Ketamine produces antidepressant effects by inhibiting histone deacetylases and upregulating hippocampal BDNF levels in a DFP-based rat model of Gulf War Illness

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Abstract

Approximately one-third of Gulf War veterans suffer from Gulf War Illness (GWI), which encompass mood disorders and depressive symptoms. Deployment-related exposure to organophosphate (OP) compounds has been associated with GWI development. Epigenetic modifications have been reported in GWI veterans. We previously showed that epigenetic histone dysregulations were associated with decreased Brain Derived Neurotrophic Factor (BDNF) expression in a GWI rat model. GWI has no effective therapies. Ketamine (KET) has recently been approved by the FDA for therapy-resistant depression. Interestingly, BDNF upregulation underlies KET's antidepressant effect in GWI-related depression. Here we investigated whether KET's effect on histone mechanisms signal BDNF upregulations in GWI. Male Sprague-Dawley rats were injected once-daily with diisopropyl fluorophosphate (DFP, 0.5 mg/kg s.c., 5-d). At 6-m following DFP exposure, KET (10 mg/kg, i.p.) was injected and brains were dissected 24-h later. Western blotting was utilized for protein expression and epigenetic studies utilized chromatin immunoprecipitation methods. Dil staining was conducted for assessing dendritic spines. Our results indicated that an antidepressant dose of KET inhibited the upregulation of HDAC enzymes in DFP rats. Furthermore, KET restored acetylated histone occupancy at the Bdnf promoter IV and induced BDNF protein expression in DFP rats. Finally, KET treatment also increased the spine density and altered the spine diversity with increased T-type and decreased S-type spines in DFP rats. Given these findings, we propose that KET's actions involves the inhibition of HDAC expression, upregulation of BDNF, and dendritic modifications that together ameliorates the pathological synaptic plasticity and exerts an antidepressant effect in DFP rats.
Significance Statement

Our research offers evidence supporting the involvement of epigenetic histone pathways in the antidepressant effects of ketamine (KET) in a rat model of Gulf War Illness (GWI)-like depression. This effect is achieved through the modulation of histone acetylation at the Bdnf promoter, resulting in elevated BDNF expression and subsequent dendritic remodeling in the hippocampus. These findings underscore the rationale for considering KET as a potential candidate for clinical trials aimed at managing GWI-related depression.
Introduction

Over a third of the First Gulf War veterans suffer from a multisystem disorder known as Gulf War Illness (GWI). The commonly reported symptoms include neurological problems such as concentration difficulties, mood alterations (e.g. anxiety and depressive symptoms), and memory dysfunctions (Committee on Gulf War and Health, 2016). While many deployment-related factors have been studied for GWI development (Steele et al., 2012; White et al., 2016), several lines of research now convincingly point towards the involvement of the organophosphate (OP) category of compounds in GWI etiology (Haley and Tuite, 2013; Tuite and Haley, 2013; Parrish and Haley, 2021; Haley et al., 2022). OPs include pesticides and chemical-warfare agent sarin (Costa, 2018), and there is evidence that GW soldiers were exposed to these compounds during deployment (Ribeiro and Deshpande, 2021). The First GW ended in 1992, and it has been over 30 years since the deployed soldiers returned state-side. However, the GWI symptoms have persisted and worsened over time (Zundel et al., 2019; Yee et al., 2020).

Researchers have developed multiple GWI rodent models by trying to mimic exposures to GW deployment-related factors (Ribeiro and Deshpande, 2021). One such model uses repeated, low-dose exposures to OP agent diisopropyl fluorophosphate (DFP) in rats (Phillips and Deshpande, 2016). These DFP-exposed rats exhibit neuronal injury and neurological morbidities similar to those reported by GW veterans and this model has been used to identify mechanisms and therapeutics for GWI treatment (Phillips et al., 2019; Zhu et al., 2020; Ribeiro et al., 2021). Pre-clinical research has implicated several mechanisms for GWI persistence, including chronic neuroinflammation, gut dysbiosis, neuronal calcium imbalances, and epigenetic alterations (Dickey et al., 2021; Ribeiro and Deshpande, 2021; Kodali et al., 2023). Epigenetics studies chemical modifications to the genome that do not alter the DNA sequence.
but can influence how genes are expressed (Jaenisch and Bird, 2003). Epigenetic mechanisms include DNA methylation, histone post-translational modifications (PTMs), and microRNAs that switch genes "on and off" in a cell (Sweatt, 2013). Exposure to environmental factors such as pesticides, metals, and endocrine disruptors are reported to produce deleterious epigenetic changes. Such modifications are further known to signal molecular pathways that mediate toxicities from such exposures (Cortessis et al., 2012; Hou et al., 2012; Collotta et al., 2013).

