Published in final edited form as:

Eur J Neurosci. 2017 October ; 46(7): 2241–2252. doi:10.1111/ejn.13624.

miR-101a-3p and Ezh2 modulate anxiety-like behavior in high-responder rats

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Abstract

A greater understanding of neural mechanisms that contribute to anxiety is needed in order to develop better therapeutic interventions. The current study interrogates a novel molecular mechanism that shapes anxiety-like behavior, demonstrating that the microRNA miR-101a-3p and its target, enhancer of zeste homolog 2 (*Ezh2*) in the amygdala, contribute to rodent anxiety-like behavior. We utilized rats that were selectively-bred for differences in emotionality and stress reactivity, showing that high novelty responding (HR) rats, which display low trait anxiety, have lower miR-101a-3p levels in the amygdala compared to low novelty responding (LR) rats that characteristically display high trait anxiety. To determine if there is a causal relationship between amygdalar miR-101a-3p and anxiety behavior, we used a viral approach to over-express miR-101a-3p in the amygdala of HR rats and test whether it would increase their typically low levels of anxiety-like behavior. We found that increasing miR-101a-3p in the amygdala increased HRs' anxiety-like behavior in the open field test and elevated plus maze. Viral-mediated miR-101a-3p over-expression also reduced expression of the histone methyltransferase *Ezh2*, which mediates gene silencing via tri-methylation of histone 3 at lysine 27 (H3K27me3). Knockdown of *Ezh2* with short-interfering RNA (siRNA) also increased HRs' anxiety-like behavior, but to a lesser degree than miR-101a-3p over-expression. Overall our data demonstrate that increasing miR-101a-3p expression in the amygdala increases anxiety-like behavior and that this effect is at least partially mediated via repression of *Ezh2*. This work adds to the growing body of evidence implicating miRNAs and epigenetic regulation as molecular mediators of anxiety behavior.

Keywords

microRNA; miR-101a-3p; Ezh2; Polycomb Repressive Complex 2; Amygdala; Anxiety

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Conflicts of Interest: The authors declare no conflicts of interest

INTRODUCTION

Anxiety disorders are characterized by excessive fear and anxiety and are both highly prevalent, with a lifetime risk for developing any anxiety disorder estimated at 28.8% in the U.S., and can cause lifelong impairment in many patients (Kessler et al., 2008). A greater understanding of the molecular mechanisms that contribute to anxiety is needed in order to develop more effective treatments. In order to elucidate genetic and environmental factors that produce an anxious phenotype, we developed a rat model of individual differences in emotionality by selectively breeding Sprague Dawley rats for disparate response to novelty (Stead et al., 2006). Rats bred for high response to novelty (HRs) vigorously explore new environments and exhibit low-levels of trait anxiety compared to rats bred for low response to novelty (LRs), which exhibit high levels of trait anxiety (Clinton et al., 2011; Cohen et al., 2015; Glover et al., 2015).

Previous work from our group suggests that HR/LR differences in anxiety-like behavior rely, in part, on differences in the amygdala (Cohen et al., 2015; Cohen et al., 2016). We recently found that low-anxiety HR rats display reduced c-Fos activation in multiple amygdalar subnuclei following electric-shock stress compared to LRs. We then used high-throughput sequencing of mRNAs and microRNAs (miRNAs) in the HR/LR amygdala to begin to interrogate molecular mechanisms that may drive their disparate behavioral and neural responses to stress (Cohen et al., 2016). miRNAs are short non-coding RNAs that control translation and/or stability of mRNA targets. Each miRNA potentially targets several genes, so co-expression of only a few miRNAs exerts powerful control over large gene networks (Mathonnet et al., 2007; Brodersen and Voinnet, 2009). miRNAs are increasingly recognized for their role in human mental illness (O'Connor et al., 2012; Hauberg et al., 2016). Single nucleotide polymorphisms associated with several miRNAs effect risk for anxiety disorders (Muinos-Gimeno et al., 2011; Hommers et al., 2015) and expression levels of other miRNAs have been shown to correlate with anxiety symptoms (Xu et al., 2016). Rodent studies show that acute and chronic stress effect miRNA expression (Meerson et al., 2010; Rinaldi et al., 2010), and rats that are vulnerable vs. resilient to developing learned helpless behavior display baseline miRNA expression differences (Smalheiser et al., 2011). Additionally, several miRNA knockout mice display alterations in anxiety-like behavior (Andolina et al., 2016; Jin et al., 2016).

