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TITLE: VIPER: Chronic Pain after Amputation: Inflammatory Mechanisms, Novel Analgesic Pathways, and Improved Patient Safety

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14. ABSTRACT
Chronic pain is a significant problem after nerve injury from trauma or surgery. Current therapies and attempts at prevention have proven largely ineffective. Through analysis of data obtained in the Molecular Signatures of Chronic Pain Subtypes study termed Veterans Integrated Pain Evaluation Research (VIPER) (W81XWH-11-2-0003) we have discovered two novel pain pathways with potential therapeutic relevance (Wnt and TGR5). In addition, we recognize that improving the safety and efficacy of existing therapies must continue to be a priority and plan to use the large pharmacogenomic database at Vanderbilt University to identify patients at risk for adverse opioid related events. The current proposal intends to study the contribution of non-neuronal immune cells (macrophages) to chronic pain while also evaluating novel analgesics in relevant animal models. The current proposal also attempts to determine the optimal patient population for opioid therapy while identifying those patients at greatest risk from opioids.

15. SUBJECT TERMS
Post-amputation pain, Phantom limb pain, Residual limb pain, neuropathic pain, novel analgesics, opioid related adverse events.

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INTRODUCTION:
Chronic pain is a significant problem after nerve injury from trauma or surgery. Current therapies and attempts at prevention have proven largely ineffective. Through analysis of data obtained in the Molecular Signatures of Chronic Pain Subtypes study termed Veterans Integrated Pain Evaluation Research (VIPER) (W81XWH-11-2-0003) we have discovered two novel pain pathways with potential therapeutic relevance (Wnt and TGR5). In addition, we recognize that improving the safety and efficacy of existing therapies must continue to be a priority and plan to use the large pharmacogenomic database at Vanderbilt University to identify patients at risk for adverse opioid related events. The current proposal intends to study the contribution of non-neuronal immune cells (macrophages) to chronic pain while also evaluating novel analgesics in relevant animal models. The current proposal also attempts to determine the optimal patient population for opioid therapy while identifying those patients at greatest risk from opioids.

KEYWORDS:
Post-amputation pain, Phantom limb pain, Residual limb pain, neuropathic pain, novel analgesics, opioid related adverse events.

Major goals of this research project

Specific Aim 1: Characterize the role of *Wnt* signaling in macrophage polarization, mouse nerve injury models and human neuroinflammation

Major Task 1: Characterize macrophage polarization changes after Wnt signaling modification in mouse macrophage cell culture. **100% COMPLETE**

Major Task 2: Determine the specific wnt pathway responsible for prevention of mechanical allodynia in a mouse model of peripheral nerve injury and correlate this with macrophage polarization state and IL-6 to IL-10 ratio. **90% COMPLETE**

Major Task 3: Characterize wnt pathway expression and DNA methylation changes in humans before and after amputation and determine the role of cytokine ratio measurement in prediction of pain phenotype. **70% COMPLETE**

Specific Aim 2: Determine the role of TGR5 in astrocyte activation and treatment of mechanical allodynia in a mouse model of neuropathic pain.

Major Task 1: Determine role of TGR5 signaling in astrocyte activation **100% COMPLETE**

Major Task 2: Determine the role of TGR5 signaling in treating mechanical allodynia in a mouse peripheral nerve injury model **85% COMPLETE**

Specific Aim 3: Use existing data from the Vanderbilt EMR and genotyping repositories to look for associations between genetic variants and pain phenotypes

Major Task 1: Preliminary analyses conducted to confirm the precise numbers of patients for whom there are sufficient data available. Validation of previously published genotype-phenotype associations. **100% COMPLETE**

Major Task 2: Discovery and validation of novel exomic variants associated with opioid adverse drug events. **35% COMPLETE**

What was accomplished under these goals?

Overall Progress

We are progressing as expected with the organization, experiments and logistics of this project.

Specific Aim 1: Characterize the role of *Wnt* signaling in macrophage polarization, mouse nerve injury models and human neuroinflammation

Major Task 1: Characterize macrophage polarization changes after Wnt signaling modification in mouse macrophage cell culture. **100% Complete**

We repeated the macrophage stimulation experiment using known wnt pathway ligands and found, again, that the non-canonical wnt pathway ligand (wnt6) strongly favors M2 phenotype in these cells.

