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Full Length Article

Inhibition of microRNA-124-3p as a novel therapeutic strategy for the treatment of Gulf War Illness: Evaluation in a rat model☆

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ABSTRACT

Gulf War Illness (GWI) is a chronic, multisymptom illness that continues to affect up to 30% of veterans deployed to the Persian Gulf during the 1990–1991 Gulf War. After nearly 30 years, useful treatments for GWI are lacking and underlying cellular and molecular mechanisms involved in its pathobiology remain poorly understood, although exposures to pyridostigmine bromide (PB) and pesticides are consistently identified to be among the strongest risk factors. Alleviation of the broad range of symptoms manifested in GWI, which involve the central nervous system, the neuroendocrine system, and the immune system likely requires therapies that are able to activate and inactivate a large set of orchestrated genes. Previous work in our laboratory using an established rat model of GWI identified persistent elevation of microRNA-124-3p (miR-124) levels in the hippocampus whose numerous gene targets are involved in cognition-associated pathways and neuroendocrine function. This study aimed to investigate the broad effects of miR-124 inhibition in the brain 9 months after completion of a 28-day exposure regimen of PB, DEET (N,N-diethyl-3-methylbenzamide), permethrin, and mild stress by profiling the hippocampal expression of genes known to play a critical role in synaptic plasticity, glucocorticoid signaling, and neurogenesis. We determined that intracerebroventricular infusion of a miR-124 antisense oligonucleotide (miR-124 inhibitor; 0.05-0.5 nmol/day/28 days), but not a negative control oligonucleotide, into the lateral ventricle of the brain caused increased protein expression of multiple validated miR-124 targets and increased expression of downstream target genes important for cognition and neuroendocrine signaling in the hippocampus. Off-target cardiotoxic effects were revealed in GWI rats receiving 0.1 nmol/day as indicated by the detection in plasma of 5 highly elevated protein cardiac injury markers and 6 upregulated cardiac-enriched miRNAs in plasma exosomes determined by next-generation sequencing. Results from this study suggest that *in vivo* inhibition of miR-124 function in the hippocampus is a promising, novel therapeutic approach to improve cognition and neuroendocrine dysfunction in GWI. Additional preclinical studies in animal models to assess feasibility and safety by developing a practical, noninvasive drug delivery system to the brain and exploring potential adverse toxicologic effects of miR-124 inhibition are warranted.

1. Introduction

Gulf War Illness (GWI) is a chronic, multisymptom illness that continues to affect up to 30% of veterans deployed to the Persian Gulf during the 1990–1991 Gulf War [Institute of Medicine, 1996; Research Advisory Committee on Gulf War Veterans' Illnesses (RAC), 2008, 2014; White et al., 2016; Sullivan et al., 2018]. Neurological health effects including concentration problems, learning and memory difficulties, and depression are among the most prominent symptoms reported; additional complaints include severe fatigue, headaches, musculoskeletal pain, gastrointestinal issues, skin rashes, poor coordination, sleep disturbances, and weakened immune function (Kang et al., 2009; Li B. et al., 2011a; Odegard et al., 2013; Smith et al., 2013). Behavioral and functional imaging studies in ill GW veterans revealed chronically altered structure and function of the hippocampus, a brain region critical for learning and memory formation (Menon et al., 2004; Vythilingam et al., 2005; Li X. et al., 2011b; Rayhan et al., 2013; Hubbard et al., 2014). Reduced white and gray matter volumes in

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cortical areas have also been reported in structural MRI studies (Chao et al., 2011; Rahan et al., 2013). More recently, functional and structural brain anomalies in GWI involving subcortical regions including the brainstem, cerebellum, and thalamus have been observed (Engdahl et al., 2016; Christova et al., 2017). Although some progress has been made, useful treatments for GWI are currently lacking after nearly 30 years, and underlying cellular and molecular mechanisms involved in its pathobiology remain poorly understood (White et al., 2016).

Epidemiological studies have consistently identified exposure to cholinesterase-inhibiting chemicals during deployment to be among the strongest risk factors for the persistent symptoms affecting GW veterans (Golomb, 2008; RAC, 2008, 2014; Steele et al., 2012; Kerr, 2015; White et al., 2016; Sullivan et al., 2018). These include the ingestion of pyridostigmine bromide (PB) pills issued as a prophylactic treatment against nerve agent attack and the overuse of insect repellants and pesticides such as DEET (*N,N*-diethyl-3-methylbenzamide) and permethrin. Detonations at Khamisiyah and other Iraqi weapons facilities may have also contributed low-level exposure to cholinesterase-inhibiting organophosphate nerve agents such as sarin (RAC, 2008, 2014). A recent study examining objective cognitive measures in GW-deployed preventative medicine personnel found that cognitive and mood functioning was most impacted in veterans with combined high pesticide and high PB exposure compared to those with high single exposures or low combined exposures to pesticides and PB (Sullivan et al., 2018). Importantly, emerging data from post-9/11 veterans deployed to Iraq and Afghanistan revealed that 37% of veterans from more recent wars in the region also suffer from chronic symptoms similar to GWI that are associated with pesticide exposure in particular, suggesting that less hazardous alternatives to pesticides should be employed in ongoing and future campaigns (DeBeer et al., 2017). Toxicity from pesticides is mediated by perturbation of ion channels and the cholinergic system, genetic damage, epigenetic modifications, endocrine disruption, mitochondrial dysfunction, oxidative stress, and other mechanisms, and is well known to be associated with chronic diseases affecting the nervous, endocrine, immune, reproductive, renal, cardiovascular, and respiratory systems (Manikkam et al., 2012; Collotta et al., 2013; Mostafalou and Abdollahi, 2013).

Epidemiological findings suggesting a role for pesticides and other neurotoxic chemicals as causative agents of GWI are supported experimentally by neuropathological and neurobehavioral changes observed in rodent models (Abdel-Rahman et al., 2002, 2004; Abdullah et al., 2011, 2012; Parihar et al., 2013; Hattiangady et al., 2014; Ojo et al., 2014; O'Callaghan et al., 2015; Zakirova et al., 2015). Our laboratory previously determined in an established rat model that exposure to physiologically relevant levels of PB, DEET, permethrin, and mild stress daily for 4 weeks resulted in long-term epigenetic alterations in the brain including a 3-fold increase in hippocampal microRNA-124-3p (miR-124) expression one year after the exposure period (Pierce et al., 2016). An upregulation of miR-124 in the hippocampus of GWI rats is consistent with other rodent studies that demonstrated impaired synaptic integrity and reduced hippocampal neurogenesis (thought to be critical for hippocampal plasticity and memory formation) associated with observed memory and mood deficits following GW exposures (Parihar et al., 2013; Hattiangady et al., 2014; Ojo et al., 2014; Megahed et al., 2015; Zakirova et al., 2015). Interestingly, increased miR-124 expression has also been observed in honey bees exposed to a field-realistic dose of the neonicotinoid insecticide thiamethoxam which has previously been shown to negatively affect memory and locomotor capacity of the bees (Shi et al., 2017). This insecticide is widely used in agriculture and acts on the nervous system through an agonistic action on nicotinic acetylcholine receptors (Shi et al., 2017).

MicroRNAs (miRNAs) are small non-coding RNAs that negatively regulate the expression of numerous target genes at the post-transcriptional level by binding to the 3′ untranslated region of miRNA targets (Salta and De Strooper, 2012). Their aberrant expression in neurodegenerative and other human diseases makes them potential drug targets, and therapeutic modulation of aberrantly expressed miRNAs via inhibitors or mimics is increasingly being considered as a treatment option (Sun et al., 2015; Saraiva et al., 2017). It is plausible that chronically elevated miR-124 levels in the hippocampus induced by a combination of GW-relevant neurotoxic chemicals and stress mediates the persistent neurological and neuroendocrine symptoms observed in veterans with GWI because miR-124 i) is the most abundant miRNA in the brain, ii) plays a critical role in the regulation of signaling molecules underlying synaptic plasticity and memory formation, iii) is involved in chronic stress, neurodegeneration, synapse morphology, neurogenesis, and neurotransmission, iv) affects a variety of glucocorticoid receptor and mineralocorticoid receptor-mediated physiological processes by reducing protein levels of these two receptors (known targets of miR-124), v) regulates mitochondrial activity and localization which are critical for maintaining energy and calcium homeostasis in neurons (mitochondrial dysfunction has been implicated in GWI), and vi) is highly conserved among species, ranging from insects to mammals (Fischbach and Carew, 2009; Vreugdenhil et al., 2009; Sober et al., 2010; Mannironi et al., 2013; Koslik et al., 2014; Pan-Vazquez et al., 2015; Sun et al., 2015; Abdullah et al., 2016; Chen et al., 2017; Shetty et al., 2017; Yardeni et al., 2018). Rodent studies determined that increased miR-124 negatively impacts memory by downregulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-mediated glutamate signaling, while inhibition of miR-124 in the hippocampus is associated with enhanced memory performance and elicits antidepressant-like effects (Dutta et al., 2013; Bahi et al., 2014). A polymorphism in the miR-124 target gene IQ motif containing GTPase activating protein 1 (IQGAP1) that impairs miR-124 binding to IQGAP1 has been shown to improve human cognitive agility (Yang et al., 2014). These findings suggest that *in vivo* inhibition of miR-124 function in the hippocampus is a promising novel therapeutic approach to improve cognition, emotion regulation, and neuroendocrine dysfunction in GWI.

In this study we used an established rat model of GWI to investigate the broad effects of miR-124 inhibition by profiling the hippocampal expression of downstream target genes important for cognition and neuroendocrine signaling after administration of a locked nucleic acid (LNA)-antisense oligonucleotide (miR-124 inhibitor) into the lateral ventricle of the brain. Therapeutic inhibition of miR-124 was evaluated 9 months after exposure to GW agents to mimic the persistent effects suffered by current veterans with GWI who are presently 28 years post-exposure.

2. Materials and methods

2.1. Chemicals

PB (≥ 98%), permethrin (analytical standard containing a 98.3% mixture of 26.7% cis and 71.6% trans isomers), and DEET (≥ 97%) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of the highest purity available from commercial vendors.