Our recent transcriptome study identified miR-101a-3p as one of the most abundant miRNAs in the amygdala and showed that HR rats had significantly lower miR-101a-3p levels in the amygdala relative to LRs (Cohen et al., 2016). A major target miR-101a-3p is the Polycomb Repressive Complex 2 (PRC2), a multi-protein complex including histone methyltransferase enhancer of zeste homolog 2 (Ezh2) (Varambally et al., 2008; Friedman et al., 2009), which mediates gene silencing via tri-methylation of histone 3 at lysine 27 (H3K27me3) (Cao et al., 2002; Marchesi and Bagella, 2013). PRC2 critically regulates gene expression during several developmental processes (Marchesi et al., 2014), including formation of brain circuits (Di Meglio et al., 2013; Neo et al., 2014), although a fairly limited number of studies have examined Ezh2 and H3K27me3 in the adult brain (Qi et al., 2014; Zhang et al., 2014; Koo et al., 2015).

The present study builds on our previous findings by measuring Ezh2 and H3K27me3 levels in the amygdala of HR/LR rats (to test whether HR/LR miR-101a expression differences are associated with predicted changes in these targets); and determining whether manipulating amygdalar miR-101a-3p and Ezh2 expression can alter anxiety-like behavior. Because we found significantly lower miR-101a-3p levels in the HR versus LR amygdala and because miR-101a-3p represses PRC2 signaling, we hypothesized (*a*) that HRs would exhibit elevated Ezh2 and H3K27me3 levels in the amygdala relative to LR; and (*b*) that increasing miR-101a-3p expression in the HR amygdala would increase their anxiety-like behavior through repression of Ezh2 and H3K27me3.

METHODS

All experiments were approved by the local Institutional Animal Care and Use Committee and conducted in accordance with National Institutes of Health guidelines on animal care and experimentation.

Animals

Adult male HR/LR rats were obtained from the 8th generations of our in-house colony (McCoy et al., 2016). Rats were pair-housed in a 12:12 light-dark cycle in a temperature- and humidity-controlled environment with food and water available ad libitum.

Viral Constructs

Adeno-associated viruses (AAVs) engineered to express either pre-miR-101a and GFP or only GFP were created by the University of Alabama at Birmingham, Neuroscience NINDS Protein Interactions Core C. pre-miR-101a was cloned downstream of GFP in the pSLIK series of vectors, MBA253 digested with BfuAI (ATCC) (Shin et al., 2006), and ligated with annealed primers to miR-101a (primers designed based on miRBase Accession Number: MI0000886) with overlapping BfuAI sites (miR-101a-F: 5'-agcgaTGCCCTGGCTCAGTTATCACAGTGCTGATGCTGTCCATTCTAAAGGTACAGTACTGTGATAACTGAAGGATGGCAC and miR-101a-R: 5'-ggcagTGCCATCCTTCAGTTATCACAGTACTGTACCTTTAGAATGGACAGCATCAGCATGTGATAACTGAGCCAGGGCAT). The resulting MBA253-pre-miR-101a clone was amplified by PCR to include the GFP provided by the MBA253 vector [miR-101a-GFP-F: 5'-AAGCTTggatccGCCGCCACCATGGTGAGCAAGGGCGAG; mir101a-GFP-R: gatatcagatctAATTGAAAAAAGTGATTTAATTTATACC]. It was cloned into the BamHI and BglII sites of AAV-MCS (Agilent, Santa Clara, CA, USA) to obtain the AAV-GFP-miR-101a vector; the clones obtained were in reverse orientation due to the compatibility of the BamHI and BglII overhangs. The vector was digested with BamHI and XbaI and the ~1100 bp GFP-miR-101a fragment was subcloned into the BamHI and XbaI sites of AAV-MCS (Agilent) to achieve proper orientation with respect to the promoter. pAAV-GFP vector was obtained from Agilent. All clones were verified by sequencing.

