FKBP5 variant rs3800373 alters FKBP5 RNA secondary structure and prevents stress-induced microRNA-320a downregulation of FKBP5, resulting in glucocorticoid resistance and increased vulnerability to chronic posttraumatic pain

Abstract

Background: Chronic pain affects more Americans than cancer, heart disease, and diabetes combined and has a reported economic impact of more than 600 billion dollars annually. Despite these alarming statistics, the pathophysiology of chronic pain is not well understood, thus precluding the development of improved or novel therapeutics. In an effort to understand the underlying pathogenesis of chronic pain development, our group recently showed that genetic variant rs3800373 in the 3'UTR of FKBP5, an important regulator of the stress response, is associated with increased chronic pain severity following trauma exposure. In the current study, we aimed to validate this finding in an additional post-trauma cohort, and to understand the mechanism by which this allele affects chronic pain states.

Methods: To validate our previous genetic association results showing increased severity of chronic pain in individuals with the minor allele at rs3800373, we used an additional prospective cohort of individuals experiencing trauma. This cohort included African American (AA, n = 930) men and women presenting to the Emergency Department (ED) following motor vehicle collision (MVC) trauma. RNA and DNA (via blood) were collected in the ED and chronic pain severity was evaluated six weeks following MVC using established questionnaires. Because rs3800373 is located in the 3'UTR of FKBP5 near a microRNA-320a binding site, we used molecular and in silico assays to test whether this allele affects binding by miR-320a, a microRNA shown to be associated with fibromyalgia, a related pain disorder.

Results: In general linear models the influence of FKBP5 rs3800373 on chronic pain severity after MVC in AA depended on the level of peritraumatic distress. Individuals who reported higher levels of peritraumatic distress and had one or more copies of the minor allele experienced more severe pain six weeks following MVC than individuals with the major allele. Analysis of microRNA expression levels from individuals in the same study showed that in distressed individuals, lower expression levels of miR-320a predict increased chronic pain six weeks following MVC. Molecular studies showed that miR-320a binds to the FKBP5 3'UTR and that in the presence of the risk allele binding is less efficient. Further, this difference in miRNA binding was shown via in silico analyses to be caused by allele dependent changes in the secondary structure conformation of the 3'UTR.

Conclusion: These results add further evidence to the role for FKBP5 in chronic pain development following trauma exposure and suggest that stress dependent regulation of FKBP5 by microRNA-320a leads to variability in post-traumatic chronic pain development.
**Introduction**

Chronic pain affects more Americans than cancer, heart disease, and diabetes combined and has a reported economic impact of more than 600 billion dollars annually.\(^1\) One of the most common causes of chronic pain is exposure to traumatic/stressful events.\(^2,3\) However, little is known regarding the pathogenesis of post-traumatic chronic pain, precluding the development of novel therapeutics for secondary intervention.

One type of traumatic event causing persistent pain development is exposure to motor vehicle collision (MVC). In the US, more than 10 million motor vehicle collisions (MVC) occur each year. A substantial proportion of individuals experiencing MVC report to Emergency Departments (EDs) for care, however more than 90% of the individuals do not have life threatening physical injury and are discharged to home after ED evaluation.\(^4\) Persistent post-MVC musculoskeletal pain (MSP) develops in 10-40% of individuals who experience such “minor” MVCs. Current evidence indicates that altered stress system signaling (e.g. glucocorticoid and catecholaminergic pathway signaling) plays an important role in the pathogenesis of post-MVC chronic pain.\(^5-10\)

Data supporting the role for stress system biology in post-MVC chronic MSP includes (1) a substantial proportion of individuals developing chronic MSP also develop neuropsychiatric diseases previously shown to be caused by trauma/stress, (2) peritraumatic distress at the time of injury is a strong predictor of chronic MSP development following MVC, (3) stress exposure in animals results in increased hyperalgesia compared to non-stressed animals, and (4) a number of genetic association studies demonstrate that polymorphisms in key glucocorticoid and catecholaminergic system genes predict post-MVC chronic MSP development.\(^7,8,10,11\)