2.2. Animals

A total of 80 male Sprague-Dawley rats (8 weeks old) underwent the 28-day GW exposure protocol described below. Rats were obtained from Charles River (Wilmington, MA) and were housed in polycarbonate cages in HEPA-filtered laminar airflow racks with wood chip bedding under controlled lighting conditions (12 h light/12 h dark). Rats were allowed free access to standard laboratory diet and tap water. The study protocol was approved by the Institutional Animal Care and Use Committee at Tripler Army Medical Center and investigators complied with the policies as prescribed in the USDA Animal Welfare Act and the National Research Council's *Guide for the Care and Use of Laboratory Animals*. Facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.3. GW exposure protocol

The exposure regimen of Abdel-Rahman et al. involving physiologically-relevant doses of dermal DEET, dermal permethrin, and oral PB followed by 5 min of restraint stress was utilized (Abdel-Rahman et al., 2002, 2004; Parihar et al., 2013; Hattiangady et al., 2014; Pierce et al., 2016). GWI rats (n = 57) were treated daily with PB (1.3 mg/kg/day, oral in water), DEET (40 mg/kg/day, dermal in 70% ethanol), and permethrin (0.13 mg/kg/day, dermal in 70% ethanol) for 28 days. This exposure regimen has been designed to simulate the daily exposure experienced by veterans during the Gulf War (Abdel-Rahman et al., 2002, 2004). GWI rats were also subjected to 5 min of restraint stress every day for the duration of the exposure period. Restraint occurred after the chemical treatment by placing the rats in a Plexiglas cylinder. Vehicle-treated control rats (n = 23) were treated with a dermal application of 70% ethanol and oral water daily for 28 days using an equal volume of vehicle as that given to GWI rats. Oral doses were given by gavage (1 ml/kg body weight), while dermal applications (about 0.1 ml) were applied to the back of the neck on a 2.5 cm^2 area preshaved with clippers. Body weight measurements were obtained weekly throughout the exposure regimen and every two weeks during the post-exposure latency period. The post-exposure latency period was 5 months for an initial group of 20 rats that were used to establish effective miR-124 inhibitor doses and 9 months for a group of 60 experimental rats to mimic the persistent effects suffered by GW veterans. Treatment groups and animal numbers are presented in Fig. 1.

2.4. Locked nucleic acid (LNA)-modified antisense oligonucleotides and intracerebroventricular (i.c.v.) infusion

Custom *in vivo* miRCURY™ LNA inhibitor probes for rno-miR-124-3p (miR-124 inhibitor) and negative control non-targeting miRNA inhibitor were obtained from Exiqon (Woburn, MA). These unconjugated oligonucleotides optimized for *in vivo* use have a modified phosphorothioate backbone to increase nuclease resistance and improve biostability and pharmacokinetic properties. At 5 months or 9 months following GW-related exposures, GWI rats were anesthetized using a combination of ketamine (75 mg/kg) and dexmedetomidine (0.25 mg/kg) administered intraperitoneally, and were placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL) with non-rupture ear bars. Rats were implanted with 28-day osmotic pumps (Alzet model 2004, 0.25 µl/hour, DURECT, Cupertino, CA) in order to achieve the continuous release of miR-124 inhibitor at concentrations of 0, 0.05, 0.1 or 0.5 nmol/day into the brain lateral ventricle. Osmotic pumps were prefilled with artificial cerebrospinal fluid (aCSF, Harvard Apparatus, Holliston, MA), miR-124 inhibitor, or negative control inhibitor diluted in aCSF and filtered for sterility. Pumps were connected to a brain infusion kit (Alzet kit 2, DURECT) made of a stainless steel cannula which was implanted into the right lateral ventricle using established stereotaxic coordinates (1.00 mm caudal and 1.40 mm lateral to bregma, with no height adjustment spacers used). The cannula was secured to the skull with cyanoacrylate adhesive after a small hole was drilled at the marked location, and each pump was implanted subcutaneously in the midscapular area of the rat's back following the manufacturer's instructions. After the cannula was firmly set in place, the scalp incision was closed with interrupted 4-0 silk sutures. Animals received a carprofen tablet (2 mg carprofen per 5 g tablet) daily for 2 postoperative days and were returned to individual cages for the remainder of the study. Correct cannula placement into the ventricular system was verified in all animals with diluted trypan blue (40 µl) injected into the catheter during brain harvest, and residual volumes measured in recovered pumps confirmed delivery of oligonucleotides.

2.5. Blood and tissue collection

Twenty-eight days after implantation of osmotic pumps, blood was drawn from the heart in deeply anesthetized rats by cardiac puncture. Blood was collected into ethylenediaminetetraacetic acid tubes and spun 10 min at 1900 × g (3000 rpm) at 4 °C. Plasma was transferred to new RNAse-free tubes, aliquoted, and stored at −80 °C until use. Rats were euthanized by intracardiac injection of a pentobarbital-based euthanasia solution, and brains were removed rapidly. Brains were dissected in half sagittally using an ice cold rat brain slicer (Zivic Instruments, Pittsburgh, PA), and the hippocampus was isolated as described previously (Pierce et al., 2016). The right hippocampus was stored in RNALater solution (Life Technologies, Grand Island, NY) at −80 °C until RNA isolation was performed and the left hippocampus was snap frozen in liquid nitrogen for protein analysis.

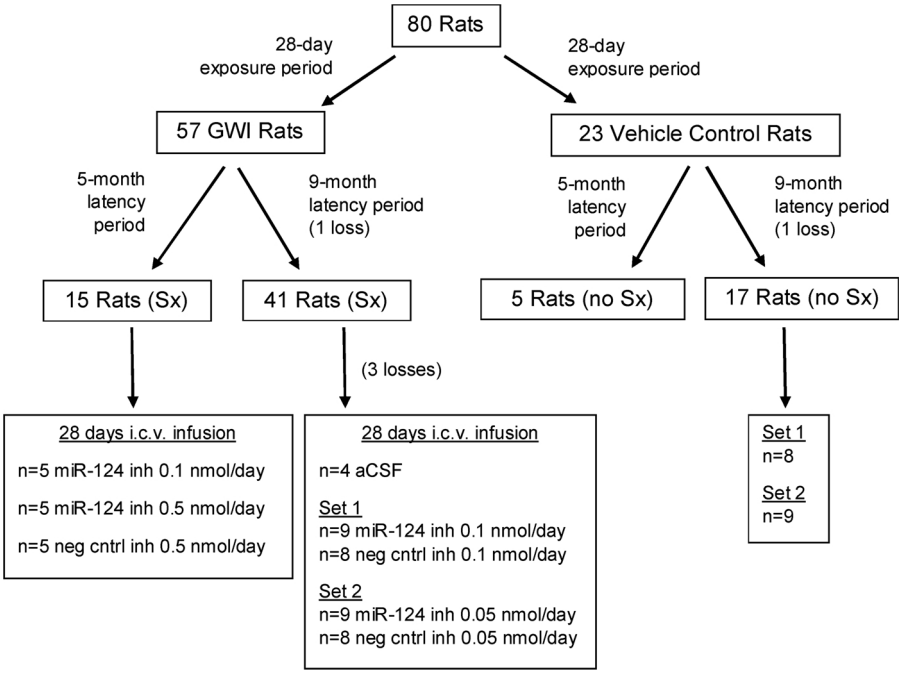

Fig. 1. Experimental design. Sx, surgery; i.c.v., intracerebroventricular; aCSF, artificial cerebrospinal fluid; inh, inhibitor; neg cntrl, negative control.

2.6. Western blot analysis of known miR-124 targets to verify functional inhibition of miR-124

In order to establish effective doses and confirm that i.c.v. infusion of miR-124 inhibitor caused functional inhibition of miR-124 in the brain, protein expression levels of known miR-124 targets that play important roles in synaptic plasticity and cognition were analyzed by western blot in GWI animals that underwent i.c.v. infusion 5 months after the GW exposure period. These included the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), the AMPA receptor subunit GluR2 (ionotropic glutamate receptor 2), and the transcription factor early growth response protein 1 (EGR-1). Hippocampi were lysed in RIPA buffer containing phenylmethylsulfonyl fluoride, protease inhibitors, and sodium orthovanadate (Santa Cruz Biotechnology, Dallas, TX) using 100 µl buffer per 23 mg tissue. Samples were homogenized on ice with a Potter-Elvehjem tissue grinder (VWR, Radnor, PA), passed through a 27-gauge needle, and finally passed through a 30-gauge needle. Lysates were then incubated on ice for 1.5 h with occasional vortexing and centrifuged 10 min at 10,000 × g (13,000 rpm) at 4 °C. After clarification, protein concentration was determined using the bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Life Technologies Corp.). Clarified lysates were boiled in 4x sample buffer, aliquoted, and frozen at −80 °C. Total protein lysate [25 µg hippocampus lysates and 10 µg control lysates (normal rat brain whole cell lysate and PC-12 whole cell lysate, Rockland Antibodies and Assays, Limerick, PA)] were subjected to 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a polyvinylidene fluoride membrane. Nonspecific binding was blocked with TBS Odyssey Blocking Buffer (LI−COR, Lincoln, NE). Blots were incubated 2 h at room temperature or overnight at 4 °C with primary antibodies against EGR-1 (rabbit polyclonal Ab191441, Abcam, Cambridge, MA, 1:5000 dilution), GluR2 (rabbit monoclonal Ab133477, Abcam, 1:10,000 dilution), GR (rabbit monoclonal Ab109022, Abcam, 1:10,000 dilution), MR (rabbit polyclonal sc-11412, Santa Cruz Biotechnology, 1:400 dilution), and tubulin as a normalization control (mouse monoclonal DMIA sc-32293, 1:15,000 dilution, 1 h incubation at room temperature). Blots were then incubated for 1 h at room temperature with secondary antibodies from LI-COR (IRDye 680 anti-rabbit IgG at 1:20,000 dilution and IRDye 800 anti-mouse IgG at 1:30,000 dilution). Visualization occurred by infrared fluorescence detection using an Odyssey Fc imaging system (LI-COR). Band densities were normalized to tubulin detected on the same blot.