For example, chronic OP exposure is associated with DNA methylation changes in humans (Paul et al., 2018). Similarly, DNA methylation alterations have also been reported in GW veterans’ blood (Trivedi et al., 2019). Alterations in DNA methylation and histone modifications have also been identified in rodent models of GWI (Pierce et al., 2016; Ashbrook et al., 2018; Ribeiro et al., 2021). Thus, epigenetic modifications represent a leading mechanism that could elucidate the onset and enduring nature of GWI.

Research into treatments for GWI has taken a mechanisms-based approach by choosing/repurposing drugs that could target the underlying pathological mechanisms to alleviate GWI symptoms (Dickey et al., 2021; Ribeiro and Deshpande, 2021; Kodali et al., 2023). One such pathological mechanism that has come to the forefront for GWI are the alterations in the brain-derived neurotrophic factor (BDNF) (Carreras et al., 2018; Kimono et al., 2020; Ribeiro et al., 2020; Brown et al., 2021; Ribeiro et al., 2021). Recently, in agreement with other models of depression (Bjorkholm and Monteggia, 2016; Zanos and Gould, 2018; Deyama and Duman, 2020), we demonstrated that in a rat model of GWI-like depression, low-dose ketamine (KET) produced a rapidly acting and sustained antidepressant effect via BDNF induction (Ribeiro et al., 2020; Zhu et al., 2020). Interestingly, there is growing evidence that KET’s antidepressant action may act via epigenetic mechanisms to induce BDNF expression (Choi et al., 2015; Choi...
et al., 2017). While a role for BDNF epigenetics (Sakata et al., 2010; Poon et al., 2021) and reduced BDNF leading to lower neuroplasticity in depression is well-established (Castren and Rantamaki, 2010; Duman and Duman, 2015; Qiao et al., 2017; Yang et al., 2020), it is not known if KET acts via epigenetic mechanisms in to alleviate depression symptoms in our rat model of GWI and is the focus of this study.

Materials and Methods:

Animals

Nine-week-old male Sprague-Dawley rats (Envigo, Indianapolis) were housed (2 per cage) in VCU’s vivarium with ad libitum access to food and water. The VCU Animal Care and Use Committee approved the studies conducted in this study.

DFP-based GWI model and KET treatment

DFP was injected in rats at the dose of 0.5 mg/kg, s.c., 1x/day, for five consecutive days, while control rats received Phosphate Buffered Saline as described previously (Phillips and Deshpande, 2016; Ribeiro et al., 2020; Zhu et al., 2020; Ribeiro et al., 2021). At approximately six months, DFP-exposed rats exhibited depressive signs using various behavioral assays. No weight differences were noted between the control and the DFP group (Phillips and Deshpande, 2016). We have previously demonstrated that a non-anesthetic dose of KET (10 mg/kg, i.p.) produces immediate (1-h) and sustained (24-h) antidepressant response in DFP rats (Ribeiro et al., 2020; Zhu et al., 2020). Interestingly, significant BDNF upregulation was seen at 24-h post-KET (Ribeiro et al., 2020), a time point by which KET is reported to no longer be present in the system (Veilleux-Lemieux et al., 2013; Zanos et al., 2016). We selected the 24-h time point for our epigenetic studies. Rats were not subjected to behavioral assessments in order to avoid
introducing potential confounding factors related to behavioral stressors that might impact the study's epigenetic outcomes.

