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14. ABSTRACT The primary aim of this work was to document genetic differences in susceptibility to organophosphorus compounds (like nerve agents) following high circulating glucocorticoids as a model of Gulf War illness in inbred mouse strains. The proposal was to study the effects of the combined exposures in 30 mouse strains to show large genetic differences of the treatment on proinflammatory cytokine gene expression (evidence of neuroinflammation) as a model of individual differences in Gulf War vets who experience the illness vs. those who do not. To date we have tested 870 mice of both sexes and from the 30 strains. As proposed, we have shown wide-genetic variability in response. Moreover, we are able at this time to nominate a likely candidate gene, Spondin 1, a gene that participates in inflammatory processes. We are now preparing our brain tissue samples for omnibus gene expression analysis. Overall, we are pleased with the progress so far.								
15. SUBJECT TERMS Gulf War illness, organophosphorus compounds, glucocorticoids, neuroinflammation, genetics, genomics, mice								
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Introduction

We are approaching the end of the first year of funding of GW160086, Genetic Basis of Individual Differences in Susceptibility to Gulf War Illness.” The objectives were to expose 30 BXD recombinant inbred mouse strains to corticosterone in their drinking water and then treat them with an irreversible cholinesterase inhibitor. The endpoints were changes in the expression of proinflammatory cytokine genes, *IL-1 β* , *IL-6* and *TNF- α* in the prefrontal cortex of the brain. To date, we have tested nearly all of the animals proposed. The next step is to send the remaining mRNA from the proinflammatory gene expression for omnibus genomic gene expression using RNA-seq. We expect that all of the work proposed will be completed by January 2019 and we will spend the rest of our time on analysis and article preparation.

Keywords

Gulf War illness, organophosphorus compounds, glucocorticoids, neuroinflammation, genetics, genomics, mice

Major goals – to show that glucocorticoids act synergistically with organophosphorus compounds (mimics Gulf War exposure) to produce neuroinflammation, the basis for sickness behaviors in those afflicted with Gulf War illness.

1. To subject male and female mice from 30 BXD recombinant inbred strains to the Gulf War animal model protocol as developed by Miller and O’Callaghan
 - a. Control animals – saline injection (i.p.) and six hours later euthanized by isoflurane and brain dissected to yield prefrontal cortex and hippocampus.
 - b. Corticosterone-treated animals. Corticosterone in drinking water (20mg%) for seven days. On eighth day, animals euthanized by isoflurane and brain dissected to yield prefrontal cortex and hippocampus.
 - c. Treatment with diisopropylfluorophosphate (DFP), an irreversible cholinesterase inhibitor – mimics exposure to nerve agents such as sarin. The animals are injected i.p. with DFP (4mg/kg) and six hours later euthanized with isoflurane, brain dissected to yield prefrontal cortex and hippocampus.
 - d. Combined corticosterone and DFP. Animals are exposed to corticosterone as in 2 above followed by DFP injection on day 8, euthanized by isoflurane and brain dissected to yield prefrontal cortex and hippocampus.

All brain tissues were frozen at -80°C and sent to Dr. O’Callaghan’s laboratory for analysis of proinflammatory gene expression (*IL-1 β* , *IL-6* and *TNF- α*) by rtPCR.

2. To perform *in silico* analysis of the rtPCR data to nominate candidate genes underlying strain differences in changes in gene expression.
3. To reanalyze prefrontal cortex mRNA for genome-wide gene expression using RNA-seq. Rationale, to query the entire genome for gene variants that underlie

individual differences in susceptibility to corticosterone-DFP effects as this may inform the human genome on individual differences in susceptibility to Gulf War illness.

3. Accomplishments

As of July 25, 2018, we have tested nearly 900 mice from 30 strains and reduced the gene expression from 28 – about 300 more need to be tested to fill the cells. We have tested enough mice to get a pretty good idea about strain-related differences in the combined effects of corticosterone and DFP vs. DFP alone. The graphs below illustrate the results so far.

Gene expression results

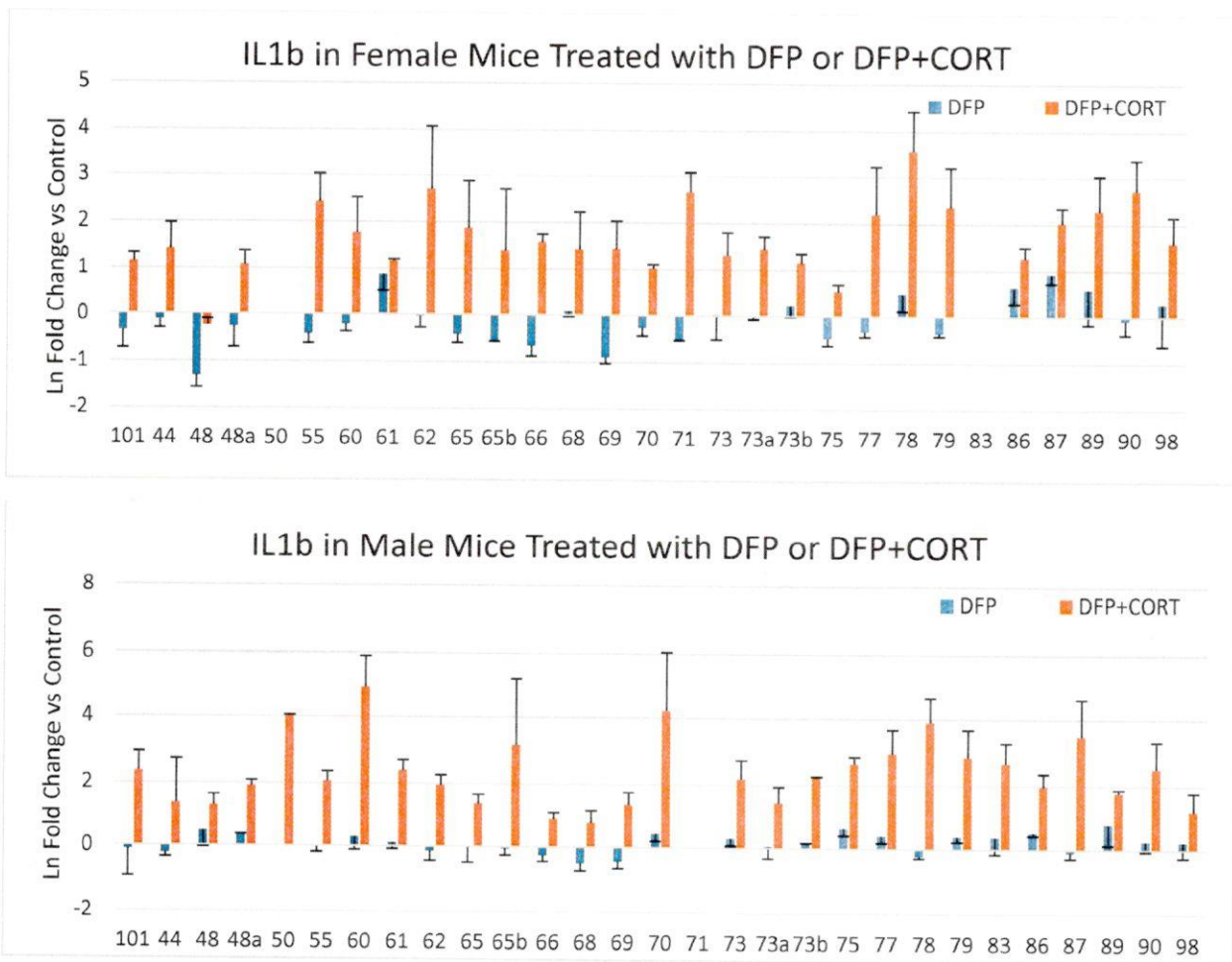


Figure1. The above bar graph shows the effect of DFP (blue bars) and DFP + corticosterone (orange bars) on the expression of IL-1 β in prefrontal cortex in 29 BXD mouse strains and in both sexes. The ordinate illustrates the extent (log2) of gene

expression compared to control and relative to GAPDH. The abscissa indicates the strain designations.

