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Epigenetic histone acetylation and *Bdnf* dysregulation in the hippocampus of rats exposed to repeated, low-dose diisopropylfluorophosphate

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Abstract

Aims: Deployment-related exposures to organophosphate (OP) compounds are implicated for Gulf War Illness (GWI) development in First GW veterans. However, reasons for the persistence of GWI are not fully understood. Epigenetic modifications to chromatin are regulatory mechanisms that can adaptively or maladaptively respond to external stimuli. These include DNA methylation and histone acetylation. DNA methylation changes have been reported in GWI but the role of histone acetylation in GWI has been less explored, despite its importance as an epigenetic mechanism for neurological disorders.

Main methods: Male Sprague-Dawley rats were exposed to OP diisopropyl fluorophosphate (DFP, 0.5 mg/kg s.c., 5-d) and 6-m later brains were dissected for hippocampus. Western blotting, activity assays and chromatin immunoprecipitation (ChIP) were utilized for epigenetic analyses. Behavior was assessed using the Forced Swim Test (FST) and the Elevated Plus Maze (EPM).

Key findings: We observed a significant upregulation in HDAC1 protein along with a significant increase in HDAC enzyme activity in the hippocampus of DFP rats. A locus-specific ChIP study revealed decreases in H3K9ac at the brain derived neurotrophic factor (*Bdnf*) promoter IV coupled with a significant decrease in BDNF protein in DFP rat hippocampus. Treatment with HDAC inhibitor valproic acid reduced HDAC activity and decreased the FST immobility time in DFP rats.

Significance: Our research suggests that epigenetic alterations to histone acetylation pathways and decreased BDNF expression could represent novel mechanisms for GWI symptomatology and may provide new targets for developing effective drugs for GWI treatment.

Introduction

Around 35% of the returning soldiers from the First Gulf War exhibit a chronic multi-symptom condition also known as Gulf War Illness (GWI), chief among which are the neurological morbidities of chronic fatigue, depression, mood disorders, and cognitive impairment [1]. While veterans were exposed to many chemicals and environmental factors in the Iraqi war theatre, chronic exposure to organophosphate (OP) category of compounds that included pesticides and accidental exposure to low levels of nerve gas sarin are considered amongst likely causes of GWI symptoms [1, 2]. Several investigators have postulated mechanistic theories for the development of GWI such as chronic neuroinflammation, oxidative stress, cell death, neurotoxicity, calcium imbalances, changes in gut microbiota among others [3-13]. However, a central question is how short-lived chemical exposures from 30-years ago could produce such a debilitating multi-system illness even when the causative factors are no longer present. This has not been satisfactorily answered.

Epigenetics could provide insights into this perplexing question. Many factors can affect epigenetic processes such as our environment, diet, and experiences [14-16]. Epigenetic mechanisms respond to these external stimuli, and the effects of such exposures can become embedded in the genome to produce long-lasting changes in cellular regulation [15-18]. There are several epigenetic mechanisms, including DNA methylation and histone modifications, that switch genes “on and off” in a cell [19, 20]. Investigators have started to address epigenetic modifications in GWI. For example, in a rat model of GWI which employed DEET + permethrin + pyridostigmine + stress, long-term epigenetic DNA methylation (5mC) modifications including increases in hippocampal but not cortical 5mC content was reported [21]. Interestingly, the DNA hydroxymethylation content (5hmC) was reduced in cortex but not in the hippocampus.

In addition, chronic alterations in microRNA (miRNA) expression including an upregulation of miR-124-3p and miR-29b-3p in the hippocampus of GWI rats were noted [21]. In another rodent model of GWI which employed corticosterone and DFP exposures in mice, early epigenetic changes including DNA methylation changes in genes related to immune and neuronal function were noted [22]. Further, a recent clinical study showed changes to DNA methylation levels in blood samples from GWI veterans [23]. While the global DNA methylation levels were similar in GWI patients and controls, over 700 differentially methylated gene promoters, which were predominantly hypermethylated were noted many of which genes being responsible for metabolism and immune system [23]. However, no studies have been conducted on the role of chronic histone modifications in GWI despite its importance as a major epigenetic mechanism for neurological disorders [24, 25].

One additional molecular factor that has come to forefront in the pathogenesis of multiple neurological disorders is the Brain Derived Neurotrophic Factor (BDNF) [26-28]. The *Bdnf* gene contains at least nine differentially regulated promoters of which promoter IV is the best characterized regulatory element [29]. Lack of *Bdnf* expression through promoter IV results in depressive phenotypes in mice [30], while epigenetic modifications at promoter IV are sensitive to stressors and can affect regulation of genes involved in monoamine neurotransmission [31, 32]. Interestingly, alterations in BDNF levels in rodent models of GWI were recently reported [33, 34]. Thus, studying the epigenetic control of *Bdnf* regulation in GWI could provide new information on molecular mechanisms underlying GWI.

Our previous studies have shown that repeated exposure to OP agent diisopropyl fluorophosphate (DFP) in rats produces hippocampal injury and behavioral signs of depression, anxiety, and memory impairment when assessed months after initial OP exposures [35, 36].

Using this DFP-based rat model of GWI-like neurological signs, we identified chronic alterations in intracellular calcium release mechanisms [37]. Subsequently, we reported reductions in BDNF levels [33] and also confirmed the antidepressant effects of ketamine [38] in this GWI-like model. Thus, this model is ideally suited to understand long-term effects of environmental factors (OP exposures) on GWI progression. In this study, we investigated histone modifications in the DFP model. We also investigated whether pharmacological blockade of the histone deacetylase (HDAC) enzyme using a prototype HDAC inhibitor valproic acid (VPA) [39-42] could help alleviate GWI-like symptoms.

Materials and Methods:

Animals

All animal use procedures were in strict accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by Virginia Commonwealth University's (VCU) Institutional Animal Care and Use Committee (IACUC) and by the Animal Care and Use Review Office (ACURO) of the United States Army Medical Research and Material Command (USAMRMC). Male Sprague-Dawley rats (Envigo, Indianapolis, IN) weighing ~300 g and 9 weeks of age were used in this study. Animals were housed two per cage at 20-22^{o} C with a 12-hour light-dark cycle (lights on 0600-01800 h) and given free access to food and water.

