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microRNAs as biomarkers of resilience or vulnerability to stress

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ABSTRACT

Identifying novel biomarkers of resilience or vulnerability to stress could provide valuable information for the prevention and treatment of stress-related psychiatric disorders. To investigate the utility of blood microRNAs as biomarkers of resilience or vulnerability to stress, microRNAs were assessed before and after 7 days of chronic social defeat in rats. Additionally, microRNA profiles of two important stress-regulatory brain regions, the medial prefrontal cortex (mPFC) and basolateral amygdala (BLA), were assessed. Rats that displayed vulnerability to subsequent chronic stress exhibited reductions in circulating miR-24-2-5p, miR-27a-3p, miR-30e-5p, miR-3590-3p, miR-362-3p, and miR-532-5p levels. In contrast, rats that became resilient to stress displayed reduced levels of miR-139-5p, miR-28-3p, miR-326-3p, and miR-99b-5p compared to controls. In the mPFC, miR-126a-3p and miR-708-5p levels were higher in vulnerable compared to resilient rats. In the BLA, 77 microRNAs were significantly altered by stress but none were significantly different between resilient and vulnerable animals. These results provide proof-of-principle that assessment of circulating microRNAs is useful in identifying individuals who are vulnerable to the effects of future stress or individuals who have become resilient to the effects of stress. Furthermore, these data suggest that microRNAs in the mPFC but not in the BLA are regulators of resilience/vulnerability to stress.

KEYWORDS: microRNA, social stress; blood; biomarker; prefrontal cortex; amygdala

Psychiatric disorders, such as post-traumatic stress disorder (PTSD) and depression, can often develop following exposure to chronic stress (Marin et al., 2011, Radley et al., 2011, McEwen, 2012, Timmermans et al., 2013). However, it is clear that some individuals are resilient to the deleterious effects of stress while others are vulnerable to these effects (Holmes and Singewald, 2013, Karatsoreos et al., 2013). Determining the neural substrates that differentiate resilient from vulnerable individuals is critical for the prevention of stress-related psychiatric disorders and for the development of novel treatment strategies for these disorders. In parallel, the identification of non-invasive biomarkers of stress vulnerability and resilience could serve as valuable tools for the identification of those individuals who are at risk for the development of psychiatric disorders and could benefit from prophylactic treatments or those individuals who are resilient to the effects of chronic social stress.

Circulating microRNAs have been well studied in cancer and cardiovascular disease, and subsets of circulating microRNAs have been identified as biomarkers for disease risk or disease sub-type (Kosaka et al., 2010, Bronze-da-Rocha, 2014). MicroRNAs, small non-coding RNAs, are important regulators of gene expression, generally repressing the expression of their target mRNAs (Bushati and Cohen, 2007). MicroRNAs in blood can be taken up by cells where they retain their transcriptional repression mechanism (Rao et al., 2013). Compared with cancer and cardiovascular disease, relatively little is known about circulating microRNAs as predictors of psychiatric disorders (Kolshus et al., 2014).

The objectives of these studies were two-fold. The first objective was to determine if microRNA profiles in blood reflect resilience or vulnerability to chronic social defeat. Using the rat resident-intruder model, we previously showed that two subpopulations of rats emerge during chronic social defeat (Wood et al., 2010). In one subpopulation, rapid latencies to submit to the resident rat are associated with increased depressive-like behaviors and

neuroendocrine dysfunction, indicating a vulnerable phenotype. The other subpopulation exhibits resistance to defeat by the resident rats, as indicated by long latencies to be defeated and no behavioral or neuroendocrine dysfunctions, indicating a resilient phenotype. We examined whether or not circulating microRNAs could reflect future resilience or vulnerability to stress, and if they could differentiate ongoing resilience and vulnerability once the phenotypes had been established. The second objective of the present studies was to determine if microRNA profiles in specific stress-regulatory brain regions are different between vulnerable and resilient rats and could therefore represent a novel mechanism through which stress might exert its effects. We assessed microRNA profiles in two brain regions important in mediating the effects of stress on behavior and cognitive function- the medial prefrontal cortex (mPFC) and the basolateral amygdala (BLA). Studies have highlighted the differential regulation of microRNAs associated with the dysfunction or differential activity in these brain regions (Im and Kenny, 2012, Hamilton et al., 2014), but little is known about the impact of chronic stress on microRNA expression in these brain regions. Overall, the results obtained here provide proof-of-principle that blood microRNAs are biomarkers that reflect vulnerability to future stress and also reflect ongoing resilience to chronic social stress. The results further indicate that microRNAs in the mPFC, but not the BLA, are potential important determinants of an individual's vulnerability to stress.

EXPERIMENTAL PROCEDURES

Subjects

Young adult male Sprague-Dawley rats initially weighing 225-275 g were utilized in these experiments (Charles-River, Wilmington, MA). Rats were singly housed and were randomly assigned to social defeat or control groups, with ad libitum access to food and water. Animals were allowed to acclimate to a 12-hour light/dark cycle (lights on at 0700 h) for at least 5 days prior to administration of stress protocols. All procedures were conducted between 0900-1100 h and were in compliance with the NIH Guide for Care and Use of Laboratory Animals and the CHOP Research Institute's Animal Care and Use Committee.

Acute social stress induces changes in circulating microRNAs

The purpose of this experiment was to assist in determining the timepoint for study of circulating microRNAs after chronic social defeat. Little is known about how rapidly microRNAs in blood can change with stress and how long-lasting these changes are. To assess the time course with which microRNAs change in response to acute stress, animals were subjected to a single social defeat. Three days prior to social defeat, tail blood was taken from each animal via tail vein snips to measure pre-stress baseline microRNA levels. All animals were then subjected to a single social defeat stress as described below. Post-stress blood was taken by tail vein snips at 1 h (n=9), 4 h (n=9), and 24 h (n=9) from the same cohort of rats. Group sizes were 9 at each timepoint except for the pre-stress timepoint where n=7 due to a lack of sufficient sample volume from two rats.

