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TITLE:

Gulf War Illness and Gut Microbiome Dysbiosis: Treatment with Probiotics and Microbiota Transfer Therapy

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CONTRACTING ORGANIZATION: Wayne State University School of Medicine

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13. SUPPLEMENTARY NOTES

None

14. ABSTRACT

The purpose of this project is to test the hypothesis that Gulf War Illness (GWI) is mediated by a pathological alteration in the gut microbiome (i.e., dysbiosis). It is known that the three primary symptom clusters of GWI (gastrointestinal alterations, central nervous system dysfunction, and chronic fatigue) can each be caused individually by an altered gut microbiome. Therefore, there is a strong likelihood that broad dysbiosis can contribute to the main symptoms of GWI in a concerted fashion. The primary scope of the project is to use a validated animal model of GWI and determine if this treatment results in a significant gut dysbiosis. Once this step is completed, the project will use probiotics and fecal microbiota transfer to re-balance the GWI-altered gut microbiome and, in turn, provide symptom relief. We treated mice with toxicants to which military personnel deployed to the Gulf were exposed (e.g., pyridostigmine bromide [PB] plus permethrin [PER]). Thereafter, mice were maintained on a normal diet or one containing high fat. The high fat diet was included because a significant number of Veterans with GWI are overweight/obese. We found that the taxonomic structure of the gut microbiome was significantly altered by the GWI agents. The high fat diet led to significant weight increases and this interacted with PB + PER to worsen the dysbiosis. When mice on the high fat diet were returned to a normal diet, they lost weight and the alterations in the gut microbiome were corrected. We also found that mice treated with PB + PER developed anxiety- and depression-like behaviors. These results confirm that GWI can be associated with a significant alteration in the gut microbiome, reflected as an increase in heterogeneity and altered community composition. These results are significant because they suggest that a life-style risk factor such as a poor diet (i.e., high in fat) can worsen and potentially extend the duration of the disease. This finding offers answers to the question of why GWI has persisted in deployed Veterans for so long after the toxicants to which they were exposed are no longer detectable in their bodies. Poor dietary habits and other life-style risk factors can mimic the effects of GWI on the gut microbiome, suggesting that the symptoms are perpetuated by these unhealthy factors.

15. SUBJECT TERMS

Gulf War Illness, gut microbiome, dysbiosis, probiotics, microbiota transfer therapy, mood alterations, depression- and anxiety-like behavior, 16S rRNA gene sequencing

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TABLE OF CONTENTS

1. INTRODUCTION: The subject of this project is a study of how Gulf War Illness (GWI) is modulated by alterations in the gut microbiome. In addition, the GWI-modified gut microbiome will the targeted for therapy using probiotics and gut microbiota transfer, in order to re-balance the GWI-disrupted microbiome. The purpose of the project is to use a validated animal model of GWI and then carry out 16S rRNA gene sequencing to determine if treatment results in alterations in the composition and structure of the gut microbiome. This project will also assess the effects of the GWI model on the development of anxiety- and depression-like behaviors in treated mice. These latter symptoms mirror the central nervous system alterations seen in Veterans with GWI. The scope of this project includes a broad assessment of how GWI can alter the gut microbiome, which then pivots to include attempts to correct the GWI-induced dysbiosis and provide symptom relief.

2. KEYWORDS: Gulf War Illness, gut microbiome, dysbiosis, probiotics, microbiota transfer therapy, mood alterations, depression- and anxiety-like behavior, 16S rRNA gene sequencing, permethrin, pyridostigmine bromide.

3. ACCOMPLISHMENTS:

○ **What were the major goals of the project?**

● Major Task 1 (Specific Aim 1): Treat mice with GWI modeling compounds (i.e., pyridostigmine bromide [PB] and permethrin [PER]).
● Major Task 2 (Specific Aim 1): Characterize gut microbiome in controls and treated mice using 16S rRNA next generation sequencing and liquid chromatography/mass spectrometry.
● Major Task 3 (Specific Aim 2): Rebalance dysbiosis using probiotics.
● Major Task 4 (Specific Aim 2): Rebalance dysbiosis using microbiota transfer (fecal transplantation).

○ **What was accomplished under these goals?**

1. Major activities:

● Treat mice in the following groups: 1) vehicle control and 2) PB + PER per Subtask 1 of Major Task 1;
● Test both treatment groups for anxiety-like and depression-like outcomes using the elevated plus maze and sucrose preference test, respectively per Subtask 2 of Major Task 1; Subtask 1
● Isolate DNA from caecum contents of control and GWI-treated mice using Qiagen QIAmp Power Fecal DNA kit per Subtask 1 of Major Task 2
● Run PCR using sequence specific bacterial primers, prepare sequencing library and generate clonal clusters through bridge amplification per Subtask 2 of Major Task 2
● Sequence DNA using our MiSeq System and carry out data and statistical analyses using software (Mothur, R) to generate cladograms, heat maps, and alpha- and beta-diversity comparisons among microbial communities of the treatment groups per Subtask 3 of Major Task 2.

2. Specific objectives:
● Determine if GWI toxicants PB and PER significant alter the structure and composition of the gut microbiome
● Determine if the GWI toxicants PB and PER result in the appearance of anxiety- and depression-like behaviors in treated mice

● Determine if a high fat diet (HF), used to simulate the fact that the majority of Veterans who deployed to the Gulf are now overweight/obese.

3. Significant results: Fig. 1 shows the body weights of mice treated with GWI or control (Con) ± HF. Both treatment groups fed the HF gained on average 10g over the 6 week test period, whereas both groups fed the normal diet (ND) gained ~3g. GWI treatment did not alter body weight in mice fed either ND or HF compared to controls. When mice initially fed a HF diet were switched to the ND for 3 weeks, both groups lost significant amounts of weight (~6-7 g). However, the Con-HF-ND group achieved a significant reduction in body weight sooner after the diet switch (post hoc Tukey's test;

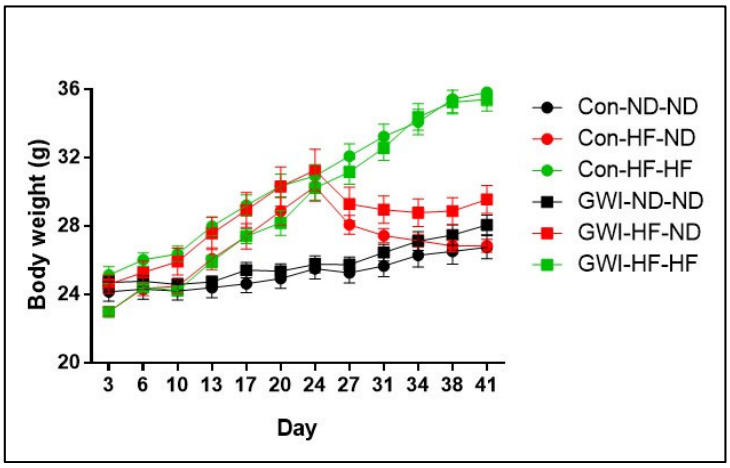

Fig. 1. Effect of diet on body weights. Mice were treated with GWI (PER + PB) or Con (control) and then fed a normal (ND) or high fat (HF) diet for 3 weeks. Thereafter, half of the mice on the HF diet (Con and GWI) were switched to ND (HF-ND) for an additional 3 weeks. Remaining GWI and Con mice were fed ND or HF diet throughout (ND-ND or HF-HF). Results are mean body weight ± SEM, N= 7-9.

$p < 0.001$ at day 27) than the GWI-HF-ND group (post hoc Tukey's test; $p < 0.001$ at day 31), and the GWI treated mice ultimately lost less weight than controls (post hoc Tukey's test; $p < 0.05$ at day 41). The main effects of time ($F_{11,528} = 64.8$, $p < 0.001$) and treatment ($F_{5,528} = 115.9$, $p < 0.001$) as well as their interaction ($F_{55,528} = 7.3$, $p < 0.001$) were significant (2-way ANOVA). These data establish that the HF led to significant gains in body weight that were of the same magnitude in controls and GWI treated mice. Both groups lost significant weight when switched back to ND, although weight loss was more pronounced among controls. Food intake paralleled body weight gain and did not differ between the control and GWI groups for either diet (not shown). GWI treatment therefore did not alter food intake or body weight gain for either diet.

Fig. 2 presents an analysis of α-diversity using the Chao-1 index as a measure of gut microbiome richness. The main effect of treatment ($F_{5,44} = 26.1$, $p < 0.0001$) was highly significant. Post hoc comparisons indicated that GWI treatment significantly reduced microbiome richness compared to controls (Tukey's test, $p < 0.05$), and that HF led to significantly decreased richness in both control (Tukey's test, $p < 0.001$) and GWI groups (Tukey's test, $p < 0.05$). Notably, when mice were shifted from HF to ND, α-diversity recovered to the levels of the appropriate treatment control and differed significantly from the respective HF-HF group (Tukey's test, $p < 0.001$ for controls and $p < 0.01$ for GWI).

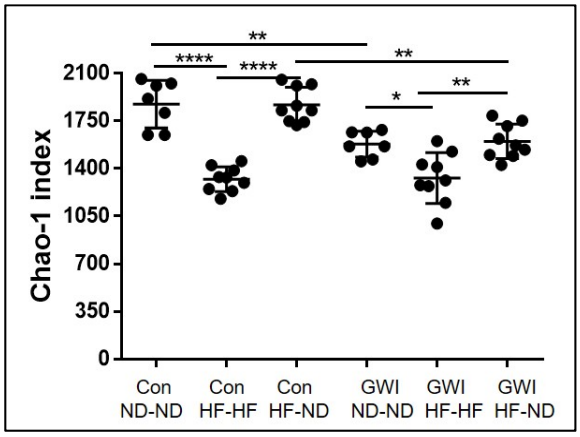

Fig. 2. Effects of GWI ± HF on α-diversity. Data are presented as Chao-1 ± SEM, N= 8-9. Con = control; GWI = PER + PB; ND = normal diet; HF = high fat diet. Symbols represent significance levels for the indicated post hoc comparisons as p < *0.05, **0.01, ****0.0001.

With respect to β-diversity, analyses based on the Jaccard index, which reflects shared microbiome membership (i.e. community composition) results showed that the OTU profiles of samples clustered together tightly according to the diet regimen, and that within diet regimen groups, samples also clustered by treatment (Fig. 3). Two-way NPMANOVA analyses revealed that the main effects of treatment ($p < 0.01$) and diet ($p < 0.0001$), as well as their interaction ($p < 0.02$), were significant. All post hoc comparisons among groups were statistically significant. It is interesting that mice in the control and GWI

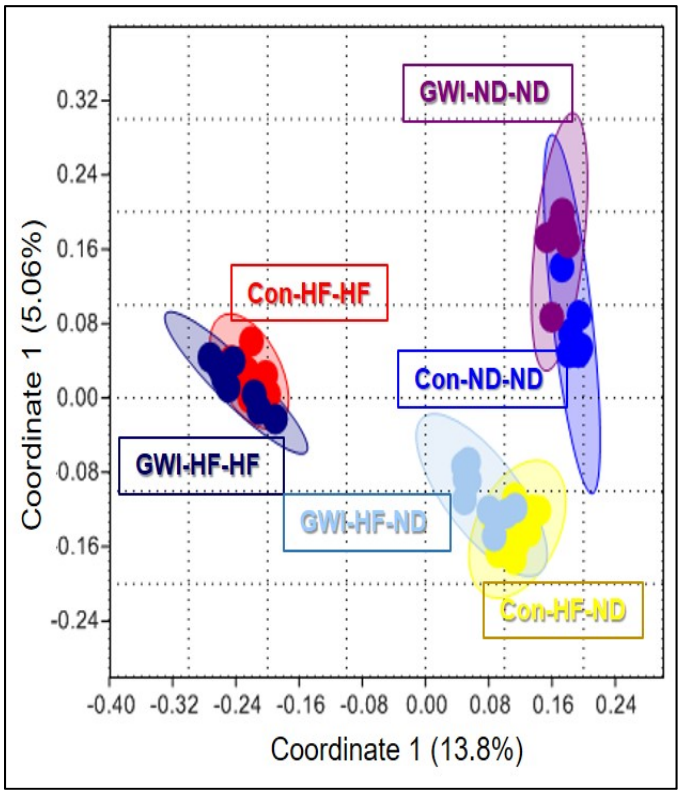

Fig. 3. Effects of treatments on β-diversity. PCoA showing differences in the similarities of the gut microbiome profiles of the study groups using the Jaccard index. Con = control; GWI = PER + PB; ND = normal diet; HF = high fat diet.

groups exposed to the HF-ND regimen clustered near the ND-ND groups on the PCoA plot, suggesting rapid recovery of the gut microbiome following a return to a ND, as was also seen above for α-diversity.

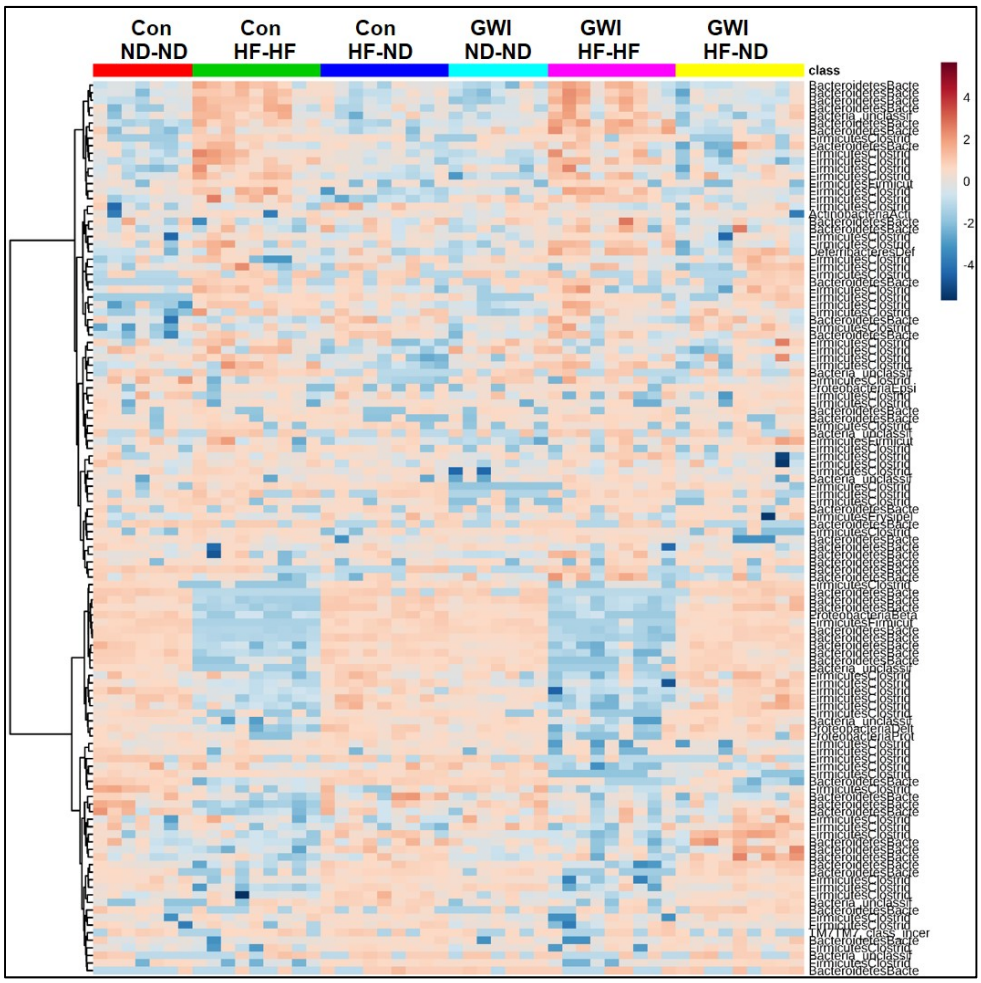

Fig. 4. Heat map illustrating patterns in OTU relative abundance among the treatment groups. All subjects in each group are arrayed in columns and bacterial taxonomies are indicated in rows. Con= control; GWI = PER + PB; ND = normal diet; HF= HF diet. Clustering along the y-axis was done using the Ward algorithm.

