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14. ABSTRACT Our societal dependence on opioid drugs as the primary therapy for severe acute and chronic pain has resulted in a broad range of problems (e.g., drug abuse, addiction), creating an urgent need to phase out the pervasive use of opioids for pain. The formidable challenge that our program will address is to establish a dynamic pipeline of diverse natural products that should provide a continuous stream of non-opioid drug leads for pain. One of the few successful non-opioid pain drugs to be launched and FDA-approved in the past decades was isolated in our labs from natural sources. Two other compounds from the same source have reached human clinical trials. Despite this record of success, what has slowed down our efforts is the complexity of the biology underlying pain. Recently, we developed a new biological platform that has accelerated the development of new ligands for new targets and pain therapy. An even greater acceleration was recently achieved in which we have married our new developments in constellation pharmacology with well-validated pain models, enabling us to compare sensory pathways in neurons that have experienced chronic pain with those in normal neurons. Together, these methods are transforming our ability to rapidly discover new targets and ligands to treat pain.					
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1. INTRODUCTION:

Our societal dependence on opioid drugs for severe acute and chronic pain has resulted in a broad range of problems, including dangerous side effects, drug abuse and addiction. Over the past 50 years, few new pain drugs with novel mechanisms of action have been approved. The major classes of pain drugs in use today were discovered from natural sources long ago. Opioids were derived from morphine, isolated from the opium poppy, and non-steroidal anti-inflammatories (NSAIDs) were inspired by aspirin, derived from plant sources, such as willow bark. Prior to isolating their analgesic compounds in the 19th century, these medicinal plants were used since antiquity to treat pain. Although plant sources of pain drugs have been explored extensively, natural products from the marine environment have not. We aim to establish a pipeline of marine natural products to provide a continuous stream of non-opioid drug leads for pain. Historically, most drugs were discovered through phenotypes elicited in animals or humans, often through natural experiments. This type of drug testing has been called phenotypic screening. For this program, we observe the effects of marine natural products on animals (chemical neuroethology) in combination with our own form of phenotypic screening (constellation pharmacology), which employs native neurons to determine the effects of drugs on nociceptive neurons. We then identify compounds that prevent, treat or reverse the effects of a neuropathic pain state *in vivo*, utilizing rodent pain models. We are screening for natural products that have the potential to inhibit neuronal excitability in pain circuitry, based on the premise that many natural compounds have been honed by evolutionary forces to interact potently and selectively with a variety of drug targets that are useful for blocking pathological pain. Each project is pursuing independent specific aims. These complementary projects encompass a broad spectrum of non-opioid pain drug targets. Project 1 will identify new pain drug targets and mechanisms. Projects 2 and 3 are providing novel compounds from marine sources to be tested as potential pain drugs. Project 4 is exploring the roles of TRP channels in acute and neuropathic pain. Project 5 is focused on developing orally bioavailable and metabolically stable inhibitors of the $\alpha 9\alpha 10$ subtype of nicotinic acetylcholine receptors (nAChRs). Blocking this receptor has already proven to alleviate pain and reverse some effects of nerve injury that result in neuropathic pain.

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

Pain; Natural Products; Conotoxins, Neuropathic Pain, Rodent Pain Models; Constellation Pharmacology; Nicotinic Receptors; Purinergic Receptors.

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?

Project 1: The over-arching goal of Project 1 is to identify novel non-opioid pain drug targets and mechanisms. The following tasks, subtasks and milestones for Project 1 were to be completed or started during the first year of the award, as summarized below from the SOW, along with dates of completion or percentage completion:

Specific Aim 1: Identify combinations of cholinergic, purinergic and bradykinin receptor subtypes that are functionally expressed and upregulated or downregulated in rat CCI and SNL models of neuropathic pain.	Timeline	Completion dates or percentage of completion
Major Task 1: Obtain and analyze transcriptomic data for DRG neurons from ipsilateral and contralateral CCI and SNL rats, 14 days after injury.	Months	
Subtask 1: Prepare mRNA/cDNA for RNA-seq and submit to Shared Huntsman/University of Utah High-Throughput Genomics and Bioinformatic Analysis Core.	1-4	March 1, 2018
Subtask 2: Analyze RNA-seq data for ipsilateral vs. contralateral DRGs from CCI and SNL rats. Evaluate genes that are significantly upregulated or downregulated, with particular emphasis on subtypes of bradykinin, purinergic and cholinergic receptors.	5-8	June 15, 2018
Major Task 2: Use subtype-selective pharmacological agents to test hypotheses of functional protein upregulation for subunits of P2X, nAChR and bradykinin receptors. Hypotheses will be based on transcript upregulation from RNA-seq data analysis.		
Subtask 1: Use subtype-selective bradykinin receptor agonists/antagonists to identify bradykinin receptor subtypes upregulated or downregulated.	8-12	September 10, 2018
Subtask 2: Use subtype-selective nicotinic acetylcholine receptor agonists/antagonists to identify nAChR subunits upregulated or downregulated.	8-16	~90% completed
Subtask 3: Use subtype-selective P2X receptor agonists/antagonists to identify P2X receptor subunits upregulated or downregulated.	12-24	~90% completed
<i>Milestone #1: Complete experimental evaluation and create a summary of the combinations of cholinergic, purinergic and bradykinin receptor subtypes that are functionally expressed and upregulated or downregulated in rat CCI and SNL models of neuropathic pain.</i>	24	RNA-seq completed. Single cell transcriptomic completed.
Specific Aim 2: Employ a panel of subtype-selective pharmacological agents to determine which nAChR and P2X receptor subtypes are functionally coupled in rat CCI and SNL models of neuropathic pain. Identify the neuronal subclasses in which such receptor combinations are expressed.		

Major Task 1: Preparation for tests of functional coupling.		
Subtask 1: Complete Specific Aim 1.	1-25	~80% completed
Subtask 2: Read all literature on functional coupling between nAChRs and P2X receptor subtypes.	1-25	Completed
Major Task 2: Test functional coupling with subtype selective pharmacology.		
Subtask 1: Use subtype-selective antagonists to isolate nAChR and P2X receptor subtypes and then use subtype-selective agonists to determine effects that are < or > additive by activating isolated pairs of nAChR and P2X receptor subtypes.	25-42	~90% completed
Subtask 2: Use histochemical staining and genetic labeling of DRG neuronal cell types to determine which neuronal cell types exhibit functional coupling between nAChRs and P2X receptors.	30-45	
<i>Milestone #2: Manuscript on functional coupling between nAChR and P2X receptor subtypes.</i>	44-48	~20% (manuscript in preparation)
Specific Aim 3: Move from rat models of neuropathic pain to reporter mouse models of neuropathic pain to identify additional receptor and ion channel subtypes that are functionally upregulated or downregulated after CCI or SNL. Use transcriptomic data from genetically labeled cell types in combination with constellation pharmacology to identify cell-type-specific changes. Correlate the magnitude of changes in functional receptor and ion channel expression with pain behavioral scores to identify the most promising potential pain targets.		Move from rat model to reporter mouse model of neuropathic pain: Completed June 2019
Major Task 1: Obtain and analyze transcriptomes for peptidergic nociceptors (GFP genetic label), non-peptidergic nociceptors (IB4 stained) and other putatively non-nociceptive DRG neurons (unlabeled) at days 3, 7 and 14 after CCI and SNL of CGRP-GFP mice. Compare ipsilateral and contralateral DRG neurons for each pain model at each time point.		~50% completed
Subtask 1: FACS sort labeled and unlabeled neurons and then prepare mRNA/cDNA for RNA-seq for each cell class. Submit cDNA for RNA-seq to Shared Huntsman/University of Utah High-Throughput Genomics and Bioinformatic Analysis Core.	12-18	Replaced with single cell transcriptome ~80% completed
Subtask 2: Analyze RNA-seq data for ipsilateral vs. contralateral DRGs from CCI and SNL mice at each time point and for each cell type (peptidergic nociceptors, non-peptidergic nociceptors and non-nociceptors). Evaluate genes that are significantly	18-24	Single-cell transcriptome initiated and 50% complete.

upregulated or downregulated, with emphasis on ion channels and GPCRs. Look for trends across multiple time points within cell types.		
Major Task 2: Test hypotheses of upregulation or downregulation of receptor and ion channel subtypes with subtype selective pharmacological agents.		
Subtask 1: Identify appropriate pharmacological tools.	20-30	~90% completed
Subtasks 2: Use pharmacological tools to test hypotheses.	21-36	~60% completed
Major Task 3: Correlate the magnitude of changes in functional receptor and ion channel expression with pain behavioral scores to identify the most promising potential pain targets.		
Subtask 1: Use subtype-selective pharmacological agents from Major Task 2 to identify trends in functional expression of receptor and ion channel subtypes that correlate with pain behavioral scores, as described in Project 1.	21-40	~60% completed
Subtask 2: Use available subtype-selective pharmacological tools for receptors or ion channels that correlate with pain behavioral scores from Subtask 2 to test for analgesic affects in mouse pain models.	36-48	~60% completed
<i>Milestone #3: Manuscript on changes in functional protein expression that correlate with behavioral scores from 3 pain models.</i>	44-48	~80% completed

Project 2:

Specific Aim 1: High priority venoms will be collected from high priority species (makes targeted prey quiescent) in substantial quantities.	Timeline	Completion dates or percentage of completion
Major Task 1: Determine venom-collection priorities through chemical neuroethology.	Months	
Subtask 1: Evaluate marine mollusks with high-priority venoms by chemical neuroethology observations in aquaria, literature reports and others' field observations.	1-12	October 2017
Subtask 2: Evaluate collection methods and locations for high priority venoms.	1-12	October 2017
Major Task 2: Collect venoms in substantial quantities from appropriate locations.		

Subtask 1: Contract with fishermen and other collectors in appropriate field locations.	1, 13, 25, 37	Collections Philippines: October 24 th - November 4 th 2017 Philippines: February 16 th –February 21 st 2018 Solomon Islands April 8 th - April 17 th 2018 Philippines: October 21 st - October 29 th , 2018 April 7 th -April 11 th , 2019 June 5 th -June 15 th , 2019
Subtask 2: Collect venoms, marine mollusks and other samples in quantity, complete import documentation and receive venoms in the lab for experiments. (Dr. Olivera will lead. Dr. Schmidt will also collect bacterial symbionts and other samples for Project 3; see below.)	1, 13, 25, 37	November 4 th 2017 February 21 st 2018 April 17 th 2018 October 29 th , 2018 April 11 th , 2019 June 15 th , 2019
Milestone #1: Collect, preserve and inventory high priority venoms (makes target prey quiescent) from high priority species in substantial quantities.	2, 14, 26, 38	First phase completed in May 2018 Second phase completed in May, 2019
Specific Aim 2: Venoms will be systematically characterized, peptides purified and synthesized. Pure peptides tested for analgesic effects in pain models.		
Major Task 1: Individual components will be assessed by constellation pharmacology and in vivo injections in mice.		

Subtask 1: Test high priority crude venoms and venom fractions each by multiple constellation pharmacology assays. <u>Baldomero Olivera</u>, Project 2 leader, will collect venoms, fractionate and provide to constellation pharmacology core for testing. <u>Shrinivasan Raghuraman</u>, Constellation Pharmacology Core leader, will test venom fractions.	4-42	First year phase completed in September 2018 Second year phase completed in September 2019*
Subtask 2: Test high priority crude venoms and venom fractions each by injection into mouse ICV and IP.	4-42	First phase completed in January 2018 Second year phase completed in September 2019*
Specific Aim 3: Conopeptides previously characterized using procedures similar to those described in specific Aims 1 and 2 will be further developed as potential pain therapeutics.		
Major Task 1: Contulakin-G, a neurotensin receptor agonist, has demonstrated anti-nociceptive activity in a wide variety of animal models. We will evaluate the possibility a pilot feasibility study on human cancer patients and conduct a human clinical trial, if possible.		
Subtask 1: Evaluate the possibility of a pilot feasibility study for Contulakin-G on human cancer patients suffering from severe intractable cancer pain with the Huntsman Cancer Institute.	1-12	90% completed **
Major Task 2: Evaluate conantokins and dw-Asprella conopeptides for analgesic properties.		
Subtask 1: Test peptides in CCI and SNL pain models for analgesic effects.	1-12	In progress ~ 80% completed

***These tasks were accomplished beyond the originally proposed scope. This is described in the text below**

****The feasibility of developing Contulakin G in partnership with Huntsman Cancer Institute has been thoroughly explored and we are presently concretely exploring what would be required to carry out a small trial on patients with severe intractable pain that is not relieved by opioids or any other drug. This project has been delayed because of COVID-19.**

Project 3:

Specific Aim 1 (Create chemical library)	Timeline	Completion dates or percentage of completion
Subtask 1: Create extract library	1-12	September 2018
Milestone 1: Obtain and process samples (yearly) (Collection activities will be led by <u>Dr. Olivera</u> as funded out of Project 2; see above. Dr. Schmidt will lead in the small molecule and bacterial harvesting as part of those activities.)	2, 14, 26, 38	First phase completed in Solomon Islands April 8 th -April 17 th 2018 Second phase to be completed in Philippines November 8-11 2019 Delayed due to COVID
Subtask 2: Purify 100 bacterial strains (yearly)	2-10	September 2018 September 2019 Delayed due to COVID
Subtask 3: Extract organs from 10 animals and array in library (yearly)	2-4	September 2018 March 2019 Delayed due to COVID
Subtask 4: Extract bacterial cultures and array in library (yearly)	3-12	September 2018 September 2019 Delayed due to COVID
Subtask 5: Chemical curation and analysis (yearly)	3-12	September 2018 September 2019 Delayed due to COVID

Milestone #2: On a yearly basis, compile a library of crude extracts from 10 marine animals and 100 bacterial strains from which chemical libraries will ultimately be produced for drug screening and testing.	12, 24, 36, 48	September 2018 September 2019 September 2020
Specific Aim 2 (Screen libraries)		
Major Task 1: Screen libraries		
Subtask 1: Screen 110 crude extract in constellation pharmacology assays per year and purify active components that may have analgesic properties.	1-12	September 2018 September 2019 September 2020
Subtask 2: CCI screen complete for prioritized extracts (3 months behind extraction schedule) 20 priority extracts/yr x 3 CCI rats x 3 sham rats = 120 rats/yr	1-12	Methodology changed - see report
Subtask 3: SNL screen complete for prioritized extracts (3 months behind extraction schedule) 20 priority extracts/yr x 3 SNL rats x 3 sham rats = 120 rats/yr	1-12	Methodology changed - see report
Major Task 2: Screen in targeted assays		
Subtask 1: TRP and P2X screens complete for all samples collected that year (Dr. Reilly will perform the screens as described in Project 4. Dr. Schmidt will lead the chemistry and assay-guided purification that is needed as part of this activity.)	1-12	September 2018 September 2019 September 2020
Milestone #3: On a yearly basis, complete a curated hit list from extracts (3 months behind extraction schedule) that have been screening in constellation pharmacology assays, CCI & SNL pain models and in target-based screens against TRP channel and P2X receptor subtypes.	12, 24, 36, 48	September 2018 September 2019 September 2020
Specific Aim 3 (Characterize compounds)		
Major Task 1: Structure determination		September 2018 September 2019 September 2020

Subtask 1: Characterize structures of at least 10 active hits annually	6-12	September 2018 September 2019 September 2020
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Project 4

Specific Aim 1: Discovery of inflammatory and pain mediators + biosynthetic origins and molecular consequences.	Timeline	Completion dates or percentage of completion
Major Task 1: <i>Treat animals and collect tissues for molecular studies.</i>		
Subtask 1: Obtain samples from pain models. Order animals, treat animals, and isolate tissues for molecular studies. (DRG tissue will be procured from treated/surgically modified animals in collaboration between Reilly lab personnel and the rodent pain models and behavioral testing core. DRG will be used for transcriptomics and metabolomics. We will use 5 animals per group: control, oxaliplatin, SNL, and CCI. We will collect tissues at 0, 1, 3, 7, and 14-days post-treatment totaling 100 mice. DRG will be divided as 2/mouse for transcriptomics assays and the remaining pooled for initial activity screens, compound enrichment, structure identification and metabolomics assays.)	1-12	~95% Completed
Major Task 2: <i>Identify direct-acting agonists, antagonists, sensitizers, and desensitizers of TRPA1, M8, V1 (tier 1 assays) receptors and perform transcriptomic analyses of neuronal tissues.</i>		
Subtask 1: Process tissues for initial activity screening assays. - Prepare crude extract fractions of animal tissue for initial cell-based screening for bioactive molecules. - Perform cell-based screening assays to identify crude fractions with TRP channel modulatory (activation/inhibition) activity. (Cell lines used: Human TRP channel over-expressing HEK-293 cells, established in the Reilly laboratory, and standardized assays.)	4-18	~85% Completed

Subtask 2: Transcriptomic analyses. - Isolate and quantify mRNA from animal tissues. - Submit RNA sample to Genomics core facility for RNA. quality assurance, library and sample preparation for transcriptomic analysis. - Interact with Bioinformatics core to generate differential expression data for genes in naïve and treated animals.	8-18	~95% Completed
Major Task 3: <i>Decipher structures and biosynthetic origins for bioactive molecules through collaboration with Projects 2 and 3 and perform tier 2 assays for TRP activity (TRPV2, V3, V4, M2)</i>		
Subtask 1: Sub-fractionate, isolate and identify bioactive molecules from crude fractions of animal tissue. - Sub-fractionate bioactive materials from Task 2. - Purify bioactive molecules of interest from crude fractions using activity guided fractionation. - Screen fractions and compounds against alternative TRP channels	12-36	~75% Completed
Subtask 2: Neurometabolome Analysis - Submit inactive and active samples from Aim 1, Task 3 for targeted and non-targeted metabolome analyses (Metabolomics Core Facility). - Perform bioinformatics analyses to identify biologically active molecules. - Purify bioactive molecules of interest from refined, active fractions using a combination of mass- and activity-guided fractionation.	12-36	~75% Completed
Subtask 3: Bioactive molecule validation assays Develop assays for targeted analyte analysis to support validation efforts in Aims 2 and 3	24-48	~50% Completed
Specific Aim 2: Discovery of novel molecules that target inflammatory and pain pathways.		
Major Task 4: <i>Perform cell-based TRP activity assays</i>		
Subtask 1: Identify natural products that activate and/or inhibit specific TRP channels or combinations of TRP channels using libraries/compounds from Projects 2 and 3.	12-48	~100% Completed; screen target met, but still ongoing

Obtain and evaluate extracts and purified compounds from Projects 2 and 3 for modulation of human TRP channels using TRP over-expressing cells.		
Subtask 2: Develop assays for newly identified molecular targets for TRP modulation and pain signaling using results from Aim 1.	24-48	~75% Completed
Major Task 5: <i>Conduct pharmacological mechanisms studies</i>		
Subtask 1: Determine binding sites and potency data for newly discovered TRP and pain pathway target modulators. - Determine EC50 and IC50 values for agonist and antagonists using established assays in hTRP channel over-expressing HEK-293 cells. - Determine if biological activity/potency is the result of targeting known ligand binding sites on targets using mutant proteins - Elucidate new pharmacological mechanisms of biological activity of the most potent molecules at their respective biological target using site-directed mutagenesis and activity assays	24-48	~75% Completed
Specific Aim 3: Test/validate novel molecules and associated targets for reduction of pain (efficacy)		
Major Task 6: <i>Tests for alterations in pain testing by novel natural product modulators of TRPs and/or related targets</i>		
Subtask 1: Provide promising molecules from Aim 2 (i.e., TRP channel agonists, antagonists, functional modulators, and/or modulators of related pain pathways from Aim 1) to Light for assessment of efficacy in pain models.	30-48	~50% Completed
Subtask 2: Collect tissues and perform molecular validation assays to confirm or refute hypotheses related to mechanisms of efficacy using methods from Aim 1.		
Major Task 7: <i>Prioritization of lead compounds for further development as therapeutics and/or newly discovered biological targets for future concentrated discovery and development efforts</i>		
Subtask 1: Identify molecules with the following criteria for future therapeutic development: - Potency <10 uM at a specific and/or multiple TRP channels - Efficacy in reducing pain perception in multiple models of pain	36-48	~50% Completed

- Contains no known “structural alerts” that could represent risk for toxicity/adverse drug effects in development studies - Is amenable to further structural modifications to optimize potency and efficacy and “fine-tuning” of PK properties for maximum flexibility of delivery		
Subtask 2: Identify biological targets with the following criteria for future drug discovery and therapeutic development efforts: - Molecules targeting the pathway show efficacy in multiple pain models - Target novelty (i.e., there are not multiple advanced drug candidates or commercial therapeutics that target the entity) - Limited known roles of the target in general physiology - Existing precedent, but limited understanding of the target in pain pathobiology	36-48	~40% Completed

Project 5:

Specific Aim 1: <i>Develop a medium-throughput assay for $\alpha 9\alpha 10$ nAChRs</i>	Timeline	Completion dates or percentage of completion
Major Task 1: Optimize production and purification of non-radioactive, iodinated peptide analog 1	Months	
Subtask 1: Optimize production and purification of non-radioactive, iodinated peptide analog 1 - Optimize reaction kinetics to favor production of monoiodo compound - Optimize HPLC procedure to obtain >98% purity - Mass spectrometry of major products	1-3	100% complete
Subtask 2: Radiolabel candidate ligand #1 - Use ^{125}I and verify radiochemical purity of product - Optimize storage conditions - Stability studies	4-6	100% complete
Subtask 3: Develop alternative radioligand candidates	7-24	100% complete

Major Task 2: Develop high expression cell line		
Subtask 1: Assess cell surface expression of HEK 293 cell lines Western blots Preliminary cell surface binding Functional assays using Ca ⁺⁺ sensing dye	1-12	100% complete
Subtask 2: Develop alternative cell lines expressing $\alpha 9\alpha 10$ CHO U937	13-42	75% complete
Specific Aim 2: <i>Design and test families of ligands that specifically interact with the $\alpha 9\alpha 10$ nAChR</i>		
Major task 1: Synthesis and testing of single loop leads.		
Subtask 1: Use synthetic biology to synthesize 100 single loop compounds	1-8	100% complete
Subtask 2: Enzymatic Modification of Templates	3-14	80% complete
Subtask 3: Chemical Derivatization	6-16	80% complete
Subtask 4: Testing of compounds using heterologously expressed receptors expressed in <i>Xenopus</i> oocytes, or if ready, by radioligand binding.	6-18	100% complete
Major Task 2: Synthesis and testing of second generation compounds by iterative fragment combination with receptor testing		
Subtask 1: Synthesis of 2 nd generation compounds based on results from Major task 1.	7-24	80% complete
Subtask 2: Staple active fragments (chemical derivatization)	6-24	100% complete
Specific Aim 3: Analysis of subtype specificity and functional effects of best 3 rd generation compounds		
Major Task 1: Off-target testing • Testing against other nAChR subtypes • Testing against non-nAChRs	9-48	75% complete
Major Task 2: Compound screening in oxaliplatin pain model	30-48	75% complete
Major Task 3: selection of lead	42	----
Major Task 3 Testing of lead in other models of neuropathic pain	42-48	-----

What was accomplished under these goals?

Major accomplishments in the year 2020 include two major publications on facile classification and identification of changes in sensory neurons underlying chronic pain. The details of these publications are listed below:

1. Giacobassi MJ et al. An integrative approach to facile functional classification of dorsal root ganglion neuronal subclasses. PNAS 117, 5494-5501 (2020)
2. Raghuraman S et al. Chronicling changes in the somatosensory neurons after peripheral nerve injury. PNAS 117 (42), 26414-26421 (2020)

Project 1, Specific Aim 1, Major Task 1: A significant accomplishment of the 1st year was to complete the RNA-seq experiments for CCI, SNL and sham-operated rats. We compared these data to our preliminary functional data reported in our grant proposal. These data were essentially in line with our functional data, insofar as accurate comparisons can be made. For example, our functional data demonstrated upregulation of bradykinin receptors and downregulation of TRPV1 channels in the injured neurons (ipsilateral) of these rodent models of neuropathic pain (relative to contralateral and sham), as shown in the following table:

RPKM					
	Ipsi-SNL	Contra-SNL	Ipsi-CCI	Contra-CCI	Average Sham
TRPV1	45.5	61.3	46.1	53.2	58.7
BDKRB2	7.7	4.5	9.2	4.1	5.2

Interestingly, there were no reads for the bradykinin B1 receptor, which is not normally expressed in rodent DRG neurons but has been reported to be upregulated in some inflammatory pain models. According to our transcriptomic and functional data, we see no evidence for upregulation of bradykinin B1 receptors in neuropathic pain models.

Another interesting finding was down-regulation of $\alpha 3$ subunits of nicotinic acetylcholine receptors (nAChRs), in line with our functional data and as shown in the table below. We previously used subtype-selective pharmacological tools (primarily α -conotoxins) to identify nAChRs expressed under normal conditions in rat and mouse DRG neurons and concluded that they are primarily $\alpha 3$ - and $\alpha 6$ -containing nAChRs. One surprise from these data was the low expression levels of

RPKM					
	Ipsi-SNL	Contra-SNL	Ipsi-CCI	Contra-CCI	Average Sham
CHRM2	10.9	10.2	14.3	11.3	12.0
CHRM3	0.8	1.3	2.6	1.8	1.3
CHRM4	0.5	0.6	0.5	0.6	0.2
CHRM5	0.5	0.4	0.3	0.4	0.4
CHRNA1	1.3	0.9	1.7	0.3	0.6
CHRNA2	0.0	0.1	0.3	0.0	0.1
CHRNA3	16.9	23.9	17.6	21.0	26.9
CHRNA4	0.2	0.2	0.2	0.6	0.3
CHRNA5	3.4	3.1	4.1	3.9	3.5
CHRNA6	14.5	19.4	18.9	14.5	22.6
CHRNA7	13.3	14.9	25.0	23.8	18.3
CHRNB1	0.4	0.3	0.1	0.1	0.2
CHRNB2	59.5	47.5	77.4	51.8	58.7
CHRNB3	7.7	10.0	12.2	36.6	11.9
CHRNB4	0.7	0.7	1.3	3.1	1.0
CHRNE	3.4	5.3	3.6	5.4	5.1

$\beta 4$ nAChRs. We had interpreted functional pharmacological data to mean that most of the nAChRs in mouse and rat DRG neurons were $\alpha 3\beta 4$ and $\alpha 6\beta 4$ heteromeric nAChRs and $\alpha 7$ homomeric nAChRs. Based on the results from uninjured DRG neurons, we may need to revisit this interpretation. However, mouse transcriptomic data suggest that $\beta 4$

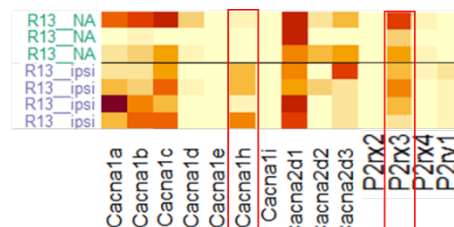
is mostly expressed in peptidergic nociceptors and the signal may be diluted in whole DRG.

