

miR-34a-mediated regulation of XIST in female cells under inflammation

Botros B Shenoda¹
Yuzhen Tian¹
Guillermo M Alexander²
Enrique Aradillas-Lopez^{2,3}
Robert J Schwartzman²
Seena K Ajit¹

¹Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, PA, USA; ²Neurology, Drexel University College of Medicine, Philadelphia, PA, USA; ³Vincera Institute, Philadelphia, PA, USA

Background: Evidence is overwhelming for sex differences in pain, with women representing the majority of the chronic pain patient population. There is a need to explore novel avenues to elucidate this sex bias in the development of chronic inflammatory pain conditions. Complex regional pain syndrome (CRPS) is a chronic neuropathic pain disorder, and the incidence of CRPS is greater in women than in men by ~4:1. Since neurogenic inflammation is a key feature of CRPS, dysregulation of inflammatory responses can be a factor in predisposing women to chronic pain.

Methods: Our studies investigating alterations in circulating microRNAs (miRNAs) in whole blood from female CRPS patients showed significant differential expression of miRNAs between responders and poor responders to ketamine treatment. Several of these miRNAs are predicted to target the long noncoding RNA, X-inactive-specific transcript (XIST). XIST mediates X-chromosome inactivation and is essential for equalizing the expression of X-linked genes between females and males. Based on the well-established role in inflammatory process, we focused on miR-34a, one of the miRNAs predicted to target *XIST*, and downregulated in CRPS patients responding poorly to ketamine.

Results: Our in vitro and in vivo models of acute inflammation and data from patients with CRPS showed that miR-34a can regulate *XIST* under inflammation directly, and through pro-inflammatory transcription factor Yin-Yang 1 (YY1). *XIST* was significantly upregulated in a subset of CRPS patients responding poorly to ketamine.

Conclusion: Since dysregulation of XIST can result in genes escaping inactivation or reactivation in female cells, further investigations on the role of XIST in the predominance of chronic inflammatory and pain disorders in women is warranted.

Keywords: long noncoding RNA, sex difference, miR-34a, inflammation, pain, XIST

Introduction

Chronic pain impacts millions of individuals and is extremely difficult to treat. Pain sensitivity differs between the sexes, and the majority of patients with chronic pain are women. Epidemiological and laboratory data provide overwhelming evidence for sex differences in pain, with women reporting more severe and more frequent bouts of pain that is more anatomically diffuse and longer-lasting compared with pain in men.^{1,2} This observation holds true even after sex-specific disorders are excluded from the analyses. However, the underlying basis for the sex bias observed in pain remains unclear. A number of factors ranging from experiential and sociocultural differences in pain between men and women, genetics, and hormone-driven sex differences in brain neurochemistry have been proposed to explain the predominance of chronic pain in women.¹ Examples of highly prevalent chronic pain syndromes occurring

Correspondence: Seena K Ajit
Pharmacology and Physiology, Drexel University College of Medicine, 245 North 15th Street, Mail Stop 488, Philadelphia, PA 19102, USA
Tel +1 215 762 2218
Fax +1 215 762 2299
Email sk52@drexel.edu

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predominantly in women include complex regional pain syndrome (CRPS), fibromyalgia, chronic fatigue syndrome, etc.

CRPS is characterized by intense pain, inflammation, altered autonomic function, abnormal motor function, and trophic changes, with an estimated 50,000 new cases in the USA each year.³ CRPS is a multifactorial disorder that cannot be explained by a single mechanism.⁴ The multitude and the complexity of the pathophysiological processes associated with CRPS are thought to be responsible for the heterogeneity of the clinical presentation and may underlie the difficulty in establishing evidence-based treatment regimens.⁵ Current therapies are limited in their effectiveness, and none are curative, leaving more than 80% of chronic CRPS patients severely disabled. The incidence of CRPS is greater in women than in men by ~4:1.⁶ We have previously identified differential expression of circulating miRNAs in whole blood from CRPS patients compared to control and between female CRPS patients with good and poor analgesic response to ketamine therapy.^{7,8} Studying the target genes of these miRNAs, and their role in regulating pain and inflammation can provide important insights on the functional significance underlying aberrant levels of circulating miRNAs. We extended our studies to understand if there is a common target for more than one miRNA, ie, to determine if a group of the altered miRNAs can target a common RNA molecule. In our bioinformatics analyses for the targets of the miRNAs differentially expressed in responders and poor responders prior to ketamine treatment using target prediction algorithms, we observed that many of these miRNAs are predicted to bind to X-inactive-specific transcript (XIST), a long noncoding RNA (lncRNA) involved in X chromosome inactivation. The inactivation of about a thousand genes on one of the two essentially identical X chromosomes in females is one of the well-known epigenetic gene silencing mechanism. However, not all the genes on the inactive X chromosome (Xi) are silenced. In humans, ~15% of X-linked genes escape from X chromosome inactivation (XCI).⁹ Thus, escape from XCI is not an absolute with either full or no expression from the Xi.¹⁰ In both humans and mice, there is variability in the extent of gene expression from Xi. There are also differences between X chromosomes and between tissues as to which genes are expressed. Therefore, escape from XCI will not only result in differences in expression between males and females, but also between tissues in the same individual (mosaic expression) and between females. This could have important implications for disease predispositions between men and women, or among women.¹⁰

Though several miRNAs are predicted to regulate XIST, we focused on miR-34a based on its role in regulating inflammatory response.^{11,12} miR-34a had an 11-fold down-regulation in poor responders relative to responders prior to treatment.¹³ In our investigations of whether miR-34a can directly regulate XIST expression, we also studied if miR-34a can indirectly regulate the expression of XIST by targeting Yin-Yang 1 (YY1). YY1 is a transcription factor with diverse and complex biological functions¹⁴ and a validated miR-34a target gene.¹⁵ As its name “Yin-Yang” implies, YY1 either activates or represses gene transcription, depending on the stimuli received by the cells and its association with other cellular factors. YY1 regulates gene expression by directly binding to its target promoters and recruiting histone and DNA modifiers.^{14,16} YY1 is known to positively regulate XIST expression,¹⁷ but its role in regulating XIST under inflammatory states is unknown. We show that miR-34a can regulate XIST under inflammation directly and through proinflammatory transcription factor YY1.