2.7. Quantitative real-time PCR to measure miR-124 levels

Quantitative PCR analysis was performed to examine expression levels of endogenous miR-124 in animals that underwent i.c.v. infusion 9 months after the GW exposure period. Hippocampi were homogenized in QIAzol lysis reagent (Qiagen, Valencia, CA) using an Omni GLH homogenizer (Omni International, Keensaw, GA), and total RNA (including miRNA) was purified using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Further purification of the miRNA-enriched fraction separate from the larger RNAs occurred with the use of an RNeasy MinElute Cleanup Kit (Qiagen). Quantity and quality of extracted RNA was determined using a NanoDrop 2000c spectrophotometer (Life Technologies) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). cDNA was generated from 500 ng RNA using the miScript II RT Kit with miScript HiSpec Buffer for mature miRNA detection (Qiagen). Quantitative real-time PCR was performed using the cDNA template (diluted 1:100) with a miScript Primer Assay for rat miR-124-3p and the miScript SYBR Green PCR Kit (Qiagen) by means of a Bio-Rad CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) per the manufacturer's instructions. Standard curves were generated in each PCR run using 10-fold dilutions enabling the determination of miRNA copy number using software from Bio-Rad. Raw threshold cycle (C_T) values from duplicate measurements of each sample were averaged and the expression of miR-124-3p was normalized to levels of the small noncoding RNA SNORD96 A (which showed the highest stability in our prior analyses) within each sample. Relative expression levels were determined by dividing the average ratio (copy number miR-124-3p/copy number SNORD96 A) of the normalized experimental samples by the average ratio of the normalized control samples to obtain a fold difference in miRNA expression between experimental groups.

2.8. mRNA expression profiling using PCR Arrays

Total RNA was extracted from the hippocampus as described above. cDNA was generated from 5 µg total RNA per array using the RT^2 First Strand Kit (Qiagen) and analyzed using the RT^2 Profiler Rat Synaptic Plasticity PCR Array, the RT^2 Profiler Rat Glucocorticoid Signaling PCR Array, and the RT^2 Profiler Rat Neurogenesis PCR Array (Qiagen) by means of a Bio-Rad CFX96 real-time PCR Detection System per manufacturer instructions. Each of these PCR arrays analyses 84 different genes known to be involved in synaptic alterations during learning and memory, signaling initiated by the glucocorticoid receptor, and neurogenesis/neural stem cell differentiation, respectively. Arrays include RNA quality controls and internal housekeeping genes to normalize the data for the amount of RNA added to each reverse transcription reaction. Expression profiles were determined using RNA purified from a single rat per array, and arrays were repeated with 5–8 different rats in each experimental group. Raw C_T values were normalized to housekeeping genes encoding hypoxanthine phosphoribosyltransferase 1 (Hprt1) and lactate dehydrogenase A (Ldha) on the array using software from Qiagen, and the analysis tool was used to perform relative quantification using the $\Delta\Delta C_T$ method to reveal statistically significant fold differences. Differentially expressed mRNAs were considered significant using a cutoff value > 2.5 fold change and $p < 0.05$.

2.9. Plasma exosome RNA sequencing (RNA-seq) analysis

Next-generation sequencing (NGS) of plasma exosome RNAs was performed in rats that underwent i.c.v. infusion 9 months after the GW-relevant exposure period and in vehicle controls i) to expand the search for miRNAs and other small noncoding RNAs that could serve as potential novel biomarkers of GWI (Pierce et al., 2016), and ii) to determine whether administration of miR-124 inhibitor into the lateral ventricle of the brain could influence circulating exosomal miRNA content. Available plasma was pooled from GWI rats infused with 0.1 nmol/day miR-124 inhibitor (n = 9) or 0.1 nmol/day negative control inhibitor (n = 6), referred to as "set 1." Plasma was also pooled from GWI rats infused with 0.05 nmol/day miR-124 inhibitor (n = 9) or 0.05 nmol/day negative control inhibitor (n = 6), referred to as "set 2." Vehicle control rats were analyzed for comparison (no GW exposures/no miR-124 inhibitor therapy; n = 14, with 7 controls analyzed with set 1 and 7 controls analyzed with set 2). Plasma samples were pooled to identify robust differences between groups and to cancel out individual variances between samples.

RNA-seq analysis on plasma samples was performed by System Biosciences (SBI; Palo Alto, CA) utilizing SBI's Exosome RNA NGS Service. Briefly, defibrination reagent (5 µl, SBI) was added to 500 µl plasma to a final concentration of 5U/ml and the solution was incubated at room temperature for 5 min while mixing. Samples were then centrifuged at 10,000 rpm for 5 min, and the supernatant (clear of clotting factors) was transferred to a clean microfuge tube. ExoQuick exosome precipitation solution (SBI) was added to the supernatant at 1:4 ratio (ExoQuick:supernatant) for 30 min at 4 °C, followed by a centrifugation step at 1500 × g for 30 min to isolate exosomes.

Exosomes were processed for total RNA isolation using the SeraMir Exosome RNA Purification Column Kit (SBI) according to the manufacturer's instructions. Measurement of small RNA concentration was determined using a Bioanalyzer 2100 instrument with the Agilent Small

RNA Kit (Agilent Technologies). Small RNA libraries were constructed with the CleanTag Small RNA Library Preparation Kit (TriLink Biotechnologies, San Diego, CA) according to the manufacturer's protocol. The final purified library was quantified with the High Sensitivity DNA Kit (Agilent Technologies). The libraries were pooled, and the 140 bp to 300 bp region was size selected on an 8% TBE gel (Life Technologies). The size selected library was quantified with the High Sensitivity DNA 1000 Screen Tape Kit (Agilent Technologies) and the TailorMix HT1 qPCR Assay (SeqMatic, Fremont, CA), followed by a NextSeq High Output single-end sequencing run at SR75 using NextSeq 500/550 High Output v2 Kit (Illumina, San Diego, CA) according to the manufacturer's instructions.

Data analysis occurred with the use of the Banana Slug Exosome RNA-seq Analysis platform (SBI) using the rat reference RGSC 5.0/rn5 assembly (UCSC Genome Bioinformatics). The web-based analysis service included library sequence quality control metrics, normalization of raw sequence reads, data analysis for relative RNA abundance and identity, and differential expression analysis. Differentially expressed plasma exosome RNAs were reported as log2 fold change > 2 with significance considered at $p < 0.01$ (adjusted for multiple comparisons).

2.10. miRNA target prediction and pathway analysis

To determine the biological significance of differentially expressed exosomal miRNAs, TargetScan Release 7.2 (http://www.targetscan.org/vert_72/, accessed July 2018) was used to generate a list of predicted mRNA targets (Agarwal et al., 2015). A cutoff of ≤ -0.3 cumulative weighted context + + score was used to exclude weak predictions. The function of predicted target genes was analyzed using the Functional Annotation Clustering Tool in the Database for Annotation, Visualization and Integrated Discovery (DAVID Bioinformatics Resource 6.8; https://david.ncifcrf.gov/, accessed July 2018; Huang et al., 2009a, 2009b). DAVID analyses were performed using *Rattus norvegicus* as the background and medium stringency settings. The predicted miRNA target genes were then analyzed for enrichment in terms of their gene ontology (GO) categories and Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathways within DAVID.

2.11. Quantification of protein biomarkers of cardiac injury in plasma

The brain and heart are highly interconnected, and it is well known that brain disorders can lead to heart damage (Ripoll et al., 2018). Because it is unknown how changes in miRNA expression in the brain may affect cardiac function, we chose to investigate plasma protein markers of cardiac injury to explore possible cardiotoxic effects of miR-124 inhibition in the brain. The simultaneous detection of cardiac troponin I, cardiac troponin T, creatine kinase (muscle type), fatty acid binding protein 3, follistatin-like protein 1, myosin light chain 3, and tissue inhibitor of metalloproteinase-1 in individual rat plasma samples was performed using the MILLIPLEX MAP Rat Cardiac Injury Magnetic Bead Panel 1 and Luminex multiplex instrument MAGPIX (Millipore Sigma, Burlington, MA) according to the manufacturer's instructions. Plasma samples were from the same animals that underwent exosome RNA-seq analysis.

2.12. Statistical analysis

Results were reported as means ± standard error of the mean for experimental and control groups. Changes in mRNA expression analyzed using the RT^2 Profiler PCR Arrays were calculated using software from Qiagen. Plasma exosome RNA-seq analysis was performed by SBI, and differentially expressed RNAs were identified using the DESeq package. Gene enrichment analysis in DAVID used a modified Fisher Exact test to determine whether genes were enriched in the annotation categories, and enrichment statistics were adjusted for multiple hypothesis testing by the Benjamini correction. All other statistical analyses were performed using SigmaPlot 11.2 software (Systat Software, Inc., San Jose, CA) with $p < 0.05$ considered significant. Body weight measurements were compared using a two-way repeated measures analysis of variance (ANOVA) followed by multiple comparison testing (Holm-Sidak method). Other comparisons among groups were made using one-way ANOVA followed by multiple comparison testing to assess differences between individual pairs of means among the groups. Comparisons between groups were made using unpaired t tests. Nonparametric tests were used when indicated.

3. Results

3.1. General health observations

Over the course of the 5-month and 9-month post-exposure periods, 1 GWI rat and 1 vehicle control rat died prior to the completion of the study due to illnesses which appeared to be unrelated to GW exposures. A veterinary pathologist determined that the vehicle control rat died from mononuclear cell leukemia at 4 months of age, while the exact cause of death was unknown for the GWI rat that died at 11 months of age, although evidence of myocardial degeneration and necrosis was present. One GWI rat was unable to undergo surgery due to an intolerance to the ketamine and dexmedetomidine injection prior to surgery (the rat experienced severe respiratory and heart rate depression). Two GWI rats died during recovery from surgery due to complications from anesthesia. The general behavior and clinical signs of health were similar between GWI and vehicle control rats, with no visible signs of neurotoxicity, pain, or discomfort. Weights between the groups were not statistically different during the GW exposure period or during the post-exposure survival period ($p > 0.05$).

Statistically significant weight loss was observed at 3 weeks (4.8% loss) and 4 weeks (7.8% loss) after implantation of 0.5 nmol/day miR-124 inhibitor pumps indicating possible off-target effects of miR-124 inhibition at a dose of 0.5 nmol/day (Fig. 2). Post-surgical weights were not different between groups infused with 0.05 or 0.1 nmol/day miR-124 inhibitor or negative control inhibitor (Fig. 2).