**Western blot studies**

Briefly, rats were euthanized, brains were removed, hippocampus was dissected, flash frozen and stored at -80°C. Antibody-specific Western blotting studies were conducted using procedures established in our lab and validated for DFP exposures (Ribeiro et al., 2020; Ribeiro et al., 2021). The blots were carefully cut between HDAC 1, 2, 3, 4, and 5, BDNF, and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) bands based on visual identification using a Ponceau S temporary stain solution (P7170, MilliporeSigma). The HDAC sections were subsequently subjected to primary antibodies with the following dilutions: 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). In the case of the BDNF section, a primary BDNF antibody at 1:1000 (ab108319, Abcam) was employed. Meanwhile, the GAPDH section was probed using a 1:20000 dilution of the primary antibody (MA5-15738, Thermo Scientific) and incubated overnight at 4°C. Subsequently, three 5-minute washes with TBST were performed. The blots were then exposed to secondary antibodies for detection, specifically 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. The BDNF blot used a horse-radish peroxidase (HRP)-coupled rabbit IgG secondary antibody (1:10000, 7074S, Cell Signaling), while the GAPDH blot utilized HRP-coupled goat anti-mouse IgG secondary antibody (1:20000, sc-2031, Santa Cruz) for 1 hour at room temperature. Following this, the blots were washed five times with TBST for 5 minutes each. To initiate chemiluminescence, 2µL
of Clarity™ Western Enhanced chemiluminescence (ECL) Substrate (170-5060, Bio-Rad, Hercules, CA) was applied, and imaging occurred 5 minutes later. The individual blot sections were then realigned and imaged using the default auto-optimal exposure or manual exposure settings for up to 5 minutes, capturing an image every 10 seconds using a ChemiDoc Touch (Bio-Rad). Image analysis was conducted using ImageLab (Bio-Rad). The intensity levels of each HDAC and BDNF band were normalized to the reference bands of GAPDH to establish a normalization factor, which was employed to calculate the normalized intensity for the HDAC and BDNF bands in each sample.

**Chromatin immunoprecipitation coupled to quantitative PCR (ChIP-qPCR)**

For each sample, chromatin was prepared from 10 mg hippocampal tissue using the TruChIP tissue kit (Covaris, Woburn, MA) and sheared using a Covaris M220 instrument according to optimized conditions as described previously (Ribeiro et al., 2021). From each sample, 4% of total chromatin was retained and crosslinks reversed to provide an input control. The remaining chromatin was divided into two equal parts, and chromatin immunoprecipitation (ChIP) was carried out in ChRIPA buffer after sequestering SDS, employing either an anti-H3K9ac antibody (39137, Active Motif, Carlsbad, CA) or a Rabbit IgG isotype control (ab171870, Abcam), in accordance with previously established protocols (Ribeiro et al., 2021). The immunoprecipitated DNA was subsequently assessed for H3K9ac occupancy at the Bdnf promoter IV region using quantitative PCR (qPCR). The primers and conditions for the qPCR were as previously described (Seo et al., 2016; Asp, 2018; Ribeiro et al., 2021).
Spine Density Analysis

For Dil staining (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) (Kim et al., 2007; Stanic et al., 2015), crystals were applied on 2-3 mm coronal slices. Slices were kept in dark for 12-24h at room temperature and then fixed with 4% PFA for 45min-1h at 4°C and cut on a vibratome at 100-150 μm thickness. Slices were mounted on glass slides, sealed, and images acquired as follows. Images of neurons are registered using an LSM 880 confocal microscope (Zeiss, NY, USA) equipped with 40x oil immersion Plan Apo objective lens (NA=1.4) and a 32-element image scanning detector (Airy). Each element corresponded to 0.2 Airy units. Fluorescence of Dil is excited with a 25 mW diode-pumped solid state (514 nm) laser (3% nominal output) and detected in 525-735 nm range. Series of optical sections (stacks) are collected with 204 nm z-step, 45 nm pixel xy size and 1.59 μs pixel dwell time. The total imaging field corresponds to 118x118 μm. Imaging parameters were set so that the 16-bit dynamic range was filled in approximately 50% to best clarify neuronal spines from the background. The raw images produced by the elements of Airy detector were combined (with nominal element shift). The reconstructed were ML deconvolution (25 iterations) using Huygens v. 19.10 (SVI, The Netherlands) and nominal microscope PSF. Background was estimated as a minimum of average intensity in 41x41 pixel region and SNR was set to 15. Using ImageJ program (NIH), spine density was measured (in this order, length of spine- head width- neck width) and reported as total spine density/10 μm.