Patients and methods

CRPS and control subjects

The study was approved by the Drexel University College of Medicine Institutional Review Board. All enrolled subjects gave written informed consent, and all patients met the clinical Budapest criteria for CRPS. For the control group, there were 14 females and 7 males. In the CRPS patient group there were 52 female patients including 19 CRPS female patients who received intravenous ketamine.^{7,8} Healthy control subjects were recruited from the general public. The demographics of control and CRPS patients are shown in Table S1. The exclusion criteria for all subjects included pregnancy, recent infection, lupus erythematosus, HIV/AIDS, rheumatoid arthritis, recent extracorporeal circulation (hemodialysis, bypass surgery, plasmapheresis), bone marrow transplant, immunosuppressive therapy, blood disorders (anemia, leukemia), thymectomy, or sarcoidosis. Eligibility for ketamine treatment, treatment protocol, and response assessment were discussed in detail before.⁷ Briefly, ketamine was infused for five and a half days in the intensive care unit of Hahnemann University Hospital (Philadelphia, PA, USA). The infusion rate began at 10 mg/h and was increased by 10 mg/h every 2 h until a maximum rate of 40 mg/h was reached. This rate was continued for 120 h and was then tapered off by 10 mg/h every 2 h. Patients were asked to rate their overall pain prior to and after the infusion using a 10-point Numerical Rating Scale and McGill Pain Questionnaire. Blood samples were

collected prior to and after ketamine infusion. As reported previously, patients were grouped as responders if following treatment their average pain score decreased by at least 50% and poor responders if their average pain score either increased or decreased <50%.⁷

Complete Freund's adjuvant (CFA)-induced inflammatory pain model

All studies were performed in accordance with the guidelines of the National Institutes of Health. The care and use of all mice were approved by the Institutional Animal Care & Use Committee of Drexel University College of Medicine. All behavioral tests were performed using 8-week-old C57BL/6 female mice purchased from Jackson labs (Bar Harbor, ME, USA). Mice were housed in 12 h light/dark cycles in a standard temperature and humidity-controlled room and had access to food and water ad libitum. Mice were habituated in the testing room 2–3 days before experiments. Baseline measurements were obtained before initiation of treatment. Twenty microliters of 50% CFA (Sigma-Aldrich, St. Louis, MO, USA) was administered by intraplantar injection into the right hind paw. Mechanical sensitivity was measured using a series of von Frey filaments (North Coast Medical). The smallest monofilament that evoked paw withdrawal responses on 3 of 5 trials was taken as the mechanical threshold.

Cell culture

HEK293 cells (American Type Culture Consortium) were maintained in complete media (Dulbecco's Modified Eagle's Medium (DMEM)) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in 5% CO₂. J774A.1 cells (female mouse macrophages, ATCC) were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. SH-SY5Y cells, (ATCC) were maintained in a 1:1 mixture of F-12K Medium (Kaighn's Modification of Ham's F-12 Medium) (Corning Inc., New York, NY, USA) and DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

RNA isolation from human and mouse blood

Human whole blood was collected in PAXgene blood RNA tubes (BD Biosciences, Franklin Lakes, NJ, USA). RNA was isolated using a PAXgene blood miRNA kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Mouse blood was collected in 2 mL RNAlater tubes (Invitrogen, Carlsbad, CA, USA). RNA isolation from whole blood was performed using the mouse RiboPure-blood RNA kit

(Ambion Inc., Foster city, CA, USA). RNA concentration was measured using Nanodrop 1000 (NanoDrop Technologies, Wilmington, DE, USA).

Quantitative real-time PCR (qPCR)

cDNA synthesis was performed from 100 to 500 ng total RNA using Maxima first strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). All primer probes were purchased from Applied Biosystems (Foster city, CA, USA). Genes tested included XIST (Hs01079824_m1), Xist (Mm01232884_m1), YY1 (Hs00998747_m1), and Yy1 (Mm00456392_m1). GAPDH/Gapdh (4326317E/4352339E) was used for normalization.

qPCR for miRNA

cDNA synthesis was performed using 40 ng total RNA and qPCR for miR-34a was performed using TaqMan microRNA assay (Assay ID 002316, Applied Biosystems) as recommended by the vendor. U6 snRNA was used for normalization (Assay ID 001973, Applied Biosystems).

Luciferase reporter assay

Human XIST cDNA clone (Origene, Rockville, MD, USA) was subcloned using the Sac I and Xho I restriction sites into the pmirGLO vector (Promega, Madison, WI, USA). HEK293 cells were transfected with XIST pmirGLO plasmid and precursor miRNA clone miR-34a (HmiR0005, GeneCopoeia) or control miRNA using Lipofectamine 2000 (Invitrogen) at 1:1 ratio according to the manufacturer's instructions for a final concentration of 100 ng DNA per well using 96 well plates. Firefly and Renilla luciferase activity were measured 24 h after transfection according to the manufacturer's protocol (Promega).