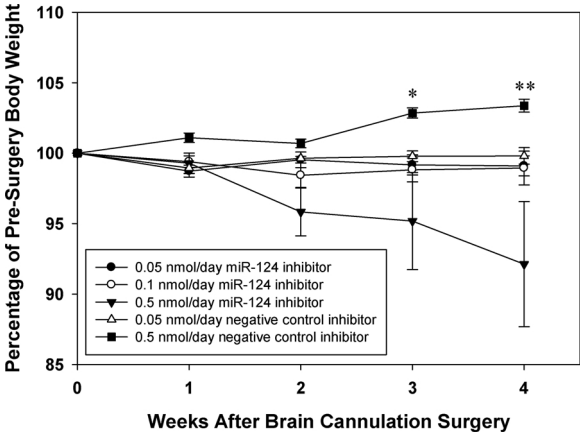

Fig. 2. Change in body weight after 28-day osmotic pump implantation in GWI rats. Weight loss was observed at 3 weeks (4.8% loss) and 4 weeks (7.8% loss) after implantation of the 0.5 nmol/day miR-124 inhibitor pumps (but not a negative control inhibitor) indicating possible off-target effects of miR-124 inhibition at a dose of 0.5 nmol/day. Two way repeated measures ANOVA, $p = 0.01$. *$p < 0.05$ 0.5 nmol/day miR-124 inhibitor vs. 0.5 nmol/day negative control inhibitor; **$p < 0.05$ 0.5 nmol/day miR-124 inhibitor vs. all other groups (post hoc pairwise multiple comparison tests); n = 5–9 per group. Animals in the 0.05 nmol/day groups had a post-exposure latency period of 9 months, while all others had a post-exposure latency period of 5 months.

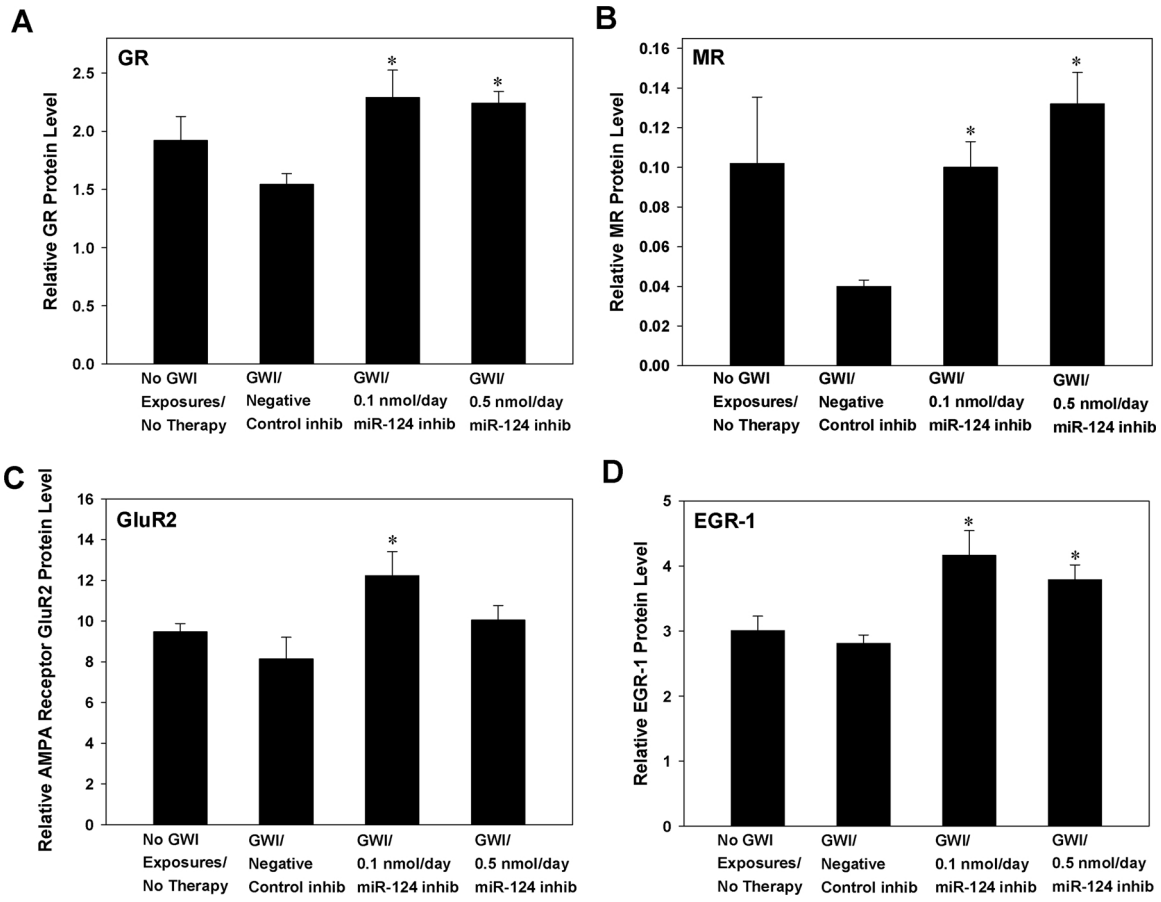

Fig. 3. Increased protein expression of miR-124 targets GR (A), MR (B), GluR2 (C) and EGR-1 (D) in the hippocampus after i.c.v. infusion of 0.1 or 0.5 nmol/day miR-124 inhibitor for 28 days in GWI rats after a post-exposure latency period of 5 months indicating functional inhibition of miR-124. ANOVA, $p = 0.03$ (A), $p = 0.03$ (B), $p = 0.04$ (C), and $p = 0.005$ (D). *$p < 0.05$ vs. 0.5 nmol/day negative control inhibitor (post hoc pairwise multiple comparison tests); $n = 5$ per group.

3.2. Intracerebroventricular infusion of miR-124 inhibitor increases protein levels of known miR-124 targets and reduces PCR-amplifiable miR-124 levels in the hippocampus

Increased protein expression of GR, MR, GluR2, and EGR-1 was observed in the hippocampus after i.c.v. infusion of 0.1 nmol/day and 0.5 nmol/day miR-124 inhibitor (but not the negative control inhibitor at 0.5 nmol/day) for 28 days after a 5-month post-exposure period indicating functional inhibition of miR-124 (Fig. 3). Of note, particularly for MR, protein expression levels tended to decrease in GWI animals receiving the negative control oligonucleotide relative to animals not exposed to GW agents, which is consistent with an elevation of miR-124 levels observed in the hippocampus of GWI rats (Pierce et al., 2016). Likewise, miR-124 levels in the hippocampus of GWI rats that underwent i.c.v. infusion of aCSF 9 months after the GW exposure regimen as determined by quantitative PCR were significantly increased (2.99-fold, $p = 0.006$) relative to vehicle control rats that did not undergo GW exposures (Fig. 4). PCR-amplifiable miR-124 levels were drastically reduced in the hippocampus of GWI rats that received 0.1 nmol/day miR-124 inhibitor (but not the negative control inhibitor or aCSF; Fig. 4). In rats infused with 0.05 nmol/day miR-124 inhibitor ($n = 9$), PCR-amplifiable miR-124 levels were also reduced by 76% in the hippocampus compared to those receiving 0.05 nmol/day negative control inhibitor ($n = 8$; Fig. 4). Data from Fig. 4 onward include animals from the 9-month post-exposure period.

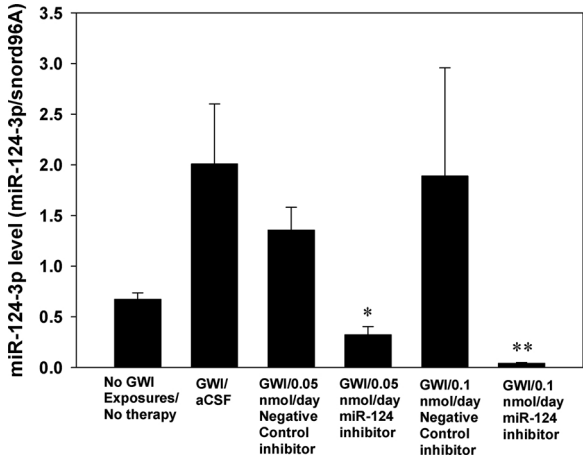

Fig. 4. Hippocampal miR-124 levels detected by quantitative PCR. GWI rats underwent i.c.v. infusion of 0.1 nmol/day miR-124 inhibitor ($n = 9$), 0.1 nmol/day negative control inhibitor ($n = 8$), 0.05 nmol/day miR-124 inhibitor ($n = 9$), 0.05 nmol/day negative control inhibitor ($n = 8$), or aCSF ($n = 4$) for 28 days. Kruskal-Wallis ANOVA on Ranks, $p < 0.001$. *$p < 0.05$ vs. aCSF and 0.05 nmol/day negative control inhibitor; **$p < 0.05$ vs. all other groups except 0.05 nmol/day miR-124 inhibitor; post hoc pairwise multiple comparison tests.

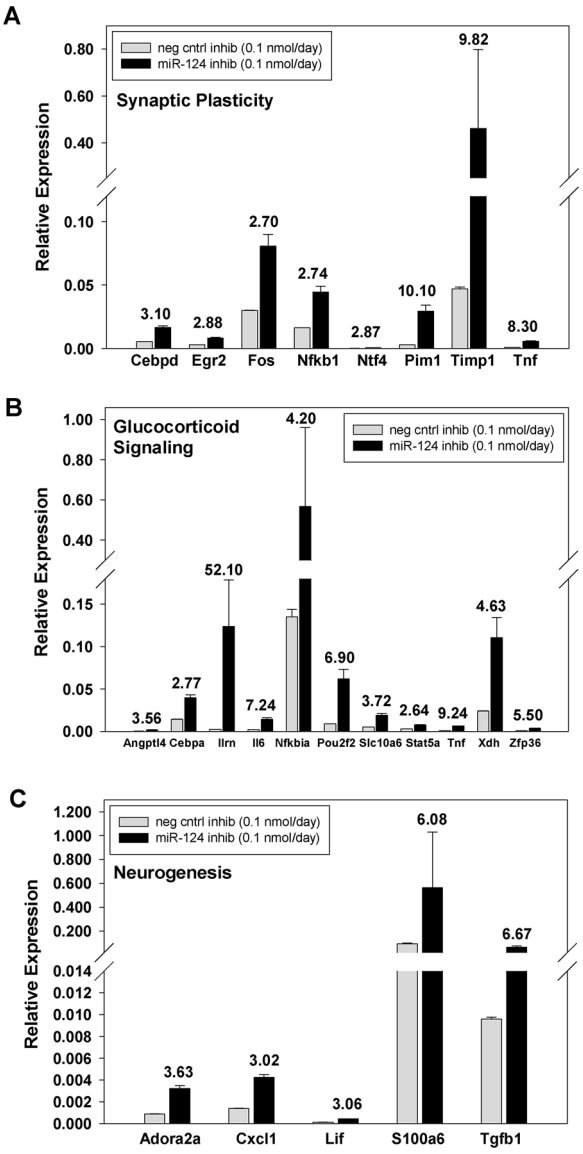

Fig. 5. Upregulated synaptic plasticity (A), glucocorticoid signaling (B), and neurogenesis (C) associated gene expression in the hippocampus of GWI rats that underwent i.c.v. infusion of 0.1 nmol/day miR-124 inhibitor for 28 days. Genes were expressed relative to housekeeping genes Hprt1 and Ldha on the array. Fold change above bars; cutoff value > 2.5 fold change and $p < 0.05$; n = 5 per group. No genes were downregulated.