DFP exposure

All the chemicals were obtained from Millipore Sigma (St. Louis, MO, USA) unless otherwise noted. Rats were injected with DFP (0.5 mg/kg, s.c.) once daily for 5-days, while control rats received DFP vehicle injections (Phosphate Buffered Saline) for the same period

[35-37]. Animal health including weight measurements were assessed daily during the exposure and for an additional seven days following DFP injections. No overt cholinergic signs such as seizures were observed during DFP administrations or subsequently after the end of treatment period. Other cholinergic signs such as diarrhea, loose stools, body cramping or behavioral changes were also not noted. Some animals (< 5%) experienced weight loss, mild porphyrin stain, and developed mild tremors after the last DFP injection and all of these signs resolved on their own within 48-h after the last DFP dose without any need for pharmacological interventions. No mortality was associated with our DFP exposure protocol. At 6-months following DFP exposures, no significant differences in weights or any seizures were observed between control and DFP exposed rats. Using a battery of behavioral assays, we have previously shown that rats display signs of depression, anxiety, and memory impairment at this time point post-DFP exposure. To prevent confounds of behavioral stressors on epigenetic outcomes, in this study, we did not subject the rats to behavioral assessments. Rats were euthanized and hippocampal tissue obtained for epigenetic studies. A separate cohort of DFP-exposed rats were utilized for valproic acid (VPA) studies as described below **(Fig. 1)**.

Western blot studies

Rats were deeply anesthetized under saturated isoflurane conditions and then euthanized by decapitation. Brains were immediately removed and placed on glass surface cooled over ice. The hippocampus was dissected out and flash frozen in liquid nitrogen and stored at -80°C until use. The hippocampus was homogenized in a buffer containing 50 mM Tris-HCl, 7 mM EGTA, 5 mM EDTA and 0.32M sucrose, together with SigmaFast protease inhibitors (S8820, MilliporeSigma, Burlington, MA) and PhosStop phosphatase inhibitors (4906845001, MilliporeSigma), using a Teflon high speed motor homogenizer. Total protein concentration was

measured using bicinchoninic acid (BCA) assay. 20 µg of total protein in 20 µL of βME-Laemmli buffer were separated on gradient 4-20% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels by electrophoresis for 45 min at 20 mA and 50 mA and then transferred onto Polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 hour. The blots were cut between HDAC 1, 2, 3, 4 and 5, BDNF, and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) bands after locating them visually (visual standard ladder) and using a Ponceau S temporary stain solution (P7170, MilliporeSigma). The HDAC sections were probed using primary HDAC antibodies, as follows: HDAC 1 at 1:1500 (PA1-860, ThermoFisher), HDAC 2 at 1:2000 (ab32117, Abcam), HDAC 3 at 1:5000 (ab32369, Abcam), HDAC 4 at 1:1000 (sc-46672, Santa Cruz) and HDAC 5 at 1:1000 (sc-133106, Santa Cruz). The BDNF section was probed using primary BDNF antibody at 1:1000 (ab108319, Abcam). GAPDH section was probed with 1:20000 (MA5-15738, Thermo Scientific), at 4^{o}C overnight, followed by three TBST washes for 5 min each. The blots were incubated with horse-radish peroxidase (HRP)-coupled goat anti-rabbit (1:10000, sc-2030, Santa Cruz) for HDAC 1, 2 and 3 or goat anti-mouse IgG secondary antibody (1:10000, sc-2031, Santa Cruz) for HDAC 4 and 5. For the BDNF blot was incubated with horse-radish peroxidase (HRP)-coupled rabbit IgG secondary antibody (1:10000, 7074S, Cell Signaling). The GAPDH blot was incubated with HRP-coupled goat anti-mouse IgG secondary antibody (1:20000, sc-2031, Santa Cruz), for 1hr at room temperature. Blots were washed 5 times TBST for 5 minutes. Blots were treated with 2µL Clarity^{TM} Western Enhanced chemiluminescence (ECL) Substrate (170-5060, Bio-Rad, Hercules, CA) to start chemiluminescence and imaged 5 minutes afterwards. The respective pieces of a blot were realigned and imaged at the default auto optimal exposure setting or manual exposure setting up

to 5 minutes, acquiring an image each 10 seconds in a ChemiDoc Touch (Bio-Rad). Images were analyzed using ImageLab (Bio-Rad). The intensity levels of each HDAC and BDNF were normalized to the reference bands of GAPDH to yield a normalization factor used to calculate the normalized intensity for HDAC and BDNF bands of each sample.

HDAC activity assay

HDAC activity was measured in rat hippocampal extracts using the Epigenase HDAC Activity Direct Assay Kit (Colorimetric) from Epigentek (Farmingdale, NY) according to the manufacturer's instructions. Briefly, cell nuclei were extracted from 20 mg sections of hippocampal tissue using the EpiQuik nuclear extraction kit (Epigentek) and nuclear protein was quantified using a BCA assay (ThermoFisher, Waltham, MA). We loaded 10 µg of nuclear protein per Epigenase HDAC activity assay. We used hippocampal extracts from equal numbers of control, DFP-treated, and VPA-treated DFP rats, with HDAC activity in each sample assayed in duplicate. Epigenase colorimetric assays were read at 450 nm on a Synergy HT (BioTek, Winooski, VT), with reference wavelength of 655 nm.

Chromatin immunoprecipitation

Chromatin was prepared using the TruChIP tissue shearing kit (Covaris, Woburn, MA). For each sample, 10 mg of hippocampal tissue was minced into 1 mm^3 fragments over dry ice, washed with 1X cold PBS and centrifuged at 200g for 5min at 4^oC. Pellets were resuspended in Covaris fixing buffer A and fixed in methanol-free formaldehyde (1% final concentration). After centrifugation, the cell pellet was washed with cold PBS before being flash frozen in liquid nitrogen and pulverized in a Covaris tissueTUBE. Preparations were lysed in Covaris lysis buffer and nuclei were sheared on a Covaris M220 instrument. Time course trials were conducted to

determine optimal formaldehyde fixation times (2 and 5 min) and shearing times (6-12 min). Of these, 5 min fixation and 8-minute shear yielded the highest percentage (>75%) of fragment sizes between 150 and 700 bp as assessed via the Agilent Bioanalyzer high sensitivity assay (Agilent, Santa Clara, CA).