Statistical analysis

All experiments were analyzed using Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Statistical differences were assessed using a two-tailed unpaired Student's *t*-test, one-way analysis of variance, and Mann–Whitney *U* test. *P*-values of <0.05, <0.01, <0.001 indicated the significant difference between relevant groups. Data are represented as mean ± standard error of the mean.

Results

Decrease in miR-34a is associated with increase in XIST

miR-34a was significantly downregulated in the blood of CRPS patients responding poorly to ketamine compared

to responders. Based on the bioinformatics prediction that miR-34a can bind *XIST*, we performed a qPCR to determine if there is an inverse correlation in expression between *XIST* and miR-34a. We first validated the qPCR for *XIST* using RNA isolated from blood samples from control male and female individuals. Figure 1A shows the relative expression of *XIST* in blood from control male (n=6) and female (n=10) donors confirming *XIST* expression in females and not males. We then performed qPCR using CRPS patient samples grouped as responders and poor responders to ketamine. Figure 1B shows that there is an inverse correlation between expression of miR-34a and *XIST* in both groups, suggesting that a decrease in miR-34a could lead to an upregulation of *XIST*. We then investigated *XIST* expression using RNA samples from all female CRPS and control individuals (CRPS patients n=40, control females n=10) we had obtained from our previous study.⁸ Figure 1C shows that though there were some patients with increased levels of *XIST*, this was not statistically significant relative to female control group. CRPS

patient population is heterogeneous and our data suggests that increased in *XIST* levels may be restricted to a subset of patients who may respond poorly to ketamine therapy.

miR-34a can bind to the lncRNA *XIST*

In addition to binding to 3' untranslated region of mRNAs, miRNA can bind to lncRNAs, and lncRNAs can compete with mRNAs for miRNA binding. Potential binding of miRNAs to *XIST* was determined using the mirSVR method.¹⁸ Experimental validation was done using a reporter assay system designed to evaluate miRNA activity quantitatively by insertion of the sequence containing predicted miRNA binding sites downstream of the reporter gene. There are at least three miR-34a binding sites in this fragment derived from NR_001564.1 and their positions are 1055, 2045, and 2417. There are additional binding sites predicted along the whole length of *XIST*.¹⁹ We cloned 5 kb fragment from 5' *XIST* downstream of the firefly luciferase gene. Relative luminescence was determined after transfecting HEK293 cells with *XIST*

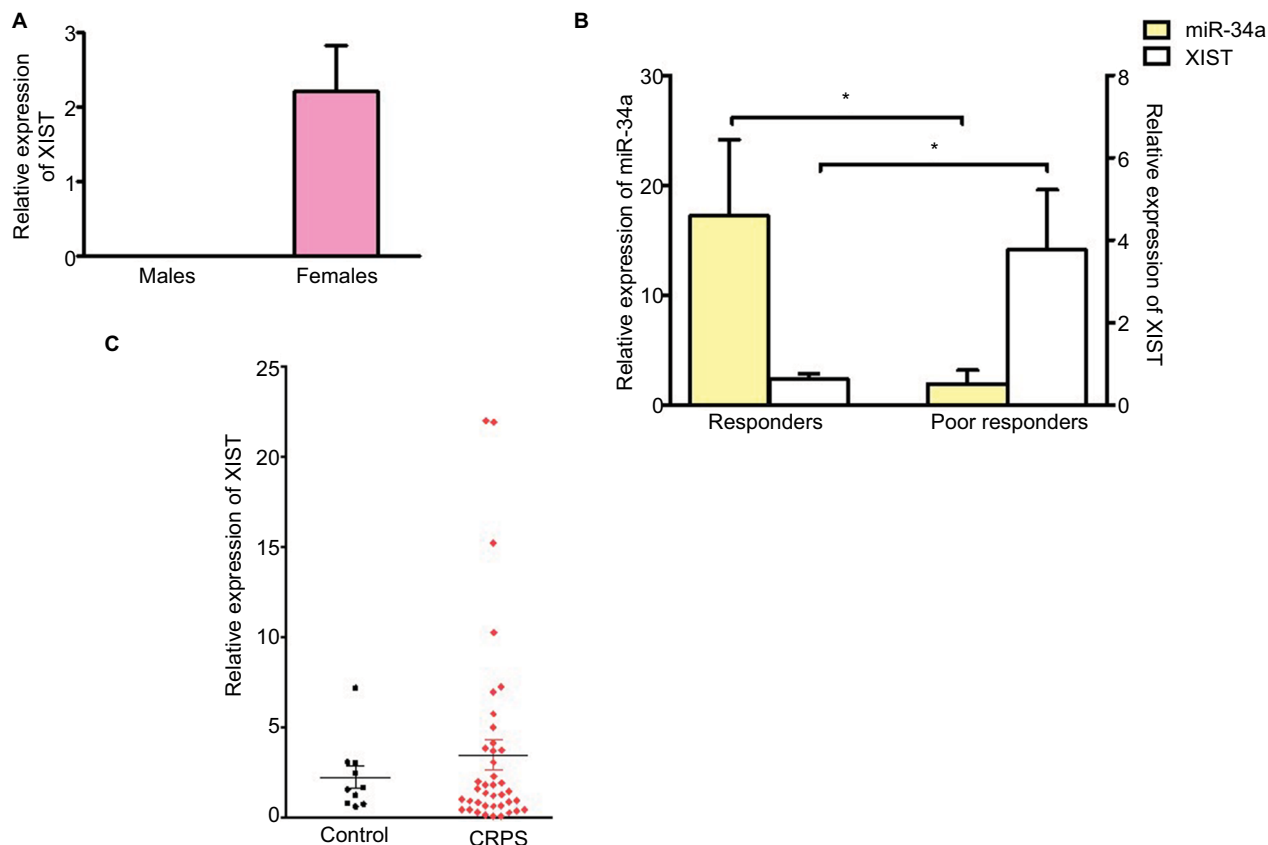


Figure 1 *XIST* is upregulated in a subset of CRPS patients and inversely correlated with miR-34a.

