom miRNA blood levels were assayed using RT-qPCR (**Figure 2d**). In secondary analyses using small RNAseq data, we also found that circulating blood miR-19a levels predicted PTWP and substantial PTSS in a sex-dependent manner (**Supplementary Table 5**).

miR-19b is expressed in a sex-dependent manner in animal models of pain and PTSS

We next evaluated whether this sex-dependent expression of miR-19b observed in our human MVC cohort was also present in animals exposed to paradigms known to produce stress-induced hyperalgesia (unpredictable sound stress model (USS)[67,68]) and altered fear learning (single prolonged stress (SPS) model,[70,80] a model of PTSS) (**Figure 3a**). Because our human studies focused on blood expression levels of miR-19b, we first examined circulating levels of miR-19b in animals. In these samples, we found results paralleling our human data: male animals exposed to these paradigms demonstrated higher levels of circulating miR-19b as compared to unstressed control animals, whereas females demonstrated lower levels of miR-19b (**Figure 3b, 3c**).

We next evaluated for sex differences in miR-19b levels in animal tissue known to be relevant to PTSS and PTWP pathogenesis. miR-19b was moderate-to-highly expressed in all tissues examined (**Supplementary Figure 2a**). When comparing expression levels between males and females, females expressed lower levels of miR-19b in all peripheral tissues (blood, DRG, peripheral nerve, and adrenal gland) but was expressed at equal levels in both sexes in all brain regions examined (amygdala, hippocampus, hypothalamus) (**Supplementary Figure 2b**). In male animals exposed to USS, miR-19b expression levels were increased 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.[23,35] 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 DRG neurons results in a decrease in miR-19b expression

The above data demonstrate that miR-19b predicts PTSS and PTWP in a sex-dependent manner in humans, and is expressed at lower levels in females than males in human blood and animal tissue (see also **Supplementary Figure 2b**). These data, together with previous data showing that miR-19b is under the transcriptional control of the main female sex hormone 17β- estradiol,[40] 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 (DRG) neurons. miR-19b expression decreased 3 hours following stimulation with 100 mM 17β-estradiol (1.23 fold lower, $p < 0.05$, **Supplementary Figure 3**) but returned to baseline expression levels 6 hours following stimulation. As a positive control, we also assessed expression of *CGRP* mRNA expression following stimulation with 17β-estradiol. Consistent with previous reports which show that *CGRP* is upregulated in response to estrogen (e.g.[81]), *CGRP* expression increased 3 and 6 hours after stimulation with 100 mM 17β-estradiol (1.90 fold higher, $p < 0.05$, **Supplementary Figure 3**).

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 targets using the DAVID online algorithm. The GO group with the highest fold enrichment was “rhythmic processes” (GO:0048511) (9.867 fold enrichment, $p = 1.5 \times 10^{-5}$, **Supplementary Table 6**); this group included well-known circadian rhythm pathway genes such as *CLOCK*, *NPAS,* and *PER1*. Interestingly, this algorithm did not categorize the miR-19b predicted target, *RORA*, as a gene involved in rhythmic processes even though it is known to play a central role in the circadian rhythm.[82] *RORA* is of interest because it has previously been associated with PTSS vulnerability.[83,84] Additionally, evidence also implicates circadian and sleep dependent processes in pain, PTSS, and related neuropsychiatric disease pathogenesis.[85-90] Overall, the results of GO bioinformatics predictions suggest that miR-19b may influence PTSS and PTWP outcomes by regulating circadian rhythm pathway genes.

Circadian rhythm gene transcripts are negatively correlated with miR-19b expression levels in individuals who develop PTSS and/or PTWP 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). Consistent with this hypothesis, among individuals experiencing MVC a negative correlation was observed between miR-19b and key circadian rhythm transcripts *RORA*, *CLOCK*, and *NPAS2* (except for men with *NPAS2*), and this relationship was stronger in individuals who developed PTSS/PTWP following MVC vs those who recovered (**Figure 4a, 4b, 4c**). The inverse relationship between miR-19b and *CLOCK* and *RORA* transcripts was also stronger in males than females, which is consistent with higher expression of miR-19b in male participants who develop PTSS/PTWP than female participants who developed PTSS/PTWP (see **Figure 2b, 2c**). Using *in vitro* dual luciferase reporter assays, we also found that, consistent with bioinformatics predictions, miR-19b could directly bind and repress each of the 3'UTRs of these genes (**Figure 4d, 4e, 4f**). Together these data suggest that the influence of miR-19b on PTSS/PTWP development is due, at least in part, to miR-19b's regulation of key circadian rhythm pathway transcripts.

Discussion

We evaluated the potential role of miRNA in the development of PTSS and PTWP using *in silicio*, *in vitro*, animal, and human data. Unbiased *in silicio* analyses implicated members of the miR-19 family in PTSS and PTWP pathogenesis, by having the highest degree of preferential predicted binding to PTSS and PTWP-associated transcripts. Circulating miR-19b levels in the immediate aftermath of MVC were found to predict both substantial PTSS and PTWP six months after trauma exposure. Interestingly, associations between miR-19b levels and PTSS and PTWP outcomes were sex-dependent, with lower levels of miR-19b predicting PTSS and PTWP in women and higher levels predicting PTSS and PTWP in men. These associations and sex differences in associations were found in animal models of PTSS and PTWP, both in blood and in tissues relevant to PTSS and PTWP pathogenesis, and may be mediated in part by 17β-estradiol. Bioinformatics analyses indicated that gene pathways associated with circadian processes implicated in the development of adverse posttraumatic neuropsychiatric sequelae such as PTSS and PTWP were most likely to be targeted by miR-19b. 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, and a negative correlation was observed between miR-19b and these transcripts in humans experiencing MVC trauma, particularly those developing PTSS or PTWP.

The molecular mechanisms accounting for the above associations between miR-19b and the development of substantial PTSS and PTWP, and sex differences in the direction of these associations, remain unknown. If miR-19b not only marks vulnerability to these outcomes, 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. For example, in males, the higher levels of miR-19b expression associated with vulnerability to PTSS/PTWP would be expected to result in increased repression of key circadian rhythm regulators such as *CLOCK*, *RORA*, and *NPAS2*, and less robust activation of the circadian rhythm pathway. In women, the lower levels of miR-19b associated with vulnerability would be expected to have the opposite effect, resulting in more robust activation of the circadian rhythm pathway. Evidence suggests that disruption in circadian rhythm processing in either direction can have large downstream effects on rhythmic gene expression across multiple tissues and bodily systems.[91-93] Thus from a global perspective it is possible that in both males and females miR-

19b expression levels that push biological systems too far from the mean in either direction are pathogenic. Further studies are needed to understand the mechanisms accounting for the association between miR-19b and PTSS and PTWP, 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,[23,35] and Wang et al. and Sakai et al. found that miR-19a was associated with chronic neuropathic pain in male rats.[36,94] In humans, Martin et al. identified miR-19a as the most differentially up-regulated miRNA in male combat veterans who developed PTSD.[25] Our findings in male animals and humans are consistent with these data, but extend them and place them in sex-specific context, as miR-19a consistently predicts substantial PTSS and PTWP 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 contribute to the lower levels of miR-19b observed in females. Although not experimentally tested in the current study, it is likely that 17-β-estradiol regulation of miR-19b is indirect, based on the fact that we did not bioinformatically identify an estrogen response element in the promoter region of miR-19b. In addition to regulation by 17-β-estradiol, it is also possible that miR-19b is regulated in a sex-dependent manner via other sex hormones or via non-hormonal mechanisms. 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.