For AAV production, 293A cells (Agilent) were transfected using calcium phosphate transfection (Clontech, Mountain View, CA, USA) with 10 µg each of pAAV-RC, pAAV-Helper, and AAV-GFP-miR-101a or pAAV-GFP. After 72 h, the transfected cells were

harvested, pelleted, resuspended in serum-free DMEM, and subjected to four rounds of freeze/thaw cycles. The AAV-containing supernatants were spun at $10,000 \times g$ for 10 min to pellet cell debris and viral supernatants were stored at -80°C . The AAVs were purified using columns according to the manufacturer's instructions (Cell Biolabs, San Diego, CA, USA), which included final buffer exchange and concentration. AAV-GFP-pre-miR-101a (referred to henceforth as AAV-miR-101a) and AAV-GFP were tittered by infectivity on HT1080 cells (ATCC) (1.5×10^8 AAV-miR-101a and 1.2×10^8 AAV-GFP infectious units/ml) and by RT-PCR (5.3×10^{11} AAV-miR-101a and 5.2×10^{11} AAV-GFP genome copies/ml).

Intracranial Surgeries to Manipulate miR-101a or *Ezh2* Expression

Intracranial surgeries were performed on 71 HR male rats (60–75 days old) as described previously (Nam and Kerman, 2016). One group of rats (N=33) were used in an experiment to overexpress miR-101a via AAV viral infusion in the amygdala; the second group (N=48) was used in an experiment to suppress *Ezh2* expression via short-interfering RNA (siRNA) infusion. Rats were anesthetized with a mixture of oxygen (1 l/min) and isoflurane (5% for induction, and 1–3% for maintenance). Following administration of carprofen (5 mg/kg sc; Pfizer Inc., New York, NY, USA) and buprenorphine (0.05–0.1 mg/kg sc; Reckitt Benckiser Pharmaceuticals, Richmond, VA, USA) for post-surgical analgesia, rats were secured in a stereotaxic frame using blunt ear bars. Skin over the skull was shaved, sterilized, and incised. Bregma and lambda were identified in order to determine stereotaxic coordinates using Angle Two software (Leica Biosystems, Richmond, IL, USA). The coordinates A/P: -2.8 , M/L: ± 4.8 , and D/V: -8.43 were used to target the left and right amygdala, respectively. Both left and right amygdala were injected with either 1 μl AAV-miR-101a (n=18; 5.3×10^{11} genome copies/ml) or AAV-GFP (n=15; 5.2×10^{11} genome copies/ml) for the miR-101a overexpression experiment or 1 μl *Ezh2* siRNA (n=26; 15 μM ; GE Dharmacon, Lafayette, CO, USA) or control siRNA (n=22; 15 μM) over 5-min. After injections the overlying skin was sutured. Rats were returned to their home cages upon full recovery from anesthesia.

Animals in the miR-101a overexpression experiment were given 2-weeks to allow for surgical recovery and adequate viral expression while animals in the *Ezh2* siRNA experiment were given 7-days to recover. After each of these recovery periods, all animals were subjected to two days of behavioral testing.

Behavioral Testing

All behavior was recorded using a computerized analysis program (Ethovision XT 8.0, Noldus, Wageningen, The Netherlands) and all testing was conducted under dim light (30 lux) between 8:00–1:00 pm. Animals were tested first on the Open Field Test (OFT) followed by the Elevated Plus Maze (EPM) 24 h later.

Open Field Test—The OFT was conducted in a 100 $\times$ 100 $\times$ 50 cm black Plexiglas box with a black floor as described (Cohen et al., 2015). At the beginning of the test, a rat was placed in a corner of the box and was permitted to explore the apparatus for 5 min. The latency to enter the center of the OF, the amount of time spent and distance traveled in the center, sides, and corners of the apparatus were quantified. The periphery was defined by a 20 cm zone

around the edge of the OF arena that was further subsided into mutually exclusive side (20×60 cm) and corner (20×20 cm) zones.

Elevated Plus Maze—The EPM test was conducted as described (Cohen et al., 2015) in a black Plexiglas EPM consisting of four elevated arms (70 cm from the floor, 45 cm long, 12 cm wide) arranged in a cross. Two opposite arms are enclosed by 45-cm-high walls (lighting 3–5 lux), and the remaining arms are open (lighting 30 lux). To start the test, rats were placed in a central square platform facing the same closed arm at the intersection of the open and closed arms provided access to all arms. The latency to enter the open arms, the amount of time spent in the open arms, closed arms, and center square, and the total distance traveled over the course of the 5-min test were recorded. An animal was considered to be in the open arm when the rat's body fully crossed out of the center square onto an open arm platform.