Notes: (A) Relative expression of *XIST* in blood from control male (n=6) and female (n=10) donors. (B) Relative expression of miR-34a and *XIST* in CRPS patients receiving ketamine therapy. Responders n=7, poor responders n=6. (C) Relative expression of *XIST* in CRPS patients (n=40), control females (n=10) and CRPS patients receiving ketamine therapy, responders n=7, poor responders n=6. Expression levels of *XIST* normalized to *GAPDH*. miR-34a expression normalized to *U6*. One-way ANOVA and Student's *t*-test were used for statistical analysis. **p*<0.05, data represent mean \pm SEM.

Abbreviations: ANOVA, analysis of variance; CRPS, complex regional pain syndrome; SEM, standard error of the mean; *XIST*, X-inactive-specific transcript.

pmirGLO plasmid and precursor miR-34a clone or control miRNA. There was a significant decrease in luciferase activity after miR-34a transfection but not the control miRNA, thereby confirming the binding of miR-34a to XIST (Figure 2A).

miR-34a can downregulate endogenous XIST

To confirm miR-34a can modulate endogenous XIST levels, this miRNA was overexpressed in female cell lines and qPCR for XIST was performed 24–48 h after transfection. We used female mouse J774A.1 cells and human SH-SY5Y cells for miR-34a transfection and used mouse- or human-specific primer probe, respectively, for qPCR. Figure 2B shows that overexpression of miR-34a can decrease *Xist* in J774A.1 cells, and Figure 2C shows downregulation of XIST in SH-SY5Y cells. These experiments provided *in vitro* confirmation of the inverse correlation of the expression of miR-34a and *XIST*.

Acute inflammation induces a decrease in miR-34a and an increase in *Xist* expression

To determine if inflammation can alter the expression of miR-34a, J774A.1 cells were stimulated with lipopolysaccharide

(LPS) (1 $\mu\text{g}/\text{mL}$ for 4 h). LPS treatment induced a decrease in miR-34a and an increase in *Xist* in J774A.1 cells, confirming the reciprocal relationship between miR-34a and *Xist* under acute inflammation (Figure 3).

Xist is upregulated and miR-34a is downregulated in mouse model of acute inflammation

To further investigate how inflammation can modulate *Xist* expression *in vivo*, we used the CFA model of acute inflammatory pain. CFA model was established in 8-week old female C57BL/6 mice by intraplantar injection of 20 μL of 50% CFA into the right hind paw. Behavioral assays assessing tactile allodynia were performed before and after CFA administration, to confirm increased sensitivity (Figure 4A). Blood samples were collected for RNA isolation and qPCR analysis at two time points representing acute and chronic inflammation. *Xist* RNA was upregulated in whole blood from CFA treated mice compared to saline injected control mice at 4 h but not after 14 days post CFA (Figure 4B). miR-34a was also downregulated in the acute inflammatory phase of the CFA model compared to blood samples from saline-treated mice. There was no significant difference miR-34a expression 14 days after CFA administration (Figure 4C).