The taxonomic identities of prominent OTUs (≥ 1.5% average relative abundance among all subjects) varied among treatment groups. These results are presented in the heat map in Fig. 4. It can be seen that the GWI and control groups displayed similar patterns of OTU expression according to diet. The most prominent differences in these groups were decreases in Bacteroidetes (see the clusters near the bottom of Fig. 4) and increases in Firmicutes (clusters near the top) in the C-HF-HF and G-HF-HF groups. Furthermore, within each diet group, differences in OTU relative abundances were evident for GWI versus controls. As reported above for community α and β diversity, as mice in the GWI and control groups transitioned from HF to the ND, patterns of OTU relative abundance appeared to "recover" toward the pattern shown in the groups fed ND throughout this experiment (i.e., ND-ND groups).

Fig. 5 presents results from linear discriminant analysis effect size (LEfSe) analysis and highlights the effect sizes of the treatments and diets on affected taxa. LEfSe compares each group to all others simultaneously and generates bar plots that include taxa that are distinctly relatively abundant in each specific treatment and diet group. LEfSe is used as a means for biomarker discovery by finding OTUs that

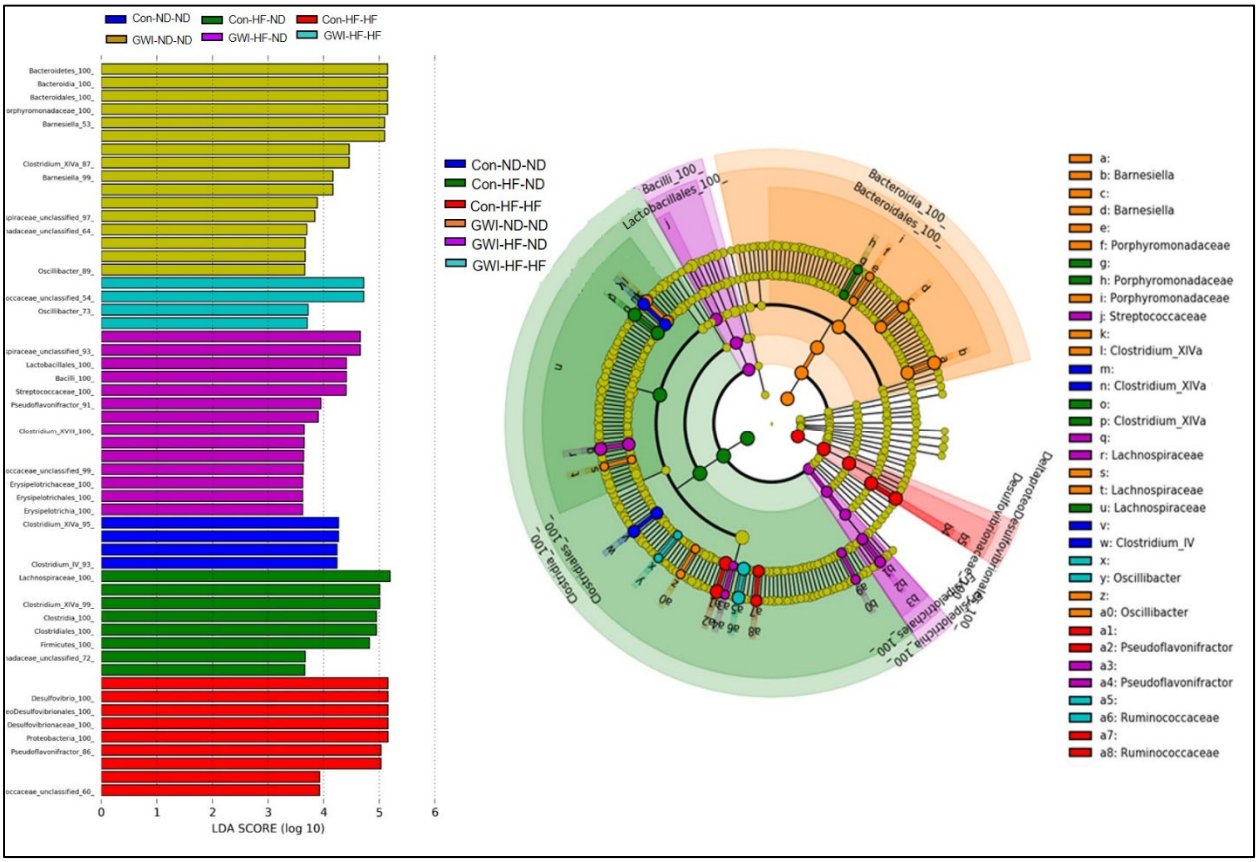

Fig. 5. Bacterial taxa that were differentially abundant across treatments. LEfSe was carried out using the Galaxy Project and the results are displayed in the bar charts (A) and the associated cladogram (B). Taxa showing different abundance values in each treatment group (according to LEfSe) are shown in the cladogram highlighted by small circles and by shading. All groups are statistically significant compared to each other (LDA > 3.6)**.** Con = control; GWI = PER + PB; ND = normal diet; HF = high fat diet.

consistently explain the differences between two or more types of microbial communities. Two main outcomes from this analysis are apparent. First, the GWI groups are demarcated by more taxonomic biomarkers than controls for each diet condition. Second, most treatment groups were distinguished by taxa in the order Clostridiales within the phylum Firmicutes (i.e., Con-ND-ND, Con-HF-ND and GWI-HF-ND). However, the GWI-ND-ND group was represented primarily by taxa in the order Bacteroidales within the phylum Bacteroidetes, the Con-HF-HF group was singularly characterized by taxa within the order Desulfovibrioales, and the GWI-HF-HF group was represented by taxa within the orders Lactobacillales and Erysipelotrichales. The HF diet shifted the predominant taxa for the GWI-ND-ND group from Bacteroidetes to Firmicutes. All of the control groups regardless of diet were distinguished by taxa within Firmicutes and the relatively most abundant taxa in the group fed a ND were in the Clostridium XIVa and IV clusters. Controls fed the HF diet were characterized by taxa within the genera Desulfovibrio and Pseudoflavonifractor and the control group shifted to a ND from the HF diet was distinguished by Porphyromonadaceae and Lachnospiraceae. Treatment- and diet-induced biomarkers were observed down to the level of family or genus as shown in the cladogram (Fig. 5).

Fig. 6 illustrates treatment effects at the phylotype level. Treatment and diet effects on specific bacterial phyla are presented as percent relative abundance. The main effect of phylum was significant ($F_{7,352} = 2616$, $p < 0.0001$) but the treatment main effect was not. The phylum X treatment interaction was also highly significant ($F_{35,352} = 50.6$, $p < 0.0001$) by two–way ANOVA. Post hoc comparisons revealed that virtually all treatment groups differed significantly from one another (p values ranging from 0.05 to

0.0001). The observed changes occurred only within the prominent phyla Firmicutes and Bacteroidetes (Fig. 6). The only groups that did not differ were Con-ND-ND vs GWI-ND-ND within Firmicutes and Con-ND-ND vs GWI-ND-ND within Bacteroidetes.

The effects of treatments and diets on taxa below the level of phylum were also probed in view of the

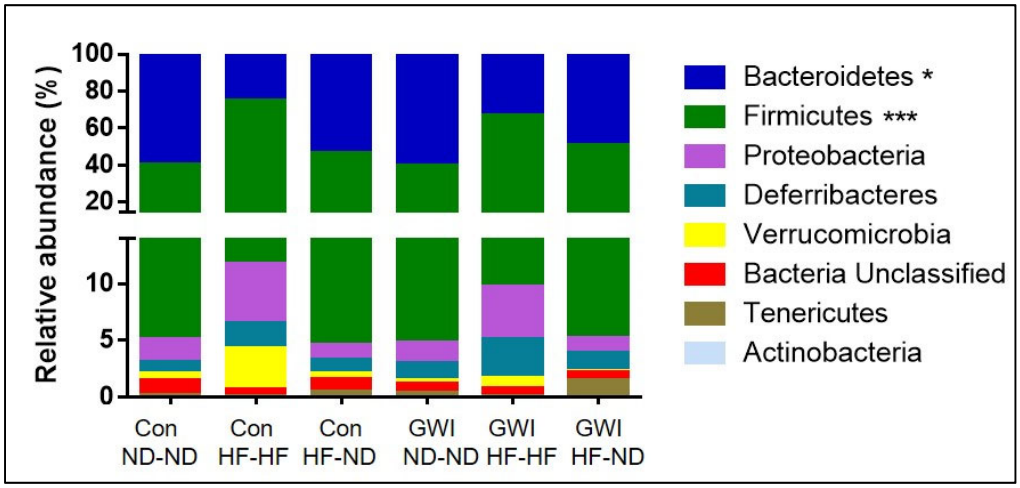

Fig. 6. Percent relative abundances of phyla in treatment and diet groups. Stacked columns for the 8 most prominent phyla are included. Con = control; GWI = PER + PB; ND = normal diet; HF = high fat diet.

likelihood that changes at the highest taxonomic level may have not reached statistical significance because of increases and decreases of equal magnitude within phyla in percent relative abundances of bacteria at lower taxonomic levels. Fig. 7 shows these results and indicates that effects at the taxonomic levels of class and order vary in a complex manner that is dependent on the combined influence of

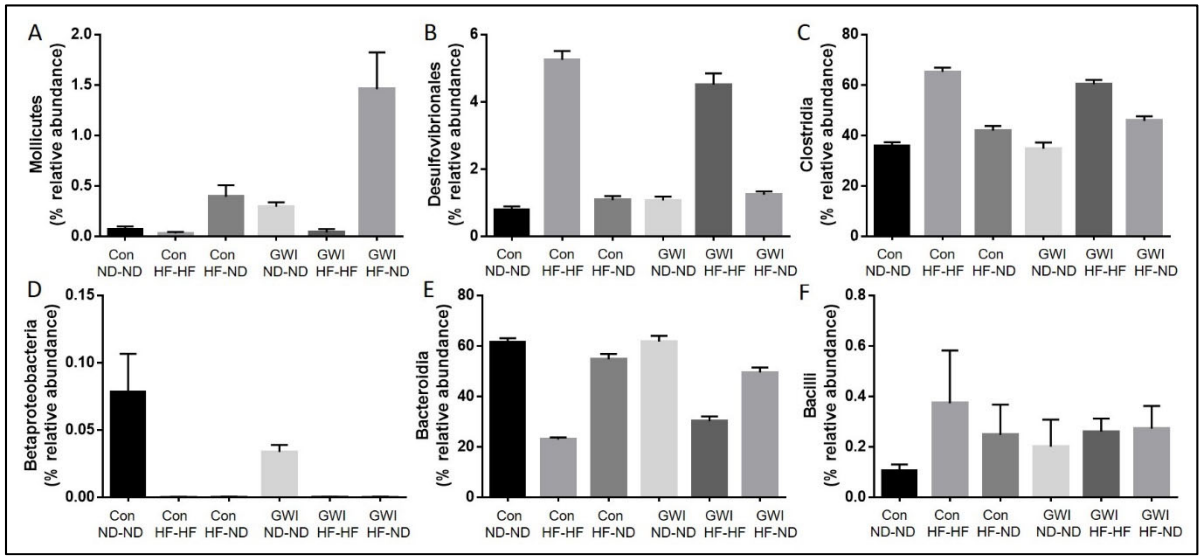

Fig. 7. Relative abundance of taxa below the level of phylum in treatment and diet groups. Results are presented as % relative abundance for each taxon. Con = control; GWI = PER + PB; ND = normal diet; HF = high fat diet.

treatment and diet. The main effect of treatment in each panel of Fig. 7 was significant by one-way ANOVA with p values ranging from 0.035 (for Bacilli) to 0.0001 (for all remaining taxa). In general, the effects of the HF on bacterial taxa were more prevalent than those of GWI treatment. The Con-ND-ND

group did not differ from the GWI-ND-ND group, whereas both control and GWI groups fed ND-ND were significantly different from the respective HF-HF groups for most taxa. The complexity of the changes are most evident for Desulfovibrionales and Clostridia, where the relative abundances of these taxa were increased in HF-HF groups compared to ND-ND groups, and in Betaproteobacteria and Bacteroidia, which were both greatly decreased in abundance in the HF-HF groups. Two additional unique changes can be seen in Fig. 7A where the abundance of Mollicutes in GWI-HF-ND group was significantly increased compared to the other groups, and in Fig. 7D where the abundance of Betaproteobacteria was significantly decreased for most groups compared to the Con-ND-ND group. Each of the OTUs from the LEfSe analysis (Fig. 5) was subjected to analysis using the Basic Local Alignment Search Tool (BLAST) in an attempt to identify taxa that were differentially abundant among treatments at the species level (i.e. the consensus sequence of the OTU had > 99% sequence identity with the sequence of a bacterial species within the BLAST taxonomy database). The results presented in Table 1 show that all groups except Con-HF-ND were represented by specific bacterial species. The Con-ND-ND group was characterized by Muribaculum intestinale whereas Fusimonas intestini was characteristic of the GWI-ND-ND group. The Con-HF-HF group was represented by Flintibacter butyricus and Bacteroides intestinalis and the corresponding GWI-HF-HF group was demarcated by Bacteroides vulgatus, Mucispirillum schaedleri and Parabacteroides goldstenii. Finally, the biomarkers Paramuribactum intestinale, Duncaniella muris and Bacteroides acidifaciens emerged in the GWI-HF-ND group.

OTU #	Phylum	Bacteria sp	Identity (%)	Group
OTU0088	Bacteroidetes	*Muribaculum intestinale*	100	Con-ND-ND
OTU0007	Firmicutes	*Flintibacter butyricus*	99.6	Con-HF-HF
OTU0075	Bacteroidetes	*Bacteroides intestinalis*	99.6	Con-HF-HF
OTU0047	Firmicutes	*Fusimonas intestini*	99.6	GWI-ND-ND
OTU0022	Bacteroidetes	*Paramuribaculum intestinale*	100	GWI-HF-ND
OTU0066	Bacteroidetes	*Duncaniella muris*	100	GWI-HF-ND
OTU0011	Bacteroidetes	*Bacteroides acidifaciens*	100	GWI-HF-ND
OTU0019	Bacteroidetes	*Bacteroides vulgatus*	100	GWI-HF-HF
OTU0013	Deferribacteres	*Mucispirillum schaedleri*	100	GWI-HF-HF
OTU0069	Bacteroidetes	*Parabacteroides goldstenii*	100	GWI-HF-HF

Finally, Fig. 8 shows that the GWI model used in this study recapitulates some of the key features of the condition, such as mood alterations. Mice treated with PER + PB showed decreased self-motivated care reflected as a shorter grooming time in the splash test compared to controls ($p < 0.05$; Fig. 1A). This is

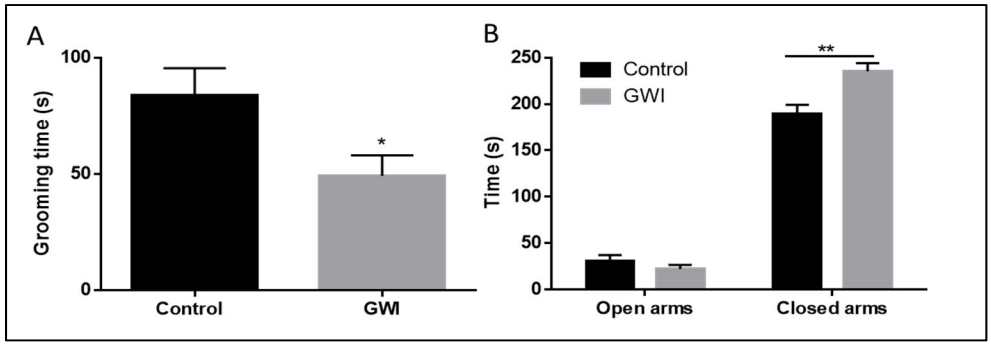

Fig. 8. Effects of treatment with Gulf War agents PER + PB on the splash test (A) and the elevated plus maze (B). Behaviors were evaluated 6 weeks after administration of the agents to corroborate that the GWI model induced some of the outcomes reported for this condition. Results are mean ± SEM, N = 5-6. Symbols represent significance levels for the indicated comparisons as $p < *0.05$, **0.01.

associated with a depression-like phenotype in rodents. Two-way ANOVA analysis of anxiety-like behaviors tested with the elevated plus maze test revealed a main effect of treatment ($F_{1,20} = 6.64$, $p < 0.05$), time in each set of arms ($F_{1,20} = 633.2$, $p < 0.0001$) and these two factors interaction ($F_{1,20} = 13.51$, $p < 0.01$). The time animals treated with PER + PB spent in the closed arms of the maze was significantly longer compared to controls ($p < 0.01$, post hoc Sidak's test), whereas no differences were found in the time spent in the open arms (Fig. 8B). These results are indicative of anxiety-like phenotype in the mice treated with PER + PB.