Vulnerable and resilient subpopulations of rats emerge after repeated social defeat

The chronic social defeat stress paradigm was modified from the resident-intruder model originally described by Miczek (Miczek, 1979), as previously published by our group (Wood et al., 2010, Berube et al., 2013, Kenworthy et al.,

2014). In brief, experimental Sprague Dawley rats were placed in the home cage of an unfamiliar Long-Evans rat and allowed to interact until the intruder displayed the supine defeat posture for approximately 3 seconds. If the rat did not display defeat, it was allowed to interact with the resident rat for a full 15 min. After display of the defeat posture or at the end of 15 min if there was no defeat, the rats were separated by a wire mesh barrier, which prevented injury and wounding from continued aggressive interactions but permitted olfactory, visual and auditory contact for the remainder of the 30 min stress. Control rats were placed in a novel cage with a wire mesh divider for 30 min. At the end of the 30 min, both experimental and control rats were returned to their home cages.

Injuries during the 15 minutes of physical interaction were minimal and infrequent. This procedure occurred every day for 7 consecutive days. The daily latencies to display the defeat posture were averaged over the 7 days. We characterized short latency rats (SL) as having an average latency score of <300 s, and long latency rats (LL) with an average latency score of >300 s, based on our previous work (Wood et al., 2010), and we confirmed this with cluster analysis. The average latency scores and body weight gains were determined. In an independent cohort of rats exposed to social defeat, behavior in the social interaction test, a validated test of anxiety-related behavior (Lapiz-Bluhm, 2008, Baudry et al., 2010), was examined. Animals were placed in an open field black box (70 cm x 70 cm) with an age-matched stimulus rat *of the same strain and of a similar size and allowed to interact for 15 min.* Each interaction was videotaped and coded for social interaction time by 2 coders who were blind to the experimental conditions.

Evaluating microRNA expression in blood before and after chronic social defeat in resilient and vulnerable animals

Three days before the initiation of the chronic social defeat paradigm, tail blood was collected from each rat via tail snips. Experimental rats were subjected to 7 consecutive days of social defeat or control novel cage exposure, described above. On Day 8, 24 h after the last social stress or control novel cage

exposure, trunk blood was collected for the post-stress timepoint *after live decapitation of the animal*. 24 h was chosen based on the *results of the acute social stress study*. Pre-stress blood samples were obtained from 6 control, 6 SL, and 6 LL rats. Post-stress blood samples were collected from 6 control, 6 SL, and 6 LL rats. A subset of significant microRNAs was validated by qPCR in another cohort of rats.

Identifying changes in microRNA expression in the mPFC and BLA in rats resilient or vulnerable to chronic social defeat.

A naïve cohort of rats was subjected to the 7-day chronic social defeat stress or novel cage exposure, as described above. Twenty-four hours after the last social defeat stress for experimental animals or novel cage exposure for controls, *animals were euthanized by live decapitation, and the mPFC and BLA were dissected from fresh tissue using brain slicer matrices and flash frozen in 2-methyl-butane*. Group sizes were 5 rats for each group (control, SL, and LL). mPFC tissue from a separate cohort of rats was utilized for individual qPCR validation of significantly altered microRNAs.

RNA extraction from blood and tissue

Whole blood samples were incubated with PAXgene RNA stabilizing reagent (PreAnalytiX, Switzerland), at a ratio of 1:2.76. Total RNA, including microRNA, was isolated from whole blood using the PAXgene Blood microRNA kit, per manufacturer's instructions (PreAnalytiX, Switzerland). Total RNA including microRNA was isolated from fresh frozen brain tissue using the miRNeasy kit (Qiagen, Hudson, NH) per manufacturer's instructions. For all samples, RNA quantity and purity was determined using the NanoDrop 1000 spectrophotometer (Thermo-Scientific, Waltham, MA).

RNA labeling and microarray analysis

The FlashTag Biotin HSR RNA labeling kit (Affymetrix, Santa Clara, CA) was used to biotin-label total RNA from blood and tissue samples. A total of 500 ng

of blood-derived RNA and 250 ng of tissue-derived RNA was analyzed on Affymetrix GeneChip microRNA 3.0 arrays. All microarrays were quantified with an Affymetrix GeneChip Scanner 3000. With array hybridization technology, gene expression measurement is limited by background at the low end of expression. Thus it is important to filter the data to remove probe sets that have hybridization intensities that are too low to be reliable. We plotted the distribution of mean expression values ($\log_2$ intensity) across all samples and eliminated any probe sets for which the $\log_2$ intensity deviated from linearity, which in our case were probe sets with a mean $\log_2$ intensity value of 2 or less. This resulted in a total of 170, 170, 123, and 213 microRNAs for analysis in pre-stress blood, post-stress blood, mPFC, and BLA, respectively. Affymetrix GeneChip microRNA 3.0 arrays use microRNA nomenclature from miRBase v.17. This nomenclature version is used in Table 2. For the main text, figures, and Table 1 we have updated the names of all significant microRNAs to match the nomenclature of the most updated miRBase v.21. We have also updated the nomenclature for any cited literature.