3.3. Inhibition of miR-124 results in increased expression of genes involved in synaptic plasticity, glucocorticoid signaling, and neurogenesis in the hippocampus

Upregulation of 8 genes important for synaptic alterations during learning and memory, 11 genes known to be important for signaling initiated by the glucocorticoid receptor, and 5 genes related to neurogenesis and neural stem cell differentiation was observed in the hippocampus of set 1 GWI rats receiving 0.1 nmol/day miR-124 inhibitor compared to 0.1 nmol/day negative control inhibitor, while no genes were downregulated (Fig. 5; Table 1; n = 5 per group). When analyses were repeated in the hippocampus of set 2 rats receiving 0.05 nmol/day miR-124 inhibitor (n = 8) compared to 0.05 nmol/day negative control inhibitor (n = 8), 4 genes important for synaptic plasticity, 8 genes involved in glucocorticoid signaling, and 2 genes involved in neurogenesis were upregulated, while no genes were downregulated (Fig. 6). Neurogenesis associated genes *S100a6* and *Tgfb1* were upregulated 3.64 fold ($p = 0.0004$) and 4.99 fold ($p = 0.001$), respectively, in rats receiving 0.05 nmol/day miR-124 inhibitor compared to 0.05 nmol/day negative control inhibitor (not shown). Although *Il10* and *Ddit4* were significantly upregulated in the hippocampus of GWI rats receiving 0.05 nmol/day miR-124 inhibitor and are presented in Fig. 6, these genes did not reach statistical significance at the 0.1 nmol/day dose due to variability (*Il10*, 5.58 fold upregulation, $p = 0.23$; *Ddit4*, 4.83 fold upregulation, $p = 0.05$). No differentially expressed genes were identified using the synaptic plasticity and glucocorticoid signaling PCR arrays when GWI rats that received the negative control inhibitor were compared to vehicle control rats (no GW exposures).

3.4. Plasma exosome RNA-seq analysis

The small RNA yield from each of the pooled plasma samples used for exosome RNA-seq analysis was 10 to 48 ng per 500 µl plasma, which is consistent with RNA yields in human plasma samples (Huang et al., 2013) and in rat plasma samples determined in a previous study (Pierce et al., 2016). Exosome RNA sizes ranging from approximately 20 nucleotides to approximately 150 nucleotides in length were used for library generation. For the sets 1 and 2 GWI/miR-124 inhibitor exosome libraries, the GWI/negative control inhibitor exosome libraries, and the vehicle control exosome libraries, the number of raw single-end reads retained after quality assessment used for mapping ranged from about 11 million to 22 million. Approximately 60–65% of reads mapped to the rat genome (the number of aligned reads ranged from about 7–15 million per sample).

Exosome RNA-seq analysis demonstrated that the circulating rat exosomes contain a broad range of RNA species. RNA species that mapped to the rat genome included noncoding RNAs [miRNAs, tRNAs, piwi-interacting RNAs (piRNAs), long noncoding RNAs (lncRNAs), and other noncoding RNAs], coding sequences, and antisense transcripts (Fig. 7). A total of 530 known miRNAs were detected in the 6 pooled samples, similar to findings from a previous study (Pierce et al., 2016). The top 20 most abundant miRNAs made up 67–75% of all mappable exosome miRNAs and are presented in Table 2.

No statistically significant differences in miRNA expression were identified between the GWI/negative control inhibitor and vehicle control (non-GWI) groups, similar to results observed between GWI and control rats one year after the 28-day exposure regimen (Pierce et al., 2016). However, 7 miRNAs were upregulated (log2 fold change > 2, $p < 0.01$) in plasma exosomes from set 1 GWI rats treated with 0.1 nmol/day miR-124 inhibitor compared to negative control inhibitor (no miRNAs were downregulated; Table 3). Six of these miRNAs (miR-1/miR-1b, miR-133a/miR-133b, miR-208b, and miR-499) are enriched in cardiomyocytes and play central roles in cardiogenesis, heart function, and pathology (Fichtlscherer et al., 2011; McGahon et al., 2013; Chistiakov et al., 2016; Navickas et al., 2016; Paiva and Agbulut, 2017). Upregulation of these (or other) miRNAs in circulation was not observed at the 0.05 nmol/day dose of miR-124 inhibitor in set 2 GWI rats (Table 4).

Evaluation of rat putative target mRNAs using TargetScan Release 7.2 identified 363 predicted gene targets of these 7 differentially expressed miRNAs. To gain functional insights into these miRNAs altered in response to i.c.v. infusion of miR-124 inhibitor at the higher dose, gene sets were constructed corresponding to biological pathways and networks underlying complex diseases using Functional Annotation Clustering analysis in the online tool DAVID. Four functional annotation clusters remained significant after the adjustment for multiple hypothesis testing using the conservative Benjamini correction (Table 5). The most highly enriched GO terms and biological pathways included cell-cell adhesion, mRNA binding and spliceosome, acetylcholine and norepinephrine neurotransmitter release cycles, and terminal bouton.

Table 1
Synaptic plasticity, glucocorticoid signaling, and neurogenesis genes upregulated in the hippocampus of GWI rats following i.c.v. infusion of miR-124 inhibitor compared to negative control inhibitor (0.1 nmol/day/28 days).

Gene	Encoded Protein; Function	References
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta; transcription factor of the basic-leucine zipper class; involved in synaptic plasticity and memory formation in neurons and neuroinflammation in glial cells; plays essential role in memory consolidation in the hippocampus	Arguello et al., 2013
Egr2	Early growth response 2; immediate-early gene that encodes an inducible transcription factor activated by stimuli that cause synaptic plasticity; increases rapidly in response to neuronal activity; plays role in cognitive functions associated with attention; reduced levels in human autism brains; downstream target of NF-kB	DeSteno and Schmauss, 2008; Swanberg et al., 2008
Fos	FBJ osteosarcoma oncogene; immediate-early gene that encodes an inducible transcription factor; elevated mRNA levels required for learning-facilitated long-term depression (LDP) important for long-term information storage in the hippocampus; reduced levels in hippocampus are associated with anxious- and depressive-like behavior in mice	Kemp et al., 2013; Ieraci et al., 2016
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; encodes NF-kB p50 subunit; increased hippocampal expression induced by antidepressant treatment in middle-aged mice is associated with improved cognitive function and reduction in depressive-like behavior; required in long-term spatial memory in hippocampus	Li et al., 2015; Oikawa et al., 2012
Ntf4	Neurotrophin 4; essential for hippocampus-dependent long-term memory and long-lasting long-term potentiation (LTP); i.c.v. infusion of neutralizing antibodies to neurotrophin 4 blocks long-term recognition memory in the rat; downregulation of Ntf4 gene expression observed in CA1 pyramidal neurons in patients with mild cognitive impairment	Xie et al., 2000; Callaghan and Kelly, 2013
Pim1	Pim1- oncogene; serine/threonine kinase whose expression is both induced by and required for LTP in the hippocampus critical for learning and memory formation; Pim1 RNA and protein are virtually undetectable in unstimulated hippocampus but strongly induced by synaptic activity, the protein being distributed to nuclei and dendrites of activated neurons	Konietzko et al., 1999
Timp1	TIMP metallopeptidase inhibitor 1; tissue inhibitor of matrix metalloproteinases 1; matrix metalloproteinase (MMP)/TIMP ratio (e.g., MMP activity) modulates neuronal plasticity during learning and memory formation by controlling extracellular proteolysis, known to play a major role in synaptic plasticity; Timp1 expression in the hippocampus is neuronal activity-dependent and is dramatically induced by stimuli leading to LTP	Chaillan et al., 2006
Tnf	Tumor necrosis factor (TNF superfamily, member 2); once considered purely inflammatory, is produced in the brain under normal conditions and is important for normal synaptic functioning; modulates synaptic strength by increasing expression of AMPA receptors; presence of TNF is required for preservation of synaptic strength at excitatory synapses	Beattie et al., 2002
Angptl4	Angiopoietin-like 4; direct glucocorticoid receptor target that participates in glucocorticoid-regulated triglyceride metabolism; produced in glial cells; appears to participate in metabolic crosstalk between glia and neurons; does not regulate lipoprotein lipase in the brain in contrast to skeletal muscle	Vienberg et al., 2014
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha; transcription factor of the basic-leucine zipper class; plays key roles in hepatic lipid and glucose metabolism; expression and function in the central nervous system are less well known; expressed in activated microglia after hypoxic-ischemic brain injury in rats; forms complex with the glucocorticoid receptor accounting for a novel pathway of glucocorticoid action	Rudiger et al., 2002
Il1rn	Interleukin 1 receptor antagonist (IL-1ra); competitively inhibits the binding of IL-1 agonists to IL-1 receptors on target cells; corticosteroids physiologically control the IL-1/IL-1ra system during inflammatory or immune processes via the glucocorticoid receptor; IL-1 has major role in neurobehavioral regulation in addition to its role in immunoregulation of inflammation; IL-1 at pathophysiological levels can produce detrimental effects on hippocampal-dependent processes; i.c.v. administration of IL-1ra induces the expression of brain-derived neurotrophic factor in the hippocampus, beneficially affects spatial memory, and partially rescues neurogenesis impairment in mice with Alzheimer's disease-like pathology	Ben-Menachem-Zidon et al., 2014
Il6	Interleukin 6; expressed in the normal central nervous system at low levels and at elevated levels in neurodegenerative and psychiatric diseases and in central nervous system infection and injury; can regulate synaptic transmission and synaptic plasticity; has neuromodulatory and neuroprotective effects in vivo; expressed in hippocampal neurons and Purkinje cells of the cerebellum; may be essential at physiological levels for neurogenesis in the adult rodent brain; IL-6 deficient mice demonstrate impaired hippocampus-dependent and –independent learning; lower IL-6 levels associated with more severe posttraumatic stress symptoms in Gulf War veterans	Groul, 2015; O'Donovan et al., 2015
Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; inhibitor of NF-kB alpha; inhibits the NF-kB signaling pathway thereby repressing inflammatory gene expression; traps activated NF-kB in inactive cytoplasmic complexes; major component of the anti-inflammatory activity of glucocorticoids; inhibitor of NF-kB alpha protein plays a role in neurite outgrowth and cell survival	Newton, 2014
Pou2f2	POU class 2 homeobox 2; POU homeodomain protein octamer transcription factor 2 (Oct-2); low-affinity, high-capacity carrier that mediates sodium-independent transport for biogenic cations including catecholamine, serotonin, histamine, and choline/acetylcholine; expressed in neurons; may play role in regulating presynaptic reuptake and recycling of choline and monoamines; interacts synergistically with the glucocorticoid receptor (which directly recruits Oct-2 to glucocorticoid- responsive promoters) to activate transcription of many genes; important determinant of the response to stress in the brain; deletion of Oct-2 in mice enhances hormonal response to acute stress and impairs function of the hypothalamic-pituitary-adrenal (HPA) axis; also implicated in anxiety, antidepressant efficacy, osmoregulation, and neurotoxicity	Prefontaine et al., 1998; Courousse and Gautron, 2015
Slc10a6	Solute carrier family 10 (sodium/bile acid cotransporter family), member 6; sodium-dependent organic anion transporter (SOAT); transports primarily sulfated steroids	Claro da Silva et al., 2013
Stat5a	Signal transducer and activator of transcription 5a; critical component of cytokine signaling pathways; important in mediating proliferative responses and energy homeostasis; the glucocorticoid receptor has been shown to interfere with the STAT signaling pathway under inflammatory conditions by interacting with STAT3 and STAT5, thereby influencing immune responses; STAT5 binding to DNA- bound glucocorticoid receptor may lead to either transcriptional repression or activation of glucocorticoid receptor target genes	Petta et al., 2016
Xdh	Xanthine dehydrogenase; enzyme that oxidizes a variety of purines, pyrimidines, and other heterocyclic nitrogeneous compounds; mammalian form of the enzyme (also known as xanthine oxidoreductase) can be converted to xanthine oxidase which produces superoxide anion and hydrogen peroxide that can promote injury; mRNA expression and activity is regulated by cytokines and dexamethasone involving the glucocorticoid receptor and STAT3; expression in brain normally low	Suzuki et al., 2015
Zfp36	Zinc finger protein 36; tristetraprolin (TTP); upregulated by glucocorticoids; RNA-binding protein that destabilizes and translationally represses inflammatory cytokine mRNAs; degrades transcripts through specific AU-rich sequences in the 3' untranslated regions; mice deficient in TTP develop a systemic autoimmune inflammatory syndrome	Shah et al., 2016