We used these optimized conditions to prepare chromatin from hippocampi of DFP-treated rats and control rats. From each sample, 2% of total chromatin was retained and crosslinks reversed to provide an input control. The remaining chromatin was split into two equal portions and ChIP carried out in ChRIPA buffer after SDS sequestration using either anti-H3K9ac antibody (39137, Active Motif, Carlsbad, CA) or 5µL of Rabbit IgG isotype control (ab171870, Abcam). Samples were first precleared with anti-rabbit IgG M280 Dynabeads (Thermo Fisher, Waltham, MA) for 1 hour followed by incubation with antibody overnight at 4ºC. The formed complex was captured using fresh Dynabeads for 4 hrs at 4ºC. The bead linked complex was captured by magnet and washed 4 times with LiCl wash buffer prior to elution. DNA was liberated from the ChIP'ed samples and input controls by incubating overnight (16 hrs) at 65ºC followed by purification using the Minelute kit (Qiagen, Hilden, Germany).

H3K9ac occupancy at *Bdnf* promoter IV was tested via quantitative PCR (qPCR) using primers and conditions previously described [43, 44]. 2.5 µL of each DNA elution (H3K9ac, IgG, and input control) per sample was used per qPCR reaction. Each sample was amplified in triplicate on a Quantstudio3 instrument (Applied Biosystems, Foster City, CA) using PowerUp SYBR Green reporter dye. A single melt peak was shown by the qPCR melt curves indicating formation of a single product. Standard curves of serially diluted rat DNA were also run for this primer set to evaluate performance and the resulting standard curve showed an excellent fit with $R^2 = 0.995$.

The mean threshold cycle (Cq) values were obtained for each sample and normalized to the dilution factor (1%) corrected Cq value (Log2 (100) =6.64) of the input control to obtain Delta Cq. Percentage of input was calculated by multiplying 100 with 2 raised to the exponent of Delta Cq. For each sample, "percentage of input" values were obtained that correspond to the degree of occupancy of that fragment of DNA by histone H3 with acetylated lysine 9.

Elevated Plus Maze (EPM)

This test assesses for signs of anxiety by considering the innate behavior of rats to prefer dark enclosed spaces over bright open spaces. An increase in open arm activity (duration and/or entries) following a drug treatment reflects anti-anxiety profile [36-38, 45]. The maze (Med Associates Inc., St. Albans, VT) was made of black polyvinyl chloride and consisted of four arms, 50 cm long x 10 cm wide, connected by a central square, 10 x 10 cm: two open without walls and two closed by 31-cm-high walls. All arms were attached to sturdy metal legs; the maze was elevated 55 cm above the floor level and was set in a dimly lit room. A video camera was suspended above the maze to record the rat movements for analysis. A video-tracking system (Noldus Ethovision XT 11) was used to automatically collect behavioral data. Briefly, rats were placed at the junction of the open and closed arms, the center of the maze, facing the open arm opposite to where the experimenter was. The video-tracking system was started after the animal was placed in the maze so that the behavior of each animal was consistently recorded for 5 min. At the end of the 5 min test session, the rat was returned to its home cage and maze cleaned with 70% ethanol and air-dried to remove any scent traces. Time spent and entries made in the various arms of EPM were calculated.

Forced Swim Test (FST)

Porsolt's modified FST was used to assess signs of behavioral despair as indicated by the amount of time an animal stays immobile during the test session [33, 36, 46, 47]. An increase in FST immobility time is often construed as an indication of despair and a decrease in FST immobility time following pharmacological intervention is regarded as the drug possessing potential antidepressant profile. Briefly, animals were placed in a glass cylindrical chamber (46cm H x 30cm D) filled with water (30 cm height, 25°C) forced to swim for 6-min. Swimming sessions were recorded for off-line analysis. Immobility was defined as the period during which the animal floats in the water making only those movements necessary to keep its head above water and was evaluated by 2-reviewers blinded to the treatment conditions. The tank was emptied and thoroughly cleaned between sessions.

Data analysis

Data were analyzed and graphs plotted using the GraphPad Prism9 software. For measuring changes in protein levels in Western blot, relative optical densities for the specific protein bands were measured, corrected for protein loading against GAPDH and then calculated as a ratio of control ± SEM. We utilized n= 5-7 rats/ group for various antibody-specific western blot studies, n= 3 rats/ group for ChIP studies, n= 5 rats/ group for HDAC activity assay, and n= 7 rats/ group for behavioral studies. To compare optical densities between control and DFP rats and to compare DFP-treatment versus controls in ChIP, a Student's *t*-test was used. A one-way analysis of variance (ANOVA) followed by post-hoc Tukey test was applied when comparing multiple groups as appropriate. In all cases, statistical significance was indicated by $*p < 0.05$.

Results

Increased HDAC1 protein in hippocampus of DFP rats

We assessed expression of various HDAC proteins in the hippocampal homogenates from DFP-exposed and age-matched control rats. As shown in Fig. 2, a significant increase in the levels of HDAC1 protein in DFP rats was observed, with densitometric analysis revealing an approximately two-fold increase in HDAC1 expression compared to age-matched control rats (n=5 rats/group, t-test, p= 0.01). No significant changes were noted in the hippocampal levels of HDAC2-4 proteins between DFP and control rats (n= 5-7 rats/group, t-test, p= 0.8, p= 0.6 and p= 0.4 respectively). A small increase in the protein levels of HDAC5 expression were noted in DFP rats, but this change was also not significantly different compared to control conditions (n=7 rats/group, t-test, p= 0.3).