Statistics

For analyses of behavior and microRNA expression by qPCR, data were analyzed by t-tests and one-way ANOVAs with p values set to <0.05 . For analyses of microarray data, normalization and quality control were completed using Affymetrix® Expression Console software (Affymetrix, Santa Clara, CA) which consists of 1) background correction, 2) robust multi-array analysis normalization (RMA) and 3) summarization. A Principal Component Analysis Plot and heat map of the overall expression values was used to determine the outliers. After removal of outliers, the final number of samples for each experiment is as follows: *for the acute stress study: pre-stress: (n=7), 1 h (n=9), 4 h (n=9), and 24 h (n=9). For the remaining studies, animals were exposed to repeated defeat.* Pre-stress blood samples: 5 control, 6 SL, and 4 LL rats. Post-stress blood samples: 6 control, 6 SL, and 6 LL rats. mPFC samples: 3 control, 5 SL, and 4 LL rats. BLA samples: 4 control, 5 SL, and 4 LL. Limma, a package

used in the statistical computing software R, was used for statistical analysis of all microarray data to identify differentially expressed microRNAs between all groups. The results were then adjusted for multiple comparisons using the Benjamini-Hochberg correction (Smyth, 2004). Any resulting microRNA with an adjusted p-value <0.1 was considered significant.

Validation of microRNAs by qPCR

In independent cohorts of rats, a subset of the circulating and mPFC-derived microRNA identified as significantly different in the microarray analyses were validated by qPCR. MicroRNA was reverse transcribed into cDNA using the TaqMan MicroRNA Reverse Transcription kit, with microRNA- specific RT primers from TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA). Small nuclear RNA U6 (Life Technologies, Carlsbad, CA) was used as the endogenous control for all microRNAs. Relative quantification results based on $2^{-\Delta\Delta C_t}$ were expressed as fold change from controls.

RESULTS

Acute social stress induces changes in circulating microRNAs up to 24 h later

MicroRNA levels in blood collected before the onset of acute social defeat (pre-stress) were compared to those in samples taken at 1 hour, 4 hours and 24 hours after termination of a single social defeat. MicroRNAs significantly different between pre-stress compared to 1, 4 or 24 h are shown in Figure 1. One hour following acute stress, 5 microRNAs were up-regulated in stressed rats: let-7a-5p, let-7c-5p, let-7i-5p, miR-106b-3p, and miR-15b-5p. Of these microRNAs, let-7a-5p, let-7c-5p, and let-7i-5p remained elevated for 4 h and 24 h after acute stress. In contrast, miR-150-5p, miR-342-3p, and miR-361-5p were suppressed 1 h after stress. MiR-342-3p and miR-361-5p remained down-regulated in stressed rats throughout the 24 h of testing. At the 4 h and 24 h timepoints, miR-26a-5p was elevated, while miR-339-5p, miR-423-3p, and miR-92a-3p were

reduced in stressed rats. Interestingly, a set of microRNAs was differentially regulated only at 4 h, but not at 24 h following stress (let-7d-5p, miR-146b-5p, miR-192-5p and miR-339-3p). Lastly, miR-125a-5p, miR-125b-5p, miR-145-5p, miR-191a-5p, miR-23a-3p, and miR-425-5p were all suppressed only 24 h following stress. These findings demonstrate that levels of circulating microRNAs can change as rapidly as 1 h post-stress and that some of these effects can persist for at least 24 h, indicating the sensitivity of the circulating microRNA profile to stress. Based on these results and because we have observed behavioral changes in the social interaction test (Figure 2C), the forced swim test, and in neuroendocrine function at 24 h after the last defeat (Wood et al., 2010), we assessed post-stress circulating and brain microRNAs at 24 h after the last defeat in subsequent experiments.

Rats exhibiting short latencies to be defeated show increased anxiety-like behavior indicating vulnerability

The average latency to be defeated (Figure 2A) in the cohorts of rats used in Experiments 2 and 3 was determined based on our previously published criteria (Wood et al., 2010). LL rats exhibited significantly higher latencies to be defeated than SL rats. Both SL and LL groups exhibited less body weight gain compared to controls (Figure 2B), indicating both were affected by the social stress. However, only the SL rats exhibited lower time spent interacting with a novel rat (Figure 2C), indicating increased anxiety-related behavior.

The circulating microRNA profile reflects vulnerability or resilience to chronic social stress

We assessed whether microRNAs in blood could reflect subsequent or ongoing vulnerability or resilience to chronic stress, using pre-stress and post-stress blood samples, respectively. *Based on results from the acute social stress study above, we assessed post-stress blood microRNAs 24 h after the last defeat.*

In blood samples taken prior to stress (pre-stress timepoint), microarray analysis revealed 6 microRNAs in which levels were significantly lower in SL rats compared to controls: miR-24-2-5p, miR-27a-3p, miR-30e-5p, miR-3590-3p, miR-362-3p, and miR-532-5p (Figure 3A). No microRNAs significantly differed between LL and control or LL and SL rats. Levels of miR-24-2-5p, miR-27a-3p, and miR-362-3p were positively correlated with the average defeat latency over the 7 days (Figures 3B-D). Four of these 6 microRNAs identified through microarrays were validated by individual qPCRs in an independent cohort of rats (Table 1). In sum, decreased levels of a small subset of microRNAs was associated with future vulnerability to the effects of chronic social stress.

In blood taken 24 hours after the last social defeat stress (the post-stress timepoint), microarray analysis revealed 4 microRNAs that were significantly lower in LL animals compared with control animals: miR-139-5p, miR-28-3p, miR-326-3p, and miR-99b-5p (Figure 4A). Expression of these 4 microRNAs identified through microarrays was validated in an independent cohort of rats by individual qPCRs (Table 1). Additionally, miR-326-3p levels were significantly correlated with the average defeat latency (Figure 4B). Thus, microRNA profiling in blood at 24 h after the last stress identified microRNAs that were associated with resilience to stress.