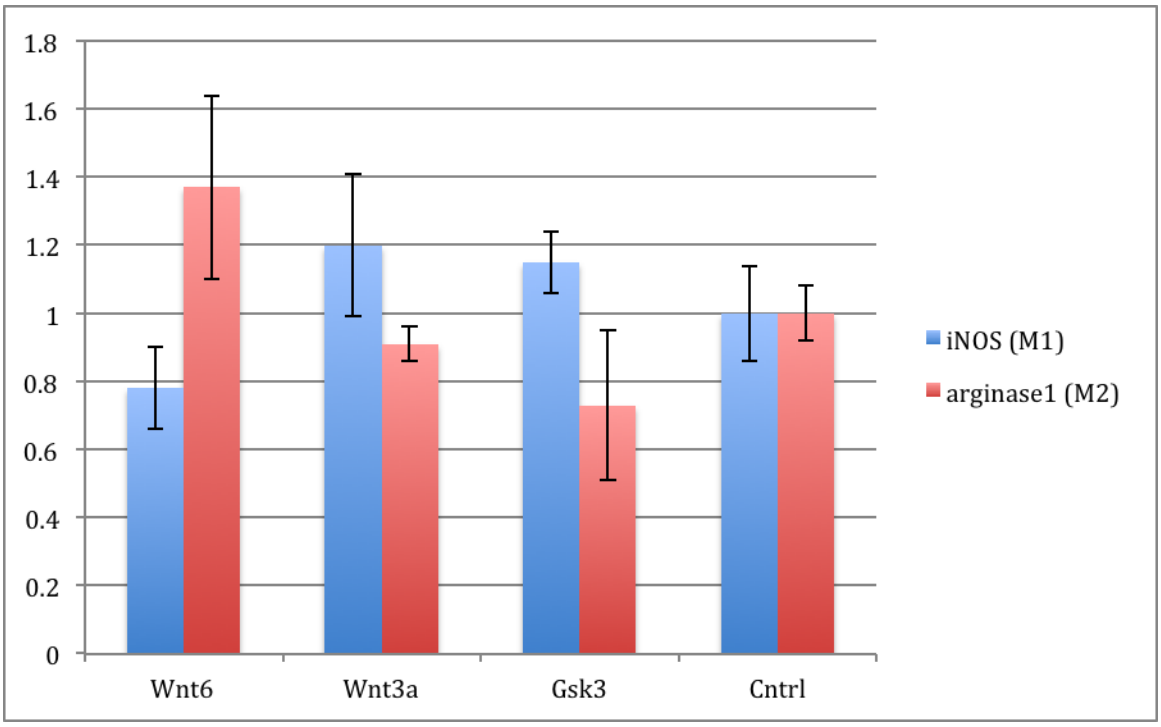


Figure 1.) Non-canonical wnt ligand Wnt6 favors M2 macrophage phenotype. Murine peritoneal macrophages were collected and cultured and treated with either wnt pathway ligands (wnt6 100ng/ml, wnt3a 100ng/ml, gsk3 inhibitor 100ng/ml or saline as control). RNA was collected and qPCR performed. Transcript levels were first normalized to GAPDH as a reference gene, and then to control for comparison to various treatments. All data are mean ± SEM (n=3, treatments and control).

Major Task 2: Determine the specific wnt pathway responsible for prevention of mechanical allodynia in a mouse model of peripheral nerve injury and correlate this with macrophage polarization state and IL-6 to IL-10 ratio. **85% COMPLETE**

We mice in the SNI model with intraperitoneal wnt agonist. Our first experiment had three groups: sham, SNI and SNI treated with wnt3a. The SNI and wnt3a groups both exhibited mechanical allodynia after surgery, but interestingly, by day 15 and 18, the wnt3a group appears to be recovering compared to the SNI group. The number of mice in each group was 5.