Decreased H3K9ac occupancy at *Bdnf* promoter

HDAC1 has been shown to directly affect histone acetylation at *Bdnf* promoter IV [48]. Therefore, we next tested if acetylation of histone 3 lysine 9 (H3K9ac) was altered at that position using ChIP coupled to qPCR. H3K9ac is one of the primary acetylation marks in promoters that is associated with activated gene expression [49]. As shown in Fig. 3, overall, the analysis showed a significant decrease in H3K9ac levels at the *Bdnf* promoter IV in DFP rats (percent input material 1.00 ± 0.04 (CTL) vs 0.64 ± 0.1(DFP), n= 3 rats/group, t-test, p=0.005).

Decreased BDNF in hippocampus of DFP rats

Western blot analysis revealed significant downregulation of BDNF protein expression in hippocampal homogenates obtained from DFP-treated rats compared to age-matched, control

rats (**Fig. 4A**). Densitometric analysis indicated that BDNF protein levels were 0.4-fold lower in DFP rats compared to age-matched controls (n=5 rats/ group, t-test, p= 0.01, Fig. 4B).

Increased HDAC activity in hippocampus of DFP rats and HDAC inhibition by VPA

We used a colorimetric assay to assess HDAC activity in hippocampal nuclear extracts from DFP rats and age-matched control rats. A significant increase in global HDAC activity in DFP rats compared to age-matched control rats was noted (13.4 ± 0.4 vs 11.9 ± 0.2 optical density/mg protein sample, respectively, n=5/group, $p<0.05$, One-Way ANOVA, Tukey test). Treatment with HDAC inhibitor valproic acid (VPA, 1 mM) significantly inhibited Global HDAC activity in VPA-treated versus untreated DFP samples (22.05 ± 0.1 vs 24.98 ± 0.3 optical density/mg protein sample, respectively, n=5 rats/group, $p<0.05$, One-Way ANOVA, Tukey test, **Fig. 5**).

Effect of epigenetic therapy on GWI behavioral signs

Having observed that VPA reduced HDAC activity in DFP-treated hippocampal extracts *in vitro*, we next investigated effects of pharmacological blockade of HDAC activity on GWI behavioral signs. Age-matched control and DFP-treated rats were treated with either saline or VPA (100 mg/kg, 1x/ day for 5-days). Twenty-four hours after the last VPA injection, rats were assessed on EPM and FST paradigms. As shown in Fig. 6, DFP-treated rats exhibited significant decreases in open-arm exploration on the EPM and also significant increases in FST immobility time compared to saline-treated age-matched control rats confirming presence of GWI-like chronic neurobehavioral morbidities upon DFP treatment (*$p<0.05$, One-way ANOVA, n= 7 rats/group). VPA treatment in DFP rats had no effect on anxiety-like behavior as evidenced by no significant changes between time-spent or entries made in the open-arm of the EPM

compared to saline-treated DFP rats (*p<0.05, One-way ANOVA, n=7 rats/ treatment group). In contrast, as shown in Fig. 6C, VPA treatment in DFP rats produced a significant decrease in immobility time on the FST compared to saline-treated DFP rats indicative of an antidepressant-like effect (*p<0.05, One-way ANOVA, n=7 rats/ group). VPA treatment in age-matched, naïve rats had no effects on EPM or FST outcomes (*p<0.05, One-way ANOVA, n=7 rats/ group).

Discussion

This study provided several salient findings. First, we observed significant increase in HDAC1 protein in DFP rats. Second, we showed that there was a significant increase in global HDAC activity in the hippocampus of DFP rats. Third, we found that there was a significant decrease in H3K9ac at the *Bdnf* promoter IV locus. Fourth, we observed a significant reduction in BDNF protein levels in DFP rats. Finally, we demonstrated that treatment with a HDAC inhibitor, VPA, not only reduced HDAC activity in DFP hippocampal homogenates but also significantly improved depressive signs in this OP DFP-based rat model for GWI-like neurological symptoms.

Epigenetic modifications can change as adaptive (or maladaptive) responses to diverse external factors. Interestingly, research has shown that these epigenetic changes can persist even in the absence of initial factors that triggered them [50]. This mechanistic scenario is like GWI, wherein a previous chemical exposure (OP) resulted in the chronic expression of GWI even when the causative environmental factors are no longer present. Environmental exposures such as pesticides, metals, and endocrine disruptors are known to trigger epigenetic modifications that activate downstream signaling pathways leading to their deleterious effects on human health [15, 16, 50-52]. For example, maladaptive epigenetic responses have been reported in stress, chronic neurodegenerative disorders, and even in drug dependence and substance abuse [24, 53-55].

Importantly, research has indicated that epigenetic alterations may mediate toxicity from pesticides and may be involved in the development of depression, [15, 50, 56] and, histone deacetylases may be among the most sensitive component of the epigenetic machinery to xenobiotic chemical exposures [57]. In agreement with these reported epigenetic modifications in response to environmental factors, this study found significant alterations in chromatin modifying histone enzymes and a significant decrease in BDNF expression within the hippocampus of DFP-exposed rats.

Histone post-translational modifications act as docking sites for other protein complexes that ultimately modulate gene activation or repression. Two enzyme families control histone acetylation: Histone acetyltransferases add acetyl moieties in the N-terminal tails of histones, thereby relaxing the chromatin and enabling transcription. Histone deacetylases (HDACs) remove acetyl groups from histones, thereby condensing chromatin and repressing gene transcription [20, 58]. Our studies showed that HDACs were being differentially modulated following DFP exposures. There are at least 11 subtypes of HDACs in mammals. HDAC1 is broadly involved in neurogenesis [59], brain development [60] and functions in several multiprotein co-repressor complexes that remodel chromatin and regulate gene expression [61]. In this study, we found that there was a global increase in HDAC activity along with an increase in HDAC1 protein. Previous studies have shown that HDAC1 activity may increase with stress [62, 63] and increased HDAC1 expression was observed in a mouse model of depression and reversed by use of the antidepressant fluoxetine [64]. While no significant changes in other HDAC subtypes were observed, a trend towards an increased HDAC5 expression was noted in DFP rats. Studies have shown increased HDAC5 in depressive models [65, 66] along with an antidepressant effect of HDAC5 inhibition in rodents [67]. An overall increase in HDAC

activity, as observed here, could imply a decrease in acetylated histones in DFP rats. We chose to look at H3K9ac because it is one of the most important acetylation marks at promoter regions that promotes gene expression. However, there are several other acetylation marks (e.g. H3K27ac, H4K12ac) that we did not assay in this study and that may change in DFP rats. Indeed, in a mouse model of GWI that utilized a high-dose DFP plus corticosterone exposures, acute changes in H3K27ac were reported [22]. Further, decreased H3K14ac, H3K23ac, and H4K16ac levels were also noted in rodent models of depression and in clinical depression [65, 68-70]. Thus, it will be interesting to study expression of other histone markers in our DFP-based GWI model.