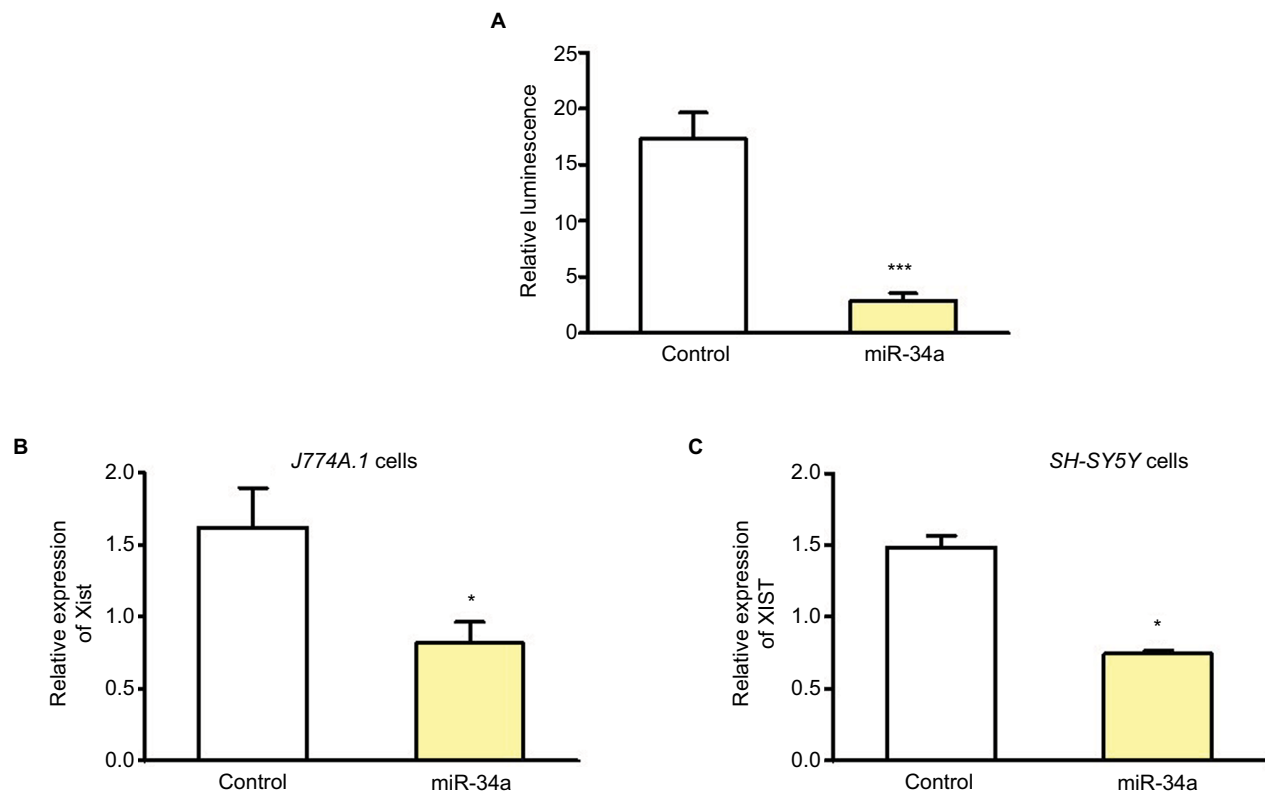


Figure 2 miR-34a can bind and regulate XIST.

Notes: (A) Relative luminescence after transfecting HEK293 cells with XIST pmirGLO plasmid with 5 kb fragment from 5'XIST cloned downstream of the firefly luciferase gene, and precursor miR-34a or control miRNA (n=5). (B) and (C) Relative expression of *Xist/XIST* in mouse J774A.1 and human SH-SY5Y cells, respectively, after miR-34a transfection. Expression of *Xist/XIST* normalized to *Gapdh/GAPDH*. Student's *t*-test used for statistical analysis * $p < 0.05$, *** $p < 0.001$. Data represent mean \pm SEM.

Abbreviations: SEM, standard error of the mean; XIST, X-inactive-specific transcript.

miR-34a can contribute to the upregulation of *XIST* indirectly by regulating the expression of *YY1*

YY1, a transcriptional activator of *XIST*,¹⁷ is a validated target of miR-34a. Hence, we investigated *YY1* expression in blood

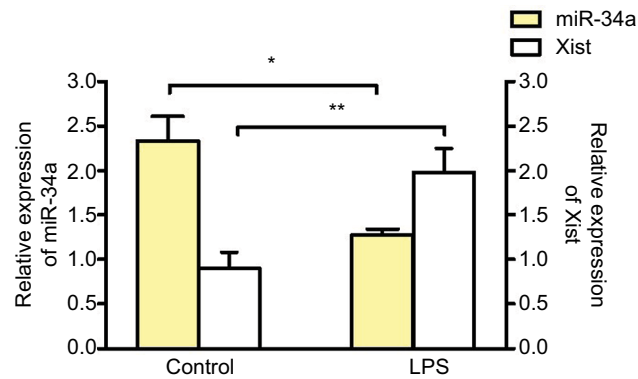


Figure 3 miR-34a regulates *Xist* under inflammation.

Notes: Relative expression of *Xist* and miR-34a in J774A.1 (mouse macrophage cell line) after LPS treatment (1 μ g/mL for 4 h) ($n \geq 3$). Expression of *Xist* normalized to *Gapdh*. miR-34a expression normalized to *U6* ($n \geq 3$). One-way ANOVA and Student's *t*-test were used for statistical analysis * $p < 0.05$, ** $p < 0.01$. Data represent mean \pm SEM.

Abbreviations: ANOVA, analysis of variance; LPS, lipopolysaccharide; SEM, standard error of the mean; *Xist*, X-inactive-specific transcript.

samples from CFA model. Figure 5A shows that similar to *Xist* levels, *YY1* was upregulated in the acute phase of inflammation in the CFA model (Figure 5A). We then investigated expression of *YY1* in CRPS patients, and there was significant upregulation of *YY1* in patients grouped as poor responders to ketamine compared to responders (Figure 5B), indicating *YY1* expression paralleled *XIST*. Thus, downregulation of miR-34a can play a dual role in *XIST* increase by regulating *XIST* directly, and indirectly by modulating *YY1*.

Discussion

Differences in miRNA signature in CRPS patients provided molecular insights distinguishing responders from poor responders to ketamine. Investigating genes targeted by these differentially regulated miRNAs can be beneficial in obtaining insight into previously unexplored mediators in CRPS. We wanted to investigate if an individual transcript can be targeted by multiple circulating miRNAs altered in CRPS. Based on bioinformatics prediction, we hypothesized that circulating miRNAs downregulated in pretreatment samples of CRPS patients responding poorly to ketamine therapy can target *XIST* resulting in upregulation of *XIST*