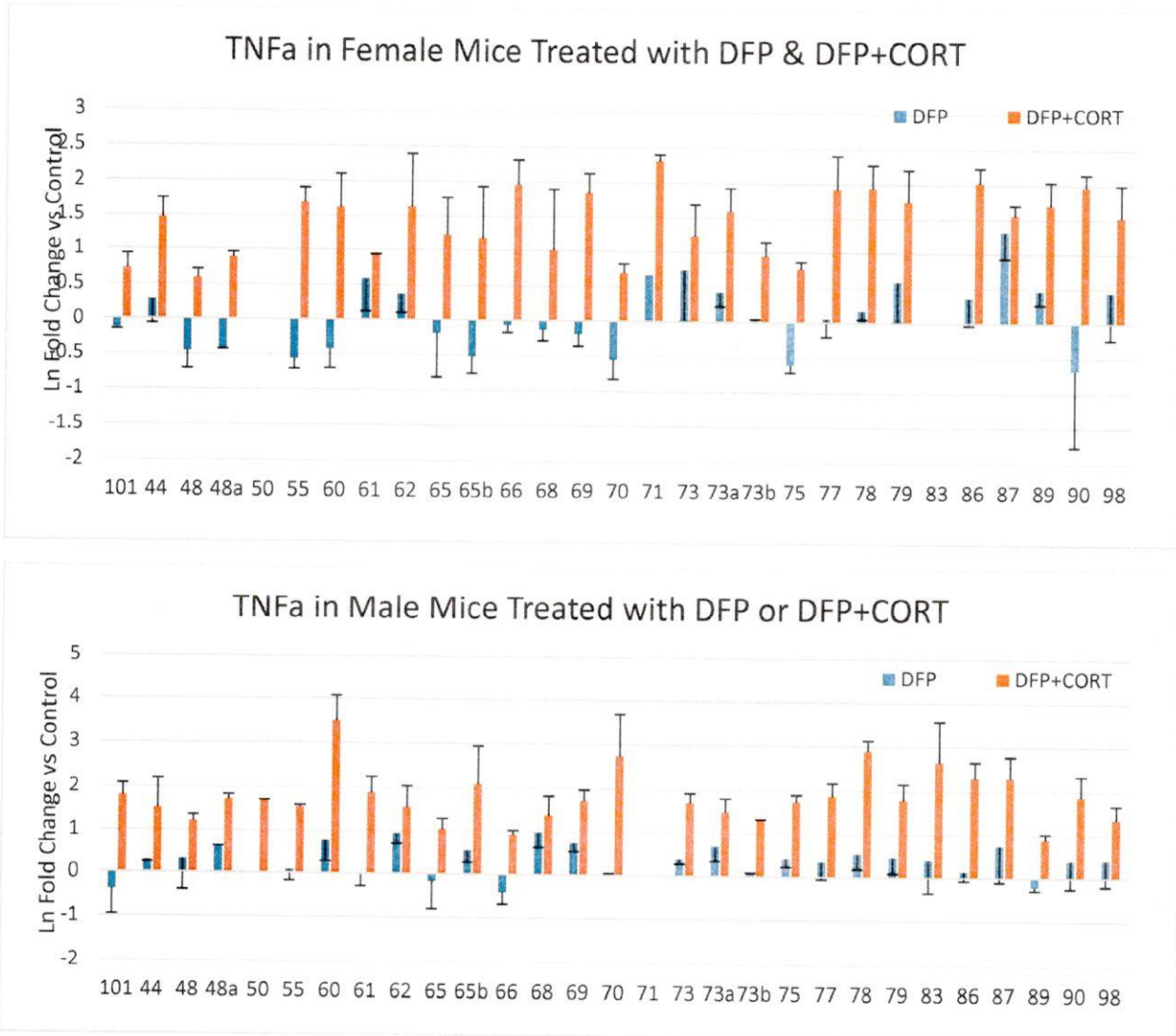


Figure 2. The above bar graph shows the effect of DFP (blue bars) and DFP + corticosterone (orange bars) on the expression of TNF- α in prefrontal cortex in 29 BXD mouse strains and in both sexes. The ordinate illustrates the extent (log2) of gene expression compared to control and relative to GAPDH. The abscissa indicates the strain designations.

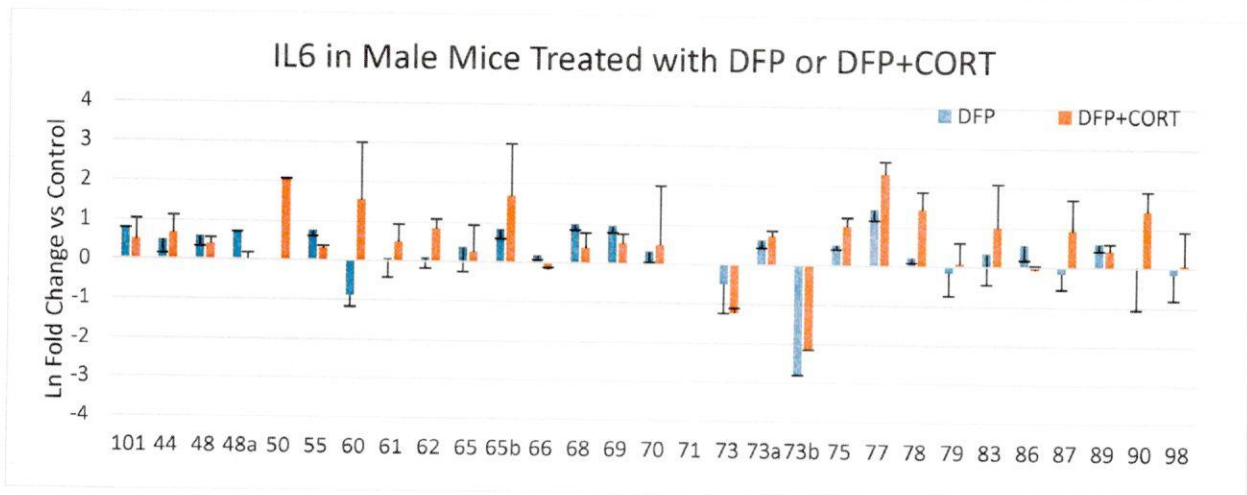
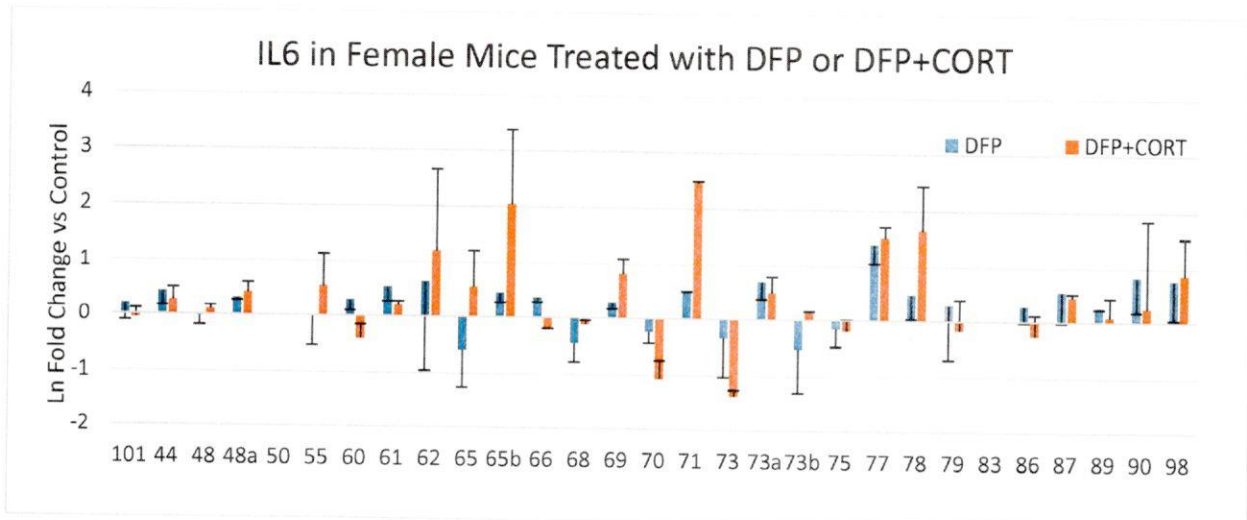


Figure 3. The above bar graph shows the effect of DFP (blue bars) and DFP + corticosterone (orange bars) on the expression of IL-6 in prefrontal cortex in 29 BXD mouse strains and in both sexes. The ordinate illustrates the extent (log2) of gene expression compared to control and relative to GAPDH. The abscissa indicates the strain designations.