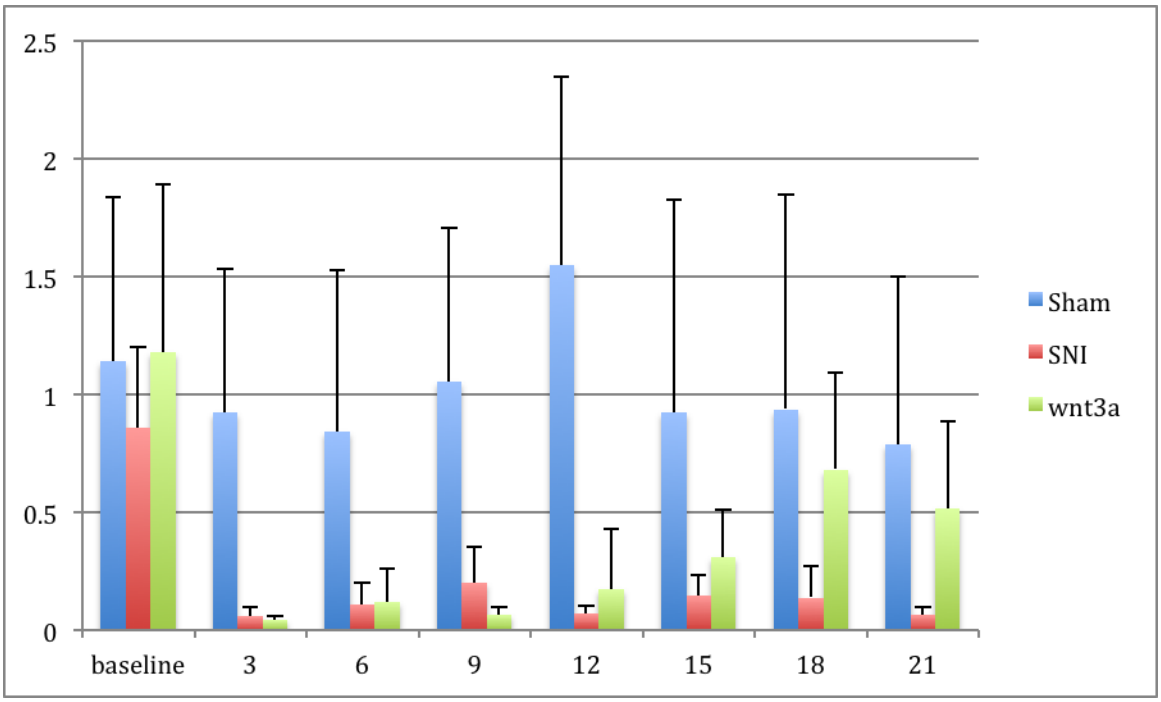


Figure 2: Mice either received sham surgery or SNI surgery. Three groups of 5 animals were tested. Sham animals maintained baseline withdrawal responses, SNI and wnt3a (100ng) treated animals developed significant allodynia with some recovery at the final timepoints. X-axis is time in days and y-axis is force in grams.

Mice treated with wnt3a in the SNI model showed late recovery from allodynia suggesting that wnt3a was able to reverse the late phase of mechanical allodynia development after peripheral nerve injury. We then did the same experiment with wnt5a and found no difference between the SNI group and the treated group suggesting that wnt5a does not play a role in preventing or treating mechanical allodynia (Figure 3 below).

