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14. ABSTRACT

This New Investigator Award to Dr. Jones funded basic science research into the genetics of why some Gulf War veterans became sick and others, all else being equal, did not. The research was carried out using a genetic reference population of mice, 30 lines of the BXD recombinant inbred strains and following the experimental model of James O'Callaghan and Diane Miller. The treatment was 7 days of corticosterone in the drinking water followed on the 8th day by injection of a sarin surrogate, diisopropylflurophosphate, an irreversible cholinesterase inhibitor. As the presumed basis for Gulf War Illness as neuroinflammation, our endpoints were differential expression of proinflammatory cytokine genes from the prefrontal cortex. We found robust strain effects on the expression of interleukin 1b and were able to nominate a candidate gene, *Spon1* as underlying individual differences in response to the treatment. Further work using RNA-seq identified another, *Ccr6.*

15. SUBJECT TERMS
Mouse, proinflammatory cytokines, organophosphates, glucocorticoids

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Introduction

Somewhere between 25 and 35% of the soldiers sent from the US and its allies to the Gulf War of 1991 returned home with a debilitating, multi-symptom illness, Gulf War Illness (GWI). Why 25-35% became sick while others, presumably experiencing the same exposures, did not, became a major target for clinical and basic research. In answering this problem, we submitted a proposal in 2016 to address the problem of individual differences in susceptibility using a genetic reference population of recombinant inbred mice. The proposal was approved and funded for $500,000 TDC for 3 years. The experimental design was to subject male and female mice from 30 BXD recombinant inbred strains to 7 days of corticosterone added to their drinking water followed by treatment with diisopropylflurophospnate (DFP, a sarin surrogate) on the 8th day. This treatment regimen modeled the presumed exposure of combat troops to organophosphate compounds while experiencing high levels of circulating glucocorticoid hormones as might be expected in a combat situation. Six hours following the DFP treatment, the animals were euthanized and frontal cortex from brain dissected for analysis of proinflammatory cytokine gene expression by rtPCR. The cytokine genes analyzed were *Il1b, Il6,* and TNFa.

All experimental work in 30 of the mouse strains was completed within 15 months and we then submitted brain samples for analysis. The results showed that *Il1b* expression in response to the treatment showed the greatest genetic differences among the mouse strains and furthermore, we were able to nominate a candidate gene, *Spon1* that underlies individual differences in susceptibility to developing GWI. Additional brain samples were prepared for next generation sequencing – RNA-seq – to Novogene for analysis.

RNA-seq analysis revealed more gene expression candidate genes including Il1b, tnfa, Nf-kappa-B and Ccr6. According to the Kyoto Encyclopedia of Genes and Genomes, gene enrichment pathways affected by the exposure include oxidative phosphorylation, fatty liver disease, Parkinson's disease, and Alzheimer's disease. Of interest is the fact that the Gulf War veterans are now in the age range of 50-60, the age range characteristic of development of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases. In fact we are now preparing a grant application that addresses risk for PD in Gulf War veterans.

To summarize, we are very pleased with the outcome of this project. The genes and gene pathways identified in this work make sense concerning GWI as a major neuroinflammatory condition. This work has also led to another project to understand why those afflicted with GWI are still sick, nearly 30 years after having been exposed. This question has led to the development of an R01 grant to NIEHS, "The genetics of epigenetic response to high circulating glucocorticoids coupled with exposure to organophosphates." The proposal received an impact score of 24 and percentile rank of 7. We were notified recently that we are on the list for funding. The total award is 3.4 million USD, including overhead and the funding period is 5 years.

Key words: glucocorticoids, organophosphates, neuroinflammation, QTL analysis, candidate gene analysis, genomic analysis.

Accomplishments

We were funded to apply genetic definition on the GWI mouse model developed by James O'Callaghan and Diane Miller. They proposed that GWI sickness behavior derived from neuroinflammation produced by exposure to the chemical environment in theater, coupled with high circulating cortisol, reflecting the stress of being in a combat situation. Specifically, they reasoned that exposure to some chemical agent was the source of the illness but enhanced by high circulating glucocorticoids, viz., cortisol. They settled on organophosphates (OPs) as the causative agent, indeed, the soldiers were exposed to an accidental release of the nerve gas, sarin as well as being exposed to chlorpyrifos, an insecticide applied to the barracks and pyridostigmine, a prophylaxis

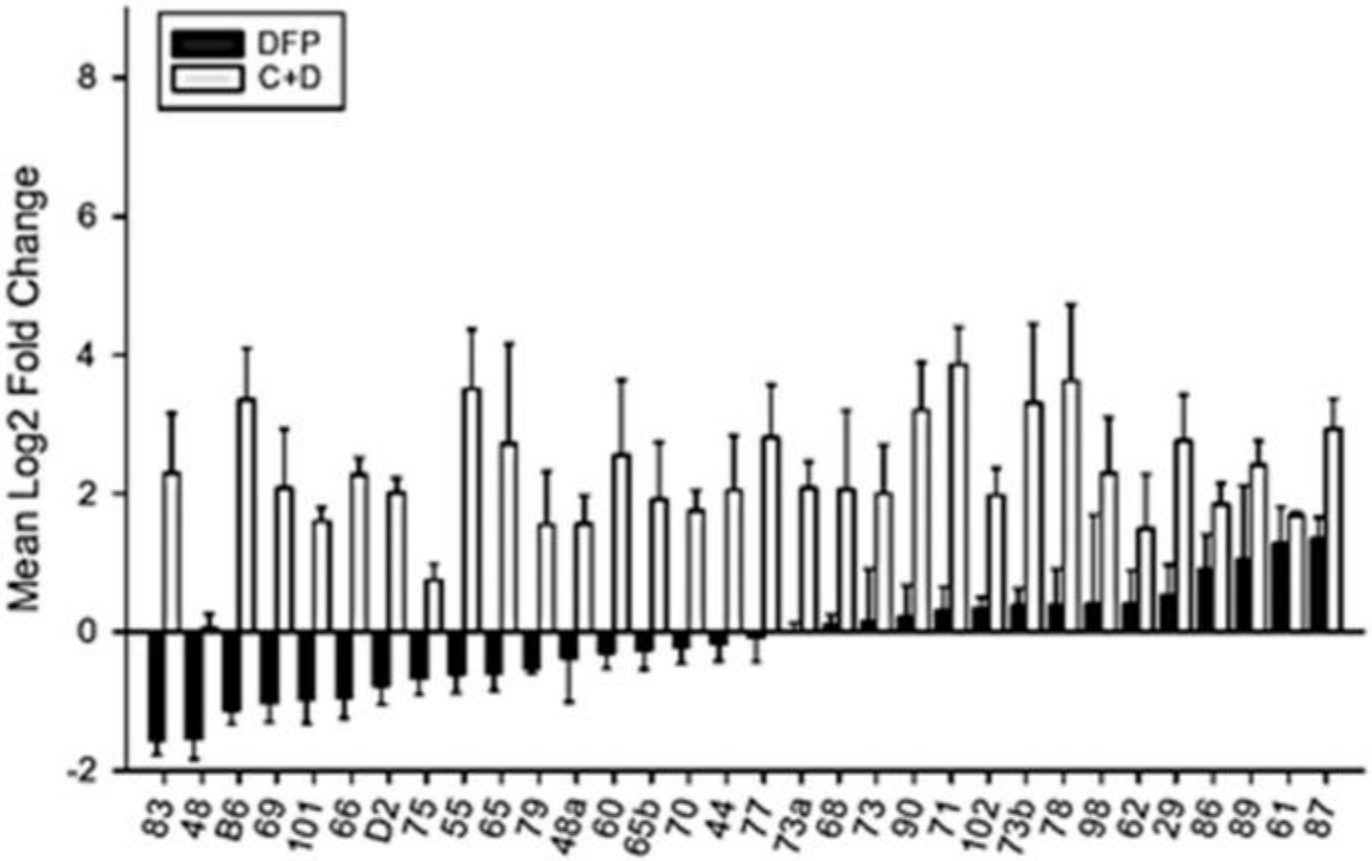
against sarin. As these three OPs constituted the preponderance of the chemical exposome for the Gulf War soldiers and other personnel. In order to mimic the etiological environment, O'Callaghan and Miller exposed mice to corticosterone (rodent glucocorticoid) followed by treatment with diisopropylfluorophosphate (DFP, an OP sarin surrogate). As they used only one mouse strain, C57BL/6J and only males, we proposed that increasing the genetic variability among test subjects would address the question why 25-35% of Gulf War personnel became sick while the rest did not.

Our protocol involved testing 30 BXD recombinant inbred strains and both sexes. Earlier work showed us that while one of the two progenitor strains was highly sensitive to the treatment, the other was less so. Also, female mice of both strains were less responsive to the treatment.

The experimental mice received 20mg% corticosterone in their drinking water for seven days and on the eight day, received 4 mg/kg diisopropylfluorophosphate intraperitoneally. A separate group of animals did not receive corticosterone and were injected with DFP. Control animals received untreated drinking water and were injected with saline. Six hours after the injection the animals were euthanized and brain dissected to yield the medial prefrontal cortex. This tissue was then analyzed for proinflammatory cytokine gene expression by rtPCR. The genes were *Il1b, Il6* and *Tnfa.* The data reported are differences in gene expression, DFP or corticosterone + DFP minus saline.

Our results showed wide variation among mouse strains and sexes in expression of all three cytokines. The figure below shows the differential response among the strains and sexes for response to DFP alone and corticosterone + DFP.