Mapping the gene expression data for IL-1 β .

We have performed preliminary genome-wide mapping for IL-1 β – the other cytokines to follow. We found that the expression of IL-1 β in the DFP+corticosterone condition to map in both sexes to an area on chromosome 7 between 110 and 115 Mb. We also found that IL-1 β expression in this condition to correlate $r=0.80$ between the sexes, so we performed a principal components analysis for males separately, females separately and sexes combined. This usually increases the signal for quantitative trait locus (QTL) mapping and that is what we observed. The QTLs for the sexes separately went from suggestive to significant (Figure 4).

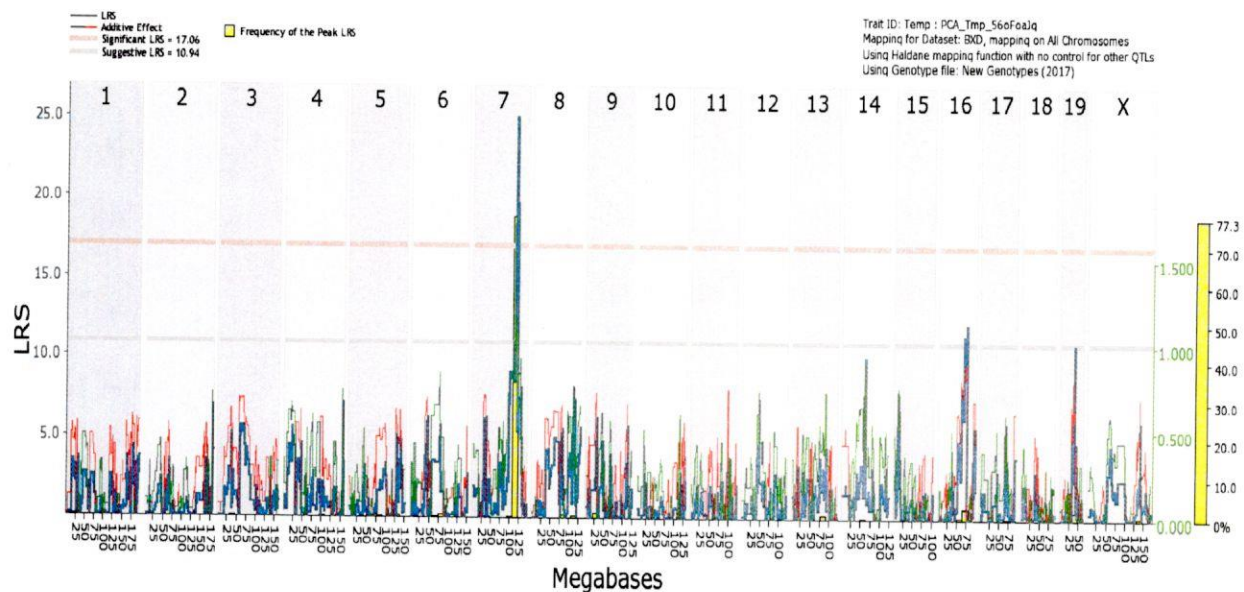


Figure 4. QTL mapping of IL-1 β expression in the DFP+corticosterone group, sexes combined.

We then searched for a plausible candidate gene in the area between 110-115 Mb on chromosome 7. The criteria for such a gene are 1) that the gene is *cis*-or self-regulated and 2) the expression of the gene is significantly correlated with the phenotype – in our case, the principal component. The search produced one candidate, Spondin 1 (*Spon1*). Figure 5 shows that this gene is *cis*-regulated (variability in its expression among the strains maps to the same location as its coding region – blue triangle). Figure 6 shows the correlation (scatter diagram) between the principal component and expression of *Spon1*. The Pearson product-moment correlation is $r=-0.755$.

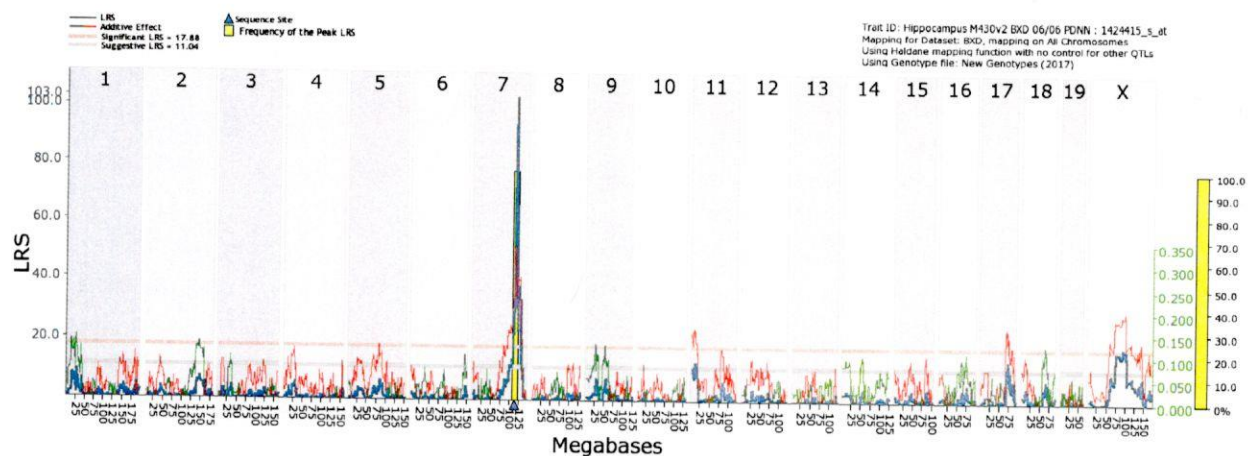


Figure 5. Genome-wide QTL mapping of the expression of *Spon1*.