We observed an increase in HDAC activity and reduced H3K9ac at *Bdnf*, so it is tempting to speculate that the former is causal of the latter. Unfortunately, however, our study cannot determine this causal link and more work will be required. From a mechanistic perspective, it is unclear how the epigenetic effects are induced and sustained over many months. The increase in HDAC activity could result from AChE inhibition by DFP or through some intrinsic property of DFP that increases HDAC expression or function. Previous studies have shown that HDAC inhibitors stimulate AChE expression and protect against OP exposure [71]. Additionally, DFP has also been reported to directly affect HDAC *in vitro* [72]. Understanding how HDAC activity is increased in response to DFP exposure will be crucial to understanding this problem.

Bdnf is known to be regulated by chromatin remodeling [73]. Here, we studied epigenetic regulation of *Bdnf* dysfunction in DFP rats in order to investigate whether this could explain some of the molecular mechanisms underlying the chronic nature of GWI symptoms. Our ChIP analysis indicated a significant reduction in H3K9ac at *Bdnf* promoter site on exon IV. H3K9ac

is associated with a relaxed chromatin conformation that allows the cellular transcriptional machinery to access DNA [49]. Therefore, a reduction in H3K9ac, as we see here, is indicative of the opposite scenario; more condensed chromatin and reduced gene expression. Indeed, reductions in BDNF expression have been reported in two different animal models of GWI [33, 34]. Reductions in BDNF levels have also been reported with depression, aging, and in various neurodegenerative conditions [26, 28, 74]. For example, decreased BDNF levels have been reported both in the animal models of depression and also in patients with clinical depression [26, 75]. Treatment with antidepressants increased BDNF levels [74, 76-80] and, recently, it was reported that BDNF signaling could be the common mechanism for antidepressant action [81]. Epigenetic regulation of BDNF seen here is important since BDNF plays a vital role in modulating synaptic plasticity and epigenetically-altered neuronal plasticity is commonly seen in multiple neurological disorders [26, 27, 77, 82-84]. Mechanistically, the ability of BDNF to affect spines is thought to underlie alterations in synaptic plasticity. For example, reduction in synaptic transmission likely due to a decrease in hippocampal synaptic spine density has been reported following sub-clinical OP chlorpyrifos exposures in mice [85]. It will be interesting to study whether dendritic remodeling underlies BDNF reductions as mechanism for pathological synaptic plasticity precipitating as GWI-like neurological signs in our DFP model.

Our results also indicated that treatment with VPA improved aspects of GWI-related depressive signs in DFP rats. We found that VPA treatment significantly reduced despair as indicated by reductions in FST immobility time but could not produce a significant improvement in anxiety-like signs in DFP rats. VPA is used as an antiepileptic drug, mood stabilizer and also in the treatment of migraine disorder [86]. VPA has pleiotropic actions [87] and is known to block voltage-gated sodium channels and increase inhibitory neurotransmitter GABA levels in

the brain [88]. In addition, studies have suggested involvement of PI3K/Akt/mTOR pathway [89] and cytoskeletal alterations [90] for antidepressant-like effects of VPA. While, these mechanisms could be at play in our model, more recently, the epigenetic actions of VPA have come to the forefront. VPA can modulate epigenome by inhibiting HDACs more specifically HDAC1 [39-42]. Moreover, HDAC inhibition has also been reported to protect against OP exposure in vitro [71]. Indeed, our results showed that VPA blocked the elevated HDAC activity in nuclear extracts from DFP rats. Interestingly, VPA has also been reported to increase BDNF expression in the brain by targeting HDAC and activating BDNF promoter IV [48]. Our future studies will investigate whether VPA treatment restores acetylated histones and increases BDNF expression in DFP rats. Pharmacologically, it remains to be seen whether adjusting VPA dosing regimen improves anxiety-like aspects of GWI signs and whether optimized VPA dosing affects cognitive status in DFP rats.

This study has some limitations. First, the DFP exposed rats used in our studies were exclusively males. While the majority of GW deployed soldiers were males, approximately 7% of GW veterans are females [91]. Recent epidemiological studies have started to investigate how GW service affected female veterans versus the male veterans [92]. Our future studies will address role of sex as a biological variable for differential epigenetic expression due to DFP exposures. Also, this study only focused on the hippocampus as a region of interest. Indeed, hippocampus is critical to studying GWI given its importance as an essential brain region for mood and memory function which are the major GWI neurological symptoms [93, 94]. Thus, our primary focus has been on hippocampus and accordingly previous studies that reported neuronal injury and calcium imbalances in this model utilized hippocampal regions [35-37]. However, other brain regions including cortex and amygdala are also implicated in neurological

dysfunction in response to organophosphate exposure. It remains to be investigated whether other brain structures also display neuronal injury, neuroinflammatory changes and epigenetic changes in the DFP-based GWI model. Changes in gut microbiota are among likely cause for GWI [13, 95, 96]. Our DFP exposure paradigm could possibly affect cholinesterase functioning in the gastrointestinal tract that could likely to have a significant impact on gut microbiota. With increased gut motility there may be a significant depletion of the initial microbiota population, with later repopulation with a different set of bacteria. While it is beyond the goals of our current studies to investigate these possibilities, additional studies are needed to address the many complexities of interpretation of the data reported here.