	RPKM				
	Ipsi-SNL	Contra-SNL	Ipsi-CCI	Contra-CCI	Average Sham
P2RX1	1.0	1.1	1.1	0.3	0.6
P2RX2	4.0	4.1	3.2	2.1	2.6
P2RX3	120.2	121.9	145.9	112.7	135.7
P2RX4	21.4	23.7	13.5	12.0	21.0
P2RX5	4.8	8.0	5.7	7.9	8.3
P2RX6	10.3	17.9	9.8	15.3	19.8
P2RX7	3.5	2.9	2.5	3.9	2.7
P2RY1	12.3	14.3	17.1	17.5	15.1
P2RY10	0.8	0.5	0.5	0.1	0.2
P2RY12	6.6	4.8	4.3	2.0	4.1
P2RY13	1.6	1.5	1.3	1.7	1.0
P2RY14	3.6	2.3	1.9	2.3	2.1
P2RY2	9.9	11.5	9.7	8.5	10.9
P2RY4	0.0	0.2	0.0	0.4	0.2
P2RY6	2.6	1.9	2.0	0.6	1.0

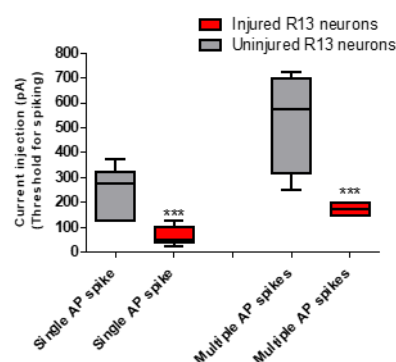
Purinergic receptor expression was more difficult to reconcile with our functional data. At day 14 post-surgery, there appeared to be upregulation of P2X3 receptor subunits in the CCI model, which is consistent with previous reports of neuropathic pain models, but this was not evident in the SNL model (see table below). These were validated by the constellation pharmacology using a potent and selective antagonist of P2X3,

Ro-51, that efficiently blocked the ATP induced calcium signals observed in injured DRG neurons.

Initially, it was unclear why many more DRG neurons are responsive to ATP in calcium-imaging assays after SNL injury than contralateral DRG neurons or DRG neurons from sham-operated animals. Both, the bulk sequence analysis and single-cell transcriptomic analysis from non-peptidergic nociceptive neurons (obtained from ipsilateral and contralateral SNL DRG cultures), revealed that the P2RX3 transcripts were not significantly different between the groups. Our single-cell transcriptomic analyses revealed the upregulation of voltage-gated calcium channels Cav3.2 in these cells, which could account for an increase in the percentage of ATP responsive neurons post injury. Our current working hypothesis is that upregulation of low-voltage activated (LVA) calcium channel encoded by the gene *CACNA1H* decreases the action potential firing threshold and increases the cellular excitability. We have initiated tandem single cell electrophysiological investigations in each cell type to monitor the effects of nerve injury on cellular excitability. As shown in figure below, nerve injury induced increased



Increased excitability of non-peptidergic (R13) neurons after nerve injury



excitability of a specific subclass of DRG neurons (R13, non-peptidergic nociceptors). Thus, application of ATP results in robust calcium signals mediated by the calcium influx through Cav3.2. We are currently investigating the single-cell transcriptomic data from Day 3,6,10 and 14 post injury to assess the levels of Cav3.2 with the progression of chronic pain.

Figure: Nerve injury increased the electrical excitability of a subset of non-peptidergic neurons (R13 cell class). The R13 cells were identified from constellation pharmacology experiments and were selected for tandem whole-cell current clamp experiments. The same cell classes were analyzed from injured DRGs (red) and cells obtained from uninjured DRGs (grey). As shown, the threshold to trigger single AP spike and multiple AP spikes significantly reduced in cells from injured R13 cell class. Data obtained from n=5 cells per group. P<0.005.

Project 1, Specific Aim 1, Major Task 2: Our goal is to identify the functional expression of cholinergic and purinergic receptor expression in uninjured rodent DRG neurons, which provides a baseline for comparison with injured DRG neurons. We have completed subtasks 1 and identified that bradykinin receptor B2 is the only bradykinin receptor subtype upregulated after SNL or CCI in rat DRG neurons.

We are currently evaluating the functional expression of nAChR and P2X subtypes upregulated or downregulated in rodent models of neuropathic pain. We have completed functional tests using selective pharmacological agents to dissect the expression of purinergic and nicotinic receptors in different cell types. The table below summarizes the results from uninjured DRGs obtained from adult mice:

Purinergic receptor tested	Agonist (concentration)	Antagonist (concentration)	Inference
P2X vs P2Y receptors	Application of ATP in calcium free media	N/A	36% neurons and 46% glia express P2Y receptors
P2X1	ATP (100uM)	NF449 (3uM)	P2X1 absent in neurons and glia
P2X2 and P2X2/3	ATP (20uM)	RO-51 (50nM)	Expression in peptidergic and non-peptidergic nociceptors
P2X4	ATP (100 uM)	5-BDBD (5uM)	Predominant expression in peptidergic nociceptors
P2X7	BzATP (30uM)	A-804598 (100nM)	Irreversible block observed in glia and AITC+ non-peptidergic nociceptors: P2X7 expressed in glia and a small subset of non-peptidergic neurons
P2Y1	MRS 2365 (100nM)	N/A	P2Y1 expressed in large diameter A-delta LTMRs and a subset of small diameter C-LTMRs

The functional data was validated by single cell transcriptomic approach. Single cell from each cell type were picked at the end of calcium imaging experiments and the table below summarizes the transcripts of purinergic receptors found in each cell type.

Cell Type (Classification ID; number of single cells picked)	P2X receptors (transcripts per million)							P2Y receptors (transcripts per million)							
	P2rx1	P2rx2	P2rx3	P2rx4	P2rx5	P2rx6	P2rx7	P2ry1	P2ry2	P2ry4	P2ry6	P2ry10	P2ry12	P2ry13	P2ry14
Proprioceptor (L1;n=16)	0.0	0.0	0.7	3.1	26.4	3.0	0.0	0.2	3.2	0.0	0.0	0.0	0.0	0.0	13.6
A-delta LTMR (L2; n=6)	0.0	0.0	0.1	18.4	1.8	0.1	0.0	20.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
peptidergic nociceptor (G9; n=5)	0.0	7.7	7.4	23.0	59.3	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
non-peptidergic nociceptor (R13; n=3)	0.0	0.0	34.2	5.4	2.8	0.0	0.0	0.2	0.7	0.0	0.0	0.0	0.0	0.0	0.7

The table shows data from 4 out of 16 cell types that we have classified so far. The next set of experiments will focus on identifying the purinergic receptor changes in these cell types after nerve injury.

Project 1, Specific Aim 2, Major Task 1 and 2: We have initiated the experiments to investigate the co-expression and functional coupling of nicotinic and purinergic receptor subtypes that are expressed in different cell types. Based on our previous results, we know that alpha 6, alpha 3, beta2/4 containing nicotinic receptors and alpha-7 nicotinic receptors are the major subtypes that are predominantly expressed by mice DRG neurons. Specific Aim 1 determined the subtypes of purinergic receptors that are expressed by different cell types. We designed experimental protocols to identify which of the nicotinic and purinergic receptors are co-expressed in different cell types of DRG neurons and which are functionally coupled.

The experiments on DRG as a model system did not reveal any significant functional coupling between nicotinic and purinergic receptors. The nerve injury induced pain is also dependent on the nerve injury induced inflammation and we explored the functional interactions between nicotinic receptors and purinergic receptors using mouse macrophages and THP-1 derived human macrophages. Our experiments revealed novel functional coupling between nicotinic receptor ($\alpha 9$ $\alpha 10$) and purinergic receptor (P2Y13).

The experiments were validated using 1) $\alpha 9\alpha 10$ antagonist RGIA5474 2) $\alpha 9\alpha 10$ knock out mice and 3) P2y13 selective antagonist MRS2211. This novel interaction between $\alpha 9\alpha 10$ and P2Y13 purinergic receptor is currently being investigated as a mechanism for alleviate inflammation and pain.

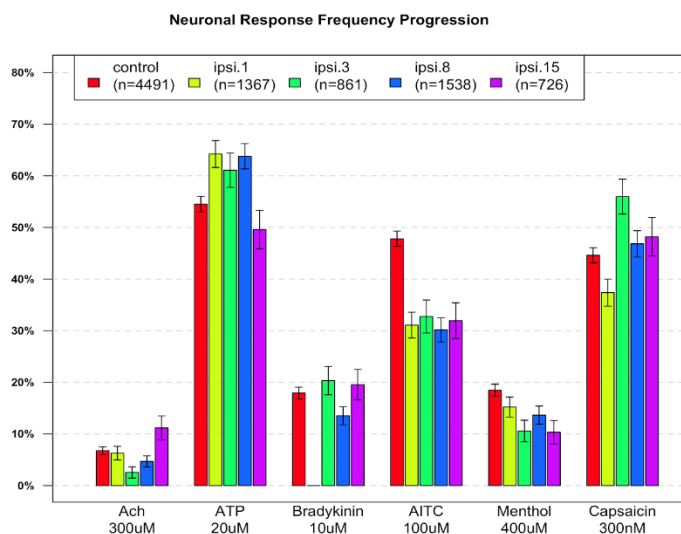
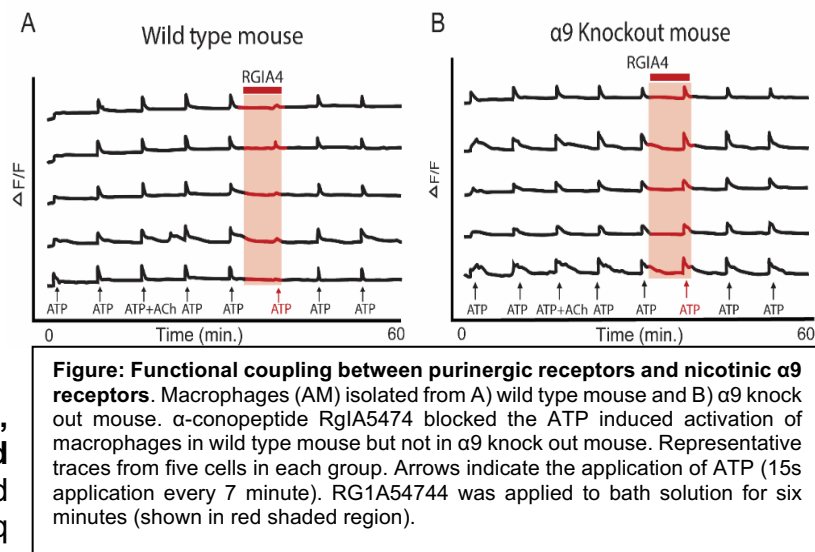
Project 1, Specific Aim 3: We made major advancements in Specific Aim 3. In June 2019, we successfully switched from rat model to mouse model of neuropathic pain. The project leader trained two students to perform CCI in mice and two technicians were trained to assess the behavioral scores.

Project 1, Specific Aim 3, Major task 1, Subtask 1 and subtask 2: We initially proposed to perform whole DRG RNA-seq and RNA-seq on pooled neurons

that express transgenic fluorescent labels (similar experiments were accomplished successfully in rats in Specific Aim 1). With recent advances in technology and cost reduction in performing single cell transcriptomics, we successfully picked 79 single cells of interest and obtained transcriptomic data from these neurons. This approach provided high content data from proprioceptors, A-delta LTMRs, a subset of non-peptidergic nociceptors and cold thermosensors. Currently we are able to classify DRG neurons into 16 cell types and the goal is to obtain single cell transcriptomic data from these cell types from uninjured mice and on days 1,3,7 and 14 post CCI.

Project 1, Specific Aim 3, Major Task 2: We began performing CCI surgeries in transgenic mice in which the CGRP expressing peptidergic neurons are labelled with

green fluorescent protein. In addition to transgenic label, we use isolectin B4-conjugated to Cy5 to label non-peptidergic nociceptive neurons. These fluorescent markers help track temporal changes in different DRG neurons following CCI. The graph below shows the changes observed in the frequency of neurons that respond to the application of various agonists at days 1,3,7 and 14 post CCI in mice. As shown in graph, increase in ATP responsive neurons was observed on day 1



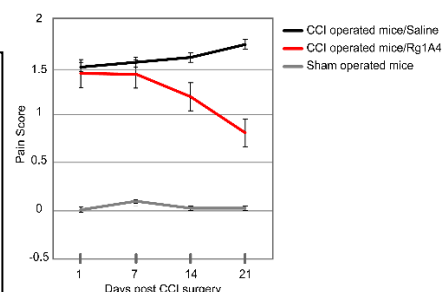
post CCI. A reduction in AITC responsive neurons was observed. These data will be tested and validated using transcriptomic and immunostaining approaches.

One notable result that we obtained was the increase in ATP responsive neurons were accounted by increase in P2X3 receptor subtype in a subset of IB4+ non-peptidergic nociceptors. This disappears after Day 7, suggesting transient changes in receptors occur that could lead to the establishment of chronic pain. As discussed earlier, the single cell transcriptomic analyses revealed that the observed changes are dependent on the expression of Cav3.2 ion channels that is currently being investigated.

Project 1, Specific Aim 3, Major task 3: Based on the results obtained from the above major task 2, we have initiated experiments to test the effects of injecting RO-51 (P2X3 antagonist) in mice. Our preliminary data suggests that blocking P2X3 on the day of nerve injury prevents the upregulation of P2X3 receptors in non-peptidergic nociceptors after 1-day post CCI. Next set of experiments will focus on testing the long term effects of injecting RO-51. In addition to RO-51, we have completed experiments with Rg1A analogs (RG1A5474) to investigate the effects on receptor changes following CCI. The data is currently being analyzed to assess the effects of RG1A5474 on nerve injury induced receptor changes in DRG neurons. We successfully tested and observed the efficacy of RG1A 5474 in alleviating chronic pain in mouse models of CCI. As shown in figure below, RG1A5474 alleviate pain behaviors in mice following CCI. The DRGs from these animals were isolated for constellation pharmacology experiments and the effect of RG1A on nerve injury

Figure: Rg1A4 alleviates pain in mouse model of CCI

Mice were assessed on days 1, 7, 14 and 21 post chronic constriction nerve injury and the guarding behavior was assigned a scale of 0 to 2 (0=no guarding; 1=mild guarding; 2=full guarding). Mice were given single subcutaneous injection of saline/Rg1A4 (40ug/kg) everyday till the day of behavioral assessment. Observer was blinded to which mice received treatment/saline injections. CCI resulted in significant pain that persisted till day 21 in saline injected animals. In contrast, CCI operate mice treated with Rg1A4 had reversal in pain phenotypes. N= 5 Rg1A4 treated and 4 saline treated animals/ group.



induced remodeling of receptors is currently being analyzed.

Additionally, we have initiated experiments to test the effects of interleukin-1 receptor agonist (IL-1beta) on the establishment of chronic pain. The single cell transcriptomic analysis revealed an up-regulation of il1r1 transcripts in non-peptidergic nociceptors after nerve injury. Following this observation, we incubated the DRG neuronal cells with 100nM IL-1beta overnight. Our preliminary results suggest that the treatment with IL1-beta increases the excitability of R13 non-peptidergic nociceptors and upregulates Cav3.2, which is in line with the observations from peripheral nerve injury. The role of nerve injury induced inflammation, remodeling of receptor constellations in DRG neurons and the effects of RG1A treatment at the molecular level is underway and will accomplished in the final year of the program.

Project 2: Overview

Unexpected and accelerated progress

The significant advances in technology, both with respect to sophisticated calcium-imaging assays, and the systematic analysis of venom transcriptomes has resulted in a new paradigm of discovery. We are now able to identify promising venom

components even in the absence of substantial amounts of venom, and without any biochemical purification from venom required. The basic strategy is to do an analysis of superfamilies of conopeptides among *Conus* species that are related; by comparing these sequences, we are able to identify peptide sequences likely to have novel and biomedically-relevant bioactivity; the peptides can then be synthesized or expressed recombinantly and then directly tested for bioactivity. Thus, in effect, the initial identification of venom peptides is carried out largely *in silico*, thereby avoiding both the necessity for collecting large amounts of each individual venom, and a comprehensive biochemical characterization.

A second general advance made possible by improved technology is a quick and much more incisive assessment of bioactivity of new compounds. This has come about because we are now able to take a complex mixture of different neuronal subtypes and deconvolute their divergent phenotypes to a taxonomic framework of all different neuronal subclasses present. We have demonstrated this for dorsal root ganglion neurons, and can assign any neuron in the lumbar DRGs into one of 16 neuronal subclasses. Our ability to be able to firmly identify neuronal subclasses allows us to quickly determine whether or not a novel venom component acts on that particular subclass, and what the nature of the activity is (for example, does it cause an increase in neuronal excitability, or does it depress the excitability). The real breakthrough is that because of the accessibility of single-cell transcriptomics, we can quickly compare the neuronal subtypes that are affected to those which are not for each individual bioactive venom component — this provides us with a pathway to identifying the molecular target of that bioactive venom component.

Thus, when we combine our increased ability to identify bioactive venom components *in silico*, with the possibility of assessing the identity of the molecular targets, the synergy gained through these two advances means that we are able to greatly improve our throughput in terms of identifying promising bioactive peptides, and characterizing the mechanism through which they act. This new paradigm bypasses several bottlenecks in the discovery and characterization of novel bioactive venom components. We had originally anticipated that the biochemical definition of interesting lead compounds from the venoms that we are focusing on would be achieved only in part of the grant; because of these technological advances, this work has gone much faster.

Thus, from *Conus rolandi* venom, instead of having only 1 characterized peptide, there are now 4 in which a thorough biochemical definition and characterization of the bioactivity were carried out. Remarkably, all of these peptides that have been purified, although distinct in sequence (and therefore presumably targeting different macromolecular components of the nervous system) cause a decrease in excitability both at the behavioral level, and at the level of individual neurons. Thus, instead of a characterization of different unpurified fractions of these venoms, what has been achieved is the biochemical definition of promising bioactive venom components, the identification of homologs in related venoms, the successful chemical synthesis of these bioactive venom components and of analogs that will provide structure/function information. It was not anticipated that this would be achieved in the first 24 months of this grant period, and we are therefore further ahead than planned in this respect.

The lopsided progress in these aspects of the project have, in many ways, reordered our priorities; thus for example, the new compounds are much more promising than the conantokin peptides that we originally proposed to characterize, and therefore it is our intention to define the bioactivity of these peptides with a higher priority.

Summary overview of Specific Aims 1 and 2.

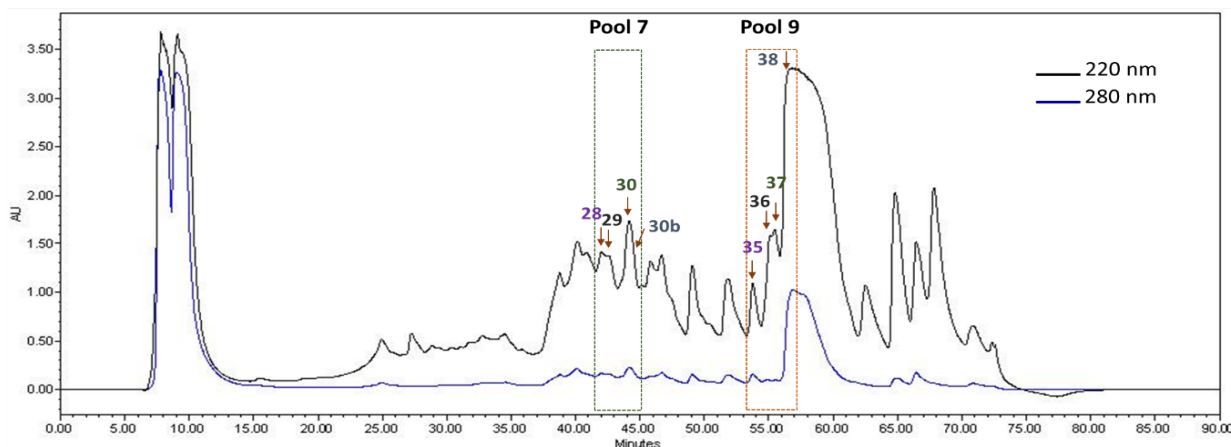
Specific Aim 1, Major Task 1, Subtask 1 & 2. Major Task 2, Subtask 1 & 2.

The aims of Specific Aim 1 have been largely achieved, and as is outlined above, advances in technology have allowed us to finesse the need for elaborate collection trips to accumulate substantial amounts of venom (which is fortunate, given that such an effort would not have been possible because of the COVID-19 pandemic). Thus, for the remaining of the grant period, we do not see the need for additional collection trips, since the largely *in silico* strategy outlined in the overview above has provided us with a vastly increased pipeline of potentially-novel peptides that need to be assessed by the core, and through Specific Aim 2. Specific Aim 2, Major Task 1, Subtasks 1 & 2.

As outlined in the overview, we now have a shortcut to determining the amino acid sequence of peptides of potential biomedical importance, and go directly to the chemical synthesis or expression of what is now an expanded peptide pool that need to be tested. These types of tests have been carried out on additional cone snail species besides the high-priority *Asprella* species that were mentioned in last year's report. The peptides related to the somatostatin receptor-targeted peptide, and to the MSRLF superfamily peptides have been synthesized and characterized, but in addition, additional families of peptides from other lineages of *Conus* and non-*Conus* Conoideans have also been assessed. Thus, we are able to survey a much wider range of venom peptides than we had anticipated because of the technological advances summarized in the Overview.

A significant amount of work was done in screening the venom (and venom fractions) from *Conus gloriamaris* (glory of the sea). The figure below shows the HPLC profile of *C. gloriamaris* venom that was tested in functional assays. Bioactive components were observed in pool 7 and pool 9 of the venom, that were further sub-fractionated to identify the bioactive peptides.

***C. gloriamaris* Preparative HPLC Chromatogram**



Solvent A: 0.1% TFA-H₂O; Solvent B: 90% CH₃CN + 0.1% TFA-H₂O

Gradient (% Solvent B): 5%-55% (50 min)_ 65% (55 min)_ 100% (60 min)_100% (62 min)_5% (67 min)_5% (87 min)

Flow rate: 7 ml/min

Project 2: The table below summarizes the results of screening venom and fractions from *C. gloriamaris*. Current work is focused on identifying the peptide sequence of fraction 9-37-22, 9-37-23. Pool 9-38 will be further sub-fractionated into 25 fractions that will be tested to identify the bioactive peptides.

	SAMPLE	ACTIVITY		SAMPLE	ACTIVITY
1	CgM Full Venom	Direct effects distributed between classes: G8, R11, R12, N16	35	CgM Pool 7-29	No Effect
2	CgM Pool 1	No Effect	36	CgM Pool 7-30	No Effect
3	CgM Pool 2	No Effect	37	CgM Pool 7-30b	No Effect
4	CgM Pool 3	No Effect	38	CgM Pool 8	No Effect
5	CgM Pool 4	No Effect	39	CgM Pool 9	Direct effects in large unlabeled G8, R11, N14 & N16
6	CgM Pool 5	No Effect	40	CgM Pool 9-35	No Effect
7	CgM Pool 6	No Effect	41	CgM Pool 9-36	No Effect
8	CgM Pool 7	Direct effects distributed between classes: G9, R11, R12, N16	42	CgM Pool 9-37	Direct Effect
9	CgM Pool 7-28	Direct effects in classes R12 & N16	43	CgM Pool 9-37-4	No Effect
10	CgM Pool 7-28-1	No Effect	44	CgM Pool 9-37-5	No Effect
11	CgM Pool 7-28-2	No Effect	45	CgM Pool 9-37-6	No Effect
12	CgM Pool 7-28-3	No Effect	46	CgM Pool 9-37-7	No Effect
13	CgM Pool 7-28-4	No Effect	47	CgM Pool 9-37-8	No Effect
14	CgM Pool 7-28-5	No Effect	48	CgM Pool 9-37-9	No Effect
15	CgM Pool 7-28-6	No Effect	49	CgM Pool 9-37-10	No Effect
16	CgM Pool 7-28-7	No Effect	50	CgM Pool 9-37-11	No Effect
17	CgM Pool 7-28-8	No Effect	51	CgM Pool 9-37-17	No Effect
18	CgM Pool 7-28-9-10	No Effect	52	CgM Pool 9-37-21	No Effect
19	CgM Pool 7-28-11	No Effect	53	CgM Pool 9-37-22	Direct Effect
20	CgM Pool 7-28-12	No Effect	54	CgM Pool 9-37-23	Direct Effect
21	CgM Pool 7-28-13	No Effect	55	CgM Pool 9-37-24	No Effect
22	CgM Pool 7-28-14	No Effect	56	CgM Pool 9-37-27	No Effect
23	CgM Pool 7-28-15	No Effect	57	CgM Pool 9-37-28	No Effect
24	CgM Pool 7-28-16	No Effect	58	CgM Pool 9-37-29	No Effect
25	CgM Pool 7-28-17	No Effect	58	CgM Pool 9-37-30	No Effect
26	CgM Pool 7-28-18	No Effect	59	CgM Pool 9-37-31	No Effect
27	CgM Pool 7-28-19	No Effect	60	CgM Pool 9-37-32	No Effect
28	CgM Pool 7-28-20	No Effect	61	CgM Pool 9-37-33	No Effect
29	CgM Pool 7-28-21	No Effect	62	CgM Pool 9-37-34	No Effect
30	CgM Pool 7-28-22	No Effect	63	CgM Pool 9-37-35	No Effect
31	CgM Pool 7-28-23	No Effect	64	CgM Pool 9-37-36	No Effect
32	CgM Pool 7-28-25	No Effect	65	CgM Pool 9-37-37	No Effect
33	CgM Pool 7-28-26	No Effect	66	CgM Pool 9-37-38	No Effect
34	CgM Pool 7-28-28	No Effect	67	CgM Pool 9-38	Direct Effects in large unlabeled neurons, R11, G8
			68	CgM Pool 10	No Effect

In addition to *Conus gloriamaris*, we initiated and successfully completed screening of *Conus geographus* venom to identify bioactive components. These are currently being followed through sub fractionation and identification of bioactive peptides that will be useful for the study of chronic pain. The figure below demonstrates our approach to identification and characterization of bioactive venom components of *Conus geographus*.