MicroRNA profiles in the mPFC, but not in the BLA, are different between vulnerable and resilient animals

We analyzed the microRNA profiles in the mPFC and BLA following chronic social defeat in an independent cohort of rats using microarray analyses. In the mPFC, two microRNAs, miR-126a-3p and miR-708-5p, were significantly up-regulated in SL rats when compared with LL rats (Figure 5A). *We performed power calculations (using the calculator available at: <http://www.uccs.edu/~lbecker/>) on the comparisons that had the smallest group sizes, the miR-126-a-3p in the mPFC. The effect size for the SL-LL comparison for miR-126-a-3p was Cohen's $d=2.53$, which corresponds to an observed power*

of greater than .95. The Cohen's $d=4.06$ for the control-SL comparison, which corresponds to an observed power of greater than .95. Other comparisons yielded similar results. Thus, the power calculations suggest that the group sizes were sufficient to observe actual significant differences in microRNA expression. Expression of both microRNAs was validated in an independent cohort of rats by qPCR, and these were negatively correlated with the average defeat latency (Figures 5C and D).

In contrast to the limited number of microRNAs identified in the mPFC, there was an abundance of differentially expressed microRNAs detected in the BLA. A total of 77 microRNAs differed between SL and control rats, while 42 differed between LL and control rats (Table 2). 41 of these 42 microRNAs (with the exception of miR-27a-3p) also differed between SL and control rats. Among the top 15 regulated microRNAs as determined by p-values, levels of miR-140-3p, miR-143-3p, miR-16-5p, miR-210-3p, miR-341, miR-376a-3p, and miR-497-5p were suppressed in the BLA of both SL and LL rats compared to control rats (Figure 6A). Alternatively, levels of let-7e-5p, let-7f-5p, miR-150-5p, miR-219a-2-3p, miR-329-3p, miR-382-5p, miR-505-5p, and miR-664-1-5p were elevated in the BLA of SL and LL rats (Figure 6A) compared to controls. BLA expression of those microRNAs that were identified in blood or mPFC as significantly different but were not in the top 15 regulated microRNAs are shown in Figure 5B to allow comparison of their levels in other tissues. Levels of miR-24-2-5p and miR-708-5p are both suppressed in the BLA of SL rats compared with control rats, whereas the level of miR-27a-3p is suppressed in the BLA of LL rats compared with control rats (Figure 6B). Overall, for those microRNAs that were significantly different between SL and control rats, expression tended to be in the same direction in LL rats compared to control rats. Importantly, there were no microRNAs significantly different between SL and LL rats. These results suggest that, in contrast to the microRNA profile of the mPFC, that of the BLA is more sensitive to the effects of chronic social stress but is not differentially affected in vulnerable compared to resilient animals.

DISCUSSION

Prevention or early diagnosis of stress-related anxiety and mood disorders could greatly reduce their onset and severity (Kearns et al., 2012, Searcy et al., 2012). Identifying novel non-invasive biomarkers of individuals who are either vulnerable or resilient to the effects of stress will aid in prevention, early diagnosis and timely treatment of such disorders. Here, we set out to investigate the potential of a novel biomarker, blood microRNAs, to reflect resilience or vulnerability to chronic stress in an animal model. Overall, our results indicate that circulating microRNAs can reflect vulnerability to future stress and identify those individuals who have become resilient to stress. Furthermore, our analysis in two important stress-regulatory neural structures indicates that microRNAs in the mPFC, but not in the BLA, are specifically associated with vulnerability or resilience to stress.

Previously, we used the assessment of coping strategies during defeat to identify rats that go on to be vulnerable or resilient to the effects of chronic social defeat (Wood et al., 2010). Here, we assessed circulating microRNAs both prior to the onset of social defeat as well as post-stress at 24 h after the last stress exposure. MicroRNA profiling in blood prior to the onset of chronic social defeat indicated that the expression of a small subset of microRNAs (miR-24-2-5p, miR-27a-3p, miR-30e-5p, miR-362-3p), that were identified in the microarray and validated in an independent cohort of rats, was decreased in rats that become vulnerable to stress. In a population of major depressive disorder patients, genetic alterations of the precursor to miR-30e-5p (pre-miR-30e), has proven to be a reproducible blood biomarker (Xu et al., 2010). MiR-30e-5p is also down-regulated in peripheral blood mononuclear cells of patients with schizophrenia, another stress-related psychiatric disease (Gardiner et al., 2012). Another microRNA that was observed to be decreased in vulnerable rats, miR-27a-3p, is decreased in peripheral blood mononuclear cells in individuals with PTSD (Zhou et al., 2014). Thus, an extension of our observation that levels of miR-27a-3p

and miR-30e-5p were decreased in rats that went on to be vulnerable is that in humans, miR-27a-3p and miR-30e-5p may be changed prior to stress exposure and subsequent onset of psychiatric symptoms. To our knowledge, these preclinical results are the first to suggest that blood microRNAs could serve as biomarkers for individuals that become vulnerable to the effects of subsequent stress.

After exposure to chronic stress, the levels of four microRNAs (miR-139-5p, miR-28-3p, miR-326-3p, miR-99b-5p), that were identified through microarrays and validated in an independent cohort of rats, were decreased in animals exhibiting resilience to defeat (LL rats). Of these, miR-99b-5p has been shown to be increased in circulation in mice 3 h after induction of mild traumatic brain injury (Sharma et al., 2014). However, in another study assessing serum microRNAs following a very different type and intensity of stress (3 days of immobilization and tail shock), a set of microRNAs distinct from those identified here were differentially expressed two weeks after stress (Balakathiresan et al., 2014). It is reasonable to expect that two weeks of recovery from stress affects a very different set of microRNAs than a timepoint more proximal to stress termination, such as the 24 h timepoint studied here. Recently, transcriptomic analysis of fibroblasts revealed a differential regulation of a panel of microRNAs and their targeted mRNAs between individuals with major depressive disorder and normal controls (Garbett et al., 2015). Thus, there is strong potential for the development of microRNAs as biomarkers for stress-related disease. Overall, the present results provide proof-of-principle that blood microRNAs are biomarkers that reflect vulnerability to future stress and can identify individuals that are resilient to ongoing stress.