Conclusions

In summary, our studies provide new information about the potential role of epigenetic regulation of gene expression in a preclinical model of GWI-like signs and its treatment. Our research for the first time demonstrated that repeated low-dose OP DFP exposures in DFP rats, which, mimicked GW deployment-related OP nerve gas exposures, were also associated with chronic alterations in the histone signaling pathway, possibly contributing to a decreased BDNF expression in the hippocampus of these DFP rats. Further, treatment with a non-specific HDAC inhibitor VPA alleviated some of the GWI-like signs in rodents which suggests that HDAC inhibitors may represent a promising new class of drugs for GWI and encourages further studies on these mechanisms.

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Figure Legends

Figure 1. Schematic of experimental studies

Nine-week old, male, Sprague-Dawley rats were exposed to DFP (0.5 mg/kg, s.c., 1x daily). Six-months later, DFP rats and age-matched, vehicle-treated control rats were euthanized, hippocampal homogenates obtained and subjected to (a) epigenetic assays or Western blot analysis. For (b) behavioral studies, another group of DFP rats received either valproic acid (VPA, 100 mg/kg, i.p., 2x daily) or saline for 5-days. Age-matched control rats also received similar treatments. On the sixth day, these rats were subjected to behavioral tests to assess for the signs of anxiety and depression.

Figure 2. HDAC protein expression in DFP rats

Western immunoblot of hippocampal protein from DFP and age-matched control (CTL) rats stained for various HDAC subtypes (A). To adjust for sample loading, the blots were stained with an antibody specific for the GAPDH protein. Densitometric analyses of the Western blotting revealed significant increases in the levels of HDAC1 (B) compared with age-matched control (Data expressed as mean optical density ratio (HDAC/GAPDH) ± SEM, *p< .05, t test, n= 5-7 rats/ group).

Figure 3. H3K9ac ChIP-qPCR results for *Bdnf* promoter IV.

Quantity of immunoprecipitated DNA at the *Bdnf* locus for each experimental group (DFP, control) is expressed as percentage of input material into the ChIP assay. The IgG results show that there was low background noise and that the ChIP findings were not driven by nonspecific immunoprecipitation effects. (percent input material 1.00 ± 0.04 (CTL) vs 0.64 ± 0.1(DFP), n= 3 rats/ group, t-test, *p<.05).

Figure 4. BDNF protein expression in DFP rats

Western immunoblot of hippocampal protein from DFP and age-matched control (CTL) rats stained for BDNF (A). To adjust for sample loading, the blots were stained with an antibody specific for the GAPDH protein. Densitometric analyses of the Western blotting revealed significant increases in the levels of BDNF (B) compared with age-matched control (Data expressed as mean optical density ratio (BDNF/GAPDH) ± SEM, *p< .05, t test, n= 5 rats/ group).

Figure 5. Increased HDAC activity in DFP rats and its inhibition by VPA

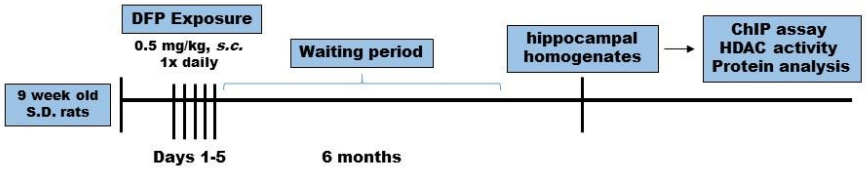
Hippocampal homogenates from age-matched control and DFP rats were assessed for global HDAC activity using a colorimetric assay that showed a significant increase in HDAC activity in specimens from DFP rats compared to control rats. In another assay, these homogenates were treated with pan-HDAC inhibitor valproic acid (VPA, 1 mM) into the assay buffer. A significant inhibition of HDAC activity was noted in the specimens from DFP rats compared to control rats (Data expressed as percent change from control HDAC activity ± SEM, *p< .05, One-way ANOVA, Tukey test, n= 5 rats/ group).

Figure 6. Effect of VPA treatment in DFP rats

Age-matched control and DFP rats were either treated with saline or VPA. Thus, four group of rats were included in the behavioral analysis. Valproic acid was administered (VPA, 100 mg/kg, 1x daily, 5 days) and behavior assessed on Elevated Plus Maze (EPM) and Forced Swim Test (FST) 24-h after the last injection. Significant differences were noted in EPM behavior between the saline-treated control rats and DFP-treated rats indicating presence of anxiety-like behavior following DFP exposures. No significant differences in the open-arm entries (A) or open-arm

time (B) were observed on EPM behavior between saline (SAL)- or VPA-treated DFP rats. Similarly, VPA-treatment did not have any significant effect on EPM performance in age-matched control rats. C. Significant differences were noted in FST immobility time between the saline-treated control rats and DFP-treated rats indicating presence of depression-like signs following DFP exposures. VPA treatment produced significant reductions in immobility times on FST in DFP rats compared to saline-treated DFP rats. Similarly, VPA-treatment did not have any significant effect on FST immobility outcomes in age-matched control rats. (Data expressed as mean ± SEM, *$p<0.05$, One-way ANOVA, Tukey test, n= 7 rats/group).