Genetic association analyses are a powerful method of assessing the potential role that biological pathways play in post-MVC chronic MSP pathogenesis. Our group recently showed that polymorphisms in the glucocorticoid receptor co-chaperone, *FKBP5*, strongly predicts chronic MSP development following MVC.\(^12\) The main polymorphism that tagged the risk haplotype for chronic MSP following MVC, rs3800373, has previously been shown to be associated with co-morbid neuropsychiatric disorders such as depression and PTSD. Despite this wealth of data implicating rs3800373 in adverse post-traumatic sequelae, little is known regarding molecular mechanisms through which this polymorphism affects the pathogenesis of chronic MSP and related disorders. However, such studies could provide insight into the molecular pathogenesis of chronic MSP and thereby identify novel targets for therapeutic intervention.

For my thesis studies, I assessed whether (1) there are allele-dependent effects of rs3800373 on glucocorticoid resistance, an established mechanism through which *FKBP5* can affect neuropsychiatric outcomes, (2) rs3800373, which is located in the 3’UTR of *FKBP5*, affects regulation by miR-320a, and (3) the ability of miR-320a to bind *FKBP5* is affected by allelic effects on RNA secondary structure. This work builds upon previous work I contributed towards in the Linnstaedt lab showing that miR-320a predicts chronic MSP development following MVC\(^9\), and also involves a collaboration with Alain Laederach’s group in the Biology Department at UNC.

**Methods**

**Study design, setting, and eligibility criteria**

A prospective longitudinal study enrolling African American individuals ≥ 18 and ≤ 65 years of age presenting to the ED within 24 hours of MVC was used to study chronic post-traumatic pain outcomes. The details of this study have been described previously.\(^13\) The study was approved by the institutional review boards at the data coordinating center (The University of North Carolina at Chapel Hill) and at all participating hospitals. Each study participant provided written informed consent before enrollment.

Eligible and consenting participants provided blood samples in the ED and completed an ED interview evaluation. Interview evaluations were performed by research assistants at the time of the ED visit using a web-based survey with explicit definitions of variables. Injury characteristics and medications administered in the ED were obtained by data extraction from the ED medical record. Six weeks after the MVC, participants completed a follow-up interview by telephone, online, or via mail.
Participant demographic characteristics (including age, gender, income, height, weight, and educational attainment) were obtained from the ED medical record and from participant self-report.

**DNA collection and genotyping**

Study personnel collected blood samples from each participant at the time of enrollment using PAXgene DNA tubes. Following DNA purification (PAXgene blood DNA kit, QIAGEN), genotyping using the Infinium Multi-Ethnic Global Array (Illumina) was performed. DNA from an individual with known genotype (NA19819, 1000 genomes) and two repeat samples were included in each genotyping batch (96 samples) to ensure genotypic accuracy and reliability. SNP rs3800373 was in Hardy-Weinberg equilibrium ($p > 0.05$) and had an excellent call rate (>99%).

**RNA collection and sequencing**

For RNA sample collection, research assistants collected blood samples 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 stored at -80°C until use. RNA concentration and purity were measured using a NanoDrop 1000 (Nanodrop Technologies, Wilmington, DE).

Small RNA sequencing: Template libraries for miRNA Next Generation Sequencing were produced from 1.0 ug total RNA using two similar protocols. An initial set of 69 samples (randomly chosen from 930 participant cohort) were prepped using an adaptation of published protocols as described previously.$^{14,15}$ A second set of 89 samples (also randomly chosen) 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$^{15}$, and were normalized using upper quartile normalization. In order to normalize potential technical biases between the two methods of library preparation, the sequencing reads from the six samples that were prepped using both methods were averaged and the difference in averages between the two methods was subtracted from the values in the second set of samples.

Total RNA (excluding miRNA) sequencing: 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 UNC High Throughput Sequencing Facility.

**Plasma collection and measurement of cortisol levels**

For plasma collection, research assistants collected blood samples in the ED at the time of enrollment using EDTA tubes. (Blood samples collected in the early morning between 5am and noon were not considered for analysis in order to avoid potential confounding by early morning spikes in cortisol levels.) To separate plasma, EDTA blood tubes were centrifuged within one hour of collection at 1,500xg for 15 minutes and plasma was frozen at -20°C until use.

