microRNA -19b acts as a sex-dependent regulatory hub for post traumatic stress symptoms and chronic widespread pain development following trauma exposure

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INTRODUCTION

In humans, chronic widespread pain (CWP) and posttraumatic stress symptoms (PTSS) are frequent sequelae of trauma that occur more commonly in women. One known trigger of CWP and PTSS is motor vehicle collision (MVC).[1,2] Molecular mechanisms mediating the development of CWP and PTSS after MVC remain poorly understood. Using *in silico,* human, animal, and molecular studies, we sought to identify microRNA (miRNA), small regulatory RNA, that may contribute to CWP/PTSS vulnerability.

In preliminary studies, using an un-biased *in silico* approach to define miRNA that preferentially target pain/PTSS pathways, we identified miR-19 as a candidate regulatory hub (Figure 1). Interestingly, previous reports have shown that miR-19b (1) modulates the behavioral response to stress[3] and (2) is regulated by the estrogen receptor alpha[4].

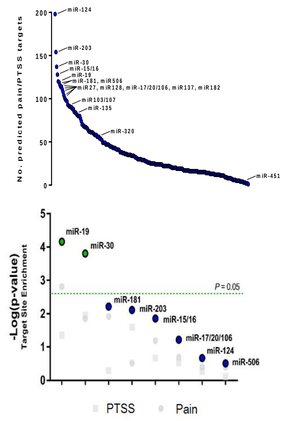
HYPOTHESES

Based on the above *in silico* data, we hypothesized that (1) circulating levels of miR-19b would predict CWP and PTSS following MVC trauma in a sex-dependent manner, (2) Similar sex-dependent expression of miR-19b would be observed in animal models of CWP/PTSS, and (3) miR-19b would regulate transcripts involved in pain/PTSS pathogenesis.

METHODS

IN SILICO: We first used an unbiased approach to identify miRNA that target gene transcripts that play an important role in pain and PTSS processing. Pain genes were identified using three published databases (n = 629 genes)[5,6,7] and PTSS genes were identified using a structured literature search. Candidate miRNAs were determined via predicted binding to the 3'UTR of these genes (TargetScan); Monte Carlo simulations (x10,000) consisting of randomly selected sets of genes were used to generate a background distribution of the number of predicted targets for each miRNA. This distribution was then used to determine miRNA that preferentially target pain/PTSS genes. **HUMAN**: The association between miR-19b expression (identified via small RNA sequencing) and the presence of CWP (ACR definition) and PTSS (Impact of Events Scale-Revised > 33) at 6 months was assessed using logistic regression analysis (n=178). **ANIMAL**: Male and Female Sprague Dawley rats (n>=12) were subjected to either the single prolonged stress protocol described previously[8] or the unpredictable sound stress protocol described previously[9]. Whole blood samples were collected using Qiagen RNAprotect Animal Blood Tubes before stress exposure and 2 weeks following stress; total RNA was isolated using RNeasy Protect Animal Blood Kits. Animals used for tissue isolation were sacrificed via live decapitation without anesthesia, after which tissue samples (Amygdala, Hippocampus) were isolated within 30 minutes. Tissue was homogenized using Bashing Beads (Zymo Research, Irvine, CA). RNA was isolated using DirectZol (Zymo Research). miR-19b was quantified using TaqMan stem-loop RT-qPCR normalized to U87. **MOLECULAR**: DRG cells were incubated in 100mM 17β-Estradiol for 3 or 6 hours. RNA was isolated and miR-19b levels were measured via RT-qPCR. For dual luciferase assays, 3'UTR constructs were made by amplifying 3'UTRs from genomic DNA, and cloning downstream of a Firefly Luciferase gene. Mutations to predicted binding sites within the cloned 3'UTR were introduced via primers using two step PCR. In dual luciferase reporter assays, miR-19b binding to the specified 3'UTRs was quantified by measuring the level of Luciferin protein produced in HEK293T cells 72 hours after transfection with 20fmol pL-SV40-Rluc (transfection control), 20fmol pL-SV40-Fluc-3'UTR, and 10nM miR-19b mimic or control mimic.

FIGURE 1. *In silico* analyses indicate that miR-19 is a strong candidate regulatory hub for both CWP and PTSS.



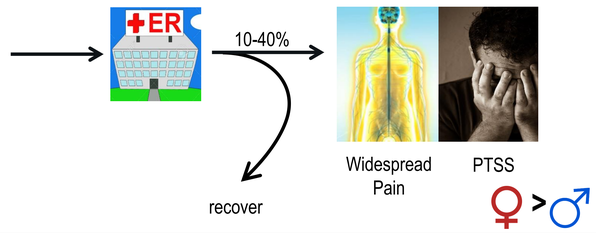


FIGURE 3. Circulating miR-19b expression levels in rats exposed to single-prolonged stress (SPS) are sex-dependent.