In our study we used the PAXgene blood RNA system to isolate total RNA including microRNA from rat whole blood, a procedure similar to what is performed for studying microRNA expression in human subjects and is considered the gold standard in terms of blood microRNA extraction procedures.

We note that the cellular sources of the total microRNA expression in our study would include the total hematocyte fraction, meaning the erythrocyte as well as the lymphocyte fractions of blood. Recent studies have focused on the role of microRNAs expressed in the lymphocyte fraction to be of relevance to a number of diseases (Smith et al., 2010, Atarod et al., 2014).

Currently there is little evidence as to whether miRNA levels in whole blood are influenced by circulating glucocorticoids. Two studies (Smith et al., 2010, Smith et al., 2013) investigating the role of glucocorticoids in lymphocyte apoptosis found that miRNAs are substantially repressed during glucocorticoid-induced apoptosis of thymocytes. This suggests that stress-induced glucocorticoids could have an important role in blood miRNA regulation, especially in upregulating apoptotic pathways in blood cells.

In our assessment of the microRNA levels in the mPFC, levels of miR-126a-3p and miR-708-5p were significantly increased in the SL rats compared to control and/or LL rats. It has been shown that restraint stress induces a change in miR-126a-3p in the mPFC (Berube et al., 2013). In the dorsal striatum, miR-126a-3p was shown to be elevated in cocaine-seeking rats (Hollander et al., 2010). Oxidative stress upregulates miR-708-5p levels in cultured hippocampal neurons, implicating this microRNA in the pathology of Alzheimer's Disease (Xu et al., 2012). Furthermore miR-126a-3p seems to affect cell growth and survival across a number cell types, including neurons (Sonntag et al., 2012), suggesting that processes related to cell growth and survival could be affected in SL rats. Future studies are necessary to determine whether or not these two microRNAs are important in producing vulnerability to chronic stress and if that is through neural processes that involve neurodegeneration, cell growth, and survival.

We found that the impact of chronic stress on microRNA expression in the BLA was much more widespread than in the mPFC, with alterations in the levels of 77 microRNAs in this brain region. The BLA is important in regulating behavioral

and neuroendocrine responses to stress (Grissom and Bhatnagar, 2009) and the structural properties of its cells are affected by repeated stress (*Padival et al., 2013*). Interestingly, the electrophysiological properties of its cells are different in resilient rats (Hetzl and Rosenkranz, 2014). However, we did not observe significant differences between SL and LL rats in the levels of any microRNA in the BLA. Thus, while other properties of BLA cells may differ between resilient and vulnerable rats, our findings specifically suggest that microRNAs in the BLA are not sensitive to the vulnerability or resilience state of the individual.

The contrasting findings between the mPFC and BLA suggest that microRNA regulation of the stress response is region-specific with microRNAs in the mPFC, but not BLA, being sensitive to individual differences in responses to stress. The current findings are supported by the results of Thompson and colleagues that demonstrated differences in microRNA expression between animals classified as high or low responders in their processing of reward and anxiety (Hamilton et al., 2014). In addition to the mPFC, microRNA regulation in the nucleus accumbens is important in mediating resilience to stress (Dias et al., 2014). Thus, microRNAs in multiple brain regions, including at least in the mPFC and nucleus accumbens, are potentially important in producing vulnerability or resilience to stress and therefore relevant to onset and exacerbation of symptoms of stress-related diseases. Alternatively, the changes in microRNA expression in the mPFC may not produce vulnerability to stress in SL rats but could occur as a consequence of developing vulnerability. Direct manipulations of microRNA expression will be necessary to parse out these two possible scenarios.

CONCLUSIONS

The major finding of the present studies was that blood microRNA profiles are associated with future vulnerability or ongoing resilience to stress. These findings provide proof-of-principle that microRNA content in blood is a fruitful biomarker for vulnerability or resilience to stress. Follow up studies in stressed

and affected patient populations are necessary to determine the validity of circulating microRNAs as biomarkers of stress-related disease. The extent of individual-to-individual variations and robustness of microRNAs in blood or brain of stressed individuals is not yet clear. However, the results observed here are encouraging due to the overlap in the microRNAs highlighted here with other studies of rodent neuropsychiatric models and affected human populations. Current efforts directed at establishing platforms for reliable and validated microRNA quantification in saliva and other biological fluids (Weber et al., 2010) may ultimately yield non-invasive methods of microRNA assessment that are more amenable for clinical and in-the-field assessment. Thus, the addition of microRNA profiling to other modalities, such as neuropsychiatric evaluation and neuroimaging, may provide a more comprehensive way to identify individuals vulnerable to stress and aid in the development of individualized prophylactic and treatment therapies.

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The authors have nothing to disclose.

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FIGURE LEGENDS

Figure 1. Acute stress elevates circulating levels of let-7a-5p, let-7c-5p, and let-7i-5p, and suppresses miR-342-3p and miR-361-5p for 24 h. Blood samples were obtained 3 days prior to as well as at 1 h, 4 h, and 24 h following acute social defeat stress. MicroRNAs with significantly different levels compared to the pre-stress timepoint are shown. Of note, levels of circulating let-7a-5p, let-7c-5p, and let-7i-5p increased 1 h following acute stress, and remained elevated for 4 h and 24 h (A). Additionally, acute stress induced a consistent and prolonged suppression of miR-342-3p and miR-361-5p (B). Other microRNAs were significantly different at one or two of the three timepoints. Asterisks denote statistical significance compared to pre-stress samples ($p < 0.1$) after Benjamini-Hochberg correction for multiple comparisons.