Of note, 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-30, miR-181, miR-15, and miR-124, have already been shown to be associated with stress-related disorders.[33,95-98] 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 PTSS or posttraumatic pain if they affect the expression of one or only a few genes, but these genes are highly influential. Examples of such miRNA include miR-135[21,34,99,100], miR-103[101,102], and miR-320[22,65,103-106].

miR-19b originates from both the miR-17-92 cluster (chromosome 13) and the miR-106a-363 cluster (X chromosome). In this study, we did not examine the role of other members of these two clusters on PTSS and PTWP outcomes following MVC. However, it is interesting to note that the miR-17/20/106 family of miRNA, that originates from the same genomic regions (and is regulated by the same promoter region), was identified as a candidate regulatory hub in *in silico* analyses. Therefore, these miRNAs might also predict PTSS/PTWP development following trauma exposure in a sex-dependent manner. As additional evidence suggests that this might be the case, using the online bioinformatics algorithm DIANA miRPath[107], these three miRNA are also predicted to preferentially target the circadian rhythm pathway.

The fact that miR-19b regulates multiple 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 strongly regulated by miR-19b in this study, has been shown to be both genetically associated[83,84,108] and not associated[109] with PTSD. Additional studies have also shown physiological relationships between the circadian rhythm and PTSD pathogenesis,[110] and literature from the pain field also implicates sleep and circadian abnormalities to pain vulnerability[86,87,111-114]. However, very few studies have examined whether there are sex-dependent differences in the contribution of the circadian rhythm pathway to PTSS/PTWP 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. Future studies examining the influence of sleep and the circadian rhythm pathway on pain and PTSS outcomes should include sex as a variable.

The many strengths of this study include being one of the largest human studies of miRNA predictors of PTSS and PTWP development in male and female trauma survivors to date, and that it used coordinated translational studies (*in silico*, human cohort, animal, and molecular/cell culture experiments) to guide and strengthen the validity of the findings. However, several limitations should be considered when interpreting this work. First, the main findings of the study have not been replicated in a second cohort of post-trauma survivors. This is an essential next step for future studies. However, this study did internally replicate the RNA sequencing findings (both technically and biologically) by examining miR-19b expression in an expanded cohort of 26 age-matched cases and controls using RT-qPCR. Second, this study did not assess the influence of 17-β-estradiol

levels on miR-19b association with PTSS/PTWP in female participants following MVC. This would have been an interesting experiment given the cell culture results demonstrating that 17-β-estradiol can negatively regulate miR-19b in neuronal 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 strong bioinformatics pathway analyses, however, it is possible that other transcripts predicted to be regulated by miR-19b are also important to the pathogenesis of PTSS/PTWP. Finally, this work was performed in only African American individuals and in one strain of rats. Therefore, whether there are also ethnic/strain differences in the association between miR-19b and PTSS/PTWP is not known.

In summary, we report the first set of evidence indicating that miR-19b is differentially regulated in males and females following trauma and that expression levels of miR-19b in the early aftermath of MVC predict PTSS and PTWP outcomes in men and women. Further, this 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.

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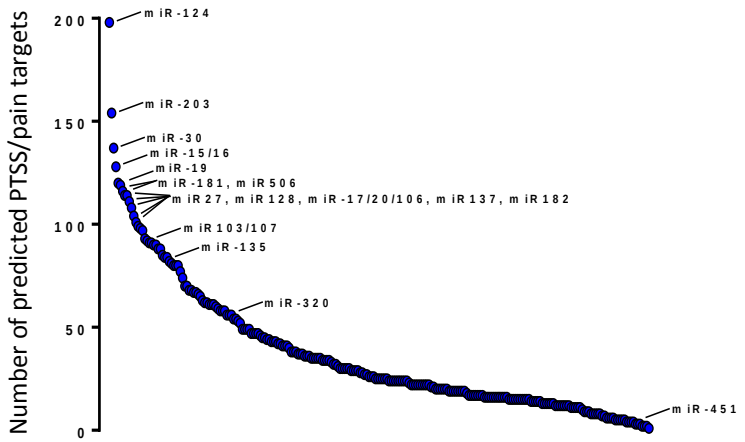
a



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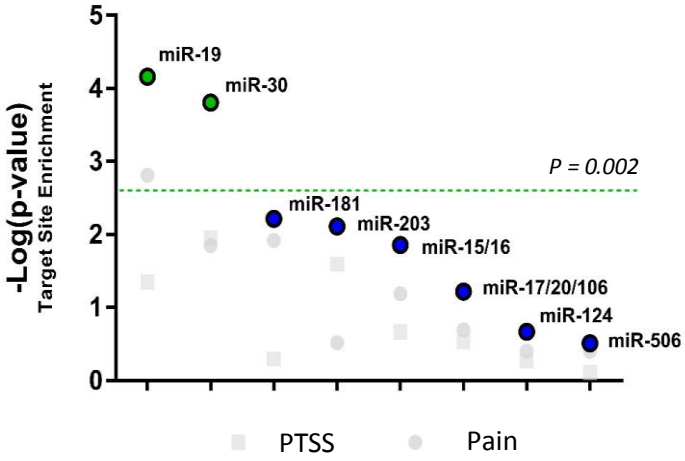


Figure 1. *In silico* analyses indicate that miR-19 is a strong candidate regulatory hub for both pain and PTSS. (a) 244 miRNA (indicated by each blue dot) target at least one pain/PTSS gene. miR-19 is predicted to target 112 of 629 pain/PTS genes. (b) Of the miRNA targeting the most pain/PTS genes, miR-19 is the strongest predicted regulatory hub for pain and PTSS, indicated by high combined values of the negative logarithm of the empirical p-value ($p < 0.002$, green dotted line) for pain (gray circles) and PTSS (gray squares).

Table 1. Baseline characteristics of study participants (n = 178)

Characteristic	
Enrolled, n	178
Age, years, mean (SD)	34 (12)
Females, n (%)	112 (63)
Education, n (%)	
HS or less	68 (39)
Some college	77 (43)
College	25 (14)
Post-college	7 (4)
Collision characteristics	
Driver, n (%)	121 (68)
Severe vehicle damage, n (%)	106 (60)
Seatbelt worn, n (%)	143 (80)
Airbag deployed, n (%)	63 (35)
Location of vehicle damage	
Front, n (%)	81 (46)
Rear, n (%)	54 (30)
Right side, n (%)	38 (21)
Left side, n (%)	29 (16)
Distress* in the early aftermath of trauma, mean (SD)	23 (11)
Overall pain in the ED (0-10 NRS), mean (SD)	7.3 (2.2)
Median time to ED, minutes	60
Number of previous life events**, mean (SD)	3.5 (2.7)
IESR score at 6 months, mean (SD)	31 (26)
Overall pain at 6 months, mean (SD)	5.2 (3.3)

*Distress measured with the peritraumatic distress inventory (scale of 0-52)
**Life events checklist (assess 16 different types of trauma plus a question about 'other trauma')

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 = 152).