Cortisol was measured using the MILLIPLEX MAP Steroid / Thyroid Hormone Magnetic Bead Panel - Endocrine Multiplex Assay (STTHMAG-21K, Millipore, Darmstadt, Germany). This assay was designed to measure cortisol, estradiol, and progesterone and have <10% cross-reactivity; samples were prepped according to the manufacturer’s specifications and assayed on a Bio-Plex 200 system (BioRad, Hercules, CA) using version 6.0 Bio-Plex Manager software. Data were exported to Excel after manual outlier removal to achieve optimal 5-Parameter Logistic fit probabilities of the standard curves.

**RT-qPCR and measurement of FKBP5 mRNA expression levels**

RT-qPCR was performed using 40 ng Total RNA from the same subset of individuals used to assess plasma cortisol levels. Random primers were used for reverse transcription as described (High Capacity Reverse Transcription Kit, Life Technologies). For qPCR, TaqMan reagents were used with transcript specific primers as instructed (Life Technologies) using the TATA-box binding protein (TBP)
gene as an endogenous control. Intron spanning primers were used for both \textit{FKBP5} (Assay ID: Hs01561006\_m1) and \textit{TBP} (Assay ID: Hs00427620\_m1).

\textit{Characterization of rs3800373}

The miRdSNP online database (http://mirdsnپ.ocr.buffalo.edu/) and TargetScan\textsuperscript{16} were used to assess whether rs3800373 directly interferes with miRNA binding and to determine miRNAs predicted to bind 300nt upstream or downstream of the SNP.

\textbf{DNA constructs}

Lentiviral constructs containing either a firefly luciferase gene (pL-SV40-GL3) or a renilla luciferase gene (pL-SV40-RLUC) were used for dual luciferase assays.\textsuperscript{17} All primers used for the creation of the following constructs are detailed in Table 2. The 3'UTR of \textit{FKBP5} was amplified from HEK293T cells using primers 1-F and 1-R. The resulting 2125bp product was cloned downstream of the firefly luciferase gene in pL-SV40-GL3 using Xhol and EcoRI restriction enzyme sites. This newly created construct, pL-GL3-FKBP5-maj, was sequenced to confirm the major allele, T, at position rs3800373 in the \textit{FKBP5} 3'UTR, and was then used as template to mutate the major allele to the minor allele, G, using the QuickChange II Site-Directed Mutagenesis Kit (Promega) and primers 2-F and 2-R. This new construct, pL-GL3-FKBP5-min, was then sequenced to confirm the presence of the minor allele and absence of introduced mutations.

A shortened version of the \textit{FKBP5} 3'UTR, "\textit{FKBP5}-truncated", was amplified from the full length 3'UTR using primers 3-F and 3-R. This shortened version is 588bp and contains only the genomic region of the 3'UTR used to predict structural changes using RNAsnp. Mutations in three nucleotide positions that were predicted to disrupt complementarity between miR-320a and the \textit{FKBP5} 3'UTR seed binding region were engineered into \textit{FKBP5}-truncated using primers 4-F and 4-R, to create a new construct termed "\textit{miR}-320a mut".

The miRNA coding regions for \textit{miR}-99a, \textit{miR}-122, and \textit{miR}-320a were amplified from HET293T cells using primers 5-F/R, 6-F/R, and 7-F/R respectively. The resulting products, approximately 400bp in length containing the the full pre-miRNA sequence, were cloned into a pcDNA backbone using EcoRI and Xhol restriction sites. The pLCE-s320a sponge construct and associated controls, pLCE and pLCE sCXCR4 were generously donated from the Cullen Lab (Duke University).

\textbf{Cell culture}

HEK293T cells were grown at 37°C, 5% CO\textsubscript{2}, in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% gentamicin. Human neuroblastoma SK-N-AS cells were cultured in DMEM with 10% FBS, 0.1 mM Non-essential Amino Acids (NEAA), and 1% gentamicin. Other neuroblastoma cell lines used to test \textit{miR}-320a and \textit{FKBP5} expression levels (SK-N-SH, BE-2C, SK-N-MC, SH-SY5Y, and IMR-32) were grown as suggested by ATCC guidelines.

