CHRONIC MILD STRESS EXACERBATES SEVERITY OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS IN ASSOCIATION WITH ALTERED NON-CODING RNA AND METABOLIC BIOMARKERS

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Abstract—The causal factors determining the onset and severity of multiple sclerosis (MS) are not well understood. Here, we investigated the influence of chronic stress on clinical symptoms, metabolic and epigenetic manifestations of experimental autoimmune encephalomyelitis (EAE), a common animal model of MS. Lewis rats were immunized for monophasic EAE with MBP_{69-88} and were exposed to chronic stress for 37 days starting 7 days prior to immunization. The exposure to stress accelerated and exacerbated the clinical symptoms of EAE. Both stress and EAE also disrupted metabolic status as indicated by trace elemental analysis in body hair. Stress particularly exacerbated chlorine deposition in EAE animals. Moreover, deep sequencing revealed a considerable impact of stress on microRNA expression in EAE. EAE by itself upregulated microRNA expression in lumbar spinal cord, including miR-21, miR-142-3p, miR-142-5p, miR-146a, and miR-155. Stress in EAE further up-regulated miR-16, miR-146a and miR-155 levels. The latter two microRNAs are recognized biomarkers of human MS. Thus, stress may synergistically exacerbate severity of EAE by altering epigenetic regulatory pathways. The findings suggest that stress may represent a significant risk factor for symptomatic deterioration in MS. Stress-related metabolic and microRNA signatures support their value as biomarkers for predicting the risk and severity of MS. © 2017 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: multiple sclerosis, stress, metabolites, elemental hair analysis, epigenetic, microRNA.

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INTRODUCTION

Multiple sclerosis (MS) is considered as an autoimmune disorder and characterized by long-lasting neuroinflammation accompanied by leukocyte infiltration into the central nervous system (CNS), destruction of CNS myelin, axonal loss and gliosis. The exact cause of MS is not known, however, causal mechanisms are believed to include genetic and environmental components. Previously studied environmental factors include availability of vitamin D (Munger et al., 2004), exposure to Epstein–Barr virus (Ascherio and Munger, 2010), and stress (Artemiadis et al., 2011).

The causal factors contributing to onset and severity of MS are not well understood; however, stress was suggested to modulate the inflammatory processes and symptoms of this disease (Huitinga et al., 2004; Heesen et al., 2007 b). Patients afflicted by MS frequently report that stress triggers relapses and exacerbates the clinical symptoms (Ackerman et al., 2002; Buljevac et al., 2003; Heesen et al., 2007a). Furthermore, psychological stress may exacerbate the course of relapsing-remitting MS (Karagkouni et al., 2013) and cause new lesions in the brain of MS patients (Mohr et al., 2004; Gold et al., 2005). Depending on its duration and severity, stress can disrupt immune functions and autoimmunity processes. In animal models, chronic stress in particular has been associated with increased vulnerability to infectious disease and autoimmunity (Godbout and Glaser, 2006; Harpaz et al., 2013), CNS inflammation (de Pablos et al., 2006) and experimental neuropathologies (Smith et al., 2008).

As a response to a stressful experience, the hypothalamic–pituitary–adrenal (HPA) axis secretes glucocorticoids, such as cortisol, which inhibits the activation, proliferation and recruitment of immune cells (Barnes, 2006). On one hand, excess glucocorticoids may suppress the immune system and reduce inflammation within the peripheral nervous system (Perez-Nievas et al., 2010). On the other hand, elevated glucocorticoid levels may actually exert pro-inflammatory actions (O'Connor et al., 2003) and prolonged stress may result in glucocorticoid receptor resistance that prevents the downregulation of inflammatory responses (Cohen et al., 2012). Dinkel et al. (Dinkel et al., 2003) reported that excess glucocorticoids increase the inflammatory response in response to neuronal injury. In line with these

complex effects, stress was shown to diminish symptoms in a common animal model of MS, experimental autoimmune encephalomyelitis (EAE) (Levine et al., 1962; Perez-Nievas et al., 2010), while other studies indicated that stress may rather accelerate EAE onset and symptoms (Chandler et al., 2002). However, no systematic study has yet linked chronic stress to the course of EAE and associated inflammatory, metabolic and potential epigenetic responses.