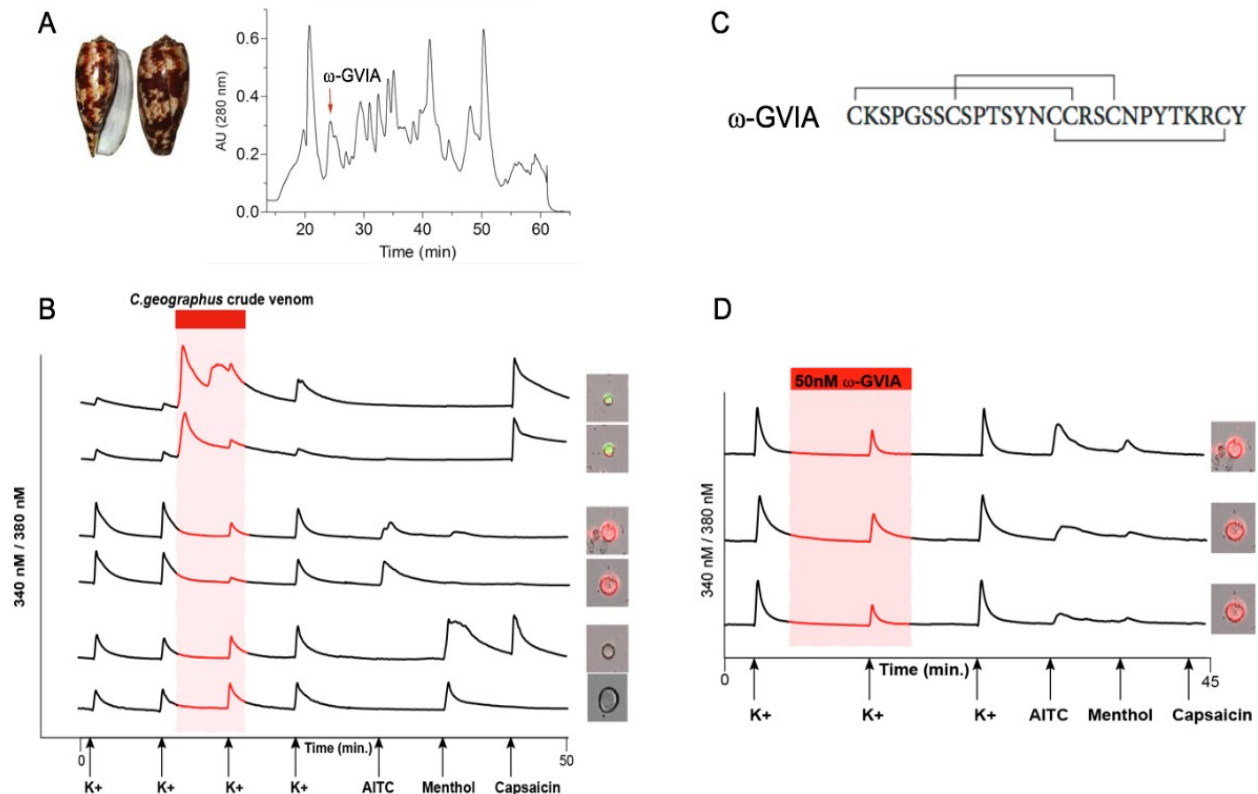


Figure: Identification of bioactive components of venom using constellation pharmacology

A: HPLC profile of crude venom obtained from *Conus geographus* B) Diverse effects of crude venom on dissociated neurons from mouse TG using constellation pharmacology. The intracellular calcium was monitored from different neuronal cell classes. Examples of three cell classes are shown. Application of crude venom elicited robust calcium influx in a subset of peptidergic nociceptors (top two trace). Crude venom blocked the depolarization induced calcium response in a subset of non-peptidergic neurons (middle two traces) and some neurons were unaffected by crude venom (bottom two trace). Neuronal identity was obtained using a combination of pharmacology, transgenic expression of fluorescent proteins and dye stains. C) The peptide sequence of one component of crude venom, ω -conotoxin GVIA. D) Application of 50nM GVIA peptide replicated the block in non-peptidergic neurons as shown. X-axis: Time (in minutes). Arrows indicate 15 second application of pharmacological agents which was washed and sequentially applied every 7 minutes. High extracellular K⁺ was applied before and after the application of crude venom. Highlighted region (in red) was monitored for the effects of crude venom. Mustard oil (allyl isothiocyanate (AITC)), menthol and capsaicin were applied for classification of neuronal types. Data was obtained from individual cell (shown to the right of each trace). n=1500 cells monitored per trial, N=10 trials.

The experiments testing different components of *C. geographus* is summarized in the table below. Each component consists of ~20 peptides that are being fractionated and purified to identify the bioactive peptides. Highlighted are the fractions that displayed bioactivity in constellation pharmacology assays.

Sample No:	Sample Code	Notes
1	C geo P1 L652	
2	C geo P1 L645 1	
3	C geo P3 L645 11 12	
4	C geo P3 L645 11 13	
5	C geo P3 L645 11 14	
6	C geo P3 L645 11 16	
7	C geo P3 L645 11 17	
8	C geo P3 L645 11 18-19	
9	C geo P3 L645 11 20-21	
10	C geo P3 L645 11 24	
11	C geo P3 L645 11 25	
12	C geo P5 L645 17	
13	C geo P5 L645 18	
14	C geo P5 L645 19	
15	C geo P5 L645 20	
16	C geo P5 L645 21	
17	C geo P7 L645 27 11	
18	C geo P7 L645 27 12	
19	C geo P7 L645 27 13	
20	C geo P7 L645 27 14	Active (block)
21	C geo P7 L645 27 15	
22	C geo P7 L645 27 16	
23	C geo P7 L645 27 17	
24	C geo P7 L645 27 18-19	
25	C geo P7 L645 27 20-21	
26	C geo P7 L645 27 22	
27	C geo P7 L645 27 23-24	
28	C geo P7 L645 27 25-26	
29	C geo P7 L645 27 27	
30	C geo P7 L645 29 5-7	
31	C geo P7 L645 29 8	Active (Block)
32	C geo P7 L645 29 9	
33	C geo P7 L645 29 10	
34	C geo P7 L645 29 11	
35	C geo P7 L645 29 12	Active (Block)
36	C geo P7 L645 29 13-14	

Sample No:	Sample Code	Notes
37	C geo P7 L645 29 15	
38	C geo P7 L645 29 16	
39	C geo P7 L645 29 17	
40	C geo P7 L645 29 18	
41	C geo P7 L645 29 19	
42	C geo P7 L645 29 20	
43	C geo P7 L645 29 21	
44	C geo P7 L645 29 24	
45	C geo P7 L645 29 25	
46	C geo P7 L645 29 26	
47	C geo P7 L645 29 27	
48	C geo P7 L645 29 28	
49	C geo P7 L645 29 29	
50	C geo P7 L645 29 30	
51	C geo P7 L645 29 31	
52	C geo P7 L645 29 32	
53	C geo P9 L645 37	Active (Direct Effects)
54	C geo P9 L645 38	Active (Direct Effects)
55	C geo P9 L645 39	
56	C geo P9 L645 40	
57	C geo P9 L652 27	
58	C geo P9 L652 28	
59	C geo P9 L652 29	Active (Block)
60	C geo P9 L652 29-11	Active (Block)
61	C geo P9 L652 29-12	
62	C geo P9 L652 29-13	
63	C geo P9 L652 29-14	
64	C geo P9 L652 29-15	
65	C geo P9 L652 29-16	
66	C geo P9 L652 29-17	
67	C geo P9 L652 29-18	
70	C geo P9 L652 29-19	
71	C geo P9 L652 29-20	

Project 2, Specific Aim 1, Major Task 1,

Subtask 1 and 2: This has largely been completed. We have detailed observations on the behavior of cone snail species of interest, and have established adequate collection methods, and identified new locations for obtaining the desired specimens. The collection of the high-priority species will continue as part of Major Task 2 throughout the grant period, but we have a clear definition of which species need to be targeted for collection.

Project 2, Specific Aim 1, Major Task 2, Subtask 1 and 2: Venom glands were collected from several species of cone snails during a field collection trip in the Solomon Islands. These include species that are not readily found in the Philippines or other areas

the PI has access to. Samples were collected under a collection and license agreement with the Solomon Islands National University. A subset of these samples has been sent for transcriptome analyses. The remainder are stored at -80C in the PI's lab until further analyses.

Project 2, Specific Aim 2, Major Task 1, Subtask 1: We initiated an assessment of venom fractions from high-priority species by Constellation Pharmacology assays. An initial purification of the bioactive venom fractions has been achieved, and the structure of several of these have been determined. Chemical synthesis of the initial tranche of peptides has been carried out.

Project 2, Specific Aim 2, Major Task 1, Subtask 2: Venoms were collected, extracted and fractionated from two high priority *Asprella* species, *Conus rolandi* and *Conus sulcatus*. Venom fractions have been injected ICV and IP in mice and phenotypic behaviors have been recorded. The venom of *Conus rolandi* contained multiple compounds that elicited a hypoactive phenotype in mice. These compounds have been further analyzed by Edman and mass spectrometric sequencing. A subset of these peptides has been made synthetically for more in-depth characterization with a particular emphasis on a group of peptides called DW-peptides. Synthetic peptides were sent to the NIH funded psychoactive drug screening facility (PDSP) in North Carolina for target determination. This group of peptides appears to target a particular subtype of the somatostatin receptor (SST4) that has recently been implicated in non-opioid pain. One of these peptides, Cont-Ro4, proved to induce analgesia in the formalin pain model. Others are currently under investigation.

One peptide from *Conus rolandi* MSRLF-Ro1.2 and one peptide from *Conus sulcatus* MSRLF-Su1.2 were sequenced by mass spectrometry. Both peptides have similar amino acid sequences. The peptides were synthesized by solid-phase-synthesis and used for intracranial injections in 16 old mice at different doses. The most evident and consistent phenotypes were recorded at 20 nmol. Both peptides induced a similar hypoactive and unresponsive phenotype. In brief, after 12 minutes, the mice became hypoactive and unresponsive to touch and sound. Twenty-five minutes after injection, the mice leaned against the wall and used their front two paws to climb the wall. Eventually, the mice appear to fall asleep against the wall with its front two paws in a vertical position. All mice recover after 2 hours.

Project 2, Specific Aim 3, Major Task 1, Subtask 1: Together with clinicians at HCI we have begun to write the study protocol, including a detailed budget for patient recruitment, treatment, observation and drug production. Additionally, we have begun to gather published and unpublished preclinical data in order to ask for a pre-IND call with the FDA. We anticipate that the FDA may suggest amendments to the proposed study and potentially ask for additional preclinical data.

Project 2, Specific Aim 3, Major Task 2, Subtask 1: As part of this project, one of the peptides from a high-priority venom (*Conus rolandi*) was tested in an animal model of acute pain for analgesic effects. We plan to test the peptide in CCI and SNL pain models because very rapid progress in our Constellation Pharmacology has indicated that the analysis will be much more revealing if instead of testing candidate peptides in rats, we test their efficacy in mice. If analgesic effects are found, then the precise mechanisms of the peptides can be evaluated in a much more thorough and refined way.

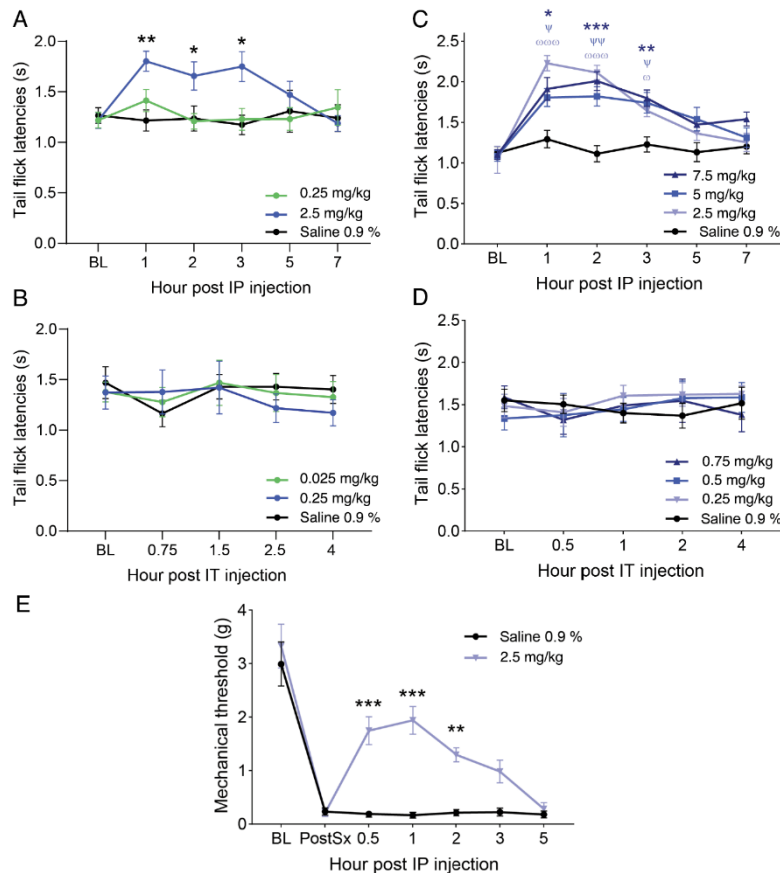
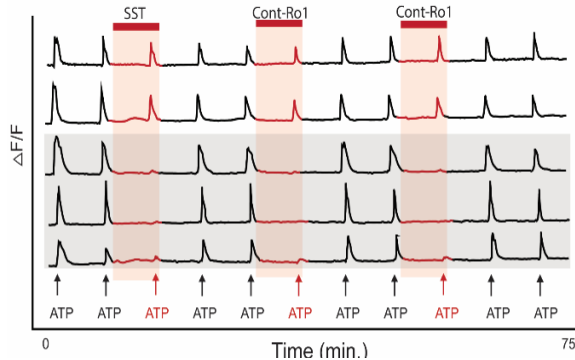


Figure: Cont-Ro1 provides analgesia in mouse models of acute pain. A-D. Sensitivity to thermal noxious stimuli was evaluated by dipping mouse tails into hot water (52°C) and recording tail flick latencies (TFLs). A. Mouse tail flick latencies after a single intraperitoneal (IP) injection of 2.5 mg/kg but not 0.25 mg/kg resulted in an increase in TFLs. TFLs were captured over a 7-hour period (n=6-7 per group, two way ANOVA with Tukey's post-hoc test, *p<0.05, **p<0.01, stars illustrate significant difference to saline condition). B. no significant changes were observed in TFLs after intrathecal (IT) injection of 0.25 mg/kg or 0.025 mg/kg. TFLs were captured over a 4 hour period (n=8 per group, two way ANOVA). C-D. TFLs were recorded after IP or IT injection of higher doses of Consomatin-Ro1. For IP injections, TFLs were captured over a 7 hour period (n=6-8 per group, two way ANOVA with Tukey's post-hoc test, */ω/ψp<0.05, **/ωω/ψψp<0.01, ***/ωωω/ψψψp<0.001, *: 2.5mg VS saline, ψ: 5mg VS saline, ω: 7.5mg VS saline). For IT injections, TFLs were captured over a 4 hour period (n=7-8 per group, two way ANOVA).E. Analgesic effect of Consomatin-Ro1 on acute post-surgical pain. Plantar incision surgery was performed on the hind paw. 24-hours post-surgery, animals were administered saline solution or 2.5 mg/kg of Consomatin-Ro1 through IP injections. Mechanical sensitivity was assessed using Von Frey filaments (n=6 per group, two-way ANOVA with Sidak's post-hoc test, black asterisks illustrate significant

differences to saline control, **p<0.01, ***p<0.001).

Analgesic effects of ContRo1 can be mediated by attenuating purinergic signaling.



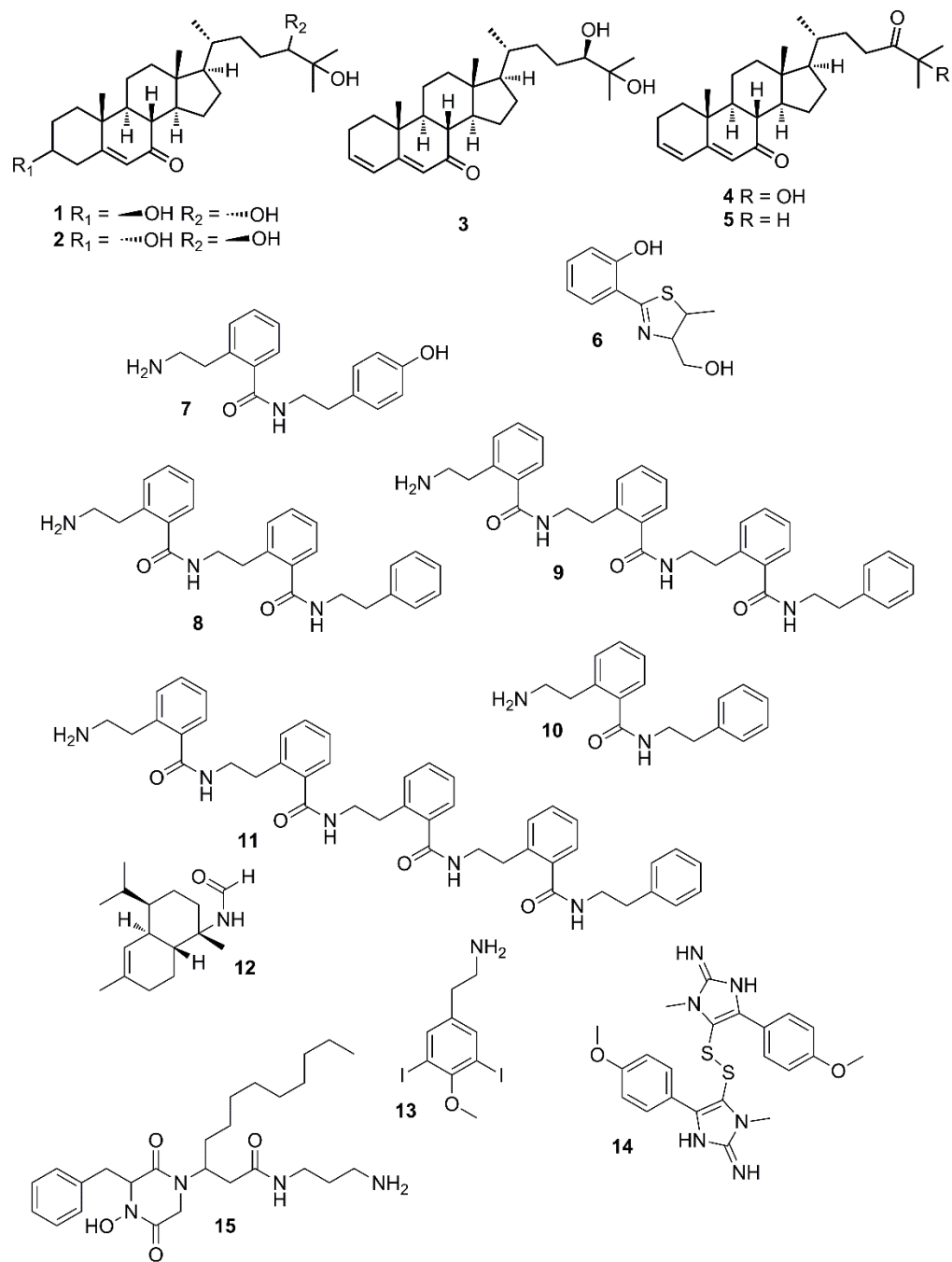
Current experiments are focused on investigating the molecular mechanisms of ContRo1 mediated analgesia. We have identified that ContRo1 binds to somatostatin receptor and we tested the effects of ContRo1 on THP-1 derived macrophages. As shown in figure, both somatostatin and ContRo1 attenuated ATP induced calcium signals. We are currently investigating this novel non-opiate molecular mechanism with functional implication in attenuating pain.

Project 3, Specific Aim 1: This aim involves creating extracts and arraying them for screening. This year, our task was completed as planned, and we achieved the milestone numbers. We were not able to perform our usual sample activity because of COVID-19. To remedy that situation to the best of our ability, we took an invaluable collection of marine sponges from the career of Chris Ireland (recently retired) and used those in a screening campaign. Moreover, in a project funded by NOAA, we have been investigating the symbiotic bacteria of marine shipworm mollusks, and we used a sample of bacteria obtained in Alabama in December, 2019, to obtain bacterial strains for screening in year 3. In this way, we hit our target numbers for the year.

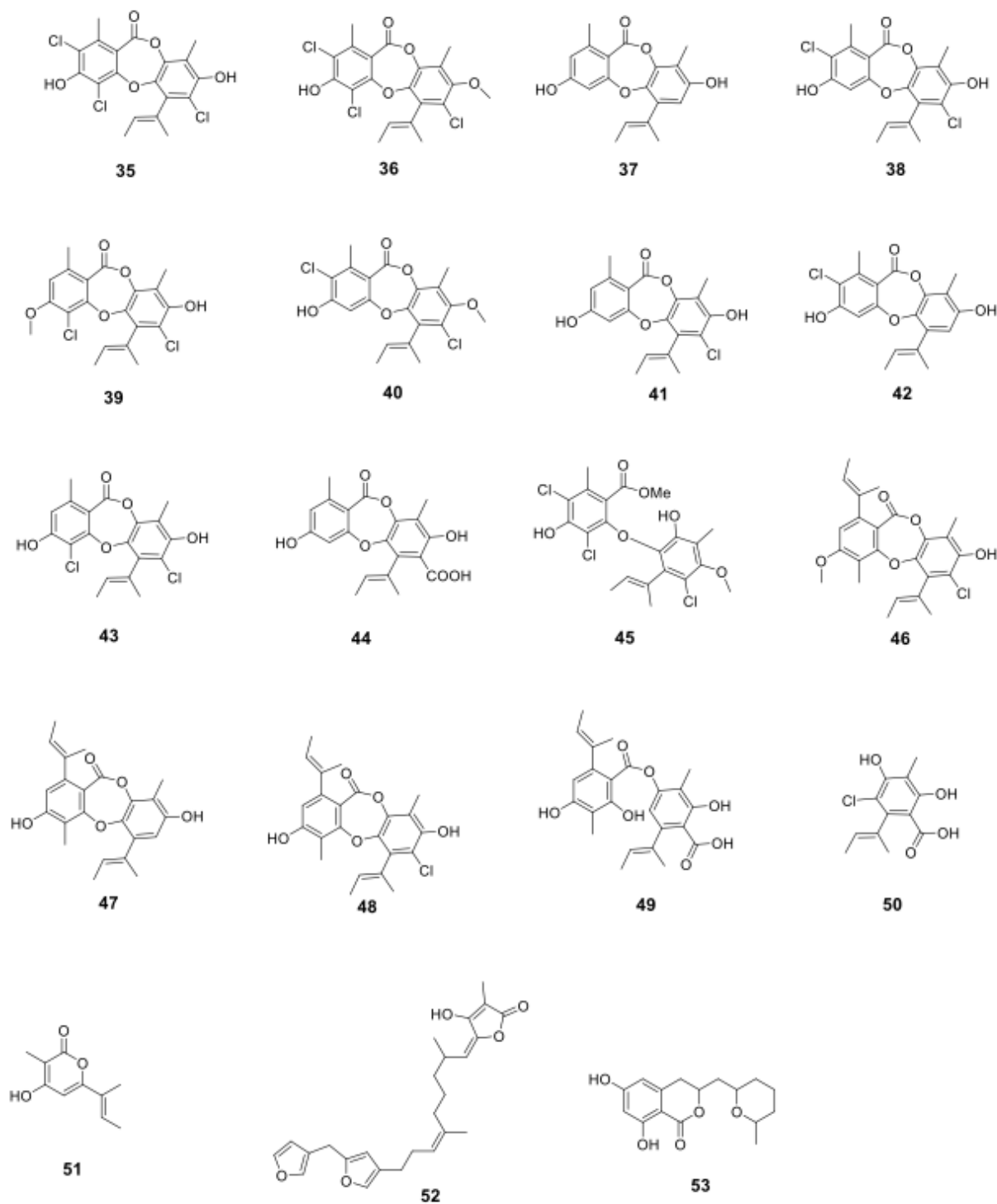
Project 3, Specific Aim 2: We aim to perform screening of the chemical libraries obtained in aim 1. We hit our target screening numbers, as shown in the table. **THIS REFERS TO THE ATTACHED TABLE.**

Project 3, Specific Aim 3: We aimed to perform activity-guided purification of active compounds and use chemical methods to characterize structures of at least 10 active hits annually. So far, in three years, we have identified 53 active hit compounds by purifying them and performing spectroscopy (see Figure 1). Many of these are new to science. Moreover, we proposed a milestone to annually move at least 2 compounds into further preclinical development. We have so far found 8 compounds that are viable hits and are investigating their properties further.