	PTSS		PTWP	
Variable[a]	OR (95% CI)[b]	p value	OR (95% CI)[b]	p value
miR-19b	0.86 (0.59, 1.27)	0.462	0.74 (0.47, 1.12)	0.190
x stress	0.99 (0.98, 1.01)	0.735	1.00 (0.99, 1.02)	0.693
x sex	1.41 (1.02, 1.95)	**0.039**	1.46 (1.04, 2.06)	**0.031**
Stress	1.06 (1.00, 1.13)	0.051	1.05 (0.99, 1.12)	0.131
Sex	0.78 (0.19, 3.10)	0.726	0.33 (0.08, 1.39)	0.131
Age	1.00 (0.97, 1.05)	0.660	1.03 (0.99, 1.07)	0.088

[a]Site was also included in the model as a categorical variable. [b]OR = odds ratio, CI = confidence interval.

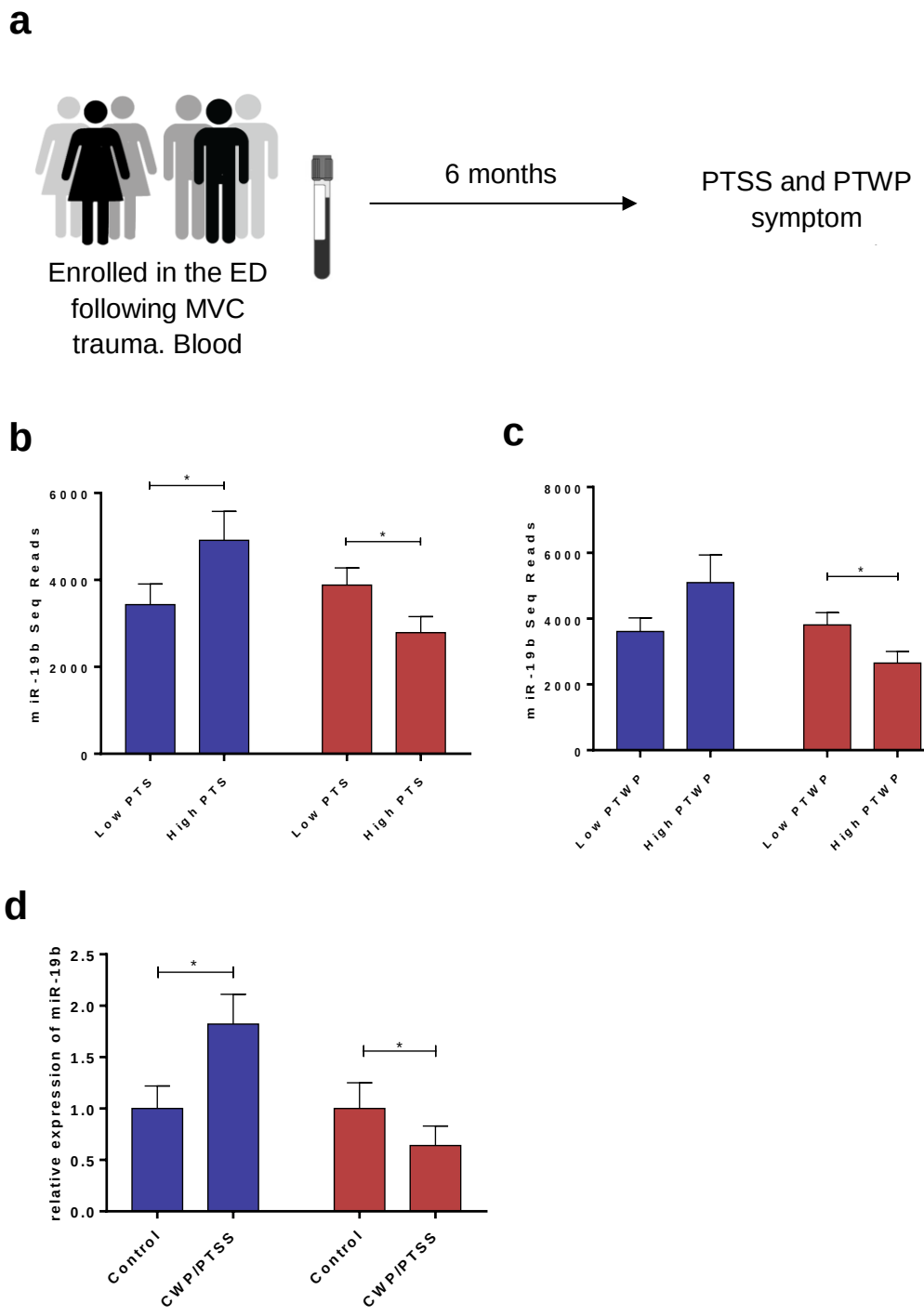


Figure 2. miR-19b is differentially expressed in male and female individuals who develop posttraumatic stress symptoms (PTSS) and/or posttraumatic widespread pain (PTWP) following motor vehicle collision (MVC). (a) Simplified study design schematic showing that women and men were enrolled in the ED following MVC, blood samples were obtained in the ED, and PTSS and PTWP outcomes were assessed at six months. (b) miR-19b expression levels (n=152) in male (blue) and female (red) participants who developed low versus high levels of PTSS following MVC as measured by miRNA sequencing. (c) miR-19b expression levels (n=152) in male (blue) and female (red) participants who develop low versus high levels of PTWP following MVC as measured by miRNA sequencing. (d) RT-qPCR data showing relative miR-19b expression levels in individuals who recover following MVC versus those who develop both PTSS and PTWP six months following MVC. Data are represented as mean $\pm$ SEM. *$p < 0.05$.