IL1b Expression in Female Mice Treated with DFP or DFP+Corticosterone



IL1b Expression in Male Mice Treated with DFP or DFP+Corticosterone

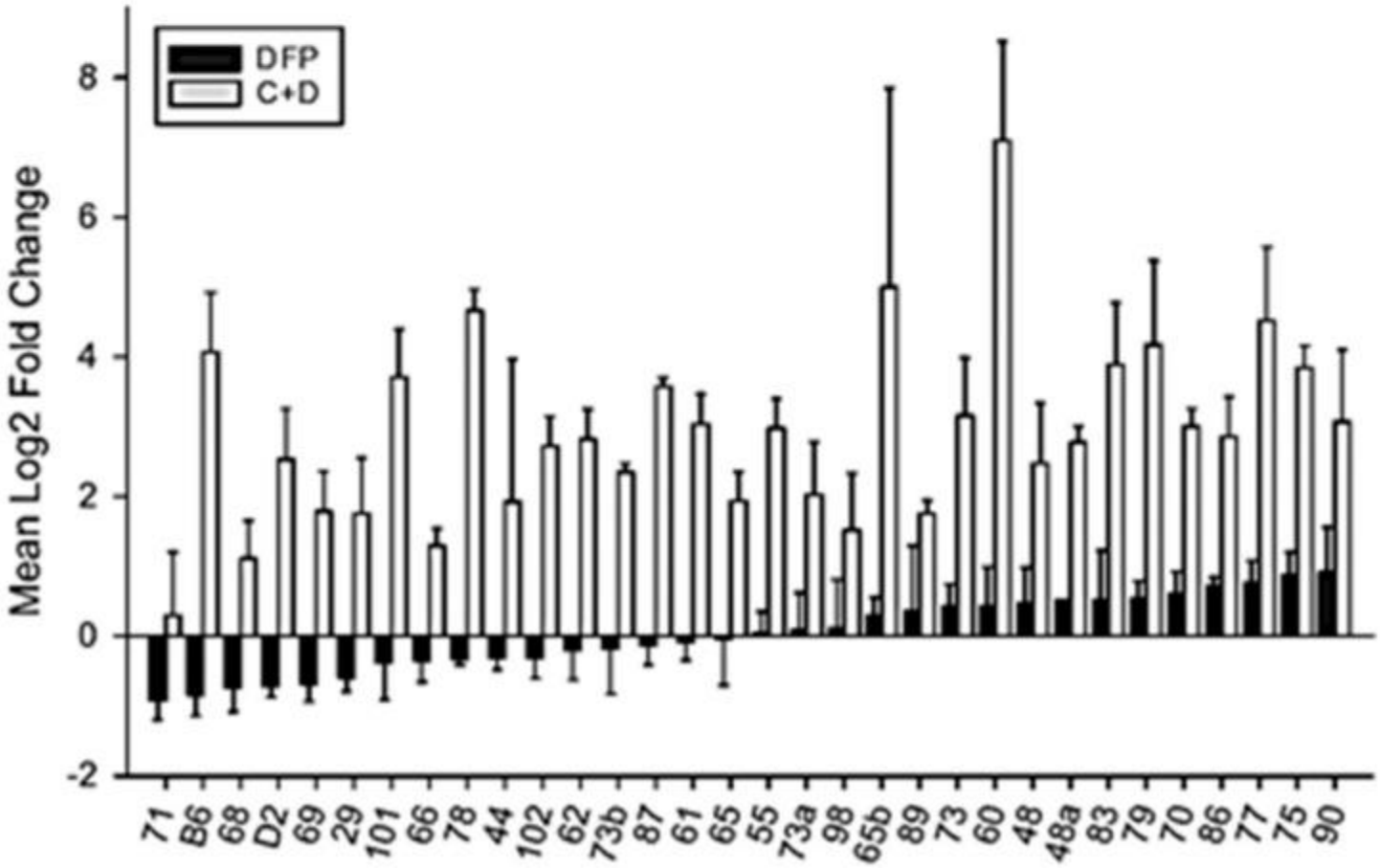


Figure 1. Mean change (±s.e.m) vs. control in *Il1b* expression in prefrontal cortex 6 h following intraperitoneal injection with diisopropylflurophosphate (4 mg/kg—black bars) or 7 days of corticosterone in the drinking water and 6 h following intraperiteoneal injection with diisopropylflurophosphate (gray bars). Top panel, females, bottom panel, males.

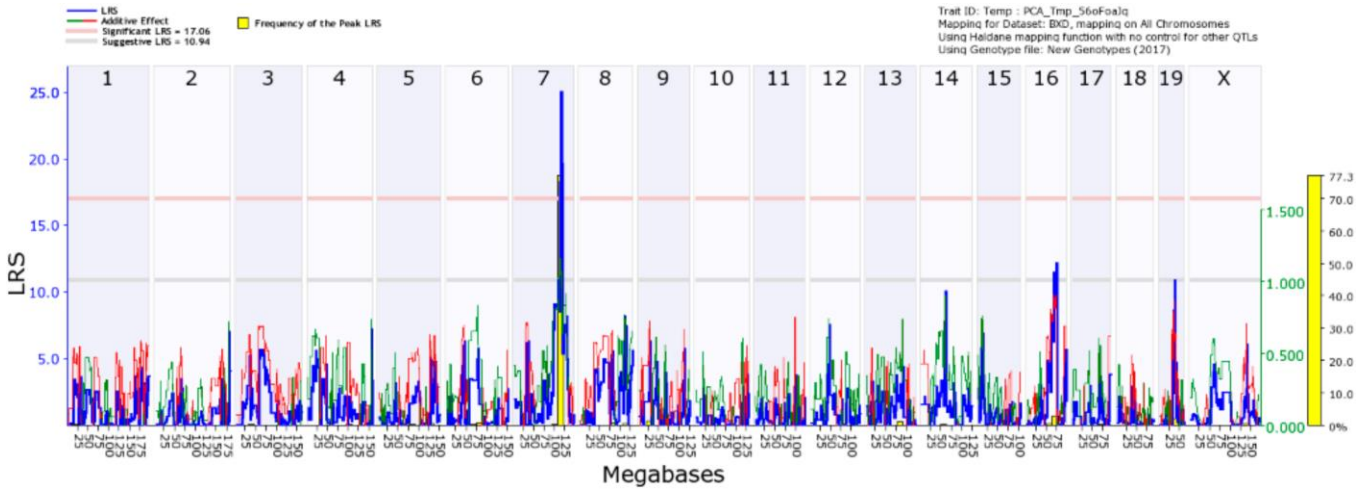


Figure 2. Quantitative trait locus map of *Il1b* expression change following 7 days of corticosterone in the drinking water and 6h following i.p. injection of diisopropylfluorophosphate (4 mg/kg). The dependent variable (eigenvariable) is the first principal component derived from males and females individually and combined. Genome-wide interval mapping was performed using GeneNetwork software. The map shows a significant peak on chromosome 7 and two suggestive peaks, one on chromosome 16 and the other on chromosome 19. The latter two peaks lacked sufficient bootstrap support (yellow bars) and thus considered spurious.

The results in Figure 1 were subjected to genetic mapping to show associations between genetic polymorphisms and gene expression response. A significant association was noted on chromosome 7 (Figure 2) and the search for a possible candidate gene in the same region yielded *Spon1* or spondin 1.

The correlation between expression of *Spon1* and expression *Il1b* in the medial prefrontal cortex is illustrated in Figure 3.

There are reported findings that draw our attention to *Spon1* as candidate gene underlying individual differences in susceptibility to GWI. *One* is the observation Spondin 1 reduces the toxicity of chemotherapeutic agents and radiation via the WNT/βcatenin pathway. The mechanism may involve changes in proinflammatory cytokine production locally and it is worth exploring whether Spon 1 might affect other inflammatory systems, including IL1 cytokines. If so, then Spon 1 and WNT/βcatenin might be appropriate targets for therapeutics for GWI. Another and more compelling reason is its effects on amyloid precursor protein. Recent genome-wide association studies of the rate of cognitive decline in Alzheimer's disease indicated *SPON1* as candidate gene associated with slower rate of cognitive decline in Alzheimer's disease one possible mechanism is spondin protein is associated with inhibition of amyloid beta while promoting synaptophysin.

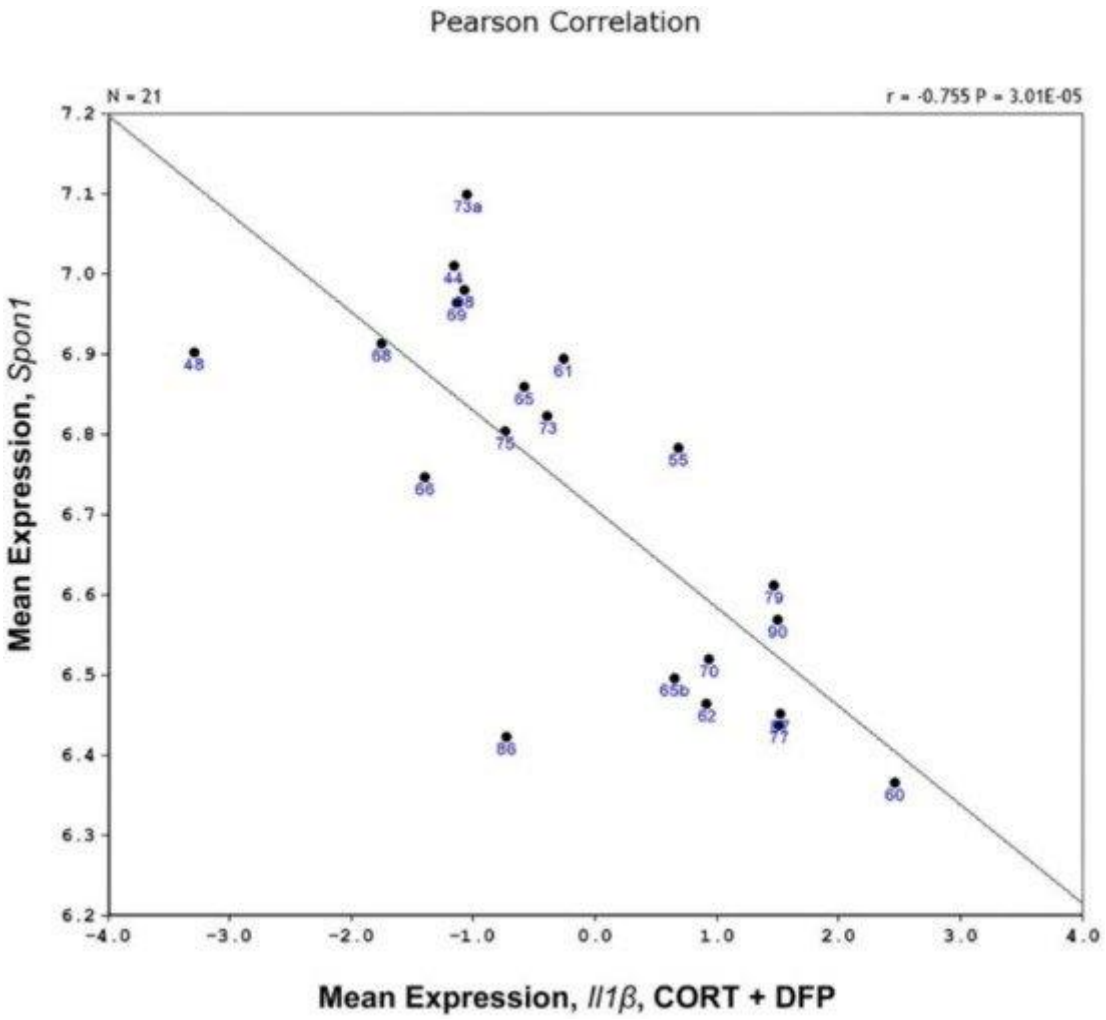


Figure 3. Scatter diagram of the association between the expression of *Spon1* and the eigenvariable relative to *Il1b* expression in mice treated with corticosterone and DFP.

Following the phenotyping experiments for expression of the proinflammatory cytokines, we extracted messenger RNA from frontal cortex tissues for the mice and sent the RNA to Novogene for RNA-seq analysis. This analyzes genome-wide gene expression to identify those genes sensitive to DFP treatment and DFP+corticosterone treatment (vs. saline control).