a) Epigenetic assays



b) Behavioral assays

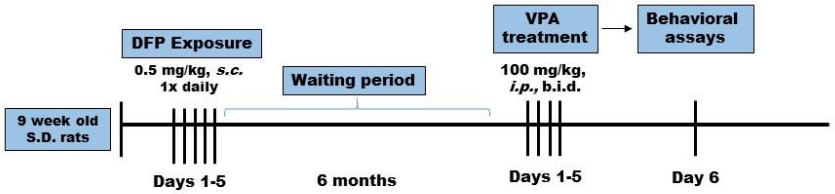
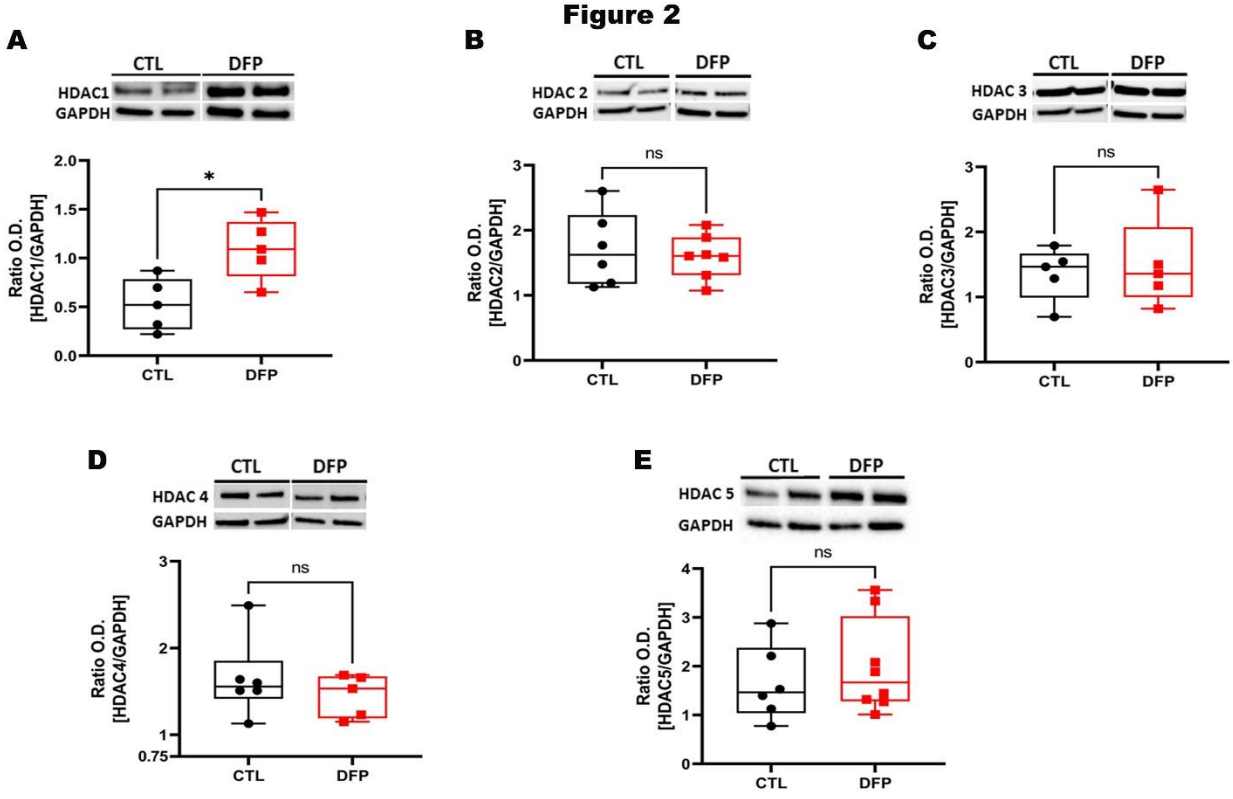


Figure 2


A
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ns
C
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GAPDH
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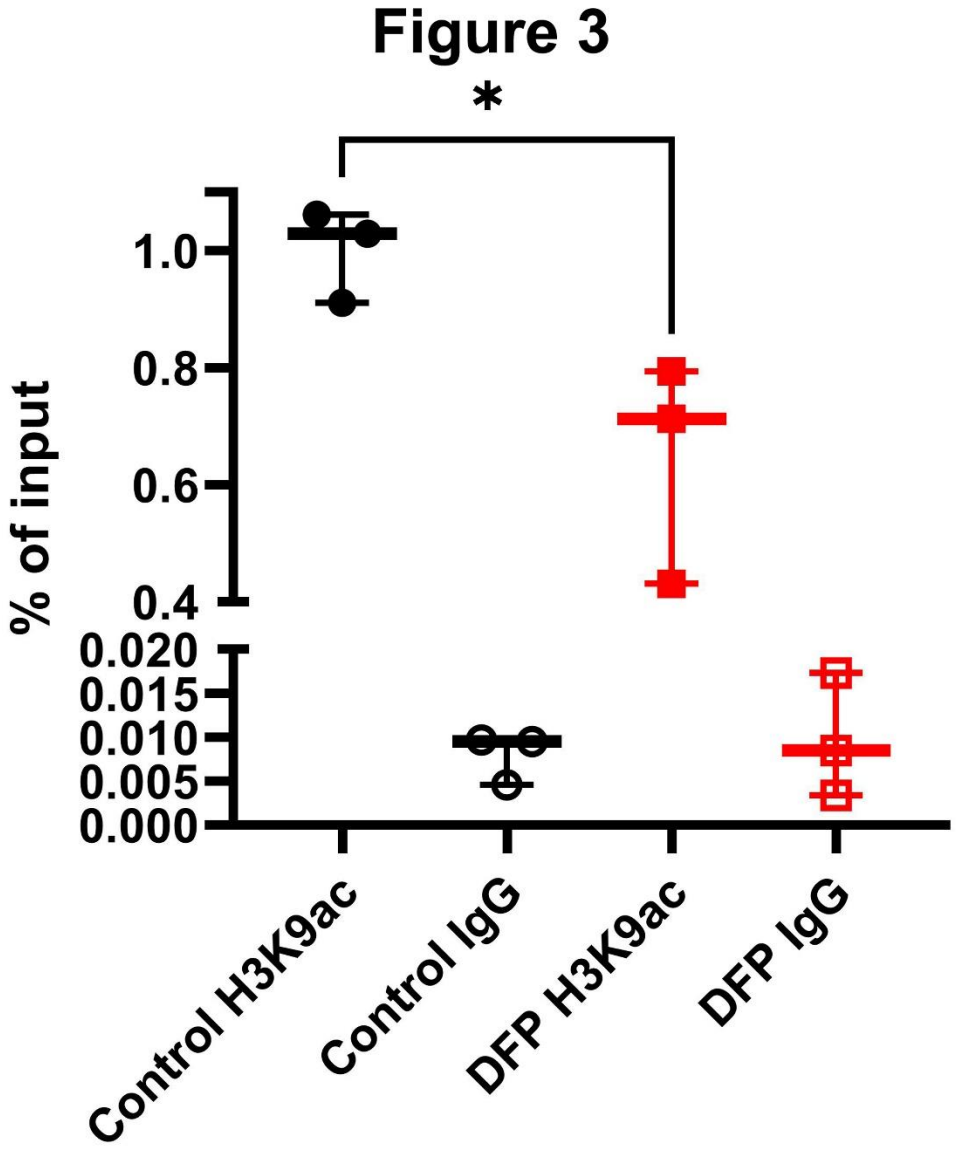
Figure 3
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DFP IgG