(*continued on next page*)

Table 1 (*continued*)

Gene	Encoded Protein; Function	References
Adora2a	Adenosine A2a receptor; G-protein-coupled adenosine receptor involved with controlling synaptic plasticity in glutamatergic synapses; plays an important role in the hippocampus, particularly in neurogenesis in the CA3 region; inhibition of the adenosine A2a receptor induces synaptic damage in rat hippocampal nerve terminals; reduction of A2a receptors in mice with traumatic brain injury decreases cognitive impairment; inhibition of Adora2a has also been shown to enhance spatial memory and hippocampal plasticity through adult neurogenesis; suggested to play an important role in the pathogenesis of anxiety disorders	Horgusluoglu-Moloch et al., 2017
Cxcl1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha); keratinocyte-derived chemokine (KC); GRO alpha; ligand of the CXCR2 receptor; in addition to its neutrophil chemotactic properties, has been shown to have neuroprotective and neurotrophic functions	Parachikova et al.,2008
Lif	Leukemia inhibitory factor; cytokine that affects the survival and differentiation of various cells in the hematopoietic and nervous systems; mRNA expression in the hippocampus is increased following kainic acid-induced seizures in rats; maternal LIF promotes fetal neurogenesis; exogenous LIF stimulates oligodendrocyte progenitor cell proliferation and enhances hippocampal remyelination; regulates glucocorticoid receptor expression in the HPA axis	Deverman and Patterson, 2012
Tgfb1	Transforming growth factor, beta 1; regulates neural progenitor cell proliferation; promotes stem cell quiescence and also promotes survival of newly generated neurons and their functional differentiation; chronic overexpression alters hippocampal structure and causes learning deficits	Kandasamy et al., 2014
S100a6	S100 calcium binding protein A6 (calcyclin); plays role in cell proliferation and differentiation, calcium homeostasis, and neuronal degeneration; specific marker of neural stem cells and astrocyte precursors in the hippocampus; highly expressed in brain neurons	Yamada and Jinno, 2014

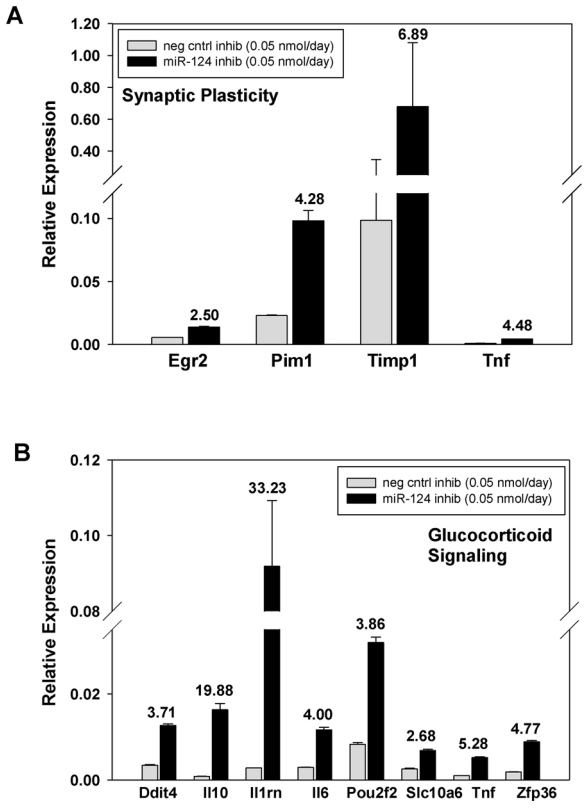

Fig. 6. Upregulated synaptic plasticity (A) and glucocorticoid signaling (B) associated gene expression in the hippocampus of GWI rats that underwent i.c.v. infusion of 0.05 nmol/day miR-124 inhibitor for 28 days. Genes were expressed relative to housekeeping genes Hprt1 and Ldha on the array. Fold change above bars; cutoff value ≥2.5 fold change and $p < 0.05$; $n = 8$ per group. No genes were downregulated.

3.5. Inhibition of miR-124 in the brain results in increased protein biomarkers of cardiac injury in plasma

To examine possible cardiotoxic effects of miR-124 inhibition in the brain, we simultaneously quantified 7 protein biomarkers of cardiac injury in plasma samples from the same rats that underwent plasma exosome RNA-seq analysis using a multiplex assay. Five of the 7 cardiac injury biomarkers (cardiac troponin I, cardiac troponin T, creatine kinase muscle type, fatty acid binding protein 3, and myosin light chain 3) were elevated in the plasma of set 1 rats that received the 0.1 nmol/day miR-124 inhibitor compared to the negative control inhibitor or vehicle control groups (Fig. 8). These biomarkers were not elevated in set 2 animals that were infused with the lower dose of 0.05 nmol/day (Fig. 8).

4. Discussion

After nearly 30 years, veterans of the 1990–1991 Persian Gulf War continue to suffer from a chronic complex illness, with cognitive dysfunction and mood deficits being among the most prevalent and debilitating symptoms. Although the etiology of GWI is unknown, epidemiological investigations and experimental studies using animal models have determined that these impairments are causally related to neurotoxic chemical exposures such as PB, permethrin, and DEET encountered during deployment (RAC, 2008, 2014; Steele et al., 2012; Kerr, 2015; White et al., 2016; Sullivan et al., 2018). Previous work in our laboratory using the same established rat model of GWI that was previously shown to develop memory and mood deficits and reduced hippocampal neurogenesis identified persistent elevation of miR-124-3p levels in the hippocampus whose numerous gene targets are involved in cognition-associated pathways and neuroendocrine function (Parihar et al., 2013; Hattiangady et al., 2014; Pierce et al., 2016). The current study provides novel evidence that *in vivo* inhibition of miR-124 function in the hippocampus upregulates the expression of genes known to play a role in synaptic plasticity, glucocorticoid signaling, and neurogenesis, and these findings warrant further investigation into miR-124 inhibition as a novel therapeutic approach to improve the cognitive and neuroendocrine dysfunction observed in GWI.

Support for miR-124 inhibition as a strategy for novel drug development for GWI is provided by observations that downregulating miR-124 in the hippocampus has been shown to enhance memory performance and elicit antidepressant-like effects in rodents (Dutta et al., 2013; Bahi et al., 2014). This strategy has also been proposed to improve synaptic and memory deficits and/or depression in patients with multiple sclerosis, major depressive disorder, amyotrophic lateral sclerosis (ALS), and Alzheimer's disease (Dutta et al., 2013; Marcuzzo et al., 2015; Roy et al., 2017; Wang et al., 2018). Behavioral studies investigating the therapeutic efficacy of a miR-124 antisense oligonucleotide to improve learning and memory and depressive- or anxiety-like behaviors in this rat model will be discussed in a separate paper.