Figure 1. Active hits discovered in this project years 1, 2, and 3.







Last year, we developed gram-scale syntheses capable of providing sufficient material of an active analog of compounds **4** and **5**, as well as compounds **6**, **8**, **9**, and **11** for full pharmacological testing and preclinical development. We previously reported syntheses of **6** and analogs in a *Journal of Natural Products* manuscript in 2017.

In the past year, the major advance that we made with these initial hit compounds is that we characterized their *in vitro* potency and mechanisms of action, and we determined their efficacy animal models. Briefly, we showed that compounds **1-5** and synthetic analog SSP3 are neurosteroids that modulate type A GABA receptors, and that they have different receptor subunit selectivity than available neurosteroids. Synthetic SSP3 exhibited antinociceptive activity in the hot plate assay. Compound **8** and analogs are primarily subtype-selective antagonists of nicotinic acetylcholine receptors (nAChRs) in peptidergic nociceptors. They are active in the formalin model of neuropathic pain. *Neurosteroids*. Using SSP3, a synthetic analogue of neurosteroids **1-5**, we demonstrate that the compounds modulate type A GABA receptors, and are selective for $\alpha 3 > \alpha 2 > \alpha 1$ subunits (Figure X). Neurosteroids have diverse mechanisms of action, focused on GABA A receptors, and they have been used clinically for diverse conditions including as anesthetics and to treat postpartum depression. The activity differs from that of anesthetic or antidepressive neurosteroids, leading . Moreover, their structures are much different than those known for other neurosteroids, expanding the chemical space available for designing novel neurosteroids for therapeutic use. In addition, we learned that SSP3 (also called compound **6**) is active in the hot plate assay, indicating a potential application in pain (Figure Y).

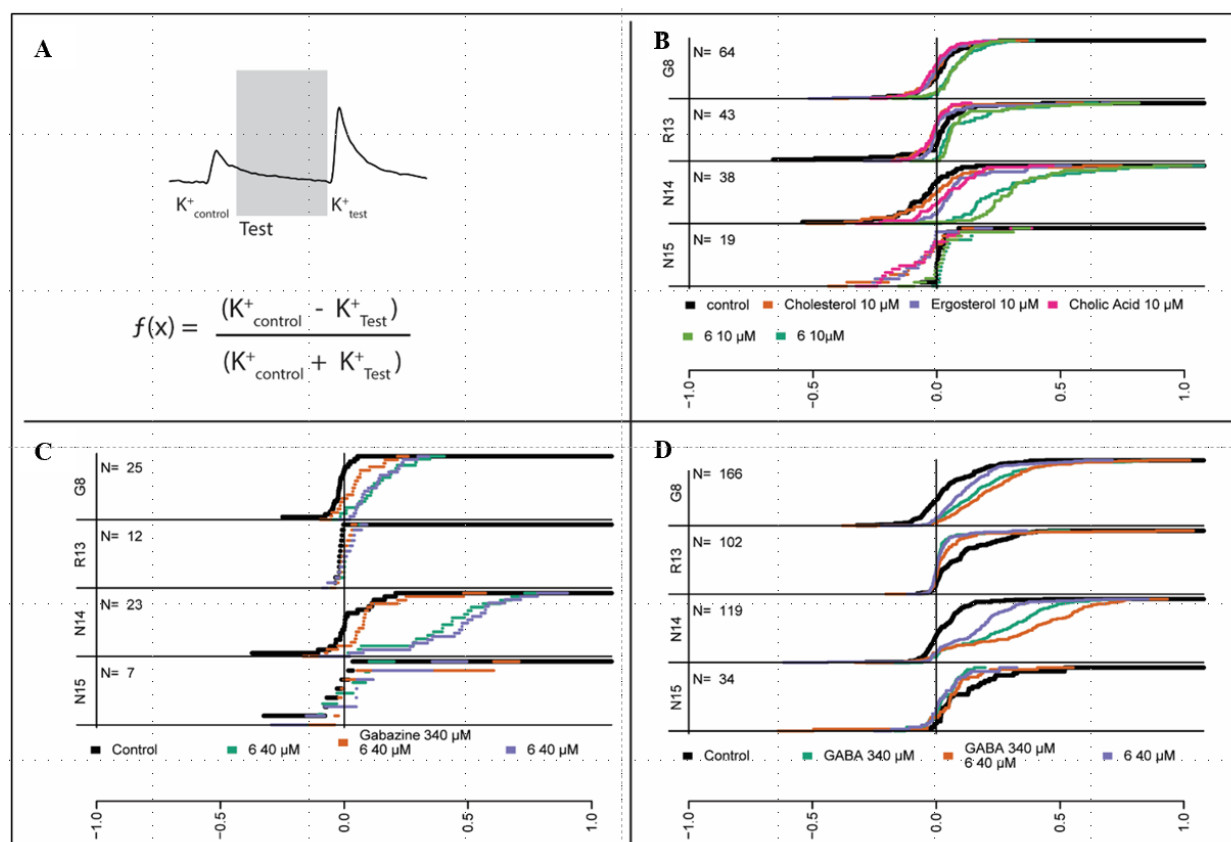


Figure X. SPP3 (described here as “compound **6**”) is a direct activator of GABA receptor in some cells and a partial allosteric modulator in others. This is part of the evidence demonstrating the mechanism of action and subtype receptor selectivity of SSP3. **(A)** The output of DRG / constellation pharmacology experiments was analyzed using this equation, leading to graphs shown in **(B-D)**. In the x-axis, the value of -1 to 1 indicates block to amplification, with 0 indicating no effect. The y-axis indicates the relative number of cells

that fall below the given value (the empirical distribution function), from 0 (bottom) to 1.0 (top). The number of cells in each statistic is provide as N= #. For example, in panel (B), 64 cells belonging to the G8 nociceptor group were used. N14 neurons are significantly amplified by compound **6** (green traces), but when compared with other sterols, these cells are not amplified. In (C) the effect of gabazine can be observed in N14 and G8 cells. While **6** causes an amplification of Ca²⁺ flux in N14 and G8 cells, when gabazine is applied the effect goes down nearly to zero. In (D), the experiment was designed to determine whether **6** has PAM activity. In the N14 neuron, **6** gives a smaller amplification event than does GABA, but the combination of **6** and GABA results in an even larger response. This effect somewhat duplicates in G8 but is not as dramatic as N14. Notably, N15 cells are not affected in panels (C) and (D).

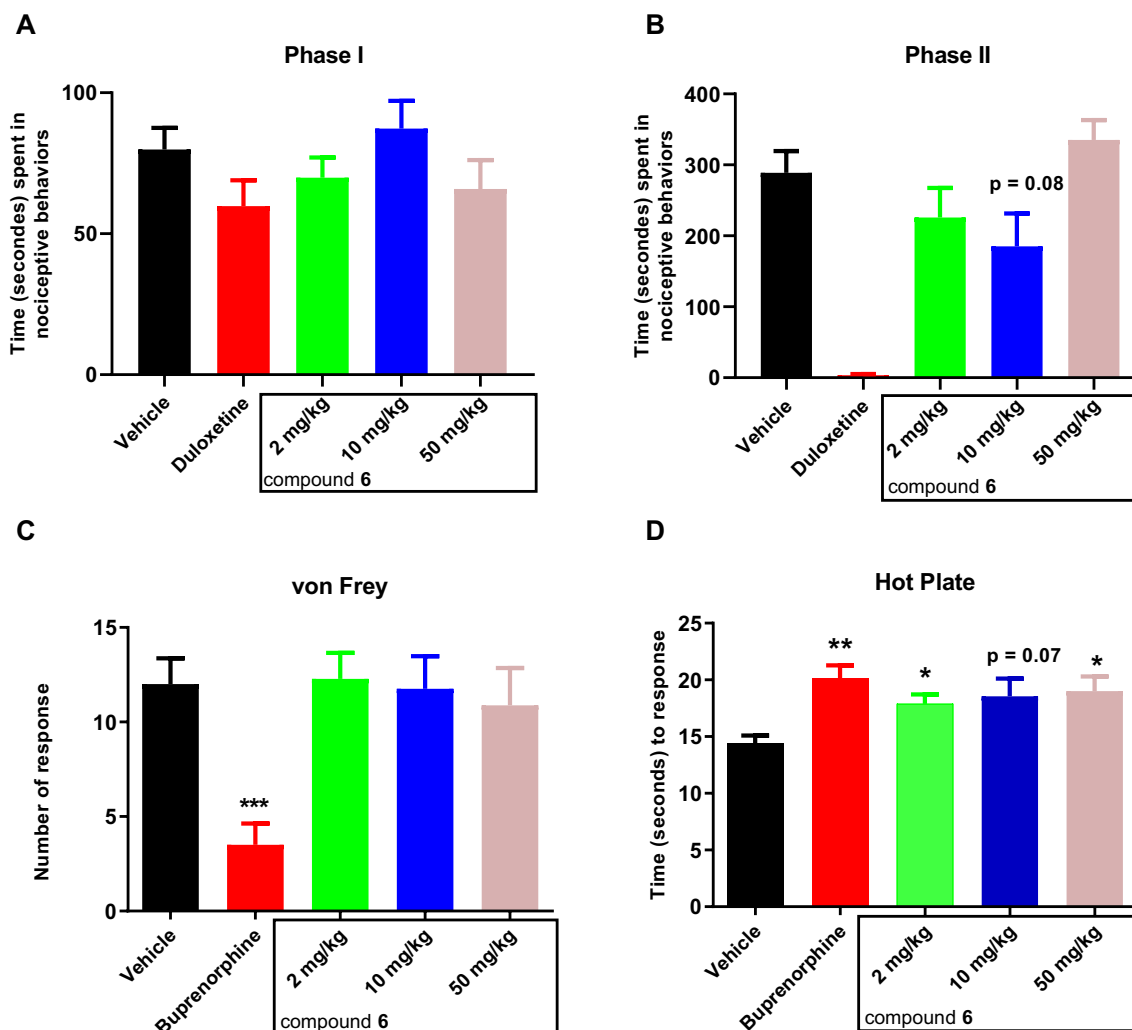


Figure Y. Pain models used to potential utility of SPP3, also known as compound **6** (2, 10, and 50 mg/kg, i.p.). In all panels, error bars indicate the mean $\pm$ the standard error of the mean, using 8 mice per group. Statistical significance was assessed by comparing text compounds to vehicle using one-way ANOVA, (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (A) and (B) represent the response to formalin in phase I (the first five min post-injection) and phase II (the time between 20-35 min post-injection). (C) In the von Frey test for mechanical nociception, a 2g stimulus was applied to each mouse 30 times, and the total number of responses (fast withdrawal, flinching or licking/biting of the paw in response to the filament contact) was recorded. (D) In the hot plate test for thermal nociception, animals were placed on a 52 °C hot plate, and the amount of time prior to nocifensive behavior was recorded.

Polyamides as new, subtype-selective nAChR antagonists. In previous years, we isolated and then synthesized new molleamine compounds, exemplified by compound **8**, as interesting and effective new chemical entities. This year, we determined the mechanism of action and antinociceptive potential of this compound series. Using constellation pharmacology (Figure Z), we determined that compound **8** specifically blocks nAChRs in nociceptors. We then demonstrated a useful effect on pain using in vivo assays (Figure AA).

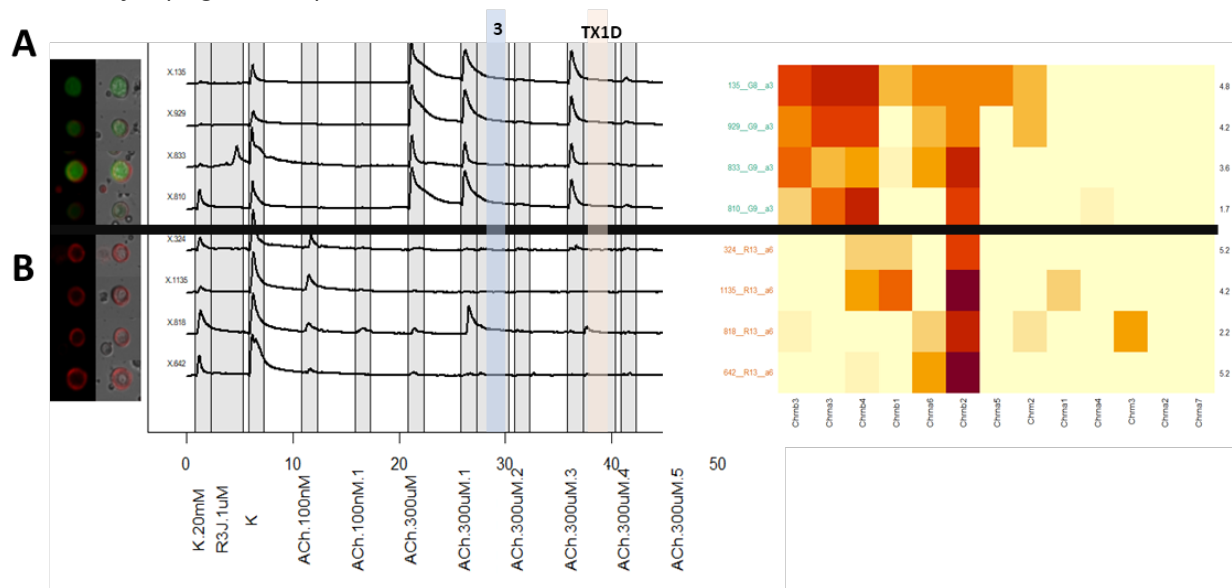


Figure Z. Comparative transcriptomic analysis of neurons that were blocked by compound **8**, referred to in this figure as “compound **3**”. This figure shows some of the evidence that demo (A) to those that were not affected by **3** (B) revealed enhanced expression of transcripts for $\alpha 3$ (Chrna3), $\beta 3$ (Chrnb3), $\beta 4$ (Chrnb4), $\beta 2$ (Chrnb2) and $\alpha 6$ (Chrna6) nicotinic acetylcholine receptor subunits. TX1D is a highly selective and potent antagonists of $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ nAChRs. Because **3** and TX1D both block the ACh responses from the same cells, **3** modulates the activity of $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ nAChRs, as does TX1D. The advantage of compound **3** is that it is a small molecule, rather than a large peptide like TX1D.

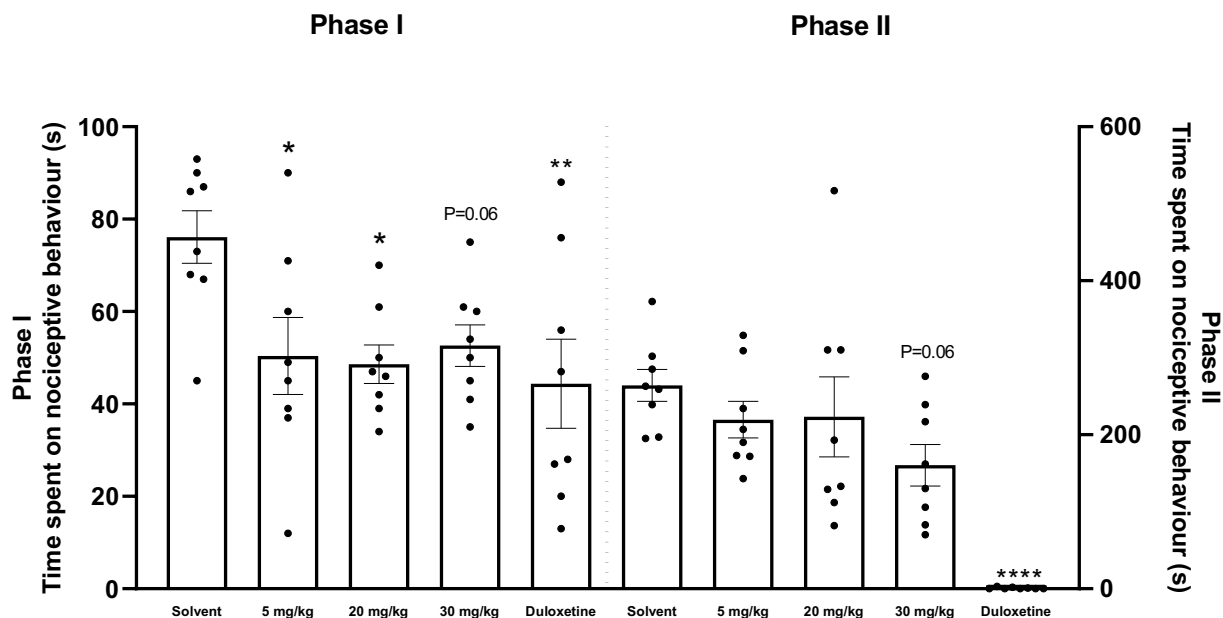


Figure AA. Compound **3** is antinociceptive in Phase I and Phase II of the formalin test. Male adult (6-8 weeks old) C57BL/6 mice were pre-treated with compound **3** (5 mg/kg, 20 mg/kg, 30 mg/kg, i.p.), vehicle (5 mL/kg, i.p.) or duloxetine (positive control drug, 10 mg/kg, 10 mL/kg, i.p.). Formalin (5% in saline) was injected into the left hind paw pad (intraplantar) after dosing; nociceptive behaviours (including flinching, shaking, biting and licking of the injected paw in the mice) were recorded for 0-35 min. Phase I represents the sum of time spent on nociceptive behaviour in the first 5 minutes immediately after formalin injection while Phase II denotes the response period 20-35 minutes after injection. Each individual animal is represented by a circle, and the average $\pm$ standard error of the mean is shown by the histogram. The compound effect versus vehicle (* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$) was evaluated by One-way ANOVA and Dunnet's multiple comparisons test ($n = 8$ mice per treatment) using Graphpad Prism 8.4.3 (Graphpad Software, San Diego, CA). Compound **3** inhibited the display of nociceptive behaviours in Phase I at doses 5 mg/kg ($P < 0.05$), 20 mg/kg ($P < 0.05$), and 30 mg/kg ($P = 0.06$). In Phase II, only the 30 mg/kg dose of compound **3** showed efficacy in reducing the nociceptive behaviour ($P = 0.06$).

Summary: In the first 3 years, we achieved the overall goals of obtaining and curating the sample extracts. We met the major goal of characterizing at least 20 hits and further investigating four promising lead compounds. We have assessed the major hits in appropriate nociception assays in mice and have drafted the first publications. In the final year, we hope to accelerate discovery of new compounds based upon the lessons we have learned in the first few years, and to fully characterize and determine the therapeutic potential of molecules discovered in previous years.

Sample Type	Number screened	Summary Table	
		Constellation Pharmacology hits	TRP Channel Assay hits
Animal	125	34	15
Cultivated bacteria	318	4	4
Total	306	36	8
Purified compounds	53	37	31

All Screening List
1. Animal Specimens

No.	Collection Code	Type of sample	Type of animal	Type of organ	Targeted Screening			Phenotypic Screening (Constellation Pharmacology Assay)
					TRPM8	TRPV1	TRPA1	
1	17-19-HB	animal extract	<i>C. geographus</i>	hypobranchial gland	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	ATP: Block: 38%, Amp: 28%
2	18-1-HB	animal extract	<i>C. ammiralis</i>	hypobranchial gland	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	ATP: Block: 78%,
3	18-2-HB	animal extract	<i>C. bandanus</i>	hypobranchial gland	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	ATP: Block: 34%, Amp: 25%
4	18-3-HB	animal extract	<i>C. eborneous</i>	hypobranchial gland	<i>antagonist</i>	<i>antagonist</i>	<i>antagonist</i>	ATP: Block: 33%, Amp: 44%, KCl: Block: 75%
5	18-3-VD	animal extract	<i>C. eborneous</i>	venom duct	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	ATP: Block: 55%, Amp: 40%

6	18-5-HB	animal extract	<i>C. leopardus</i>	hypobranchial gland	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	ATP: Block: 48%, Amp: 48%
7	18-7-HB	animal extract	<i>C. textile</i>	hypobranchial gland	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	ATP: Block: 35%, Amp: 16%
8	18S-1a	animal extract	<i>C. litteratus</i>	Whole body	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	ATP: Block: 58%, Amp: 38%
9	18S-2	animal extract	<i>Corilcella nigra</i>	Whole body	<i>inactive</i>	<i>antagonist</i>	<i>inactive</i>	KCl: Block: 80%
10	SI-024K	animal extract	<i>Phylidiella sp.</i>	Whole body	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	KCl: Block: 81%
11	SI-223L	animal extract	<i>Pleurobranchus sp.</i>	Whole body	<i>inactive</i>	<i>antagonist</i>	<i>inactive</i>	Base line jump after the sample application due to pigmentation in the sample. The extract will be retested if needed.
12	SI-043X	animal extract	sponge	Whole body	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	ATP/K: Very big direct effect
13	SI-036L	animal extract	sponge	Whole body	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	ATP/K: Very big direct effect
14	18-8-HB	animal extract	<i>C. tessulatus</i>	hypobranchial gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Amplification of K responses
15	18-9-HB	animal extract	<i>C. vexillum</i>	hypobranchial gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Amplification of K responses
16	SI-001H	animal extract	Hippospongia	Whole body	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Amplification of K responses
17	SI-133H	animal extract	Tunicate (polycarpa?)	Whole body	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Amplification of ATP responses
18	SI-312M	animal extract	Tunicate	Whole body	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Amplification of K responses
19	SI-002K	animal extract	Ascidian	Whole body	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Amplification of K responses

20	SI-028S	animal extract	Tunicate	Whole body	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Amplification of K responses and some direct effects
21	18-4-VD	animal extract	C. generalis	venom duct	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Baseline jump
22	18-6-VD	animal extract	C. litoratus	venom duct	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
23	18-6-HB	animal extract	C. litoratus	hypobranchial gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	ATP: Few amplification
24	18-7-VD	animal extract	C. textile	venom duct	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
25	18-8-VD	animal extract	C. tessulatus	venom duct	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
28	17-01-HB	animal extract	Oliva sericea	hypobranchial gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
29	17-01-HP	animal extract	Oliva sericea	heptopancharease gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
30	17-12-HB	animal extract	Oliva miniacea	hypobranchial gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
31	17-12-HP	animal extract	Oliva miniacea	heptopancharease gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
32	17-20-HP	animal extract	C. bullatus	heptopancharease gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
33	17-22-HB	animal extract	C. chicoreus	hypobranchial gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
34	17-22-HP	animal extract	C. chicoreus	heptopancharease gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	ATP: block 3%; IDE 2%; DE 13%
35	17-14-HP	animal extract	C. arenatus	heptopancharease gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
36	17-14-VD	animal extract	C. arenatus	venom duct	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
37	17-14-HB	animal extract	C. arenatus	hypobranchial gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive

38	17-14-SG	animal extract	C. arenatus	snout gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
39	17-17-VD	animal extract	C. textile	venom duct	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
40	17-18-VD	animal extract	C. omaria	venom duct	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
41	17-19-VD	animal extract	C. geographus	venom duct	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
42	17-20-HB	animal extract	C. bullatus	hypobranchial gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
43	17-21-VD	animal extract	C. rolani	venom duct	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	K: block 37% ATP: 15%block
44	482W	animal extract	tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
45	463H	animal extract	tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
46	236P	animal extract	tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
47	397H	animal extract	tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
48	17-25-HB	animal extract	C. tribblei	hypobranchial gland	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
49	SI-072S	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
50	SI-123K	animal extract	D. molle	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
51	SI-130W	animal extract	Tunicate (Leptoclinides like)	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
52	SI-135L	animal extract	Leucetta sponge	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
53	SI-147M	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive

54	SI-148P	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
55	SI-153X	animal extract	Tunicate (Lissoclinum vareu)	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
56	SI-154Y	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	K: Block of K pulses
57	SI-329X	animal extract	D. molle	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
58	SI-330Y	animal extract	D. molle	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
59	SI-340X	animal extract	Porifera (Leucetta)	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
60	SI-387K	animal extract	D. molle	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
61	SI-462Y	animal extract	D. molle	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
62	SI-484Y	animal extract	Ascidian	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	K: Amplification
63	SI-397H	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
64	SI-390P	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
65	SI-231Y	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
66	SI-334M	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
67	SI-255K	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	ATP: Few Amplification
68	SI-237S	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive
69	SI-236P	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	ATP: Few amplification

70	SI-387K	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	ATP/K: Few amplification
71	SI-232H	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	K: few block ATP: very big direct effect
72	SI-470U	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
73	SI-245L	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	K: few blocks
74	SI-402U	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	ATP/K: Few amplification
75	SI-042W	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	K: few blocks ATP: Direct effects
76	SI-150T	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	K: several reversible blocks
77	SI-383W	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	K: several reversible blocks
78	SI-361W	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	ATP: few blocks
79	SI-358S	animal extract	Tunicate	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	inactive
80	SI-251W	animal extract	Cones snail	whole	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	K: few amplifications
81	5012T	animal extract	muricidae	whole	inactive	inactive	inactive	<i>to be tested</i>
82	5170Y	animal extract	pholad sp	whole	inactive	inactive	inactive	<i>to be tested</i>
83	5026X	animal extract	bryozoan	whole	inactive	inactive	inactive	<i>to be tested</i>
84	5017H	animal extract	bugula sp.	whole	antagonist	inactive	inactive	<i>to be tested</i>
85	5108M	animal extract	pholads	whole	inactive	inactive	inactive	<i>to be tested</i>