4. Other achievements: All stated goals for this period of activity were met and the data was positive in outcome. The method used to model GWI was based on a validated and approved model that involves treatment of mice with PB + PER. After treatment, the contents of the caecum were removed and DNA was extracted. The DNA was used to construct a sequencing library and the library was subjected to 16S rRNA gene sequencing on an Illumina MiSeq system. Behavioral assays used to assess anxiety- and depression-like behavior were the elevated plus maze and the splash test, respectively, both of which have been extensively validated in published work.

○ **What opportunities for training and professional development has the project provided?** Nothing to report.

○ **How were the results disseminated to communities of interest?** The results collected up to the present time on this project have been published. The citation is: Angoa-Perez, M., Zagorac, B., Francescutti, D.M., Winters, A.D., Greenberg, J.M., Ahmad, M.M., Manning, S.D., Gulbransen, B.D., Theis, K.R. and Kuhn, D.M. Effects of a high fat diet on gut microbiome dysbiosis in a mouse model of Gulf War Illness. Scientific Reports, 10 (1):9529. doi: 10.1038/s41598-020-66833-w, 2020.

○ **What do you plan to do during the next reporting period to accomplish these goals?** In the next reporting period we will carry out Subtask 4 (Major Task 2 on SOW) which is to homogenize caecum tissue and use liquid chromatography/mass spectrometry to determine the levels of short chain fatty acids (e.g., butyrate, acetate, propionate, valerate) and selected gut- and CNS-active large neutral amino acid phenyl derivatives (e.g., p-cresol, indoxyl sulfate, phenylacetylglutamine). In addition, we will progress to Major Task 3 which involves rebalancing the GWI-modified gut microbiome using probiotics and microbiota transfer therapy.

4. IMPACT

○ **What was the impact on the development of the principal discipline of the project?** The findings of this project so far have added substantiation to the possibility that the multi-symptom disorder referred to as GWI could be based in a significantly altered gut microbiome. Numerous other health disorders included diabetes, obesity, hypertension, developmental disorders and neurodegenerative diseases have now been linked to an altered gut microbiome. Therefore, our results extend GWI to this growing list of health conditions that have been linked to dysbiosis. In addition, the findings that a high fat diet can accentuation the effects of PB + PER on the gut microbiome establishes that life-style risk factors can worsen and possibly perpetuate the symptoms of GWI. Of significance is the finding that a dietary intervention can correct or re-balance the effects of a high fat diet on GWI-induced alterations in the gut microbiome.

○ **What was the impact on other disciplines?** Our findings were published very recently so it is not yet possible to determine their impact on other disciplines.

○ **What was the impact on technology transfer?** Nothing to report.

○ **What was the impact on society beyond science and technology?** As above, the impact of our recently published findings are hard to gauge now. However, we did participate in the recent CDMRP-VA Conference on "Gulf War Illness 2020 State of the Science Conference" held August 18-19, 2020. This virtual conference included a panel of Gulf War Veterans all of whom listened intently and provided valuable feedback and suggestions for future work on GWI therapies. Awareness of our results could impact the broader Veterans community by suggesting that unhealthy life-style risk factors such as obesity, smoking and alcohol consumption could complicate existing health conditions in Veterans by accentuating the modification in the gut microbiome. Attention to these results could lead to a change in behavior (e.g., eating a healthier diet, reduce smoking and drinking) that has the possibility of improving a health condition through the application of non-drug and non-invasive "therapies".

5. CHANGES/PROBLEMS:

○ **Changes in approach and reasons for change**: No changes.

○ **Actual or anticipated problems or delays and actions or plans to resolve them**: The one unanticipated problem was the closure of our research institutions as a result of the Corona virus pandemic. We have now been approved to resume research activities using a wide variety of approaches that will minimize increased infection. These include intensive health screen upon entry into the facility, wearing PPE, social distancing and careful de-contamination of all instruments and lab benches at the beginning and end of the workday. There were no other problems.

○ **Significant changes in the use or care of human subjects, vertebrate animals, biohazards, and/or select agents**: There were no deviations, unexpected outcomes or changes in IACUC protocol approvals.

○ **Significant changes in the use or care of human subjects**: Not applicable.

○ **Significant changes in use of care of vertebrate animals**: No changes.

○ **Significant changes in use of biohazards and/or select agents**: No changes.

6. PRODUCTS

○ **Publications, conference papers and presentations**

● **Journal publications:** Angoa-Perez, M., Zagorac, B., Francescutti, D.M., Winters, A.D., Greenberg, J.M., Ahmad, M.M., Manning, S.D., Gulbransen, B.D., Theis, K.R. and Kuhn, D.M. Effects of a high fat diet on gut microbiome dysbiosis in a mouse model of Gulf War Illness. Scientific Reports, 10 (1):9529. doi: 10.1038/s41598-020-66833-w, 2020. CDMRP support acknowledged.

● **Books or other non-periodical, one-time publications**: None

● **Other publications, conference papers, and presentations**: Kuhn, D.M. Effects of a high fat diet on gut microbiome dysbiosis in a mouse model of Gulf War Illness. CDMRP-VA Gulf War Illness 2020 State of the Science Conference (virtual), August 18-19, 2020. CDMRP support acknowledged.

○ **Website(s) or other Internet site(s)**: None

○ **Technologies or techniques**: None

○ **Inventions, patent applications, and/or licenses**: None

○ **Other products**: None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

○ **What individuals have worked on the project?**

Name	Role	Person months	Contribution	Funding
Donald M. Kuhn	PI	2.4	PI, design, data interpretation, manuscript prep and revision	This award
Mariana Angoa-Perez	Co-investigator	1.8	Design, data analysis, bioinformatics, manuscript prep and revision	This award
Kevin R. Theis	Collaborator	1.2	Design, data analysis, bioinformatics, manuscript prep and revision	This award

○ **Has there been a change in the active other support of the PD/PIs or senior/key personnel since the last reporting period?** Nothing to report.

○ **What other organizations were involves as partners?**

- **Describe partner organizations**
  - **Organization Name:** Michigan State University
  - **Location of Organization**: East Lansing, Michigan
  - **Partners contribution to the project:**
    - **Collaboration**

8. SPECIAL REPORTING REQUIREMENTS

○ **Collaborative awards:** None

○ **QUAD charts**: Not applicable- no changes from original

9. APPENDICES

- **Journal article**
- **PI CV**

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Effects of a high fat diet on gut microbiome dysbiosis in a mouse model of Gulf War Illness

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Gulf War Illness (GWI) is a chronic health condition that appeared in Veterans after returning home from the Gulf War. The primary symptoms linked to deployment are posttraumatic stress disorder, mood disorders, GI problems and chronic fatigue. At first glance, these symptoms are difficult to ascribe to a single pathological mechanism. However, it is now clear that each symptom can be linked individually to alterations in the gut microbiome. The primary objective of the present study was to determine if gut microbiome dysbiosis was evident in a mouse model of GWI. Because the majority of Gulf War Veterans are overweight, a second objective was to determine if a high fat diet (HF) would alter GWI outcomes. We found that the taxonomic structure of the gut microbiome was significantly altered in the GWI model and after HF exposure. Their combined effects were significantly different from either treatment alone. Most treatment-induced changes occurred at the level of phylum in Firmicutes and Bacteroidetes. If mice fed HF were returned to a normal diet, the gut microbiome recovered toward normal levels in both controls and GWI agent-treated mice. These results add support to the hypotheses that dysbiosis in the gut microbiome plays a role in GWI and that life-style risk factors such as an unhealthy diet can accentuate the effects of GWI by impacting the gut microbiome. The reversibility of the effect of HF on the gut microbiome suggests new avenues for treating GWI through dietary intervention.

Soon after the end of hostilities in the Gulf War (August 1990–April 1991), a series of health issues began emerging in Gulf War Veterans and have persisted to the present day. The health issues reported are a perplexing and complex constellation of symptoms now known as Gulf War Illness (GWI). Over the past two decades, the Institute of Medicine has completed a series of studies on GWI and Health and the most recent review concluded that "Evidence is sufficient to conclude that a causal relationship exists between being deployed to the Gulf War and a health outcome" (p. 3[1]). When considering all symptoms that have been reported to be part of GWI, posttraumatic stress disorder was the only condition judged to have sufficient evidence of a causal relationship. The other symptoms for which evidence was sufficient to establish an association with deployment were mood disorders (anxiety, depression), GI symptoms (irritable bowel syndrome [IBS], dyspepsia) and chronic fatigue syndrome[1]. These disparate outcomes make it difficult to attribute GWI to a single mechanism until consideration is given to the gut microbiome.

The GI system of humans and most other mammals is inhabited by a very large number of bacteria, viruses, fungi and archaea. Collectively, these microorganisms make up the gut microbiome. It has been estimated that the gut contains 100 trillion cells and these cells express >150-fold more unique genes than the human genome[2]. The commensal members of the gut microbiome support human health but disruption in it has been implicated in a large number of clinical and physiological disorders [see[3–5] for reviews]. Several conditions linked to enteric

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dysbiosis are reminiscent of GWI. First, IBS[6,7] and dyspepsia[8] are emerging as prototypical forms of gut dysbiosis. Second, the CNS symptoms associated with GWI (general anxiety, PTSD and depression) are frequently co-morbid with IBS and other inflammatory conditions of the bowel[9,10]. Third, chronic fatigue/fibromyalgia has also been linked to altered microbiome composition[11,12]. Therefore, the three main symptom clusters of GWI can be linked individually to gut dysbiosis, suggesting the possibility that a disrupted microbiome underlies all three. Indeed, a very small number of recent studies has confirmed that the gut microbiome is altered in Gulf War Veterans[13] and in animal models of GWI[14–16].

It remains perplexing that the symptoms of GWI are so chronic. In this preliminary report, we hypothesize that life-style risk factors, and specifically an unhealthy diet, could contribute to the persistence of GWI symptoms. It is known that Gulf War Veterans are often overweight or obese, both of which contribute to chronic health conditions[17,18]. Moreover, it is well known that a fat-laden diet causes dysbiosis within the human gut microbiome[19,20], alters GI transit[21] and can contribute to chronic low-grade gut inflammation (see[22] for review). Animal studies have reported that energy dense[23] and fat- or sugar-enriched diets[24] not only cause significant alterations in the gut microbiome and fat accumulation but can also lead to changes in memory, brain inflammation and gut-brain communication. Germ-free mice colonized by fecal transfer from obese mice[25] or obese humans[26] develop significant increases in body fat, showing the importance of the microbiome in obesity. In this study, mice were exposed to a GWI model (pyridostigmine bromide (PB) and permethrin (PER)) and then fed either a normal diet (ND) or high fat diet (HF) to mimic conditions in Veterans with GWI. The results confirm that the gut microbiome is altered in an animal model of GWI and reveal that a HF further alters the dysbiotic gut microbiome in this model.

Materials and Methods

Animal model of GWI. An established mouse model of GWI, as effectively employed by Crawford and colleagues[27–30], was used in the present studies. This model has been extensively validated[31] and has been deemed a GWI-relevant animal model in The Gulf War Illness Landscape (https://cdmrp.army.mil/gwirp/pdfs/GWIRP_Landscape.pdf) published by the DoD GWI Research Program. Male C57BL6/J mice (8 weeks of age) were purchased from Envigo (Indianapolis, I.N.) and housed individually in a room with constant temperature and humidity and with alternating 12 hr periods of light and darkness. All mice used in these studies were from the same cohort and assignment to treatment groups was random. Half of the mice were injected with 50 µl of GWI agents in final doses of 0.7 mg/kg of pyridostigmine bromide (PB) and 200 mg/kg of permethrin (PER) solubilized in 100% dimethyl sulfoxide (DMSO). Drug solutions were further diluted with sterile physiological saline to a final DMSO concentration of 3% just prior to intraperitoneal injection. The other half served as controls and received intraperitoneal injections of 3% DMSO in sterile physiological saline. Injections were administered once daily for 10 days. Several studies consistently show that in rodents, exposure to PER + PB results in neurobehavioral alterations (i.e. anxiety and mood impairment) that are similar to symptoms reported by Veterans with GWI[28]. Thus, anxiety and depression-like behaviors were tested as specified below. During treatment, all mice were given ad libitum access to water and normal rodent laboratory chow ((ND); D12450K with 10 kcal% from fat, Research Diets, New Brunswick, NJ). On the last day of treatment, the GWI and control groups were split into 3 same sized groups (N = 7–9 mice per group) and fed the following diet regimens: one group on a ND and two groups on a high fat diet ((HF); D12451 with 45% kcal from fat, Research Diets, New Brunswick, N.J.) known to induce obesity in mice[32,33]). After 3 weeks, one of the HF fed groups was switched back to a ND while the two other groups were continued on their original HF or ND for an additional 3 weeks. Hereafter, the treatment/diet groups are referred to as Con-ND-ND, Con-HF-HF and Con-HF-ND for controls and GWI-ND-ND, GWI-HF-HF and GWI-HF-ND for PER + PB treated mice. To validate the GWI model at the specific post-treatment time of 6 weeks that mice were exposed to diets, the Con-ND-ND and GWI-ND-ND groups were evaluated for depression- (splash test) and anxiety- (elevated plus maze) like behaviors prior to sacrifice. These are two of the core components of mood disorders present in individuals with GWI[1]. The splash test was performed according to our previously reported work[34]. Briefly, this test involves spraying a 10% sucrose solution onto the dorsal coat of the mouse in its home cage. This mildly sticky solution induces self-grooming, and the time the mouse spends grooming is considered a direct measure of self-motivated care. The elevated plus maze was also performed according to our previous reports[35]. In this test, the time spent in both the open and closed arms of the maze was recorded for each mouse in 5 min sessions using a motion-sensitive digital video camera and EZ Video freeware Software (Ezvid, Inc, Los Angeles, CA; https://www.ezvid.com/ezvid_for_windows). Mice were sacrificed by decapitation and the contents of the caecum were harvested and frozen at −80°C. Stressors such as noise and handling by multiple persons were avoided and mice were monitored daily for signs of distress or injury until the experimental endpoint. The Institutional Care and Use Committee of Wayne State University approved the animal care and experimental procedures (IACUC 17-08-0307). All procedures were also in compliance with the *NIH Guide for the Care and Use of Laboratory Animals* and were conducted in compliance with ARRIVE guidelines and under IACUC-approved protocols.