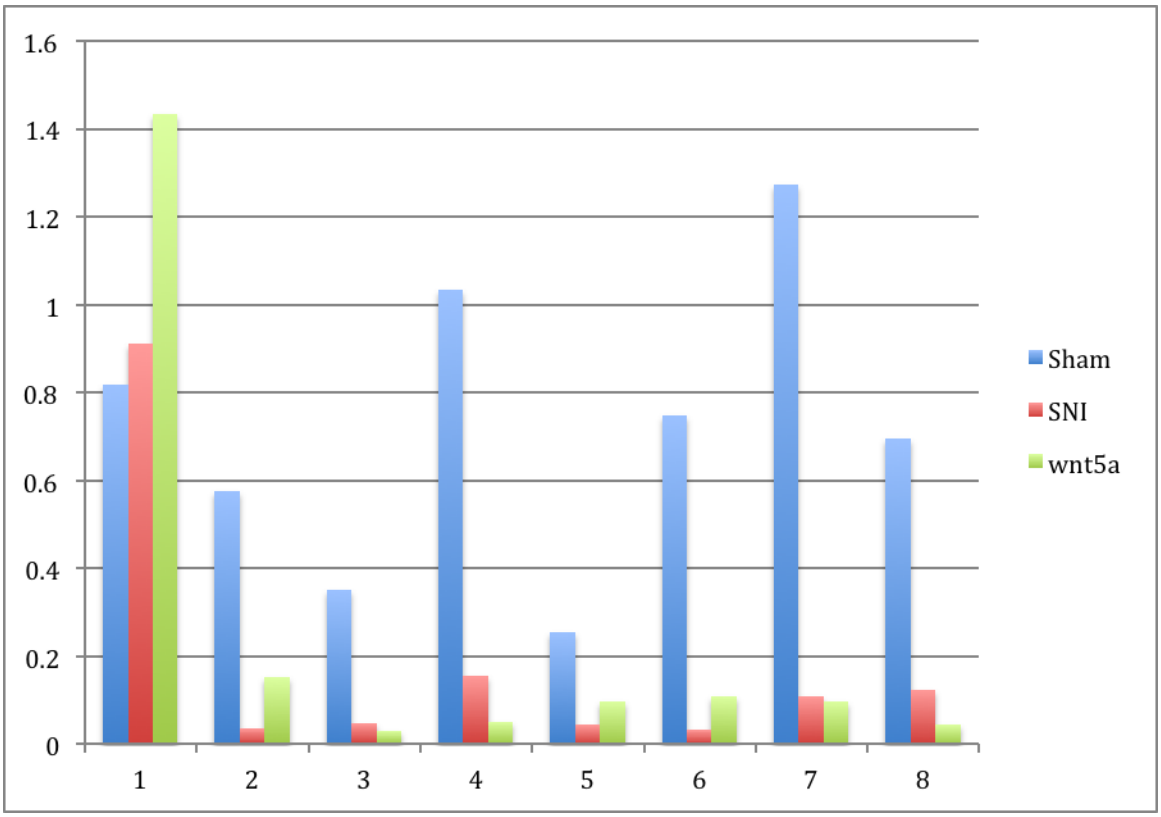


Figure 3: Mice either received sham surgery or SNI surgery. Three groups of 5 animals were tested. Sham animals maintained baseline withdrawal responses except for days , SNI and wnt5a (100ng) treated animals developed significant allodynia with no recovery at the final timepoints. X-axis is time period of 3 days with time period 1 equal to baseline, time period 2 to 3 days post-op and so on. Y-axis is force in grams.

Specific Aim 2: Determine the role of TGR5 in astrocyte activation and treatment of mechanical allodynia in a mouse model of neuropathic pain.

Major Task 1: Determine role of TGR5 signaling in astrocyte activation **100% COMPLETE**

Major Task 2: Determine the role of TGR5 signaling in treating mechanical allodynia in a mouse peripheral nerve injury model **85% COMPLETE**

Dr. Bedocs and his research assistant traveled to my Duke lab for training on mouse behavioral testing They also have final ACURO approval to do animal work and are currently beginning to validate the wnt results in the SNI model of peripheral nerve injury.

Specific Aim 3: Use existing data from the Vanderbilt EMR and genotyping repositories to look for associations between genetic variants and pain phenotypes

Major Task 1: Preliminary analyses conducted to confirm the precise numbers of patients for whom there are sufficient data available. Validation of previously published genotype-phenotype associations. **100% COMPLETE**

We have extracted data of >30,000 patients from the BioVU DNA databank who were treated with intravenous opioids after a major surgical intervention, and have phenotyped them via links to EMR records with regards to our primary outcome: patients who experienced an inpatient opioid-related ADE (respiratory depression as indexed by naloxone administration) and controls (patients