86	5176S	animal extract	pholads sp	whole	inactive	inactive	inactive	<i>to be tested</i>
87	5047W	animal extract	plannarian	whole	inactive	inactive	inactive	<i>to be tested</i>
88	ES19-02	animal extract	Phyllidia varicosa	whole	inactive	inactive	inactive	<i>to be tested</i>
89	ES19-29	animal extract	Chromodoris lochi	whole	antagonist	antagonist	inactive	<i>to be tested</i>
90	ES19-30	animal extract	Phyllidia sp.	whole	inactive	agonist	inactive	<i>to be tested</i>
91	QM-1	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
92	QM-2	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
93	QM-3	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
94	QM-4	animal extract	sponge	whole	antagonist	antagonist	<i>to be tested</i>	<i>to be tested</i>
95	QM-5	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
96	QM-6	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
97	QM-7	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
98	QM-8	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
99	QM-9	animal extract	sponge	whole	antagonist	inactive	<i>to be tested</i>	<i>to be tested</i>
100	QM-10	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
101	QM-11	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>

102	QM-12	animal extract	sponge	whole	inactive	antagonist	<i>to be tested</i>	<i>to be tested</i>
103	QM-13	animal extract	sponge	whole	inactive	antagonist	<i>to be tested</i>	<i>to be tested</i>
104	QM-14	animal extract	sponge	whole	inactive	antagonist	<i>to be tested</i>	<i>to be tested</i>
105	QM-15	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
106	QM-16	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
107	QM-17	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
108	QM-18	animal extract	sponge	whole	antagonist	antagonist	<i>to be tested</i>	<i>to be tested</i>
109	QM-19	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
110	QM-20	animal extract	sponge	whole	antagonist	inactive	<i>to be tested</i>	<i>to be tested</i>
111	QM-21	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
112	QM-22	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
113	QM-23	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
114	QM-24	animal extract	sponge	whole	antagonist	antagonist	<i>to be tested</i>	<i>to be tested</i>
115	QM-25	animal extract	sponge	whole	antagonist	antagonist	<i>to be tested</i>	<i>to be tested</i>
116	QM-26	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
117	QM-27	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>

118	QM-28	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
119	QM-29	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
120	QM-30	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
121	QM-31	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
122	QM-32	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
123	QM-33	animal extract	sponge	whole	antagonist	antagonist	<i>to be tested</i>	<i>to be tested</i>
124	QM-34	animal extract	sponge	whole	inactive	inactive	<i>to be tested</i>	<i>to be tested</i>
125	QM-35	animal extract	sponge	whole	inactive	antagonist	<i>to be tested</i>	<i>to be tested</i>

2. Bacterial isolates

No.	Collection Code	Type of sample	Targeted Screening			Phenotypic Screening (Constellation Pharmacology Assay)
			TRPM8	TRPV1	TRPA1	
1	1704-02-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	A subset of small diameter capsaicin responsive neurons showed good amplification to both KCl & ATP pulse to be tested to be tested Deconvolute activity: ATP block: 1% ATP amp: 14% K block: 3% K amp: 28% to be tested to be tested
2	1704-01-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	
3	1708-09-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	
4	1708-11-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	
5	1708-07-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	
6	1708-08-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	

7	1708-03-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
8	1708-10-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
9	1701b-03-ISP2	bacterial extract	<i>inactive</i>	<i>agonist</i>	<i>inactive</i>	to be tested
10	1708-02-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
11	1708-04-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
12	1708-05-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
13	1708-06-ISP2	bacterial extract	<i>antagonist</i>	<i>inactive</i>	<i>inactive</i>	to be tested
14	1724-Inquisitor-HB-01-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
15	1724-Inquisitor-HB-03-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
16	1724-Inquisitor-HB-04-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
17	1724-Inquisitor-HB-05-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
18	1701a-02-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
19	1724-Inquisitor-HB-02-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
20	1724-Inquisitor-HB-07-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
21	1706-01-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
22	1706-02-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
23	1706-03-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
24	1706-04-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
25	1705-02-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested

26	1712-03-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
27	MSI-Inquisitor- VD-01-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
28	1724- Inquisitor-HB- 09-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
29	1708-01-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
30	1704b-01- ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
31	1724- Inquisitor-HB- 08-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
32	1724- Inquisitor-HB- 11-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
33	1724- Inquisitor-HB- 10-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
34	1707-01-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
35	1705-01-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
36	1701b-04- ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
37	1701a-05- ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
38	1701a-01- ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
39	1701b-02- ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
40	1701b-01- ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
41	1701b-05- ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested

42	1701b-06-ISP2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
43	17-unknown-10-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
44	17-unknown-08-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
45	1709b-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 0% ATP amp: 35% K block: 0% K amp: 53%
46	1709b-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
47	17-unknown-07-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
48	1715-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
49	1705a-04-MA	bacterial extract	<i>antagonist</i>	<i>inactive</i>	<i>inactive</i>	to be tested
50	1705a-03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
51	1705a-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
52	1705a-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
53	1709a-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
54	17-inquisitor-HP-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
55	17-inquisitor-HP-06-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
56	17-inquisitor-HP-03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
57	1724-inquisitor-HB-03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
58	1724-inquisitor-HB-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
59	1724-inquisitor-VD-03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested

60	1712-06-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 5% ATP amp: 10% K block: 3% K amp: 7%
61	1712-05-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 0% ATP amp: 35% K block: 0% K amp: 53%
62	1712-04-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
63	1712-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
64	1712-03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 5% ATP amp: 10% K block: 3% K amp: 7%
65	1707b-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
66	1707b-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 1% ATP amp: 14% K block: 3% K amp: 28%
67	17-inquisitor- VD-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
68	1712b-03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
69	1712b-04-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
70	1707a-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
71	1707a-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
72	1722-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
73	1701-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
74	1701-03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
75	1701-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
76	1705b-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
77	1706-05-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
78	1706-06-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
79	17-unknown- 01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
80	17-unknown- 02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested

81	17-unknown-03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
82	17-unknown-04-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
83	17-unknown-05-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
84	17-unknown-06-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
85	17-inquisitor-VD-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
86	17-inquisitor-VD-04-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
87	1706-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
88	1706-04-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
89	1704-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
90	1704-04-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
91	1723-babylonia-HP-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
92	1709b-03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
93	1715-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
94	1715-03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
95	1709b-04-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
96	1723-babylonia-HP-03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
97	1723-babylonia-HP-04-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
98	1723-babylonia-HP-05-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested

99	1723-babylonia-HP-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
100	1709a-04-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
101	1709a-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
102	1712b-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
103	1724-inquisitor-VD-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
104	1709b-05-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
105	1708-01-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
106	17-unknown-09-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
107	1702-04-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
108	1708-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
109	17-inquisitor-HP-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
110	1704-03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
111	1706-05-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
112	1705c-05-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
113	1704-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
114	1705c-04-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
115	1706-02-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
116	1702-03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
117	17-slimy-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
118	17-inquisitor-VD-03	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
119	17-unknown-11	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
120	17-unknown-12	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
121	17-inquisitor-HP-05	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested

122	3976P.M.01.0 2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
123	3965P.M.02.0 3	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
124	3960Y.M.01.0 2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
125	3975M.M.01.0 2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
126	3965P.M.02.0 1	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
127	3975M.M.01.0 3	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
128	3960Y.M.01.0 3	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
129	3965P.M.02.0 2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
130	3974L- M.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
131	3974L- M.02.04	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
132	3960Y.01.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
133	3974L- M.02.03	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
134	3977S.I.01.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
135	3977S.I.01.04	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
136	3975M.M.01.0 1a	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
137	3977S.I.01.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
138	3977S.I.01.05	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
139	3975M.R.01.0 3	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
140	3973K.R.01.0 1	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested

141	3975M.R.01.0 4	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
142	3976P.R.01.0 2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
143	3975M.R.01.0 2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
144	1723 babylonia-HP- 03-MA	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
145	3976P.R.01.0 6	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
146	3973K.R.01.0 4	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
147	3973K.R.01.0 2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
148	3975M.R.01.0 5	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
149	3976P.R.01.0 4	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
150	SI- 303S.M.02.05	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
151	SI-358S- R.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
152	SI-303S- M.02.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
153	SI- 079K.M.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
154	3965P.I.01.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
155	SI- 303S.M.02.04	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
156	SI-079K- 2.I.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>antagonist</i>	<i>inactive</i>

157	SI-360U.M.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 3% ATP amp: 42% K block: 7% K amp: 41%
158	SI-360U.M.02.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
159	3965P.I.01.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
160	SI-463H.M.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 3% ATP amp: 42% K block: 7% K amp: 41%
161	3975M.R-I.01.01	bacterial extract	<i>antagonist</i>	<i>antagonist</i>	<i>antagonist</i>	Deconvolute activity: ATP block: 3% ATP amp: 42% K block: 7% K amp: 41%
162	3974L.R-I.01.02	bacterial extract	<i>inactive</i>	<i>antagonist</i>	<i>inactive</i>	<i>inactive</i>
163	SI-168L.M.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
164	3961H.R-I.01.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
165	SI-079K-2.I.02.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
166	SI-382U.M.02.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
167	3971Y.M.01.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
168	SI-357P-M.02.05	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
169	SI-079K.M.02.03	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
170	SI-382U.M.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
171	3968U.R-I.01.04	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
172	3965P.I.01.03	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested

173	SI-014L.M.02.03	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
174	3968U.R-I.01.05	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
175	3961H.R-I.01.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
176	3977S.R-I.01.04	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
177	3968U.R.I.01.03	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
178	3965P.R.01.06	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
179	3973K.M.02.05	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 11% ATP amp: 43% K block: 17% K amp: 11%
180	SI-014L.M.02.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 4% ATP amp: 1% K block: 9% K amp: 7%
181	3973K.M.02.04	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 4% ATP amp: 1% K block: 9% K amp: 7%
182	SI-071P.R-I.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 4% ATP amp: 1% K block: 9% K amp: 7%
183	3969W.R.02.01	bacterial extract	<i>inactive</i>	<i>antagonist</i>	<i>inactive</i>	to be tested
184	3971Y.M.02.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	ATP block: 7% ATP amp: 7% K block: 13% K amp: 4%
185	SI-454L-I.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	ATP block: 8% ATP amp: 4% K block: 0% K amp: 24%
186	SI-382U.M.02.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	ATP block: 6% ATP amp: 2% K block: 12% K amp: 1%

187	SI-022Y.R-I.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	ATP block: 9% ATP amp: 7% K block: 1% K amp: 35%
188	SI-079K-2-R-I.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
189	3965P-R-I.02.02	bacterial extract	<i>inactive</i>	<i>antagonist</i>	<i>inactive</i>	<i>inactive</i>
190	SI-010X.M.01.03	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
191	SI357P.M.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>
192	SI-357P.S-I.01.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 3% ATP amp: 8% K block: 12% K amp: 16%
193	SI-068L.I.02.08	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 3% ATP amp: 8% K block: 12% K amp: 16%
194	SI-365K.R-M.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 3% ATP amp: 8% K block: 12% K amp: 16%
195	SI-327U.M.02.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 3% ATP amp: 8% K block: 12% K amp: 16%
196	SI-071P.M.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 3% ATP amp: 5% K block: 32% K amp: 1%
197	SI-136M.M.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 3% ATP amp: 5% K block: 32% K amp: 1%
198	SI-074U.M.02.03	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 3% ATP amp: 5% K block: 32% K amp: 1%

199	SI-022Y-R-I.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 3% ATP amp: 5% K block: 32% K amp: 1%
200	3975M-R.01.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
201	SI-360U.M.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
202	SI-3965P.R-I.02.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
203	SI-357P.S.01.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	tested
204	SI-463H.I.02.01	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	Deconvolute activity: ATP block: 11% ATP amp: 43% K block: 17% K amp: 11%
205	SI-309T.M.02.02	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>inactive</i>	to be tested
206	SI-327U.M.02.03	bacterial extract	to be tested	to be tested	to be tested	to be tested
207	SI-327U.S-I.01.01	bacterial extract	to be tested	to be tested	to be tested	to be tested
208	SI-357P.I.01.01	bacterial extract	to be tested	to be tested	to be tested	to be tested
209	SI-334M.S.01.01	bacterial extract	to be tested	to be tested	to be tested	amplification of K pulses
210	SI-073T.S.01.01	bacterial extract	to be tested	to be tested	to be tested	to be tested
211	SI-359P.R.I.02.01	bacterial extract	to be tested	to be tested	to be tested	to be tested
212	SI-304T.I.02.01	bacterial extract	to be tested	to be tested	to be tested	to be tested
213	SI-035K.M.02.04	bacterial extract	to be tested	to be tested	to be tested	to be tested

214	SI-019U.M.02.05	bacterial extract	to be tested	to be tested	to be tested	to be tested
215	SI-019U.M.02.01	bacterial extract	to be tested	to be tested	to be tested	to be tested
216	SI-011Y.M.02.02	bacterial extract	to be tested	to be tested	to be tested	to be tested
217	SI-020W.M.02.01	bacterial extract	to be tested	to be tested	to be tested	to be tested
218	SI-068L.I.02.01	bacterial extract	to be tested	to be tested	to be tested	to be tested
219	SI-454L.R-I.02.02	bacterial extract	to be tested	to be tested	to be tested	to be tested
220	SI-035K.I.01.02	bacterial extract	to be tested	to be tested	to be tested	to be tested
221	3965P.R-I.02.01	bacterial extract	to be tested	to be tested	to be tested	to be tested
222	SI-010X-M.01.02	bacterial extract	to be tested	to be tested	to be tested	to be tested
223	SI-382U.M.02.01	bacterial extract	to be tested	to be tested	to be tested	to be tested
224	SI-035K.M.02.03	bacterial extract	to be tested	to be tested	to be tested	to be tested
225	SI-074U.M.02.06	bacterial extract	to be tested	to be tested	to be tested	to be tested
226	SI-018T.I.02.01	bacterial extract	to be tested	to be tested	to be tested	to be tested
227	3962K.R.01.03	bacterial extract	to be tested	to be tested	to be tested	to be tested
228	3962K.R.01.02	bacterial extract	to be tested	to be tested	to be tested	to be tested
229	SR01812	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>

230	SR01813	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
231	SR01814	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
232	SR01815	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
233	SR01816	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
234	SR01817	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
235	SR01818	bacterial extract			<i>to be tested</i>	<i>to be tested</i>
236	SR01819	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
237	SR01820	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
238	SR01821	bacterial extract	to be tested	to be tested	<i>to be tested</i>	Showed some block in KCl
239	SR01822	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
240	SR01823	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
241	SR01824	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
242	SR01825	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
243	SR01826	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
244	SR01827	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
245	SR01828	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>

246	SR01829	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
247	SR01830	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
248	SR01831	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
249	SR01832	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
250	SR01833	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
251	SR01834	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
252	SR01835	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>

253	SR01836	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
254	SR01837	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
255	SR01838	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
256	SR01839	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
257	SR01840	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
258	SR01841	bacterial extract	to be tested	to be tested	<i>to be tested</i>	<i>to be tested</i>
259	SR01842	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
260	SR01843	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
261	SR01844	bacterial extract	inactive at 50 ug/mL	Slight activity	<i>to be tested</i>	*inactive at 50 ug/mL

262	SR01845	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
263	SR01846	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	Inactive (50 ug/mL)
264	SR01847	bacterial extract	to be tested	to be tested	<i>to be tested</i>	
265	SR01848	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
266	SR01849	bacterial extract	to be tested	to be tested	<i>to be tested</i>	
267	SR01851	bacterial extract	Slight activity	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
268	SR01852	bacterial extract	to be tested	to be tested	<i>to be tested</i>	
269	SR01854	bacterial extract	Slight activity	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
270	SR01855	bacterial extract	to be tested	to be tested	<i>to be tested</i>	Inactive (50 ug/mL)
271	SR01856	bacterial extract	to be tested	to be tested	<i>to be tested</i>	
272	SR01857	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
273	SR01858	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
274	SR01859	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
275	SR01860	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
276	SR01863	bacterial extract	inactive at 50 ug/mL	inactive at 50 ug/mL	<i>to be tested</i>	*inactive at 50 ug/mL
277	SR01865	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>

278	SR01866	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
279	SR01867	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
280	SR01868	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
281	SR01871	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
282	SR01872	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
283	SR01873	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
284	SR01875	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
285	SR01877	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive (50 ug/mL)
286	SR01878	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
287	SR01884	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	Inactive (50 ug/mL)
288	SR01885	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
289	SR01886	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
290	SR01887	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
291	SR01888	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
292	SR01892	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
293	SR01893	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>

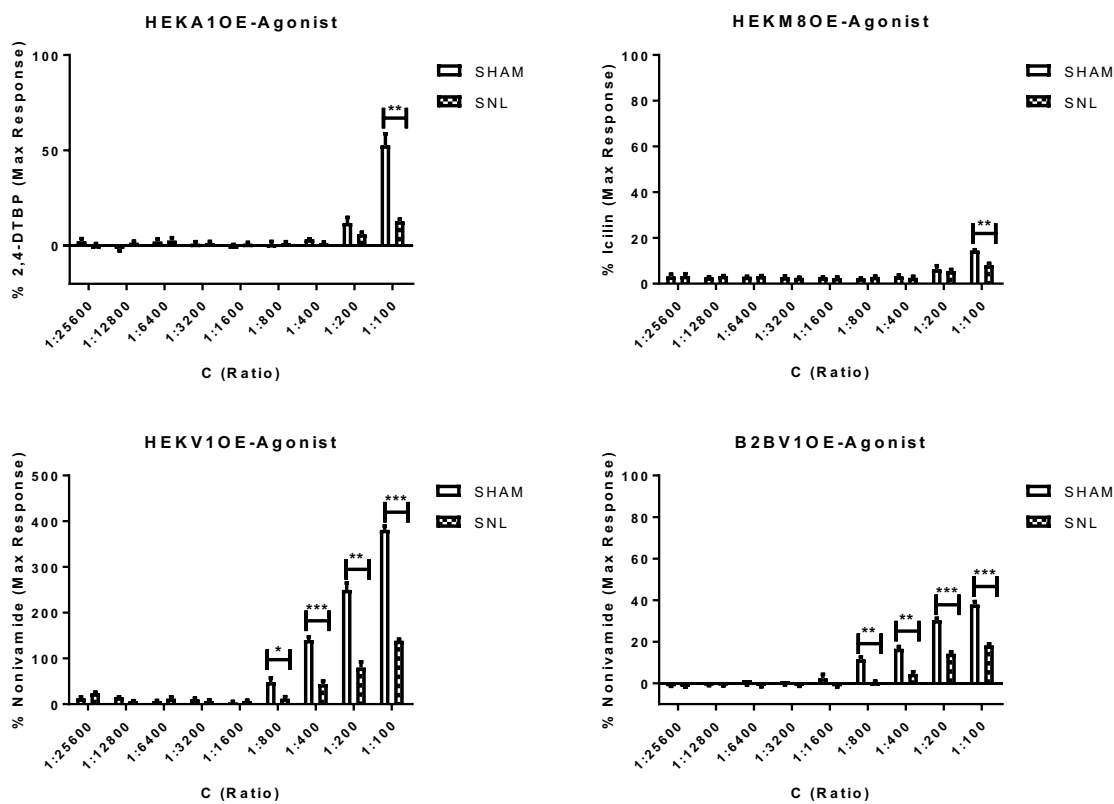
294	SR01894	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
295	SR01895	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
296	SR01896	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
297	SR01897	bacterial extract	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>	<i>to be tested</i>
298	ES-19-G.HP-P1	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
299	ES-19-4Pb	bacterial extract	antagonist	antagonist	<i>to be tested</i>	<i>Amplify KCl response</i>
300	ES-19-5Ma	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
301	ES-19-30PGa	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
302	ES-19-30PGb	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
303	ES-19-41PGa	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
304	ES-19-41PGb	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
305	ES-19-02Ra	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
306	ES-19-04Pa1	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
307	ES-19-04Pa2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
308	ES-19-05Ia	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
309	ES-19-06PGa	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>

310	ES-19-23Ia	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
311	ES-19-23PGa	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
312	ES-19-27Ia	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
313	ES-19-27Ra	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
314	ES-19-27Pa	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
315	ES-19-31PGa	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
316	ES-19-33PGa	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>
317	ES-19-G.HP-P2	bacterial extract	<i>inactive</i>	<i>inactive</i>	<i>to be tested</i>	<i>to be tested</i>

Project 4, Aim 1, Major Task 1: The goal of Aim 1 is to discover inflammatory and pain mediators + biosynthetic origins and molecular consequences. **Major Task 1, Subtask 1** involved obtaining tissue samples from pain models to identify small molecules that affect TRP channel function during the time-dependent development of chronic pain phenotypes. Through collaboration with Projects 1 and 2, we have made substantial progress on this discovery component of the project and have several leads to pursue. This element of the study has essentially been completed (95%), although we are regularly collecting tissues to validate leads on pain targets and chemical mediators of pain, as outlined under the subsequent aims and tasks.

Project 4, Aim 1, Major Task 2, Subtask 1: We have obtained several sets of DRG and spinal cord tissue from sham surgery and SNL mice from the pain testing core, including samples from different days. Currently, most of our data are from 14-day post SNL animals due largely to the availability of these tissues. Analysis of the samples for TRP channel modulation are well underway and we have observed significant differences in the ability of tissue extracts to modulate the function of TRPA1, M8, and V1. Most notable, tissue collected proximal to a SNL injury has previously been found to have slight agonist activity at TRPV1, while at 14-days post SNL, we observe inhibition of TRPA1, M8 and V1 associated with tissue collected from the site of injury (**Figure 1**).

P4 Figure 1: Crude extracts of sham vs. SNL spinal cords differentially activate TRPA1, M8, and V1. Initial results show a decrease in TRP channel specific bioactive lipids in SNL mice at 14-days post-surgery which suggests a significant alteration in lipid signaling profiles after SNL.



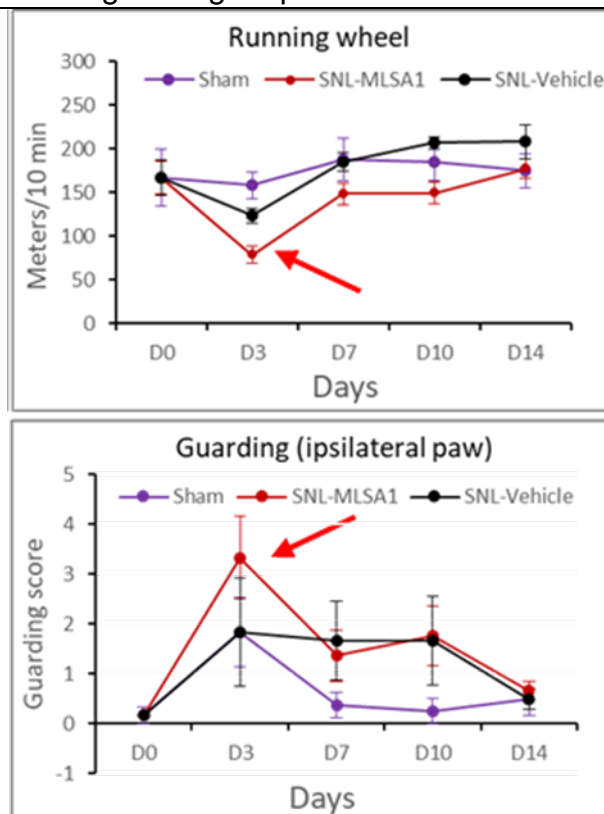
We speculate this suggests that there is dynamic regulation of a pool of bioactive lipids that are produced upon injury and are deliberately decreased as injury progresses in an effort to attenuate the extent of injury due to inflammation. Further, consistent with our hypothesis, this effect may be mediated by CYP enzymes that influence the formation and/or degradation of bioactive lipids that interact with TRPs to promote inflammation and pain. This element of the study is also nearing completion (~85%).

Project 4, Aim 1, Major Task 2, Subtask 2: We have also performed transcriptome analysis of DRGs following injury. Specifically, transcriptomic analysis of DRGs from two chronic pain models (SNL and formalin hind-paw injection; n=4/group; harvested at 0, 1, 3, and 7-days post injury) has been ~95% completed in collaboration with the Dr. Light and the pain testing core. We have not yet assayed other (oxaliplatin and CCI) pain models as a recent publication has provided a comprehensive comparison and transcriptomes for multiple pain models in DRGs (Bangash et al 2018). These data sets have been interrogated by our group and compared to our own datasets. Our assessments have confirmed that the timing of the pain phenotypes is somewhat technique dependent, and that in our hands the phenotypes develop more rapidly than that described by some laboratories. Additionally, there appears to be limited, albeit potentially significant overlap among models, and in some cases overlap between the published dataset and our own datasets in terms of deregulation of specific targets of interest. To date, we have focused our work on transcriptome data generated among our program project members and have prioritized studies to evaluate changes of interest obtained from our own data set revealing changes in lipid metabolizing genes and a novel, poorly understood TRP channel, TRPML2.

TRPML2 is of interest to us because it is not well understood and it may play a role in chronic pain due to its time-dependent up-regulation in both SNL and formalin models. However, TRPML2 has been shown to play a role in inflammation, specifically the release of CCL2 from macrophages. The compound ML-SA1 is a known non-specific activator of TRPML1, 2 and 3, and to further investigate a potential role of TRPML2 in pain, in collaboration with the pain testing core, we tested whether ML-SA1 would alter the development of pain phenotypes in the SNL model. Male 8 week old C57B/L6 mice were used in these experiments. ML-SA1 was administered daily IP ~200 µg/g of mouse body weight (average animal was ~30g) for 14 days. Animals in sham and SNL-only groups were injected with vehicle (5% DMSO in MCT oil). Before injections, baseline temperature preference and running wheel tests were performed. Testers were blind to animal surgeries and study compound treatments. Animals were tested again on days 3, 7, 10, and 14 post surgery with guarding behaviors, von Frey test of mechanical allodynia, temperature preference, and voluntary running wheel. The results are represented in **Figure 2**. No appreciable changes in Place Preference or von Frey were observed with ML-SA1 vs vehicle. In running wheel and guarding tests, ML-SA1 appeared to worsen the pain phenotype. This data can be interpreted 2 ways, using a non-specific agonist which targets all of the TRPML family of receptors results in a worsening of the pain phenotype, or that a targeted antagonist rather than an agonist may be beneficial in targeting pain. To further characterize this response, we have cloned TRPML2 and have developed a TRPML2-overexpressing HEK-293 cell line to support screening for selective agonists and antagonists derived from both tissue extracts and natural product libraries, provided by Drs. Olivera and Schmidt. We are also culturing macrophages which

naturally express these channels to further understand how they may function to alter pain phenotypes through sensitization or inhibition of TRPs. Finally, a more specific TRPML2 agonists, MLSA2-1 has been reported. We have synthesized this compound to support our work on TRPML2.