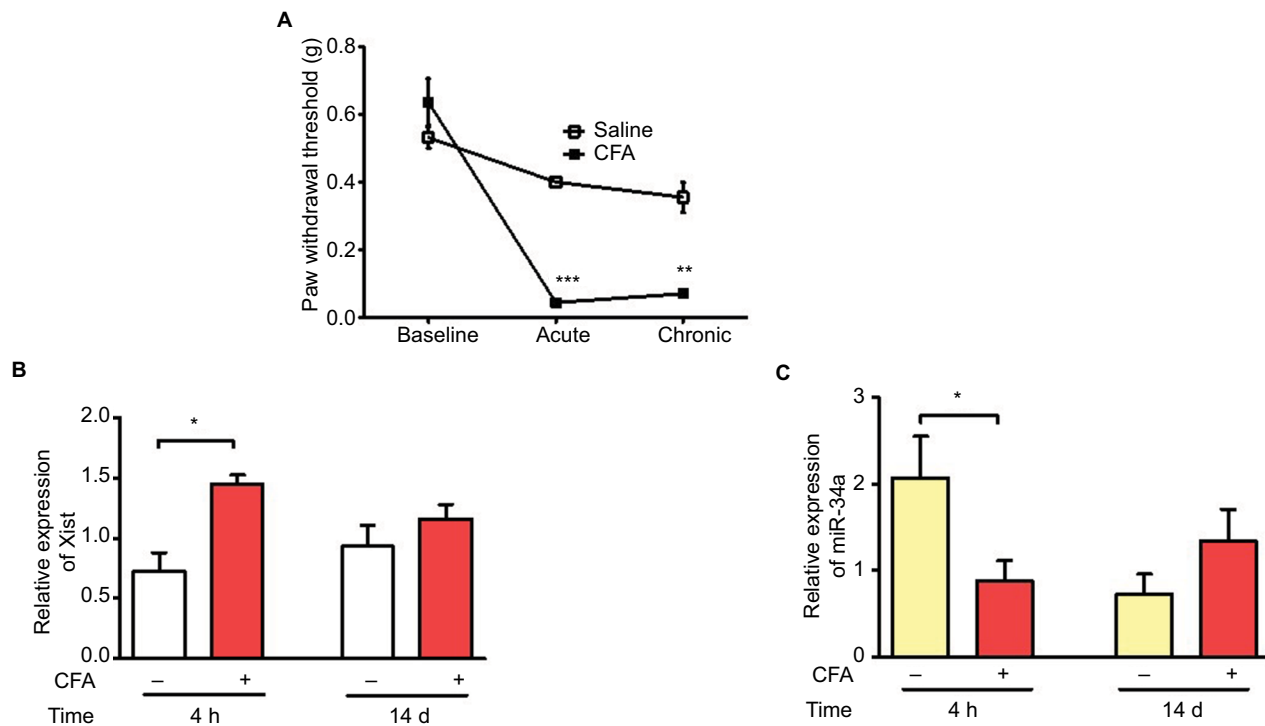


Figure 4 Upregulation of *Xist* and downregulation of miR-34a in blood samples from CFA model of inflammatory pain.

Notes: (A) CFA-induced mechanical hypersensitivity assessed using von Frey filaments in female C57BL/6 mice ($n = 3-4$). Paw withdrawal threshold dropped significantly by 4 h (acute inflammatory phase) and remained low at 14 days (chronic inflammatory phase). (B) Relative expression of *Xist* in female C57BL/6 mice injected with CFA or saline into the hind paw ($n = 3-4$). Blood samples collected at 4 h and 14 days were used to determine the expression of *Xist* during acute and chronic inflammatory phases respectively. (C) Relative expression of miR-34a in blood under acute and chronic inflammation in female C57BL/6 mice injected with CFA or saline ($n = 3-4$). One-way ANOVA and Student's *t*-test were used for statistical analysis except for mice mechanical hypersensitivity in which Mann-Whitney *U* test was used * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, data represent mean \pm SEM.

Abbreviations: ANOVA, analysis of variance; CFA, complete Freund's adjuvant; SEM, standard error of the mean; *Xist*, X-inactive-specific transcript.

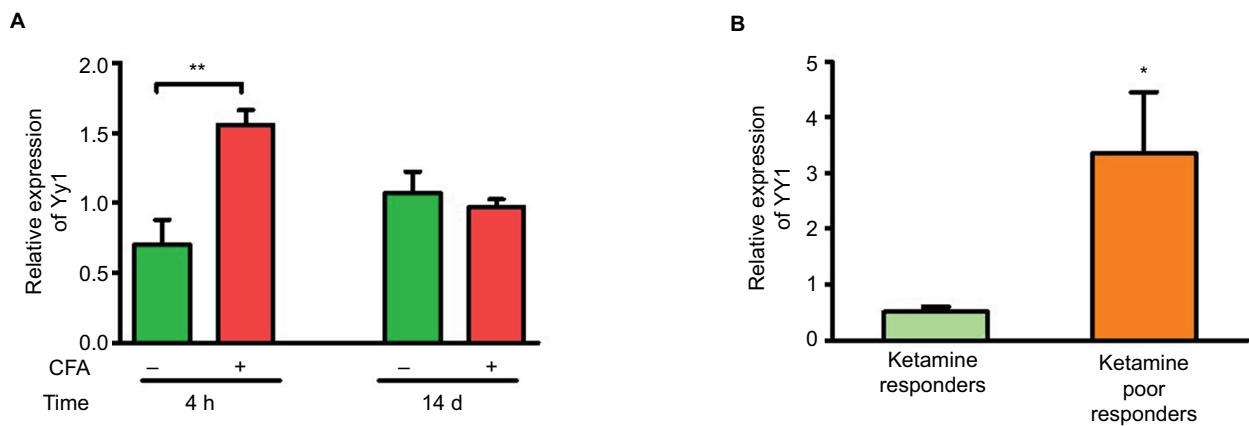


Figure 5 Upregulation of *Yy1* parallels *Xist*.

Notes: (A) Relative expression of *Yy1* in female C57BL/6 mice injected with CFA or saline (n=3-4). (B) Relative expression of *YY1* in CRPS patients receiving ketamine therapy, responders n=7, poor responders n=6. Expression of *Yy1/YY1* normalized to *Gapdh/GAPDH*. One-way ANOVA and Student's *t*-test were used for statistical analysis **p*<0.05, ***p*<0.01. Data represent mean \pm SEM.