Microbiome analysis. DNA was extracted from caecum contents (~200 mg wet weight) using QIAamp PowerFecal DNA kits and sample DNA concentrations were determined using a Qubit 4 Fluorometer (range 70–100 ng/µl). Samples were sequenced in duplicate on an Illumina MiSeq system using a 2 × 250 cycle V2 kit with Illumina reagents and Illumina sequencing procedures detailed by Kozich and colleagues[36]. The 16S rRNA gene primers targeted the V4 region of the gene (forward primer: 5′-GTGCCAGCMGCCGCGGTAA-3′; reverse primer: 5′-GGACTACHVGGGTWTCTAAT-3′). The 16S rRNA gene sequences from the paired fastq files were trimmed, screened and aligned using mothur[37], in accordance with the MiSeq SOP established by Schloss and colleagues (https://www.mothur.org/wiki/MiSeq_SOP). After de-multiplexing and quality control (e.g., truncating reads with >2 adjacent low quality base calls; discarding reads containing any ambiguous base

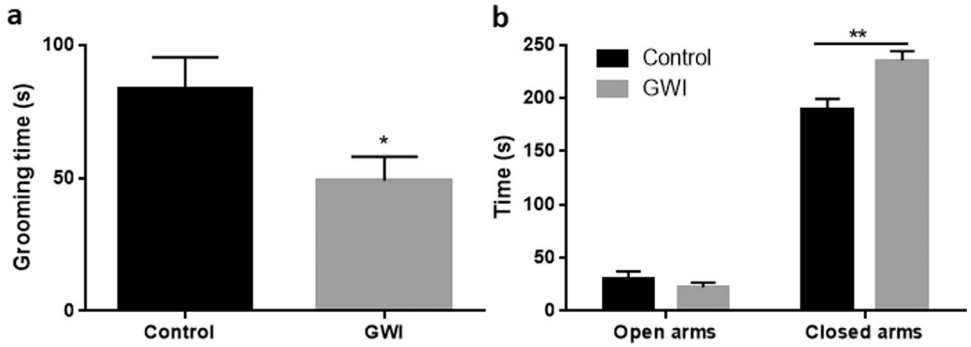

Figure 1. Effects of treatment with Gulf War agents PER + PB on the splash test (**A**) and the elevated plus maze (**B**). Behaviors were evaluated 6 weeks after administration of the agents to corroborate that the GWI model induced some of the outcomes reported for this condition. Results are mean ± SEM, N = 5–6. Symbols represent significance levels for the indicated comparisons as $p < *0.05$, **0.01.

calls in surviving sequences), sequences were binned into operational taxonomic units (OTUs) based on percent sequence identity (97%). The OTUs were taxonomically classified in mothur, and the bacterial community data were thereafter visualized and statistically analyzed using PAST software (v3.20[38]). Microbiome diversity was characterized in terms of α-diversity using the Chao1 (i.e. community richness) and Shannon and Simpson (1-D) (i.e. community heterogeneity) indices. The number of sequences and Good's coverage values were analyzed using one-way ANOVA. β-diversity was assessed using the Jaccard (i.e. shared composition) and Bray-Curtis (i.e. shared structure) indices based on relative abundance data. High-dimensional class comparisons were carried out with linear discriminant analysis effect size (LEfSe) in an on-line interface[39] using default parameters except that the minimum LDA score was set to 3.6. Heat maps were generated using MetaboAnalyst 4.0[40].

Data analysis and statistics. Data from splash test was analyzed with an unpaired student's t test using GraphPad Prism (v6.07) for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). Time spent in each set of arms of the elevated plus maze was analyzed by two-way ANOVA and subsequent Sidak's multiple comparison tests. Food and body weight data were analyzed with two-way ANOVA followed by Tukey's *post hoc* tests using Prism The indices for α-diversity were obtained using PAST software (v3.20; free software for scientific data analysis, Oyvind Hammer, Natural History Museum, University of Oslo, Norway; https://folk.uio.no/ohammer/past/). The results were analyzed statistically with a one-way ANOVA and subsequent Tukey's *post hoc* comparisons, using Prism. The indices for β-diversity were also calculated, and statistical analyses were carried out, using PAST software as well. The results were analyzed using a two-way NPMANOVA, and *post hoc* comparisons were made using one-way NPMANOVAs. Taxonomic distributions at the phylum level (treatment X phylum) and lower taxonomic levels (treatment X time) were analyzed with a two-way ANOVA followed by *post hoc* comparisons using Tukey's tests in GraphPad Prism.

Results

Effects of HF on food intake and body weight in a model of GWI. Figure 1 shows that the GWI model used in this study recapitulates some of the key features of the condition, such as mood alterations. Mice treated with PER + PB showed decreased self-motivated care reflected as a shorter grooming time in the splash test compared to controls ($p < 0.05$; Fig. 1A). This is associated with a depression-like phenotype in rodents. Two-way ANOVA analysis of anxiety-like behaviors tested with the elevated plus maze test revealed a main effect of treatment ($F_{1,20} = 6.64$, $p < 0.05$), time in each set of arms ($F_{1,20} = 633.2$, $p < 0.0001$) and these two factors interaction ($F_{1,20} = 13.51$, $p < 0.01$). The time animals treated with PER + PB spent in the closed arms of the maze was significantly longer compared to controls ($p < 0.01$, *post hoc* Sidak's test), whereas no differences were found in the time spent in the open arms (Fig. 1B). These results are indicative of anxiety-like phenotype in the mice treated with PER + PB.

Figure 2 A shows food intake measures for all groups and analysis by two-way ANOVA revealed significant main effects of time ($F_{11,484} = 72.71$, $p < 0.0001$), treatment ($F_{5,44} = 65.82$, $p < 0.0001$) and their interaction ($F_{55,484} = 5.57$, $p < 0.0001$). The GWI agent-treated group displayed a significantly higher food intake of the ND compared to controls fed equally (*post hoc* Tukey's test; $p < 0.0001$). The consumption of HF impacted the food intake as Con-HF-HF mice had a lower intake compared to Con-ND-ND mice (*post hoc* Tukey's test; $p < 0.0001$) and to Con-HF-ND (*post hoc* Tukey's test; $p < 0.0001$). Con-ND-ND mice did not differ from Con-HF-ND. In mice treated with GWI agents, both groups fed with HF showed a decreased intake compared to mice fed with ND (*post hoc* Tukey's tests for both GWI-HF-HF and GWI-HF-ND vs GWI-ND-ND; $p < 0.0001$). No differences were detected when comparing the GWI-HF-HF group to the GWI-HF-ND mice. While HF was associated with a lower food intake, body weight followed the opposite trend (Fig. 2B). Both treatment groups fed the HF (Con-HF-HF and GWI-HF-HF) gained on average 10 g over the 6 week test period, whereas both groups fed the ND (Con-ND-ND and GWI-ND-ND) gained ~3 g. Body weight was not altered by treatment with GWI agents as Con-ND-ND was not different from GWI-ND-ND, and Con-HF-HF was not different from GWI-HF-HF mice. When mice initially fed a HF diet were switched to the ND for 3 weeks, both groups lost significant amounts of weight (~6–7 g). However, the Con-HF-ND group achieved a significant reduction in body weight sooner after

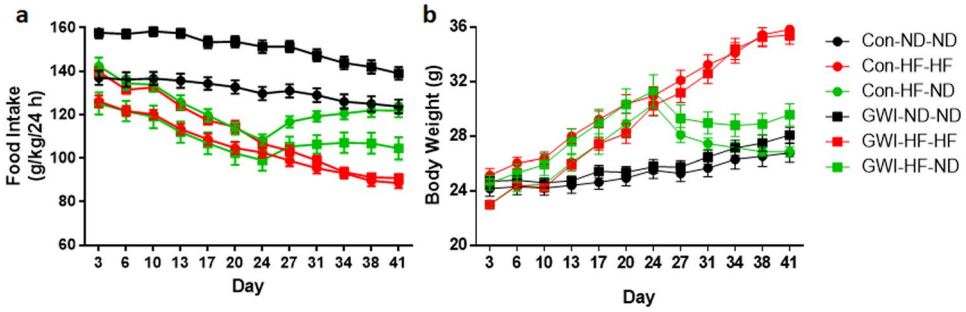

Figure 2. Effect of diet on food intake (**A**) and body weight (**B**). Mice were treated with GWI agents or Con (control) and then fed a normal (ND) or high fat (HF) diet for 3 weeks. Thereafter, half of the mice on the HF diet (Con and GWI) were switched to ND (HF-ND) for an additional 3 weeks. Remaining GWI and Con mice were fed ND or HF diet throughout (ND-ND or HF-HF). Food intake measures were calculated based on food consumption (g), mouse body weight (kg) for a 24 h period and reported as g/kg/24 h. Results are mean ± SEM, N = 7–9.

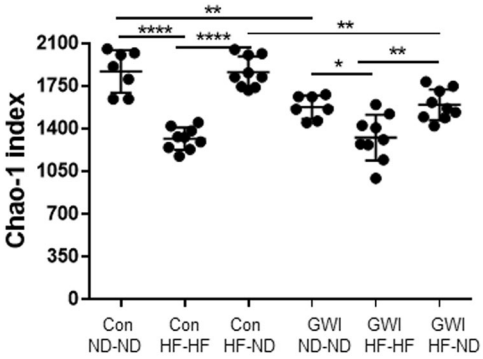

Figure 3. Effects of GWI ± HF on α-diversity. Data are presented as Chao-1 ± SEM, N = 8-9. Con = control; GWI = PER + PB; ND = normal diet; HF = high fat diet. Symbols represent significance levels for the indicated *post hoc* comparisons as $p < *0.05$, **0.01, ****0.0001.

the diet switch (*post hoc* Tukey's test; $p < 0.001$ at day 27) than the GWI-HF-ND group (*post hoc* Tukey's test; $p < 0.001$ at day 31), and the GWI agent-treated mice ultimately lost less weight than controls (*post hoc* Tukey's test; $p < 0.05$ at day 41). The main effects of time ($F_{11,528} = 64.8$, $p < 0.001$) and treatment ($F_{5,528} = 115.9$, $p < 0.001$) as well as their interaction ($F_{55,528} = 7.3$, $p < 0.001$) were significant (2-way ANOVA). These data establish that the HF led to significant gains in body weight that were of the same magnitude in controls and mice treated with GWI agents. Both groups lost significant weight when switched back to ND, although weight loss was more pronounced among controls.

Effects of treatment with GWI agents and HF on the gut microbiome at the OTU level. The number of sequences obtained were as follows: 117,212 ± 7,509 for Con-ND-ND, 103,432 ± 17,384 for Con- HF-HF, 128,772 ± 9,319 for Con-HF-ND, 100,369 ± 10,433 for GWI-ND-ND, 111,781 ± 32,363 for GWI-HF-HF, and 128,371 ± 32,694 for GWI-HF-ND. There were no statistically significant differences among these groups with respect to sequence numbers. Good's coverage values ± SD were the following: 99.63 ± 0.043 for Con-ND-ND, 99.7 ± 0.056 for Con-HF-HF, 99.67 ± 0.025 for Con-HF-ND, 99.63 ± 0.025 for GWI-ND-ND, 99.71 ± 0.06 for GWI-HF-HF, and 99.69 ± 0.079 for GWI-HF-ND.

Figure 3 presents an analysis of α-diversity using the Chao-1 index as a measure of gut microbiome richness. The main effect of treatment ($F_{5,44} = 26.1$, $p < 0.0001$) was significant. *Post hoc* comparisons indicated that treatment with GWI agents significantly reduced microbiome richness compared to controls (Tukey's test, $p < 0.05$), and that HF led to significantly decreased richness in both control (Tukey's test, $p < 0.001$) and GWI agent-treated groups (Tukey's test, $p < 0.05$). Notably, when mice were shifted from HF to ND, α-diversity recovered to the levels of the appropriate treatment control and differed significantly from the respective HF-HF group (Tukey's test, $p < 0.001$ for controls and $p < 0.01$ for GWI).

Results of α-diversity analyses based on the Simpson (1-D) index indicated that, while the heterogeneity of the gut microbiome did not differ between GWI agent-treated mice and controls, gut microbiome heterogeneity was consistently highest in HF mice whereas there were no consistent effects of treatment on gut microbiome heterogeneity using the Shannon index (Supplementary Fig. S1). With respect to β-diversity, analyses based on the Jaccard index, which reflects shared microbiome membership (i.e. community composition) results showed that the OTU profiles of samples clustered together tightly according to the diet regimen, and that within diet regimen

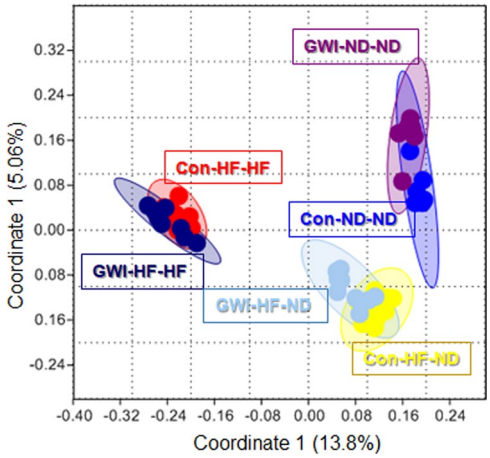

Figure 4. Effects of treatments on β-diversity. PCoA showing differences in the similarities of the gut microbiome profiles of the study groups using the Jaccard index. Con = control; GWI = PER + PB; ND = normal diet; HF = high fat diet.

groups, samples also clustered by treatment (Fig. 4). Two-way NPMANOVA analyses revealed that the main effects of treatment ($p < 0.01$) and diet ($p < 0.0001$), as well as their interaction ($p < 0.02$), were significant. All *post hoc* comparisons among groups were statistically significant (Supplementary Table S1). It is interesting that mice in the control and GWI agent-treated groups exposed to the HF-ND regimen clustered near the ND-ND groups on the PCoA plot, suggesting rapid recovery of the gut microbiome following a return to a ND, as was also seen above for α-diversity. Results for β-diversity using the Bray-Curtis index, which reflects overall microbiome structure (i.e. not just membership), were similar to those for the Jaccard index (Supplementary Fig. S2).

The taxonomic identities of prominent OTUs (≥1.5% average relative abundance among all subjects) varied among treatment groups. These results are presented in the heat map in Fig. 5. It can be seen that the GWI agent-treated and control groups displayed similar patterns of OTU expression according to diet. The most prominent differences in these groups were decreases in Bacteroidetes (see the clusters near the bottom of Fig. 5) and increases in Firmicutes (clusters near the top) in the Con-HF-HF and GWI-HF-HF groups. Furthermore, within each diet group, differences in OTU relative abundances were evident for the GWI agent-treated versus controls. As reported above for community α and β diversity, as mice in the GWI agent-treated and control groups transitioned from HF to the ND, patterns of OTU relative abundance appeared to "recover" toward the pattern shown in the groups fed ND throughout this experiment (i.e., ND-ND groups).

Figure 6 presents results from linear discriminant analysis effect size (LEfSe) analysis and highlights the effect sizes of the treatments and diets on affected taxa. LEfSe compares each group to all others simultaneously and generates bar plots that include taxa that are distinctly relatively abundant in each specific treatment and diet group. Segata *et al.*[39] propose LEfSe as a means for biomarker discovery by finding OTUs that consistently explain the differences between two or more types of microbial communities. Two main outcomes from this analysis are apparent. First, the groups treated with GWI agents are demarcated by more taxonomic biomarkers than controls for each diet condition. Second, most treatment groups were distinguished by taxa in the order Clostridiales within the phylum Firmicutes (i.e., Con-ND-ND, Con-HF-ND and GWI-HF-ND). However, the GWI-ND-ND group was represented primarily by taxa in the order Bacteroidales within the phylum Bacteroidetes, the Con-HF-HF group was singularly characterized by taxa within the order Desulfovibrioales, and the GWI-HF-HF group was represented by taxa within the orders Lactobacillales and Erysipelotrichales. The HF diet shifted the predominant taxa for the GWI-ND-ND group from Bacteroidetes to Firmicutes. All of the control groups regardless of diet were distinguished by taxa within Firmicutes and the relatively most abundant taxa in the group fed a ND were in the *Clostridium XIVa and IV* clusters. Controls fed the HF diet were characterized by taxa within the genera *Desulfovibrio* and *Pseudoflavonifractor* and the control group shifted to a ND from the HF diet was distinguished by Porphyromonadaceae and Lachnospiraceae. Treatment- and diet-induced biomarkers were observed down to the level of family or genus as shown in the cladogram (Fig. 6).

Effects of treatment with GWI agents and HF on the gut microbiome at the phylotype level. Figure 7 illustrates treatment effects at the phylotype level. Treatment and diet effects on specific bacterial phyla are presented as percent relative abundance. The main effect of phylum was significant ($F_{7,352} = 2616$, $p < 0.0001$) but the treatment main effect was not. The phylum X treatment interaction was also significant ($F_{35,352} = 50.6$, $p < 0.0001$) by two–way ANOVA. *Post hoc* comparisons revealed that virtually all treatment groups differed significantly from one another (p values ranging from 0.05 to 0.0001). The observed changes occurred only within the prominent phyla Firmicutes and Bacteroidetes (Fig. 7). The only groups that did not differ were Con-ND-ND vs GWI-ND-ND within Firmicutes and Con-ND-ND vs GWI-ND-ND within Bacteroidetes. The results of all pairwise statistical tests for % relative abundance of Firmicutes and Bacteroidetes among treatment groups are presented in Supplementary Table S2.