Tissue Collection and Molecular Analyses

For HR/LR comparisons, 75-day old male rats were sacrificed ($n=12$ /phenotype) by rapid decapitation, brains were removed, flash frozen, and stored at -80°C until further processing. For the miR-101a overexpression and *Ezh2* siRNA experiments, all animals were sacrificed in the same manner 24 h following the last behavioral test.

RNA Extraction—Amygdala samples were dissected and RNA was extracted using the miRNeasy Mini Kit (Qiagen, Germantown, MD, USA). cDNA was created using the miScript RT Kit II (Qiagen, Germantown, MD, USA) and stored at -20°C until processing for quantitative Real-Time PCR (qRT-PCR).

Protein Extractions—Whole cell protein extractions were performed on amygdala samples in RIPA lysis buffer, quantified by BSA assay (Thermo Scientific, Waltham, MA), and stored at -80°C until processing for Western Blot.

Quantitative Real-Time PCR—cDNA was used for qRT-PCR as described (Clinton et al., 2011) on a StepOne Plus (Applied Biosystems, Grand Island, NY, USA). Primers for the mRNAs *Ezh2* (F-GGAGACGATCCTGATGAAAGAG; R-CTTCTGCTGTGCCCTTATCT), *Gapdh* (F-ACCTTTGATGCTGGGGCTGGC; R-GGGCTGAGTTGGGATGGGGACT), and *GFP* (F-ATGGTGAGCAAGGGCGAGGA; R-TTTACGTCGCCGTCCAGCTCGA) were purchased from Integrated DNA Technologies (Coralville, Iowa, USA). Primers for the small RNAs *rno-miR-101a-3p* (Ca. #: MS00012950), *pre-miR-101a* (Ca. #: MP00006993), and *Snord61* (Ca. #: MS00033705) were ordered from Qiagen.

Western Blot—Protein fractions were added to Laemmli sample buffer and loaded onto SDS-PAGE gels (4–20% acrylamide and 12% acrylamide for histones; Bio-Rad, Hercules, CA, USA) for protein gel electrophoresis. Immunoblots were later incubated in primary antibodies overnight at 4°C followed by 1 h incubation with secondary antibodies and visualization on an Odyssey Infrared Imaging System (LI-COR). Following imaging, blots were put in a stripping buffer if necessary (1:50 NaOH) for 10 min and re-blocked prior to incubation with the primary antibody for the housekeeping protein (actin or histone H3) for

2 h. Secondary antibody incubation and imaging was performed in the same manner. The following primary antibodies (all of rabbit host) were used: anti-H3K27me3 (1:4000; Millipore #07-449), anti-Histone H3 (1:4000; Abcam #ab1791), anti-Ezh2 (1:2000; Abcam #ab3748), and anti-Actin (1:2000; Abcam #ab1801). Secondary antibodies were donkey anti-rabbit IRDye 800CW fluorescent antibodies (1:20,000; LI-COR #925-32213).

GFP Histology—Two-weeks following intracranial surgery, HR rats that were injected with either AAV-miR-101a (N=3) or AAV-GFP (N=3) were not exposed to behavioral testing, but instead were deeply anesthetized with sodium pentobarbital (150 mg/kg i.p.) and then transcardially perfused with ~100 ml of physiological saline followed by ~300 ml of 4% paraformaldehyde. Brains were extracted, post-fixed overnight, cryoprotected in 20% sucrose, and processed for immunohistochemistry as described in previous studies (Kerman et al., 2006). Briefly, brains were sectioned coronally on a freezing microtome at a thickness of 40 μ m. Sections were washed in phosphate buffered saline (PBS) (pH 7.2), blocked in a PBS solution containing 1% BSA, 1% normal goat serum, and 0.3% Triton X-100 for 1 h, and incubated overnight in chicken anti-GFP antibody (1:500; Invitrogen A1026) at 4 °C. The next day, sections were again washed in PBS, incubated in secondary antibody (goat anti-chicken IgY, Alexa Fluor 488; Invitrogen A11039) for 2 h at room temperature, and mounted on slides and coverslipped using Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Images were taken using an Olympus BX-UCB microscope (Center Valley, PA, USA) equipped with a motorized stage (96S100-LE; Ludl Electronic Products, Hawthorne, NY, USA), fluorophore-specific fluorescent filter sets, and a cooled mono CCD camera (Orca R2; Hamamatsu, Boston, MA, USA).