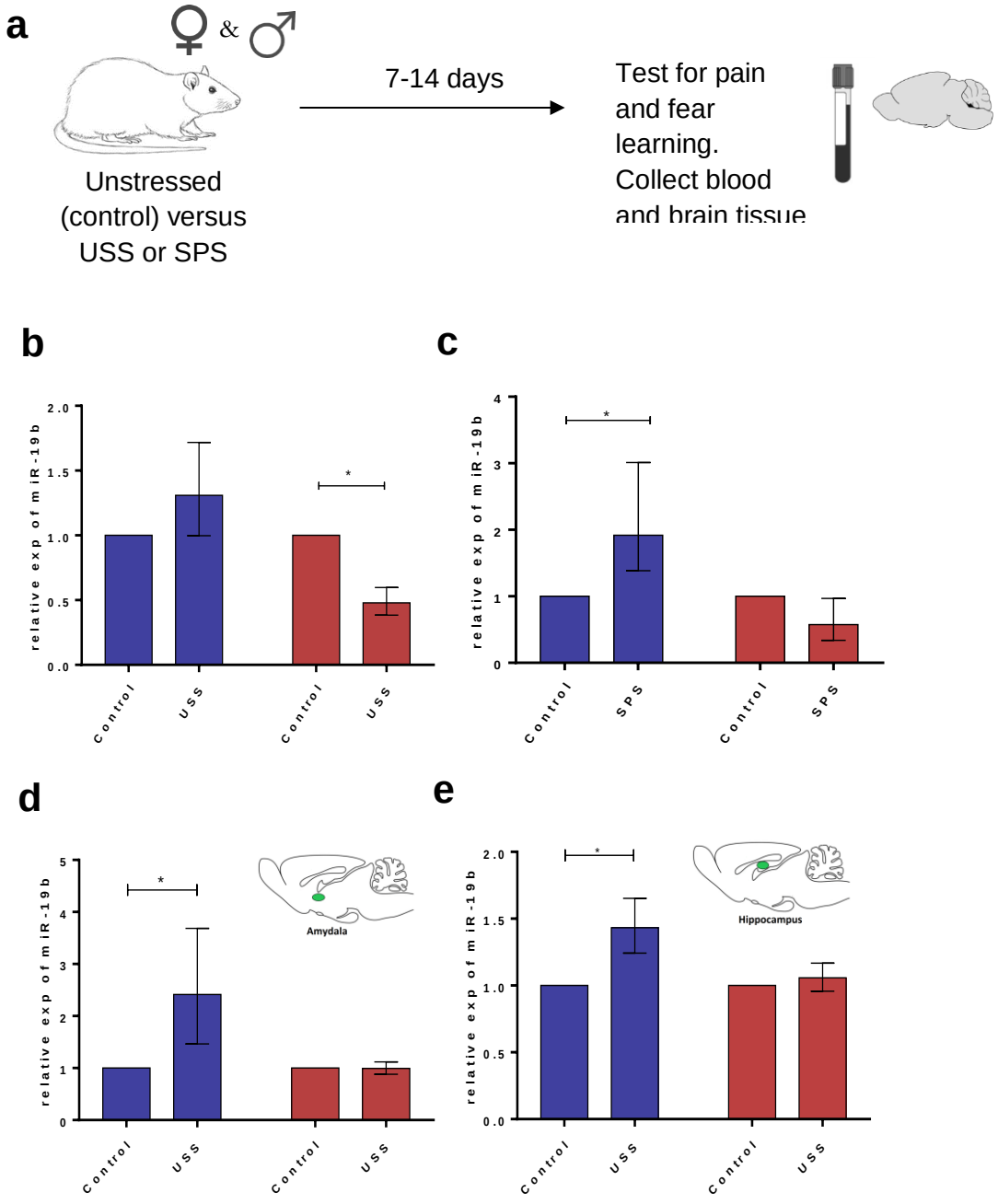


Figure 3. miR-19b is expressed differently in male and female rats following stress exposure. Sex-dependent association of miR-19b with pain and PTS symptoms in humans and animals. (a) Schematic diagram of the animal protocol used to assess sex-dependent differences in miR-19b expression. Male and female Sprague Dawley rats were either unstressed or exposed to unpredictable sound stress (USS) or single prolonged stress (SPS). All animals were assessed for hyperalgesia or fear conditioning 14 or 7 days following stress exposure, and blood and brain were isolated immediately following. (b) Circulating miR-19b expression levels in male (blue, n=12) and female (red, n=12) rats unstressed or exposed to USS. (c) Circulating miR-19b expression levels in male (blue, n=28) and female rats (red, n=19) unstressed or exposed to SPS. (d) miR-19b expression levels in the amygdala of male (blue, n=12) and female rats (red, n=12) unstressed or exposed to USS. (e) miR-19b expression levels in the hippocampus of male (blue, n=12) and female rats (red, n=12) unstressed or exposed to USS. Data are represented as mean ± SEM. *p < 0.05