Epigenetic regulation, through components such as microRNAs (miRNAs), may critically affect immune system activation, neurogenesis and myelin formation in MS (Baek et al., 2008; Selbach et al., 2008; Junker, 2011; Junker et al., 2011). We hypothesized that stress will alter the characteristic miRNA signatures of EAE and offer a new experience-dependent biomarker of MS. In the present study, we used MBP-induced EAE as a rat model of hallmark neuropathological features of MS to determine if chronic mild stress may act as a potentially predisposing and accelerating factor in MS. In addition to the classic motor symptoms, we examined effects of stress on inflammatory and metabolic functions in EAE in relation to miRNA deep sequencing profiles. Thus, the present study is the first to investigate a multi-level signature of stress and EAE including up-stream epigenetic and down-stream metabolic biomarkers.

EXPERIMENTAL PROCEDURES

Animals

Thirty-one female Lewis (LEW) rats (56–70 days old; Charles River) were used. The animals were housed in groups of two or three under standard environmental conditions (12:12 h light/dark cycle with lights on at 7:30 AM). Animals had access to food and water *ad libitum*. All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the institutional Animal Care Committee of the University of Lethbridge.

Experimental design

The experiment design allowed detailed neurobehavioral assessment over a 37-day period followed by metabolic and spinal cord miRNA changes on day 30 post-EAE induction. Baseline testing prior to EAE induction included mechanical allodynia to test pain ("Baseline"). Animals were randomly assigned to one of the following experimental groups: (1) CFA/MBP-inoculation (EAE $n = 9$); (2) inoculated with CFA/MBP and experienced restraint stress for a total of 37 days (EAE + Stress $n = 8$); (3) CFA inoculation and exposure to stress for 37 days (Stress $n = 7$); and (4) CFA inoculation only (Control $n = 7$). Starting seven days prior to EAE immunization, stress groups (15 Lewis rats: EAE + Stress $n = 8$ and Stress $n = 7$) experienced daily restraint stress for 20 min in the morning hours (between 8:00 and 10:00 AM) for 37 consecutive days, up to day 30 post-immunization. Rats were monitored daily for weight loss and neurological signs using the 5-Point Scale as described below. Post-immunization

testing included mechanical allodynia tests on day 5 after immunization ("Pre"), onset of EAE signs ("Onset"), peak of EAE clinical severity ("Peak"), first day of full recovery from EAE ("Recovery"), and 10 days following full recovery ("Post").

Stress protocol

Animals were placed individually in transparent Plexiglas cylinders (5-cm inner diameter) that maintained them in a standing position without compression of the body (Metz et al., 2005). Ventilation was possible through perforated ends of the container.

Induction of EAE

Seventeen rats were immunized with guinea pig (GP) myelin basic protein (MBP) (segment 69–88: YGSLPQKSQRSQDENPVVHF) obtained from GenScript (Piscataway, NJ) for monophasic EAE. EAE was induced by subcutaneous immunization with GP MBP$_{69-88}$ emulsified in Freund's adjuvant (Becton Dickonson Co., NJ) at the base of the tail. Freund's adjuvant was supplemented with 4-mg/ml heat killed *Mycobacterium tuberculosis* H37Ra (Becton Dickonson Co., NJ) to make complete Freund's adjuvant (CFA). The final concentration of CFA in the emulsion was 1 mg/ml. Stress and control rats were treated as sham controls with CFA only.

Behavioral testing

Behavioral assessment was performed from video recorded data by observers blind to experimental groups and treatments.

Monitoring clinical EAE symptoms using the 5-Point Scale

Rats were weighed and examined daily for neurological signs using the classic 5-Point Scale according to previously published criteria (Stromnes and Goverman, 2006). Signs of EAE were graded on the following 5-point scale: Grade 0, no clinical signs; Grade 1, paralyzed tail; Grade 2, loss of limb coordination; hind limb paresis; Grade 3, both hind limbs paralyzed: Grade 4: forelimbs paralyzed; Grade 5, moribund (Stromnes and Goverman, 2006).

Mechanical allodynia

A set of calibrated von Frey hair monofilaments were used daily to assess sensitivity to punctate mechanical stimuli as a measurement of pain sensitivity (Olechowski et al., 2009, 2013). Rats were placed in a clear Plexiglas chamber on an elevated wire mesh screen. Calibrated von Frey hair filaments were applied to the plantar surface of each hind paw in the ascending order of bending force (range: 2.0–100.0 g). Each hair was applied 5 times per paw, and the number of nocifensive responses (vigorous shaking, prolonged lifting, licking or biting of the stimulated paw) was recorded. The monofilament which produced nocifensive responses

greater than 60% of the time was recorded as the mechanical nociceptive threshold.