Because the observed differences in % relative abundance occurred within the Firmicutes and Bacteroidetes phyla, and in light of the findings that the ratio of Firmicutes/Bacteroidetes (F/B) is higher in obese and

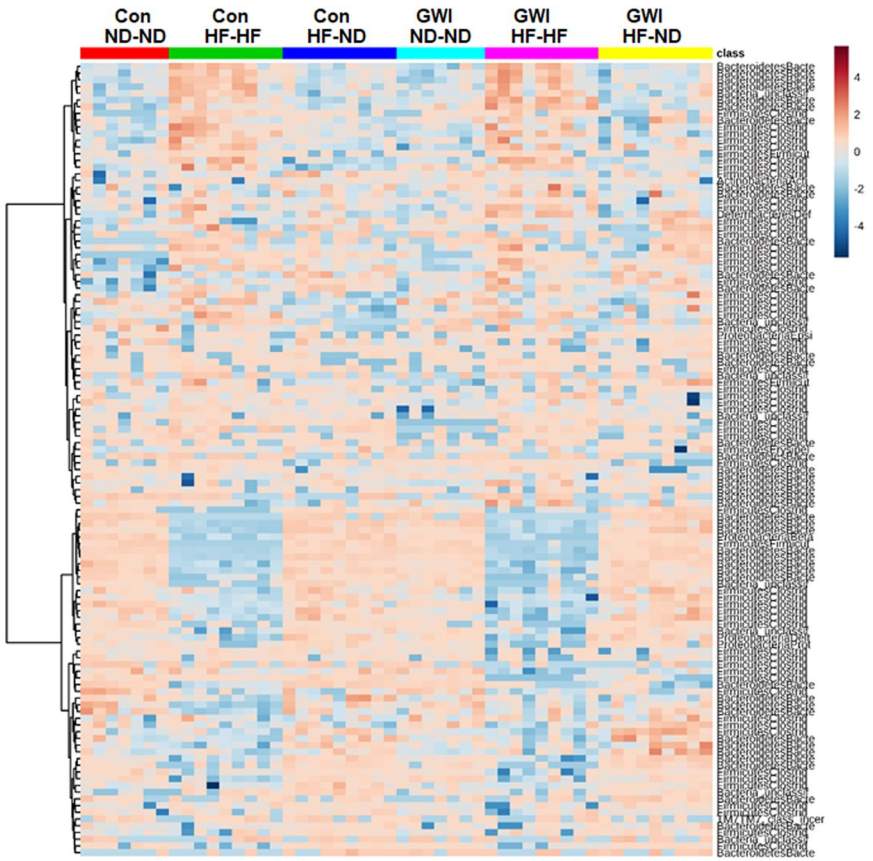

Figure 5. Heat map illustrating patterns in OTU relative abundance among the treatment groups. All subjects in each group are arrayed in columns and bacterial taxonomies are indicated in rows. Con = control; GWI = PER + PB; ND = normal diet; HF = HF diet. Clustering along the y-axis was done using the Ward algorithm.

overweight humans than in lean controls[41], we calculated this ratio for all treatment groups and the results are presented in Fig. 8. The main effect of treatment was significant ($F_{5,44} = 50.8$, $p < 0.0001$). Specifically, the HF diet caused significant increases in the F/B ratio for controls and GWI treated mice (Tukey's test, $p < 0.0001$ for both). The increase in the F/B ratio was significantly greater in the control mice than the GWI agent-treated mice (Tukey's test, $p < 0.001$). When groups fed the HF were shifted to the ND, the F/B ratio decreased to levels observed in the respective ND-ND controls (Tukey's test, $p < 0.0001$ for both).

Effects of treatment with GWI agents and HF on taxa below the level of phylum. The effects of treatments and diets on taxa below the level of phylum were also probed in view of the likelihood that changes at the highest taxonomic level may have not reached statistical significance because of increases and decreases of equal magnitude within phyla in percent relative abundances of bacteria at lower taxonomic levels. Figure 9 shows these results and indicates that effects at the taxonomic levels of class and order vary in a complex manner that is dependent on the combined influence of treatment and diet. The main effect of treatment in each panel of Fig. 9 was significant by one-way ANOVA with p values ranging from 0.035 (for Bacilli) to 0.0001 (for all remaining taxa). In general, the effects of the HF on bacterial taxa were more prevalent than those of GWI-agents treatment. The Con-ND-ND group did not differ from the GWI-ND-ND group, whereas both control and GWI agent-treatment groups fed ND-ND were significantly different from the respective HF-HF groups for most taxa. The complexity of the changes are most evident for Desulfovibrionales and Clostridia, where the relative abundances of these taxa were increased in HF-HF groups compared to ND-ND groups, and in Betaproteobacteria and Bacteroidia, which were both greatly decreased in abundance in the HF-HF groups. Two additional unique changes can be seen in Fig. 9A where the abundance of Mollicutes in GWI-HF-ND group was significantly increased compared to the other groups, and in Fig. 9D where the abundance of Betaproteobacteria was significantly decreased for most groups compared to the Con-ND-ND group.

Each of the OTUs from the LEfSe analysis (Fig. 6) was subjected to analysis using the Basic Local Alignment Search Tool (BLAST) in an attempt to identify taxa that were differentially abundant among treatments at the species level (i.e. the consensus sequence of the OTU had >99% sequence identity with the sequence of a bacterial species within the BLAST taxonomy database). The results presented in Table 1 show that all groups except Con-HF-ND were represented by specific bacterial species. The Con-ND-ND group was characterized by *Muribaculum intestinale* whereas *Fusimonas intestini* was characteristic of the GWI-ND-ND group. The

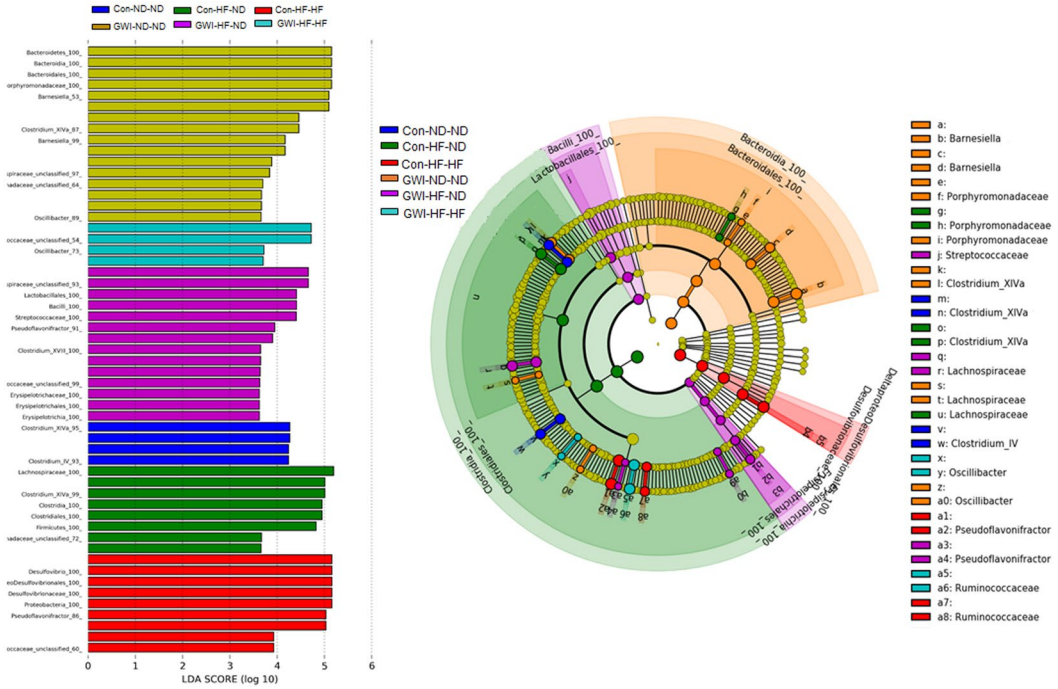

Figure 6. Bacterial taxa that were differentially abundant across treatments. LEfSe was carried out using the Galaxy Project and the results are displayed in the bar charts (**A**) and the associated cladogram (**B**). Taxa showing different abundance values in each treatment group (according to LEfSe) are shown in the cladogram highlighted by small circles and by shading. All groups are statistically significant compared to each other (LDA > 3.6). Con = control; GWI = PER + PB; ND = normal diet; HF = high fat diet.

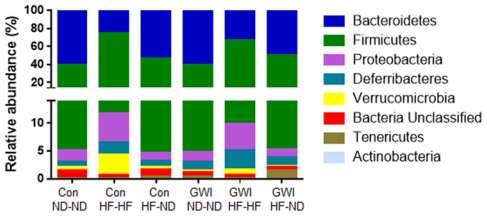

Figure 7. Percent relative abundances of phyla in treatment and diet groups. Stacked columns for the 8 most prominent phyla are included. Con = control; GWI = PER + PB; ND = normal diet; HF = high fat diet.

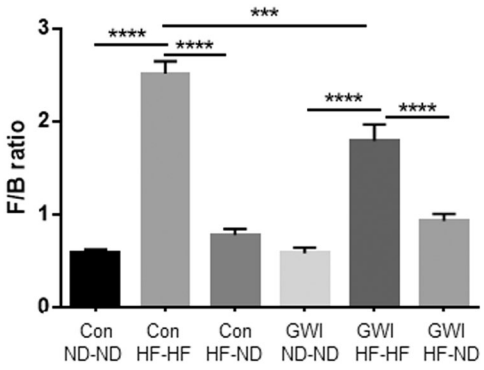

Figure 8. Firmicutes to Bacteroidetes (F/B) ratio in treatment and diet groups. Results are presented as means + SEM for each treatment and diet. Symbols represent significance levels for the indicated *post hoc* comparisons as p < : ***0.001, ****0.0001. Con = control; GWI = PER + PB; ND = normal diet; HF = high fat diet.

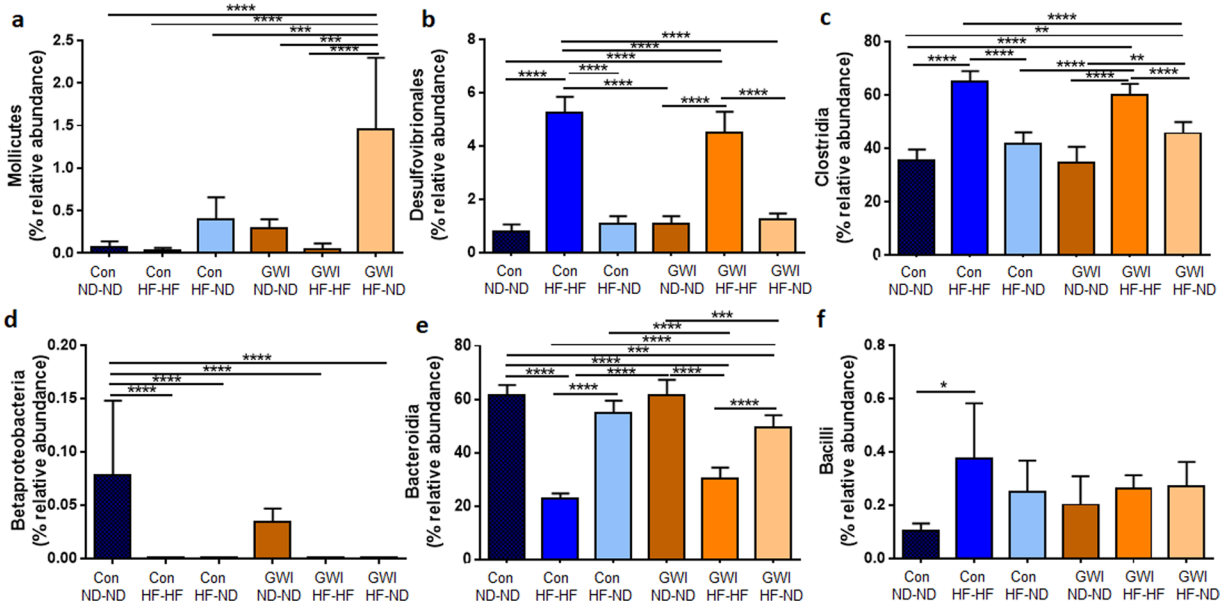

Figure 9. Relative abundance of taxa below the level of phylum in treatment and diet groups. Results are presented as % relative abundance for each taxon. Con = control; GWI = PER + PB; ND = normal diet; HF = high fat diet. Symbols represent significance levels for the indicated *post hoc* comparisons as p < : *0.05, **0.01, ***0.001, ****0.0001.

OTU #	Phylum	Bacteria sp	Identity (%)	Group
OTU0088	Bacteroidetes	*Muribaculum intestinale*	100	Con-ND-ND
OTU0007	Firmicutes	*Flintibacter butyricus*	99.6	Con-HF-HF
OTU0075	Bacteroidetes	*Bacteroides intestinalis*	99.6	Con-HF-HF
OTU0047	Firmicutes	*Fusimonas intestini*	99.6	GWI-ND-ND
OTU0022	Bacteroidetes	*Paramuribaculum intestinale*	100	GWI-HF-ND
OTU0066	Bacteroidetes	*Duncaniella muris*	100	GWI-HF-ND
OTU0011	Bacteroidetes	*Bacteroides acidifaciens*	100	GWI-HF-ND
OTU0019	Bacteroidetes	*Bacteroides vulgatus*	100	GWI-HF-HF
OTU0013	Deferribacteres	*Mucispirillum schaedleri*	100	GWI-HF-HF
OTU0069	Bacteroidetes	*Parabacteroides goldstenii*	100	GWI-HF-HF

Table 1. Bacterial species identified by BLAST analysis.

Con-HF-HF group was represented by *Flintibacter butyricus* and *Bacteroides intestinalis* and the corresponding GWI-HF-HF group was demarcated by *Bacteroides vulgatus, Mucispirillum schaedleri* and *Parabacteroides goldstenii*. Finally, the biomarkers *Paramuribactum intestinale, Duncaniella muris* and *Bacteroides acidifaciens* emerged in the GWI-HF-ND group.

Discussion

The goal of the present study was to determine if a HF would interact with PER and PB to further alter the gut microbiome in a mouse model of GWI. The rationale for this pilot study was the fact that a majority of Gulf War Veterans are overweight or obese[17,18], and that fat-laden diets can lead to changes in memory, GI and brain inflammation and gut-brain communication[19,20,23,24]. In this regard, it was important to rule out that an increased caloric consumption of the HF diet rather than its fat component itself was responsible for the observed effects. Thus, the energy density for the groups fed with HF versus ND was calculated. According to manufacturer's specifications, the energy density for the ND is 3.8 Kcal/g, whereas for the HF it is 4.7 Kcal/g. Using an average of the intake of each diet group over the entire experiment, the caloric densities were surprisingly higher for the ND groups (490.96 Kcal for Con-ND-ND and 530.1 Kcal for GWI-ND-ND) than for the HF groups (443.1 Kcal for Con-HF-HF and 444.4 Kcal for GWI-HF-HF). This is evidence that the number of calories was not the causative factor for the effects we reported. The experimental results established that PER and PB caused a significant dysbiosis, as did exposure to a HF, and their combined effects led to an altered gut microbiome that was significantly different from the effect of either treatment alone. These results are even more impactful when considering the relatively short-term period over which mice were fed the HF (i.e., 3 or 6 weeks). Consumption of the HF for only three weeks caused significant increases in body weight in groups treated with PER + PB or controls compared

to mice maintained on a ND. Two additional observations link these effects to alterations in the gut microbiome as a mediating factor. First, the Gulf War agents PER and PB did not alter water intake or the amount of food consumed on either diet. Second, when mice in both treatment groups were shifted from the HF to a ND for three additional weeks, mice treated with PER + PB lost significantly less weight than controls.