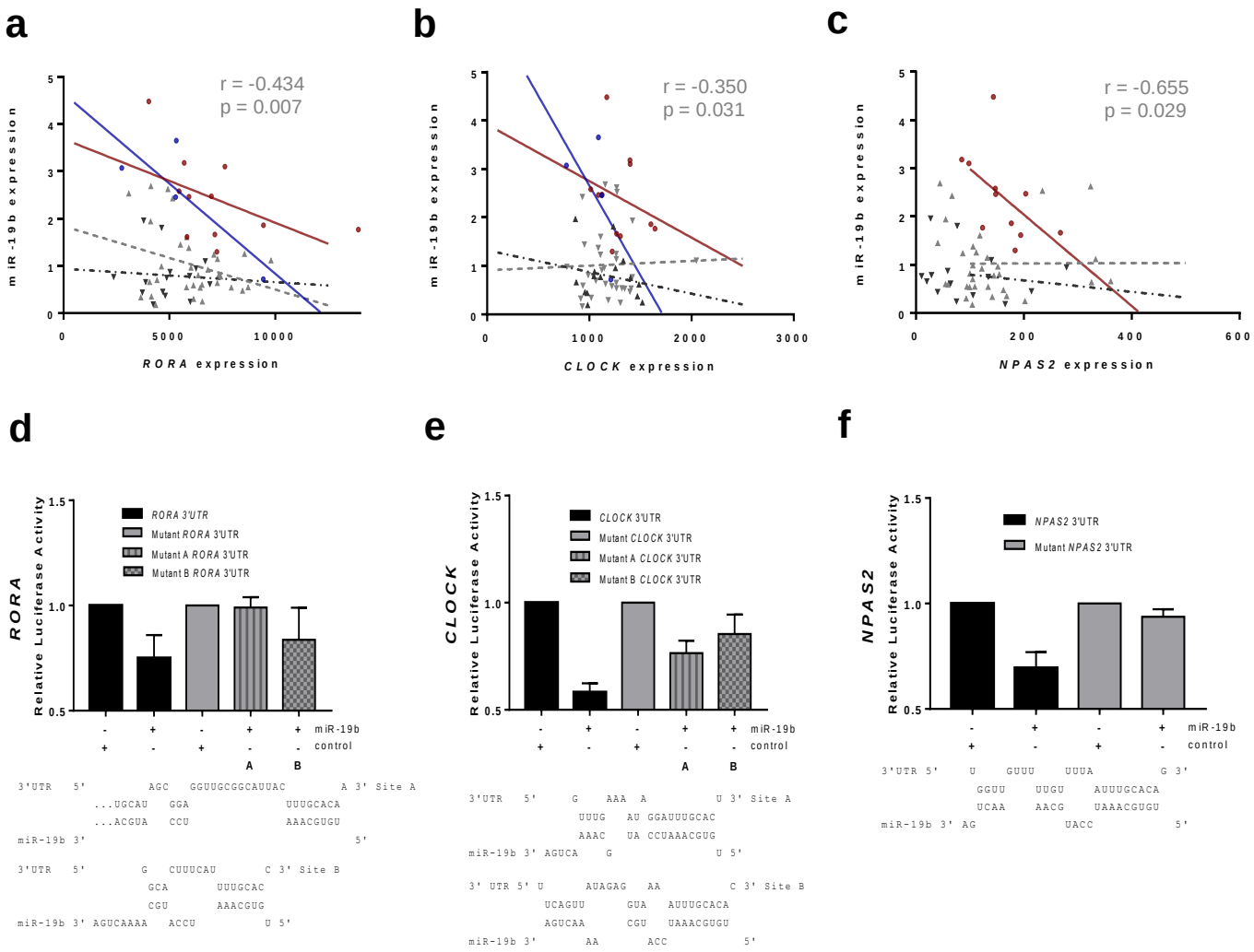


Figure 4. Evidence for miR-19b regulation of circadian rhythm genes in human cohort samples and in cell culture studies. (a and b) Correlation between circulating miR-19b expression levels and circulating mRNA expression levels in male (blue) and female (red) participants in the early aftermath of MVC (n=90) (a) miR-19b correlation with *RORA* mRNA in individuals who developed PTWP 6 months following trauma exposure (colored) versus those who recovered (gray), (b) miR-19b correlation with *CLOCK* mRNA, and (c) miR-19b correlation with *NPAS2* mRNA. Expression levels represented on the y-axis represents RNA seq reads (x1000). Pearson correlation coefficients (r) and p values were calculated using bivariate analyses for male and female participants together (except for *NPAS2*, which is for female only), but regression lines are representative of males and females separately. (d-f) Dual luciferase reporter assays examining direct binding of miR-19b to (d) *RORA,* (e) *CLOCK,* and (f) *NPAS2*. 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 as indicated. Predicted miR-19b – target hybrids are shown below each graph; *RORA* and *CLOCK* are predicted to have two miR-19b binding sites each.

Supplementary Table 1. Pain-associated genes (n = 560) compiled from three established databases (PainNetworks,[42] Algynomics,[43] and PainGenes[44]). These genes were used in bioinformatics analyses to determine which miRNA are predicted to target the genes vs. random sets of genes.

ABCC4 ACE2 ACHE ACPP ACSL1 ADAM11 ADAMTS5 ADCY5 ADCYAP1 ADCYAP1R1 ADM ADORA2A ADORA2B ADRA1A ADRA1B ADRA1D ADRA2A ADRA2C ADRB1 ADRB2 ADRB3 ADRBK1 ADRBK2 AGTR2 ALOX12 ANO1 ANO3 APP AQP1 AQP4 ARL5B ARRB1 ARRB2 ASIC1 ASIC2 ATF1 ATF3 ATF6B ATP1A1 ATP1B3 ATP2B1 ATP6V1A ATP6V1B2 ATP6V1G2 AVPR1A AVPR2 BACE1 BAMBI BDKRB2 BDNF BHLHE22 BTG2 CA8 CACNA1A CACNA1B CACNA1E CACNA1G CACNA1H CACNA2D1 CACNA2D2 CACNA2D3 CACNB3 CACNG2 CALB1 CALCRL CALM2 CAMK2A CAMK2B CAMK4 CAT CCL2 CCL3 CCL4 CCR1 CCR7 CD274 CD38 CD4 CD40 CDH11 CDK5 CDK5R1 CDKN1A CHRM1 CHRM2 CHRM3 CHRM5 CHRNA5 CHRNA7 CHRNB2 CHRNB4 CHUK CLCN6 CLOCK CNGA3 CNR1 COL11A2 COL9A1 COMT COQ10A CPN1 CREB1 CRHR1 CRHR2 CRIP2 CRYAA CSF2 CSF2RB CSK CSNK1A1 CSNK1E CTSB CTSS CX3CL1 CXCL5 CXCR3 CXCR4 CYBB CYP19A1 CYP2D6 CYP3A4 DAB1 DBH DBI DICER1 DISC1 DLG2 DLG4 DPCR1 DPP4 DRD1 DRD2 DRGX DTNBP1 DUSP6 ECE2 EDN1 EDNRA EDNRB EFNB1 EFNB2 EGF EGFR EGR1 EGR3 ENPP2 EPB41L2 EPHB1 EPHB2 EPHB3 EPHB4 EPHB6 EPHX2 ERBB2 ERBB4 EREG ESR1 ESR2 ETV1 EZR F2R F2RL1 F2RL2 FAM19A4 FGF2 FKBP5 FLOT1 FMR1 FOS FOSB FOSL2 FOXN1 FRMPD4 FSTL1 FYN GABARAPL1 GABBR1 GABBR2 GABRA4 GABRA6 GABRB1 GABRB2 GABRB3 GABRG2 GAD1 GALR1 GBP1 GBP2 GCH1 GDNF GFAP GFRA2 GHR GHRHR GJA1 GLRA1 GLRA2 GLRA3 GLRA4 GLRB GNAO1 GNAQ GNAZ GNB2L1 GNG5 GPR3 GPR55 GPSM3 GRASP GRIA1 GRIA2 GRIA4 GRIN1 GRIN2A GRIN2B GRIN2D GRIN3A GRIN3B GRK5 GRK6 GRK7 GRM1 GRM2 GRM4 GRM5 GRN GRP GUCY1B3 HCN1 HCN2 HCRT HDAC4 HDC HIF1A HINT1 HMGB1 HMOX2 HN1 HNRNPD HNRNPU HOXB8 HRH1 HSD17B8 HSPA8 HSPA9 HTR2A HTR2C IAPP ICA1 IER3 IFNG IFRD1 IGF1 IKBKAP IKBKB IKBKE IL10 IL13 IL17A IL1A IL1R1 IL1RAP IL1RN IL2 IL21R IL22RA2 IL2RA IL2RB IL33 IL4 IL6 IL6ST IL7R INADL IRF8 ITPR1 JUN KCNA1 KCNA2 KCND2 KCNIP3 KCNJ11 KCNJ3 KCNJ5 KCNJ6 KCNJ8 KCNJ9 KCNK10 KCNK2 KCNK3 KCNK9 KCNS1 KCNT1 KCTD17 KIF1A KIT KLF11 KLF7 KRAS L1CAM LAMA4 LEP LEPR LGALS1 LMX1B LPAR1 LPAR3 LPAR5 LRP1 LTA LTB LYN LYST MAOA MAOB MAP2K1 MAP2K3 MAP3K8 MAPK1 MAPK10 MAPK11 MAPK14 MAPK3 MAPK8 MAPK9 MAPT MCCD1 MCF2L MDC1 MECP2 MGLL MIF MME MMP24 MPDZ MPZ MSNMTDH MTHFR MUC21 MYD88 NAV2 NBL1 NCAM1 NDN NEDD4L NF1 NFE2L2 NFKB1 NFKBIA NFKBIZ NGF NGFR NLGN2 NLRP3 NOS1 NOVA1 NOX1 NPEPPS NPPC NPTX1 NPY1R NPY2R NR2C2 NR2F6 NR3C1 NR4A1 NRG1 NRM NT5E NTF3 NTRK2 NTRK3 NTSR1 NTSR2 OPRK1 OPRL1 OSM OXTR P2RX2 P2RX3 P2RX5 P2RX7 P2RY1 P2RY13 P2RY2 P2RY6 PACSIN1 PAK7 PAWR PBX2 PCSK2 PCSK6 PDGFB PDYN PER1 PER2 PIK3CA PIK3CB PIK3CG PIP5K1A PIP5K1B PIP5K1C PIRT PLA2G4A PLCB1 PLCB3 PLCB4 PLCG1 PLCL1 PLP1 PMP22 PNOC POMC POU5F1 PPARA PPARG PPP1R1B PPP1R9B PPP3CA PPP3R1 PPP3R2 PPT2 PRKAA2 PRKACA PRKACB PRKAG2 PRKAR1B PRKCA PRKCB PRKCD PRKCE PRKD1 PRKD3 PRKG1 PRLR PRNP PROK2 PRRT1 PRX PSMB8 PSMB9 PTAFR PTGDR PTGER2 PTGER3 PTGER4 PTGFR PTGIR PTGS1 PTGS2 PTN PTPRZ1 RAB5A RABGGTA RAD52 RAF1 RAP1A RASD2 RELA RELN RET RGS2 RGS4 RGS9 RNF5 RPS6KA3 RUNX1 RUNX2 RUNX3 RXRB S100A10 S100B S1PR3 SCD SCN10A SCN1A SCN2A SCN2B SCN3A SCN5A SCN8A SCN9A SESN2 SET SGK1 SHC1 SIGMAR1 SLC12A2 SLC12A5 SLC12A6 SLC15A2 SLC17A6 SLC17A7 SLC17A8 SLC18A2 SLC1A3 SLC29A1 SLC32A1 SLC39A7 SLC6A1 SLC6A11 SLC6A3 SLC6A4 SLC6A6 SOD2 SPARC SPP1 SPTLC1 SRD5A1 SRR STARD13 STAU1 STAU2 STK39 STOML3 STX1A TAC1 TAC4 TACR1 TACR3 TAP1 TAP2 TCF19 TCIRG1 TGFB1 TH THBS4 TLR3 TLR4 TLR5 TLR9 TMSB10 TMSB4X TNF TNFAIP3 TNFRSF1A TNFRSF1B TNXB TRIM10 TRIM26 TRPA1 TRPM3 TRPV2 TRPV3 TRPV4 TUBB TYRP1 UCP2 VDR VEGFA VIP VPS4A VPS4B VPS52 WNK1 YWHAZ YY1 ZEB2 ZFAND5 ZFAND6