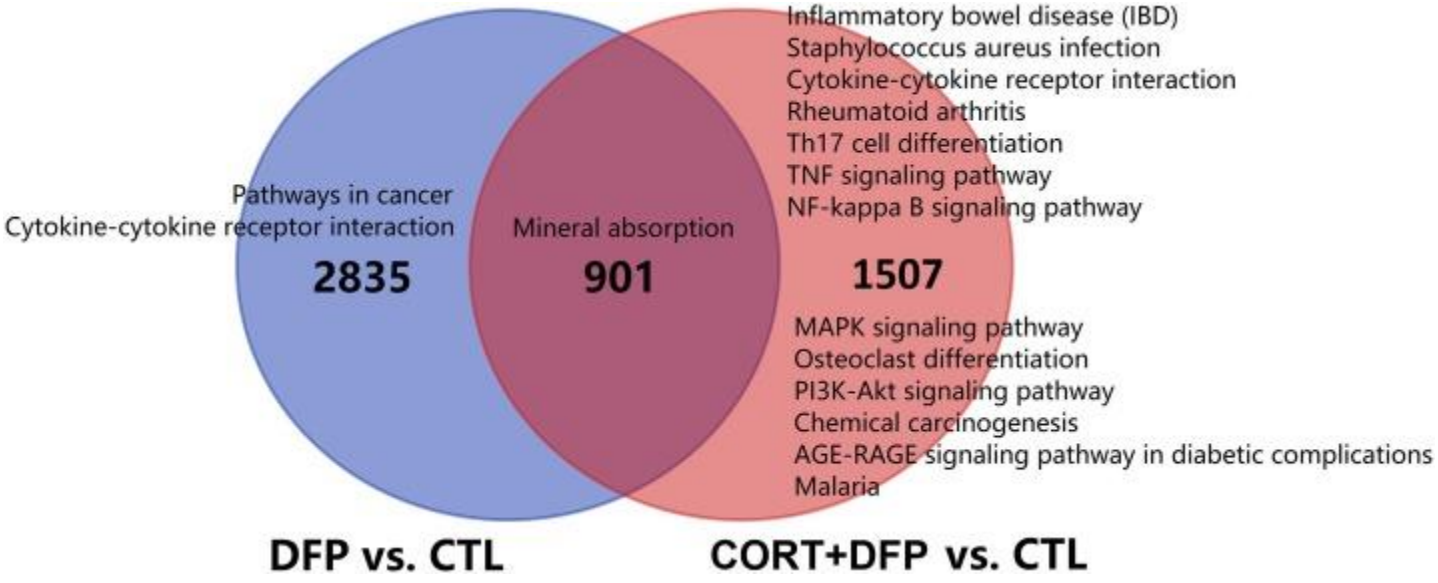


Figure 4. Venn diagrams showing the overlaps for the Differentially Expressed Genes between DFP vs. CTL and CORT + DFP vs. CTL. The numbers indicated common or unique genes for those two groups. For each gene set, Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis was conducted with WebGestalt (http://www.webgestalt.org/) and the significant enriched terms (FDR < 0.05) were listed alongside the diagrams.

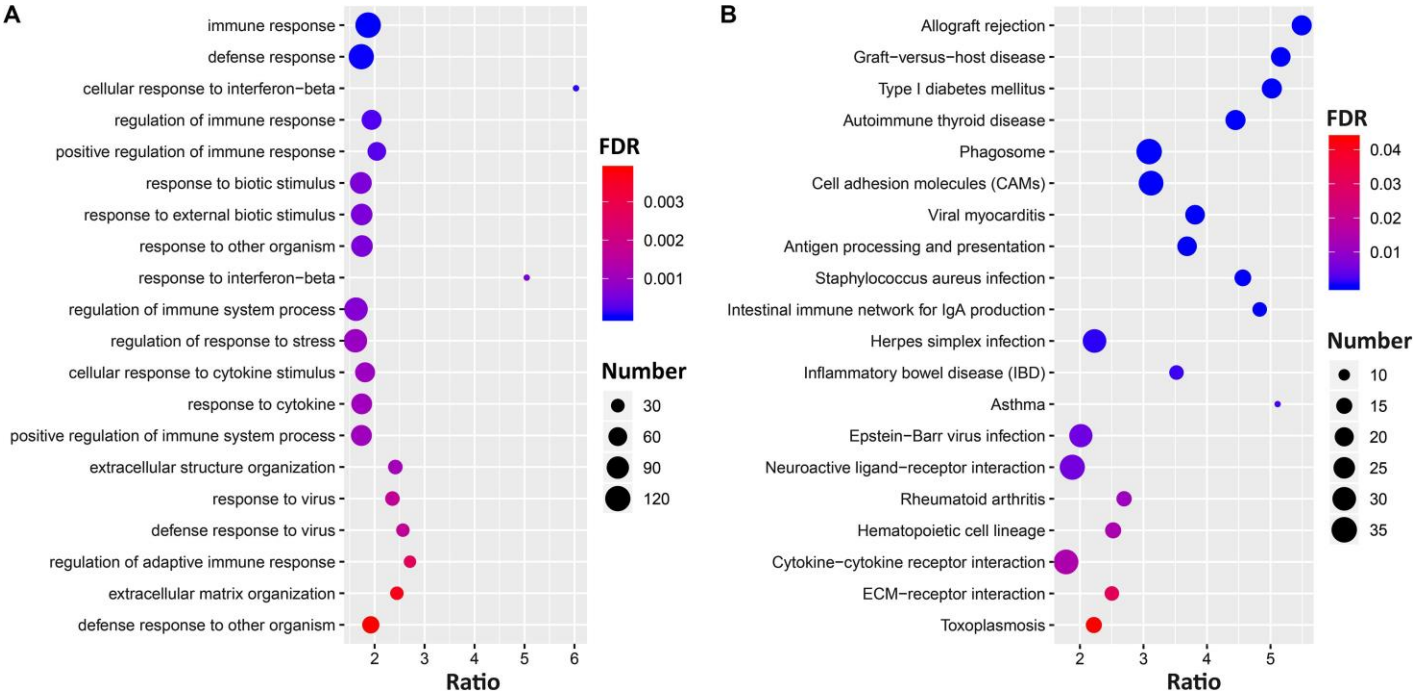


Figure 5. The gene set enrichment analysis of *Ccr6* correlated differentially expressed genes. The bubble plot shows the top 20 GO **(A)** and KEGG pathways **(B)** of *Ccr6* correlated genes in the CORT+DFP group.

Since the beginning of the funding period we have accomplished the following:

- Oral presentation at the Complex Traits Community meeting in Glasgow, 2017
- Three peer-reviewed papers
    - Jones BC, Miller DB, Lu L, Zhao W, Ashbrook DG, Xu F, Mulligan MK, Williams RW, Zhuang D, Torres-Rojas C, O'Callaghan JP. Modeling the Genetic Basis of Individual Differences in Susceptibility to Gulf War Illness. Brain Sci. 2020 Mar 2;10(3):143. doi: 10.3390/brainsci10030143. PMID: 32131477; PMCID: PMC7139661.
    - Xu F, Ashbrook DG, Gao J, Starlard-Davenport A, Zhao W, Miller DB, O'Callaghan JP, Williams RW, Jones BC, Lu L. Genome-wide transcriptome architecture in a mouse model of Gulf War Illness. Brain Behav Immun. 2020 Oct;89:209-223. doi: 10.1016/j.bbi.2020.06.018. Epub 2020 Jun 20. PMID: 32574576.
    - Gao J, Xu F, Starlard-Davenport A, Miller DB, O'Callaghan JP, Jones BC, Lu L. Exploring the Role of Chemokine Receptor 6 (*Ccr6*) in the BXD Mouse Model of Gulf War Illness. Front Neurosci. 2020 Aug 14;14:818. doi: 10.3389/fnins.2020.00818. PMID: 32922257; PMCID: PMC7456958.

NIEHS Funding to follow up on the chronic nature of the disease

A. Research Support Available

Source	Grant No	Title of Research	Principal Investigator	Dates	Total Award Amount
NIH-NIEHS	ES 031656	Genetics of epigenetic response to high circulating glucocorticoids and organophosphorous compounds	Byron C. Jones, UTHSC James P. O'Callaghan CDC-NIOSH	09/01/2020-08/31/2025	

Impact

Using a mouse model, we have identified several genetic and biochemical pathways that underlie individual differences in susceptibility to exposure effects similar to what the soldiers in the Gulf War experienced. This should have the following effects on the disease. The genes and pathways that we identified in the mouse are highly conserved across mammalian species and as the mouse genome is more than 90% syntenic with that of the human genome, what we observe in our mouse model has great potential for translation. The implications are that we can identify biomarkers that are associated with increased risk for such exposure and second, we can identify pathways to target for prophylaxis and treatment.

The impact extends beyond the Gulf War. Organophosphate compounds are used widely for insect control, parathion, malathion, and chlorpyrifos are three examples of agents to which humans come in contact frequently.

Changes/Problems

We were able to conduct the research exactly as written and we experienced no problems in execution.

Products

This was one of our more productive projects recently our output included the following:

- Oral presentation at the Complex Traits Community meeting in Glasgow, 2017
- Three peer-reviewed papers
    - Jones BC, Miller DB, Lu L, Zhao W, Ashbrook DG, Xu F, Mulligan MK, Williams RW, Zhuang D, Torres-Rojas C, O'Callaghan JP. Modeling the Genetic Basis of Individual Differences in Susceptibility to Gulf War Illness. Brain Sci. 2020 Mar 2;10(3):143. doi: 10.3390/brainsci10030143. PMID: 32131477; PMCID: PMC7139661.
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In addition to the papers and grant application, all of our phenotype and RNA-seq data are available on our database website, www.GeneNetwork.org this database is free for access to anyone and contains summary statistical data from our work. Raw data are also available upon request.

Participants and Other Collaborating Organizations

Name	Lu LU
Project Role	Co-I
Researcher Identifier	LULULU - era Commons Name
Nearest person month worked	2
Contribution to Project	Dr. Lu organized the RNA-seq and bioinformatics
Funding Support	This grant at 10% effort

Name	James P O'Callaghan – CDC-NIOSH
Project Role	Co-I
Researcher Identifier	CDCFOCALLAGHAN
Nearest person month worked	2
Contribution to Project	Dr. O'Callaghan organized the rtPCR analyses
Funding Support	No need as Dr. O'Callaghan is a Federal employee

Name	
Project Role	Diane B. Miller – CDC-NIOSH
Researcher Identifier	N/A
Nearest person month worked	2
Contribution to Project	Dr. Miller assisted with the preparation of the proposal but succumbed to cancer prior to initiation of the project
Funding Support	No need as Dr. Miller was a Federal employee
Name	David Ashbrook
Project Role	Co-I
Researcher Identifier	DASBROO - era Commons Name
Nearest person month worked	2
Contribution to Project	Dr. Ashbrook helped to rganize the RNA-seq and bioinformatics
Funding Support	Departmental support
Name	Fuyi Xu
Project Role	Research Assistant
Researcher Identifier	**n/a** as Dr. Xu is a postdoc
Nearest person month worked	**1**
Contribution to Project	Preparation of information for 2 manuscripts
Funding Support	Departmental support

Name	Jun Gao
Project Role	Research Assistant
Researcher Identifier	**n/a** as Dr. Gao is a postdoc
Nearest person month worked	**1**
Contribution to Project	Preparation of information for 2 manuscripts
Funding Support	Departmental support

Our other colleagues are Diane Miller and James O'Callaghan at CDC, NIOSH. Both of these individuals assisted in the conception and preliminary studies stage of the proposal and later, rtPCR analysis of proinflammatory cytokine gene expression from out prefrontal cortex samples. As both are federal employees, no salary support was requestee

Much to our great sorrow, Dr. Miller succumbed to cancer during the first year of our funding, but Dr. O'Callaghan continued the work and continues to collaborate with us to this day.

MDPI brain sciences

MODELING THE GENETIC BASIS OF INDIVIDUAL DIFFERENCES IN SUSCEPTIBILITY TO GULF WAR ILLNESS

Byron C. Jones, Diane B. Miller, [...], and James P. O'Callaghan

Additional article information

Abstract

Between 25% and 30% of the nearly one million military personnel who participated in the 1991 Persian Gulf War became ill with chronic symptoms ranging from gastrointestinal to nervous system dysfunction. This disorder is now referred to as Gulf War Illness (GWI) and the underlying pathophysiology has been linked to exposure-based neuroinflammation caused by organophosphorous (OP) compounds coupled with high circulating glucocorticoids. In a mouse model of GWI we developed, corticosterone was shown to act synergistically with an OP (diisopropylflurophosphate) to dramatically increase proinflammatory cytokine gene expression in the brain. Because not all Gulf War participants became sick, the question arises as to whether differential genetic constitution might underlie individual differences in susceptibility. To address this question of genetic liability, we tested the impact of OP and glucocorticoid exposure in a genetic reference population of 30 inbred mouse strains. We also studied both sexes. The results showed wide differences among strains and overall that females were less sensitive to the combined treatment than males. Furthermore, we identified one OP-glucocorticoid locus and nominated a candidate gene—*Spon1*—that may underlie the marked differences in response.