\textbf{Dual luciferase reporter assays}

Binding of \textit{miR}-320a to \textit{FKBP5} 3'UTR constructs described above was assessed using FLUC and RLUC-based indicator vectors, pL-SV40-GL3 and pL-SV40-RLUC (also described above). HEK293T cells were co-transfected with 120fmol pC-320, 5fmol pL-SV40-RLUC, and 30fmol of either pL-GL3-FKBP5-maj or pL-GL3-FKBP5-min using Fugene 6 Transfection Reagent (Promega). Seventy-two hours after transfection cells were collected and lysed. RLUC and FLUC levels were measured on a SpectraMax microplate reader (Molecular Devices) using substrates from a dual luciferase reporter assay kit as described (Promega); FLUC values were normalized to RLUC values and relative luciferase activity calculated between compared samples.

\textbf{Generation of neuroblastoma cells stably knocked down for \textit{miR}-320a expression}

To knock down \textit{miR}-320a expression in SK-N-AS cells, we stably transduced the cells with pLCE-s320, a \textit{miR}-320 sponge construct. Such “sponge” constructs have been previously described.\textsuperscript{18} Viral transductants for pLCE-s320 were produced in HEK293T cells using a third generation lentiviral packaging system\textsuperscript{19} consisting of pMD2-VSVG, pRSV-REV, pMDLgp, and either pLCE-s330 (‘s320’),
pLCE (GFP control), or PLCE-sCXCR4 (‘sCXCR4’, a control sponge). Each sponge construct contained the GFP gene. HEK293T cells were transfected using Fugene 6 Reagent (Promega). 48 hours after transfection, viral media was collected, filtered, and concentrated using Amicon Ultra centrifugal columns, then added to the culture medium atop SK-N-AS cells, and integration of sponge sequence was monitored by GFP expression. Cells with the top 30% mean GFP fluorescence were sorted using FACS analysis (ARIA II) and grown in culture until sufficient numbers of cells were available for RNA isolation via TRIzol (Life Technologies) or for protein isolation via RIPA buffer (Pierce).

**RT-qPCR and Western blotting**

For analysis of FKBP5 mRNA expression, RNA was first treated with DNasel (New England Biolabs) to remove genomic DNA. Random primers were used for reverse transcription as described (High Capacity Reverse Transcription Kit, Life Technologies) and SYBR reagents were used for qPCR with transcript specific primers as instructed (Life Technologies). Primer sequences for detecting FKBP5 mRNA, 8-F and 8-R are shown in Table 2. Endogenous control primers were designed against GAPDH, 9-F and 9-R; their sequences are also shown in Table 2.

To compare FKBP5 protein levels, concentrations of protein lysate were first measured with a BCA Protein Assay (Pierce) and equal amounts of protein (5µg per lane) were loaded onto a 4-12% Bis-Tris gel (LifeTechnologies) and analyzed by Western blotting. Primary rabbit anti-FKBP5 was from Santa Cruz Biotechnology (sc-28983), and primary rabbit anti-β-actin was from Cell Signaling Technology (13E5, #4970) both at a 1:1000 dilution. Secondary anti-rabbit IgG HRP was from Cell Signaling Technology (#7074) at a 1:2000 dilution. Signals were developed using ECL Western Blotting Substrate (Pierce) and signal intensities quantified using NIH supported software, ImageJ.

**Analyses**

Correlation analyses were bivariate linear regression models performed using SPSS statistical software (IBM Corporation, Armonk, NY, U.S.A) Pearson correlation coefficients (r) and p values were derived from these analyses. Mann Whitney analyses were used to determine significance of differential microRNA binding to FKBP5 3'UTR between constructs with either major or minor alleles.

**Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE)**

SHAPE data was obtained in vivo using the Yoruban male cell line 19098 (1000 Genomes - Coriell Institute for Medical Research), which is heterozygous for rs3800373. This cell line is lymphoblastoid in nature and grows in suspension. Approximately 50 million cells were spun down and resuspended in 1 mL of folding buffer (100mM Na-HEPES, 8.0 pH, 100mM NaCl and 10mM MgCl₂) and supplemented with 400U murine RNase inhibitor (NEB). Cells were kept on ice and sonicated at 10% power for three 10 second intervals (Fisher Scientific Sonic Dismembrator Model 500). The lysate was incubated at 37C for 10 minutes. SHAPE treatment was carried out over five minutes with an addition of DMSO or three separate additions of 1-methyl-7-nitroisatoic anhydride (1M7) with a final concentration of 30 mM 1M7, 10% DMSO. The DMSO alone provides a background control. RNA was extracted from the lysate and depleted of ribosomal RNA (ThermoFisher Trizol LS, 5PRIME PhaseLock Heavy, Invitrogen Purelink RNA columns, and ThermoFisher Scientific RiboMinus™ Eukaryote System v2 from Life Technologies). SHAPE-MaP has been previously described. Briefly, reverse transcription was performed with SuperScript II under special conditions (Mn²⁺ buffer) to convert adducts into mutations (ThermoFisher Scientific SuperScript II, NEB random nonamers). Primers were designed specific to the 3' region of the mRNA containing rs3800373 (Supplementary Table 1), which was amplified by PCR (NEB Q5 HotStart). Secondary PCR was performed to add TruSeq barcodes. An Illumina MiSeq Sequencing Platform Sequencing was used to sequence the treated and background samples as a paired end, 2x300 multiplex run.
Allele-specific sorting and SHAPE profile calculation
Sequencing reads were aligned to the FKBP5 3′ UTR using bowtie2 (v2.2.9)\textsuperscript{23}. The aligned reads were then split into separate FASTQ files by the allele at the SNP position. For each allele, the SHAPE profile of the amplified region was derived from the allele-specific sorted sequencing reads, using the ShapeMapper pipeline (v1.2)\textsuperscript{22}. The SHAPE reactivity at each position corresponds to the difference between the mutation rate in the treatment condition and the mutation rate in the background control, normalizing the mutation rates by a multiplier so that the distribution of SHAPE reactivities matched that of a reference mRNA.

Results

Participants
Baseline characteristics of participants in the AA MVC study are shown in Table 1. Of 930 participants enrolled, 766 individuals provided DNA, were genotyped, and completed 6-week pain questionnaires. Total RNA was sequenced from blood samples collected from 96 participants, microRNA from 172 participants, and cortisol from plasma of 80 participants. Due to only a subset of participants having plasma collected, not all participants overlapped with sequencing/cortisol data. For each subset of participants, most individuals were women, with an average age of 35 years and had at least high school education (Table 1).

rs3800373 allele affects glucocorticoid resistance
In order to determine whether the risk allele at rs3800373 affects glucocorticoid resistance, correlations between FKBP5 expression and (1) circulating cortisol or (2) NR3C1 mRNA levels were determined (Figure 1). In individuals with two copies of the major allele, there was no correlation between FKBP5 mRNA levels and circulating cortisol levels (Figure 1A), however the correlation was significant for individuals with one or more copies of the minor allele (p=1.2 x 10\textsuperscript{-4}) (Figure 1B). Additionally, the correlation between FKBP5 mRNA levels and NR3C1 mRNA expression levels was not significant for individuals with two copies of the major allele (Figure 1C), but was significant for those with one or more copies of the minor allele (p=8.3 x 10\textsuperscript{-7}) (Figure 1D).

The genomic locus of rs3800373 is located in the 3′ UTR and affects miR-320a binding
The location of rs3800373 in the 3′ UTR of FKBP5 (Figure 2A) made it an ideal candidate as a functional SNP based on the immense regulatory roles established for the 3′ UTR region of RNA transcripts.\textsuperscript{24,25} The seed site for miR-320a is also broadly conserved across mammals (Figure 2B). We used luciferase assays to test binding to the predicted miRNA seed regions (miR-99a, 122, and 320a) (Figure 2C). The presence of the minor allele had a significant effect on miRNA binding only for miR-320a. miR-320a binds the 3′ UTR of FKBP5 containing the minor allele less efficiently (p = 0.025) than it binds the 3′ UTR with the major allele. Figure 2D shows mutational analyses which rule out the possibility that other binding sites apart from the one in the SNP-related binding region of the FKBP5 3′ UTR are responsible for miR-320a binding, indicated by the restored luciferase signal upon mutation of the miR-320a seed site.