Abbreviations: ANOVA, analysis of variance; CFA, complete Freund's adjuvant; CRPS, complex regional pain syndrome; SEM, standard error of the mean; *Xist*, X-inactive-specific transcript; *YY1*, Yin-Yang 1.

in poor responders. *XIST* is a 17-kb spliced, polyadenylated transcript that is retained in the nucleus coating the X chromosome in *cis*, rendering it inactive. Since CRPS is a female-predominant disorder, we sought to investigate if *XIST*, a lncRNA expressed only in female cells, has a role beyond X-chromosome inactivation, and whether differential expression of miRNAs in female CRPS patients can contribute to differences in target gene expression between responders and poor responders to ketamine.

It has been shown that miRNAs and lncRNAs have a regulatory role over each other.²⁰ We pursued target validation studies focusing on miR-34a. As previously reported, CRPS patients responding poorly to ketamine showed a downregulation of miR-34a,⁷ and is involved in regulating inflammatory and immune functions.^{11,21} Our previous studies have shown a role for miR-34a in the negative regulation of CRHR1 and CRH-mediated pro-opiomelanocortin expression.¹³ To further elucidate the different ways miR-34a could be influencing molecular underpinnings observed in CRPS, we investigated miR-34a-mediated regulation of *XIST*. We confirmed that miR-34a can bind and regulate the expression of *XIST* in both human and mouse female cell lines. We observed an upregulation of *XIST* in blood samples from poor responders indicating inverse correlation in expression of miRNA and its target gene. Based on these observations, we conclude that miR-34a is a potential regulator of *XIST* in a subset of CRPS patients. Additional studies in larger patient cohort are needed to further elucidate the biomarker utility of *XIST* expression as a predictor for ketamine response.

Our studies in J774A.1 female mouse macrophage cell line showed an upregulation of *Xist* and a corresponding decrease in miR-34a upon LPS stimulation. miR-34a is

known to be involved in many inflammatory and immune functions. miR-34a level was reduced in LPS-treated RAW 264.7 macrophages, and transfection of miR-34a mimics diminished proinflammatory responses, evidenced by lower levels of M1 cytokines TNF and IL-6. Mechanistically, miR-34a targeted Notch1, which is needed for LPS-mediated production of proinflammatory cytokines in macrophages.¹¹ A previous study reporting the binding of *XIST* with miR-34a suggested that *XIST* can act as a sponge for miR-34a allowing the upregulation of other miR-34a targets such as the transcription factor E2F3.²² Our studies in CFA model also showed a decrease in miR-34a and an upregulation of *Xist* under acute inflammation. Collectively, these data indicate that miR-34a dysregulation can inversely affect *XIST* expression under acute inflammation in vitro and in mouse model, and under chronic inflammatory pain in a subset of CRPS patients resistant to ketamine.

Several factors regulate *Xist* expression during initiation of XCI.²³ It has been shown that *YY1* upregulates *XIST* expression¹⁷ and miR-34a negatively regulate *YY1*.²⁴ *YY1* plays an important role in analgesia and inflammation. *YY1* has a role in the initiation of transcription of the mu opioid receptor gene²⁵ and COX-2²⁶ in lymphocytes. Transcriptional regulation of miRNA genes expressed during monocytic differentiation is mediated by *YY1*.²⁷ In addition, *YY1* plays a crucial role in the B-cell development and immune function.²⁸ *YY1* binds to the promoter regions of several cytokine genes in T-cells.²⁹ Studies also showed that *YY1* mutant mice displayed reduced inflammatory pain sensitivity on the formalin test, increased sensitivity to systemic and spinal (but not supraspinal) morphine analgesia, and greatly increased endogenous (swim stress-induced)

opioid analgesia.³⁰ YY1 mRNA expression and protein was upregulated in rheumatoid arthritis patients and in mice with collagen-induced arthritis. Inhibition of YY1 reduced inflammation in the collagen-induced arthritis model.³¹ This recent study also showed that YY1 can positively regulate IL-6 transcription by binding to the promoter region of the IL-6 gene. It has been shown that NF- κ B regulates the expression of YY1,³² which in turn can regulate the transcription of XIST.¹⁷ In CRPS, there was significant upregulation of YY1 in patients grouped as poor responders to ketamine compared to responders. Thus, downregulation of miR-34a can play a dual role in XIST increase by regulating XIST directly, and indirectly by modulating YY1. XIST is predominantly a nuclear lncRNA and mature miRNAs are usually present in the cytoplasm. It is known that reciprocal regulations exist between nuclear lncRNA and miRNA.^{33,34} This reciprocal interaction underscores the rich and complex regulation of gene expression mediated by ncRNAs.²⁰ Though miR-34a can regulate XIST directly, YY1 can be an important factor in the signaling cascade. Although the well-studied function of XIST is related to its fundamental role in XCI, some reports suggest that it can have other functions unrelated to the silencing of the second X chromosome. XIST expression is higher in several tumors, including ovarian cancer,³⁵ non-small-cell lung cancer,³⁶ glioblastoma,³⁷ breast cancer,³⁸ and hepatocellular carcinoma,³⁹ indicating that XIST might act as a potential diagnostic biomarker for these cancers. Gene expression profiling of rheumatoid arthritis synovial cells showed a 40-fold overexpression of XIST compared to control.⁴⁰ This was reversed by treatment with prednisolone, suggesting a role for XIST in rheumatoid arthritis, a disease predominantly affecting women.