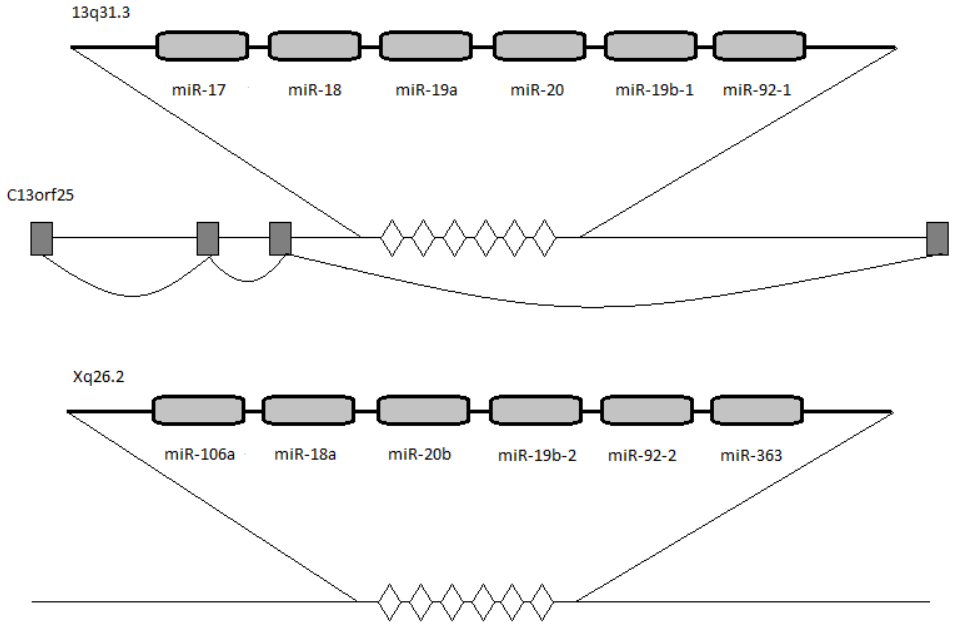
Supplementary Table 2. PTS- (or related neuropsychiatric disorder) associated genes[a] (n = 154) identified using a systematic literature review as described in methods. These genes were used in bioinformatics analyses to determine which miRNA are predicted to target the genes vs. random sets of genes.

ADRA1A, ADRA1B, ADRA2A, ADRA2B, ADRA2C, ADRB2, ADRBK2, ARRB2, COMT, SLC6A2, TH CNR1, FAAH, ARNTL, BHLHB2, CCK, CCKAR, CLOCK, HCRTR1, NPAS2, OPN4, PER1, PER2, PER3, RORA, RORB, TIMELESS, TEF, DBH, DRD1, DRD2, DRD3, DRD4, DBI, GABBR1, GABRA1, GABRA2, GABRA3, GABRA6, GABRB1, GABRB2, GABRB3, GAD1, GAD2, SLC6A11, ANK3, CAMK2A, DAO, DAOA, GRIK1, GRIK4, GRIN1, GRIN2A, GRIN2B, GRM1, SLC1A1, ADCYAP1, ADCYAP1R1, CCKBR, CLPS, CPS1, CRH, CRHBP, CRHR1, CRHR2, FKBP5, GAL, NPY, NPY1R, NPY2R, NPY5R, NR3C1, NR3C2, SLC18A1, TULP1, ACE1, ACHE, F5, IL10, IL17, IL18, IL1A, IL1B, IL1R2, IL1RN, IL2, IL6, IL8, TNF, TNFRSF1A, OPRD1, OPRK1, OPRL1, OPRM1, PDYN, PENK, PNOC, POMC, BDNF, OXT, OXTR, PRL, TAC1, HTR1A, HTR1B, HTR2A, HTR2C, HTR3A, HTR3B, MAOA, MAOB, SLC6A3, SLC6A4, TPH1, TPH2, AMTN, ARAP3, CACNA1C, CSMD1, CTSF, DICER1, DGKH, EGFR, FGF1, FGF2, FGFR2, GDA, GFOD1, HYI, ITIH1, JAM3, KCNT2, KLHL13, KIT, MAMDC1, MCTP1, MYO5B, NFIA, PDE4D, PLSCR4, PTN, PTPRG, REG3A, RBMS3, SORCS2, STAB1, SYNE1, TCF4, TDRD9, TECTA, TMEM16C, TMEM16D, TRDN, TSPAN8, VGCNL1