Figure 2. Vulnerable rats are characterized by increased anxiety-like behaviors. (A) Latencies to be defeated were averaged over the 7 days of stress and are shown for the animals in the chronic stress studies in which microRNAs were assessed. Based on our previous work (Wood et al., 2010) and cluster analysis, animals exhibiting average latencies of < 300 s were classified as short latency (SL) and rats exhibiting average latencies of > 300 s were classified as long latency (LL) subpopulations. (B) Both SL and LL rats are similarly responsive to social stress as measured by a suppression in body weight gain compared to controls. (C) However, the SL rats spend less time interacting with a novel rat *compared to controls*, indicating increased anxiety-like behavior. These data and our previously published work (Wood et al., 2010) suggest that SL rats are vulnerable to chronic social stress but that LL rats are resilient to the effects of stress. Data represent mean $\pm$ SEM. Asterisks denote statistical significance ($p < 0.05$).

Figure 3. A panel of circulating microRNAs is suppressed in rats that become vulnerable to subsequent chronic stress. MicroRNA expression was evaluated by microarray in blood samples from rats 3 days prior to onset of chronic social defeat. (A) Of the 170 circulating microRNAs that were above background expression and subsequently analyzed, miR-24-2-5p, miR-27a-3p, miR-30e-5p, miR-3590-3p, miR-362-3p, and miR-532-5p were suppressed in SL vulnerable rats *compared to controls*. Expression of three of these microRNAs, miR-24-2-5p (B), miR-27a-3p (C), and miR-362-3p (D) significantly correlated with average latencies to be defeated in the chronic social defeat paradigm. Data represent mean $\pm$ SEM. Asterisks denote statistical significance compared to control samples ($p < 0.1$) after Benjamini-Hochberg correction for multiple comparisons.

Figure 4. MiR-139-5p, miR-28-3p, miR-326-3p, and miR-99b-5p are suppressed in the blood of rats resilient to chronic stress. 24 h after the completion of chronic social defeat, microRNA levels in blood were evaluated. Of the 170 circulating microRNAs that were above background expression and subsequently analyzed, LL rats had reduced levels of miR-139-5p, miR-28-3p, miR-326-3p, and miR-99b-5p *compared to control rats*. (B) In an independent cohort of SL and LL rats, there was a significant correlation between average latency score and miR-326-3p levels by qPCR. Data represent mean $\pm$ SEM. Asterisks denote statistical significance compared to control samples ($p < 0.1$) after Benjamini-Hochberg correction for multiple comparisons.

Figure 5. Levels of miR-126-a-3p and miR-708-5p are elevated in the mPFC of rats vulnerable to chronic social defeat. After 7 days of social defeat, analysis of microRNAs in the mPFC indicated that SL rats exhibited increased levels of miR-126-a-3p (A, left) compared to LL rats and increased levels of miR-708-5p (A, right) compared to LL and control rats. *The mPFC tissue collected is shown in Panel B.* Image is taken from Paxinos and Watson, 4th edition. In a separate cohort of rats, qPCR was used to validate these findings and indicated significant negative correlations in levels of both microRNAs with average latency to be defeated (C, D). Data represent mean $\pm$ SEM. Asterisks denote statistical significance ($p < 0.1$) after Benjamini-Hochberg correction for multiple comparisons.

Figure 6. MicroRNAs differentially expressed in the BLA of SL and LL rats compared to control rats. Out of the total of 213 microRNAs that were expressed above background and further analyzed, 77 significantly differed between SL and control rats, and 42 significantly differed between LL and control rats. Overall, the expression in SL and LL rats was similar for all microRNAs examined and no significant differences between these two groups were observed. Shown here are the top 15 significantly expressed microRNAs (based on p-values) between control rats and SL or LL rats (A). Panel B shows levels of 3 microRNAs in the BLA for which expression was also significantly affected in the blood or in the mPFC. *Panel C shows the BLA tissue examined in this study.* Image is taken from Paxinos and Watson, 4th edition. Data represent mean $\pm$ SEM. Asterisks denote statistical significance compared to control samples ($p < 0.1$) after Benjamini-Hochberg correction for multiple comparisons.

Figure 1.

A

B

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

miR	SL	LL
Pre-stress Blood		
miR-24-2-5p	-1.35 ± 0.13*	-1.10 ± 0.49
miR-27a-3p	-1.53 ± 0.22*	-0.95 ± 0.88
miR-30e-5p	-1.46 ± 0.19*	-0.31 ± 0.58
miR-362-3p	-1.56 ± 0.18*	-1.05 ± 0.55
Post-stress Blood		
miR-28-3p	-0.83 ± 0.51	-1.69 ± 0.31*
miR-99b-5p	-3.42 ± 0.49*	-3.74 ± 0.88*
miR-139-5p	-0.55 ± 0.5	-1.45 ± 0.23 ⁺
miR-326-3p	0.52 ± 0.63	-2.44 ± 0.84*

Table 1. To validate the results from the microRNA microarrays, the expression of a subset of significantly regulated microRNAs in the pre-stress blood and post-stress blood was analyzed by qPCR in independent cohorts of control, SL and LL rats. Fold Change in expression of each microRNA in SL and LL rats compared to controls are shown with +/- SEM.