Synaptic plasticity genes upregulated by miR-124 inhibition at a dose which did not appear to have off-target cardiotoxic effects

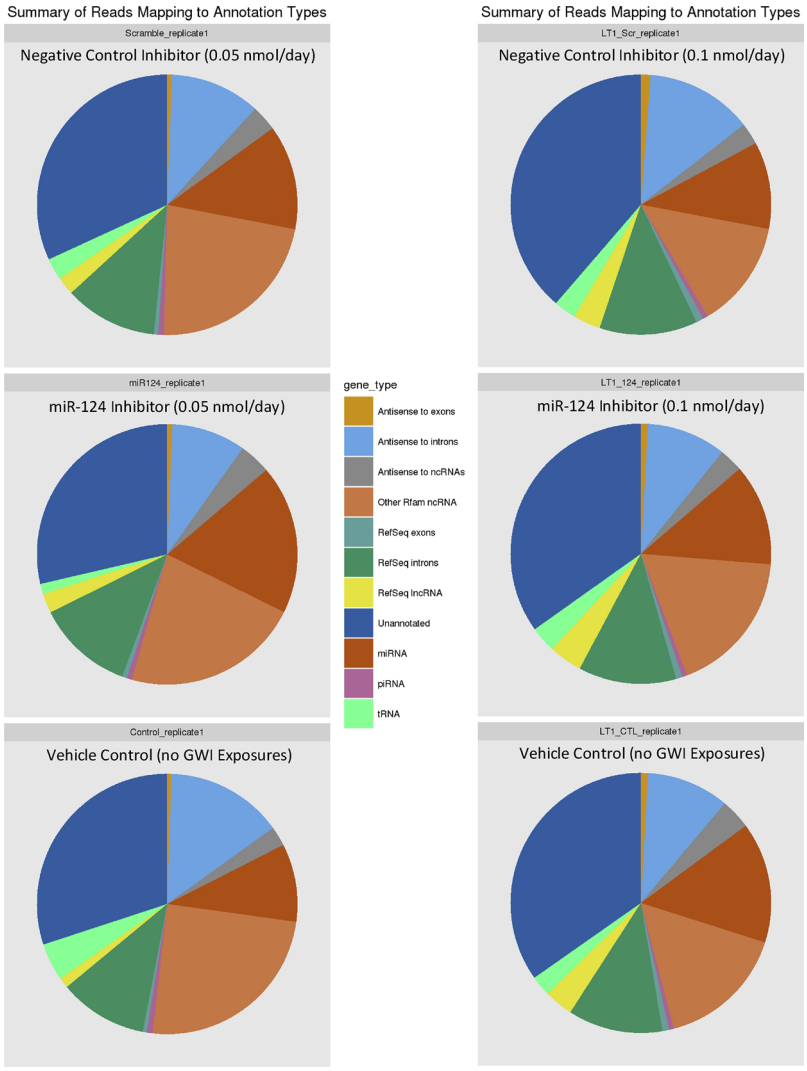

Fig. 7. Plasma exosome small RNA expression summary. Pie charts summarize the number of reads mapping to each annotation type in each pooled sample.

Table 2
The twenty most abundant miRNAs among the plasma exosome RNAs from pooled samples (0.1 nmol/day/28 days miR-124 inhibitor and negative control inhibitor).

miRNA	Raw Read Counts GWI/miR-124-3p inhibitor	Raw Read Counts GWI/negative control inhibitor	Raw Read Counts Controls (non-GWI)
rno-miR-191a-5p	735801	539316	1108685
rno-miR-143-3p	313303	209750	153566
rno-miR-192-5p	275402	239267	342209
rno-miR-22-3p	270518	110838	161767
rno-miR-26a-5p	194399	99746	137874
rno-miR-133a-3p	177237	9533	8307
rno-miR-30a-5p	170904	78189	80456
rno-miR-10b-5p	151248	67418	71265
rno-miR-148a-3p	137775	78706	133967
rno-miR-320-3p	132571	65003	97981
rno-miR-16-5p	127729	65883	73551
rno-let-7c-5p	125932	60279	84824
rno-miR-122-5p	121907	100486	182517
rno-miR-142-5p	113228	62040	75033
rno-miR-30d-5p	106807	44696	53839
rno-let-7b-5p	101754	56241	78694
rno-miR-25-3p	97944	50434	57529
rno-miR-10a-5p	96510	81602	51512
rno-miR-128-3p	90024	42630	126590
rno-let-7i-5p	87389	44646	70276

Only miR-133a-3p was not among the top 20 most abundant miRNAs for the 0.1 nmol/day negative control inhibitor and control (non-GWI) groups. Those miRNAs in bold were also found to be among the top 20 most abundant plasma exosome miRNAs in rats receiving 0.05 nmol/day inhibitors and/or in rats from a previous study (Pierce et al., 2016).

Table 3
Upregulated miRNAs in plasma exosomes from GWI rats treated with miR-124 inhibitor compared to negative control inhibitor (0.1 nmol/day/28 days).

miRNA	Log2 Fold Change	Normalized Abundance Negative Control Inhibitor	Normalized Abundance miR-124 Inhibitor	P Value
rno-miR-1-3p	3.72	3992	52702	< 0.0001
rno-miR-1b	3.58	3335	39819	< 0.0001
rno-miR-133a-3p	3.65	11008	138524	< 0.0001
rno-miR-133b-3p	3.63	283	3510	< 0.0001
rno-miR-208b-3p	3.73	755	10037	< 0.0001
rno-miR-499-5p	3.32	234	2342	0.0001
rno-miR-201-5p	3.79	64	878	0.0002

Table 4
Plasma exosome miRNAs from GWI rats treated with 0.05 nmol/day miR-124 inhibitor compared to 0.05 nmol/day negative control inhibitor for 28 days. These miRNAs were upregulated at a dose of 0.1 nmol/day (Table 3) but were not at elevated at this lower dose.

miRNA	Log2 Fold Change	Normalized Abundance Negative Control Inhibitor	Normalized Abundance miR-124 Inhibitor	P Value
rno-miR-1-3p	1.9	1799	6613	0.58
rno-miR-1b	1.2	1460	3396	1.0
rno-miR-133a-3p	1.3	5005	12198	1.0
rno-miR-133b-3p	1.2	180	404	1.0
rno-miR-208b-3p	ND	ND	ND	ND
rno-miR-499-5p	0.68	215	346	1.0
rno-miR-201-5p	−1.2	243	104	1.0

ND, not determined.

(0.05 nmol/day/28 days) included *Egr2*, *Pim1*, *Timp1*, and *Tnf*. *Egr2* is immediate-early gene that encodes an inducible transcription factor activated by stimuli that cause synaptic plasticity (DeSteno and Schmauss, 2008; Swanberg et al., 2008). Furthermore, *Egr2* is known to play a role in cognitive functions associated with attention, and reduced levels have been detected in the brains of human autism patients (DeSteno and Schmauss, 2008; Swanberg et al., 2008). *Pim1* is a serine/threonine kinase critical for learning and memory formation, and its mRNA and protein levels are strongly induced by synaptic activity in the hippocampus (Konietzko et al., 1999). *Timp1* expression in the hippocampus is neuronal activity-dependent and is dramatically induced by stimuli leading to long-term potentiation; it modulates neuronal plasticity during learning and memory formation by controlling extracellular proteolysis, known to play a major role in synaptic plasticity (Chaillan et al., 2006). *Tnf*, once considered purely inflammatory, is produced in the brain under normal conditions, is important for normal synaptic functioning, and causes an increase in the expression of AMPA receptors thereby modulating synaptic strength (Beattie et al., 2002).

Several genes involved in glucocorticoid signaling were upregulated in the hippocampus after i.c.v. infusion of miR-124 inhibitor, as expected. Of note is *Ilrn*, whose expression levels were highly induced (33-fold increase) at the reduced dose of miR-124 inhibitor (0.05 nmol/day). *Ilrn* encodes the interleukin 1 receptor antagonist (IL-1ra) which competitively inhibits the binding of IL-1 agonists to IL-1 receptors on target cells. IL-1 at pathophysiological levels plays a major role in neurobehavioral regulation in addition to its role in inflammation; i.c.v. infusion of IL-1ra has been shown to increase the expression of brain-derived neurotrophic factor in the hippocampus, improve spatial memory, and enhance neurogenesis in an Alzheimer's disease mouse model (Ben-Menachem-Zidon et al., 2014). Another interesting finding is *Il6*, which was increased 4-fold in rats i.c.v. infused with 0.05 nmol/day miR-124 inhibitor. Interleukin 6 (IL-6)-deficient mice have been shown to demonstrate impaired hippocampus-dependent and hippocampus-independent learning, and lower IL-6 levels have been associated with more severe posttraumatic stress symptoms in GW veterans (Groul, 2015; O'Donovan et al., 2015).

Evaluation of the beneficial effects of miR-124 inhibition on mitochondrial function in GWI rats was beyond the scope of this investigation, but future studies are warranted. Mitochondrial dysfunction has been proposed as a causative factor in the etiology of GWI, particularly in the context of exposures to pesticides and PB during deployment, since mitochondria are thought to be a target for environmental toxicants (Koslik et al., 2014; Chen et al., 2017; Yardeni et al., 2018). Biomarkers of mitochondrial dysfunction have been observed in ill GW veterans and in rodent models of GWI (Koslik et al., 2014; Abdullah et al., 2016; Chen et al., 2017; Shetty et al., 2017). Overexpression of miR-124 has been shown to negatively impact

Table 5
Functional Annotation Clustering analysis for predicted pathways targeted by differentially expressed plasma exosome miRNAs.