Supplementary Table 3. Primers used in the study

Primer	Sequence	Construct
F-CLOCK	CATGACCTCGAGTTACAGGTGTGAGCACCTTTCC	pL-SV40-GL3-CLOCK-3'UTR
R-CLOCK	CATGACGCGGCCGCACCTCCAGTCCAAGAGACTGAT	pL-SV40-GL3-CLOCK-3'UTR
F-NPAS2	CATGACCTCGAGAGTCGGGACACAATCAGCTT	pL-SV40-GL3-NPAS2-3'UTR
R-NPAS2	CATGACGCGGCCGCTGGAATGTTGGCTGGCATGA	pL-SV40-GL3-NPAS2-3'UTR
F-RORA	ACTACTCTCGAGTCTTTTGGTGATCGGGGTCA	pL-SV40-GL3-RORA-3'UTR
R-RORA	ACTACTGCGGCCGCTTCCCTCACCCTCCTATCCA	pL-SV40-GL3-RORA-3'UTR
F-CLOCK-mutA	AGGATTGGTACCGCTTTATTTTAGGTGGCTG	pL-SV40-GL3-CLOCKmutA-3'UTR
R-CLOCK-mutA	GCGGTACCAATCCTATTTTCAAACATAATTG	pL-SV40-GL3-CLOCKmutA-3'UTR
F-CLOCK-mutB	AAATTGGTACCCAAAACATCTTAGGCACTTT	pL-SV40-GL3-CLOCKmutB-3'UTR
R-CLOCK-mutB	TTTGGGTACCAATTTTACCTCTATAACTGAAAC	pL-SV40-GL3-CLOCKmutB-3'UTR
F-NPAS2-mut	TAATTGGTACCGCTACACAGAGGAAATAACT	pL-SV40-GL3-NPAS2mut-3'UTR
R-NPAS2-mut	GTAGCGGTACCAATTAAAACAAAAACAACCATT	pL-SV40-GL3-NPAS2mut-3'UTR
F-RORA-mutA	TTACTTGGTACCACTAGCTCTTTGTTTCATGA	pL-SV40-GL3-RORAmutA-3'UTR
R-RORA-mutA	TAGTGGTACCAAGTAATGCCGCAACCTCCGCT	pL-SV40-GL3-RORAmutA-3'UTR
F-RORA-mutB	TCATTTGGTACCTTTTTCTTTAAATTAAATGC	pL-SV40-GL3-RORAmutB-3'UTR
R-RORA-mutB	AAAAGGTACCAAATGAAAGTGCCTTATCAATT	pL-SV40-GL3-RORAmutB-3'UTR