Statistical Analyses

Data were analyzed with Prism 6.0 software (GraphPad Software, San Diego, CA). qPCR data was analyzed by comparing CTs, difference in threshold between the gene of interest and housekeeping gene (*Gapdh* for mRNA and *Snord61* for miRNA). Western blots were analyzed using ImageJ software (NIH, Bethesda, MD), where band densities were determined using area under the curve of each lane's optical density histogram. Bands for H3K27me3 were normalized to total Histone 3, while bands for *Ezh2* were normalized to actin; HR/LR, AAV-miR-101a/AAV-GFP, and *Ezh2* siRNA/Control siRNA groups were compared via one-way Student's t-test or Welch's corrected t-test when there was a significant difference ($p < 0.05$) between the variances of the two groups. OFT and EPM data were compared in the same manner. For all tests, significance was set at $p < 0.05$ and results are presented as mean $\pm$ SEM.

RESULTS

HR rats display reduced expression of miR-101a-3p and increased expression of *Ezh2* and H3K27me3 in the amygdala compared to LR rats

Using a high-throughput sequencing approach, we previously found reduced miR-101a-3p expression in the amygdala of HR rats compared to LR rats. miR-101a-3p is a known regulator of PRC2 that represses the function of *Ezh2* (Varambally et al., 2008; Friedman et al., 2009), the histone methyltransferase that mediates gene silencing by trimethylating H3K27 (Fig.

1A) (Cao et al., 2002). Using qRT-PCR we confirmed a significant reduction in miR-101a-3p in the amygdala of HR rats compared to LRs ($p < 0.05$; Fig. 1B). Consistent with miR-101a-3p's reported role in repressing translation of *Ezh2*, we found that while there was no change in *Ezh2* mRNA (Fig. 1C) there was significantly more Ezh2 protein in HR compared to LR amygdala ($p < 0.01$; Fig. 1D). HRs also displayed greater levels of H3K27me3 compared to LRs ($p < 0.001$; Fig. 1E), indicating greater functional output of Ezh2 activity.

Viral-mediated miR-101a overexpression in the amygdala increases HR rats' anxiety-like behavior

A separate cohort of HR rats received intra-amygdala injections of either AAV-miR-101a (to overexpress miR-101a-3p) or AAV-GFP (control) and were tested on two rodent tests of anxiety, the OFT and EPM. On the OFT, there was no difference in total distance moved between the two groups (Fig. 2A) indicating that the viruses did not differentially affect locomotion. HR rats injected with the miR-101a-overexpressing virus displayed increased time spent in the corners of the OFT ($p = 0.058$), a reduction in time spent in the center ($p < 0.01$), and fewer center entries ($p < 0.01$; Fig. 2B–D) compared to the control group. On the EPM, there was also no group difference in total distance moved (Fig. 2E). AAV-miR-101a-injected HRs spent more time in the closed arms ($p < 0.05$), less time in the open arms ($p < 0.05$), and had fewer open arm entries ($p < 0.05$; Fig. 2F–H) compared to AAV-GFP injected rats. These results indicate that overexpressing miR-101a in HR rats' amygdala led to increased anxiety-like behavior across both the OFT and EPM.

Viral expression was confirmed through visualization of GFP for both the AAV-GFP and the AAV-miR-101a viruses (Fig. 3A). Using qRT-PCR, we confirmed that HRs injected with the AAV-miR-101a virus had greater expression of pre-miR101a ($p < 0.05$) and mature miR-101a-3p ($p < 0.01$) compared to controls. There was no GFP expression differences between the two experimental groups (Fig. 3B–D). We also showed that miR-101a overexpression caused a reduction in Ezh2 protein levels ($p < 0.05$; Fig. 3E) and reduced H3K27me3 levels ($p = 0.057$; Fig. 3F), which is consistent with miR-101a's known role in repressing Ezh2 expression and functional output.

Knockdown of Ezh2 increases HR rats' anxiety-like behavior

In order to determine if the anxiogenic effects of miR-101a overexpression in the HR amygdala are mediated through repression of Ezh2 by miR-101a-3p, another group of HR rats received intra-amygdalar injections of either an Ezh2 targeting siRNA or a control siRNA. *Ezh2* knockdown did not affect behavior in the OFT, with no group differences in distance moved, time in corners, time in center, or center entries into the OF (Fig. 4A–D). On the EPM there were no differences in total distance moved or time in the closed arms (Fig. 4E–F). However, animals that received the Ezh2 siRNA spent less time in the open arms ($p < 0.05$) and had fewer open arm entries ($p < 0.05$; Fig. 4G–H). Overall these data showed that *Ezh2* knockdown in the amygdala of HR rats had a weaker anxiogenic effect compared to miR-101a overexpression as it increased anxiety-like behavior on the EPM, but not the OFT.