Keywords: BXD mice, recombinant inbred strains, candidate gene, DFP, neuroinflammation, corticosterone

1. Introduction

In 1991 the USA sent about 700,000 military personnel, joined by another 200,000 from allied nations, to the Persian Gulf to counter the invasion of Kuwait by Iraq. Of those who participated in the conflict, 25–30% developed a multi-symptom malaise, Gulf War illness GWI [1,2]. Symptoms range from gastrointestinal complaints, to lethargy, cognitive lapses, and depression. As described by Dantzer and colleagues [3], sickness behaviors are likely linked to activation of macrophages and microglia, and subsequent neuroinflammation via production of proinflammatory cytokines such as *Tnfa*, IL1β, and IL6. GWI thus has the features of a neuroimmune disorder [4,5,6,7]; but causes are otherwise poorly understood. The leading

suspected causes are chemicals to which the personnel were exposed. These include depleted uranium, cholinesterase inhibitors used as insecticides or as prophylactics against nerve agents (e.g., chlorpyrifos and pyridostigmine bromide) and even small amounts of sarin. The evidence points to exposure to the irreversible cholinesterase inhibitors chlorpyrifos and the nerve gas, sarin [8]. The former is an insecticide applied to living quarters and the latter was inadvertently released from ammunition dumps during their destruction by allied troops. GWI also presents two puzzles. The first is why only 25–30% became sick while all else being equal, the rest did not. This leads to the hypothesis that differential susceptibility to developing Gulf War illness is a gene-environment problem. To date, there has been only limited effort to address this problem. Georgeopolis and colleagues [6] proposed brain synchronicity indices as biomarkers of GWI and addressed individual differences in susceptibility as related to various HLA alleles. Alternatively, Steele and colleagues [9] differentiated individuals who had high butyrylcholinesterase activity from those with low activity. They demonstrated that those with the low activity and treated with pyridostigmine (a prophylaxis against organophosphate toxicants such as sarin) are at greater risk for developing GWI in response to wartime exposure than those evincing high activity. Nevertheless, at this time, there have been no published studies using GWAS or other genetic methods to delineate differential susceptibility to GWI. The second is why the illness has persisted for so long—nearly 30 years. The aim of this work is to address the former, using a genetic reference population of mice. An animal model of GWI was developed by O'Callaghan and colleagues [10]. The model involved exposing C57BL/6J (B6) mice to an irreversible cholinesterase inhibitor, diisopropylflurophosphate (DFP, a sarin surrogate). They proposed that exposure to DFP was necessary but not sufficient to produce neuroinflammation. Rather, high concentrations of circulating glucocorticoids (cortisol) as would be expected in a combat zone [11] were required as well. They showed that expression of proinflammatory cytokine genes was greatly enhanced by the co-exposure to DFP and corticosterone, the major glucocorticoid in rodents, compared to DFP alone [10]. Using this "two hit" treatment model, we will begin to address why all exposed troops did not become sick by expanding the testing of mice from the B6 strain to the genetic reference population of recombinant inbred (RI) strains derived from B6 and DBA/2J (D2) mouse strains (BXD) and both sexes. There are currently 150 extant BXD RI strains [12,13]. All have been genotyped and sequenced. The BXD family have an extensive phenome of over 7000 traits, including phenotypes for behaviors, immune parameters, neurochemistry, and pharmacology of alcohol and other drugs of abuse. Gene expression data have been recorded for numerous tissues including several brain regions. Moreover, there are ~6 million genetic variants (SNPs, insertions, deletions, duplications, etc.) that segregate in the family [13]. The strains are appropriate for systems genetics/systems biology analysis [14], genetic mapping and genetic correlations of parameter means, and thus constitute an ideal platform for toxicogenomic research [15]. All data are available at www.genenetwork.org. GeneNetwork exists in two forms, GN1 and GN2 [16]. GN2 is an expansion and refinement of the features of GN1. A tutorial of how to use GN1 may be found here: http://gn1.genenetwork.org/tutorial/ppt/index.html. The tutorials here can provide the basis for working in Gn2. Here, we report variability in cytokine gene expression following exposure to corticosterone (CORT) and DFP in 30 BXD recombinant inbred mouse strains and in both sexes.

2. Materials and Methods

2.1. Animals

The subjects for this study were male and female mice from 30 BXD.

Recombinant inbred strains. The animals were between 2 and 4 months of age at testing and 5–8 animals per strain, sex and treatment group were used. All animals were obtained from the breeding colony at the UTHSC vivarium. All animals had access to water and food ad libitum and controlled climate at 20 ± 2 °C and 35% relative humidity. All procedures were approved by the UTHSC animal care and use committee, approval code 17-022.0B, on March 30, 2017. Our target number of animals was 1200 (30 strains X 2 sexex X 4 treatments X n = 5); however, 1050 samples proved to have RNA suitable for analysis.

2.2. Materials

The following drugs and chemicals were obtained from the sources indicated: DFP, (Sigma, St. Louis, MO, USA), CORT (Steraloids, Inc., Newport, RI, USA). All reagents were analytical grade.

3. Experimental Design

3.1. Treatment Groups

The animals were divided into four treatment groups as follows:

1. Control. Plain tap water for fluid, saline injection and euthanized 6h after injection by cervical dislocation followed by decapitation. The brain was removed, and the frontal cortex dissected, weighed and placed on dry ice and stored at −80 °C until assay for cytokine gene expression.

2. CORT group. These animals received tap water containing 20 mg% CORT dissolved in 0.6% (*v/v*) EtOH vehicle for 8 days. On the 8th day, the animals were injected with saline, euthanized 6h after injection by cervical dislocation followed by decapitation. The brain was removed, and the frontal cortex dissected, weighed and placed on dry ice and stored at −80 °C until assay for cytokine gene expression.

3. DFP group. These animals received plain tap water for fluid and were injected with 4 mg/kg DFP, i.p. 6 h after injection, the animals were euthanized by cervical dislocation

followed by decapitation. The brain was removed, and the frontal cortex dissected, weighed and placed on dry ice and stored at −80 °C until assay for cytokine gene expression.

4. CORT-DFP (C + D), group. These animals received tap water containing 20 mg% CORT dissolved in 0.6% (*v/v*) EtOH vehicle for 8 days. On the 8th day, the animals were injected with 4 mg/kg DFP, i.p. 6 h after injection, and the animals were euthanized by cervical dislocation followed by decapitation. The brain was removed, and the frontal cortex dissected, weighed and placed on dry ice and stored at -80 °C until assay for cytokine gene expression.

An abbreviated version of the design is presented in Table 1.

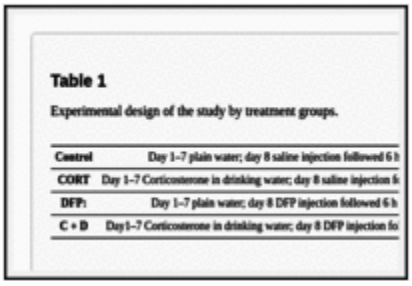

Table 1

Experimental design of the study by treatment groups.

Control	Day 1–7 plain water; day 8 saline injection followed 6 h
CORT	Day 1–7 Corticosterone in drinking water; day 8 saline injection fo
DFP	Day 1–7 plain water; day 8 DFP injection followed 6 h
C + D	Day1–7 Corticosterone in drinking water; day 8 DFP injection fo

Table 1
Experimental design of the study by treatment groups.

3.2. RNA Isolation, cDNA Synthesis and rtPCR

QPCR was used to analyze expression of mRNA for the proinflammatory cytokines, Il1β, Il6, and *Tnfa* in brain samples (medial prefrontal cortex). All procedures are described by O'Callaghan et al. [10] and Locker et al. [8]. Total RNA was isolated from medial prefrontal cortex at 6 h after DFP exposure. Real-time PCR analysis of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and of the proinflammatory mediators, TNFα, IL-6, and IL-1β was performed in an ABI7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) in combination with TaqMan chemistry. Relative quantification of gene expression was performed using the comparative threshold ($\Delta\Delta C_T$) method. Changes in mRNA expression levels were calculated after normalization to GAPDH. The ratios obtained after normalization are expressed as fold change over corresponding saline-treated controls.

3.3. Data Analysis

Transcript abundance for *Il1β, Il6,* and *Tnfa* obtained by qPCR were LOG_2 transformed and analyzed by analysis of variance for a three between-subjects variables (strain, sex, treatment) experiment. Main effects and interactions were considered statistically significant at $\alpha = 0.05$. Genetic mapping and quantitative trait loci (QTL) analyses were conducted using GeneNetwork

software (http://www.genenetwork.org; [16]). The data of greatest importance to this study are from the CORT-DFP treatment group; however, we also present the findings from the CORT alone and DFP alone treatment groups.

4. Results

4.1. Corticosterone Consumption

Average consumption of CORT added to the drinking water over the seven days varied widely (Figure 1). Analysis of variance revealed strain and sex main effects ($F_{33,524} = 12.12$, $p < 0.001$; $F_{1,524} = 91.45$, $p < 0.001$, respectively). Overall, females (GeneNetwork ID 21265) consumed more corticosterone than did the males GeneNetwork ID 21273). The interaction between strain and sex was also significant ($F_{33,524} = 1.77$, $p < 0.01$). We also evaluated the effect of variability in corticosterone consumption on expression of the three cytokines by conducting analysis of covariance and reporting the adjusted means.

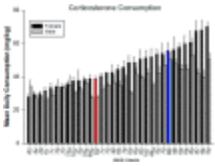

Figure 1

Corticosterone consumption. Male and female BXD mice were given 20mg% (*w/v*) corticosterone in their drinking water as sole liquid source for seven days prior to i.p. treatment with 4 mg/kg diisopropylflurophosphate. Data are mean consumption per day ± ...

4.2. Gene Expression in Response to Treatments

4.2.1. IL1b

We observed wide variability in the effect of CORT on expression of *Il1b* (Figure 2). ANOVA revealed a significant main effect for strain ($F_{32,122} = 3.61$, $p < 0.001$). The main effects for sex and the sex X strain interaction were not significant ($F < 1$ for both). Interestingly, CORT increased the expression of this cytokine in BXD strains 78 and 79 in males and 60 and 89 in both sexes.

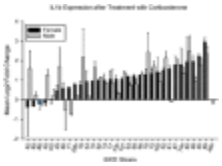

Figure 2

Mean change (±s.e.m) vs. control in *Il1b*expression in prefrontal cortex following 7 days of corticosterone in the drinking water and 6h following intraperitoneal injection with normal saline.

DFP produced inconsistent effects on *Il1b* expression (Figure 3); however, the combination of DFP with CORT increased the expression in nearly all strains, thus supporting the observation of O'Callaghan and colleagues [10] that CORT enhances the expression of this cytokine and others in the prefrontal cortex. Analysis of variance revealed significant effects for strain, sex, and treatment ($F_{33,843} = 2.56$, $p < 0.001$; $F_{1,843} = 4.67$, $p < 0.04$; $F_{3,843} = 253.31$, $p < 0.001$ respectively) on *IL1b* expression. The strain X treatment, sex X treatment and sex X strain interactions were also significant ($F_{97,843} = 1.40$, $p < 0.02$: $F_{3,843} = 3.76$, $p < 0.02$; $F_{32,843} = 2.51$, $p < 0.001$, respectively). Analysis of covariance showed no significant effect of CORT consumption ($F_{1,250} < 1$).