Our observation raises the question as to why the upregulation of Xist in CFA model was limited to 4 h but not at 14 days post CFA administration. In a separate study, we investigated the molecular mechanisms underlying Xist upregulation in mouse macrophage cell line. Our unpublished data show that under acute inflammation XIST can have a protective anti-inflammatory effect. These findings indicate that the role of XIST differ under acute and chronic inflammation. Based on the protective anti-inflammatory role for XIST under acute inflammation, we postulate that a greater incidence of acute inflammation in men could be due to the absence of XIST in male cells. However, in chronic painful disorders, XIST may have a proinflammatory role. A recent report showed upregulation of Xist in spinal cord from rat chronic constriction injury model of neuropathic pain,

and silencing Xist alleviated both mechanical and thermal hyperalgesia.⁴¹

X chromosome is known to contain the largest number of immune-related genes in the whole human genome and a higher density of miRNAs compared to autosomes and the Y-chromosome.⁴²⁻⁴⁴ This highlights the importance of tight control and complex regulation of gene expression from the X chromosome. Any aberration in X-linked gene inactivation⁹ from either abnormal escape or increased inactivation, mediated by lncRNAs or epigenetic modifications, can result in aberrant immune and inflammatory response. We postulate that epigenetic changes including histone modifications and DNA methylation in female cells and miRNA alterations may predispose immune cells to heightened chronic inflammatory response. Dysregulation of *XIST* can result in genes escaping inactivation or reactivation in female cells.⁴⁵ We postulate that under chronic inflammatory pain disorders, aberrant expression of XIST could affect the sites it coats on the inactive X chromosome. This in turn can influence the silencing or inactivation of genes encoded by the X chromosome. Mechanistic studies on how the expression of proinflammatory genes and transcription factors can be influenced by changes in XIST levels and localization under acute and chronic inflammation are needed to validate this. Understanding the tissue- or cell-specific aberrations and temporal regulation is crucial for further understanding the role of XIST in acute inflammation and chronic pain. We propose a dual and opposing role for *XIST* in acute and chronic inflammatory and pain disorders predominant in women that warrants further investigations. Though it is early to assume that miR-34a-induced XIST regulation would directly help in treating or preventing pain, our studies link lncRNA XIST to ketamine treatment resistance in a female-predominant chronic inflammatory pain disorder CRPS. XIST is essential for dosage compensation in female cells, and thus manipulating XIST directly or downregulating it completely will not be a feasible therapeutic intervention strategy. However, higher levels of XIST as we observed in a subset of CRPS patients, in addition to being a potential biomarker, can be modulated by altering miRNAs such as miR-34a. miRNAs are fine tuners of gene regulation and can induce nuanced knockdown of its target mRNAs. Downregulation of Xist by siRNAs was recently shown to decrease pain hypersensitivity⁴¹ in mouse model of neuropathic pain. miR-34a is a tumor suppressor and currently in Phase 1 clinical trials for cancer.⁴⁶ Thus, miRNA-mediated downregulation of XIST could be a potential avenue for pain relief.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table SI The demographics of control and CRPS patients

Controls	Sex	Age (years)	Weight (lb)	Height (cm)	BMI
C1	F	42	120	63	21.25
C2	F	37	134	67	20.99
C3	F	40	240	67	37.59
C4	F	24	113	62	20.67
C5	F	34	124	66	20.01
C6	F	39	157	67	24.59
C7	F	29	132	62	24.14
C8	F	47	140	65.5	22.94
C9	F	59	117	60	22.85
C10	F	41	110	65	18.30
C11	M	68	220	76	26.78
C12	M	63	190	71	26.50
C13	M	56	235	78	27.15
C14	M	43	165	70	23.67
C15	M	49	186	75	23.25
C16	M	23	165	67	25.84

Patients	Ketamine response	Age (years)	Weight (lb)	Height (cm)	BMI	
P1	NA	F	40	128	65	21.30
P2	NA	F	65	164	67	25.68
P3	NA	F	47	233	65	38.77
P4	NA	F	63	102	58	21.32
P5	NA	F	33	142	62	25.97
P6	NA	F	31	170	62	31.09
P7	NA	F	35	230	66	37.12
P8	NA	F	41	155	67	24.27
P9	NA	F	32	165	64	28.32
P10	NA	F	54	212	71	29.56
P11	NA	F	27	128	62	23.41
P12	NA	F	51	120	62	21.95
P13	NA	F	58	272	64	46.68
P14	NA	F	60	165	69	24.36
P15	NA	F	38	147	60	28.71
P16	NA	F	48	140	65	23.29
P17	NA	F	49	175	66	28.24
P18	NA	F	28	102	61	19.27
P19	NA	F	44	99	64	16.99
P20	NA	F	23	163	61	30.80
P21	NA	F	38	160	64	27.46

(Continued)

Table SI (Continued)

Patients	Ketamine response	Age (years)	Weight (lb)	Height (cm)	BMI	
P22	NA	F	68	154	63	27.28
P23	NA	F	58	191		
P24	NA	F	43	145	68	22.04
P25	NA	F	57	155	63	27.45
P26	NA	F	33	166	68	25.24
P27	NA	F	45	160	61	30.23
P28	Responder	F	51	200	64	34.33
P29	Responder	F	49	298	67	46.67
P30	Responder	F	51	176	68.5	26.37
P31	Responder	F	39	194	67	30.38
P32	Responder	F	43	260	63	46.05
P33	Responder	F	65	165	61	31.17
P34	Responder	F	49	173	63	30.64
P35	Poor responder	F	43	175	63	31.00
P36	Poor responder	F	69	152	61	28.72
P37	Poor responder	F	35	150	66	24.21
P38	Poor responder	F	45	160	61	30.23
P39	Poor responder	F	56	145	63	25.68
P40	Poor responder	F	33	166	68	25.24

Abbreviation: CRPS, complex regional pain syndrome.

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