miR-320a regulates endogenous expression of FKBP5 in neuroblastoma cells
The SK-N-AS neuroblastoma cell line was transduced with either a control sponge or one specific to miR-320a. Upon isolation of cellular RNA and protein, levels of FKBP5 were assessed. Cells which were treated with the s320 sponge showed higher relative expression levels of FKBP5 mRNA and FKBP5 protein as compared to cells transformed with the control sponge (Figure 2E).

miR-320a expression correlates with FKBP5 expression in participants with the major allele
Bivariate analyses on mRNA and miRNA sequencing data showed a correlation between FKBP5 and miR-320a expression levels in individuals with the major allele (r= -0.475 p=0.026). Individuals with at
lease one copy of the minor allele did not show correlation between these RNAs ($r=0.144$, $p=0.349$). (Figure 2F)

**FKBP5 RNA adopts different secondary structures in the 3’UTR depending on the allele at rs3800373**

Figure 2A showed that rs3800373 is not located in a miRNA seed region. However, luciferase assays showed that the allele at rs3800373 affects miR-320a binding efficiency, suggesting some other mechanism through which the SNP alters miR-320a binding. Preliminary in silico data from RNAsnp predicted allele-dependent RNA secondary structures for the 3’UTR containing the minor allele versus the major allele ($p = 0.0136$, data not shown). This data suggests that rs3800373 might be a riboSNich.26 At the miR-320a binding site, in vivo SHAPE data showed higher SHAPE reactivity in mRNA transcripts containing the major allele versus transcripts with the minor allele (Figure 3A, gold trace). Predicted 3’UTR structures are shown in Figures 3B and 3C.

**Discussion**

The results of this study helped to further solidify that the SNP rs3800373 is predictive of chronic pain development following trauma. The process we predict to be responsible for this is through the development of glucocorticoid resistance in affected individuals. In her review27, Binder summarizes the normal function of the HPA Axis and how FKBP5 fits into the process. Following stress exposure, the HPA axis is responsible for the release of glucocorticoids which have a wide array of systemic effects, including anti-inflammatory action. Circulating cortisol enters cells and binds to the glucocorticoid receptor (GR) which then serves as a transcription factor upon translocation into the nucleus. The result is upregulated transcription of genes important for the body’s response to stress, including those involved in feedback loops responsible for terminating the response. One of the genes involved in feedback is FKBP5, a co-chaperone of the GR that when bound, reduces cortisol binding affinity to GR and the efficiency of the complex’s nuclear translocation. Although FKBP5 production is initially caused by the GR to help terminate the stress response, dysregulation of FKBP5 production results in overly high levels of FKBP5. Increased levels of FKBP5 lead to increases in both circulating cortisol and cellular GR through disruption the negative feedback that typically results from GR-cortisol binding. Resistance to circulating corticoids results could result in a state of chronic inflammation and be a potential source of chronic pain.

Our determination of glucocorticoid resistance relies on the assertion that it is result of increased FKBP5 levels which leads to increased levels of circulating cortisol as well as glucocorticoid receptor. The results in Figure 1 help to show this as a probable cause. Only in patients with the minor allele were the levels of these FKBP5, NR3C1, and cortisol correlated. However, if increased FKBP5 levels are responsible for glucocorticoid resistance, there needs to be a mechanism for stressful encounters to attenuate its expression under normal conditions. This mechanism could be through FKBP5 repression by miR-320a. Since our results showed that the minor allele reduces miR-320a binding efficiency through changes in the mRNA secondary structure, this would account for more uncontrolled increases of FKBP5 protein in individuals with the risk allele. Additionally, we would expect that for individuals with similar levels of miR-320a expression, those with the minor risk allele at rs3800373 would have increased levels of FKBP5. Our results did show that, in fact, as expected in patients with the major allele, there is an expected negative correlation between miR-320a expression and FKBP5 levels. This correlation is lost in individuals with the minor allele, suggesting that miR-320a has lost its ability to regulate FKBP5 expression. This result, in conjunction with, previous publications which show decreased levels of miR-320a as predictive of chronic pain development9 suggest that expression of miR-320a may be stress induced and normally play a role in protection from glucocorticoid resistance. Patients with lower levels of miR-320a or an inability to respond to increasing miR-320a would in turn have higher FKBP5 expression and be at risk for developing chronic pain.