a



b

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miR-19a     -UGUGCAAAUCUAUGCAAAACUGA-- 13q.31.3
miR-19b-1   -UGUGCAAAUCCAUGCAAAACUGA-- 13Q.31.3
miR-19b-2   -UGUGCAAAUCCAUGCAAAACUGA-- Xq26.2
```

Supplementary Figure 1. miR-19 genomic loci and sequences. a) miR-19a and miR-19b-1 are expressed as part of the miR-17-92 polycistronic miRNA cluster on chromosome 13. miR-19b-2 is expressed as part of the miR-106a-363 miRNA cluster on the X chromosome. b) the sequence of miR-19a and miR-19b miRNAs are identical except for one nucleotide that is shown in bold font. The miR-19 seed sequence is greyed.

Supplementary Table 4. miR-19b expression levels in male and female individuals who develop Posttraumatic Stress symptoms (PTSS) or Posttraumatic Widespread Pain (PTWP) six months after Motor Vehicle Collision versus those who recover.

	Male (n = 58)		Female (n = 95)	
	miR-19b expression in individuals developing PTSS or PTWP/ recover (Fold Diff)[a]	p value[b]	miR-19b expression in individuals developing PTSS or PTWP/ recover (Fold Diff)[a]	p value[b]
PTSS	5099 / 3579 (1.42)	0.039	2787 / 3906 (-1.40)	0.044
PTWP	4915 / 3396 (1.45)	0.113	2648 / 3832 (-1.45)	0.026

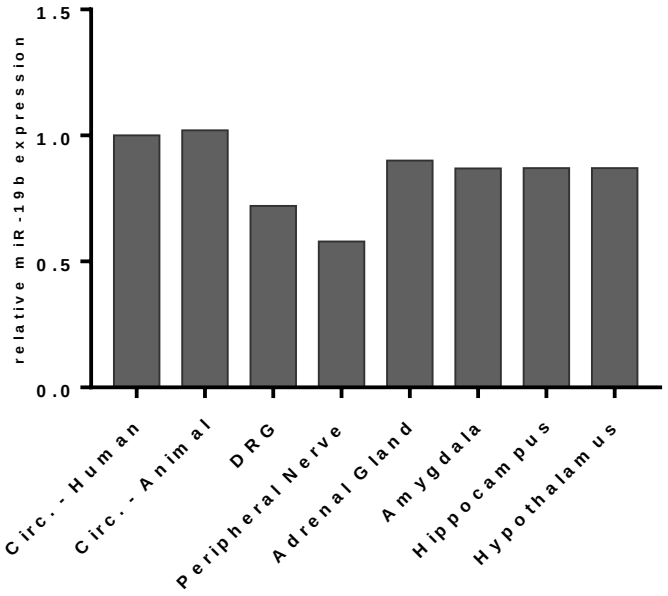
[a]Fold difference was determined by dividing the mean sequencing reads in individuals who developed PTSS or PTWP by the mean sequencing reads in individuals who recovered. [b]p values were determined using Student's T-tests.

Supplementary Table 5. Logistic regression models examining the relationship between Emergency Department levels of miR-19a expression and Posttraumatic Stress Symptoms (PTSS) or Posttraumatic Widespread Pain (PTWP) six months after Motor Vehicle Collision (n = 152).

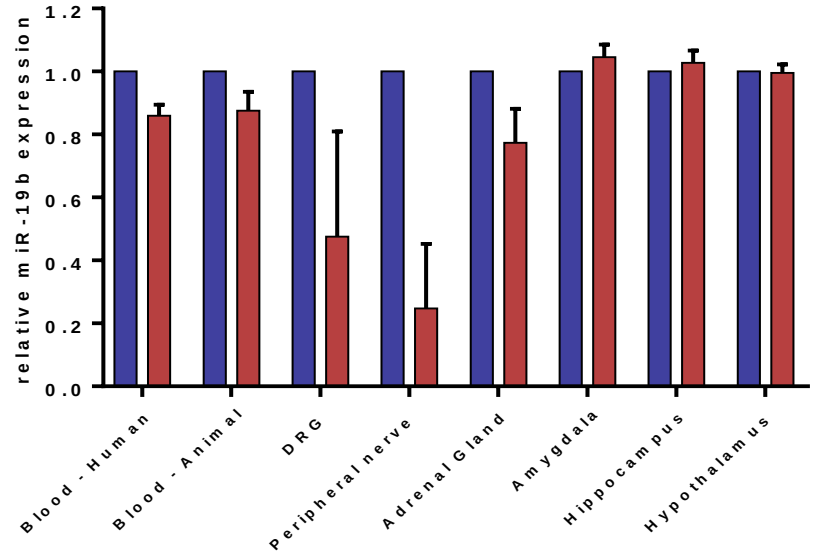
	PTSS		PTWP	
Variable[a]	OR (95% CI)[b]	p value	OR (95% CI)[b]	p value
miR-19a	0.84 (0.34, 2.09)	0.704	0.53 (0.19, 1.49)	0.228
x stress	0.99 (0.95, 1.02)	0.418	1.00 (0.97, 1.04)	0.831
x sex	2.65 (1.11, 6.32)	**0.028**	2.43 (1.05, 5.65)	**0.039**
Stress	1.07 (1.01, 1.13)	0.016	1.05 (0.99, 1.11)	0.066
Sex	0.82 (0.23, 2.95)	0.762	0.42 (0.12, 1.52)	0.187
Age	1.01 (0.97, 1.05)	0.615	1.03 (0.99, 1.07)	0.094

[a]Site was also included in the model as a categorical variable. [b]OR = odds ratio, CI = confidence interval.

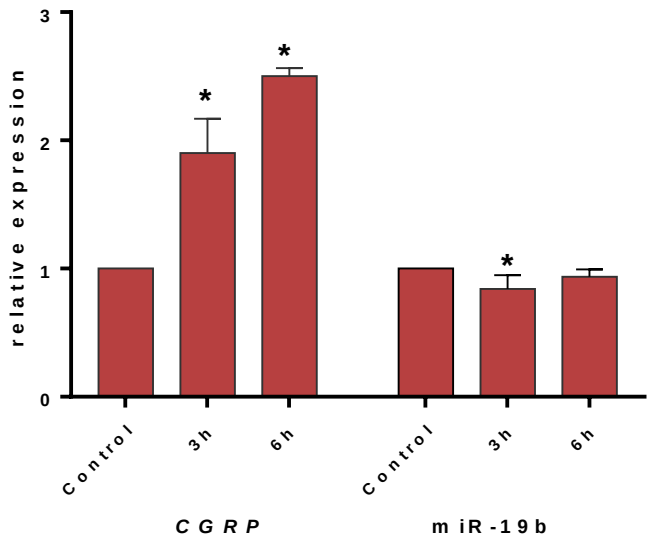
a



b



Supplementary Figure 2. Relative expression of miR-19b in blood and tissue samples relevant to pain/stress/PTS. a) Blood expression levels ("Circ") were measured in male and female human (n = 26) and animal (n ≥ 12) samples; tissue expression levels were measured in rats only. Expression levels of miR-19b were assayed using RT-qPCR and expressed as normalized average cycle threshold values subtracted from the total number of cycles. Therefore, the lowest levels of expression are in DRG and peripheral nerve tissues. b) Relative expression of miR-19b in male (blue bars) and female (red bars) human and animal tissues. Results are the average of technical duplicates and standard errors represent error across all humans and animals assayed. DRG = dorsal root ganglion.



Supplementary Figure 3. Effect of 17β-estradiol stimulation of primary dorsal root ganglia neurons on miR-19b expression levels. *CGRP* mRNA expression levels were measured as a positive control. miR-19b expression levels decreased 3 hours following stimulation with 17β-estradiol while *CGRP* mRNA expression levels increased. Expression levels of each RNA were measured using RT-qPCR; miR-19b expression changes were also validated using a microarray. Error bars represent the average of biological duplicates performed in technical duplicate. Statistical significance (* $p < 0.05$) represents significant difference in expression relative to baseline ("control", no estradiol added).

Supplementary Table 6. Gene Ontology* relationships of miR-19b predicted targets (from pain and posttraumatic stress symptoms gene lists)

Gene Ontology	# Genes	Fold Enrichment	P value	List of genes
GO:0048511 rhythmic process	8	9.867	$1.5x10^{-5}$	NPAS2, EGR3, EREG, GRIN2A, PER1, CHRNB2, CLOCK, OPN4
GO:0050896 response to stimulus	43	1.938	$2.9x10^{-6}$	PPARA, IL1R1, ERBB4, IL6ST, NPY2R, DICER1, GJA1, KIT, EDNRB, PLCL1, NPAS2, TNFRSF1B, KRAS, CNR1, IL1RAP, VPS4B, PER1, STK39, PRKACB, PRKAA2, PLCB1, GHR, SGK1, PIK3CB, MAP2K3, NF1, ESR1, GRIN2A, IGF1, CNGA3, HDAC4, MAPK1, GRM4, HIF1A, ADRB1, GNAQ, EREG, MAPK14, CHRNB2, MAPK8, CACNA1C, CLOCK, OPN4
GO:0032502 developmental process	37	1.856	$7.4x10^{-5}$	ZFAND5, PPARA, GDA, ERBB4, DICER1, ANO1, GJA1, KIT, RORA, EPHB3, EDNRB, NPAS2, KRAS, EGF, RUNX3, GHR, KLF7, EGR3, PIK3CB, YY1, NF1, SCN2A, EFNB2, FMR1, GRIN2A, IGF1, HDAC4, MAPK1, HIF1A, ADRB1, EREG, GNAQ, MAPK14, CHRNB2, MAPK8, SCN8A, CDH11
GO:0051179 localization	35	1.846	$1.5x10^{-4}$	ZFAND5, PPARA, SCN3A, GABRB2, GLRA3, ANO1, NR3C2, GJA1, ATP6V1B2, KIT, KCNK10, EDNRB, KRAS, ANO3, VPS4B, GHR, TRPM3, SGK1, SLC6A11, SCN2A, FMR1, WNK1, GRIN2A, CNGA3, KCNK2, MAPK1, GRM4, HIF1A, SLC17A6, KCNJ6, SLC6A6, CHRNB2, SCN8A, CLCN6, CACNA1C
GO:0032501 multicellular organismal process	48	1.771	$5.8x10^{-6}$	ZFAND5, PPARA, GDA, ERBB4, GABRB2, GLRA3, DICER1, ANO1, NR3C2, GJA1, RORA, KIT, EPHB3, EDNRB, NPAS2, KRAS, CSF2RB, PLCB1, EGF, RUNX3, GHR, KLF7, EGR3, PIK3CB, YY1, MAP2K3, NF1, SCN2A, EFNB2, FMR1, GRIN2A, IGF1, CNGA3, HDAC4, MAPK1, GRM4, ADRB1, HIF1A, SLC17A6, GNAQ, EREG, MAPK14, CHRNB2, MAPK8, SCN8A, CACNA1C, CDH11, OPN4
GO:0065007 biological regulation	67	1.413	$2.1x10^{-5}$	PPARA, FOSL2, IL6ST, DICER1, GJA1, RORA, KCNK10, EDNRB, IL1RAP, PIK3CA, CSF2RB, PRKACB, PLCB1, GHR, EGR3, PIK3CB, YY1, MECP2, ESR1, GRIN2A, WNK1, CNGA3, HNRNPU, MCTP1, MAPK1, GRM4, ADRB1, HIF1A, GNAQ, EREG, MAPK8, TNFAIP3, CLOCK, ZFAND6, IL1R1, ERBB4, NPY2R, NR3C2, KIT, EPHB3, PLCL1, NPAS2, TNFRSF1B, KRAS, CNR1, HNRNPD, PER1, ETV1, PRKAA2, EGF, RUNX3, KLF7, MAP2K3, NF1, RAF1, IGF1, HDAC4, SLC17A6, MAPK14, GRK6, RAP1A, CHRNB2, SCN8A, CACNA1C, CLCN6, NFIA, OPN4

*determined using DAVID: https://david.ncifcrf.gov/home.jsp