Animals were sacrificed 24 h after EPM testing and knockdown of *Ezh2* in the amygdala was confirmed ($p < 0.05$; Fig. 5A). We also showed that *Ezh2* knockdown caused a reduction in total H3K27me3 in the amygdala ($p < 0.05$; Fig. 5B)

DISCUSSION

The HR/LR rat lines provide a useful tool for studying genetic and molecular factors that contribute to anxiety-like behavior. Selective breeding for high vs low locomotor response to novelty produced the behaviorally distinct HR and LR lines, which differ in a number of behavioral domains, including trait anxiety. LR rats display greater anxiety-like behavior compared to HRs on several behavior tests, including the elevated plus maze, open field test, and light dark box (Stead et al., 2006; Clinton et al., 2014; Cohen et al., 2015; Glover et al., 2015), and these differences are observable as early as the 25th day of life (Clinton et al., 2011). Since the HR/LR behavioral phenotypes are highly heritable and apparent early in life, our research group has sought to identify genetic and epigenetic factors that shape their behavior. We recently used next-generation sequencing to identify numerous miRNA and mRNA expression differences in the amygdala of HR/LR rats (Cohen et al., 2016). The miRNA miR-101a-3p emerged as an abundant miRNA in the amygdala that was expressed at significantly higher levels in LR versus HR rats. Our current study confirmed that HRs exhibit lower miR-101a levels in the amygdala compared to LRs as well as greater levels of *Ezh2* and H3K27 (*Ezh2*'s functional output); this is consistent with the notion that LRs' elevated miR-101a-3p levels impinge on PRC2/*Ezh2* function and hinder H3K27me3 (relative to HRs). We therefore tested the hypothesis that increasing miR-101a-3p in the HR amygdala would increase their typically low levels of anxiety-like behavior. We found that viral-mediated miR-101a-3p overexpression in the HR amygdala increased their anxiety-like behavior on the EPM and OFT. Overexpressing miR-101a-3p also reduced levels of its target, the histone methyltransferase *Ezh2*, and H3K27me3. Using an siRNA approach, we found that direct knockdown of *Ezh2* also caused an increase in HRs' anxiety-like behavior, but to a lesser degree than miR-101a-3p over-expression.

Polycomb group proteins (like the PRC2) play an important role in gene silencing through post-translational modification of histone tails. miR-101a-3p is known to negatively regulate PRC2 by inhibiting translation of multiple PRC2 components including embryonic ectoderm development (*Eed*), suppressor of zeste (*Suz12*), and *Ezh2*, the histone methyltransferase that silences gene expression via tri-methylation H3K27 (Varambally et al., 2008; Cao et al., 2010). PRC2/*Ezh2* function is required for a number of developmental processes (Chakrabarty et al., 2007; Marchesi et al., 2014), including maintaining embryonic and adult stem cells, likely due to its role in repressing developmental transcription factor expression (Pietersen and van Lohuizen, 2008). Given their roles in stem cell maintenance, miR-101a and *Ezh2* have been widely studied in the context of cancer. For instance, miR-101a expression decreases and *Ezh2* expression increases during progression of prostate cancer (Varambally et al., 2008) and bladder cancer (Friedman et al., 2009). The *Ezh2* inhibitor tazemetostat is currently undergoing clinical trials to test safety and efficacy in treating several different cancers (Kurmasheva et al., 2016).