Higher shape reactivity translates to increased flexibility in RNA structure. Multiple previous reports have demonstrated that increased RNA flexibility makes a the structure more accessible to microRNA
binding. Therefore SHAPE data demonstrates that rs3800373 is a ribosnitch and that RNA structures in a representative YRI individual show increased flexibility in the miR-320a seed binding site when the UTR contains a T allele. The increased flexibility would suggest a more accessible seed region for miR-320a binding, represented visually by Figures 3B and 3C. This shows that changes in secondary structure could be the mechanism by which rs3800373 alters miR-320 binding.

As evidence increases for the importance of miRNA in regulating pain development, studies like this will hopefully shed insight on key ways in which miRNA regulation is disrupted and leads to a disease state. The fact that the miR-320a seed site is so broadly conserved across mammals suggests it could play an important evolutionary role. In this study, we showed that SNPs have the ability to alter mRNA secondary structure in such a way that miRNA binding is affected.

There are additional ways in which chronic pain might manifest. Data from rat models has shown that prolonged stress exposure leads to dysregulation of normal stress pathway signaling resulting in nociceptor sensitization and increased hyperalgesia. Sensitization was also achieved through overstimulation of the glucocorticoid receptor through direct, chronic administration of cortisol. This would process would also be consistent with our assessment that adverse outcomes following MVC are a result of the stress associated with the trauma. The question remains then how to connect the two phenomena, glucocorticoid resistance and nociceptor sensitization. It is uncertain if the two processed are mutually exclusive or if they complement each other. It is possible that glucocorticoid resistance could lead to chronically elevated cortisol levels and eventual peripheral nociceptor sensitization and chronic pain development. However, more studies on the specifics of these processes would be necessary.

One limitation to our study is the relatively small sample size for many of our sequencing analyses. Another is that the only tissue type we can work with in human samples is blood. Rat models of pain are widely used, and expanding some of the findings in this study to a rat model could help provide more insight on the sequence of events leading from trauma exposure to chronic pain development.

Further studies could be done to try and confirm that changes in secondary structure are in fact what alters miR-320a binding.

**Conclusion**

This study provided evidence for a potential functional mechanism connecting a genetic association to its eventual outcome. The minor allele at rs3800373 is predictive of pain development following stress exposure. The change in the allele from a T to a G results in a change in secondary structure that could be responsible for the observed decrease in miR-320a binding efficiency to the *FKBP5* 3'UTR. Previous studies which provide evidence that miR-320a expression is stress induced, and by interfering with this protective measure, the minor allele at rs3800373 puts individuals at risk for glucocorticoid resistance and chronic pain development.
Table 1. Baseline characteristics of participants in the AA MVC study

<table>
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<tr>
<th>Characteristic</th>
<th>Genotyping</th>
<th>mRNA</th>
<th>microRNA</th>
<th>Cortisol</th>
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<tr>
<td>Enrolled, n</td>
<td>930</td>
<td>96</td>
<td>172</td>
<td>80</td>
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<tr>
<td>Age, years, mean (SD)</td>
<td>35.1 (12.7)</td>
<td>30.5 (10.3)</td>
<td>34.6 (11.8)</td>
<td>32.3 (10.4)</td>
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<td>Females, n (%)</td>
<td>578 (62.2)</td>
<td>66 (68.8)</td>
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<td>Education, n (%)</td>
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<td>8-11 yrs</td>
<td>71 (7.6)</td>
<td>2 (2.0)</td>
<td>10 (5.8)</td>
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<td>3 (3.1)</td>
<td>5 (2.9)</td>
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<td>Some college</td>
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<td>42 (43.8)</td>
<td>77 (44.8)</td>
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<td>College graduate</td>
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<td>15 (15.6)</td>
<td>25 (14.5)</td>
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<td>Post-graduate studies</td>
<td>36 (3.9)</td>
<td>3 (3.1)</td>
<td>6 (3.5)</td>
<td>3 (3.8)</td>
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<tr>
<td>Overall pain severity, 0-10 NRS, mean (SD)</td>
<td>2.2 (3.3)</td>
<td>1.1 (2.4)</td>
<td>1.6 (2.9)</td>
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<td>Month prior to MVC</td>
<td>7.2 (2.1)</td>
<td>7.1 (2.2)</td>
<td>7.2 (2.1)</td>
<td>7.4 (1.8)</td>
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<td>Week 6</td>
<td>6.0 (2.9)</td>
<td>4.8 (4.1)</td>
<td>5.5 (3.4)</td>
<td>6.2 (2.5)</td>
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<td>Peritraumatic distress in the ED, mean (SD)</td>
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<td>24.3 (11.6)</td>
<td>23.3 (11.5)</td>
<td>23.5 (11.5)</td>
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Table 2. Primers for DNA Constructs and SYBR qPCR analyses