Tissue collection

After completion of behavioral tests, rats were anesthetized and blood samples were collected by cardiac puncture between days 31 and 33 post-EAE immunization using heparinized catheters and collection tubes. All samples were collected at the same time of day. The rats were then sacrificed by intracardiac infusion with 0.2 ml of sodium pentobarbital (Euthansol; CDMV Inc., QC). After cardiac arrest, the animals were rapidly decapitated and cervical spinal cord segments were collected and flash-frozen for further miRNA profiling. In addition, hair samples were also collected and stored at room temperature for further analysis.

Deep sequencing miRNA analysis

Total RNA was isolated from cervical spinal cord segments from Stress + EAE, EAE, Stress Only and Naïve control rats ($n = 3$ each) as previously described (McCreary et al., 2016). Illumina TruSeq Small RNA sequencing kits were used for library preparation. The samples were given individual barcodes. Barcoded small RNA libraries were loaded on a single flow-cell lane and sequenced using an Illumina GAIIx genomic analyzer (Illumina, CA). Briefly, base calling and demultiplexing was completed using CASAVA 1.8.1 software pipeline with default settings. Short read quality was examined using FastQC software (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/).

Since miRNAs are mostly shorter than 26 bp, a substantial portion of short reads was expected to contain part of the adapter sequence; adapters were trimmed using Cutadapt software (http://code.google.com/p/cutadapt/). Adapters were trimmed from 3′ ends using Cutadapt software with options set to retain only sequences between 17 and 27 nt since this was the expected range of miRNA length. FastQC quality check was performed and counting was performed using standalone MicroRazerS version 1.0 (Emde et al., 2010). Count data from MicroRazerS was supplied to DESeq bioconductor package for statistical comparisons (Anders and Huber, 2010). On average >55% of trimmed reads were mapped (allowing 1 mismatch) to hairpin miRNA sequences. miRNAs having less than 5 reads on average were removed from further analysis. Quality check of data was done and the report showed that at least two of the biologically replicated samples cluster together. Results were filtered for differentially expressed miRNAs using p-values ($p < 0.05$, corrected for multiple testing using the Benjamini–Hochberg method), and false discovery rate (FDR) $\leq 10\%$.

Hair trace elemental analysis

Approximately 0.5 g of hair was collected from the abdomen and back from each rat post-mortem. To account for metal trace contamination by the collection process, fabric was cut with the same pair of scissors used for hair collection and values were subtracted from the experimental data. Samples were stored in 2-ml Eppendorf tubes at room temperature. Hair trace elemental analysis was performed by CanAlt Health Laboratories (City, ON, Canada). Briefly, about 300 ± 5 mg of each sample was transferred into tared, labeled centrifuge tubes, and the exact weight was recorded. To each sample digestion tube, 3.0 ml of reagent-grade nitric acid was added. Samples were incubated for 25 min and then subjected to acid microwave digest, in order to stabilize the elements of interest. The digested solution was analyzed for amounts of mineral element and trace metals by inductively coupled plasma mass spectrometry. Sample results were quantified by comparison with calibration solutions of known concentrations.

Statistical analysis

Statistical analyses were carried out using SPSS version 21.0 software (IBM, NY). Based on random assignment of animals to groups, statistical differences were compared between groups by a one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) post hoc test repeated measures analysis of variance (RM ANOVA) with LSD post hoc test, and t-test as necessary. In addition, if Mauchley's W test indicated violation of sphericity, the degrees of freedom were corrected using the Greenhouse–Geisser test. Statistical significance was set at $p < 0.05$. For miRNA data t-values were calculated with p-values below a p-value (< 0.10). All data are presented as mean ± standard error of the mean (SEM).

RESULTS

EAE reduces body weight

To reflect the physical impact of the chronic mild stress protocol body weight was determined daily throughout the 30-day time course after EAE induction. A significant effect of Group was found in body weight ($p < 0.05$). A significant reduction in body weight was observed in the EAE group ($p < 0.05$, LSD post hoc) and also in the EAE + Stress group ($p < 0.01$, LSD post hoc) compared to Controls (Fig. 1).