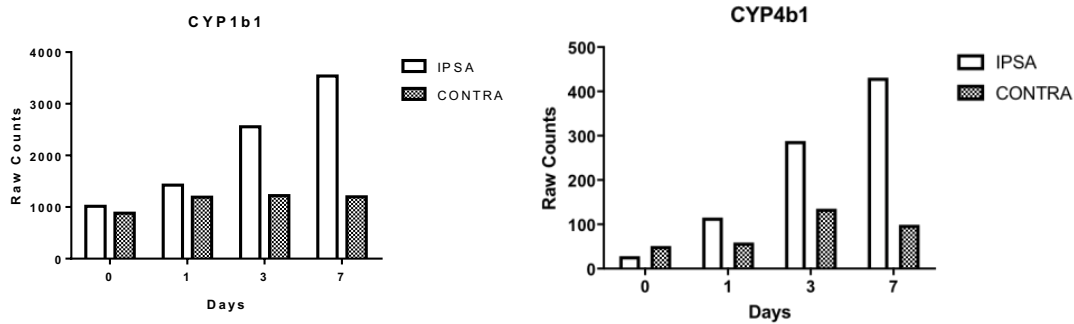
P4 Figure 2. Effects of TRPML1-3 activation, using the non-selective agonist ML-SA1, on chronic pain phenotypes. Note the apparent exacerbation of pain behaviors in the running wheel and ipsilateral guarding responses.



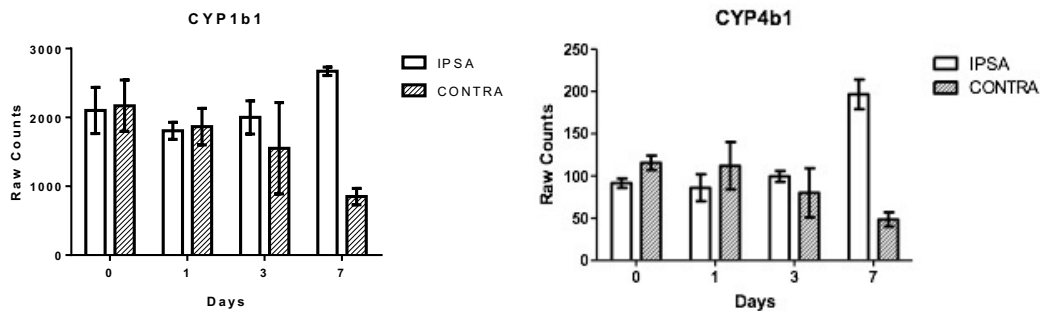
Through analysis of the transcriptomics data, we have also observed time-dependent changes in lipid metabolism enzymes following SNL and formalin injury. Specifically, *Cyp1b1* and *Cyp4b1* (**Figure 3**) which are known to oxidize arachidonic acid to produce multiple bioactive lipids involved in inflammation, including lipids that modulate TRPV1. Follow up experiments to further understand P450 gene induction in conjunction with the lipid analyses are currently underway. Studies include developing new cell models to over-express *Cyp1b1* and *Cyp4b1* to assess their modulatory effects on lipid metabolism which could alter a pain phenotype through interactions with TRP channels, as well as to create models for screening novel metabolic inhibitors that may impact pain severity.

P4 Figure 3: Expression of *Cyp1b1* (left side) and *Cyp4b1* (right side) mRNA in mouse DRGs following SNL (Top figures) or formalin (bottom figures) induced pain.

SNL



Formalin

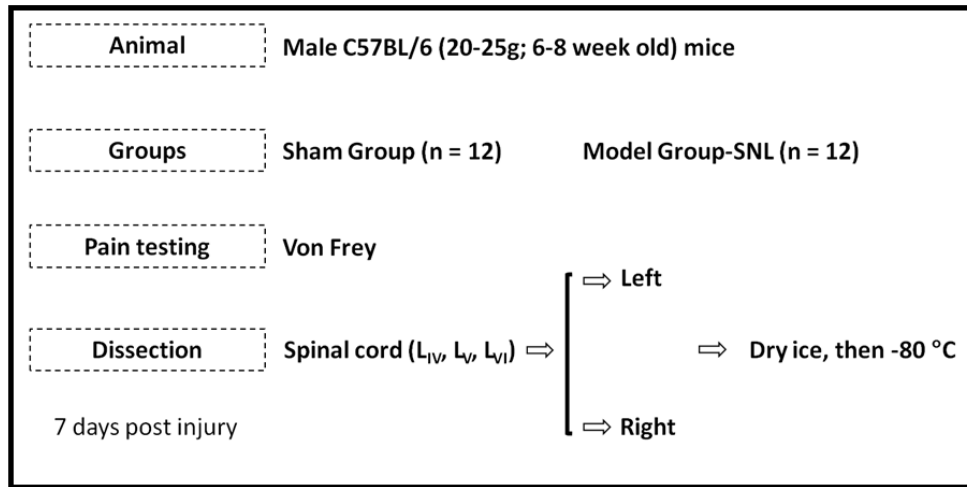


Project 4, Aim 1, Major Task 3, Subtask 1: Efforts are ongoing to isolate components of the tissue extracts from above, that alter TRP channel activity. We have learned that this is a challenging task due to the inherent instability of the bioactive molecules in the extracts and have to a large extent abandoned the activity-guided fractionation approach, in favor of a more direct approach involving neurometabolomics analysis, as outlined under **Project 4, Aim 1, Major Task 3, Subtask 2**.

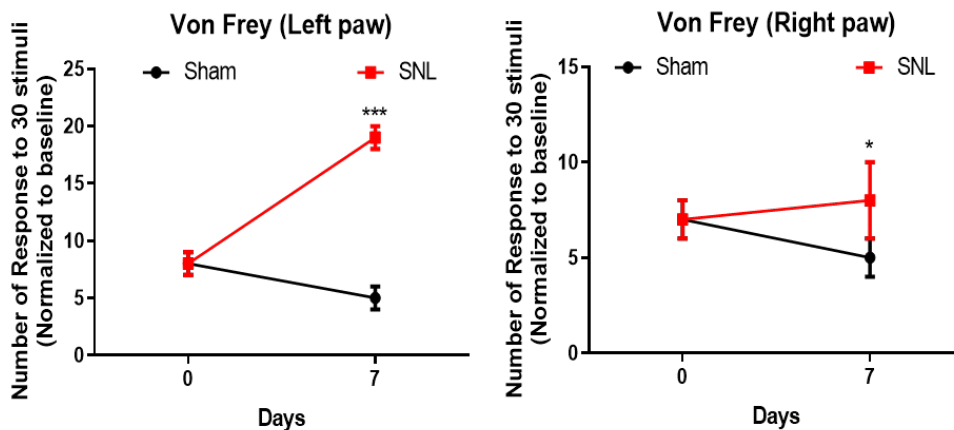
Project 4, Aim 1, Major Task 3, Subtask 2: We have performed a large scale neurometabolomics study using spinal cord tissues collected under Aim 1, Major Task 1, Subtask 1. Spinal cords were used because the material present in DRGs is limited. First, mice were subjected to either SNL or sham operations, and pain was verified in these mice. The experimental design and the verification of pain in these mice is shown in **Figure 4A and B**.

P4 Figure 4A and B: Experimental design of the neurometabolomics study (A), and verification of the chronic pain phenotype in SNL-injured mice (B).

A



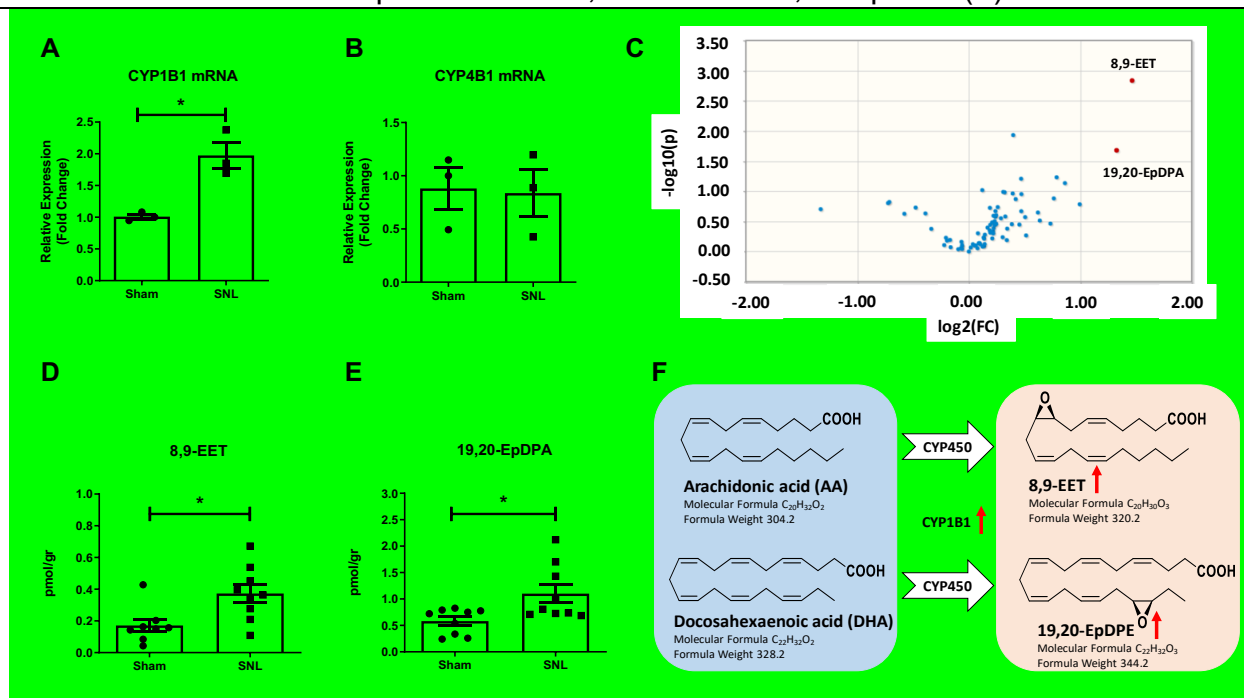
B



We then investigated whether the expression of *Cyp1b1* and *Cyp4b1* increased in the spinal cord as observed in the transcriptomics data set. A significant increased expression of *Cyp1b1*, but not *Cyp4b1* was observed (**Figure 5A and B**). After verification of the pain phenotype and the induction of *Cyp1b1*, the tissue samples were prepared and sent for analysis of oxylipins (CYP metabolites) to the West Coast Metabolomics Center at UC Davis. We have received and processed the analytical data, and have identified two highly up-regulated lipids in the spinal cord tissue of SNL mice when compared to that of sham operated mice; 8,9-EET and 19,20-EpDPA, which are CYP-derived epoxide metabolites of arachidonic acid (AA) and docosahexaenoic acid (DHA) respectively (**Figure 5C-F**).

P4 Figure 5: *Cyp1b1*, but not *Cyp4b1* mRNA expression was induced in SNL-injured mouse spinal cord tissue (A and B). Volcano plot showing metabolomics analysis of lipids in the spinal cord tissue of injured and sham operated mice. A statistically significant

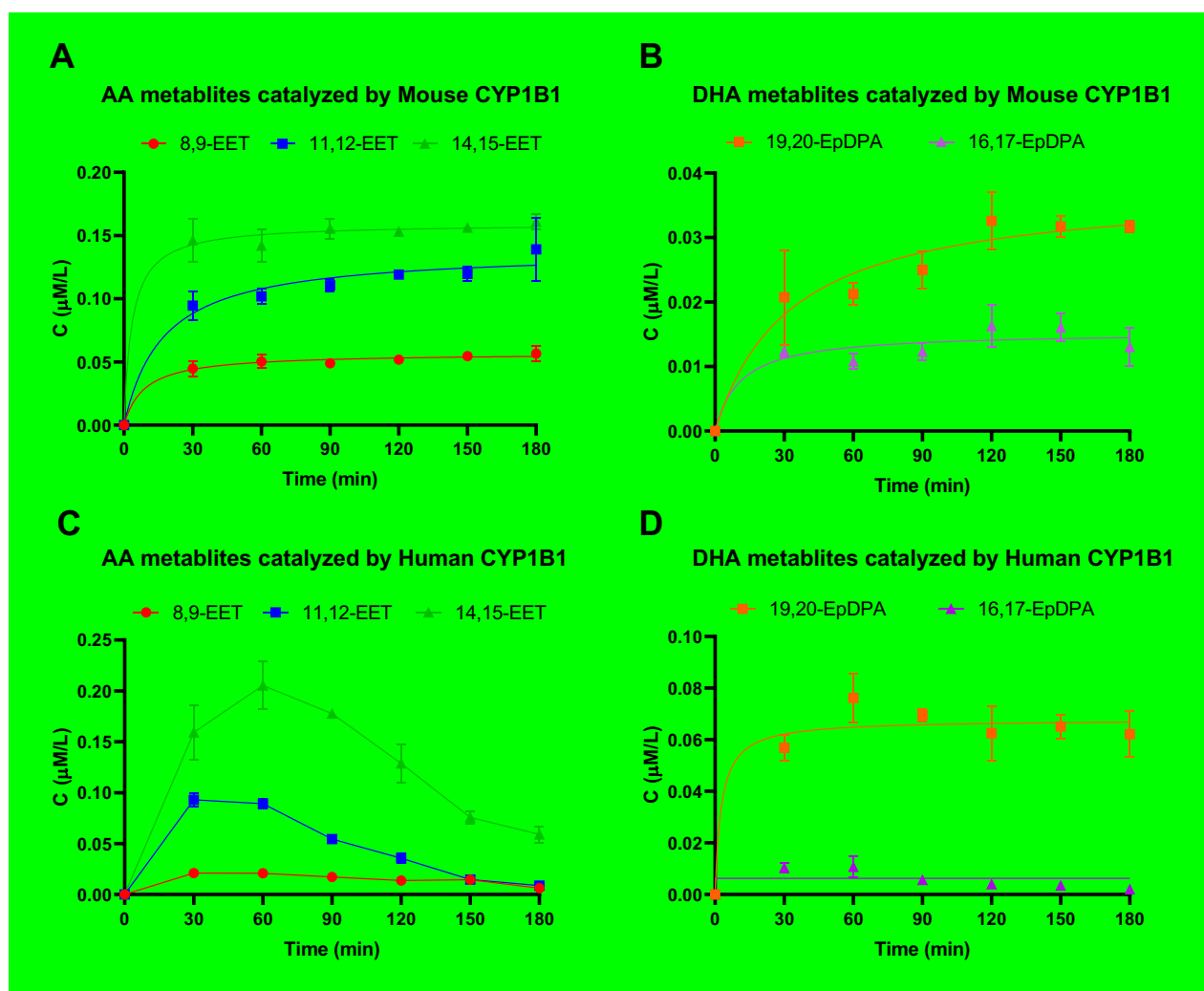
increase in 8,9-EET and 19,20-EpDPA in injured spinal tissue was observed (C-E). Metabolic scheme for the production of 8,9-EET and 19,20-EpDPA (F).



It is known that multiple CYP enzymes metabolize ω -6/ ω -3 fatty acids to lipid epoxides such as the epoxyeicosatrienoic acids (EETs), or ω -hydroxides such as the hydroxyeicosatetraenoic acids (HETEs), which have pro-inflammatory properties. We hypothesized, based on our transcriptomic and metabolomics data sets that 8,9-EET and 19,20-EpDPA might occur at higher levels in the spinal cords of mice as a result of *Cyp1b1* induction, and that this change may be involved in the development of chronic pain.

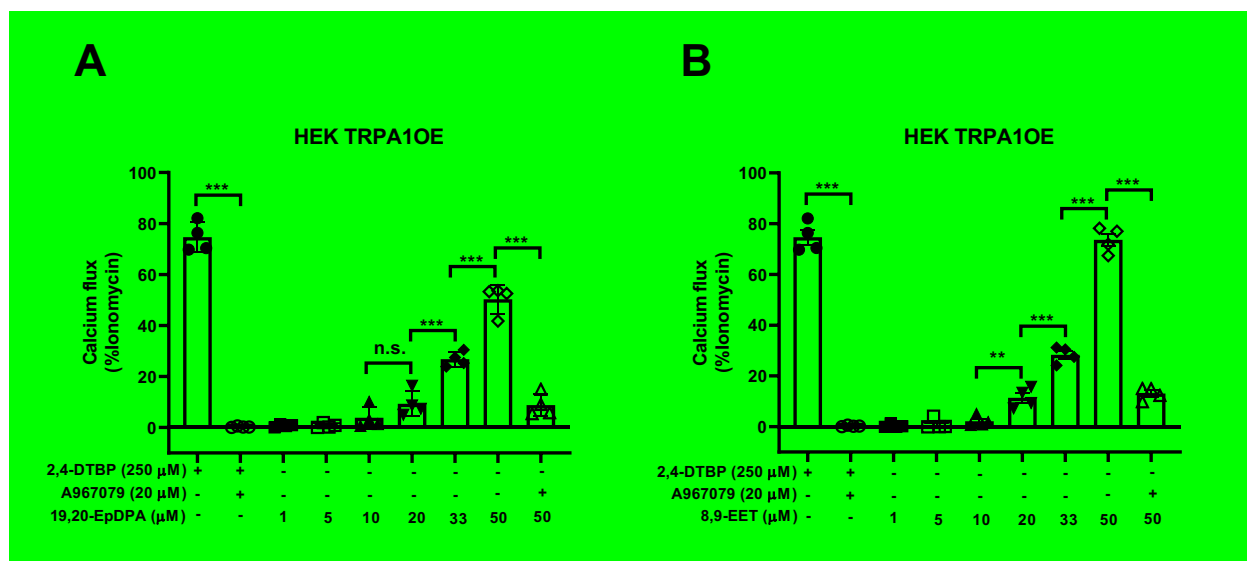
Project 4, Aim 1, Major Task 3, Subtask 3: To date, CYP1B1/*Cyp1b1* had not been shown to produce 8,9-EET and 19,20-EpDPA from AA and DHA, respectively. To determine if CYP1B1/*Cyp1b1* could produce 8,9-EET and 19,20-EpDPA, we performed *in vitro* metabolism studies of AA and DHA using human and mouse CYP1B1/*Cyp1b1* supersomes (i.e., recombinant CYP1B1/*Cyp1b1* enzyme co-expressed with CYP P450 reductase and cytochrome b5), respectively. The production of these metabolites and other related EETs and EpDPAs was quantified using LC-MS/MS. For mouse *Cyp1b1*, the main epoxides of AA were 14,15-EET, 11,12-EET and 8,9-EET, whereas the primary epoxides of DHA are 19,20-EpDPA and 16,17-EpDPA (**Figure 6 A and B**). Similarly, 14,15-EET, 11,12-EET and 8,9-EET were the primary metabolites of AA generated by human CYP1B1, and 19,20-EpDPA was the dominant metabolite of DHA using human CYP1B1 (**Figure 5 C and D**).

P4 Figure 5. Both mouse *Cyp1b1* (A and B) and human CYP1B1 (C and D) metabolize AA to form 8,9-EET (left side of panel) and DHA to 19,20-EpDPA (right side of panel).



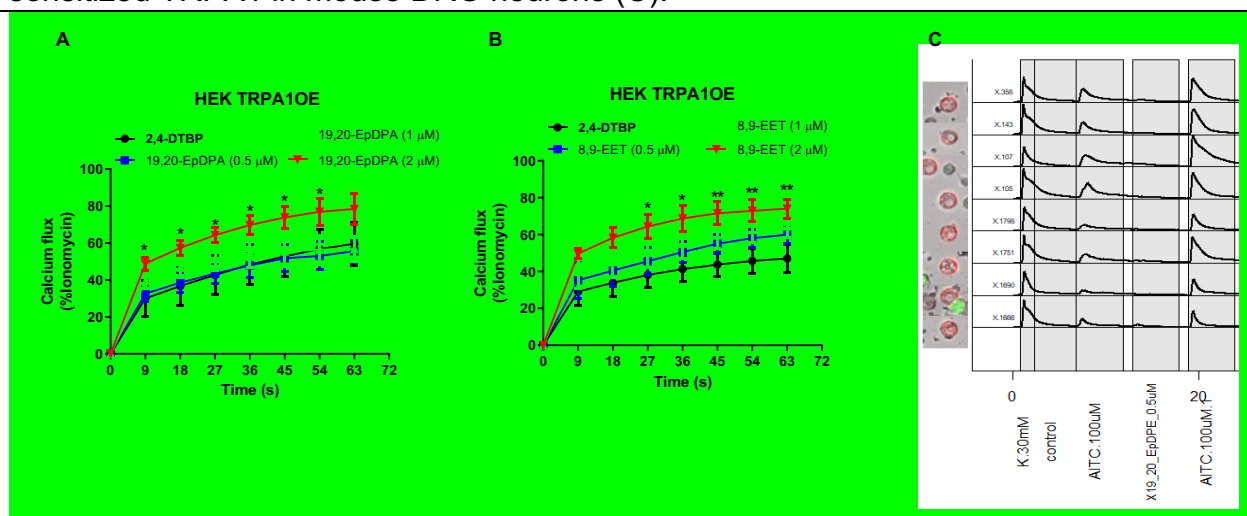
A long-term goal of **Project 4, Aim 1, Major Task 3, Subtask 3** was to understand how CYP-derived lipids affected the pain phenotypes. Accordingly, we characterized the effects of these oxidized lipids on pain-related signaling involving the activation of TRP channels. Both 8,9-EET and 19,20-EpDPA were evaluated as TRP agonists and antagonists in HEK-293 cells over-expressing human TRPA1, V1, V4 and M8. In agonist assays, 19,20-EpDPA and 8,9-EET at 20-50 μ M activated TRPA1, but not TRPV1, V4 or M8. The activation of TRPA1 by 19,20-EpDPA and 8,9-EET was dose-dependent and was attenuated by A967079, a potent and selective TRPA1 antagonist (**Figure 6**).

P4 Figure 6: The effects of 19,20-EpDPA (A) and 8,9-EET (B) on TRPA1 channel calcium flux in HEK-293 cells over-expressing TRPA1.



Inhibition of TRPs by 8,9-EET and 19,20-EpDPA was not observed, suggesting that these lipids are likely not the compounds in the crude extracts of DRGs/spinal cord that suppress TRPA1 and V1, as shown in Figure 1. However, both 19,20-EpDPA and 8,9-EET (2 μ M) sensitized TRPA1 in both TRPA1 overexpressing HEK-293 cells at concentrations well below those required for direct activation (2 μ M vs 20 μ M) (**Figure 7A and B**), which in the context of pain could amplify the contribution of TRPA1 to inflammation and the severity of pain phenotypes that occur at sites of injury. Additionally, 19,20-EpDPA sensitized TRPA1 in isolated mouse DRG neurons, determined using the constellation pharmacology assay (**Figure 7C**); the effect of 8,9-EET had already been reported for rat TRPA1.

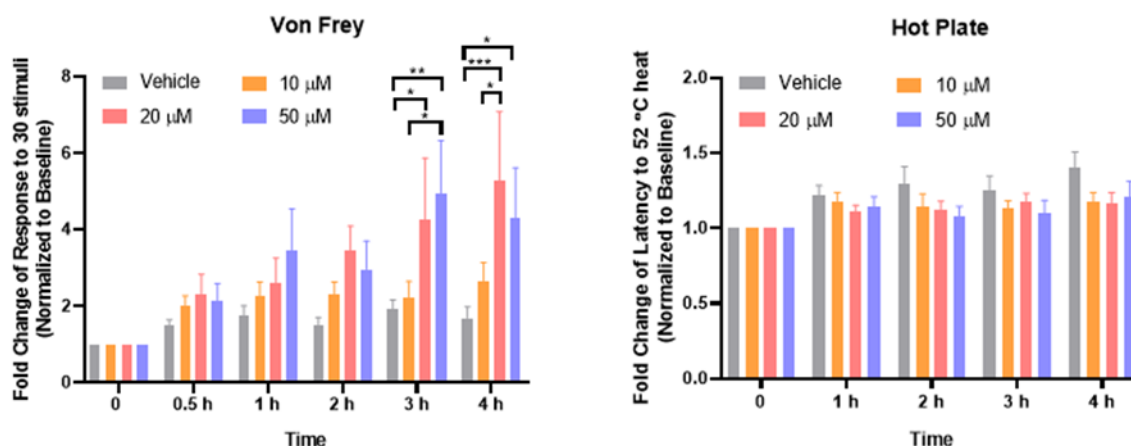
P4 Figure 7: 19,20-EpDPA (A) and 8,9-EET (B) sensitized in TRPA1 overexpressing HEK-293 cell responses to a prototypical TRPA1 agonist (A and B), while 19,20-EpDPA sensitized TRPA1 in mouse DRG neurons (C).



Similarly, a brief exposure (5 min) to 0.5 μ M 9,10-EpOME, another CYP-derived lipid found to be elevated in the metabolomics dataset, was capable of sensitizing TRPV1-dependent calcium influx in 7.6% of DRG neurons (not shown).