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<tbody>
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<td>1-F</td>
<td>ATGATGCTCGAGGTGAATCGCCCTCTCAATGG</td>
<td>XhoI</td>
<td>To make 2125 bp FKBP5 insert</td>
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<tr>
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<td>EcoRI</td>
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<td>2-F</td>
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<td>Quickchange to create minor allele</td>
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<tr>
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<td>XhoI</td>
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<tr>
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<td>Nael</td>
<td>Mutate miR-320 site to Nael</td>
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<td>CTCCCTAAAATTTCCTCGAATGC</td>
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Figure 1. Associations between circulating levels of FKB5 mRNA and circulating cortisol levels (a, b) and between circulating levels of FKB5 mRNA and circulating NR3C1 mRNA levels (c, d). These associations show high correlation in individuals with at least one copy of the minor allele at rs3800373 (b, d) versus those with no copies of the minor allele (a, c). Increased levels of FKB5 are associated with increased cortisol and glucocorticoid receptor mRNA (NR3C1), in individuals with the minor allele, suggesting development of glucocorticoid resistance. Values for Pearson correlation coefficients (r) and p values were derived using bivariate analyses.
Figure 2. miR-320a (1) binds the FKBP5 3'UTR in an allele dependent manner and (2) alters FKBP5 expression levels in vivo. (a) rs3800373 is located in the 3'UTR of FKBP5. The three nearest predicted microRNA binding sites are for miR-320a, miR-122, and miR-99a. (b) The miR-320a binding site in FKBP5 is broadly conserved across mammals. (c) miR-99a, -122, and -320a directly bind to the FKBP5 3' UTR. Extent of miR-320a binding to the FKBP5 3'UTR is significantly less in the presence of the minor allele versus the major allele (gray bar, control; white bar with dots, major allele; black hashed bar, minor allele) (p = 0.025). There was no significant difference in binding between FKBP5 3'UTR with the major versus the minor allele for miR-99a and miR-122. (d) mutation of the miR-320a seed site eliminates binding of the FKBP5 3'UTR by miR-320a. (e) In neuroblastoma cells knocked down for miR-320a (s320) FKBP5 mRNA and protein expression are increased relative to control cells. (f) miR-320a expression is correlated with FKBP5 expression in African American individuals with the major allele but not with the minor allele at rs3800373.
Figure 3. *In silico* data from RNAsnp show that the predicted FKBP5 3'UTR mRNA secondary structure is different depending on the identity of the allele at rs3800373 (a). The secondary structure with the major allele (left) has more loop regions than the structure with the minor allele (right), suggesting greater accessibility for miRNA binding. The miR-320a binding site is indicated in black (with a red arrow) and SNP rs3800373 is indicated by a blue arrow. *In vivo* SHAPE analysis of the FKBP5 3' UTR in an African-derived (YRI) cell line heterozygous for rs3800373. (b) SHAPE results for RNA containing the major allele (T, gold trace) vs RNA containing the minor allele (G, blue trace). The SNP and predicted binding locations for miR-320a and miR-122 are indicated. (c, d) Predicted minimum free energy structures for FKBP5 3'UTR RNA with the major allele vs the minor allele based on SHAPE data. The predicted RNA secondary structure is different depending on the identity of the allele at rs3800373.