Data analysis

To compare spine counts, optical densities, and histone occupancy between experimental groups, a one-way analysis of variance (ANOVA) followed by a posthoc Tukey test was applied and graphs plotted (Prism 10). p < 0.05 was considered as significant.
Results

KET downregulates the increased HDAC protein expressions in the hippocampus of DFP rats

As shown in Fig. 1, compared to control rats, densitometric analysis revealed a significant increase in HDAC1 and HDAC5 expression in DFP rats but no significant changes in HDAC2-4 proteins were noted. HDAC expressions in DFP rats treated with KET (10 mg/kg, i.p. at 24-h) were then assessed. As shown in Fig. 1, in the presence of KET, there were significant reductions in the elevated HDAC1, HDAC3, and HDAC5 protein levels noted in DFP rats, and these protein expressions were restored to levels seen under control conditions. However, HDAC2 and HDAC4 protein expressions were not significantly different between DFP+SAL vs. DFP+KET rats (n= 6-8 rats/group, One-way ANOVA, posthoc Tukey test, *p<0.05). Together, these results indicated that repeated DFP exposures were associated chronic increases in HDAC protein expressions. Further, a single KET injection at the antidepressant dose significantly downregulated these DFP-induced increased HDAC expressions.

KET upregulates the DFP-induced reduced H3K9ac occupancy at Bdnf promoter

Histone 3 lysine 9 (H3K9ac) acetylation levels at the Bdnf promoter IV were approximately two-fold greater in DFP+KET rats compared to DFP+SAL rats (Fig. 2, n= 4 rats/group, One-way ANOVA, posthoc Tukey test, *p<0.05). KET restored Bdnf promoter IV H3K9ac to approximately 85% of normal levels observed in control rats that did not receive DFP (Fig. 2). H3K9ac is considered one of the key acetylation marks found in promoters, and it is typically associated with the activation of gene expression. (Tessarz and Kouzarides, 2014).

These results indicated that KET treatment in DFP rats increased H3K9ac levels at Bdnf promoter IV.
KET upregulates diminished hippocampal BDNF levels in DFP rats

In agreement with our previous studies (Ribeiro et al., 2020; Ribeiro et al., 2021), hippocampal BDNF protein expressions were significantly downregulated in DFP rats and were 0.46-fold lower compared to control rats (Fig. 3). BDNF protein levels in DFP-KET group were ~1.7 fold greater and significantly different compared to BDNF protein levels noted in saline-treated DFP rats but not different from control rats (*p<0.05, n= 8 rats, One-way ANOVA, Fig. 3). These results indicated that KET dose that exhibited an antidepressant response at a timepoint when it is no longer present in vivo is associated with significant increases in BDNF expressions that were chronically downregulated following repeated DFP exposures.

Effect of KET on dendritic spine densities and spine-type distribution

Dendritic spine densities in the hippocampus from DFP rats were significantly lower than those from control rats. The administration of KET (10 mg/kg, i.p.) 24 hours after initial treatment significantly improved the reduced spine densities in the hippocampus of DFP-exposed rats. These spine density values became comparable to those observed in control rats (*p<0.05, n= 3-4 rats per conditions with 10 images/ rat, One-Way ANOVA, Fig. 4A, B). We next investigated the distribution of dendritic spine types under these three conditions. As shown in Fig. 4C, as compared to naïve control rats, significant reductions in T-type spines and significant increases in S-type spines were noted in DFP rats (p=0.02 for T type and p=0.003 for S type). In contrast, KET treatment caused profound redistribution of spine type, evidenced by a significant upregulation in T-type spines in DFP rats compared to DFP + SAL rats (*p<0.0001). These increases were not significantly greater than control conditions (p= 0.3). Concomitantly, KET treatment also reduced the number of S-type spines compared to DFP-SAL treatment (p<0.0001) but not naïve conditions (p= 0.09). There was no effect of KET treatment on M-type spines.
compared to both naïve and DFP + SAL condition (*p<0.05, n= 3-4 rats per condition with 10 images/ rat, Two-way ANOVA, Fig. 4B, C). Together, these results indicated that DFP exposures produced chronic decreases in spine densities and affected spine-type distributions with a significant increase in S-type spines. KET treatment in DFP rats significantly boosted spine numbers and significantly increased T-type spines at 24-h post KET.