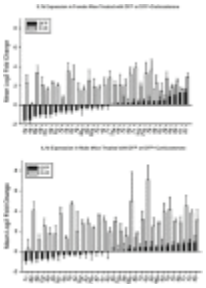

Figure 3

Mean change (±s.e.m) vs. control in *Il1b*expression in prefrontal cortex 6 h following intraperitoneal injection with diisopropylflurophosphate (DFP, 4 mg/kg—black bars) or 7 days of corticosterone in the drinking water and 6h following ...

Interestingly, CORT-DFP was the only condition that showed no decreases in expression (Figure 3) and in the same treatment condition; females (GeneNetwork ID 21195) were less affected in increased gene expression than males (GeneNetwork ID 21200).

4.2.2. IL6

We observed strain and sex-related variability in the effects of CORT on expression of *Il6* (Figure 4). It appears that males in only one BXD strain (BXD75) showed an increase in expression of this cytokine.

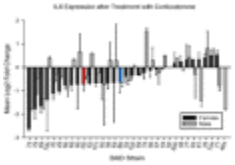

Figure 4

Mean change (±s.e.m) vs. control in *ll6*expression in prefrontal cortex following 7 days of corticosterone in the drinking water and 6h following intraperitoneal injection with normal saline.

Analysis of variance revealed significant main effects for strain and sex ($F_{32,112} = 1.92$, $p < 0.01$; $F_{1,112} = 5.92$, $p < 0.02$), The strain X sex interaction was not significant ($F < 1$). Overall, females showed a greater effect of CORT than did males.

We observed inconsistent effects of DFP and DFP+CORT on expression of *Il6* (Figure 5). Effects for females are seen on the top panel and data for males on the bottom panel. Analysis

of variance revealed significant effects for strain, sex, and treatment ($F_{33,843}$ = 2.51, p < 0.001; $F_{1.843}$= 7.91, p < 0.006; $F_{3,843}$ = 16.01, p < 0.001 respectively). None of the 2-way interactions was significant (F < 1 for all) and the three-way interaction was also not significant (F < 1). As with *Il1b*, females were less sensitive to the CORT + DFP effect on Il6 gene expression. Analysis of covariance showed no significant effect of CORT on *Il6*gene expression ($F_{1,249}$ = 2.12, p > 0.1).

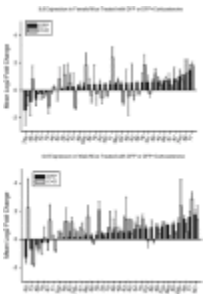

Figure 5

Mean change (±s.e.m) vs. control in *Il6*expression in prefrontal cortex 6 h following intraperitoneal injection with diisopropylflurophosphate (4 mg/kg—black bars) or 7 days of corticosterone in the drinking water and 6h following intraperiteoneal ...

4.2.3. Tnfa

CORT produced variable effects on *Tnfa*expression (Figure 6) among the strains, compared to controls. Strain means can be found in GeneNetwork for males (GeneNetwork ID 21234) and females (GeneNetwork ID 21228) respectively. The data are Log_2means for gene expression by qPCR.

Figure 6

Mean change (±s.e.m) vs. control in *Tnfa*expression in prefrontal cortex following 7 days of corticosterone in the drinking water and 6h following intraperitoneal injection with normal saline.

ANOVA revealed a significant main effect for strain ($F_{32,111}$ = 5.31, p < 0.01). Effects for sex or strain X sex interaction were not significant ($F_{1,111}$ = 1.83, p < 0.20; $F_{30,111}$ = 1.98, p < 0.35).

DFP and DFP + CORT also affected *Tnfa* expression in prefrontal cortex (Figure 7). Analysis of variance revealed significant effects for strain, sex, and treatment ($F_{33,843}$ = 3.51, p < 0.001; $F_{1.843}$ = 17.26, p < 0.001; $F_{3,843}$ = 269. 75, p < 0.001 respectively). The strain X treatment, sex X treatment and sex X strain interactions were also significant ($F_{97,843}$ = 1.52, p < 0.002; $F_{3,843}$ = 3.30, p < 0.02; $F_{32,843}$ = 2.19, p < 0.001, respectively). Analysis of covariance showed a significant effect of CORT consumption on CORT+DFP gene expression ($F_{1,249}$ = 5.10, p < 0.03). As with the other two cytokines, females were less sensitive to the combined effects of CORT+DFP. Accordingly, Figure 7 presents adjusted means for expression following CORT +

DFP treatment. The data for females are presented in the top panel and the data for males in the bottom.

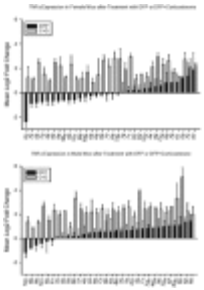

Figure 7

Mean change (±s.e.m) vs. control in *Tnfa*expression in prefrontal cortex 6 h following intraperitoneal injection with diisopropylflurophosphate (4 mg/kg—black bars) or 7 days of corticosterone in the drinking water and 6 h following intraperiteoneal ...

4.3. Mapping of IL1β Response to CORT + DFP

Of the three cytokines, *Tnfa* and *Il1β* showed significant strain (genetic) X treatment interactions. Mapping *Il1β*response, we observed a suggestive quantitative trait loci (QTL) on chromosome 7 between 110 and 115 Mb for males and females. When we combined the male and female data by principal component analysis (PCA), the signal became significant (Figure 8). We then searched for possible candidate genes that underlie the individual differences in *Il1β* response to CORT+DFP using (*Hippocampus Consortium M430v2 (Jun06) PDNN Genenetwork accession no. GN112*). In this database, the overlap of strains with our study included 21 strains. The main criteria are 1) that the gene be *cis*-regulated (not absolutely necessary) and 2) the expression of the gene be correlated with variability in the phenotype (principal component Figure 9). One gene stood out, *Spon1* (Spondin 1). The protein is secreted by the floor plate during neurogenesis and is involved in cell adhesion, axon guidance, metal binding (Ca), LPS-related inflammation, amyloid precursor protein degradation, negative regulation of amyloid beta production.

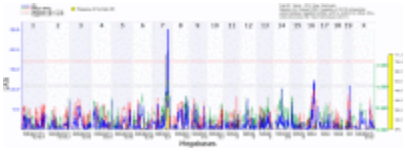

Figure 8

Quantitative trait locus map of *Il1b* expression change following 7 days of corticosterone in the drinking water and 6h following i.p. injection of diisopropylflurophosphate (4 mg/kg). The dependent variable (eigenvariable) is the first principal component ...

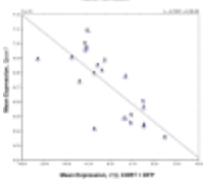

Figure 9

Scatter diagram of the association between the expression of *Spon1* and the eigenvariable relative to *Il1b* expression in mice treated with corticosterone and DFP.

In investigating the pattern of expression across the gene between the parental B6 and D2 strains, we noticed that the 3′ UTR region of the gene (Figure 10) showed greater expression in the B6 strain (in blue extreme right side of figure) compared to the D2 strain. This part of the gene is important because it usually contains regulatory sequences that affect expression.

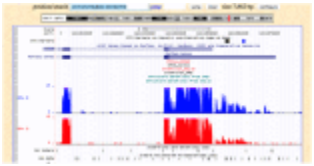

Figure 10

Diagram of *Spon1* expression from 5′ to 3′ UTR (left to right) the top (blue) figure is C57BL/6J and the bottom (red) figure is DBA/2J. The figure shows greater expression in the 3′ UTR for C57BL/6J compared to that for DBA/2J.

5. Discussion

Our work presented here is the first of its kind to show genetic-based differential susceptibility in a mouse model of GWI that may underlie the symptoms observed in ill Gulf War veterans. This work also shows sex differences with females being less sensitive to the effects of the exposure than males. Moreover, our results demonstrate once again the efficacy of our model in terms of CORT enhancing the effect of DFP on expression of proinflammatory cytokine genes. Please note that this work addresses the acute response of proinflammatory cytokines to CORT + DFP as proof-of-principle. GWI is a chronic disease and those so afflicted have suffered symptoms for nearly 30 years. Study of the chronic effects, susceptibility and treatments is compelling and likely related to epigenetics of genes identified here. Indeed, Trevedi and colleagues [17] showed DNA methylation changes in monocytes from GWI affected veterans in genes related to immune function, thus supporting the hypothesis of neuroimmune sequelae of GWI. In mice, Ashbrook et al. [18] showed genome-wide histone modification and DNA methylation in the frontal cortex. Many of the genes so modified are related to myelin production in oligodendrocytes and thus may be related to the observation of reduced white matter in frontal cortex of GWI veterans.

The importance of this work is that candidate genes identified in the mouse have high probability of overlapping with the human genome [19,20] because of conserved function (gene homology) and biological pathways.

In comparing the phenotypic responses, *Il1β* and *Tnfa*were better indices of the treatment effect of DFP following corticosterone than was *Il6*, in agreement with our prior findings [10]. Both of the former showed the expected main effects, but more importantly the interactions between strain and treatment, and sex by treatment. None of the 2-way interactions for Il6 were significant.

We were also able to nominate a candidate gene for *Il1β*response to treatment. *Spon1* met both criteria of *cis*-regulation and tight correlation with the response. *Spon1*is of interest because it is

involved with axon guidance, WNT/βcatenin signaling and protects against chemotherapy toxins [20,21], cognitive problems [22], circadian rhythms [23] and TGF-β- inflammatory response [24]. We observed differential expression of the distal 3′ UTR between C57BL/6J and DBA/2J (Figure 10). This region contains binding sites for several RNA-binding proteins. Higher expression of the B allele relative to the D allele may lead to differential transport of this mRNA in neurons across BXD strains. The B2 SINE polymorphism in the 3′ UTR of Comt is an example the key role of these types of non-coding splice variants [25].

There are other things that draw our attention to *Spon1*. *One* is the observation by Zhao and colleagues [21] that R-Spondin 1 reduces the toxicity of chemotherapeutic agents and radiation via the WNT/βcatenin pathway [26]. The mechanism may involve changes in proinflammatory cytokine production locally and it is worth exploring whether Spon 1 might affect other inflammatory systems, including IL1 cytokines. If so, then Spon 1 and WNT/βcatenin might be appropriate targets for therapeutics for GWI. Another and more compelling reason is its effects on amyloid precursor protein. Recent genome-wide association studies of the rate of cognitive decline in Alzheimer's disease indicated SPON1 as candidate gene associated with slower rate of cognitive decline in Alzheimer's disease [27]. Hafez and colleagues [28] proposed from their study in mice that one possible mechanism is *Spon1* associated with inhibition of amyloid beta while promoting synaptophysin.

6. Conclusions

We have demonstrated wide, genetic and sex variability in susceptibility to developing GWI following combined exposure to high circulating glucocorticoids and organophosphorus compounds that inhibit cholinesterase irreversibly. By locating a plausible candidate gene that underlies the strain differences in a genetic reference population of mice, we may have identified a possible factor underlying individual differences to the conditions that produce GWI, especially as concerns cognitive difficulties in a genetically defined subpopulation of humans. The study has its limitations. First, we were constrained by budgetary considerations to studying just three proinflammatory cytokine genes. A broader panel of these genes would give a more complete view. Second, we were again limited to studying 30 BXD strains. By studying more strains, we would expect to discover more candidates and with increased precision in mapping. We did perform whole genome RNA-seq analysis and those data will be submitted for publication separately.