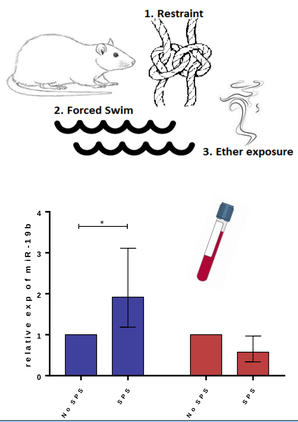


FIGURE 4. Circulating, amygdalar, and hippocampal miR-19b expression levels in rats exposed to unpredictable sound stress (USS) are sex-dependent.

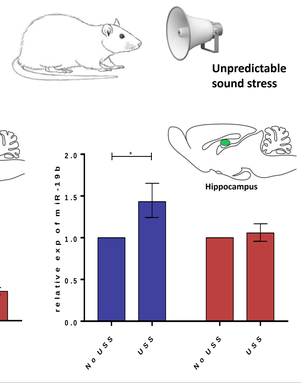


FIGURE 5. miR-19b expression levels decrease in response to 17β-estradiol stimulation in female rat primary neuronal cells

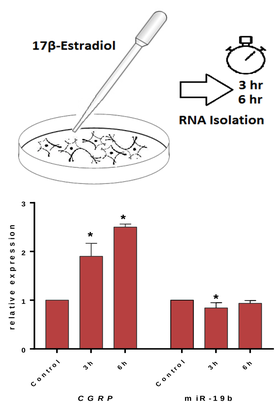
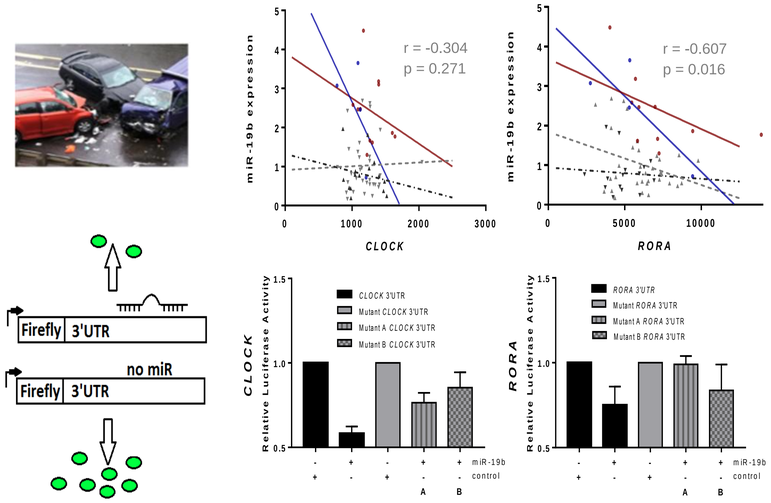


FIGURE 6. Circadian rhythm genes are over-represented in targeting by miR-19b. Two examples of core components of the circadian rhythm targeted by miR-19b are *CLOCK* and *RORA*. These regulatory relationships were determined based on inverse relationships observed between miR-19b and *CLOCK*, *RORA* in MVC participants (top), and by *in vitro* dual luciferase assays measuring direct binding of each 3'UTR by miR-19b (bottom).



RESULTS

IN SILICO: *In silico* analyses indicated that miR-19 is predicted to regulate more genes implicated in pain/PTSS pathogenesis than would expected based on chance (n = 112/629, p < 0.05, Figure 1).

HUMAN: miRNA samples were obtained from study participants enrolled in the Emergency department following MVC (n=178). All individuals were African Americans between the ages of 18-65 (Table 1). Circulating expression levels of miR-19b were associated with both CWP and PTSS in a sex-dependent manner. In males, higher expression of miR-19b was associated with CWP/PTSS. In contrast, in females, lower expression of miR-19b was associated with CWP/PTSS development following MVC (Figure 2).

ANIMAL: miR-19b expression was sex-dependent. Animals exposed to single prolonged stress or unpredictable sound stress showed similar sex-dependent miR-19b expression as observed in humans (Figure 3 and 4). The same pattern was observed for circulating miRNA as well as miRNA in the amygdala and hippocampus (Figure 4).

MOLECULAR: miR-19b expression levels in female primary rat neuronal DRG cultures decreased after 3 hours of incubation with 17β-Estradiol (Figure 5).

Bioinformatics analysis of gene ontology relationships between predicted miR-19b targets indicated an enrichment in the circadian rhythm pathway (data not shown). RNA expression levels of key circadian rhythm genes, *CLOCK* and *RORA,* are inversely correlated with miR-19b expression levels in MVC participants (Figure 6, top). Binding assays indicated that the 3'UTRs of these genes are direct targets of miR-19b (Figure 6, bottom).

CONCLUSIONS

miR-19b expression is associated with the development of CWP and PTSS following MVC trauma. Sex-dependent differences in miR-19b levels suggest that this miRNA may mediate sex differences in CWP/PTSS vulnerability after common traumatic events. Further, the role of miR-19b in CWP/PTSS pathogenesis might be mediated by dysregulation of the circadian rhythm pathway. Additional studies are needed to better understand the role of miR-19b in the development of CWP and PTSS following MVC in women and men.

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