- * indicates significantly different from Control $p < 0.05$
- + indicates a trend towards significance from Control $p < 0.1$

Table 2

Table 2: Significant microRNA expression in the basolateral amygdala in response to chronic social stress. Animals were subjected to a seven day chronic social defeat stress. If the average latency to defeat over the seven days was less than 300 seconds, animals were classified as short latency (SL) animals. If the average latency was longer than 300 seconds, animals were classified as long latency (LL) animals. Evidence suggests that SL rats are vulnerable to stress whereas LL rats are resilient to stress. Control animals were introduced to a novel cage on each of the seven days. Fresh tissue punches were taken 24 hours after the last social defeat stress. Expression levels are shown for microRNAs that were statistically significant between pairs of groups and are shown as the average Log₂ [Intensity] with the standard error. Data was analyzed in R using the Benjamini-Hochberg correction for multiple comparisons. The p-value was set at $p < 0.1$. Only the miRNAs with overall average expression values over 2 and adjusted p-values < 0.1 are shown here. miRBase v.17 nomenclature is used here, whereas all other figures, tables, and main text use the updated miRBase v.21.

SL vs Ctl							
ProbeSetID	Ctl		SL		LL		Adj. p-value
	Average	SEM	Average	SEM	Average	SEM	
let-7a	10.225	0.383	11.728	0.172	11.383	0.305	0.019
let-7b	12.723	0.261	13.398	0.018	13.398	0.060	0.063
let-7d	11.737	0.309	12.565	0.044	12.438	0.169	0.054
let-7e	11.814	0.172	12.879	0.015	12.633	0.193	0.016
let-7f	5.797	0.526	8.070	0.206	7.576	0.386	0.012
let-7i	8.331	0.470	9.240	0.132	9.002	0.036	0.086
miR-106b-star	4.350	0.225	2.604	0.334	2.218	0.756	0.053
miR-124	13.179	0.297	12.336	0.152	12.480	0.161	0.063
miR-124-star	4.180	0.367	2.068	0.247	2.291	0.576	0.018
miR-125a-5p	10.635	0.079	11.460	0.068	11.265	0.094	0.018
miR-127-star	5.457	0.679	2.917	0.645	4.036	0.534	0.038
miR-128	9.210	0.257	10.097	0.118	9.931	0.098	0.037
miR-129	5.153	0.210	5.913	0.257	5.840	0.057	0.089
miR-129-2-star	6.318	0.186	7.093	0.135	7.267	0.130	0.046
miR-130a	6.587	0.099	3.462	0.682	3.924	0.878	0.021
miR-132	10.621	0.199	11.287	0.122	11.135	0.146	0.074
miR-135a-star	4.026	0.327	5.324	0.423	5.837	0.485	0.098
miR-137-star	5.177	0.241	6.464	0.168	5.728	0.225	0.018
miR-138	11.728	0.332	10.775	0.150	10.903	0.286	0.065
miR-140-star	8.735	0.311	7.242	0.115	7.605	0.134	0.009
miR-143	8.350	0.611	5.967	0.358	6.245	0.208	0.016
miR-145	10.113	0.637	8.578	0.130	8.435	0.178	0.037
miR-150	6.930	0.388	8.413	0.050	8.257	0.225	0.016
miR-151	8.104	0.193	8.913	0.060	8.943	0.113	0.030
miR-151-star	6.511	0.238	7.219	0.148	6.829	0.071	0.068
miR-152	3.987	0.884	1.941	0.545	2.123	0.399	0.072
miR-15b	2.252	0.602	3.866	0.493	3.726	0.529	0.099
miR-16	9.012	0.321	7.065	0.191	7.017	0.416	0.012

miR-17-5p	6.512	0.141	5.227	0.285	5.677	0.300	0.027
miR-181a-1-star	2.955	0.462	1.507	0.168	1.809	0.414	0.040
miR-181c-star	3.345	0.499	1.651	0.110	2.525	0.438	0.025
miR-187	5.855	0.417	2.893	0.670	3.400	0.745	0.023
miR-20a	4.664	0.170	3.035	0.548	3.594	0.517	0.063
miR-210	5.432	0.528	2.126	0.329	2.659	0.439	0.003
miR-219-2-3p	6.595	0.295	7.649	0.276	8.360	0.089	0.046
miR-22	8.417	0.238	6.600	0.499	7.096	0.365	0.030
miR-24-2-star	3.436	0.532	2.026	0.156	2.448	0.108	0.030
miR-29b	3.160	0.425	1.557	0.246	1.522	0.146	0.018
miR-30a	6.647	0.131	4.301	0.693	4.189	1.003	0.067
miR-30b-5p	4.876	0.300	2.860	0.560	2.715	0.633	0.046
miR-324-3p	6.759	0.120	5.112	0.676	5.952	0.376	0.074
miR-328a	7.033	0.061	8.073	0.199	7.953	0.124	0.018
miR-329	5.483	0.190	7.552	0.140	7.334	0.329	0.003
miR-330	4.614	0.586	2.922	0.411	2.811	0.649	0.085
miR-339-3p	3.257	0.395	1.839	0.169	2.425	0.495	0.045
miR-339-5p	3.816	0.176	2.430	0.303	2.308	0.415	0.037
miR-341	5.162	0.823	2.688	0.395	2.234	0.392	0.025
miR-344a-3p	8.295	0.441	7.003	0.216	7.072	0.204	0.038
miR-344b-2-3p	3.888	0.709	2.129	0.379	2.103	0.300	0.053
miR-345-5p	4.729	0.312	2.806	0.452	3.569	0.415	0.025
miR-352	3.192	0.515	4.836	0.245	4.047	0.227	0.025
miR-370	4.950	0.355	3.564	0.424	4.595	0.577	0.100
miR-376a	3.739	0.758	1.517	0.236	1.167	0.192	0.019
miR-378	7.604	0.333	6.515	0.259	6.785	0.322	0.066
miR-382	7.834	0.222	9.409	0.107	9.417	0.220	0.004
miR-409-5p	5.889	0.402	3.720	0.700	4.217	0.614	0.055
miR-434	7.175	0.232	8.191	0.169	7.969	0.102	0.025
miR-434-star	3.925	0.403	1.982	0.506	2.021	0.542	0.043
miR-466b-1-star	7.854	0.243	8.950	0.314	9.067	0.197	0.050
miR-466b-2-star	3.784	0.461	5.354	0.342	5.500	0.318	0.038
miR-485	7.725	0.185	8.880	0.215	8.835	0.163	0.019
miR-490-star	3.504	0.478	1.983	0.259	2.491	0.458	0.046
miR-497	5.064	0.397	1.645	0.370	1.869	0.364	0.003
miR-500	5.016	0.240	2.764	0.403	3.565	0.620	0.019
miR-503	2.822	0.408	1.742	0.143	2.028	0.225	0.050
miR-505-star	5.299	0.138	6.857	0.237	6.494	0.288	0.012
miR-542-5p	3.241	0.718	1.647	0.269	1.848	0.213	0.054
miR-551b	4.997	0.985	2.307	0.432	2.383	0.545	0.037