Author Contributions

J.P.O., D.B.M. and B.C.J. formulated the concept and designed the research, B.C.J. wrote the paper, W.Z. and D.Z. set up and ran the experiments, F.X. and D.G.A. helped with the discussion, M.K.M. and R.W.W., L.L. helped with the editing and C.T.-R. helped with the figures. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

All authors declare no conflicts of interest.

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EXPLORING THE ROLE OF CHEMOKINE RECEPTOR 6 (*CCR6*) IN THE BXD MOUSE MODEL OF GULF WAR ILLNESS

Jun Gao, Fuyi Xu, [...], and Lu Lu

Additional article information

Associated Data

Supplementary Materials
Data Availability Statement

Abstract

Gulf War illness (GWI) is a chronic and multi-symptomatic disorder with persistent neuroimmune symptomatology. Chemokine receptor 6 (CCR6) has been shown to be involved in several inflammation disorders in humans. However, the causative relationship between *CCR6* and neuroinflammation in GWI has not yet been investigated. By using RNA-seq data of prefrontal cortex (PFC) from 31 C57BL/6J X DBA/2J (BXD) recombinant inbred (RI) mouse strains and their parental strains under three chemical treatment groups – saline control (CTL), diisopropylfluorophosphate (DFP), and corticosterone combined with diisopropylfluorophosphate (CORT+DFP), we identified *Ccr6* as a candidate gene underlying individual differences in susceptibility to GWI. The *Ccr6*gene is *cis*-regulated and its expression is significantly correlated with CORT+DFP treatment. Its mean transcript abundance in PFC of BXD mice decreased 1.6-fold ($p < 0.0001$) in the CORT+DFP group. The response of *Ccr6*to CORT+DFP is also significantly different ($p < 0.0001$) between the parental strains, suggesting *Ccr6* is affected by both host genetic background and chemical treatments. Pearson product-moment correlation analysis revealed 1473 *Ccr6*-correlated genes ($p < 0.05$). Enrichment of these genes was seen in the immune, inflammation, cytokine, and neurological related categories. In addition, we also found five central nervous system-related phenotypes and fecal corticosterone concentration have significant correlation ($p < 0.05$) with expression of *Ccr6*in the PFC. We further established a protein-protein interaction subnetwork for the *Ccr6*-correlated genes, which provides an insight on the interaction of G protein-coupled receptors, kallikrein-kinin system and neuroactive ligand-receptors. This analysis likely defines the heterogeneity and complexity of GWI. Therefore, our results suggest that *Ccr6* is one of promising GWI biomarkers.

Keywords: *Ccr6*, GWI, DFP, CORT, BXD strain, RNA-seq, neuroinflammation

Introduction

Gulf War illness (GWI) is the term used to describe a chronic and multi-symptomatic disorder affecting returning military veterans of the 1990–1991 Gulf War (Binns et al., 2014). The symptoms of GWI vary somewhat among individuals and typically include unexplained fatigue, chronic diarrhea musculoskeletal pain, headaches, cognitive dysfunction, rashes and respiratory problems, gastrointestinal, and dermatologic complaints (White et al., 2016; Maule et al., 2018). Although some views ascribed GWI to post-traumatic stress disorder (PTSD) or psychiatric condition to the consequence of wars, accumulated evidence shows that GWI is a neuroimmune disorder resulting from chemical exposures and the physiological stressors incurred in the war theater (O'Callaghan et al., 2015; White et al., 2016). Animal model behavioral data mirror GWI neurobehavioral deficits in terms of impaired memory and cognition, as well as increased anxiety and depressive-like mood (Abdullah et al., 2011; Parihar et al., 2013; Hattiangady et al., 2014; Zakirova et al., 2015; Carreras et al., 2018; Carpenter et al., 2020).

The neurotoxicant exposures encountered by GW military personnel during deployment, including carbamates, organophosphates (OPs), and other pesticides; OP nerve agents (sarin/cyclosarin); and pyridostigmine bromide (PB) (White et al., 2016; Maule et al., 2018). Accumulated neuroimaging studies have demonstrated abnormalities in the brains of veterans with GWI (Binns et al., 2014) including strong evidence for neuroinflammation (Alshelh et al., 2020). Brain pathology of reduced white and gray matter volumes also can be detected nearly two decades later in sarin and cyclosarin-exposed ill GW veterans (Chao et al., 2011). Studies revealed that brain chemistry is abnormal mainly in prefrontal cortex (PFC) and different subregions that mediate various characteristics of the chronic pain, such as sensory and affective dimensions, anxiety and depression (Apkarian et al., 2005). Changes in neurotransmitters, gene expression, glial cells, and neuroinflammation occur in the PFC during acute and chronic pain, which result in alterations to its structure, activity, and connectivity (Ong et al., 2019). Moreover, cortical regions involved in fatigue, pain, and hyperalgesia, also have been reported to be associated with diminished white matter integrity in GW veterans (GWV) (Rayhan et al., 2013). However, heterogeneous symptom presentation and lack of biomarkers in PFC that identify a distinct pathophysiological process in GWI still remain challenging.

Chronic inflammation is a component of the pathophysiology of GWI (Johnson et al., 2016). The sarin surrogate diisopropylfluorophosphate (DFP), an irreversible acetylcholinesterase (AChE) inhibitor, results in brain-wide neuroinflammation that is markedly enhanced in the mouse model by prior exposure to CORT (O'Callaghan et al., 2015; Locker et al., 2017; Koo et al., 2018). High circulating glucocorticoids exaggerates the neuroinflammatory response as measured by the expression of genes for multiple cytokines and chemokines (e.g., *Tnf-α*, *Il6*, *Ccl2*, *Il-1β*, *Lif*, and *Osm*) (O'Callaghan et al., 2015; Jones et al., 2020). Neuroinflammation disorder is induced by chemical exposure and has been linked to cytokine-induced 'sickness' behavior of GWI in veterans (Dantzer and Kelley, 2007; Dantzer et al., 2008; O'Callaghan et al., 2015); however, the underlying causes have not been fully elucidated.

Chemokine receptor 6 (CCR6) contributes to steady-state cell chemotaxis in supporting immunity and regulating immune homeostasis during inflammation (Ranasinghe and Eri, 2018). Genetic associations have been identified between *CCR6* polymorphisms and immune system disorders in humans including rheumatoid arthritis (RA) and Crohn's disease (Cheng et al., 2015; Julian et al., 2017). GWI is also characterized by gastrointestinal disorders such as inflammatory bowel disease (IBD) like Crohn's disease (Ranasinghe and Eri, 2018; Seth et al., 2019). In addition, RA is reported to overlap with specific druggable components of GWI, and some immunosuppressants have been approved by the Food and Drug Administration (FDA) as the best available candidates for treating GWI symptoms (Craddock et al., 2015). However, the causative relation between *CCR6* and GWI has not been reported yet.

The C57BL/6J X DBA/2J (BXD) recombinant inbred (RI) mouse strains, which are unique mosaic of alleles derived from the parental C57BL/6J (B6) and DBA/2J (D2) strains have been constructed as a high precision genetic reference population for systems genetics in unraveling the genetic architecture of polygenic traits (Ashbrook et al., 2019). The BXD family consists of more than 150 BXD fully inbred strains that segregate for ~6 million genetic variants and thus can be used as an informative murine genetic reference panel. The application of the BXD strains provides a unique mouse model to investigate the role of *Ccr6* in individual differences to GWI susceptibility.

In this study, we assessed the expression of *Ccr6* in the PFC of the GWI BXD model with different chemical treatments. Furthermore, we sought to identify the eQTL for *Ccr6*, analyze correlated genes and potential pathways, and to construct a protein-protein interaction (PPI) subnetwork that may contribute to individual differences in GWI.

Materials and Methods

Animals

Four hundred-nine mice from 31 BXD strains and their parental strains (B6 and D2) were used in this study. The animals were randomly chosen at 2–4 months of age at testing and 2–3 animals per strain, sex and treatment group were used (Supplementary Data 1). All animals were housed in individually ventilated cage (IVC) system in the Animal Care Facility at the University of Tennessee Health Science Center (UTHSC, Memphis, TN, United States). The vivarium is a temperature (20 ± 2°C) and humidity (35%) controlled environment under a 12 h light/12 h dark cycle. The animals had free access to food and water throughout the experiment. Nine days before the euthanasia, every mouse was single caged, and received corresponding treatment after 2 days adaptation. The euthanasia was carried out in a separate procedure room. All animal procedures were carried out in accordance with the UTHSC guidelines on the humane treatment of experimental animals and with the explicit approval of the Institutional Animal Care and Use Committee (IACUC).

Treatment Groups

The experimental animals were divided into three treatment groups (Jones et al., 2020) as follows:

- (1)

Control group (**CTL**): These strains received plain tap water for fluid (Day 1–7). On the 8th day, the animals were injected with saline (0.9% NaCl) and euthanized by cervical dislocation 6 h after injection.

- (2)

Diisopropylfluorophosphate group (**DFP**): These strains received plain tap water for fluid (Day 1–7). On the 8th day, the animals were injected with 4 mg/kg DFP, i.p., 6 h after injection, the animals were euthanized by cervical dislocation followed by decapitation.

- (3)

Corticosterone + Diisopropylfluorophosphate group (**CORT+DFP**): These strains received tap water containing 20 mg% CORT dissolved in 0.6% (v/v) EtOH vehicle for 8 days. On the 8th day, the animals were injected with 4mg/kg DFP, i.p., 6 h after injection, the animals were euthanized by cervical dislocation followed by decapitation.

The chemicals DFP (Sigma, St. Louis, MO, United States), CORT (Steraloids, Inc., Newport, RI, United States) and other reagents were analytical grade.

Tissue Collection

The mice were euthanized by cervical dislocation followed by decapitated that is descripted in our previous publication (Jones et al., 2020) and the whole brain was immediately removed from the skull. The PFC was dissected with a 90° cut 1 mm from the posterior edge of olfactory bulb and another 90° cut 2 mm caudal from the first cut. The PFC was weighed and snap frozen in dry ice bath with isopentane and stored at −80°C until RNA extraction.

RNA-Seq and Data Processing

Total RNA was extracted from 20 mg frozen PFC tissue per sample using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration and purity of the RNA was measured using NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States). The RNA integrity (RIN) was assessed using Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, United States). 1 mg qualified RNA (per sample) with OD260/280 >

1.8, OD260/230 > 2.0, and RNA Integrity Number (RIN) >8.0 was used for library preparation and sequencing. The RNA-seq libraries were prepared using the NEBNext® Ultra RNA Library Prep Kit at Novogene Corporation Inc. Paired-end sequencing was performed on an Illumina Novaseq Platform (Illumina, San Diego, CA, United States) by reading 150 bases at each end of a fragment. Overall, each library generated an average of 40 million raw reads.

Raw reads, stored in fastq format, were filtered by removing the adaptor and low-quality reads for further analysis. To generate clean reads, allowing for reads containing over 50% bases with quality greater than 5 and less than 10% "N" bases to be included. The clean reads were then mapped onto the mouse reference genome (version: GRCm38) using the STAR aligner (v2.5.0a) (Dobin et al., 2013). FeatureCount (v0.6.1) (Liao et al., 2014) program was used to get the gene level reads count based on the gene model annotation file downloaded from the Ensembl genome browser[1]. Raw read count was normalized by DESeq2 R package (v1.22.2) (Love et al., 2014) and batch was added as a covariate for data normalization. Differential expression of *Ccr6* was calculated between the three groups (DFP vs. CTL, CORT+DFP vs. CTL, and DFP vs. CORT+DFP) by unpaired *t*-test.

Analysis of Variance (ANOVA)

A two between-subjects variables (strain, treatment) design was used to assess the main effects and interaction on *Ccr6* transcript abundance using ANOVA function in R software (R Core Team, 2013). The accepted level of significance for all tests was $p < 0.05$.