To determine whether 19,20-EpDPA might induce pain hypersensitivity *in vivo*, we injected the lipid in the hind paws of wild-type C57BL/6N mice and measured the mechanical (**Figure 8A**) and thermal response (**Figure 8B**) for 4 h post-injection. 19,20-EpDPA caused significant mechanical hypersensitivity at 3 and 4 h after injection, but not thermal hypersensitivity in mice. To summarize, we believe that induction of Cyp1b1 in injured neurons of mice leads to an increase in Cyp1b1 and the production of 8,9-EET and 19,20-EpDPA. This likely contributes to acute pain via the activation of TRPA1. However, we still need to determine the effect of 8,9-EET and 19,20-EpDPA in chronic pain, to determine if pharmacological inhibition or manipulation of Cyp1b1 or other epoxygenases and/or TRPA1 may be therapeutic targets. Follow up experiments to further understand the role of TRPA1 and CYP1B1 gene induction in chronic pain are currently underway, including plans to study *Cyp1b1*-deficient (i.e., knockout) mice.

P4 Figure 8: 19,20-EpDPA caused significant dose- and time-dependent mechanical (left panel), but not thermal hypersensitivity (right panel) in mice.



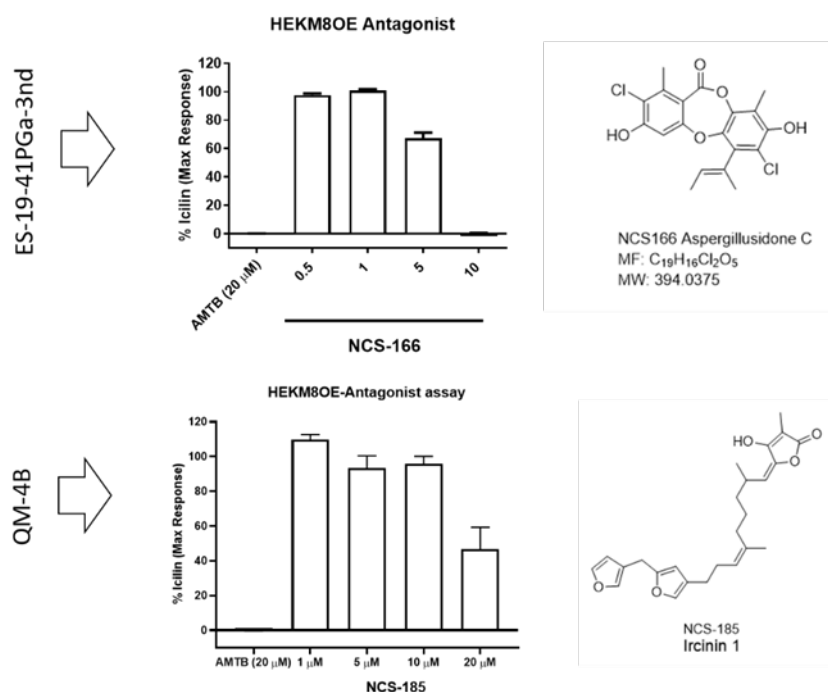
Project 4, Specific Aim 2, Major Task 4, Subtask 1: The goal of Aim 2 is to identify natural products that modulate TRP channels (Major Task 4, subtask 1), to develop assays to study newly characterized molecular targets from Aim 1 (e.g., TRPML2 and CYPs; Major Task 4, Subtask 2), and to conduct pharmacological mechanism studies of novel TRP channel (or other target) modulators (Major Task 5, Subtask 1). To date progress in this area has been excellent. In collaboration with Dr. Schmidt, we have performed >1000 tests for novel natural products that activate or inhibit TRP channels (Major Task 4, Subtask 1), which is summarized under Project 3. For many we have also performed mechanistic studies to identify binding site interactions (Major Task 5, Subtask 1). However, many of these newly discovered modulators do not show novel mechanisms of action at the TRPs, fail to meet selectivity criteria, and/or fail to meet potency and safety criteria to warrant further study in animals, partially addressing **Major Task 7, Subtasks 1 and 2**).

One exciting lead is a TRPV3 amplifier (I-211-4; and iodo-tyramine derivative), meaning it is not a direct agonist, but a compound that increases the sensitivity of TRPV3 to agonists. Based on recent studies in our laboratory, this compound, via interactions with TRPV3 may have potential as a therapeutic for promoting injury repair as well as an inhibitor of cancer cell growth; TRPV3 plays fundamental roles in determining dermal and

airway epithelial cell differentiation, manifesting as a growth accelerant at early stages of injury repair, but a cytostatic mechanism at later points. We have not yet moved forward with the idea that this compound could have benefit in cancer chemotherapy, but this is an idea we hope to pursue in the future.

Some new, promising leads as TRPM8 inhibitors include aspergillusidone C isolated from ES19-41PGa-3nd and ircinin 1 derived from QM-4B. These molecules represent novel TRPM8 inhibitors (**Figure 9**) and they show a degree of selectivity for TRPM8; they do not inhibit TRPV1

P4 Figure 9: Dose-dependent inhibitor effects of aspergillusidone C (10 μ M; top panel) and ircinin 1 (20 μ M; bottom panel) on calcium flux in TRPM8-overexpressing HEK-293 cells.



As outlined above, we are also currently developing models to study what we believe are novel and potentially important pain-related targets (TRPML2, CYP1B1, CYP4B1; Major Task 4, Subtask 2). This work will be the focus of this year's research.

Project 4, Aim 3: The goal of Aim 3 is to validate novel molecules and associated pain targets in pain models. **Major Task 6, Subtask 1** is to provide molecules to the pain testing core, while **Subtask 2** is to perform mechanistic validation studies to support Subtask 1 findings. Finally, **Major Task 7, Subtasks 1 and 2** are to prioritize lead compounds and targets for subsequent in-depth study. In general, this element of the study is progressing well, in collaboration with Project 3, but is still in its early stages. We have worked with Dr. Schmidt to identify a number of promising compounds interacting with TRPs, and a number of these compounds have been tested in animal models with varying effects on measures of pain. This element of the study is obviously important, and we continue to push forward in an effort to meet our initial testing goals.

As an example, and as outlined above, we have pursued TRPML2 and have observed that activation of TRPML2 (and/or other TRPML receptors) using ML-SA1, may

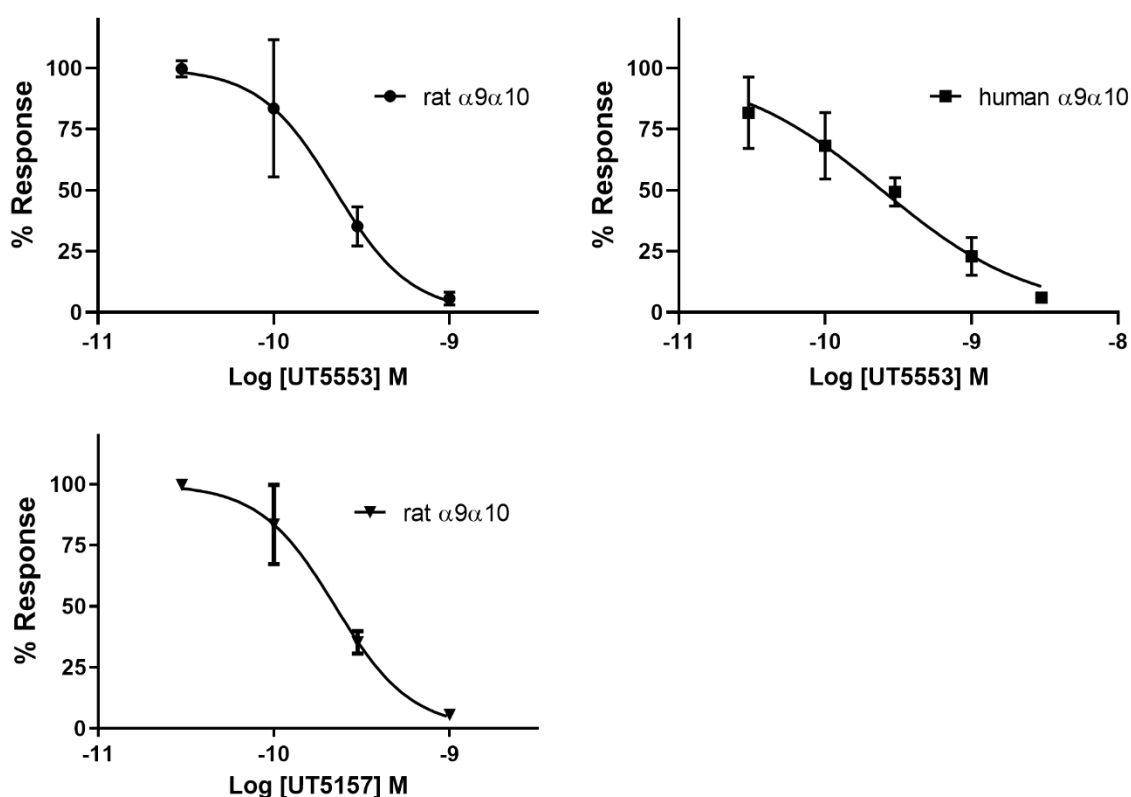
exacerbate pain presumably through an enhancement of pro-inflammatory signaling at the sites of injury (**Major Task 6, Subtasks 1 and 2**). Obviously, this is not our intention, but these data suggest that TRPML2 may play a role in pain development, perhaps through activation by endogenous ligands which we will attempt to discover over the next year. Further, inhibition of TRPML2 may be beneficial in attenuating pain. As such, we have developed key tools to identify novel TRPML2 antagonists, which currently do not exist, and we have begun efforts to identify TRPML2 modulators using screening assays. This further addresses **Major Task 7, Subtasks 1 and 2**.

Another area that we are focusing on is the use of CYP1B1/*Cyp1b1* inhibitors, addressing the hypothesis that *Cyp1b1* induction, and subsequent production of 8,9-EET and 19,20-EpDPA, may be critical in acute pain, via activation of TRPA1, and important in the development of chronic pain phenotypes. We have developed cell-based tools for screening *Cyp1b1* activity and will begin screening compounds as novel inhibitors pending the outcome of studies that will determine if *Cyp1b1* induction is detrimental, as we hypothesize, beneficial (perhaps through TRPA1 desensitization), or irrelevant.

Project 5, Specific Aim 1, Major Task 1, Subtask 1: We have completed this aim. We are able to reliably moniodinate a candidate compound. The product has the correct observed molecular mass.

Project 5, Specific Aim 1, Major Task 1, Subtask 2-3: We have successfully radiodinated compound. The oxidizing agent, chloramine T was used to covalently attach an iodine to a tyrosine residue. Monoiodo product was purified by reverse-phase HPLC. Stability studies were completed for monoiodinated compounds. Lyophilized peptide was dissolved in storage buffer and assessed initially (day 0) and after 3 and 8 weeks and 1 year. Both were stable for 1 year in solution at 4 °C, making them very suitable for wider use.

Potency of the peptides was assessed on rat and human nAChRs. The nAChRs were heterologously expressed in *Xenopus* oocytes which were voltage clamped and exposed to acetylcholine. Concentration-response analysis was performed to determine the IC₅₀s of the peptides.



The IC₅₀ for 5157 was 0.63 nM for the rat receptor. The IC₅₀ for 5553 was 0.22 nM for rat and 0.24 nM for the human receptor.

Project 5, Specific Aim 1, Major Task 2, Subtask 1: We have made several cell lines. Thus far, signal is too small to be considered reliable. We are therefore taking a number of approaches to boost expression. These include trying immortalized cell lines derived from tissues where the receptors are known to express. We have fluorescently tagged the receptors to be able to monitor expression levels. We are using a subunit chimera approach as a second strategy.

Specific Aim 1, Major Task 2,

In order to deliver and coordinately co-express multiple genes in a cell several choices are available. Cells can be transfected with multiple plasmids or the co-expression can also be carried out by having a vector in which the coding sequences of two proteins are separated by an internal ribosome entry site (IRES). However, in both these instances it is difficult to ensure balanced co-expression. When more than two proteins have to be delivered it is difficult to implement the second option. An alternate co-expression strategy is based on a design used by many viruses to express several proteins as a polyprotein. A small peptide (referred to as 2A) around 18 amino acids is introduced between two proteins to allow self-processing of polypeptides. This type of sequence has been designated as CHYSEL (cis-acting hydrolase element). We have configured our expression vectors using the following CHYSEL sequences. F2A, from foot-and-mouth disease virus, E2A, from equine rhinitis A virus), P2A, from porcine teschovirus-1 2A, and T2A, from thosea asigna virus 2A.

We have constructed an expression cassette:

I. UCOE Promoter Human α 9 P2A Human α 10 T2A GFP.

3XN-FLAG at the N-terminus

C-Streptavidin Binding Peptide at the C-terminus

II. UCOE Promoter Human α 9 P2A Human α 10 T2A GFP.

3XC-FLAG at the C-terminus

C-Strptavidin Binding Peptide at the C-terminus

The vector also encodes a gene for the resistance to zeocin.

The rationale for the vectors is that cells are first selected using resistance to zeocin and then sorted for the expression of GFP. When the receptor is expressed on the cell surface the cells can then be sorted using the fluorescently labelled antibody to FLAG. An additional sorting is possible using streptavidin bound magnetic beads. (The streptavidin binding peptide is expected to protrude from the cell surface.) Cells attached to the magnetic beads can be isolated, released using biotin and propagated. UCOE is a ubiquitous chromatin-opening element to prevent silencing of the integrated vector.

Results:

Though 70% of the zeocin resistant cells express GFP only around 2% of the cells can be sorted using FLAG antibody. The process in principle can be repeated to obtain a pure population of surface-expressing receptors.

In progress:

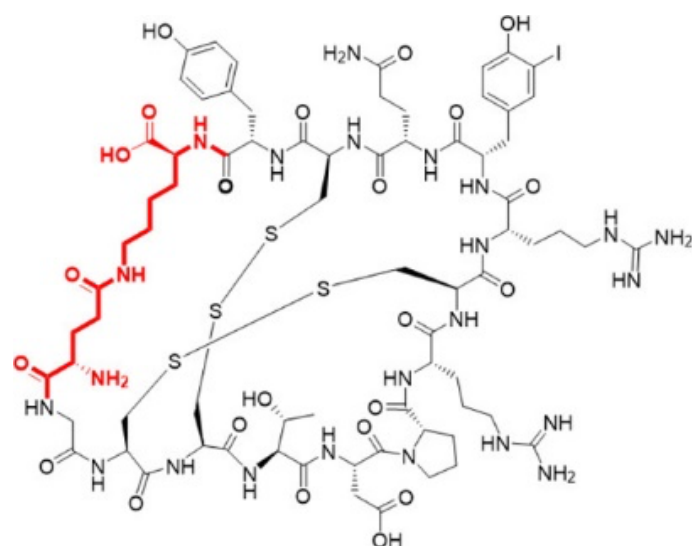
There has been a recent report indicating that membrane proteins TMIE and CHAT (choline acetyltransferase) dramatically enhances the expression and activity of α 9 α 10 receptors (1). We are at present engineering our vector to express both TMIE and CHAT using a different promoter. We anticipate being able to obtain efficient expression of active channels via this means.

Project 5, Specific Aim 2, Major Task 1, 2, 3 and 4: Overview -

During the past year we developed conformationally constrained peptide analogs as stable antagonists.

Native α -conotoxin Rg1A suffers from two weaknesses. First, the peptide has two disulfide bonds and hence three possible disulfide isomers. Only the globular isomer is active. The globular isomer is susceptible to disulfide scrambling resulting in production of a ribbon and bead isomers

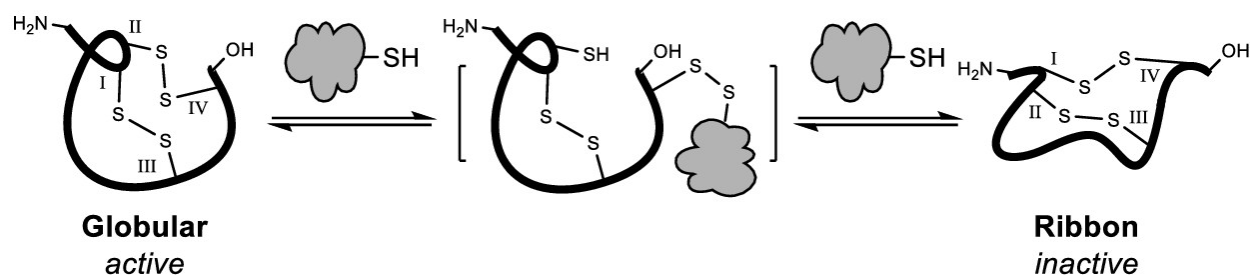
The second issue is that the native peptide has relatively low potency (~ 400 nM) for the human form of the $\alpha 9\alpha 10$ nAChR. New analogs have overcome both of these barriers. To overcome disulfide scrambling we have employed head to tail cyclization to build peptide analogs that are resistant to disulfide exchange.



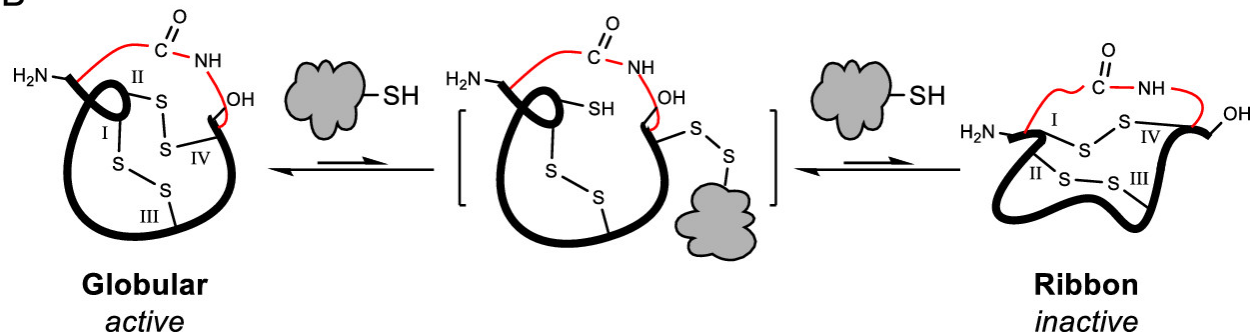
h $\alpha 9\alpha 10$ nAChR IC₅₀ = 3.4 nM
In vivo Pain Relieving
Excellent Subtype Selectivity
Enhanced Human Serum Stability
Suppressed Disulfide Scrambling

We utilized side-chain cyclization to enhance peptide stability in an attempt to rigidify the active conformation while retaining binding activity.

A

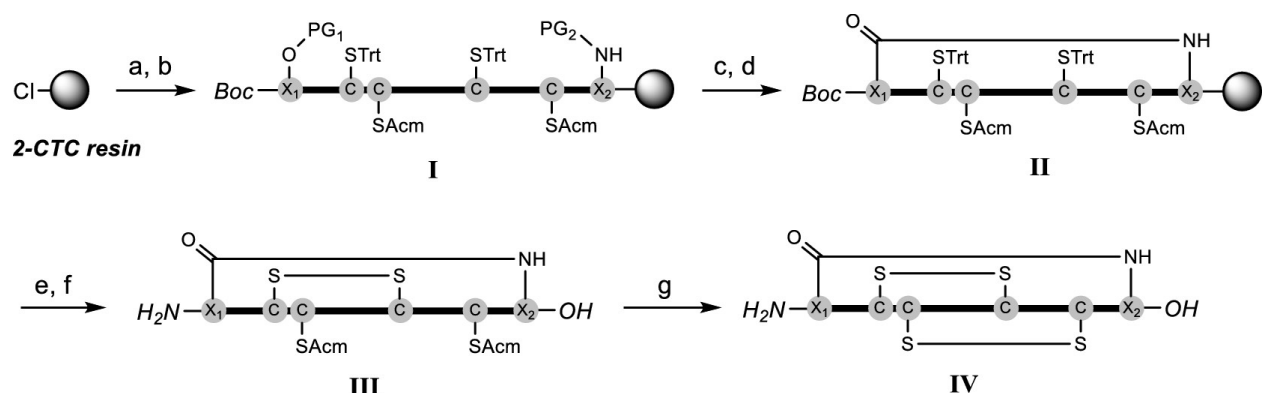


B



Concept of this research. (A) Rapid conformation equilibrium between globular (active) and ribbon (inactive) conformations. (B) Constrained conformation disfavors conformation changes and suppresses disulfide scrambling.

The Synthetic route for creating these analogs is shown below.



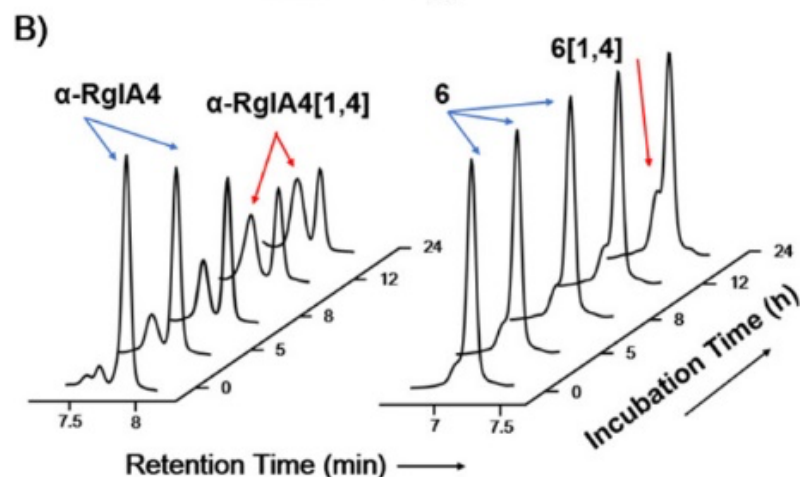
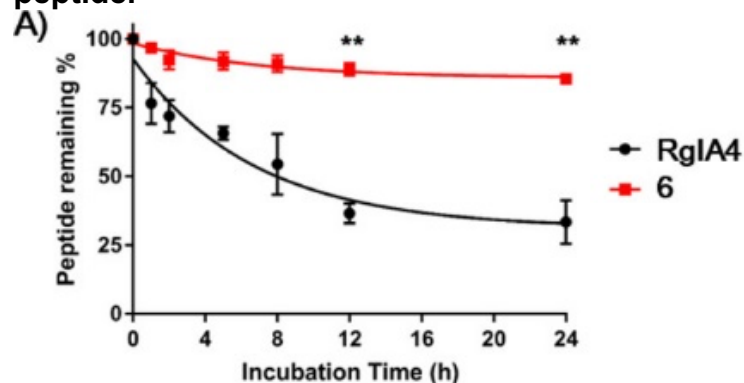
(a) Fmoc-SPPS. (b) Boc_2O , DIEA, DCM. (c) Method 1 for normal tyrosine containing sequences ($\text{PG}_1 = \text{allyl}$, $\text{PG}_2 = \text{aloc}$): $\text{Pd}(\text{PPh}_3)_4$, DMBA, DCM; method 2 for 3-iodo-tyrosine containing sequences ($\text{PG}_1 = \text{Dmb}$, $\text{PG}_2 = \text{ivDde}$): 5% Hydrazine in DMF. (d) PyBOP, DIEA, HOBT, DMF. (e) $\text{TFA}:\text{H}_2\text{O}:\text{TIPS}:\text{EDT} = 95:2:2:1$ (v/v) then RP-HPLC. (f) 0.02 M Na_2HPO_4 (pH 8.0), air. (g) I_2 , AcOH, H_2O . PG = protecting group.

These peptides were shown to have varying degrees of high potency on the target $\alpha 9\alpha 10$ nAChR.

Inhibition of Human $\alpha 9\alpha 10$ nAChR IC_{50} Values for α -RglA4 and Synthesized Analogues (1–6)^a

Peptide	Sequences ^b	IC_{50} (nM) ^c	95% CI (nM)
RglA4	GCCTDPRC(Cit)(iY)QCY [^]	1.5	0.5-2.5
1	[D GCCTDPRC(Cit)YQCY(Dap)] [^]	172	98.4-301
2	[D GCCTDPRC(Cit)YQCY K] [^]	540	348-837
3	[E GCCTDPRC(Cit)YQCY K] [^]	15.2	11.4-20.4
4	[E (^b A)CCTDPRC(Cit)YQCY K] [^]	33.7	20.9-54.3
5	[E GCCTDPRC(Cit)(i Y)QCY K] [^]	5.9	3.4-10.0
6	[E GCCTDPRC R (i Y)QCY K] [^]	3.4	2.6-4.4

Serum stability tests were conducted and demonstrated superior stability of this peptide.