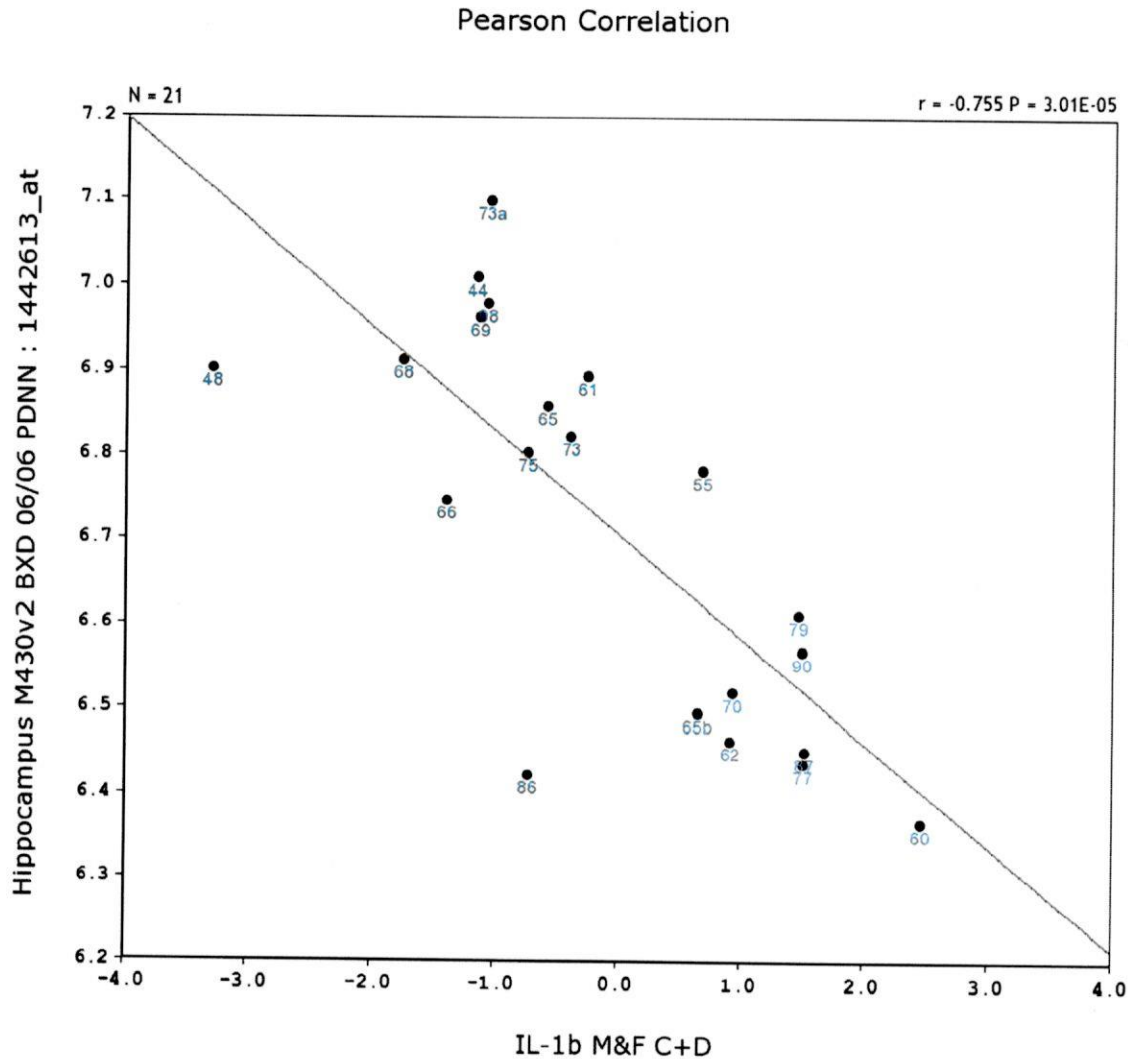


Figure 6. Scatter diagram showing the association between the principal component, sexes combined for exposure to DFP+corticosterone (X-axis) and expression of Spondin 1 (Y-axis).

In hippocampus, there is evidence for differential expression of both long and short isoforms of the 3' UTR of *Spon1*. The longer 3' UTR has higher expression from the B haplotype (all 3000+ nt of 3' UTR), whereas the short 3' UTR (about 1500 nt) has higher expression from the D haplotype. There is a third isoform that does not express the last 10 exons, and this version has higher expression in the B haplotype.

The longer 3' UTR may be responsible for the higher sensitivity to DFP+CORT in the strains carrying the B allele (Figure 7).

treatment. Because the mouse and human genomes are more than 90% syntenic, our mouse findings should inform the human situation.

4. IMPACT

Impact on the development of the principal discipline.

The results showed us the next step of developing this project. Our next step will be to study the genetics of the epigenetics of the exposures. This will help to explain why the illness persists for so long and the basis for individual differences in persistence.

Impact on other disciplines.

Nothing to report

Impact on technology transfer

Nothing to report

Impact on society beyond science

Nothing to report yet – waiting for final results and gene expression results

5. CHANGES/PROBLEMS

Nothing to report

6. PRODUCTS

One presentation to the Complex Traits Consortium meeting in Glasgow Scotland – the PPT presentation is appended

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name/organization	James O'Callaghan/CDC-NIOSH
Role	Co-Investigator
Identifier	Jdo5@cdc.gov
Nearest person month worked	4
Contribution	Coordinated rtPCR analysis of brain tissue
Funding support	CDC-NIOSH

Name/Organization	Lu Lu/UTHSC
Role	Co-Investigator
Identifier	Llu@uthsc.edu
Nearest person month worked	2
Contributions	Preparation of brain tissues for gene expression by RNAseq
Funding support	GW160086

Strain Differences in Proinflammatory Cytokine Gene Expression in Prefrontal Cortex Following High Glucocorticoid- Organophosphate Exposure in BXD Mice

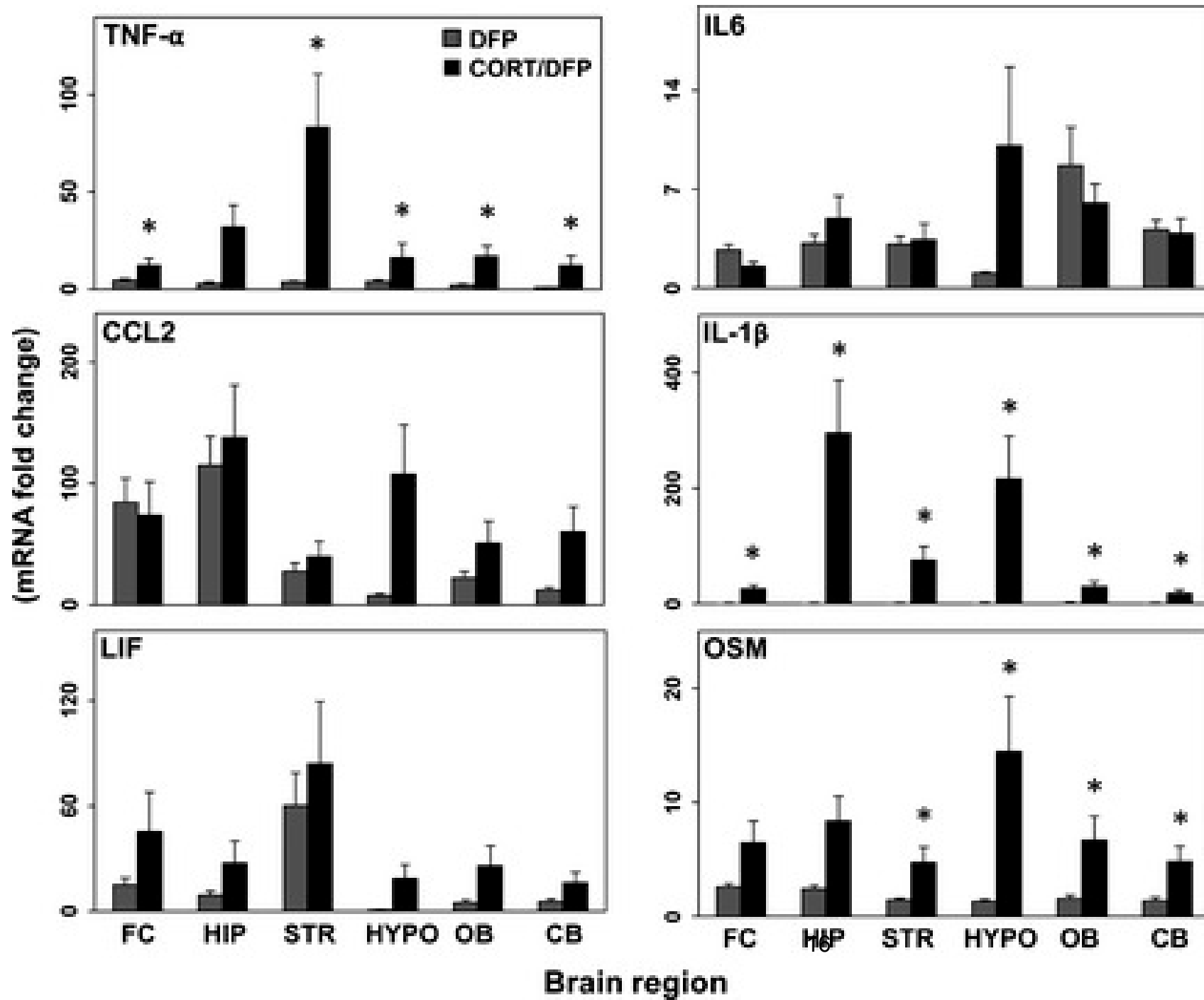
Byron C. Jones
University of Tennessee Health Science Center
Memphis, Tennessee, USA