**Discussion**

Epigenetic changes have been documented in both GWI veterans and animal models designed to simulate GWI, highlighting the potential role of epigenetics in the pathophysiology of this condition. For example, changes to DNA methylation levels have been reported in a rat GWI model (Pierce et al., 2016) and in GWI veterans' blood samples (Trivedi et al., 2019). Alterations in the histone acetylation pathways have also been reported in DFP-based GWI rat models (Ashbrook et al., 2018; Ribeiro et al., 2021). Epigenetic dysregulation has been implicated in clinical depression in human studies (Li et al., 2019), while preclinical models of depression have shown increased HDAC expression and decreased H3K9ac at *Bdnf* promoter IV following depressogenic stimuli (Sakata et al., 2010; Sakata and Duke, 2014; Poon et al., 2021).

In agreement with these findings and our previous studies (Ribeiro et al., 2021), we report that DFP exposures were associated with significant upregulation in hippocampal HDAC 1 and HDAC 5 expression. Furthermore, a substantial reduction in histone H3K9ac levels was observed at the *Bdnf* promoter IV site in rats exposed to DFP indicating that the HDAC upregulation and associated deacetylation of histone moieties were causing repression of *Bdnf* transcription leading BDNF protein reductions in GWI.
BDNF is considered a biomarker for depression and a transducer of antidepressant effects (Bjorkholm and Monteggia, 2016). Reductions in BDNF levels have been reported in clinical depression (Dell'Osso et al., 2010), in animal models of depression (Berton et al., 2006; Murinova et al., 2017; Martis et al., 2019), and GWI animal models (Carreras et al., 2018; Kimono et al., 2020; Ribeiro et al., 2020; Brown et al., 2021; Ribeiro et al., 2021). Similarly, the antidepressant response, including the one mediated by KET treatment, correlates with BDNF upregulation in depressed patients (Haile et al., 2014) and in GWI laboratory models of depression (Ribeiro et al., 2020). Considering the profound epigenetic modifications seen in GWI (Pierce et al., 2016; Ashbrook et al., 2018; Ribeiro et al., 2021), it is essential to identify how they affect BDNF transcription and whether KET acts via epigenetic pathways to upregulate BDNF.

Our current studies indicate that KET significantly lowered the HDAC 1, 3, and 5 expressions in DFP rats. Furthermore, KET treatment restored the occupancy of histone moieties at BDNF transcription sites and caused a significant upregulation in BDNF expression. These data suggest that KET acts via epigenetic histone signaling pathways to enhance BDNF levels in DFP rats. There is evidence that KET's antidepressant effect may, at least in part, be due to its effects on histone acetylation pathways (Choi et al., 2015). More specifically, HDAC1 and HDAC5 have been shown to affect histone acetylation at Bdnf promoter IV (Yasuda et al., 2009; Choi et al., 2017). KET may elicit phosphorylation-dependent nuclear export of HDAC5 (Choi et al., 2015), which may be a key mechanism in explaining our findings. Since DFP causes increased HDAC activity in the hippocampus (Ribeiro et al., 2021), the increased nuclear export of HDACs elicited by KET could attenuate the deacetylation of Bdnf promoter IV, allowing the
histone acetylation levels to normalize. Thus, by removing the HDAC suppressive effect, Bdnf expression can increase and stimulate neuronal plasticity.

Neuronal plasticity results from dynamic interconnections between neurogenesis, axonal branching, dendritic spines, and synaptic connections (Qiao et al., 2017; Laine and Shansky, 2022). Spines are neuronal protrusions and the primary recipients of excitatory signals and contain the molecular apparatus necessary for synaptic plasticity (Nimchinsky et al., 2002; Gipson and Olive, 2017). Consequently, spine morphology and density alterations profoundly affect neuronal plasticity and disease outcomes, including depression (Duman and Duman, 2015; Qiao et al., 2016). Our studies indicated that repeated low-dose DFP administrations produced chronic reductions in hippocampal spine density. In agreement with our findings, repeated, low-dose OP chlorpyrifos exposures have also been reported to decrease spine density and reduce synaptic transmission in mice at 3-m post OP exposures (Speed et al., 2012). Our studies further demonstrated that KET treatment that produced a rapid and long-lasting antidepressant action (Ribeiro et al., 2020; Zhu et al., 2020) were associated with a significant increase in spine numbers at 24-h post-treatment. In agreement with our findings, KET (10 mg/kg, 24h post-treatment) has been reported to ameliorate the decreased spine number in medial pre-frontal cortex in a chronic unpredictable stress model in mice (Li et al., 2011). Similar results have also been found for KET enantiomers. Thus, both R-KET (Zhang et al., 2019) and S-KET (Treccani et al., 2019) are reported to promote rapid formation of spines in rodent models of depression.