who did not have an ADE). We used a similar case-control phenotyping approach for two secondary outcomes: presence of a rapid response team code and administration of a motility drug (to index constipation). Using the International Classification of Disease, 9th Edition, we identified and pulled information on patient-related risk predictors of opioid-related ADEs. Since genotyping of the DNA of the ≈30,000 surgical patients was performed using the MEGAex genotyping platform, a full genome-wide association analysis (GWAS) analysis was not possible. Given the state of the field, we felt that a full unbiased GWAS approach was optimal, so in the past year we have worked to use imputation to derive full GWAS genetic data for our sample. BioVU is currently finalizing the imputation process, and based on our personal communication with the BioVU project manager, the imputed data has just been released, enabling GWAS analyses to address our aims. In the interim, we conducted a preliminary study using existing phenotypes in our dataset potentially relevant to ADEs, in particular, respiratory insufficiency, failure and arrest. Our search identified 18 distinct genes that have shown a statistically significant association with the aggregate of respiratory insufficiency, failure and arrest. Of these genes, adipocyte plasma membrane associated protein gene (*APMAP*), showed the strongest association with a combination of respiratory insufficiency, failure and arrest. Subsequently, using the United Kingdom Biobank, in a set of 500,000 subjects, we have been able to validate the above described association between *APMAP* gene and the occurrence of respiratory insufficiency, failure and arrest. Now that we have the imputed data, we have begun conducting discovery GWAS analysis as it was proposed in the above described Aim 3, which will be followed by a validation analysis in the National Institutes of Health sponsored Database of Genotypes and Phenotypes.

Major Task 2: Discovery and validation of novel exomic variants associated with opioid adverse drug events. **35% COMPLETE**

The discovery portion of the study has been delayed because Vanderbilt has access now to imputed megachip data which will allow GWAS scale discovery inste. This data is now available and being processed.

What opportunities for training and professional development has the project provided?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Pain medicine is limited by the limited number of new analgesics and adverse effects of opioids. Over the past year we have demonstrated that activation of the canonical wnt pathway does not prevent mechanical allodynia in mice but hastens recovery. Activation of the non-canonical pathway does not. Surprisingly, non-canonical wnt agonists shifter macrophage phenotype to the M2 domain which is somewhat contradictory to the peripheral nerve injury data but highlights the complexity of macrophage phenotyping. Although M2 phenotype had previously been considered the “good” pro-resolution phenotype, this dichotomous way of classifying macrophages may be far too simplistic.

Also, we were excited to find a novel gene associated with opioid-related adverse events in the Vanderbilt patient population and look forward to validating this finding using the megachip data.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

CHANGES/PROBLEMS:
The Vanderbilt portion of the project involving discovery of novel variants predicting risk of opioid related adverse events was delayed in order to wait for a much more powerful dataset to be available. This is now available and the work being performed under the approved extension.

Changes in approach and reasons for change
There were no significant changes in approach.

Actual or anticipated problems or delays and actions or plans to resolve them
No delays or anticipated problems

Changes that had a significant impact on expenditures
No significant changes on expenditures

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
No changes or deviations

PRODUCTS:

1. *Published: Chamessian A, Young M, Qadri Y, Berta T, Ji RR, Van de Ven T. Transcriptional Profiling of Somatostatin Interneurons in the Spinal Dorsal Horn. Scientific Reports, 2018 May 1;8(1):6809.*

Books or other non-periodical, one-time publications.
Nothing to report

Other publications, conference papers, and presentations.
Nothing to Report

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Other Products
Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?
Name: Thomas Van de Ven
Project Role: Principal Investigator
Nearest person month worked: 4.58

Contribution to Project: Coordinates all aspects of the project and assumes overall responsibility for its success.

Name: Ru-Rong Ji
Project Role: Co Investigator
Nearest person month worked: 0.48
Contribution to Project: He is responsible for interpreting and troubleshooting the proposed animal behavioral testing and cell culture experiments and his lab provides deep expertise in all experimental procedures

Name: Sarah Crews
Project Role: Program Manager
Nearest person month worked: 1.80
Contribution to Project: Overall project manager for all aspects of the proposal, including coordination of the biological samples, shipment of samples between sites and data organization, and ensures that the supplies are ordered and available

Name: Thomas Buchheit
Project Role: Co Investigator
Nearest person month worked: 0.24
Contribution to Project: Works closely with Dr. Van de Ven on all aspects of the project
Funding Support: Other resources

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report

What other organizations were involved as partners?
Organization Name: Vanderbilt University Medical Center
Location of Organization:1161 21st Avenue South, Nashville, TN 37232-2520
Partner's contribution to the project: Collaborated in the research

Organization Name: Henry M. Jackson Foundation for the Advancement of Military Medicine Inc.
Location of Organization: 6720 A Rockledge Drive, Bethesda, MD 20817
Partner's contribution to the project: Collaborated in the research

SPECIAL REPORTING REQUIREMENTS
QUAD CHARTS:
Attached

APPENDICES:
Attachment 1- Quad Chart

VIPER II: Chronic Pain After Amputation: Inflammatory Mechanisms, Novel Analgesic Pathways, and Improved Patient Safety.