Exposure to Organophosphate Compounds and High Circulating Glucocorticoids as Major Etiological Factors in Gulf War Illness

- ▶ In 1990-1991 the USA and allies invaded Iraq in response to Iraq's invasion of Kuwait.
- ▶ 700,000 US troops and another 200,000 from allies were sent to the Persian Gulf
- ▶ Upon return to their home countries, approximately 30% of the veterans complained of chronic, debilitating malaise, including fatigue, headaches, joint pain, indigestion, insomnia, dizziness, respiratory disorders, and memory problems.
- ▶ The nature of the disease was thought to be neuroinflammation based on the exposure to various chemicals experienced by the troops. These included depleted uranium, smoke from burning oil wells and ammunition dumps and organophosphorus compounds - triple exposure.
 - ▶ A. Insecticides in barracks - malathion
 - ▶ B pyridostigmine as prophylaxis against nerve agents (e.g. sarin)
 - ▶ C exposure to small amounts of sarin itself

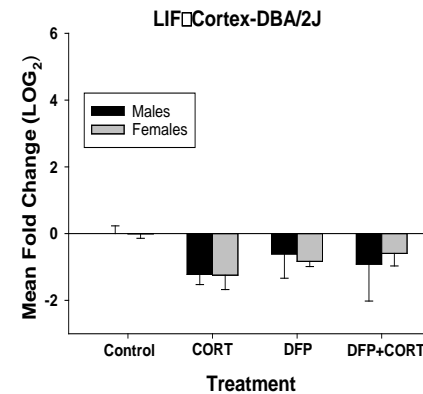
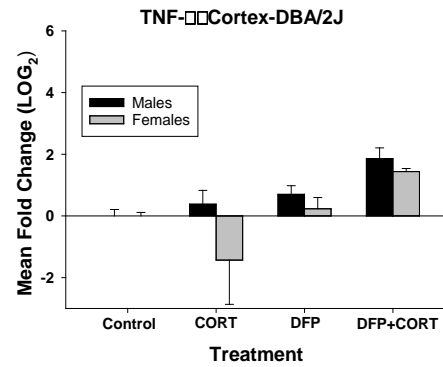
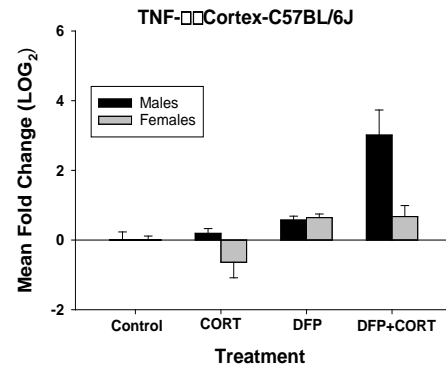
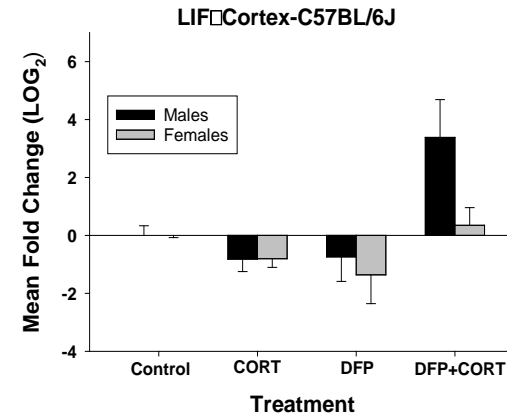
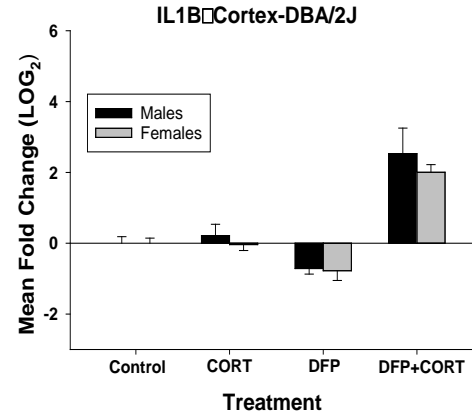
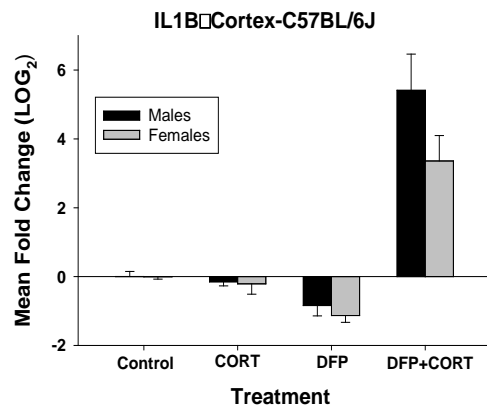
What Else?

- ▶ Diane Miller and James O'Callaghan nominated the OPs as major toxicants, but exposure by itself was insufficient to cause the malaise.
- ▶ They reasoned that being in a combat theatre would raise circulating cortisol, a possible contributing factor.
- ▶ Accordingly, they developed a mouse model in the C57BL/6J strain
 - ▶ Corticosterone in the drinking water (20 mg%) for 7 days
 - ▶ Treatment with diisopropylfluorophosphate (DFP, a sarin surrogate), an irreversible cholinesterase inhibitor, 4 mg/kg, i.p. followed by sacrifice 6h afterwards
- ▶ As indices of neuroinflammation they measured changes in expression of proinflammatory cytokine genes, IL-1 β , TNF- α , IL-6, CCL2, LIF, OSM
- ▶ They reported that CORT greatly exacerbates the expression of the proinflammatory cytokine genes, thus supporting the hypothesis that it takes high circulating glucocorticoids plus exposure to OPs to produce GWI.



So What?

- ▶ Of the troops sent to the Persian Gulf, approximately 30% came home sick.
- ▶ What about those who did not, everything else being equal?
- ▶ Could the difference in susceptibility be related to genetic constitution?
- ▶ As the C57BL/6J (B6) strain is one of the founders of the BXD family of recombinant inbred strains, we tested the other founder strain, DBA/2J (D2) and both sexes.
- ▶ The results showed the D2 strain to be less sensitive than the B6 and females of both strains to be less sensitive than the males.

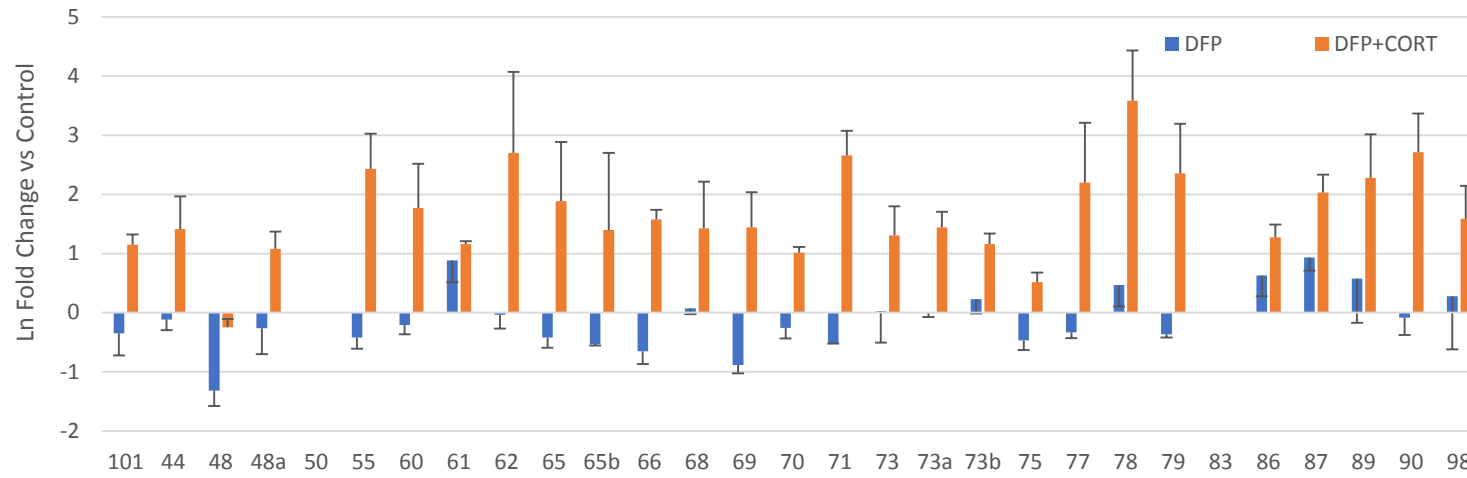


Preliminary study using both founder strains for the BXD recombinant panel. These data show that the DBA/2J strain is less sensitive to the treatment regimen than the C57BL/6J strain (the strain used by O'Callaghan). The data also show that overall, females of both strains are less sensitive to the treatment than males. These are the data that supported our application for New Investigator funding.