Term	No. of Genes	P Value	Benjamini P value
Functional Annotation Cluster 1 Enrichment Score: 4			
GO:0098609 cell-cell adhesion	14	0.000047	0.04
GO:0098641 cadherin binding involved in cell-cell adhesion	14	0.000087	0.0076
GO:0005913 cell-cell adherens junction	14	0.00025	0.0087
Functional Annotation Cluster 2 Enrichment Score: 2.9			
GO:0003729 mRNA binding	12	0.000042	0.0061
GO:0000166 nucleotide binding	17	0.00011	0.0079
KEGG_PATHWAY spliceosome	10	0.00022	0.035
Functional Annotation Cluster 3 Enrichment Score: 2.87			
REACTOME_PATHWAY acetylcholine neurotransmitter release cycle	5	0.00018	0.049
REACTOME_PATHWAY norepinephrine neurotransmitter release cycle	5	0.00023	0.032
Functional Annotation Cluster 4 Enrichment Score: 2.03			
GO:0043195 terminal bouton	10	0.00012	0.0069

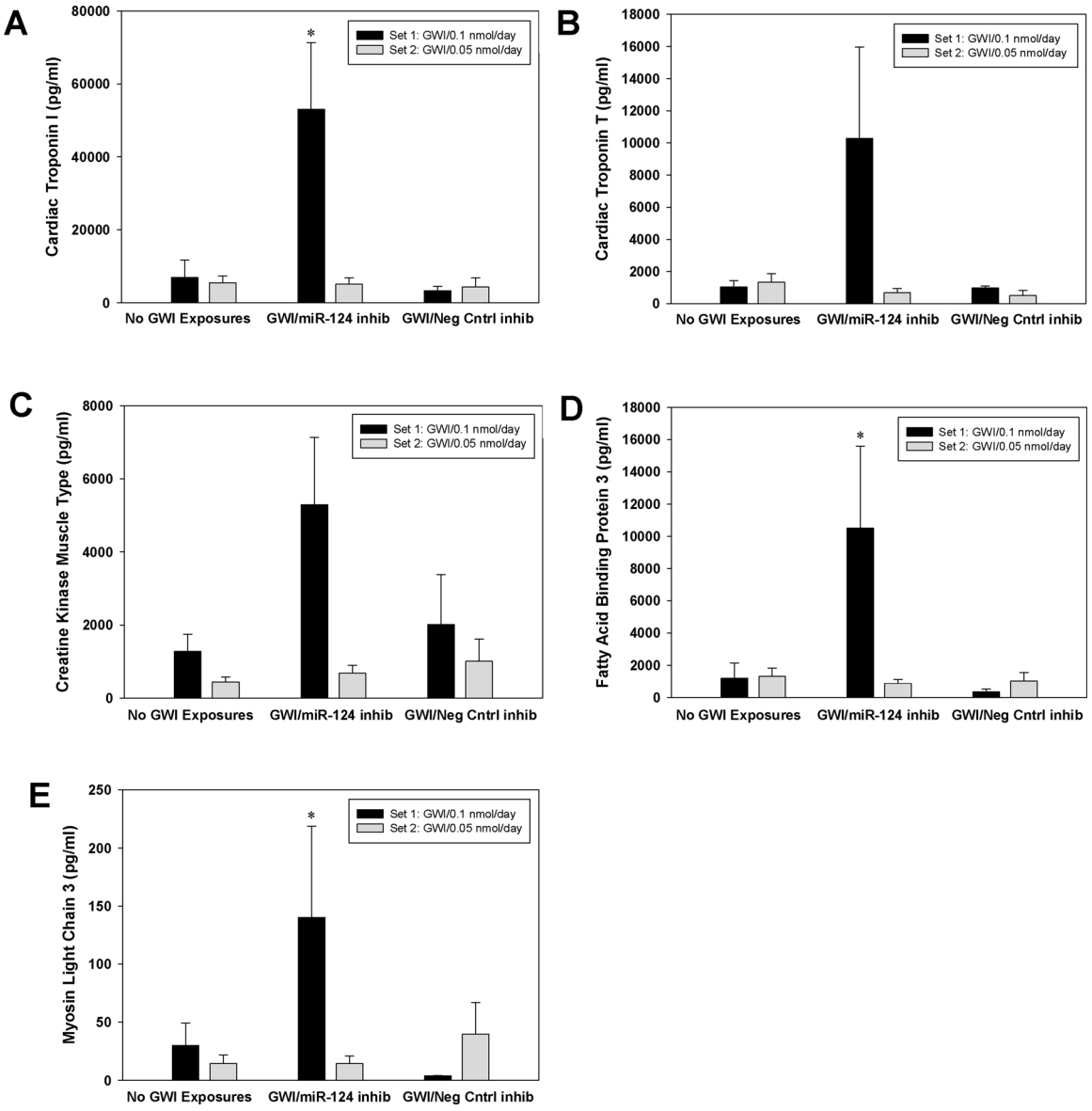

Fig. 8. Elevated plasma levels of cardiac troponin I (A), cardiac troponin T (B), creatine kinase muscle type (C), fatty acid binding protein 3 (D), and myosin light chain 3 (E) in GWI rats that underwent i.c.v. infusion of 0.1 nmol/day miR-124 inhibitor for 28 days. Cardiac injury markers were not elevated in rats receiving 0.05 nmol/day miR-124 inhibitor. Kruskal-Wallis ANOVA on Ranks for set 1 rats receiving 0.1 nmol/day, $p = 0.02$ (A), $p = 0.08$ (B), $p = 0.07$ (C), $p = 0.03$ (D), and $p = 0.045$ (E). *$p < 0.05$, 0.1 nmol/day miR-124 inhibitor vs. 0.1 nmol/day negative control inhibitor (post hoc pairwise multiple comparison tests); $n = 6–9$ per group.

mitochondrial activity in motor neurons, which is consistent with other studies demonstrating mitochondrial impairments in ALS patients and increased levels of miR-124 in several brain regions including the hippocampus of ALS mice at late disease stage (Marcuzzo et al., 2015; Yardeni et al., 2018). A higher incidence of ALS among GW veterans, farmers, and soccer players has been associated with exposure to organophosphates (Horner et al., 2008; Merwin et al., 2017).

Significant weight loss was experienced by rats treated with 0.5 nmol/day miR-124 inhibitor for 28 days, indicating toxic effects exerted at this high dose. The absence of body weight reduction in rats treated with 0.1 and 0.05 nmol/day suggests reduced toxicity at lower doses. Possible off-target cardiotoxic effects of miR-124 inhibition were revealed at a dose of 0.1 nmol/day/28 days as indicated by highly elevated plasma protein cardiac injury markers including cardiac troponins I and T, widely used to detect ischemic cardiac injury in patients with acute coronary syndromes (ACS; Mahajan and Jarolim, 2011). Increases in circulating cardiac troponins may also be indicative of cardiomyocyte necrosis secondary to conditions other than ACS most frequently including non-ACS cardiovascular, renal, hypertensive, or infectious causes (Harvell et al., 2016). Although cardiac tissue morphology was not characterized in this study, plasma levels of cardiac troponins observed in rats administered the 0.1 nmol/day dose of miR-124 inhibitor were consistent with serum levels associated with histological evidence of myocardial injury (interstitial edema, basement membrane damage, myocardial degeneration, apoptosis, necrosis, and cell membrane damage) induced by isoproterenol in Sprague-Dawley rats (Zhang et al., 2008). Plasma exosome RNA sequencing analysis from the same animals showed an upregulation of 7 miRNAs in circulation, 6 of which are cardiac-specific (miR-1/miR-1b, miR-133a/miR-133b, miR-208b, and miR-499), are highly expressed in the myocardium, play central roles in heart function and remodeling, and have been shown to be significant diagnostic and/or prognostic biomarkers for progressing stages of cardiovascular disease in human patients, actually outperforming cardiac troponin T (Fichtlscherer et al., 2011;

McGahon et al., 2013; Chistiakov et al., 2016; Navickas et al., 2016; Paiva and Agbulut, 2017). In pathologic conditions, dysregulated expression of miR-1, miR-133a, miR-208a/b, and miR-499 may lead to progressive heart failure associated with arrhythmia, hypoxia, ischemia, left ventricular dilation, fibrosis, and myocardial necrosis (Chistiakov et al., 2016).

Functional classification of predicted gene targets of the upregulated miRNAs in animals receiving a dose of miR-124 inhibitor that may have elicited cardiac injury revealed that the most significant terms and interrelated pathways identified included cell-cell adhesion, mRNA binding and spliceosome, acetylcholine and norepinephrine neurotransmitter release cycles, and terminal bouton. Cell-cell adhesion in cardiac myocytes is essential for the heart to function as an electromechanical syncytium, and alterations in cell-cell adhesion are observed in heart failure (McCain et al., 2012). Norepinephrine is a predominant endogenous neurotransmitter released mainly from nerve terminals of the sympathetic nerve and can raise blood pressure by vasoconstrictive effects, while acetylcholine is released from parasympathetic nerves and works antagonistically on the endothelium to induce vasodilation (Sheng and Zhu, 2018). Overactivation of the sympathetic nervous system can lead to myocardial injury and result in the increase of norepinephrine at the terminal nerve endings. Chronic sympathetic stimulation can lead to enlargement of the left ventricle by myocyte enlargement, interstitial growth, and remodeling (Sheng and Zhu, 2018).

Because the nervous, cardiovascular, and renal systems are highly interconnected, it is feasible that excessive inhibition of miR-124 in the brain in animals that underwent i.c.v. infusion of 0.1 nmol/day/28 days caused inappropriate activation of MR (a known target of miR-124) in the cardiovascular regions of the brain, thereby inducing an increase in sympathetic nerve activity and cardio-renal dysfunction (de Kloet et al., 2000; Boudoulas et al., 2017; Ripoll et al., 2018). The amount of receptor protein is an important determinant for the magnitude of MR- and GR-mediated responses, and MR:GR imbalance is thought to cause dysregulation of the hypothalamic-pituitary-adrenal axis, disruption of cardiovascular homeostasis, and cognitive and mood impairments (Gomez-Sanchez, 2014). These findings suggest that miR-124 levels in the brain should be tightly regulated. Importantly, a reduced dose of miR-124 inhibitor (0.05 nmol/day/28 days) did not elicit elevated plasma protein and miRNA cardiac injury markers but did induce an upregulation of genes important for synaptic plasticity, glucocorticoid signaling, and neurogenesis in the hippocampus. Future studies are needed to investigate the effects of brain miR-124 inhibition on cardiovascular and renal function.

5. Conclusion

Alleviation of the symptoms of multifactorial diseases such as GWI that affect the central nervous system, the neuroendocrine system, and the immune system likely requires therapies that are able to activate and inactivate a large set of orchestrated genes. Pharmacological modulation of dysregulated miRNAs, which have many targets within cellular networks, has been shown to be well tolerated based on animal models and short-term clinical trials in patients (van Rooij and Kauppinen, 2014). Findings from this study demonstrated that modulating the expression of miR-124-3p in the brain *in vivo* using an antisense oligonucleotide holds promise as a novel therapeutic strategy to treat the cognitive and neuroendocrine dysfunction exhibited in GWI. Because inhibition of miR-124 has also been proposed as a strategy for treating depression, Alzheimer's disease, multiple sclerosis, and ALS, results from this study may have broader implications for patients with these conditions and for people increasingly exposed to pesticide mixtures (Dutta et al., 2013; Bahi et al., 2014; Marcuzzo et al., 2015; Roy et al., 2017; Wang et al., 2018). Before this therapeutic approach can be utilized in patients, additional preclinical studies must be conducted to assess feasibility and safety by developing a practical, noninvasive drug delivery system to the brain and exploring potential adverse toxicologic effects of miR-124 inhibition on the cardiovascular and renal systems.

Transparency document

The Transparency document associated with this article can be found in the online version.

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