Stress exacerbates EAE symptom severity

Rats immunized with MBP_{69-88} were scored daily using the 5-point scale for signs of neurological impairment (Fig. 2A). There was a significant effect of Day ($F(2.45,66.17) = 21.42$, $p < 0.0001$), and Day × Group interaction ($F(7.35,66.17) = 7.83$, $p < 0.0001$). EAE animals developed clinical signs of disease between day 10 and day 16 post immunization. In EAE + Stress animals the clinical severity of EAE was greater between day 11 and 14 post immunization in comparison to animals with EAE treatment only (Fig. 2A). EAE animals initially presented with partial paralysis of the tail (Grade 0.5) and later progressed to a paralyzed tail and/or loss in coordinated movements (Grade 1) and hind limb paresis (Grade 2). EAE
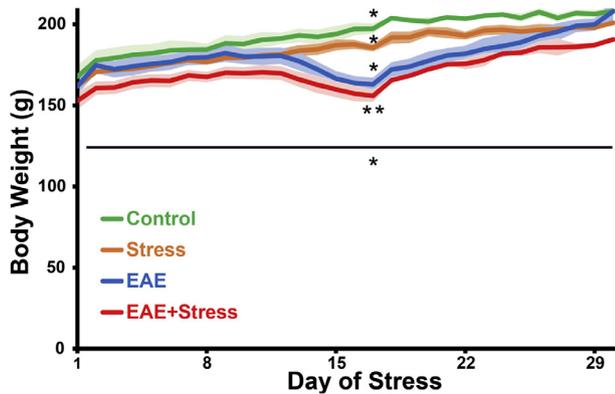
Fig. 1. EAE caused changes in body weight. Exposure to EAE caused a significant reduction in body weight in EAE ($p < 0.05$) and Stress + EAE ($p < 0.01$) animals compared to naïve Controls. Groups include naïve Controls, $n = 7$; Stress only, $n = 7$; EAE, $n = 9$; Stress + EAE, $n = 8$. Asterisks indicate significances: $^*p < 0.05$, $^{**}p < 0.01$. Error bars represent means ± SEM.

+ Stress animals initially presented with partial paralysis of the tail (Grade 0.5) and progressed to loss in coordinated movements, hind limb paresis and/or both hind limbs paralyzed (Grade 2, or 3, respectively). Therefore, EAE + Stress animals presented with more severe clinical symptoms than the EAE group. Accordingly, there were significant differences between the EAE ($p < 0.01$, LSD post hoc test) and EAE + Stress ($p < 0.01$, LSD post hoc test) groups in comparison to Stress or naïve Control animals. Conversely, there was a non-significant trend for an overall group difference between EAE and EAE + Stress animals ($p = 0.05$, LSD post hoc test).

EAE represents a critical determinant of pain threshold

Pain threshold was inferred based on the mechanical nociceptive response to von Frey hair stimulation of the hindpaw (Fig. 2B). There was a Phase effect ($F(3.35, 87.15) = 9.55$, $p < 0.001$), and Phase × Group interaction ($F(10.06, 87.15) = 4.88$, $p < 0.001$). Interestingly, Stress animals showed increased sensitivity to pain at baseline in comparison to Controls ($F(3, 27) = 1.81$, $p < 0.05$) indicating that acute stress increased mechanical sensitivity. By contrast, higher pain thresholds were seen during the peak phase of symptoms in EAE ($F(3, 27) = 5.32$, $p < 0.05$), and in EAE + Stress ($F(3, 27) = 5.32$, $p < 0.05$) animals in comparison to Stress animals. During the post phase of disease, EAE animals demonstrated a reduced pain threshold in comparison to Stress animals ($F(3, 27) = 1.52$, $p < 0.05$).