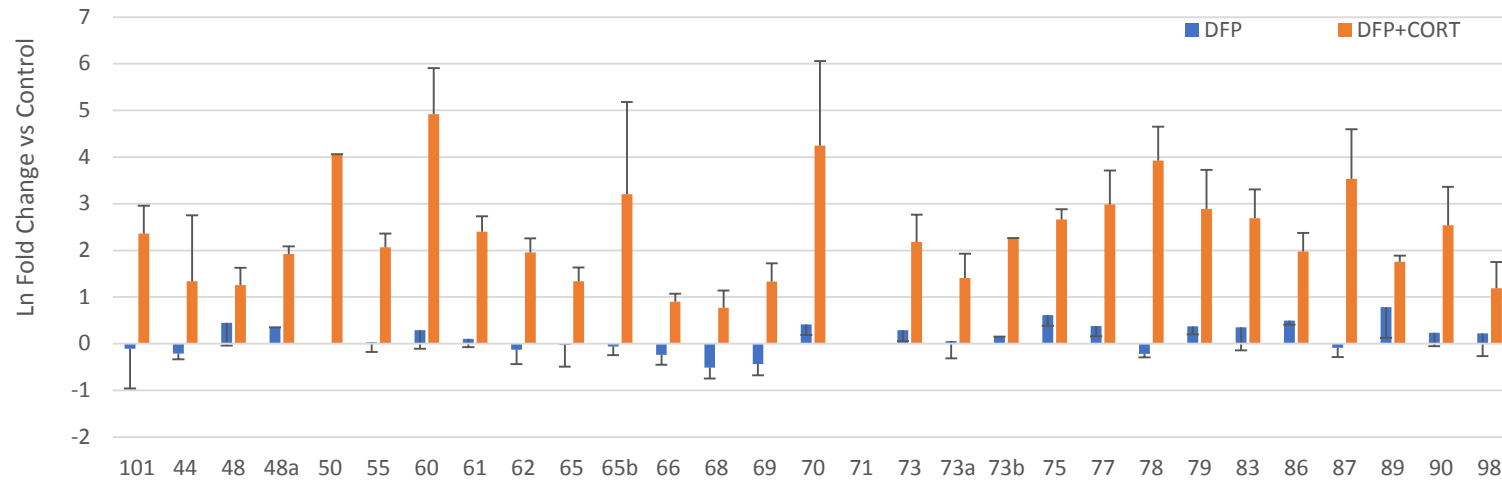
The Next Step: Test BXD Strains

- ▶ Now that we have shown strain and sex differences in susceptibility to the exposures, the next step is to test a number of BXD RI strains.
- ▶ We applied for and received funding from the Department of Defense to study the effects of DFP and DFP+CORT in 30 BXD strains. This fits the budget and the minimum necessary for genetic mapping to nominate candidate genes.
- ▶ To date, we have tested male and female mice from 28 BXD strains and are nearly finished with the experimental work.

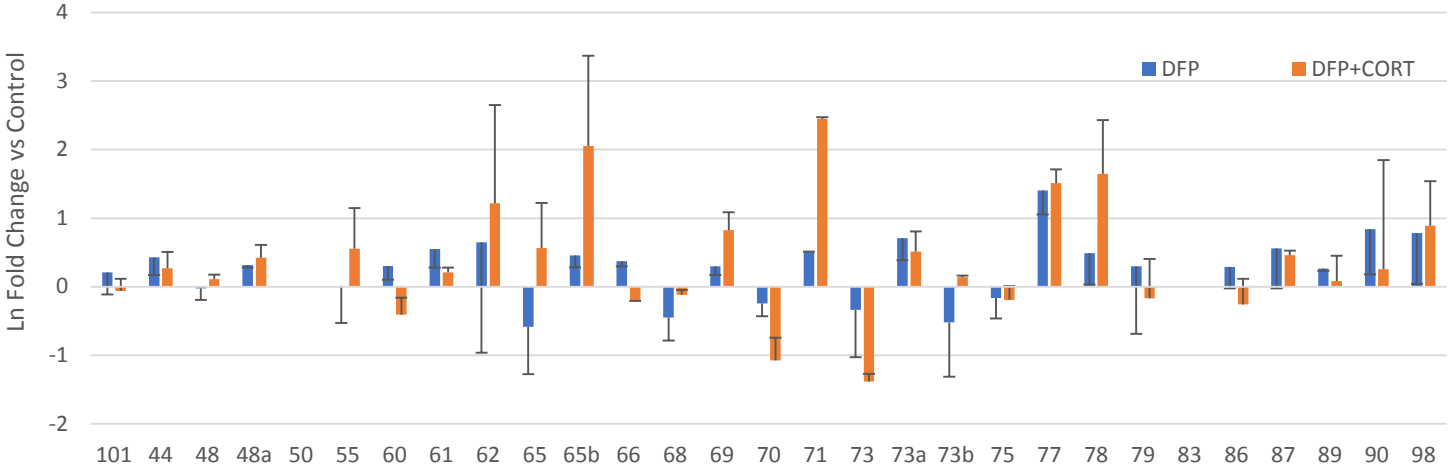
IL1b in Female Mice Treated with DFP or DFP+CORT



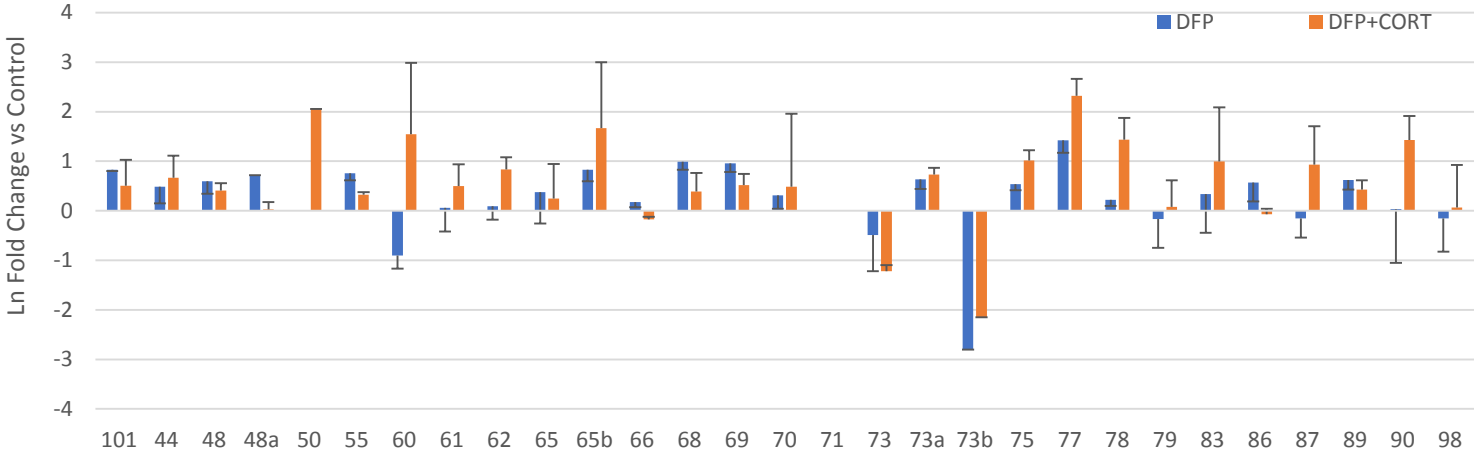
IL1b in Male Mice Treated with DFP or DFP+CORT