Dendritic spines fall into three major types: Mushroom (M-type) is a more mature form and, given their bigger size, provides a greater surface area for synaptic connections. M-type spines are thought to be sites of long-term memory storage. Thin (T-type) is highly plastic and are considered “learning” spines, while Stubby (S-type) is considered immature and possibly
form because of disappearance of other spine types (Nimchinsky et al., 2002; Gipson and Olive, 2017). Our studies indicated significant reductions in T-type spines in DFP rats, in agreement with studies reporting, decreases in T- spines with depression (Wang et al., 2013; Duman and Duman, 2015; Zhuang et al., 2019). Similarly, a significant increase in immature stubby spines was also noted in DFP rats. KET treatment restored the spine density, boosted the number of highly plastic T-type spines, and lowered the number of immature spines. In a prior study utilizing a rat model of isolation stress, it was reported that KET treatment reversed the reduced densities of thin spines, although it did not have a similar effect on mushroom spines (Sarkar and Kabbaj, 2016). This observation has led to the hypothesis that the swift restoration of spine formation induced by KET may be a contributing factor to its antidepressant effects (Moda-Sava et al., 2019). The role of BDNF in supporting spine growth and maturation (Ji et al., 2010; Kellner et al., 2014; Zagrebelsky et al., 2020), and promoting neuronal plasticity is well-established (Castren and Rantamaki, 2010; Karpova, 2014; Qiao et al., 2017; Yang et al., 2020). Specifically, BDNF is required for maintenance of the spine phenotype (Zagrebelsky et al., 2020). The ability of BDNF to affect spine densities and maturation may underlie the altered synaptic plasticity seen in depression, and KET's effect on upregulating BDNF levels could underlie its action on dendritic modifications and long-lasting antidepressant activity in DFP rats.

Our studies have few limitations. We acknowledge that a lack of females in our experimental design is a shortcoming of our research. The frequency of GWI symptoms is reportedly higher in women GW veterans and studies indicate that they may exhibit GWI symptoms with greater severity (Coughlin et al., 2017; Sullivan et al., 2020). Validation of our findings in female rats and in multiple GWI models will be a critical component of future studies. Additionally, we did not assess behavioral improvement in KET-treated rats before using
them for epigenetic studies and spine assessments. We have previously shown that KET treatment produced a rapidly acting and sustained antidepressant effect in DFP rats (Ribeiro et al., 2020; Zhu et al., 2020). To prevent the effect of stress due to behavioral testing, in this study we chose to study epigenetic and dendritic modifications at the same KET dose and at the same time points when we observed a potent antidepressant response. Lastly, we did not assess changes in the markers of synaptic plasticity following KET treatment in the DFP rats. Our results that KET is causing dendritic remodeling would be further strengthened if functional improvement in synaptic plasticity could also be demonstrated. Additional studies are needed to support these conclusions from our study.

It is important to also identify potential side-effects, abuse concerns, and possible withdrawal effects of KET therapy (Bonaventura et al., 2021). Clinical studies have reported that KET therapy could be associated with some side-effects such as blurred vision, headache, dizziness, dissociation, and elevated blood pressure. These effects occurred immediately after KET initiation but resolved shortly thereafter (Short et al., 2018; Acevedo-Diaz et al., 2020). Interaction between KET and opioid receptors are also reported (Williams et al., 2018). Reports have indicated a role of opioid receptor activation in the anti-suicidality actions of KET (Williams et al., 2019). Any changes in opioid receptor following the cessation of KET therapy could potentially raise the risk of suicide tendency.

GWI is a multisymptomatic condition with no effective therapies. GWI neurological morbidities of depression are particularly challenging to treat, and there is a greater prevalence of depressive symptoms in GW veterans (Blore et al., 2015; Maule et al., 2018). Our data demonstrates a role for BDNF epigenetics for the persistence of GWI symptoms particularly depression. Furthermore, our results indicate that KET is acting via epigenetic histone
mechanisms that involves effects on BDNF upregulation and dendritic remodeling to produce a long-lasting antidepressant response in DFP rats. The FDA has approved KET for therapy-resistant depression. Studies in rat models of GWI have demonstrated a rapid and long-lasting antidepressant effect of KET (Ribeiro et al., 2020; Zhu et al., 2020), similar to the human experience. Our studies here provide mechanistic data into KET’s antidepressant action in a pre-clinical model of GWI. This raises the possibility that KET could also be an effective therapeutic for hard-to-treat GWI-related depression and should be trialed in GWI veterans.
Acknowledgements

Data Availability Statement

The authors declare that all the data supporting the findings of this study are contained within the paper.