In addition, significant changes in pain threshold were seen during baseline and the pre-symptomatic phase of EAE ($p < 0.05$, paired t-test), onset and peak ($p < 0.05$, paired t-test), and peak and recovery phase of EAE ($p < 0.001$, paired t-test) in the EAE group. Likewise, EAE + Stress animals showed a significant difference in pain threshold during pre and onset ($p < 0.05$, paired t-test), onset and peak ($p < 0.05$,
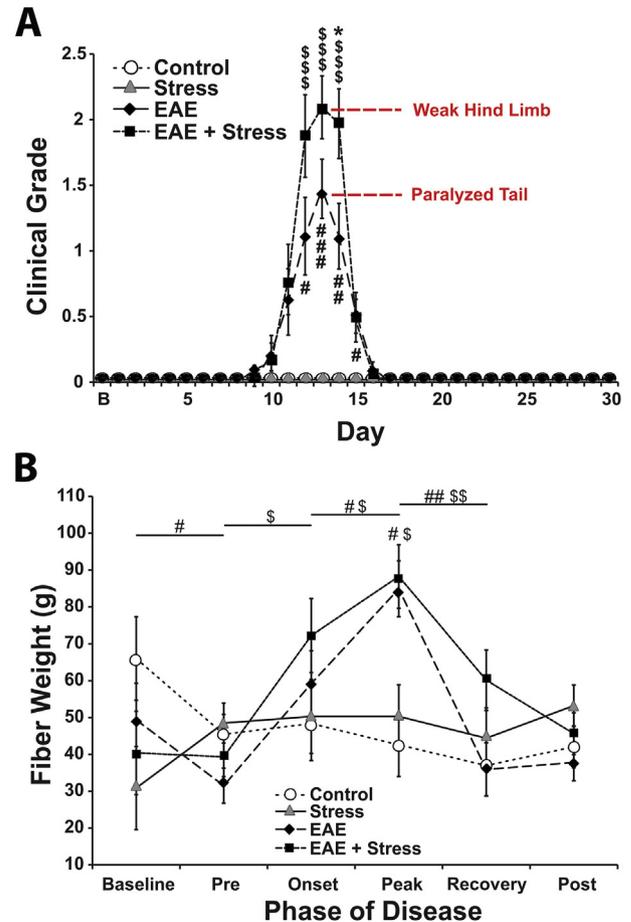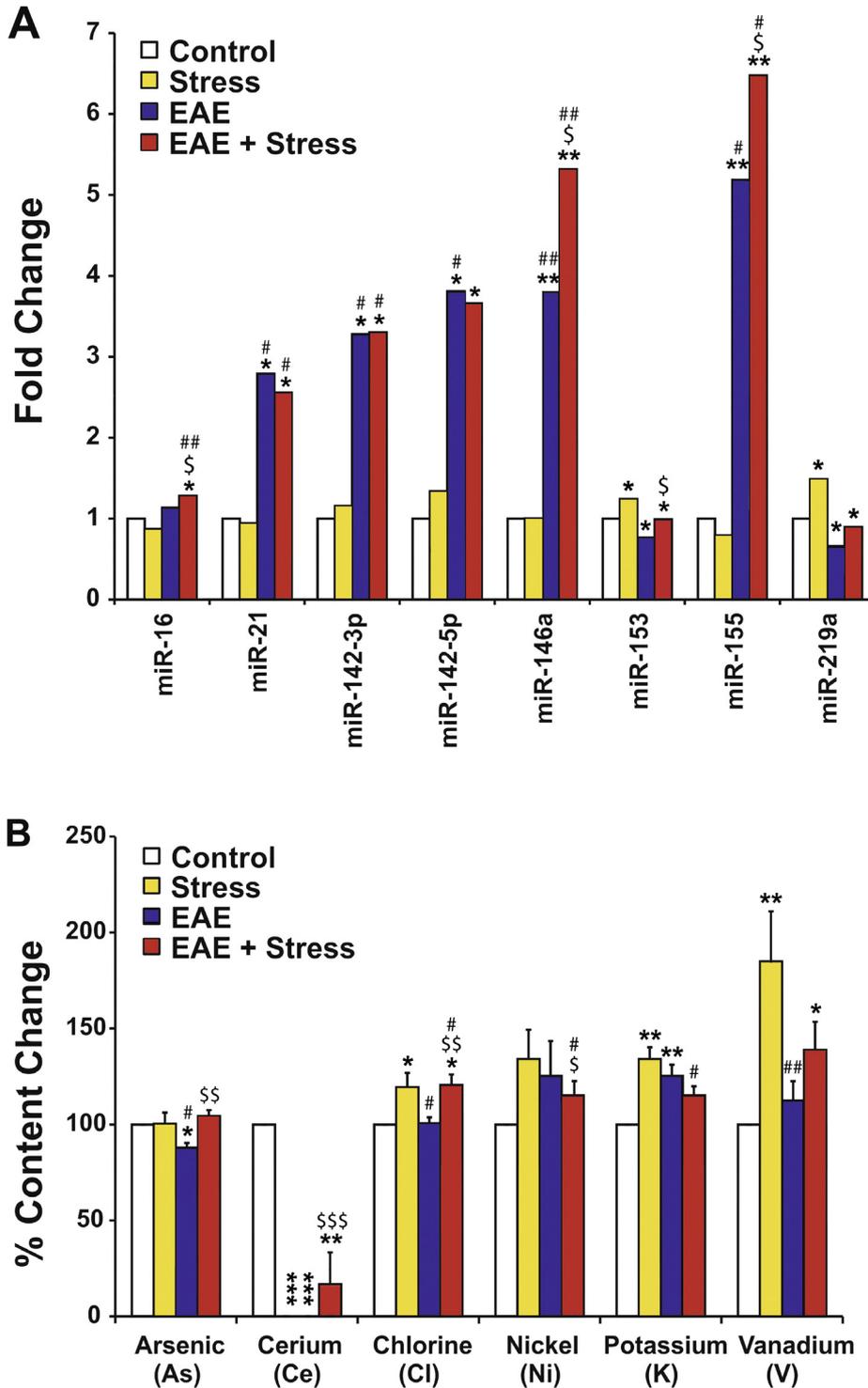