PI: Van de Ven, Thomas **Org:** Duke University **Award Amount:** $1,500,000

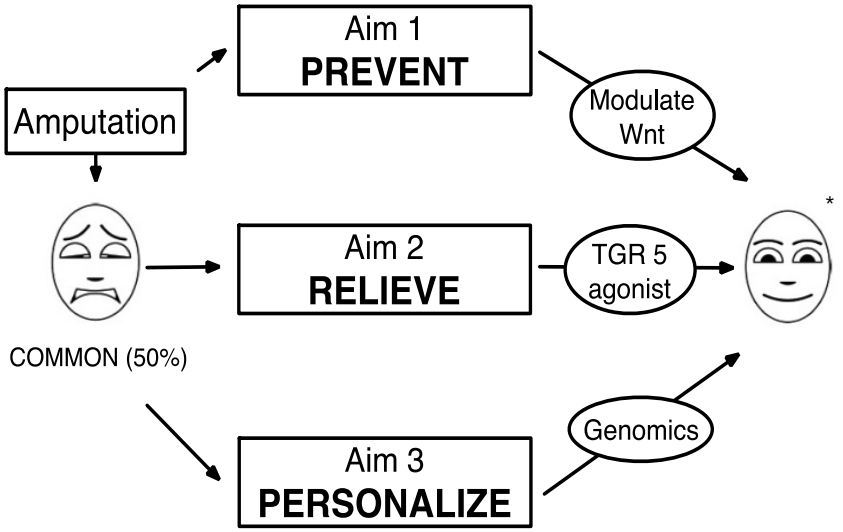
Study Aims

Problem: Current therapies for residual limb pain are ineffective or produce significant side effects.

Hypotheses: 1) Biomarkers found in the Veterans Integrated Pain Evaluation Research Study (VIPER) will lead to novel analgesics. 2) Pharmacogenomic profiling will improve the safety and effectiveness of current analgesics.

Approach

Convergent analysis of VIPER data show both the TGR5 and Wnt pathways to be important in chronic residual limb pain. We will:
1) Define the role of Wnt signaling in inflammation and mechanical allodynia after nerve injury using cell culture and animal models.
2) Test the effectiveness of TGR5 pathway agonists for the treatment of allodynia after nerve injury using animal models.
3) Use human pharmacogenomic predictors to improve the safety and effectiveness of current opioid treatments.



* Adapted from Defense & Veterans Pain Rating Scale (DVPRS)

Timeline and Cost

Activities CY	16	17	18	19
Aim 1: Wnt - Cell culture, animal behavioral testing and cytokine measurement.				
Aim 2: TGR5 – Animal behavioral testing and cell culture experiments				
Aim 3: Pharmacogenomic analysis				
Reports (▮) and Manuscripts (◆)		▮	◆ ▮	◆ ▮
Estimated Total Budget ($K)	$200K	$500K	$500K	$300K

Goals/Milestones

CY16 Goals
- ✓ Begin macrophage polarization and astrocyte activation experiments
- ✓ Begin designing data capture for pharmacogenomic analyses
- ✓ Begin animal behavioral TGR5 experiments

CY17 Goals
- ✓ Begin wnt pathway human gene expression analysis
- ✓ Complete cell culture experiments
- ✓ Begin pharmacogenomic validation experiments
- ✓ Begin pharmacogenomic discovery experiments

CY18 Goals
- ✓ Complete ELISA and macrophage phenotype experiments
- ✓ Continue animal behavioral testing
- ❑ Continue pharmacogenomic discovery experiments
- ❑ Complete one manuscript

CY19 Goals
- ❑ Complete all experiments
- ❑ Complete two manuscripts
- ❑ Develop follow-on studies and apply for follow-on funding