PER + PB and diet each caused significant alterations in the taxonomic makeup of the gut microbiome. The predominant changes in OTU structure occurred within the Firmicutes and Bacteroidetes phyla. This pattern was expected in light of the fact that the mouse gut microbiome is dominated by these two phyla[42]. Treatment with PER + PB caused a complex set of alterations in α-diversity. In both GWI agent-treated and control mice, those fed HF diets throughout the duration of the experiment exhibited gut microbiomes with reduced richness. Nevertheless, the gut microbiomes of all mice in the experiment remained OTU-rich, with Chao1 index values exceeding 1000. This high degree of OTU-richness resulted in high values for microbiome heterogeneity as well, with Simpson (1-D) and Shannon index values exceeding 0.93 and 4.0, respectively. The heterogeneity of gut microbiomes from HF-HF mice exceeded that of ND-ND mice in both GWI agent-treated and control groups based on the Simpson index, but not the Shannon index. These data suggest that although HF led to a reduction in the OTU-richness of the gut microbiome, the OTUs that were present in the guts of HF-treated mice were more evenly distributed in their relative abundances than were the OTUs in the gut microbiomes of ND mice.

Treatment with PER and PB and the HF each led to significant alterations in the complexity of the gut microbiome. The OTUs for the different diet conditions clustered together tightly and apart from the other groups. Mice fed the HF throughout (HF-HF) were most distant from mice fed a ND throughout (ND-ND) on the PCoA plot. Interestingly, when GWI and control mice were shifted from the HF to a ND (HF-ND), both groups clustered nearest to their respective ND-ND groups, suggesting a partial recovery in β-diversity after the dietary shift. Nevertheless, within each diet condition cluster, the GWI agent-treated mice differed significantly from controls. These results emphasize the fact that a life-style risk factor such as a HF can accentuate the effects of PER and PB on community diversity and establish the reversible nature of this effect after return to a ND.

LEfSe analysis identified numerous bacterial taxa that were differentially abundant among treatment groups and these taxonomic "biomarkers" varied substantially between the GWI agent-treated mice and those exposed to dietary shifts. The gut microbiome in mice fed the ND throughout were dominated by *Clostridium XIV* whereas the mice treated with PER + PB were dominated by *Barnesiella* and Porphyromonadaceae. The HF resulted in a large increase in the predominant taxa for both GWI and control mice. For instance, the GWI agent-treated mice on a HF were most defined by *Enterococcus, Clostridium*, Porphyromonadaceae, *Oscillibacter* and Proteobacteria whereas controls were dominated by *Clostridium XIV*, Ruminococcaceae, Erysipelotochaceae, *Barnsiella*, Lachnospiraceae and Actinobifidobacteriales. As seen above in treatment-induced alterations in community diversity, the shift from a HF to a ND led to a reduction in the number of defining taxa for both GWI agent-treated mice and their controls. Many of the differentially abundant taxa that emerged in the HF-HF groups (by comparison to the ND-ND groups) were not evident in the HF-ND mice for both GWI and control groups although the number of remaining taxa was greater than that seen in the ND-ND groups.

The individual OTU's that were identified in the LEfSe analysis were compared to 16S rRNA gene sequence data in the NCBI data base using BLAST in an attempt to identify bacterial species that were markers for the present treatment groups. A total of 10 species were matched with 99.6 to 100% sequence identity with 3 species linked to the Con-ND-ND and Con-HF-HF groups and 7 linked to the GWI agent-treated groups in all dietary conditions. Of these, 7 species were from the Bacteroidetes phylum, 2 were from Firmicutes and 1 was from Deferribacteres. Some interesting parallels to GWI can be seen in the identified species. For instance, *Flintibacter butyricus*, which was a marker for the Con-HF-HF group is increased in mice fed bile acids and a dietary fat[43]. *Mucispirillum schaedleri* was relatively most abundant in the GWI-HF-HF group and is known to be expanded in the gut under inflammatory conditions accompanied by reactive oxygen/nitrogen stress[44]. The GWI-HF-ND group was characterized by *Bacteroides acidifaciens* and *Duncaniella muris*. *B. acidifaciens* can ameliorate metabolic disorders such as diabetes and obesity and is expanded in lean phenotypes of the atg7 knockout mouse[45]. When mice fed a HF supplemented with resistant starch, the starch caused significant improvements in the intestinal health of obese mice and was associated with expansion of *D. muris*[46].

It is not yet possible to draw direct associations between a GWI model and HF to specific gut microbiome alterations. This can be attributed to several different factors. First, rodent models are probably limited in the extent to which they mimic the conditions to which Gulf War Veterans were exposed during their deployment. Second, GWI is a heterogeneous disorder making it difficult to link it to changes in specific taxa. For example, increases in Proteobacteria have been linked to gut inflammatory conditions[47] including a preliminary study of GWI[13]. While our present results showed significant increases in Proteobacteria, in the Con-HF-HF group, this increase did not quite reach statistical significance in the GWI agent-treated groups. The present results did document a significant increase in the F/B ratio for groups fed the HF-HF diet (both controls and GWI) in agreement with data from humans with IBS[6]. A more recent meta-analysis suggests that at least IBS is characterized at the genus level by decreases in *Lactobacillus* and *Bifidobacterium* and increased levels of *Escherichia coli* and *Enterobacter* (both in the Proteobacteria phylum) without changes in Bacteroidetes and *Enterococcus*[6]. Both of these outcomes are not fully recapitulated in Veterans with GWI[13] or in rodent models of this disorder[14–16], including the results of the present study. Third, GWI is not IBS and likely encompasses a different set of pathological alterations such that some Veterans with GWI have GI disturbances while others do not[1,13].

The present results stand in contrast to a recent study showing gut microbiome alterations in a mouse model of GWI[14]. Alhassan and colleagues demonstrated that mice treated with Gulf War agents plus corticosterone showed a significant increase in OTU richness and higher percent relative abundances for Firmicutes and Tenericutes over Bacteroidetes at the level of phylum. In contrast, we observed a reduction in OTU richness with GWI treatment in both the ND-ND and HF-ND groups and we did not observe increases in the relative abundance of Tenericutes in GWI agent-treated mice. These discrepancies may reflect differences in the Gulf War models used,

the use of different survival times post-treatment, and the lack of a stress-only group in the Alhassan *et al.*[14] study. Despite the differences in these two preclinical studies, the possibility that gut microbiome alterations may play a role in the symptoms of GWI is strengthened by the recent report of dysbiosis in Gulf War Veterans[13].

The present study has several strengths. First, it adds support to the hypothesis that gut microbiome dysbiosis contributes to the symptoms of GWI. Second, it is the first characterization of the effect of a life-style risk factor–a diet rich in fat–on the alterations in the gut microbiome caused by PER + PB. Life-style risk factors that contribute to poor health could play important roles in extending the duration and severity of the symptoms of GWI and may help explain how the symptoms of GWI persist for so long after PER and PB levels have dropped below detection in Gulf War Veterans[1]. Third, we show that the interaction between treatment with GWI agents and diet is significant, such that their combined effects on the gut microbiome are greater than either treatment alone. Fourth, the present study shows that the enhancement of gut microbiome dysbiosis by a HF in a model of GWI is reversible and leaves open the possibility that dietary modifications or other non-invasive treatments that alter the gut microbiome (e.g., probiotics, antibiotics) may provide relief from the symptoms of this chronic multi-system disorder.

Our study has three primary limitations. First, it is a molecular microbiology study without experiments designed to link gut microbiome alterations in a GWI model to changes in GI (e.g., leakiness, inflammation). Second, this project had a single post-treatment survival time; future experiments should include exposure to a HF for longer periods of time (e.g., 3–6 months) to evaluate the impact on severity and chronicity of GWI symptoms. Third, it cannot yet be determined if the observed effects of the GWI agents are due to direct effects on the gut microbiome or to indirect effects via modulation of the immune and/or nervous systems.

In summary, additional studies on the role of the gut microbiome in GWI are called for in light of emerging findings that significant enteric dysbiosis has been documented in Veterans with GWI as well as in animal models of this disorder. Each of the major symptom clusters of GWI has been linked individually to alterations in the gut microbiome so it is plausible that an altered gut microbiome could contribute to all major symptoms of this disorder. It is clear that the symptoms of GWI persist long after the toxicants to which military personnel were exposed in the Gulf War (e.g., PER and PB) have been removed from the body. Therefore, emphasis should also be placed on assessing various life-style risk factors for their ability to potentiate and/or extend the chronicity of the symptoms of GWI. There is no medically validated or effective treatment for GWI and if additional substantiation can be gathered for a role for gut microbiome dysbiosis, new and non-invasive therapies that target restoration of the gut microbiome in Veterans with GWI (e.g., probiotics, dietary interventions, fecal transplantation) could be tested as therapies.

Data availability

The MiSeq 16S rRNA gene sequence data generated in the current study will be made available upon request.

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Competing interests

The authors declare no competing interests.

Additional information

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CURRICULUM VITAE

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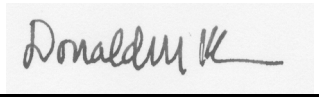

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National Heart, Lung, and Blood Institute
National Institutes of Health
Bethesda, MD

Feb 1986-Nov 1992 Director, Laboratory of Neurochemistry and
Director of Research
Lafayette Clinic
Detroit, Michigan

Aug 1993-Jun 1994 Visiting Professor
Department of Molecular Genetics and
Howard Hughes Medical Institute
University of Texas Southwestern Medical Center at Dallas, Texas

MAJOR PROFESSIONAL SOCIETIES

- American Society for Neurochemistry (ASN)
- American Society for Pharmacology and Experimental Therapeutics (ASPET)
- International Drug Abuse Research Society (IDARS)
- Society for Neuroscience (SFN)
- Federation of American Societies for Experimental Biology (FASEB)
- National Neurotrauma Society (NNTS)
- International Behavioral Neuroscience Society (IBNS)

HONORS AND AWARDS

- National Research Service Award, National Heart Lung and Blood Institute (Sponsor: Dr. Walter Lovenberg), 1976

- Mead-Johnson American College of Neuropsychopharmacology (ACNP) Travel Award, San Diego, California, 1981

- Merck, Sharpe and Dohme Visiting Scholar, Department of Medicine (Host: Dr. J. Chalmers), Flinders Medical Center, Bedford Park, South Australia 5042, 1982

- Vector Laboratories Outstanding Young Investigator Award in Neurochemistry, 1983

- FASEB Travel Award, IUPHAR International Congress of Pharmacology London, England, 1984

- Alexander von Humboldt Fellow, J.W. Goethe University (Host: Prof. Dr. H. Zimmermann), Frankfurt am Main, Federal Republic of Germany, Member of AvH 1985- present

- Wayne State University, Neuroscience Research Award, 1986

- Research Career Scientist, Department of Veterans Affairs, 2006-present

- Research Excellence Award 2012, Wayne State University School of Medicine

- Federal Employee Recognition Award, 2017, Detroit Federal Executive Board

LICENSES

- Drug Enforcement Administration Schedule 1 Controlled Substances
- Drug Enforcement Administration Schedule 2, 2N, 3, 3N, 4, and 5 Controlled Substances
- State of Michigan Schedule I Controlled Substance License
- State of Michigan Research Laboratory Controlled Substance License

SERVICE

Wayne State University

Department of Psychiatry and Behavioral Neurosciences

- Executive Education Committee (EEC) Ex-Officio as Graduate Officer, CCN
- Executive Research Directors Committee (ERDC) Ex-Officio consultant as Graduate Officer, Cellular and Clinical Neurobiology PhD program
- Graduate Officer and Chairman of the Graduate Committee, Cellular and Clinical Neurobiology PhD Program, Department of Psychiatry, Wayne State University, Jan. 1996- Jan. 1998.
- Faculty Promotion and Tenure Committee
  Elected member, Sep 1997 -Sep 2001
  Re-elected Jan 2006- 2010
  Re-elected Mar 2016-present
- Masters of Science in Psychiatry Program
  Ex-Officio member of Program Committee
  Ex-Officio member of Graduate Program
- Protocol Review Committee, Appointed member Oct. 1999 to 2000
- Departmental Leadership Committee, Basic Scientist Representative, Jan. 2012 to present
- Committee to Engage Medical Students and Undergraduates in Departmental Activities (Chair), June 2012-present
- Departmental Research Committee, Nov. 2011-present
- Asselin Award Committee, June 2012-present
- Departmental Publications Committee, July 2012-present
- Departmental New Investigator Research Grants Committee (Co-Chair), Oct. 2012-present
- Departmental Chair's Committee on Funding Innovative Pilot Projects, Dec. 2012-present
- Translational Neuroscience Program (PhD), Steering Committee, Jun. 2014- present
- Translational Neuroscience Program Assessment and Performance, Wayne State University Compliance Assist, Office of the Provost, June 2016-present
- Psychiatry Resident's Summer Seminar Program, Course Director and Lecturer, May 2016-present
- Departmental Faculty Search Committee October 2018- present

School of Medicine

- Wayne State University School of Medicine, Interdisciplinary Biological Sciences PhD Program Executive Committee (Departmental representative)
- Member, IBS Systems Biology Curriculum Subcommittee
- Director, Neurosciences Component of the Systems Biology Course
- Member, School of Medicine Task Force on Graduate Assistantships
- Member (Appointed by Dean of the School of Medicine), Chair Search Committee for the Department of Psychiatry and Behavioral Neurosciences, 2003
- Member (Elected by faculty), Department of Psychiatry & Behavioral Neurosciences Committee, 5-Year Departmental Review, 2007.

- Wayne State University Department Faculty Developmental Liaison Group (Departmental Representative), March 2012-present
- Member (elected) of the Wayne State University School of Medicine Hearing Panel, Office of the Dean, Aug. 2016 – Aug 2017
- Chair, School of Medicine Departmental 5 Year Review, Department of Pathology

University

- Member, OVPR Research Focus Group for Development of School of Medicine Strategic Plan
- Member, Wayne State University Division of Laboratory Animal Resources (DLAR) Advisory Panel, Feb. 2015- present
- Search Committee, Wayne State University, Office of the Vice President for Research, Division of Laboratory Animal Resources Attending Veterinarian Candidate Search Committee, Oct, 2016- Nov. 2017
- Member, Wayne State/VA Joint Committee on Human and Animal Research Activities, Mar. 2018- present

Affiliate Medical Organizations

- Member, John D. Dingell VA Medical Center R&D Committee
  Member, January 2001-January 2002
  Chair, January 2002 to January 2004
  Member, January 2005 to June 2014
  Chair, June 2014 to January 2017
- Member, John D. Dingell VA Medical Center Research Review Committee
  Chair, June 2012 - June 2014
  Member, June 2014 - June 2018
  Chair, August 2018 - present
- Member, John D. Dingell VA Medical Center Search Committee for Assistant Chief of Staff, Research & Development Service, Sep 2016- Jan 2017
- Assistant Chief of Staff, Research & Development Service (Acting), Jan 2017- Feb. 2018
- Deputy Assistant Chief of Staff, Research & Development Service, Mar 2018-present
- Member of the Board, The Metropolitan Detroit Research and Education Foundation (MDREF; VA), May 2017-present
- Member. Clinical Executive Committee, John D. Dingell VA Medical Center, Jan. 2017-Feb. 2018
- Member, Affiliation Partnership Council, John D. Dingell VA Medical Center, Jan. 2017-present

Scholarly Service

Grant Review Committees

- Ad hoc reviewer for the Neurosciences Research Review Committee of the National Institutes of Mental Health and for the Behavioral and Neurosciences Review Committee of the National Institutes of Health (1985).

- Ad hoc reviewer for the Program in Neural Mechanisms of Behavior and for the Program for Developmental Neuroscience of the National Science Foundation (1988).

- Ad hoc reviewer for the Drug Abuse Biomedical Research Review Committee Pharmacology II Subcommittee (DABR3), National Institute on Drug Abuse (1992-1995).

- Full member of the Drug Abuse Biomedical Research Review Committee NIDA-C, National Institute on Drug Abuse (1994-1998).

- Ad hoc reviewer for the National Institute on Alcohol Abuse and Alcoholism, Office of Scientific Affairs, Contract Review Unit (1995-1998).

- Ad hoc reviewer of scientific grant applications for the Medical Research Council of Canada and for the Netherlands Organization for Scientific Research, Council for Medical and Health Research (Nov. 1999).