Fig. 2. Stress altered the time course of the severity of clinical symptoms and mechanical allodynia. (A) Clinical severity across 30 days revealed more severe symptoms among EAE + Stress animals compared to EAE alone. Stress exacerbated clinical symptoms of EAE from causing tail paralysis to causing hind limb paralysis. (B) Sensitivity to mechanical allodynia determined by various von Frey hair fiber weights. EAE and EAE + Stress animals showed reduced sensitivity at the peak of clinical symptoms. Symbols indicate significant differences: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, EAE vs. Control or paired comparisons, respectively; $^*p < 0.05$, EAE + Stress vs. EAE; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, EAE + Stress vs. Control. Error bars represent means ± SEM.

paired t-test), and peak and recovery phases of EAE ($p < 0.001$, paired t-test).

Interactions of EAE and stress dysregulated spinal miRNA expression

Deep sequencing analysis of the cervical spinal cord revealed 301 expressed mature miRNAs. Fig. 3A shows all miRNAs that were differentially expressed as a function of stress and/or EAE. In EAE compared to naïve Controls, five miRNAs were up-regulated, including miR-21-5p, miR-142-3p, miR-146a-5p, miR-142-5p, miR-155-5p (FDR ≤ 5%; see Fig. 3A). Stress + EAE compared to Control rats showed elevated expression of miR-21-5p, miR-142-3p, miR-146a-5p, miR-142-5p, and miR-155-5p. EAE compared to Stress rats showed up-regulated miR-21-5p, miR-142-3p,

miR-146a-5p, miR-142-5p, and miR-155-5p, whereas miR-219a-5p and miR-153-3p were down-regulated (Fig. 3A).

In Stress + EAE compared to Stress rats, miR-21-5p, miR-142-3p, miR-146a-5p, miR-142-5p, miR-155-5p and miR-16-5p were elevated (Fig. 3A). In turn, in Stress + EAE compared to EAE rats, miR-16, miR-146a, miR-153, miR-155, miR-196b-5p, miR-345-3p, miR-129-2-3p were up-regulated, and miR-153-3p, miR-140-3p and miR-673-3p were down-regulated ($p < 0.05$).

Stress and EAE result in metabolic imbalance

Hair trace elemental analysis (Fig. 3B) revealed that EAE reduced arsenic ($F(3,14) = 6.04$, $p < 0.05$) and cerium ($F(3,14) = 12.76$, $p < 0.001$) content levels, and increased potassium ($F(3,14) = 5.10$, $p < 0.01$) contents relative to naïve Controls. EAE + Stress decreased nickel and potassium levels in comparison to Stress and EAE treatments alone ($F(3,14) = 3.25$, $p < 0.05$; $F(3,14) = 5.10$, $p < 0.05$, respectively). Vanadium (V) levels were increased in EAE + Stress compared to Controls ($F(3,14) = 6.64$, $p = 0.01$). EAE + Stress also raised arsenic and chlorine deposition compared to EAE alone ($F(3,14) = 5.52$, $p < 0.01$; $F(3,14) = 6.04$, $p < 0.001$, respectively). Stress depleted cerium content levels in comparison to Controls ($F(3,14) = 12.76$, $p < 0.001$) while at the same time raising chlorine, potassium and vanadium levels ($F(3,14) = 5.52$, $p < 0.05$; $F(3,14) = 5.10$; $p < 0.01$, ($F(3,14) = 4.81$, $p < 0.01$, respectively). These findings suggest that stress and EAE synergistically alter fundamental metabolic functions leading to altered hair elemental accumulation.