In the brain, *Ezh2* has been shown to play a role in neural stem cell differentiation. *Ezh2* is expressed at high levels in neural stem cells and its expression decreases during differentiation into neurons and astrocytes, but not oligodendrocytes (Sher et al., 2008); *Ezh2* over-expression in astrocytes promotes de-differentiation towards a neural stem cell-like state (Sher et al., 2011). Conditional *Ezh2* knockout in neural stem cells results in mice with impaired spatial learning and memory and contextual fear learning and memory as well as decreased progenitor cell proliferation (Zhang et al., 2014). While *Ezh2* clearly regulates a number of key neurodevelopmental processes (Di Meglio et al., 2013; Neo et al., 2014), it continues to serve important functions in mature neurons, particularly in regards to cellular plasticity (Zhang et al., 2014). Morphine exposure causes reduced expression of *Bdnf* in the ventral tegmental area by recruiting *Ezh2* and increasing H3K27me3 at the *Bdnf* promoter, and *Ezh2* over-expression combined with morphine exposure increased animals conditioned place preference to the drug (Koo et al., 2015). In the cerebellum, *Ezh2* restricts dendrite morphogenesis through H3K27me3-mediated repression of *Bdnf* (Qi et al., 2014). Other work shows that *Ezh2* expression is sensitive to external stimuli, such as stress (Hunter et al., 2009; Xu et al., 2012) and neural activity, which transiently increases *Ezh2* and H3K27me levels (Reynolds et al., 2015; Palomer et al., 2016). Thus, while *Ezh2* has traditionally been considered in the context of cell development and differentiation, recent evidence clearly shows numerous important roles in the adult brain, particularly in the ability of neurons to respond to environmental signals.

Selectively-bred HR rats, which are known to display low levels of anxiety-like behavior, exhibit reduced miR-101a-3p expression together with increased *Ezh2* and H3K27me3 levels in the amygdala compared to LRs that characteristically display high levels of behavioral inhibition and anxiety-like behavior. Since *Ezh2* maintains expression to varying degrees in mature neurons, astrocytes, and oligodendrocytes, it is unclear from our current data which cell type(s) may be contributing to the observed HR/LR differences. In our viral manipulation experiment, miR-101a-3p over-expression was limited to neurons through the use of AAV vectors, which selectively transduce neurons but not glia (Mason et al., 2010; Doherty et al., 2011), and the *Ezh2* knockdown experiment using Accell siRNA technology likely predominantly affected neurons since this tool was previously shown to selectively transduce mature neurons but not glia (Nakajima et al., 2012). Consequently, the observed anxiogenic effects caused by miR-101a over-expression and *Ezh2* suppression are likely the result of alterations in neural function. Future work will determine if knockdown or inhibition of miR-101a-3p and/or over-expression of *Ezh2* in the amygdala can produce anxiolytic effects (either in high anxiety-prone LRs or normative rates). We will also continue to investigate the genetic and epigenetic differences between HR and LR rats that may contribute to their disparate stress reactive and behavioral phenotypes. Chromatin immunoprecipitation studies will determine differences in genomic binding locations of *Ezh2* and H3K27me3 between HRs and LRs. These locations could be compared to changes in *Ezh2* and H3K27me3 sites that occur following miR-101a-3p overexpression and/or *Ezh2* knockdown. Ultimately such analyses could potentially identify downstream genes and pathways that are responsible for the miR-101a-3p and *Ezh2* driven changes in anxiety-like behavior.

In conclusion, viral mediated miR-101a-3p over-expression in the amygdala of low-anxiety HR rats increases their anxiety-like behavior and reduces *Ezh2* and H3K27me3 levels. Direct *Ezh2* knockdown also increases anxiety-like behavior, but not to the same extent as miR-101a-3p over-expression. Thus, increasing the expression of miR-101a-3p in the amygdala produces an anxiogenic effect that is at least partially mediated via the repression of *Ezh2* and its functional output, H3K27me3. Overall, these studies point to a novel molecular mechanism that may contribute to individual differences in anxiety-like behavior. They also add to the growing body of evidence supporting a role for mechanisms once considered to be exclusive to development and differentiation, such as miRNA and epigenetic regulation, in the adult brain and in underlying behavioral phenomena.

Acknowledgments

This study was funded by R01MH105447-01 (SMC), R01MH097909 (FDL), NIGMS MSTP 5T32GM008361 and NINDS 5T32NS061788 (which supported JLC and WMW). Viral constructs were produced by the UAB Neuroscience NINDS Protein Interactions Core C. (P30 NS047466)

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SIGNIFICANCE STATEMENT

We identify the microRNA miR-101a-3p and its gene target enhancer of zeste homolog 2 (*Ezh2*) in the amygdala as novel molecular factors that shape anxiety-like behavior. Selectively-bred rats that display low trait anxiety show reduced miR-101a-3p and increased *Ezh2* expression in the amygdala compared to rats that display high trait anxiety. Viral-mediated miR-101a-3p over-expression in the amygdala increased anxiety-like behavior in low trait-anxiety rats and reduced levels of *Ezh2*. Knockdown of *Ezh2* with short-interfering RNA also increased anxiety-like behavior, but to a lesser extent than miR-101a-3p overexpression. We show that amygdalar expression of miR-101a-3p contributes to anxiety-like behavior and that this effect is at least partially mediated via repression of *Ezh2*.