- Ad hoc reviewer for Neurological Sciences and Disorders B (NSD-B), National Institute of Neurological Disorders and Stroke (Aug. 2000- Aug. 2002).

- Full member of Molecular, Developmental, and Cellular Neuroscience-4 (MDCN-4) Study Section, Center for Scientific Review, NIH (Feb. 1998-June 2002).

- Full member, Integrative, Functional, and Cognitive Neuroscience (IFCN-7) Study Section (Feb. 2002- Feb. 2006).

- Full member, American Federation for Aging Research Scientific Board (Dec. 2001-Dec. 2004).

- Reviewer, Alzheimer's Association Grant Review Committee (Mar. 2002-Mar. 2004).

- Ad hoc reviewer, Integrative, Functional, and Cognitive Neuroscience (IFCN-4) Study Section (June 2002- June 2004).

- Full member, Neurobiology-A Merit Review Subcommittee, Department of Veterans Affairs (June 2004- June 2008).

- Ad hoc reviewer, Special Emphasis Panel NIMH ZMH1 BRB-S, Molecular Markers and Mechanisms of HIV-Associated Dementia, National Institute on Mental Health (July 2004).

- Reviewer, Agency for Science, Technology & Research, Biomedical Research Council (Singapore), Extramural Grant Program (June 2004).

- Reviewer, Philip Morris External Research Program (July 2005-Nov. 2007)

- Ad hoc reviewer, Special Emphasis Panel NIMH ZMH1-ERB-Y, ADHD and Long-Term Psychostimulant Therapy (March 2005).

- Ad hoc reviewer, Neurobiology of Motivated Behavior (NMB) Study Section (June 2005- June 2006).

- Ad hoc reviewer, NIMH-ERB-L-04, Silvio Conte Centers for Depression and Anxiety (Feb. 2006).

- Ad hoc reviewer and Committee Chair, MDCN-L 02S, Biophysics and Neuronal Processes 1 (Apr. 2006).

- Full member, Neurobiology of Motivated Behavior (NMB) study section (June 2006-June 2010)

- Ad hoc reviewer, NIMH-ERB-L-03, Silvio Conte Centers for Collaborative Neuroscience Research (Mar 2007)

- Full member and Deputy Chair, ZRG1 MDCN-E, Review of Neuroscience AREA-R15 Grant Applications (Nov. 2011- Nov. 2019; Chair Feb. 2020 - present)

- Ad hoc reviewer, ZRG1 IFCN H 02M, Member conflict reviews (Jan. 2012)

- Full member, Department of Veterans Affairs, RRDB 1, Brain Injury (Dec. 2011-Dec. 2013)

- Ad hoc reviewer, ZRG1 BBBP-J 92 study section (Sep. 2012)

- Ad hoc reviewer, ZDA1 GXM-A (14) 1 study section to review NIDA CEBRA grants (Nov. 2012)

- Ad hoc reviewer, ZDA1 SXC-E (13), NIDA Cutting-Edge Basic Research Awards (CEBRA) grant application online IAR review (Mar. 2013)

- Ad hoc reviewer, ZDA1 MXL-F (08) 1, NIDA EUREKA proposal telephone review (Jul. 2013)

- Ad hoc reviewer, ZDA1 SXC-E (13), NIDA Cutting-Edge Basic Research Awards (CEBRA) grant application online IAR review (Apr. 2015)

- Ad hoc reviewer, Department of Veterans Affairs, RRD6 Aging & Neurodegenerative Diseases Merit Review Panel (Aug 2016-present)

- Ad hoc reviewer, National Science Center, Poland, Panel NZ7- Influence of New Psychoactive Drugs, grant application online review, Oct 2016

- Ad hoc reviewer, ZRG1 IFCN-L (56), NIDA Synthetic Psychoactive Drugs and Strategic Approaches to Counteract Their Deleterious Effects Review Panel, Nov. 2017

-Ad hoc reviewer, Department of Veterans Affairs, RRD8, Career Development Program Panel 1, telephone reviewer, Aug. 2019- present.

- Ad hoc reviewer, Department of Veterans Affairs, RRD7, Research Career Scientist Award Applications, Aug. 2020- present.

Service for Peer Reviewed Journals Journal/Editorial Activity

- Editorial Board Membership
  Journal of Neurochemistry (1998-2010)
  Neurochemistry International (1984-1994)
  Pteridines (1988-1995)

- Review of Manuscripts
  Behavioural Brain Research
  Biological Psychiatry
  Brain Research
  Brain Research Bulletin
  Depression and Anxiety
  Drug and Alcohol Dependence
  European Journal of Pharmacology
  Experimental Neurology
  FASEB Journal
  FEBS Letters
  Free Radical Biology and Medicine
  Journal of Biological Chemistry
  Journal of Pharmacology and Experimental Therapeutics
  Journal of Neurochemistry
  Journal of Neuroinflammation
  Journal of Neurological Sciences
  Journal of Neurotrauma
  Journal of Neuroscience
  Journal of Neuroscience Research
  Molecular Neurobiology
  Molecular Pharmacology
  Neurobiology of Disease
  Neuropsychopharmacology
  Neuroscience
  Neurotoxicology
  Neurotoxicology and Teratology
  Pharmacology, Biochemistry and Behavior
  Psychopharmacology
  Synapse

Other Service

- Councilor, Michigan Society for Neuroscience Chapter, Wayne State Representative, Sep. 2000- Sep 2002

TEACHING

Years at Wayne State University: 30

Years at other universities
- Princeton University: 1 (Postdoctoral Fellow; Dr. B. Jacobs)
- The George Washington University: 6 (Adjunct Faculty while member of NIH Intramural Research Program)
- J.W. Goethe University (Frankfurt, Germany): 1 (Alexander von Humboldt Fellow; Dr. H. Zimmermann)
- University of Texas, Southwestern Medical Center at Dallas: 1 (Sabbatical; Dr. T. Sudhof)

Teaching at Wayne State (Graduate students)

- PYC 701- Neurobiology I: Lectures on Neurotransmitter Release, Synaptic Morphology, and Serotonin Neurochemistry.
- PYC 751- Neurochemical Pharmacology of Monoamine Neurons: Lectures on Protein Biochemistry and Physiological Regulation of Tyrosine Hydroxylase, Protein Biochemistry and Physiological Regulation of Tryptophan Hydroxylase, and Physiological Definition of Serotonin Neuronal Systems.

- PYC 756- Advanced Topics in Behavioral Pharmacology: Course Leader and Coordinator with lectures on operant control of behavior and the behavioral analysis of drug action, and behavioral and biochemical models of psychiatric diseases.

- PHC 750- Neuropharmacology I: Serotonin Neurochemistry and Neuropharmacology. Department of Pharmacology, Wayne State University School of Medicine.

- IBS 7050- Systems Biology-Neurobiology- Two credit hour course taught as part of the combined interdisciplinary biomedical curriculum in all School of Medicine PhD programs.

- PYC 7010- Molecular Neuropsychopharmacology- Lectures on pre-synaptic organization, essential elements of exocytosis and endocytosis, and vesicle structure; lectures on genetic polymorphisms and microarrays in neuropsychopharmacology.

- PYC 760 – Advanced topics course on emerging concepts in Parkinson's Disease and other neurodegenerative conditions with a focus on microglial activation and mediation as a cause of neuronal damage.

- PYC 7595 - The Gut Microbiome and Translational Neuroscience- starting Fall 2020 semester

(Course director M. Angoa-Perez; co-director D.M. Kuhn)

Teaching at Wayne State (Residents/Fellows)

- Psychiatry Resident's Summer Seminar Program, 2016-present

Mentorship

Name	Status	Dates	WSU/VA	Clinical or Basic Research	Current Position or Activity
William A. Wolf	Predoctoral	1981-1985 (PhD)	WSU	Basic	Hines VAMC and Adjunct Professor, Department of Anatomy & Cell Biology, University of Illinois at Chicago, Chicago, IL
Patricia A. Johanson	Predoctoral (F31 funded)	1990-1993 (PhD)	WSU	Basic	Senior Clinical Publications Lead, AstraZeneca Pharmaceuticals, Philadelphia, PA
Carroll M. D'Sa	Predoctoral	1994-1996 (PhD)	WSU	Basic	Business Systems Analyst, Yale Center for Clinical Investigation, Yale University School of Medicine, New Haven, CT
Krishnamoorthy Sankaran, PhD	Postdoctoral	1989-1991	WSU	Basic	Head Chemist, City of Detroit, Dept. Water and Sewerage, Detroit, MI
Ulrike Berresheim, MD	Postdoctoral	1990-1991	WSU	Basic	Private medical practice, Anesthesiology and Pain Management, St. Ulrich a.P., Tirol, Austria
Ellen Zaija, MD	Postdoctoral	1990-1991	WSU	Basic	Private medical practice, Radiation Oncology, Milwaukee, WI
William A. Wolf, PhD	Postdoctoral	1990-1992	VA	Basic	Hines VAMC and Adjunct Professor, Department of Anatomy and Cell Biology, University of Illinois at Chicago, Chicago, IL
Barbara Gibbs, PhD	Postdoctoral	1990-1991	WSU	Basic	Senior Patent Attorney, Office of Technology Commercialization, Purdue University, West Lafayette, IN
Panos Z. Anastasiadis, PhD	Postdoctoral	1994-1996	WSU	Basic	Professor of Cancer Biology (Tenured), Mayo Clinic, Jacksonville, FL
Samuel U. Park	Predoctoral (F31 funded)	1999-2007	WSU	Basic	
Cheryl W. Aretha, PhD	Postdoctoral (F32 funded)	1998-2000	WSU	Basic	Professor, Biology Department, Macomb Community College, Macomb, MI
Mark Ritter, MD	Postdoctoral	2000-2001	WSU	Basic/Clinical	Resident, Psychiatry & Internal Medicine, WSU School of Medicine
Mahdieh Sadidi	Predoctoral	1999-2004 (PhD)	WSU	Basic	Postdoctoral Fellow, Michigan State University, East Lansing, MI
Stacey (Sakowski) Jacoby	Predoctoral	2000-	WSU	Basic	Deputy Managing Director, Alfred Taubman Medical

		2006 (PhD)			Research Institute, University of Michigan SOM, Ann Arbor, MI
David M. Thomas, PhD	Postdoctoral and NIH KO1 mentor	2002-2005	VA	Basic	Professor (Tenured), Department of Biological Sciences and Assistant Dean for Medical Education, Oakland University William Beaumont School of Medicine, Rochester Hills, MI
Pamela VandeVord, PhD	Mentor on VA Career Dev. Award	2007-present	VA	Basic	Professor (Tenured), School of Biomedical Engineering and Sciences, Virginia Polytechnic Institute & State University, Blacksburg, VA
Alana Conti, PhD	Mentor on NIH KO1	2012-2014	WSU/VA	Basic	Associate Professor (Tenured), Department of Neurosurgery, WSU School of Medicine
Michael J. Kane, PhD	Postdoctoral	2011-2013	WSU/VA	Basic	Adjunct Assistant Professor, Neuroscience Program, Temple University, Philadelphia, PA
Mariana Angoa-Perez, PhD	Postdoctoral	2009-present	WSU/VA	Basic	Postdoctoral Research Associate, WSU School of Medicine
Nieves Herrera-Mundo, PhD	Postdoctoral	2012-2014	WSU/VA	Basic	Postdoctoral Fellow, Biological Sciences, National Autonomous University of Mexico, Mexico City MX
John H. Anneken, PhD	Postdoctoral	2013-present	WSU/VA	Basic	Postdoctoral Research Associate, WSU School of Medicine
Denise I. Briggs, PhD	Pre- and Postdoctoral	2012-2016	WSU/VA	Basic	PhD, May 2016, Department of Neurosurgery, Stanford University School of Medicine
John A. Rotondo	Predoctoral	2014-2015	WSU/VA	Basic	Student in MD/PhD program, WSU School of Medicine
Denise I. Briggs, PhD	Pre- and Postdoctoral	2012-2016	WSU/VA	Basic	PhD, May 2016, Department of Neurosurgery, Stanford University School of Medicine

Theses and Dissertations directed

- William A. Wolf, PhD dissertation, Studies on the Mechanisms which Regulate Serotonin Release, Department of Pharmacology, The George Washington University School of Medicine, June 1985.

- Patricia J. Johansen, PhD dissertation, Activation and Phosphorylation of Brain Tryptophan Hydroxylase by Protein Kinases, Cellular and Clinical Neurobiology Program, Wayne State University School of Medicine, August 1993.

- Carrol D'Sa, PhD dissertation, Regulation of Brain Tryptophan Hydroxylase, Cellular and Clinical Neurobiology Program, Wayne State University School of Medicine, July 1998.

- Mahdieh Sadidi, PhD dissertation, Molecular Footprints of Neurotoxicity: Posttranslational Modifications of Tyrosine Hydroxylase, Cellular and Clinical Neurobiology Program, Wayne State University School of Medicine, Dec. 2004.

- Stacey Sakowski, PhD dissertation, Biochemistry and Molecular Biology of Tryptophan Hydroxylase, Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, June, 2006.

- Denise I. Briggs, PhD Dissertation, Cognitive, Psychiatric and Neuropathological Outcomes of Repetitive Mild Traumatic Brain Injury, Translational Neuroscience Program, Wayne State University School of Medicine, PhD March 2016.

- John Rotondo, MS, Basic Medical Sciences, Wayne State University School of Medicine, 2014.

- Charles Fisher, MS, Basic Medical Sciences, Wayne State University School of Medicine, 2014.

- David Shaheen, MS, Basic Medical Sciences, Wayne State University School of Medicine, 2014.

- Julia Solarewicz, MS, Department of Physiology, Wayne State University School of Medicine, 2015

- Alhassan Dhia, MS, Basic Medical Sciences, Wayne State University School of Medicine, 2014.

- Helen Wu, MD/PhD Program, PhD Dissertation Committee member, Translational Neuroscience Program, Wayne State University School of Medicine, PhD May 2016.

- Muzamil Arshad, MD/PhD Program, PhD Dissertation Committee member, Translational Neuroscience Program, Wayne State University School of Medicine, PhD August 2016.

- Hamilton Trinh, M1 Honors Student thesis, Wayne State University School of Medicine, 2016.

- Krithika Muthkumaran, Department of Chemistry and Biochemistry, University of Windsor, External PhD Dissertation Examiner Sep 2016.

- Andrew Neff, Translational Neuroscience PhD Program, Dissertation Committee member, Wayne State University School of Medicine, PhD March 2018.