Authorship Contributions

Participated in research design: McClay and Deshpande.

Conducted experiments: Ribeiro-Davis, Hawkins, Al Saeedy, Jahr

Performed data analysis: Ribeiro-Davis, McClay, Deshpande.

Wrote or contributed to the writing of the manuscript: McClay, Deshpande.
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Footnote:

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2. No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends

Figure 1. Effect of KET on HDAC protein expression in DFP rats

A. Hippocampal western immunoblots stained for various HDAC subtypes (HDAC1-5) from saline-treated, age-matched naïve rats (naïve + SAL), saline-treated DFP rats (DFP + SAL), and ketamine-treated DFP rats (DFP + KET). Densitometric analyses of the Western blotting revealed significant increases in the levels of HDAC1 (B) and HDAC5 (F) compared with age-matched control that were restored to control levels in KET-treated DFP rats. No significant differences in HDAC2 (C), HDAC3 (D), or HDAC4 (E) levels were noted in saline-treated DFP rats compared to saline-treated naïve rats. KET treatment did not affect HDAC2 (C) or HDAC4 (E) levels but significantly reduced HDAC3 (D) levels in DFP rats (Data expressed as mean optical density ratio (HDAC/GAPDH) ± SD, *p< 0.05, one-way ANOVA, Tukey test, n= 6-8 rats/group).

Figure 2. H3K9ac ChIP-qPCR results for Bdnf promoter IV.

A significant reduction in H3K9ac occupancy was noted in saline-treated DFP rats compared to saline-treated naïve rats. KET-treatment in DFP rats restored H3K9ac occupancy and this effect was significantly higher than DFP + SAL but not different than naïve + SAL condition (n= 3-4 rats/group, One-way ANOVA, Tukey-test, *p<0.05). Data expressed as percent input material (quantity of immunoprecipitated DNA at the Bdnf locus) The minimal IgG values indicate low background noise and lack of nonspecific immunoprecipitation in ChIP findings.

Figure 3. Effect of KET treatment on BDNF protein expression in DFP rats

A. Western immunoblot of hippocampal protein from saline-treated, age-matched naïve rats (naïve + SAL), saline-treated DFP rats (DFP + SAL), and ketamine-treated DFP rats (DFP +
KET) stained for BDNF. **B.** Densitometric analyses of the Western blotting revealed significant decreases in the levels of BDNF in DFP + SAL rats compared with naïve + SAL rats. KET caused a significant increase in BDNF protein in DFP rats compared to saline treated DFP rats (Data expressed as mean optical density ratio (BDNF/GAPDH) ± SD, *p< .05, One-way ANOVA, Tukey test, n= 8 rats/ group).

**Figure 4. Hippocampal spine changes in DFP rats and the effect of KET treatment.**

**A.** Representative Dil staining showing dendritic spines in hippocampal slices from naïve + SAL, DFP + SAL, and DFP + KET conditions. Tightly packed spines along the dendrite are visible in naive (left), while spines in DFP (middle) are further spaced out. KET treatment (right) was associated with robust spinogenesis indicated by greater number of spines. **B.** Quantification of spine density. Significant reductions in spine density in DFP + SAL rats compared to naïve + SAL were noted. KET-treatment caused a significant increase in spine density in DFP rats compared to DFP + SAL (*p<0.05, One-way ANOVA, Tukey-test, n= 3 rats/ group, 10 neurons/rat and about 1000 spines counted/ group). **C.** Histogram depicting changes in spine diversity in DFP rats evidenced by significant increases in S-type and significant decreases in T-type spines, but not M-type spines compared to naïve + SAL rats. KET-treatment in DFP rats was associated with a significant upregulation T-type spines and a significant downregulation in S-type spines compared to DFP + SAL rats (*p<0.05, **p<0.01, ****p<0.0001 Two-way ANOVA, n=3 rats/ conditions, 10 neurons/rat and about 1000 spines counted/ group). Bar scale = 10μM.