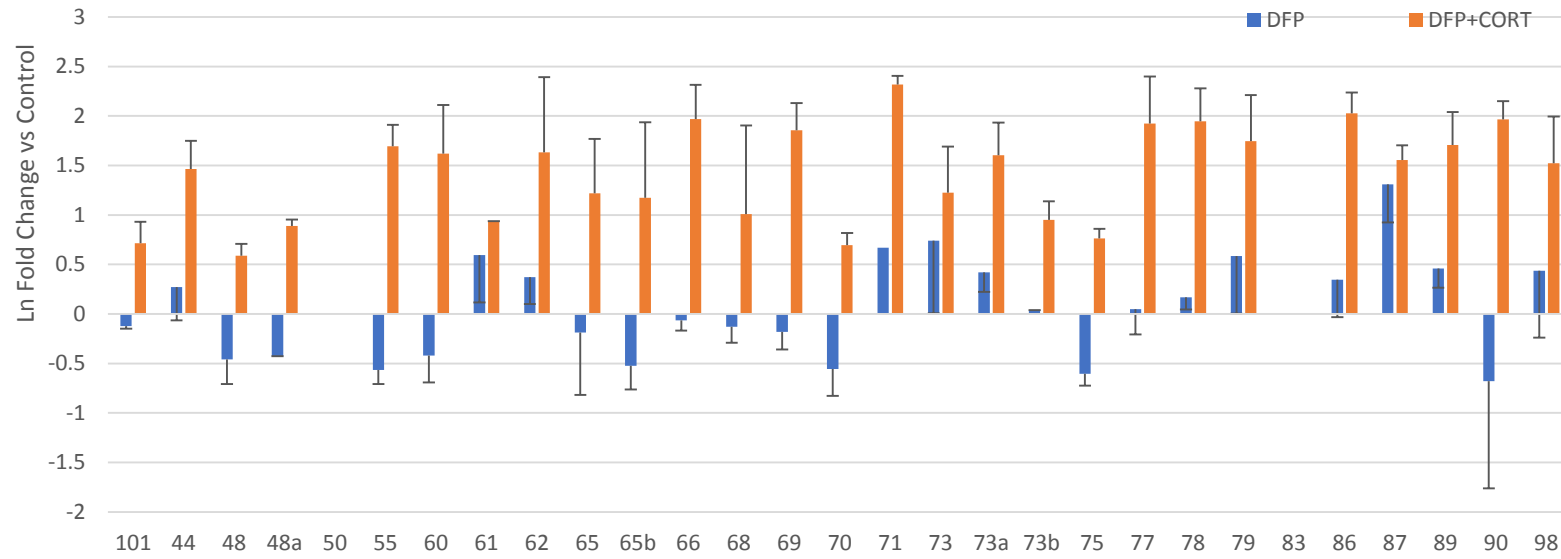
IL6 in Female Mice Treated with DFP or DFP+CORT



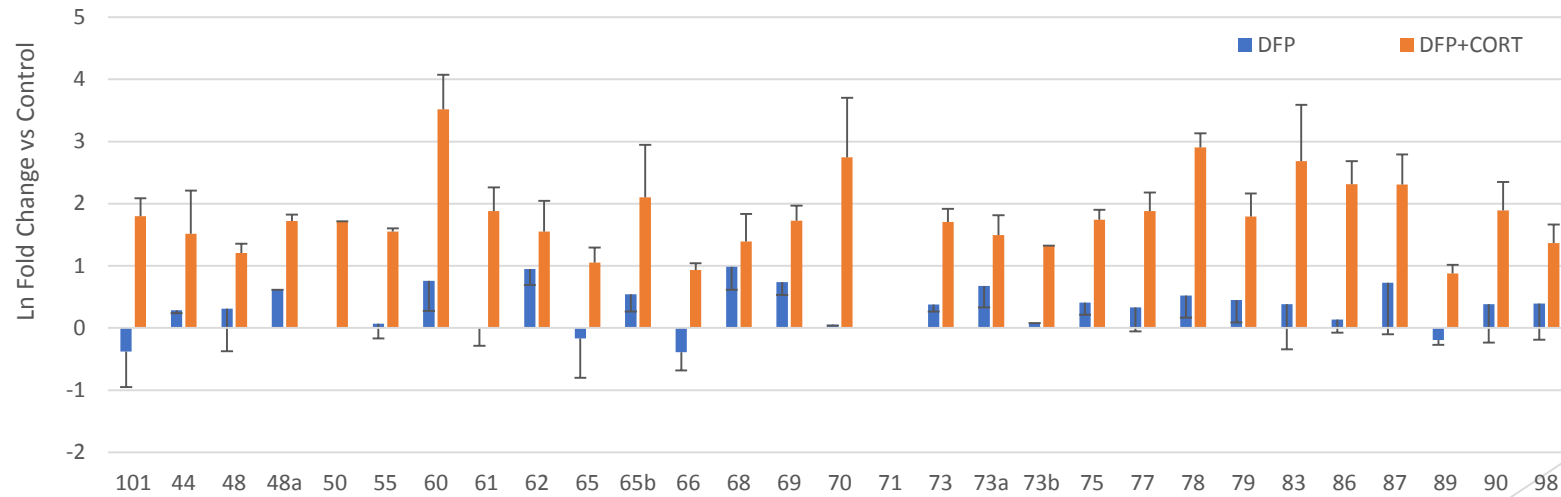
IL6 in Male Mice Treated with DFP or DFP+CORT



TNF α in Female Mice Treated with DFP & DFP+CORT



TNF α in Male Mice Treated with DFP or DFP+CORT



Summary of initial findings

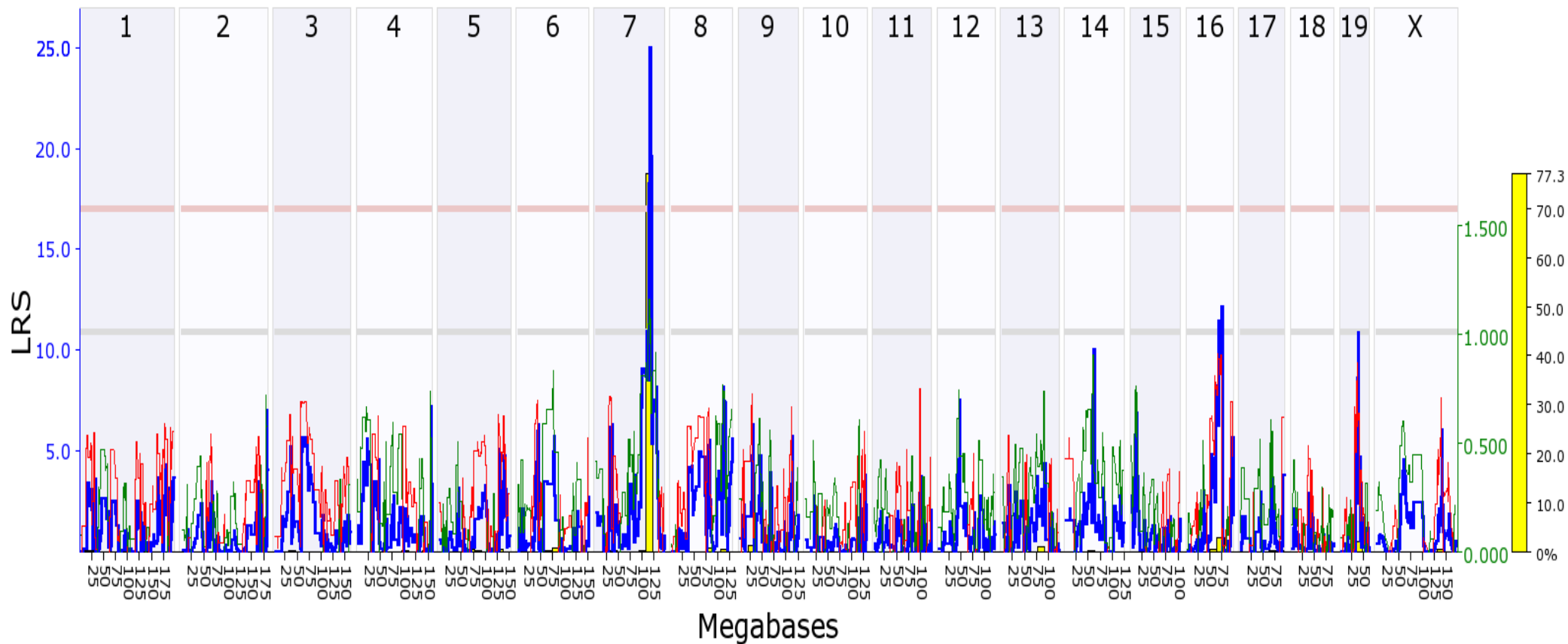
- ▶ We have demonstrated wide-ranging genetic variability in the effects of DFP + CORT on the expression of the proinflammatory cytokine genes IL-1 β , IL-6 and TNF- α . IL-1 β and TNF- α appear to give more consistent results than IL-6.