Fig. 3. Stress is associated with epigenetic and metabolic biomarkers of autoimmunity and MS. (A) MicroRNA analysis of spinal cord tissue. In particular, expression of miR-21, miR-142-3p, miR-142-5p, miR-146a, miR-146 and miR-155 was elevated in both EAE and EAE + Stress animals in comparison to stress only or control animals. Stress in EAE animals exacerbated miR-16, miR-146a, and miR-155. Note that miR-146a and miR-155 are recognized biomarkers of human MS. (B) Hair trace element analysis as indicator of metabolic status. EAE reduced hair arsenic and cerium content and elevated potassium levels. Stress in naïve controls and in EAE rats particularly elevated chlorine and vanadium deposition. Data are shown as percentage of control values. Symbols indicate significant differences: $^*p < 0.05$, $^{**}p < 0.01$ compared to Control group; $^\$p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ EAE + Stress vs. EAE; $^\#p < 0.05$, $^{\#\#}p < 0.01$ compared to Stress group. Error bars represent means ± SEM.

DISCUSSION

The specific triggers of human MS are largely unknown. As one of the most potent modulators of immune and metabolic functions, stress may be a critical determinant of MS onset and severity. Here we determined if chronic mild stress affects hallmark behavioral, metabolic and epigenetic manifestations of MS in an EAE rat model. Exposure to chronic stress beginning prior to EAE induction exacerbated the clinical symptoms but not pain sensitivity. A new approach to study fundamental metabolic functions using hair trace elemental analysis revealed that stress elevated deposition of chlorine and cerium in EAE. EAE alone reduced the deposition of arsenic and cerium. Importantly, stress had large synergistic effects with EAE on central epigenetic regulatory pathways involving miRNA expression in the cervical spinal cord. In particular, miR-146a and miR-155, both regarded as biomarkers of human MS, appeared particularly responsive to stress and showed significant differences beyond changes linked to EAE alone. These findings support the hypothesis that stress may critically exacerbate EAE severity by altering upstream epigenetic regulation that is reflected in downstream metabolic markers. These findings propose that stress may also serve as a risk factor in human MS.

The present data are in line with previous observations showing that stress has long-lasting effects on sensorimotor functions (Metz et al., 2001, 2005), pain threshold (Olechowski et al., 2009, 2010, 2013) and the clinical severity of neurodegenerative disease (Smith et al., 2008; Babenko et al., 2012). Moreover, stress drastically exacerbated the clinical severity of EAE symptoms. These findings are in line with previous observations that stress may worsen EAE symptoms (Chandler et al., 2002), while others indicated that stress may diminish severity of clinical symptoms in EAE (Levine et al., 1962; Perez-Nievas et al., 2010). Reasons for this variation may be linked to severity, type and duration of the stressor and the rodent strain used (Metz et al., 2001, 2005). The present study used chronic exposure to repeated mild psychological stress starting prior to immunization as a protocol which bears particular ecological validity considering its prevalence in the human population (Wood et al., 2010; Agyei et al., 2014).

Stress-associated changes in EAE animals were also reflected in metabolic markers, such increased chlorine, cerium and vanadium content. Hair follicles grow for approximately 7–20 days in rats (Ambeskovic et al., 2013). Therefore, hair collected in the present study represents an assessment of cumulative changes during clinical EAE symptoms. In stressed EAE rats the accumulation of chlorine may indicate altered immune competence (Exon et al., 1987). Reduced hair levels of vanadium have been implicated in human MS patients (Ryan et al., 1978), however, the present data suggest that these levels are elevated as a function of stress. Vanadium is proposed to actively modify immune functions, specifically by increasing the level of circulating B and T cells (Mravcova et al., 1993). Cerium levels on the other hand were generally decreased by EAE treatment. Cerium has been associated with expression of

inflammatory cytokines (Sang et al., 2014) and up-regulated IL-1β (Sang et al., 2014). Changes in cerium content may therefore reflect inflammatory activity during stress and symptomatic EAE. Further research on the role of trace elements as predictive and/or causative biomarkers in EAE and MS is needed to be able to identify potential pathogenic metabolic pathways or diagnostic biomarkers.