Figure 1. High novelty responding (HR) rats display reduced miR-101a-3p, *Ezh2*, and H3K27me3 expression compared to low novelty responding (LR) rats in the amygdala (a) Schematic representation miR-101a-3p's role in repressing gene transcription. miR-101a-3p inhibits the translation of several proteins that comprise the Polycomb Repressive Complex 2 (PRC2), including the histone-methyltransferase enhancer of zeste homolog 2 (*Ezh2*), embryonic ectoderm development (Eed), and suppressor of zeste (Suz12). *Ezh2* mediates the functional output of the PRC2 since it silences target gene expression via tri-methylation of Histone 3 at Lysine 27 (H3K27me3). (b) Quantitative Real-Time PCR for miR-101a-3p showed that HRs have reduced expression in the amygdala compared to LR rats ($p < 0.05$). (c) There was no difference in *Ezh2* mRNA between HRs and LR rats. (d) HRs displayed greater *Ezh2* protein levels compared to LR rats ($p < 0.01$). (e) HRs also displayed greater H3K27me3 levels compared to LR rats ($p < 0.001$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 2. Viral-mediated miR-101a over-expression in the HR amygdala increased anxiety-like behavior on the Open Field Test (OFT) and the Elevated Plus Maze (EPM)

(a) There was no difference in total distance moved between HR rats injected with AAV-miR101a and control rats injected with AAV-GFP. (b) miR-101a overexpression lead HRs to spend more time in corners of the OFT ($p=0.06$), (c) spend less time in the center ($p<0.01$), and (d) make fewer center entries ($p<0.01$) compared to the control group. (e) There were no group differences in overall activity in the EPM. (f) miR-101a overexpression lead HRs to spend more time in the closed arms ($p<0.05$), (g) spend less time in the open arms ($p<0.05$), and (h) show fewer open arm entries ($p<0.05$) compared to the AAV-GFP control animals.

* $p<0.05$, ** $p<0.01$

Figure 3. Viral-mediated miR-101a-3p over-expression lead to reduced *Ezh2* and H3K27me3 levels in the HR amygdala

(a) Viral expression of AAV-miR101a and AAV-GFP. (b) HR rats that received intra-amygdalar injections of AAV-miR101a showed increased expression of pre-miR101a ($p < 0.05$) and (c) mature miR-101a-3p ($p < 0.01$) compared to animals injected with AAV-GFP. (d) There were no differences in GFP mRNA between AAV-miR101a and AAV-GFP animals. (e) Viral-mediated miR-101a over-expression caused a reduction in *Ezh2* protein expression ($p < 0.05$) and (f) H3K27me3 ($p = 0.057$) in the amygdala of HR rats. * $p < 0.05$, ** $p < 0.01$

Figure 4. siRNA knockdown of *Ezh2* in the HR rat amygdala increased anxiety-like behavior in the EPM

(a) Injection of short-interfering (siRNA) targeting *Ezh2* into the amygdala of HR rats did not alter the total distance moved, (b) time spent in corners, (c) time spent in the center, or (d) the number of center entries in the Open Field Test (OFT) compared to rats injected with control siRNA. (e) *Ezh2* knockdown did not alter total distanced moved or (f) time spent in the closed arms on the elevated plus maze (EPM) compared to control rats. (g) *Ezh2* siRNA did reduce HR rats' time spent in the open arms ($p < 0.05$) and (h) the number of open arm entries ($p < 0.05$) compared to control siRNA-treated animals. * $p < 0.05$

Figure 5. Injection of siRNA targeting *Ezh2* in the amygdala reduced *Ezh2* protein levels and its functional output, H3K27me3

(a) Animals injected with siRNA targeting *Ezh2* displayed reduced expression of *Ezh2* protein ($p < 0.05$) and **(b)** reduced *Ezh2* output, trimethylation of histone 3 at lysine 27 (H3K27me3) levels ($p < 0.05$) compared to animals injected with control siRNA. * $p < 0.05$