Mapping II-1 β

- ▶ II-1 β showed suggestive quantitative trait loci (QTLs) for both sexes on chromosome 7 at 114 Mb.
- ▶ The principal component derived from each sex separately and both sexes (mean) together produced a significant QTL at the same location

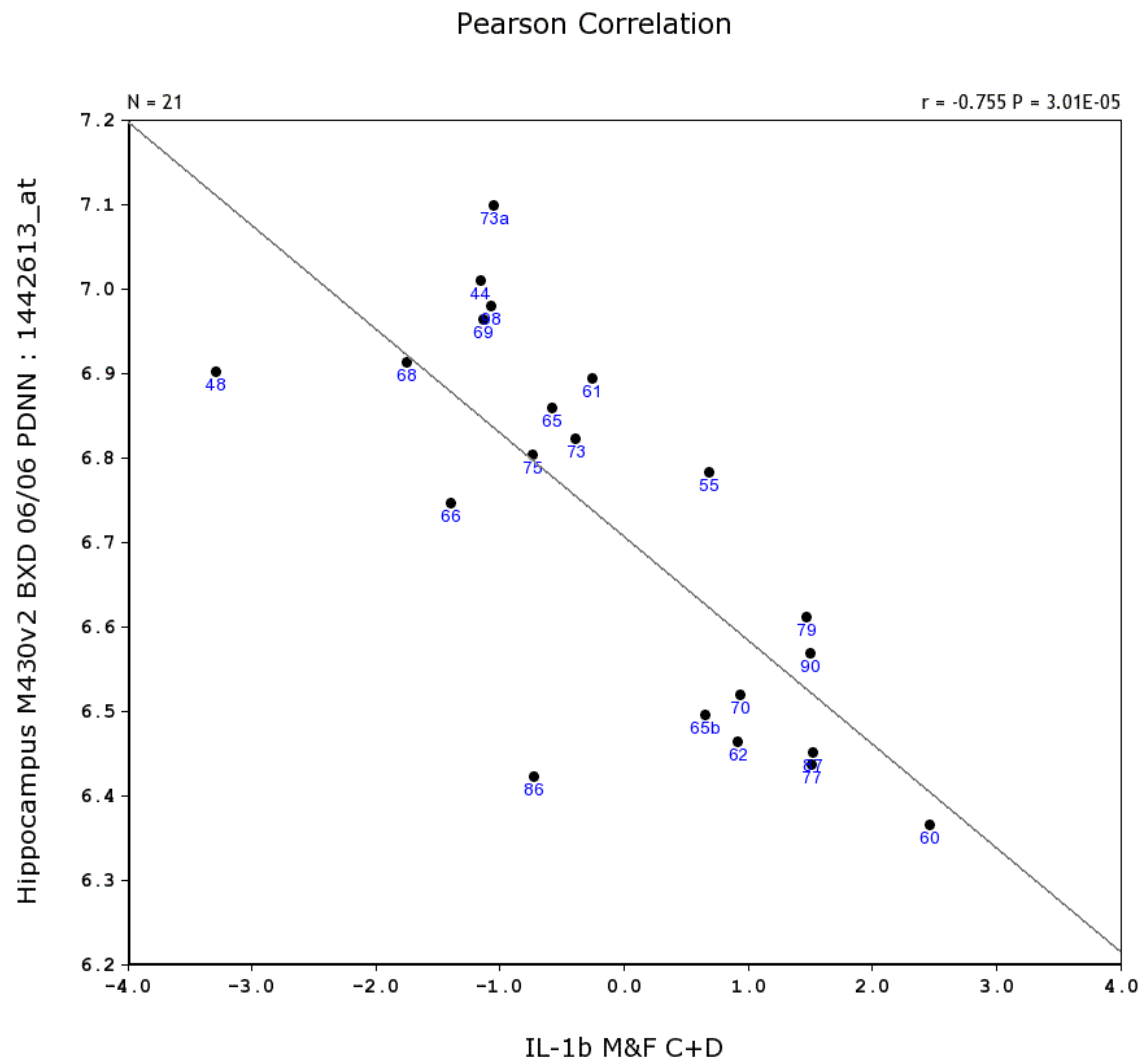
— LRS
 — Additive Effect
 — Significant LRS = 17.06
 — Suggestive LRS = 10.94
 ■ Frequency of the Peak LRS

Trait ID: Temp : PCA_Tmp_56oFoaJq
 Mapping for Dataset: BXD, mapping on All Chromosomes
 Using Haldane mapping function with no control for other QTLs
 Using Genotype file: New Genotypes (2017)



Locating Possible Candidate Gene(s)

- ▶ We searched the area between 110 and 115 Mb on chromosome 7 in hippocampus (prefrontal cortex data are from the early BXD panel; we are using the advanced intercross group).
- ▶ Criteria for possible candidate gene: 1) expression is correlated with our phenotype (principal component); 2) gene is *cis*-regulated.
- ▶ Spondin 1 is at 114Mb, is *cis*-regulated and its expression is correlated with the PC.
- ▶ Spondin 1 protein functions:
 - ▶ Cell adhesion
 - ▶ Axon guidance
 - ▶ Metal binding (Ca)
 - ▶ Stimulated by LPS and participates in inflammation



Scatter plot showing association between the principal component and spondin 1 expression in hippocampus.²⁷

What we have shown

- ▶ Priming by CORT of DFP effect on expression of pro-inflammatory cytokine genes shows wide, genetic variation.
- ▶ In our hands, IL-1 β and TNF- α are better indices of the effects of the co-exposure than IL-5.
- ▶ Mapping of the IL-1 β response shows a significant QTL and likely candidate gene.

Where do we go from here?

- ▶ Because GWI has persisted for nearly 30 years in some individuals, what is the genetic basis for this persistence? Genetic differences in epigenetic modification of gene expression?
- ▶ We have a proposal in preparation for submission to the DoD. For this work, we will examine histone modification (H3K27ac) and DNA methylation changes following CORT+DFP treated male and female mice from the same 30 strains.
- ▶ The anticipated outcome will be identification of genes whose modifications produce long-lasting or permanent changes in expression and hence, genes that underlie the persistent symptoms of GWI

Dramatis Personae

- ▶ James O'Callaghan, CDC, NIOSH
- ▶ Diane Miller, CDC, NIOSH
- ▶ Lu Lu, UTHSC
- ▶ David Ashbrook, UTHSC

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 - ▶ Daming Zhuang, UTHSC
 - ▶ Melinda McCarty, UTHS
- ▶ Funding
 - ▶ Congressionally Directed Medical Research Program, Department of Defense Grant # GWI 160086