Heritability Estimation

Broad sense heritability (h^2) is a concept that summarizes how much of the variance in a quantitative trait is due to variation in genetic factors. It was calculated from the ANOVA results using the following formula (Hegmann and Possidente, 1981): 0.5 VA/(0.5 VA + VE), where VA is the additive genetic variance (variances of the strain means) and VE is the average environmental variance (variance within strains). The factor of 0.5 in this formula was applied to adjust for the 2-fold increase in the additive genetic variance among the inbred strains relative to outbred populations (Lu et al., 2018).

eQTL Mapping and Sequence Variants Analysis

eQTL mapping is a regression analysis to determine the relationship between differences in a trait and differences in alleles at markers across the genome. The eQTL mapping of *Ccr6* in three groups (CTL, DFP, and CORT+DFP) were conducted through the WebQTL module on GeneNetwork website[2] according to the published methods (Mulligan et al., 2017; Williams and Williams, 2017). The input expression values of *Ccr6* was normalized with TPM (transcripts per million) method (Wagner et al., 2012; Vera Alvarez et al., 2019) and log2 (TPM + 1)

transformed. Simple interval mapping yielded a likelihood ratio statistic (LRS) score, providing us a quantitative measure of confidence of linkage between the observed phenotype and a genomic region. The genome-wide significance ($p < 0.05$) for each eQTL was determined with 1000 permutation tests.

Single nucleotide polymorphisms (SNPs) and insertion-deletions (InDels) in the *Ccr6* gene and its surrounding up- and down-stream regions between the B6 and D2 were extracted from the Mouse Genome Project database[3] (Keane et al., 2011; Yalcin et al., 2011).

Gene-Phenotype Correlation Analysis

To assess the relationship between the expression of *Ccr6*and related traits across the BXD cohort, we queried the BXD archival phenotypes from the GeneNetwork and analyzed for Pearson product-moment correlation to the expression of *Ccr6* in PFC. The top 500 Pearson product-moment correlations were filtered and $p < 0.05$ were considered significant.

Gene-Gene Correlation Analysis

In order to identify the *Ccr6* correlated genes across the PFC transcriptomes in the treatment groups, we conducted Pearson product-moment correlations of the strain means between the expression of *Ccr6* and the expression of all the other genes across the mouse genome to produce sets of genetically correlated genes on GeneNetwork. Genes significantly correlated with expression of *Ccr6* ($p < 0.05$) were used for the gene set enrichment analysis, in which, Riken cDNA clones, intragenic sequences, and predicted genes were eliminated.

Gene Set Enrichment Analysis

Gene set enrichment analysis was performed to investigate the gene ontology (GO, biological processes) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of the *Ccr6* correlated genes. We submitted the gene set of each treatment group to the Webgestalt website[4] (Liao et al., 2019) for analysis. The *p*-value generated from the test was automatically adjusted to account for multiple comparisons using the Benjamini and Hochberg correction (Benjamini and Hochberg, 1995). A minimum overlap of five genes and False Discovery Rate (FDR) < 0.05 was required to determine the genes significantly overrepresented in those categories.

Protein-Protein Interactions (PPI) Analysis

PPI analysis of the *Ccr6* correlated genes was based on the online STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (Szklarczyk et al., 2019), which contains

known and predicted PPIs information by consolidating known and predicted protein-protein association data for a large number of organisms (Szklarczyk et al., 2016). In this study, we first constructed the PPI network by extracting the target gene lists from the database with required the highest score of confidence interaction of 0.9. Then Markov Cluster Algorithm (MCL) clustering was used for subnetwork construction, in which the inflation parameter used a default setting 3. The narrowed subnetwork genes were further used for GO and KEGG analysis to gain insight into the biological functions and pathways of *Ccr6*correlated genes.

Results

Ccr6 Expression Across the BXD Strains

In this study, a total of 409 mice were used for PFC harvest and expression profiling across the treatments. Overall, the expression of *Ccr6* in CORT+DFP group showed significant decrease in most of the BXD strains (Figure 1A). However, this effect was not consistent for DFP treatment, in which *Ccr6* mRNA levels decreased in 18 strains (e.g., BXD29, BXD83, BXD65), but increased in the rest of 15 strains (e.g., BXD66, D2, BXD48). This finding further supports the assertion of O'Callaghan et al. (2015) that exposure to OPs plus high circulating glucocorticoids may be an essential condition for GWI. Next, we compared the expression of *Ccr6* between the different treatment groups. Results showed that *Ccr6*significantly decreased in the CORT+DFP group when compared with CTL group (Fold change = 1.60, $p < 0.0001$) and DFP group (Fold change = 1.42, $p < 0.05$) (Figure 1B), respectively.

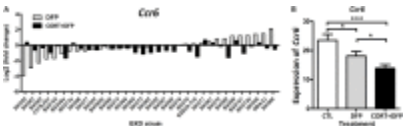

FIGURE 1

The expression of *Ccr6* across the BXD strains. **(A)** The relative fold change of *Ccr6*expression in the DFP and CORT+DFP groups compared to the CTL across the BXD RI strains. **(B)** Comparison of the expression (Mean ± SEM) of *Ccr6* between the treatments ...

In order to determine the effects of strain and treatment on the expression of *Ccr6* further, we conducted two way ANOVA which showed both factors have significant effects on the *Ccr6* expression [Treatment: $F_{(2, 310)} = 22.20$, $p < 10E\text{-}10$; Strain: $F_{(32, 310)} = 18.15$, $p < 3E\text{-}16$]. The strain × treatment interaction was also significant [$F_{(64, 310)} = 2.40$, $p < 4.0E\text{-}7$]. In addition, we calculated the heritability (h^2) for each treatment group with $h^2 = 0.29$ for CTL, 0.27 for DFP and 0.22 for CORT+DFP, suggesting both genetic and environmental factors contribute to the expression differences of *Ccr6* among the BXD strains.

eQTL Mapping and Sequence Variants of *Ccr6*

Ccr6 is located on chromosome 17 at 8.236 Mb of mice. Interval mapping indicated a genome-wide significant eQTL with a LRS of 53.5 in CTL (Figure 2A), 34.6 in DFP (Figure 2B), and

26 in CORT+DFP (Figure 2C) on chromosome 17 at 7.713 Mb. This locus is located 0.5 megabases (Mb) upstream of *Ccr6*, indicating that *Ccr6* is *cis*-regulated in the PFC for all three groups (Figure 2). Next, we grouped the mice according to their genotype (B and D type) at the QTL peak position (rs48543649, Chr 17 at 8.199 Mb) which is near the physical position of *Ccr6*. Statistical analysis revealed that the mRNA levels of *Ccr6*showed a significant difference ($p < 0.0001$) between B and D alleles in all three treatment groups (Figures 2D–F), with mice carrying the D allele evincing higher expression level of *Ccr6*.

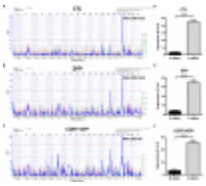

FIGURE 2

eQTLs mapping of *Ccr6*. eQTLs mapping demonstrates *Ccr6* is *cis* regulated in the prefrontal cortex mRNA. **(A)** CTL (Max likelihood ratio statistic score (LRS) = 53.5), **(B)** DFP (Max LRS = 34.6), **(C)** CORT+DFP (Max LRS = 26.0). Chromosome number can be found ...

Ccr6 is *cis* regulated, which means that sequence variants within or nearby *Ccr6* likely affect its expression. Therefore, we explored nearby genetic variants using the database of Mouse genome project[6]. We identified 31 SNPs and 3 InDels (Table 1) between the parental strains B6 and D2, in which one is a synonymous variant (rs49056705), three are 5′ UTR variants (rs33886456, rs33640330, and rs33573638), and the rest of them are located within 5000 bp upstream of *Ccr6*. We also identified one trans-eQTL achieved statistical significance in DFP group, which located on Chr14 at 100–110 Mb. This eQTL interval contains 90 genes, of which, *Kctd12*and *Mycbp2* correlated with *Ccr6* ($p < 0.05$), *Slain1* and *Ednrb* harbor nonsynonymous mutations, suggesting they could be upstream candidate regulators.

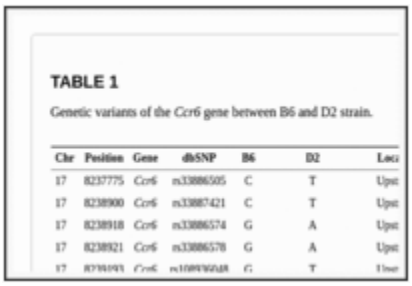

TABLE 1

Genetic variants of the *Ccr6* gene between B6 and D2 strain.

Chr	Position	Gene	dbSNP	B6	D2	Loc
17	8237775	*Ccr6*	rs33886505	C	T	Upst
17	8238900	*Ccr6*	rs33887421	C	T	Upst
17	8238918	*Ccr6*	rs33886574	G	A	Upst
17	8238921	*Ccr6*	rs33886578	G	A	Upst
17	8239093	*Ccr6*	rs33886618	G	T	Upst

TABLE 1

Genetic variants of the *Ccr6* gene between B6 and D2 strain.

Genetic Correlations Between *Ccr6* and Archival Phenotypes From Our Database in GeneNetwork Website

To our knowledge, GWI is a chronic disease with significant neurological pathophysiology. In our mouse model, exposure to CORT+DFP treatment increased expression of proinflammatory cytokine genes, which is consistent with the neuroimmune basis of GWI (O'Callaghan et al., 2015). Additionally, our results show that CORT+DFP has the greatest effect on the expression of *Ccr6*. The question then becomes does CORT+DFP-related expression of *Ccr6* associate with other central nervous system (CNS) phenotypes? After multiple testing correction (FDR <

0.05), we obtained a total of 117 CNS related phenotypes that were significantly correlated with the expression of *Ccr6* in the CORT+DFP group ($P < 0.05$) (Supplementary Data 2). We listed five CNS-related phenotypes in BXD RI strains, including brain to body weight ratio (Figure 3A), novel open field behavior (Figure 3B), anxiety assay (Figure 3C), acoustic startle response (Figure 3D), learning and memory (Figure 3E), as well as fecal corticosterone concentration (Figure 3F) significantly correlated ($p < 0.05$) with the expression of *Ccr6*. These correlated phenotypes can be found on the GeneNetwork website with the access numbers of 17494, 11530, 12365, 13355, 20585, and 20113, respectively.

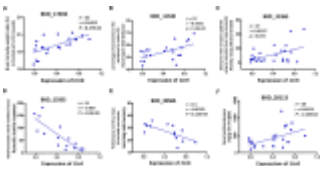

FIGURE 3

Phenotype Pearson correlation analysis with the expression of *Ccr6*. Five BXD central nervous system (CNS) related phenotypes including brain to body weight ratio **(A)**, novel open field behavior **(B)**, anxiety assay **(C)**, acoustic startle response **(D)**, and ...

Gene Set Enrichment Analysis

To understand the biological processes and gene pathways of *Ccr6* correlated genes, we performed correlation analysis and identified 1755, 7193, and 3996 genes that are significantly correlated ($p < 0.05$) with *Ccr6* in the CTL, DFP, and CORT+DFP group, respectively. After removing Riken cDNA clones, intragenic sequences, predicted genes, 806 (CTL), 3983 (DFP), and 1473 (CORT+DFP) genes were separately submitted to Webgestalt web site[2] for gene function enrichment analysis.