Altered metabolic functions may be directly linked to altered epigenetic regulation of gene expression and immune status (Ahmed et al., 2009; Pearce and Pearce, 2013). Some of the underlying mechanisms that potentially mediate the impact of stress on EAE symptoms were investigated through miRNA profiling. Deep sequencing revealed that EAE up-regulated miR-21, miR-142-3p, miR142-5p, miR-146a, and miR-155 and moderately down-regulated miR-153, and miR-219a. Stress in EAE particularly exacerbated miR-16, miR-146-a and miR-155 expression, while stress alone had no effect on these miRNAs. Notably, the present findings reproduce epigenetic hallmark features of human MS, as expression of miR-21, miR-142-3p, miR-142-5p, miR-146a, and miR-155 were found to be increased in active human MS lesions (Junker et al., 2009; Koch et al., 2013). miR-219 expression, on the other hand, is increased in inactive MS lesions (Junker et al., 2009) and associated with oligodendrocyte regulation and myelin maintenance (Li and Yao, 2012). miR-21 promotes Th17 cell differentiation and mediates EAE symptoms (Murugaiyan et al., 2015; Wang et al., 2016). Treatment with anti-miR-21 oligonucleotide is able to reduce the clinical severity of EAE (Murugaiyan et al., 2015). In addition, miR-21 and miR-146a are important regulators of Treg-triggered immune-suppression (Zhou et al., 2015). miR-142-3p, which inhibits IL-10 translation (Ding et al., 2012) along with miR-16 and miR-155 may represent suitable biomarkers of therapeutic response in MS (Arruda et al., 2015).

miR-21, miR-142, miR-146a, and miR-155 are believed to target genes regulating CD47 expression (Junker et al., 2009). miR-155 in particular has been described as a multifunctional non-coding RNA in pathological processes, including inflammation and response to elevated oxidative stress (Jacometo et al., 2015; Wang et al., 2015; Yang et al., 2015). Inhibition of miR-155 activity has been shown to exert angiogenic and neuroprotective properties (Caballero-Garrito et al., 2015) and to reduce expression of the integrin-associated protein CD47 in active MS lesions (Junker et al., 2009). CD47 inhibits the phagocytic activity of macrophages (Oldenborg et al., 2000; Yamao et al., 2002; Ishikawa-Sekigami et al., 2006) and cytokine production of dendritic cells (Latour et al., 2001). CD47 and its receptors are involved in the pathogenesis of autoimmune EAE by suppressing infiltration of pathogenic Th17 cells (Gao et al., 2016). Moreover, in human MS, miR-155 expression is increased in peripheral circulating CD14+ monocytes, which act as a co-receptor for the detection of pathogens (Junker et al., 2009; Moore et al., 2013).

On the other hand, miR-155 is able to up-regulate pro-inflammatory cytokine responses in CNS-resident myeloid cells and impairs adaptive immune responses

(Moore et al., 2013). Thus, although miR-155 may have potentially protective functions by preventing phagocytosis by macrophages, the present clinical findings suggest that its pro-inflammatory role may be dominating in the EAE model (Junker et al., 2009). This hypothesis is supported by the observation that mice lacking miR-155 do not develop EAE mainly due to defective T cell activity in the immune response (Lind and Ohashi, 2014).

Notably, miR-155 is elevated by stress (Lecchi et al., 2016) and it is also affected by various other environmental influences (Faraoni et al., 2009; Jacometo et al., 2015). miR-155 has therefore become an epigenetic biomarker of human MS while the present findings also confirm that this marker is highly responsive to stress in the EAE condition (Leung and Sharp, 2010; Babenko et al., 2012). Similar interactions may also apply to miR-146a, given that this miRNA was overexpressed by EAE and further elevated by stress. Its particular role in the control of Toll-like receptor and cytokine signaling (Taganov et al., 2006) as a function of stress still remains to be investigated in the EAE model.

CONCLUSIONS

Using an EAE rat model, the present findings suggest that chronic mild stress may critically influence clinical symptoms of MS through altering metabolic and epigenetic pathways. Stress may regulate epigenetic hallmarks of MS, in particular miR-146a, and miR-155, which may present a mechanism how environmental factors interact with pathogenic processes involved in MS. Adverse experience, via epigenetic regulatory mechanisms, may therefore potentially represent a risk factor in MS. The present findings provide new insights into experience-dependent modulation of demyelination and suggest new metabolic and epigenetic signatures of autoimmune processes that may provide a new opportunity to identify predictive biomarkers and therapeutic targets for MS.

ONE SENTENCE SUMMARY

Experience-dependent mechanisms alter epigenetic and metabolic biomarkers of MS and exacerbate clinical symptoms in an animal model.

Competing interests—*The authors declare that they have no competing interests.*

Authors' contributions—*BG, VWY, AL and GM conceived and designed the study. BG, OB and IG performed the experiments and acquired the data. BG, VS, OB, IK, AL and GM participated in the data analysis. BG, VS, AL and GM prepared the manuscript. All authors read and approved the final version of the manuscript.*

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