Figure 4

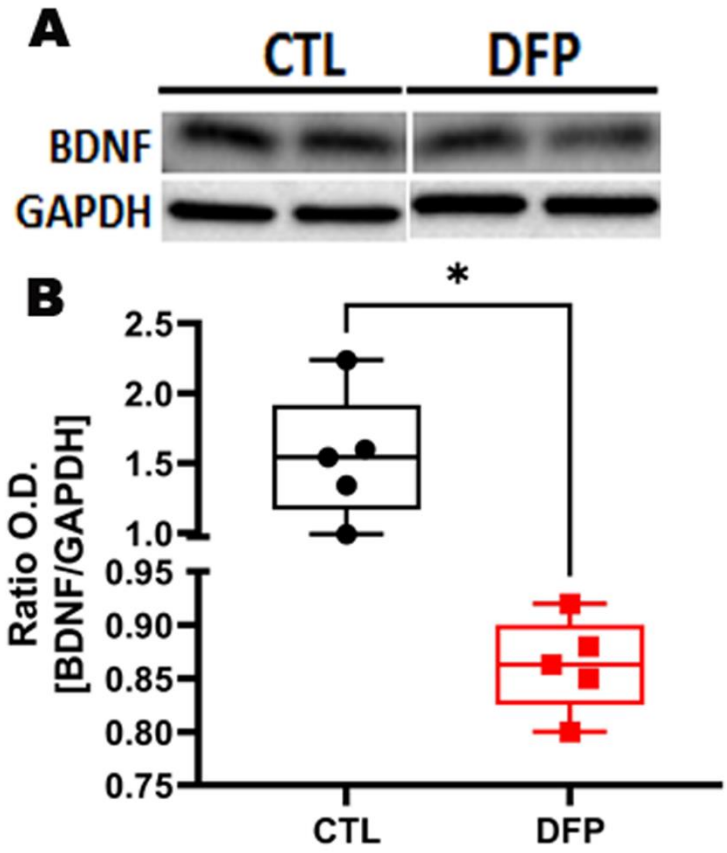
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Figure 5

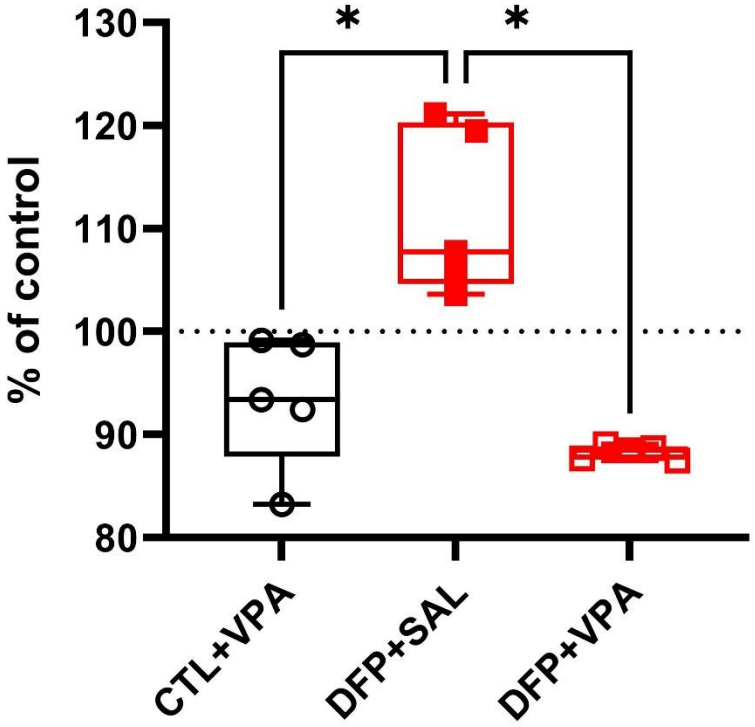
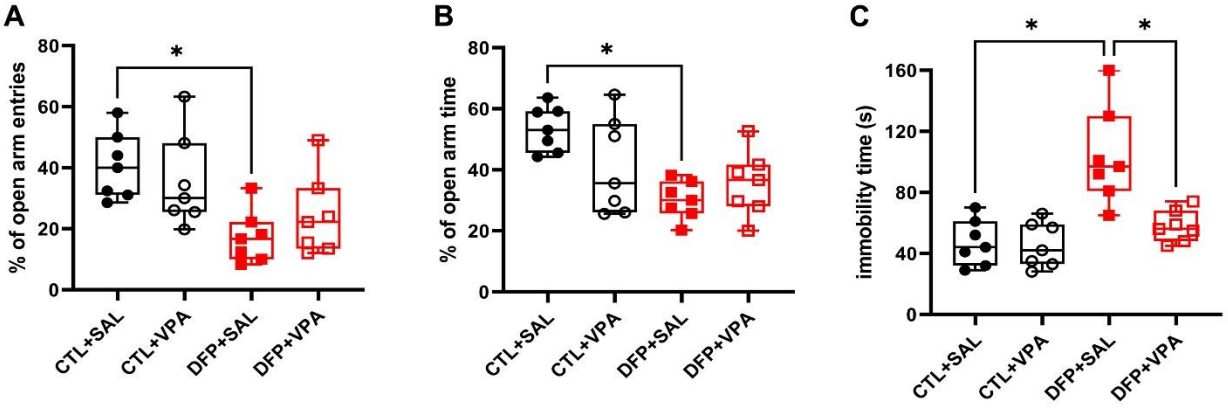


Figure 6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