The gene set enrichment results (Supplementary Data 3) showed *Ccr6* correlated genes were significantly enriched in immune and inflammation-related GO terms in the CTL group. For the DFP group, the enrichment results demonstrate a high degree of neurological association with the *Ccr6* correlated genes. For the CORT+DFP group, we obtained a total of 47 significantly enriched GO terms (FDR < 0.05) and 23 KEGG pathways (FDR < 0.05). The top 20 GO and KEGG categories are listed in Figure 4. Of which, immune, inflammation, and cytokine terms were further highlighted.

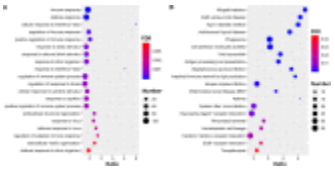

FIGURE 4

The gene set enrichment analysis of *Ccr6*correlated genes. The bubble plot shows the top 20 GO **(A)** and KEGG pathways **(B)** of *Ccr6* correlated genes in the CORT+DFP group.

Protein-Protein Interactions (PPI) Subnetwork for *Ccr6* Correlated Genes

To further dissect the potential interactions of the *Ccr6*correlated genes in the CORT+DFP group, we uploaded the above 1473 genes correlated to *Ccr6* into STRING[5] to search for PPI. By performing MCL clustering, we identified a Ccr6 PPI subnetwork, which includes 38 genes (Figure 5). These genes are highly inter-connected (interaction score ≥ 0.9).

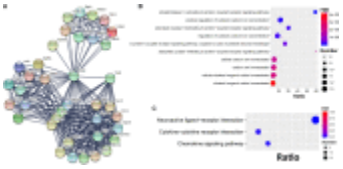

FIGURE 5
PPI subnetwork of *Ccr6* correlated genes. **(A)**Ccr6 PPI subnetwork includes 38 genes with the interaction score ≥0.9. The nodes represent genes while edges represent PPIs between two genes. Bubble plot of the Top 10 GO **(B)** and 3 KEGG **(C)** enrichment ...

Next, we performed GO and KEGG enrichment analysis for these 38 genes. The top 10 significant GO terms (FDR < 0.05) showed that those genes are mainly involved in G protein-coupled receptor signaling pathways (Oprd1, Ednra, Nmur1, Ntsr2, Fpr1, F2rl2, Lpar1, Fpr3, Gna14, Fpr2, Grm4, Sstr5, Grm6, Ptgfr, Cxcr3, and Mtnr1a) and cytosolic calcium ion concentration (Xcl1, Ednra, Fpr1, Ccl1, Ccr3, Ccr6, F2rl2, Lpar1, Ptgfr, Fpr3, Kng2, and Fpr2) (Figure 5B). The top 10 KEGG pathways (Figure 5C) had 3 achieve significance (FDR < 0.05), including one neuroactive ligand-receptor interaction pathway (Ltb4r2, Oprd1, Npffr1, S1pr5, Ednra, Grm4, Nmur1, Sstr5, Ntsr2, Fpr1, F2rl2, Grm6, Lpar1, Ptgfr, Mtnr1b, Fpr3, Mtnr1a, and Fpr2), and two cytokine-related pathways (Xcl1, Ccl1, Ccr3, Ccr6, Bmp15, Cxcr3, and Xcr1).

Discussion

Compared to nondeployed veterans, at least one fourth of the 697,000 U.S. veterans suffered from GWI when they returned from the theater of operations (Binns et al., 2014). These veterans also reported higher rates of amyotrophic lateral sclerosis (ALS) (Coffman et al., 2005; Horner et al., 2008), brain cancer (Barth et al., 2009), repeated seizures, neuralgia or neuritis, stroke (Kang et al., 2009), and migraine headaches (Unwin et al., 1999; Kang et al., 2000; Steele, 2000; Gray et al., 2002). Accumulating Studies clearly supports the links between adverse neurological outcomes and chemical exposures of GWV (Binns et al., 2014; White et al., 2016). Deployed GWV had significantly lower scores on tests of verbal memory, verbal learning, motor speed, and attention than nondeployed due to the pesticides and PB exposures (Toomey et al., 2009). Sarin/cyclosarin exposed GWV showed signs of reduced total gray and white matter volumes in the brain compared to unexposed controls and worse on a continuous performance test of attention (Chao et al., 2011).

Accurate diagnosis and treatment of GWI patients require an in-depth understanding of the cause of the disease. Although some of the individual differences in susceptibility to GWI may be explained by different exposures or different dose effects, much of it cannot be, and leaves

genetics as a significant contributor to individual differences in susceptibility and response to the exposures. The BXD mouse strains put the investigator at great advantage for systems genetics analysis of complex traits such as GWI and those traits that have modest heritability (Williams et al., 2001). In this study, we used BXD strains to explore the etiologic agents and pathways that underlie the "sickness" behavior of GWI. Indeed overall, we have supported the notion that this disease is the result of genetic–environment interaction.

Our results indicate *Ccr6* as one reasonable candidate gene that underlies individual differences in susceptibility to GWI. In addition, we also found five CNS-related phenotypes that show the wide-ranging effects of GWI. Furthermore, we identified 31 SNPs and 3 InDels that differ in response to CORT+DFP between B6 and D2 inbred strains. *CCR6* may turn out to be a target of therapeutic approaches to GWI.

The results of gene set enrichment analysis highlighted the categories of *Ccr6* correlated genes related to immune, inflammation, cytokine, and neurological aspects. Accumulating evidence suggest that *Ccr6* plays a major role in driving T-helper differentiation in inflammatory diseases and maintaining leukocyte homeostasis (Ranasinghe and Eri, 2018). *Ccr6* regulates the migration of inflammatory and regulatory T cells (Th17 and Treg), which play opposite roles in autoimmune diseases (Yamazaki et al., 2008). Although *Ccr6*-mediated Th17 migration to inflamed tissues may be important for driving CNS inflammation, *Ccr6* expression is deemed to be more critical to Treg cells than to Th17 cells, because this subset suppresses inflammatory T cell proliferation and promotes disease resolution (Ranasinghe and Eri, 2018). The use of *Ccr6*$^{-/-}$ mice in experimental autoimmune encephalomyelitis (EAE) study, an animal model of brain inflammation for the study of human CNS diseases characterized by mononuclear cell infiltration and demyelination, showed delayed disease onset and more neurological damage and increased mortality compared to wild-type mice (Villares et al., 2009). Severe phenotype in *Ccr6*$^{-/-}$ EAE mice is linked to increased inflammatory activity in target tissues. This suggests that *Ccr6* is necessary for Treg recruitment and initiates a feedback anti-inflammatory mechanism which compensatorily downregulates the CNS inflammatory activity (Yamazaki et al., 2008; Villares et al., 2009). Although the immune regulation mechanism of *CCR6* has not been fully elucidated, the *CCR6/CCL20* axis is an important chemokine receptor-ligand and may present a therapeutic target for the treatment of human disorders (Ranasinghe and Eri, 2018).

As we understand, GWI is a complex trait with underlying gene-environment and likely gene-gene interactions. Indeed, we identified a Ccr6 PPI subnetwork that includes 38 genes enriched in G protein-coupled receptor (GPCR) signaling pathways and cytosolic calcium ion concentration signaling pathway. Astrocytes and microglia are the most prominent target cells for inflammation in the CNS. Their responses upon activation include downregulation of ATP-induced Ca^{2+} signaling, G protein activities and release of pro-inflammatory cytokines (Hansson et al., 2018). Many druggable targets for treatment of common diseases involve GPCRs that mediate therapeutic effects of ~34% of the marketed drugs (Hauser et al., 2018). Further understanding of genetic factors and regulation networks of *Ccr6* is likely to advance drug treatment of GWI in the future.

Another gene worth noting is Kng2, that appears as a hub in the Ccr6 PPI subnetwork, implicating that the Kallikrein-kinin system (KKS) mediate the pathophysiological features of neurological disorders, including GWI. Despite a paucity of literature on Kng2, pharmacological research in mice and human genetic analyses suggest that the KKS may regulate anxiety (Nokkari et al., 2018; Rouhiainen et al., 2019). On the other hand, *Ccr6* correlated genes in PFC were observed to be significantly enriched in neuroactive ligand-receptor interaction pathways, of which, three formylpeptide receptors (Fpr1, Fpr2, Fpr3) are critical mediators of myeloid cell trafficking in microbial infection, inflammation, and immune responses (Krepel and Wang, 2019). Fpr2 also proved to mediate anxiety as shown by effects reported for Fpr agonists (Zhao et al., 2016). Although the role of the Ccr6 PPI subnetwork genes and their contribution to the neuroinflammation of GWI are not fully elucidated, it provides a new insight to the complexity of GWI.

Limitations

A suitable animal model of GWI should show evidence of the illness acutely and have it persist to model the entire 30-year course of the symptoms exhibited by ill veterans. The data in the present manuscript models the acute condition. We also have an extension of this model that represents the chronic "primed" inflammatory condition (manuscript in internal review). The chronic model is based on the paradigm of systemic challenge with lipopolysaccharide (LPS) (Kelly et al., 2018). It is important to subsequently investigate the changes of *Ccr6*mRNA levels at later time points in the chronic model to verify the possibility that *CCR6* can be a marker for GWI or similar ailment. Given the complexity and heterogeneity of GWI, we cannot exclude the existence of other regulatory elements. For example, genes such as *Tnf-α*, *Il6*, *Il1β*, and *Spon1* may also be involved in the neuroinflammatory response of GWI (Jones et al., 2020).

Conclusion

In this study, we identified *Ccr6* involvement in the neuroimmune response to CORT+DFP treatment in the BXD mouse model of GWI. Genetic factors and treatments both impact on the expression of *Ccr6* in PFC, which may contribute to CORT+DFP neuroinflammation in BXD strains. In humans, *CCR6*-mediates the migration of inflammatory and regulatory T cells and regulates CNS inflammation, which indicates it may be a promising therapeutic target of GWI. Our study also suggests the polymorphisms of *Ccr6* and synergy interaction of the related GPCRs, KKS system, and neuroactive ligand-receptor may contribute to the heterogeneity and complexity of GWI and related sickness behaviors.

Data Availability Statement

The datasets generated for this study can be found in the online repositories. The names of the repository/repositories and accession number(s) can be found below: www.genenetwork.org, GN880(http://gn1.genenetwork.org/webqtl / main. py ? FormID = sharinginfo &GN _AccessionId = 880), GN881 (http://gn1.genenetwork.org/webqtl/main.py?FormID= sharinginfo&GN_AccessionId=881), and GN882 (http://gn1. genenetwork . org /w ebqtl / main . py ? FormID = sharinginfo & GN _AccessionId=882).

Ethics Statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

Author Contributions

LL, BJ, DM, and JO'C conceived the study and oversaw the execution of the experimental work. JG and FX performed data analysis and prepared the figures and tables. LL, JG, and FX wrote the manuscript. BJ, LL, JO'C, and AS-D edited the manuscript. All authors read and approved the final version of the manuscript.

Disclaimer

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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[1]https://useast.ensembl.org/

[2]www.genenetwork.org

[3]http://www.sanger.ac.uk/science/data/mouse-genomes-project

[4]http://www.webgestalt.org

[5]https://string-db.org/

[6]https://www.sanger.ac.uk/science/data/mouse-genomes-project

[7]http://www.webgestalt.org

Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00818/full#supplementary-material

DATA S1

Sample information.

Click here for additional data file.(21K, XLSX)

DATA S2

Ccr6 correlated phenotypes in BXD mice.

Click here for additional data file.(78K, XLS)

DATA S3

Enrichment results of *Ccr6* correlated genes.

Click here for additional data file.(229K, XLS)

Article information

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