miR-652-star	2.745	0.329	1.605	0.180	2.271	0.387	0.054
miR-664-1-star	2.475	0.431	4.742	0.255	4.453	0.166	0.004
miR-664-2-star	5.913	0.112	7.262	0.244	7.115	0.272	0.018
miR-672	6.723	0.138	7.955	0.160	7.834	0.250	0.016
miR-674-3p	3.292	0.457	4.718	0.325	4.689	0.203	0.041
miR-708	6.764	0.543	4.977	0.494	5.489	0.530	0.063
miR-92b	7.117	0.226	7.919	0.211	7.721	0.096	0.063
miR-93	7.201	0.041	6.275	0.122	6.605	0.218	0.023
miR-99a	8.252	0.108	7.441	0.198	7.401	0.128	0.045
LL vs Ctl							
ProbeSetID	Ctl		SL		LL		Adj. p-value
	Average	SEM	Average	SEM	Average	SEM	
let-7a	10.225	0.383	11.728	0.172	11.383	0.305	0.080
let-7e	11.814	0.172	12.879	0.015	12.633	0.193	0.059
let-7f	5.797	0.526	8.070	0.206	7.576	0.386	0.046
miR-106b-star	4.350	0.225	2.604	0.334	2.218	0.756	0.046
miR-124-star	4.180	0.367	2.068	0.247	2.291	0.576	0.046
miR-125a-5p	10.635	0.079	11.460	0.068	11.265	0.094	0.074
miR-129-2-star	6.318	0.186	7.093	0.135	7.267	0.130	0.046
miR-130a	6.587	0.099	3.462	0.682	3.924	0.878	0.065
miR-135a-star	4.026	0.327	5.324	0.423	5.837	0.485	0.059
miR-140-star	8.735	0.311	7.242	0.115	7.605	0.134	0.046
miR-143	8.350	0.611	5.967	0.358	6.245	0.208	0.046
miR-145	10.113	0.637	8.578	0.130	8.435	0.178	0.046
miR-150	6.930	0.388	8.413	0.050	8.257	0.225	0.046
miR-151	8.104	0.193	8.913	0.060	8.943	0.113	0.046
miR-16	9.012	0.321	7.065	0.191	7.017	0.416	0.017
miR-187	5.855	0.417	2.893	0.670	3.400	0.745	0.075
miR-210	5.432	0.528	2.126	0.329	2.659	0.439	0.017
miR-219-2-3p	6.595	0.295	7.649	0.276	8.360	0.089	0.017
miR-27a	6.070	0.312	5.079	0.319	3.481	0.733	0.034
miR-29b	3.160	0.425	1.557	0.246	1.522	0.146	0.037
miR-30b-5p	4.876	0.300	2.860	0.560	2.715	0.633	0.065
miR-328a	7.033	0.061	8.073	0.199	7.953	0.124	0.046
miR-329	5.483	0.190	7.552	0.140	7.334	0.329	0.011
miR-339-5p	3.816	0.176	2.430	0.303	2.308	0.415	0.046
miR-341	5.162	0.823	2.688	0.395	2.234	0.392	0.035
miR-344a-3p	8.295	0.441	7.003	0.216	7.072	0.204	0.075
miR-344b-2-3p	3.888	0.709	2.129	0.379	2.103	0.300	0.088
miR-376a	3.739	0.758	1.517	0.236	1.167	0.192	0.025

miR-382	7.834	0.222	9.409	0.107	9.417	0.220	0.011
miR-434	7.175	0.232	8.191	0.169	7.969	0.102	0.098
miR-434-star	3.925	0.403	1.982	0.506	2.021	0.542	0.076
miR-466b-1-star	7.854	0.243	8.950	0.314	9.067	0.197	0.065
miR-466b-2-star	3.784	0.461	5.354	0.342	5.500	0.318	0.047
miR-485	7.725	0.185	8.880	0.215	8.835	0.163	0.046
miR-497	5.064	0.397	1.645	0.370	1.869	0.364	0.009
miR-505-star	5.299	0.138	6.857	0.237	6.494	0.288	0.046
miR-551b	4.997	0.985	2.307	0.432	2.383	0.545	0.068
miR-664-1-star	2.475	0.431	4.742	0.255	4.453	0.166	0.017
miR-664-2-star	5.913	0.112	7.262	0.244	7.115	0.272	0.046
miR-672	6.723	0.138	7.955	0.160	7.834	0.250	0.046
miR-674-3p	3.292	0.457	4.718	0.325	4.689	0.203	0.075
miR-99a	8.252	0.108	7.441	0.198	7.401	0.128	0.065