GRANTS, CONTRACTS, AND OTHER FUNDING

Active National/International Grants and Contracts

Role: Principal Investigator, Percent effort 20%, IK6RX002419
Title: Research Career Scientist Award
Source: Department of Veterans Affairs (VA) Rehab R&D
2006-2023
Total direct costs:

Role: Principal Investigator, Percent effort 20%, I01RX000458
Title: Role of TPH2 and 5HT Neuronal Loss in Non-motor Symptoms of Parkinson's
Source: Department of Veterans Affairs (VA) Rehab R&D Merit Award
2016-2020
Total direct costs: $1,080,000

Role: Co-Principal Investigator, Percent effort 5%, PI: Jason Mateika
Title: 5HT modulation of arousal and chemoreflex responses in intact and SCI mice
Source: Department of Veterans Affairs (VA) Rehab R&D Merit Award
2018-2022
Total direct costs: $980,000

Role: Principal Investigator, Percent effort 5%, IS1BX004395
Title: ShEEP Request for an Illumina MiSeq System
Source: VA Office of Research & Development, Shared Equipment Award Program
2018-2019
Total direct costs: $117,000

Role: Principal Investigator, Percent effort 20%, I01BX004340
Title: Delayed and Progressive Emergence of CTE- and Psychiatric-like Pathologies after Repetitive Mild TBI
Source: Department of Veterans Affairs (VA) Basic Laboratory R&D Merit Award
2019-2023
Total direct costs: $940,000

Role: Principal Investigator, Percent effort 20%, GW170034
Title: Gulf War Illness and Gut Microbiome Dysbiosis: Treatment with Probiotics and Fecal Transplantation
Source: Department of Defense, Congressionally Directed Medical Research Program
2019-2021
Total direct costs: $230,000

Role: Principal Investigator, Percent effort 20%, 1I01BX004757-01A1
Title: Gulf War Veterans' Illnesses: Symptom Chronicity via Interactions of Diet and Lifestyle Risk Factors with the Gut Microbiome
Source: Department of Veteran's Affairs (VA), Basic Laboratory R&D Merit Award

2020-2024
Total direct costs: $1,368,788

Pending National/International Grants and Contracts

Role: Principal Investigator, Percent effort 20%, R21DA048191
Title: Synthetic Psychoactive Cathinones and the Gut Microbiome: Potential Target to Counteract Drug Deleterious Effects
Source: NIH, National Institute on Drug Abuse
2020-2022
Total direct costs: $275,000

Role: Principal Investigator, Percent effort 25%, R21DA049548
Title: The Gut Microbiome Influences Cocaine and Heroin Self Administration, Extinction and Relapse
Source: NIH, National Institute on Drug Abuse, Cutting Edge Biological Research Application
2019-2021
Total direct costs: $275,000

Previously funded Grants and Contracts

Role: Principal Investigator, Percent effort 20%, R21DA034692
Title: βeta-ketoamphetamines: Window to the Neurotoxic Mechanisms of Methamphetamine
Source: NIH/NIDA Cutting Edge Basic Research Award
2015-2018
Total direct costs: $230,000

Role: Principal Investigator, Percent Effort: 100%, F32 HL0245
Title: Control Mechanisms for Serotonin Synthesis in Brain
Source: NIH/NHLBI
1976-1978
Total direct costs: $65,000

Role: Principal Investigator, Percent effort: 100%, NHLBI
Title: Intramural Research Program, Section on Biochemical Pharmacology, National Heart Lung & Blood Institute, National Institutes of Health
Source: NIH/NHLBI
1978-1986
Total direct costs: ~$900,000 (NIH Intramural funding)

Role: Principal Investigator, Percent effort: 10%
Title: Small Grant in Neurosciences Award
Source: Wayne State University School of Medicine
1986-1987
Total direct costs: $10,000
Role: Mentor, Percent effort: 5%

Title: Office of the Dean of the Medical School, Dean's Postdoctoral Recruiting Award (PI: Dr. W.A. Wolf)
Source: Wayne State University School of Medicine
1989-1990
Total direct costs: $20,000
Role: Principal Investigator, Percent Effort 20% R03 MH02365
Title: Tryptophan Hydroxylase: Purification and Production of Antibodies
Source: NIH/NIMH
1989-1990
Total direct costs: $31,000

Role: Principal Investigator, Percent effort 20%
Title: Differential Loss of Tyrosine Hydroxylase from the Striatum in Parkinson's Disease
Source: United Parkinson Foundation
1990-1991
Total direct costs: $25,450

Role: Principal Investigator, Percent effort 20%, R01 DA006219
Title: Cocaine and Serotonin Neurochemistry
Source: NIH/NIDA
1991-1995
Total direct costs: $1,006,004

Role: Principal Investigator, Percent effort 5%
Title: Small Instrumentation Grant Program
Source: Alcohol, Drug Abuse, and Mental Health Administration (administered through Wayne State University School of Medicine
1991-1992
Total direct costs: $21,000

Role: Mentor, Percent effort 5%
Title: Office of the Dean of the Medical School, Dean's Postdoctoral Recruiting Award (PI: Dr. B. Gibbs)
Source: Wayne State University School of Medicine
1992-1993
Total direct costs: $20,000

Role: Mentor, Percent effort 5%, F31 MH010230 National Research Service Award (Predoctoral)
Title: Tryptophan Hydroxylase: Regulation by Protein Kinases (PI: Patricia Johansen)
Source: NIH/NIMH
1992-1994
Total direct costs: $23,000

Role: Principal Investigator Percent effort 20%, R55 NS030833 (Shannon Award)
Title: Regulation of Brain Tryptophan Hydroxylase

Source: NIH/NINDS
1992-1995
Total direct costs: $300,000

Role: Principal Investigator, Percent effort 10%,
Title: Amphetamine Neurotoxins, 5-HT Neurons, and Nitric Oxide
Source: Wayne State University Office of Neuroscience Programs GETIN Grant
1993-1994
Total direct costs: $25,000

Role: Principal Investigator, Percent effort 5%
Title: Depression and Defects in Serotonin Neurochemistry
Source: Department of Psychiatry and Behavioral Neurosciences, Joe Young Sr. Research Grant
1994-1995
Total direct costs: $18,500

Role: Principal Investigator, Percent effort 5%
Title: Targeted Disruption of the Gene for Tryptophan Hydroxylase: Production of a Serotonin Deficient Knock Out Mouse as a Model for Psychiatric Disease
Source: Department of Psychiatry and Behavioral Neurosciences, Joe Young Sr. Research Grant
1994-1995
Total direct costs: $15,000

Role: Principal Investigator, Percent effort 10%
Title: Genetic Modification of Human Fibroblasts to Express Tyrosine Hydroxylase: Development of a Graft for Gene Therapy of Parkinson's Disease
Source: National Parkinson Foundation
1995-1997
Total direct costs: $80,000

Role: Principal Investigator, Percent effort 5%
Title: Neurotoxic Amphetamines, Radicals & 5HT Neurons
Source: NIH/NIEHS Center Grant (Center for Molecular and Cellular Toxicology with Human Applications Pilot Project; PI-Raymond F. Novak)
1996-1997
Total direct costs: $10,000

Role: Mentor, Percent effort 5%
Title: Office of the Dean of the Medical School, Dean's Postdoctoral Recruiting Award (PI: Dr. C. Aretha)
Source: Wayne State University School of Medicine
1999-2000
Total direct costs: $20,000

Role: Principal Investigator (Mentor), Percent effort 5%, R13 Conference Grant
Title: Neurotoxicity of Amphetamines and Related Stimulants

Source: NIH/NIDA and American Society for Pharmacology and Experimental Therapeutics
1999-2000
Total direct costs: $25,000

Role: Principal Investigator, Percent effort 10%
Title: Tyrosine hydroxylase as a cytotoxic protein in Parkinson's disease
Source: Parkinson's Disease Foundation
1999-2000
Total direct costs: $35,000

Role: Principal Investigator, Percent effort 5%
Title: Neuroprotective properties of pramipexole
Source: Pharmacia-Upjohn
1999-2000
Total direct costs: $20,000

Role: Principal Investigator, Percent effort 20% I01
Title: Neurotoxic Amphetamines, Proto-oncogenes, and Apoptosis
Source: Department of Veterans Affairs (VA) Basic Laboratory R&D Merit Award
1998-2002
Total direct costs: $592,000

Role: Principal Investigator, Percent effort 5%
Title: Paraquat, Dopamine-quinones & Parkinson's Disease
Source: NIH/NIEHS Center Grant (Center for Molecular and Cellular Toxicology with Human Applications Pilot Project; PI-Raymond F. Novak)
2000-2001
Total direct costs: $20,000

Role: Principal Investigator, Percent effort 10%
Title: Serotonin Knockout Model of Neurodevelopmental Disorders
Source: Children's Research Center of Michigan, Children's Hospital of Michigan, Wayne State University School of Medicine
2000-2002
Total direct costs: $50,000

Role: Mentor, Percent effort 5% F32 National Research Service Award (Postdoctoral)
Title: Molecular Markers of Methamphetamine Neurotoxicity (PI: Dr. C. Aretha)
Source: NIH/NIDA
2000-2002
Total direct costs: $30,000

Role: Principal Investigator, Percent effort 20%, R01 MH057743
Title: PKC Signaling and the Treatment of Bipolar Disorder
Source: NIH/NIMH
2000-2003

Total direct costs: $489,000

Role: Mentor, Percent effort 5% F31 DA006067 National Research Service Award (Predoctoral)
Title: The Role of Dopamine in Methamphetamine Toxicity (PI: Samuel Park)
Source: NIH/NIDA
2000-2003
Total direct costs: $58,000

Role: Principal Investigator, Percent effort 20%, R01 DA013753
Title: Microarray Analysis of Human Cocaine Addicts
Source: NIH/NIDA
2000-2004
Total direct costs: $800,000

Role: Principal Investigator, Percent effort 10%, T32 DA007310
Title: Neuroscience Training in Drug Abuse Training Grant
Source: NIH/NIDA
2000-2006
Total direct costs: $954,000

Role: Principal Investigator, Percent effort 20%, K05 DA014692
Title: Molecular Neurobiology of Drug Abuse Senior Scientist Career Development Award
Source: NIH/NIDA
2002-2007
Total direct costs: $600,000

Role: Principal Investigator, Percent effort 20%
Title: Microglia as Primary Mediators of Nerve Agent Neuropathy
Source: Department of the Army, Medical Chemical and Biological Defense Research Program
2006-2008
Total direct costs: $220,000

Role: Principal Investigator, Percent effort 20%, I01
Title: Brain Injury by Blast Overpressure: Role of Microglial Activation
Source: Department of Veterans Affairs (VA) Basic Laboratory R&D Merit Award
2007-2011
Total direct costs: $520,000

Role: Principal Investigator, Percent effort 20%, R01 DA017327
Title: Methamphetamine Neurotoxicity and Microglial Activation
Source: NIH/NIDA
2005-2012
Total direct costs: $1,050,000

Role: Principal Investigator, Percent effort 20%, R01 DA010756
Title: Neurotoxic Amphetamines, Radicals & 5HT Neurons

Source: NIH/NIDA
2002-2013
Total direct costs: $1,815,000

Role: Principal Investigator, Percent effort 20%, I01 RX000375
Title: TBI & Alcohol Abuse: Co-occurring Conditions that Enhance Brain Damage
Source: Department of Veterans Affairs (VA) Rehab R&D Merit Award
2012-2016
Total direct costs: $1,000,000

PUBLICATIONS

Peer-Reviewed Publications

1. **Kuhn, D.M.**, Greenberg, I., and Appel, J.B. Differential effects on lever choice and response rate produced by d-amphetamine. Bull. Psychonom. Sci. 3, 119-120, 1974.

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41. Briggs, D., Angoa-Perez, M., and **Kuhn, D**. Studies of repetitive mild TBI: Animal model of sports-related head impact. Soc. Neurosci., 589.27, G37, 2015.

42. Anneken, J.H., Angoa-Perez, M., Sati, G., Crich, D., and **Kuhn, D.M.** Differential dopaminergic toxicity of bath salt intermediates in mice: Implications for the mechanism of methamphetamine toxicity. Soc. Neurosci., 317.05, K10, 2015.

43. Ghoddoussi, F., Briggs, D.I., **Kuhn, D.M.** and Galloway, M.P. Proton magnetic resonance spectroscopy assessment of the neurochemical profile in mice genetically depleted of brain serotonin. Soc. Neurosci., 218.10, 2016.

44. Khan, A.R., Hansen, B., Iversen, N.K., Olesen, J.L., Angoa-Perez, M., **Kuhn, D.M.**, Jespersen, S.N. Functional and microstructural alterations in the rat hippocampus after repetitive mild traumatic brain injury. International Society for Magnetic Resonance in Medicine, June 2018.

45. Kuhn, B.N., Roberts, A.T., Angoa-Perez, M., Zagorac, B., **Kuhn, D.M.**, and Kalivas, P.W. Individual variation in the vulnerability and resilience to opioid dependence. Soc. Neurosci., 417.16, 2019.

46. Kuhn, B.N., Cannella, N., Robers, A.T., Lunert, V., Ubaldi, M., Tambalo, S., Bifone, A., Angoa-Perez, M., Zagorac, B., Hardiman, G., Solberg-Woods, L., Kuhn, D.M., Ciccocioppo, R. and Kalivas, P. Individual variation in addition-related behaviors contributes to opioid dependence vulnerability. NIDA Genetics and Epigenetics Cross-Cutting Research Team (GECCRT) Meeting, January 2020.

Invited Lectures/Presentations (selected)

1. Microglial activation and drug-induced neurotoxicity: Nexus between HIV- and methamphetamine-induced neuronal damage, Invited lecture, NIMH/NIDA Conference on HIV and Substance Abuse, Bethesda, MD, March 2006.

2. Methamphetamine-induced neurotoxicity: Cross-talk between microglia and dopamine nerve endings reveals novel mechanisms of drug-induced neuronal damage, Invited lecture, Department of Pharmacology, Boston University School of Medicine, Boston, MA, May 2006.

3. Microglial activation as a specific marker for neurotoxicity, Invited platform lecture, Experimental Biology, Washington, DC, August 2006.

4. Microglial-neuronal crosstalk: How the innate immune system of the CNS is tricked into damaging neurons, Invited lecture, Department of Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, MI, March 2007.

5. Modulation of dopamine neuronal function by peroxynitrite: Dopamine as a molecular switch between nitrosative and oxidative stress, Invited lecture, Sun Health Research Institute, Phoenix, AZ, April 2007.

6. Microglial-neuronal crosstalk: How the innate immune system of the CNS is tricked into damaging neurons, Invited lecture, Department of Neuroscience, Medical University of South Carolina, Charleston, SC, May 2007.

7. Regulation of serotonin function by TPH2: Protein kinases and the UPP interact to determine enzyme stability, Invited lecture, Department of Pharmacology and Toxicology, Michigan State University School of Medicine, East Lansing, MI, Nov. 2008.

8. Role of microglial activation in drug-induced neurodegeneration, Invited platform lecture, Experimental Biology, New Orleans, LA, April 2009.

9. The role of non-neuronal cells and dopamine in drug-induced neurotoxicity, Invited lecture, Institute of Biomedical Investigations, National Autonomous University of Mexico, Mexico City, Mexico, Aug. 2009.

10. The brain without serotonin: Targeted deletion of the TPH2 gene uncovers a complex physiological and behavioral phenotype, Invited lecture, Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Detroit, MI, Nov. 2009.

11. Role of dopamine and non-neuronal cells in methamphetamine-induced neurotoxicity, Invited lecture, Oregon Health Sciences University and Portland VA, Portland, OR, Mar. 2010.

12. Serotonin and psychiatric illness: New views from mice lacking TPH2, Invited lecture, Institute of Environmental Health Sciences, Wayne State University, Detroit, MI, Nov. 2010.

13. TBI and alcohol comorbidity: Interactions that complicate long-term outcome, Invited Lecture, NIH/VA/DoD Interagency Conference on TBI, Washington, DC, Jun. 2011.

14. The emerging problem of repetitive mild traumatic brain injury: Basic research perspectives and challenges, Invited Lecture, Michigan Psychiatric Society, Lansing, MI, Nov. 2012.

15. Animal models of sports-related head injury: Bridging the gap between preclinical research and clinical reality, Invited lecture, Department of Neurosciences, University of Toledo College of Medicine and Life Sciences, Toledo, OH, Dec. 2013.

16. Repetitive mild TBI: Challenges for investigation, detection and treatment, Invited lecture, Department of Neurology, Henry Ford Health System, Detroit, MI, Feb. 2015.

17. Interactions between repetitive mild TBI and alcohol: Does intoxication alter neuropathological outcomes of head injury, Invited speaker, Research Society on Alcoholism, New Orleans, LA, Jun. 2016.

18. Life Without Brain Serotonin: A New Look at an Old Neurotransmitter, Invited seminar, Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario, Canada, Nov. 2016.

19. Synthetic Psychoactive ("bath salts") Drugs and Neurotoxic Amphetamines: Chemical Relatives with Very Different Modes of Action on the Brain, Invited Seminar, Department of Pharmacology, School of Pharmacy, University of Toledo, Toledo, OH, Feb. 2017.

20. Synthetic Psychoactive ("bath salts") Drugs and Neurotoxic Amphetamines: Chemical Relatives with Very Different Modes of Action on the Brain, Invited symposium speaker, International Behavioral Neuroscience Society Annual Meeting, Hiroshima, Japan, June 2017.

21. Gulf War Veterans' Illness: Symptom Chronicity via Interactions of Diet and Lifestyle Risk Factors with the Gut Microbiome. CDMRP-VA State of the Science Conference (Virtual) on Gulf War Illness, August 18-19, 2020.