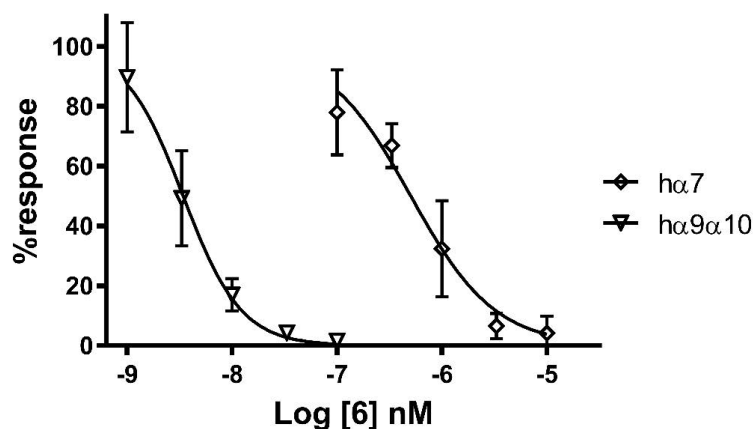


Stability of α -RglA4 and analogue 6 in human serum. (A) Human serum stability comparison. Values are the mean $\pm$ SD of three separate replicates. ** $p < 0.01$ for

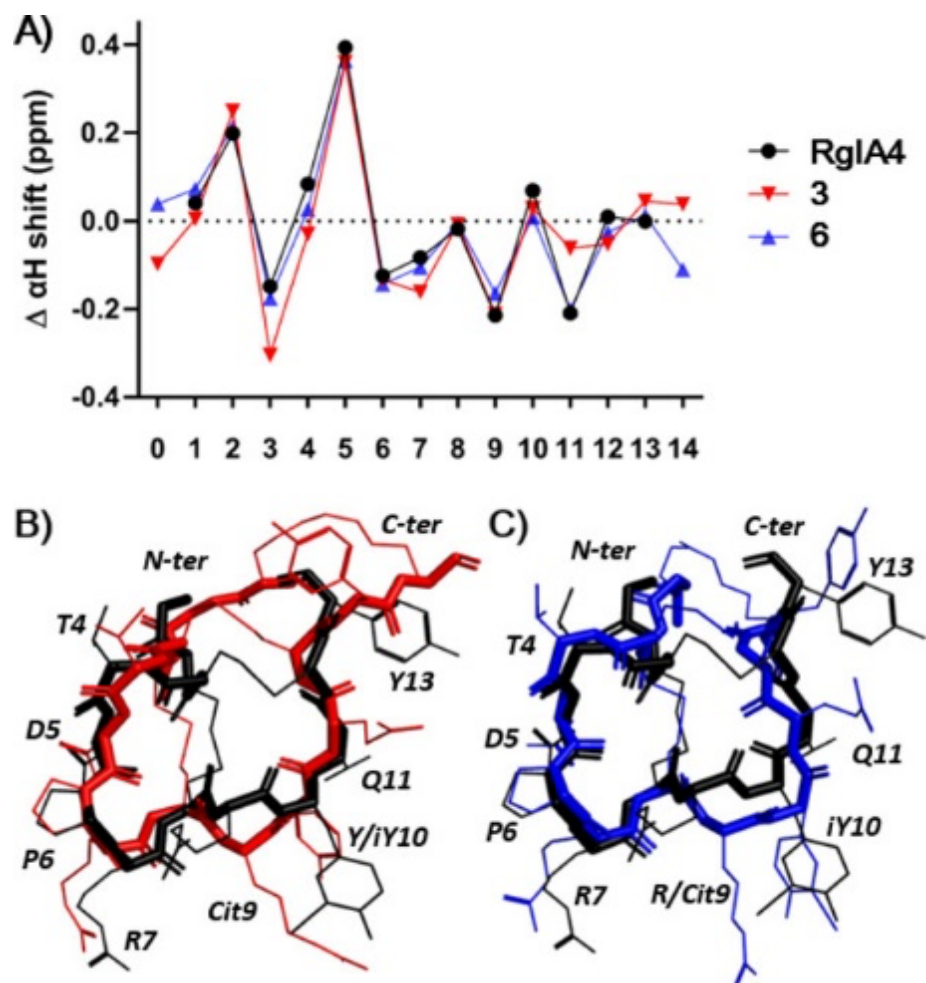
analogue 6 against RglA4, Student's *t* (unpaired) test. (B) Representative RP-HPLC trace of disulfide scrambling at different time points in human serum at 37 °C (sample concentration = 0.1 mg/mL in 90% human serum).

Project 5, Specific Aim 3, Major Task 1: - off target testing against other nAChR subtypes. We have demonstrated that the resulting analog 6 is highly selectivity for the $\alpha 9\alpha 10$ nAChR subtype.

nAChR	IC ₅₀ , Human
$\alpha 9\alpha 10$	6.1 nM
$\alpha 2\beta 2$	>10 μ M
$\alpha 2\beta 4$	>10 μ M
$\alpha 3\beta 2$	>10 μ M
$\alpha 3\beta 4$	>10 μ M
$\alpha 4\beta 2$	>10 μ M
$\alpha 4\beta 4$	>10 μ M
$\alpha 7$	0.50 μ M
$\alpha 1\beta 1\delta \epsilon$	>10 μ M



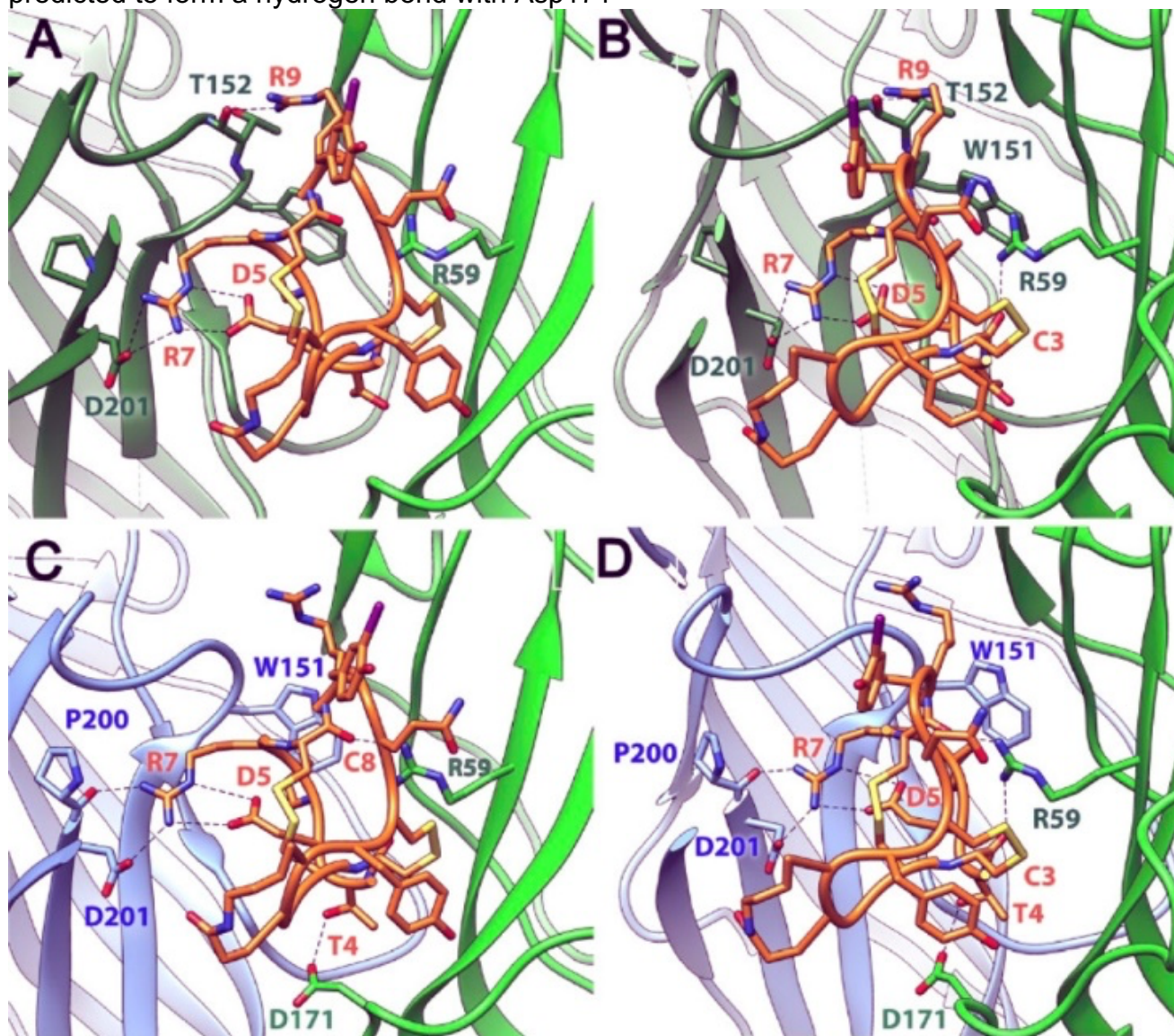
Analogues were applied to *Xenopus* oocytes heterologously expressing human nAChRs and the response to acetylcholine measured. To further understand ligand activity, we conducted NMR studies to determine 3D structure.



NMR study of α -RglA4, analogue **3**, and analogue **6**. (A) Secondary H α chemical shift overlay. Residue no. 0 represents for Glu and no. 14 for Lys. (B) Superposition of the representative NMR solution structure of (B) α -RglA4 (black) and **3** (red) (C) α -RglA4 (black) and **6** (blue).

In addition, a docking model of analogue **6** to the receptor based on Rosetta protein-protein docking calculations was generated to help inform SAR efforts. We found that key binding interactions revealed by the α -RglA/ α 9(+) crystal structure (PDB 6HY7) were predicted by Rosetta to also be present in the case of analogue **6** binding with α 9/ α 10 nAChR.¹⁵ Specifically, analogue **6** residues Asp5 and Arg7 form an intramolecular salt bridge, with the remaining amine on Arg7 hydrogen-bonding to the backbone carbonyl of receptor residue Pro200 on both the α 9(+) and α 10(+) surfaces, an interaction that is nearly identical to the reported crystal structure (Figure 5A, 5C). Additionally, Pro6 in analogue **6** forms a CH₂- π interaction with Trp151 on loop-B. Further interactions show some variation between the two receptor surfaces studied here, however these apparent differences, which place some potential interacting partners barely beyond the hydrogen-bond cutoff distance, may be overstated by this model as it does not take in to account any potential induced-fit conformational changes in the receptor backbone positions upon ligand binding. Our results indicated additional interactions between Arg9 and the backbone oxygen of Thr152 in the case of the α 9(+)/ α 9(-) interface. Receptor residue

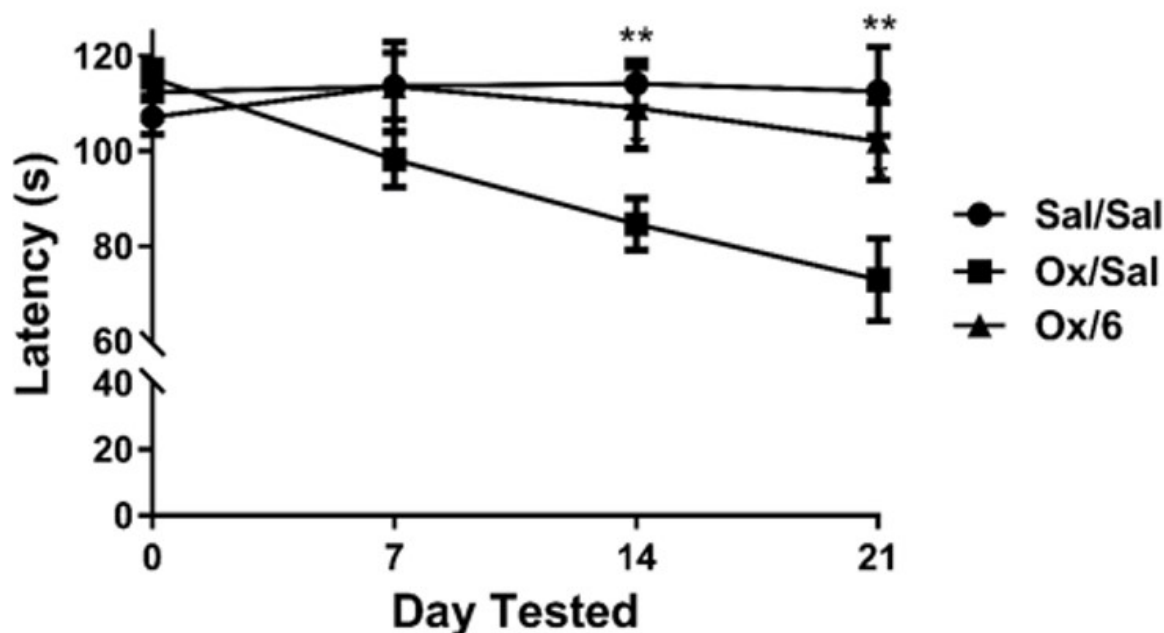
Arg59 forms hydrogen bonds with the backbone oxygen of analogue **6** residue Cys3 for both $\alpha 10(+)/\alpha 9(-)$ and $\alpha 9(+)/\alpha 9(-)$ and Cys8 for $\alpha 10(+)/\alpha 9(-)$ only. Finally, residue Thr4 is predicted to form a hydrogen bond with Asp171



Selected binding model from docking of NMR structure ensemble of analogue **6** into a homology model of human (A,B) $\alpha 9(+)/\alpha 9(-)$ and (C,D) $\alpha 10(+)/\alpha 9(-)$ nAChR interface using RosettaDock. $\alpha 9$ -ECD is shown in green, $\alpha 10$ -ECD is shown in light blue, and analogue **6** is shown in orange. Key binding residues are shown as stick representation with oxygen, nitrogen, and sulfur atoms in red, blue, and yellow, respectively. Dashed lines indicate hydrogen bonds formed between analogue **6** and receptors. The $\alpha 9$ -ECD structure was generated from the RgIA-bound X-ray crystal structure (PDB 6HY7), and the $\alpha 10$ -ECD was generated from the previously reported homology model based on the same structure.

Specific Aim 3, Major Task 2.

We tested the most potent $\alpha 9\alpha 10$ peptide analog in the chemotherapy-induced (oxaliplatin) neuropathy model. Eighty microgram/kg was injected s.c. once per day, 5 days per week x 3 weeks. The peptide was effective in preventing the development of cold allodynia (a major complication of this therapy). We have recently published the results of these studies (2).



Analogue **6** prevents chemotherapy-induced neuropathic pain on cold-plate testing. Dose = 80 $\mu\text{g/kg}$ (s.c.). Values are expressed as the mean $\pm$ SEM ($n = 8$) for each experimental determination. $**p < 0.01$ for Ox/6 versus Ox/Sal, one-way ANOVA with Dunnett's multiple comparison test. Sal = saline; Ox = oxaliplatin; SD = standard deviation.

References

- Gu S, Knowland D, Matta JA, O'Carroll ML, Davini WB, Dhara M, Kweon HJ, Bredt DS. Hair cell $\alpha 9\alpha 10$ nicotinic acetylcholine receptor functional expression regulated by ligand binding and deafness gene products. *Proc Natl Acad Sci U S A*. 2020;117(39):24534-44. Epub 2020/09/16. doi: 10.1073/pnas.2013762117. PubMed PMID: 32929005; PMCID: PMC7533656.
- Zheng N, Christensen SB, Blakely A, Dowell C, Purushottam L, McIntosh JM, Chou DH. Development of Conformationally Constrained α -RglA Analogues as Stable Peptide Antagonists of Human $\alpha 9\alpha 10$ Nicotinic Acetylcholine Receptors. *J Med Chem*. 2020;63(15):8380-7. Epub 2020/07/01. doi: 10.1021/acs.jmedchem.0c00613. PubMed PMID: 32597184.

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

All personnel who have contributed to this award have had opportunities for additional training and development through their work on the project.

Kate Tsourmas has been a laboratory technician on Project 1 for 4.5 months of year 1 and plans to be with us for 1 or 2 more years before starting a PhD program in neuroscience. We are training her in all of the scientific background and laboratory techniques that are required for accomplishing the specific aims of Project 1. This training

will contribute significantly to her future scientific career. She recently joined the PhD neuroscience program at UC Irvine and is working on neuroimmune interactions in neuropathologies.

Several undergraduate researchers have been trained by this project. One of these, Mario Giacobassi, was accepted in medical school and is currently at University of Wisconsin.

Three more undergraduates (Cristoval Urcino, Luke Hansen and Sabrina Kozel) have been well trained. They are currently applying to medical schools and we anticipate that the undergraduate researchers (or those who have recently finished their B.S. degrees) in our laboratory will likely enter a variety of biomedical professions, and go on to either professional schools or Ph.D. programs. Sabrina Kozel recently joined as a research assistant at Children's Hospital in LA.

We recently admitted two trainees in our lab. Victor Chua (graduate student) and Matias Giglio (postdoctoral researcher). They will be trained on neurobiology of chronic pain and will contribute to various aspects of Project 1 and Project 2.

Chemists brought into the project are specifically training in aspects of neuroscience and assay implementation that will be highly useful in their future careers in interdisciplinary chemical biology.

How were the results disseminated to communities of interest?

Undergraduate and graduate students involved in the project presented some of their work at Society for Neuroscience conference, spring brain conference and pain symposium at the University of Utah. The findings were presented at international conference (monsoon brain conference) and postdoc in neuro seminar series. The major findings of the project were published in the Proceedings of National Academy of Sciences, USA (PNAS) and other reputed scientific journals. The major findings from projects that will be disseminated are described below:

In Project 2, we will continue to characterize the peptides from *Asprella* venoms; any that demonstrate analgesic activities will be prioritized. Since we observed striking behavioral effects in the venom from *Conus rolandi*, we will follow it further with molecular characterization.

In project 3, we will continue our push our initial hits into further development by incorporating further animal assays and mechanism of action studies.

In Project 4, we will continue to mine transcriptome and neurometabolome data to identify potential endogenous TRP agonist/antagonists, as well as biosynthetic pathways leading to their production following injury. We will focus our efforts on identifying commonly deregulated genes associated with chronic pain phenotypes as well as the identification of bioactive substances in the neurometabolome, as we have been doing with 8,9-EET and 19,20-EpDPE. We will move forward with specific findings by creating screening tools to identify pain pathway inhibitors and to identify and characterize natural products that interact with the proteins that show deregulation as a consequence of nerve injury/the chronic pain phenotype as well as targets of endogenous molecules that we find through the neurometabolome and TRP screening assays. We also will specifically determine the role of *Cyp1b1* and *Trpa1* in regulating chronic pain in mice.

In Project 5 we will continue to optimize the molecules developed during the last reporting period. For the miniature peptides the focus will be on potency. For the peptides similar in size to the conopeptides, the focus will be on peptide stability.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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

The work that has been carried out in the first year of this award has demonstrated how to prioritize which extracts or biological mixtures to examine in order to identify components that are likely to affect pain circuitry. Our work has also led to the further development of the technology for identifying the molecular target of a bioactive peptide.

What was the impact on other disciplines?

The combination of the results in between the projects shows a path for both assessing bioactivity at the cellular level, but unexpectedly, also for determining whether off-target effects are likely. The combination of constellation pharmacology, molecular genetics, targeted single-cell transcriptomics and single-cell electrophysiology has opened avenues to identify molecular targets relevant to disease progression and identifying relevant ligands.

We believe that our work will transform the field of natural products and will require researchers to identify cell-specific molecular targets for every novel natural product ligand (in addition to the information on bioactivity of the natural products). In the field of disease biology, our work will stimulate and foster discussions on identifying drugs for different stages of the disease (in contrast to current approach where the drugs are targeted to established disease states). This class of drugs can be interventional/preventative therapeutics will be a paradigm shift in the field of drug discovery and drug development.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

The prevalence of chronic pain in U.S. military veterans is 4-5 fold higher than the general U.S population and 1 in 3 returning military troops, who sustained a deployment related concussion, report chronic pain and persistent post traumatic headache. The prevalence of chronic pain is reported to be significantly higher in troops deployed during Operation Iraqi Freedom (OIF) and Operation Enduring Freedom in Afghanistan (OEF). This is largely attributed to the blast exposures from improvised explosive devices. Although the advancements in war wound medical care has significantly increased the survival rates of the military troops, the impact of trauma chronically affects the veterans. Additionally, with the current pandemic, the cases of opiate induced respiratory depression also have also gone up, stressing the urgent need to find non-opiate alternatives for chronic pain management.

The outcomes of our research program will provide a list of molecular targets for the treatment of chronic pain. These will also be tremendously useful for the management

of other ailments including post traumatic headache and migraine and will help develop a robust pipeline of non-opiate therapeutics that will benefit the U.S. veterans and general public alike. We have requested funding to test our library of marine natural product therapeutics for the management of post traumatic headache and migraine in an expansion award. Our lab has developed one of the non-opiate FDA approved drug for intractable pain, Ziconotide. We recently developed additional drug leads that act via different molecular mechanisms. The application of these drugs go beyond the treatment of chronic pain and include the treatment of post traumatic headache and migraine.

- 5. CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

Changes in approach and reasons for change

Project 1: No major changes to report. In the last year report, we indicated changes related to the advancement in technology and cost reduction. We had initiated single cell transcriptomics (in addition to the whole DRG RNA-seq that was initially proposed) and this provided remarkable data collection useful for the identification of cell-specific molecular changes with the progression of chronic pain.

Project 3: Our partners have modified the assays applied, and so we have focused increasingly on using recent developments in constellation pharmacology. The results have been good, in terms of providing us with the anticipated number of hits and new compounds.

Project 4: No major changes to report, with the exception that activity guided fractionation of DRGs/spinal cord tissue will be secondary to metabolomics analysis of tissue extracts that may contain TRP agonists and antagonists, as described above.

Project 5: No changes except as noted in Aim 2 subtask 1. We modified our methods from the proposed because of new advances in the field.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

Publications, conference papers, and presentations

Report only the major publication(s) resulting from the work under this award.

Journal publications.**Project 1, 2 and 4:**

Giacobassi MJ, Leavitt LS, Raghuraman, S, Alluri R, Chase K, Finol-Urdaneta RK, Terlau H, Teichert RW, Olivera BM. (2020) An integrative approach to the facile functional classification of DRG neuronal subclasses. Proc Natl Acad Sci U S A., 117 (10) 5494-5501.

Raghuraman S, Xie JY, Giacobassi MJ, Tun JO, Chase K, Lu D, Teichert RW, Porreca F, and Olivera BM. (2020) Chronicling changes in the somatosensory neurons after peripheral nerve injury. Proc Natl Acad Sci U S A., 117 (42) 26414-26421.

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.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Project 1

Name:	Shrinivasan Raghuraman
Project Role:	Project 1 leader
Researcher Identifier:	00745804
Nearest person month worked:	3.0
Contribution to Project:	Dr. Shrinivasan Raghuraman is a research assistant professor. He worked closely with Dr. Teichert and helped establish the surgical suite for animal assays described in the project. He is leading and managing all aspects of Project 1 of the DoD grant investigating cell-specific molecular changes that occur in sensory neurons in rodent models of chronic pain. He works on constellation pharmacology platform and combines it with histochemical and electrophysiological techniques to investigate cellular and molecular changes that occur after nerve injury. Besides performing the molecular characterization, he also performs surgeries in rodents to create animal models of chronic pain. Dr. Raghuraman trains undergraduate and research personnel on constellation pharmacology platform and surgical techniques.
Funding Support:	NA

Name:	Lee Leavitt
Project Role:	Laboratory Specialist
Researcher Identifier:	00591788
Nearest person month worked:	6.0
Contribution to Project:	Lee Leavitt has assists in training new laboratory personnel to conduct and analyze data for calcium-imaging experiments to identify functional expression purinergic, cholinergic and bradykinin receptor subtypes in uninjured and injured DRG neurons. He has also conducted many of these experiments and analyzed data.
Funding Support:	Lee Leavitt is also supported by funding from an NIH grant

Name: Kate Tsourmas
Project Role: Laboratory Technician
Researcher Identifier: 00019559
Nearest person month worked: 4.5
Contribution to Project: Kate Tsourmas conducts calcium-imaging experiments to identify functional expression purinergic, cholinergic and bradykinin receptor subtypes in uninjured and injured DRG neurons. She has conducted many of these experiments and analyzed data for many of these experiments. She also conducts behavioral assays for rodent pain models.
Funding Support: NA

Name: Kevin Chase
Project Role: Statistical Data Analysis
Researcher Identifier: 00031821
Nearest person month worked: 2.7
Contribution to Project: Kevin Chase performs statistical data analysis for most experiments conducted in this project. Each experiment generates a large amount of data because the calcium-imaging responses of hundreds of neurons are monitored in each experiment, using various structural and functional markers to classify neurons into different subclasses or cell types. He has helped to automate some aspects of the complex data analysis.
Funding Support: Kevin Chase is also supported by funding from an NIH grant.

Name: Cristoval Urcino
Project Role: Undergraduate Researcher
Researcher Identifier: 00758742
Nearest person month worked: 2.25
Contribution to Project: Cristoval Urcino prepares cell cultures from rodent dorsal-root ganglia (DRG) for calcium-imaging experiments. Cristoval is also assisting other lab personnel with experiments and data analysis.
Funding Support: NA

Name: Luke Hansen

Project Role:	Lab Specialist
Researcher Identifier:	01196433
Nearest person month worked:	2.5
Contribution to project:	Luke Hansen is trained by Dr. Raghuraman, Dr. Alan Light to perform live surgeries on rodents. He is also trained to prepare cell cultures from rodent DRG for constellation pharmacology experiments. He provides the test samples from injured DRGs to the constellation pharmacology core for molecular characterization and identification of novel ligands for pain management.

Project 2

Name:	Baldomero Olivera
Project Role:	Principal Investigator of the Grant and Project 2
Researcher Identifier:	00034901
Nearest person month worked:	2.63
Contribution to Project:	Dr.Olivera leads the entire Grant and also Project 2.
Funding Support:	Dr. Olivera is also supported by funding from an NIH grant.

Name:	Joanna Gajewiak
Project Role:	Research Assistant Professor
Researcher Identifier:	00440664
Nearest person month worked:	6.0
Contribution to Project:	Dr. Gajewiak conducts all peptide synthesis, folding, purification and analysis of synthetic peptides required by Project 2.
Funding Support:	Dr. Gajewiak is also supported by funding from an NIH grant.

Name:	Julita Imperial
Project Role:	Research Associate Professor
Researcher Identifier:	00073769
Nearest person month worked:	5.0
Contribution to Project:	Dr. Imperial coordinates all efforts for storage/retrieval of venoms and venom fractions, purification of peptides from venom, mass

spectrometry and sequencing of peptides for Project 2.
Funding Support: Dr. Imperial is also supported by funding from an NIH grant.

Name: Shrinivasan Raghuraman
Project Role: Research Assistant Professor
Researcher Identifier: 00745804
Nearest person month worked: 3
Contribution to Project: Dr. Shrinivasan Raghuraman is a research assistant professor. His responsibilities for Project 2 include training lab personnel on constellation pharmacology platform to screen bioactive marine natural products. He designs experimental protocols for testing natural products target towards molecular targets relevant to chronic pain.
Funding Support: NA

Name: Manju Karthikeyan
Project Role: Senior Lab Specialist
Researcher Identifier: 00303661
Nearest person month worked: 6
Contribution to Project: Manju Karthikeyan was trained by Dr. Raghuraman to conduct constellation pharmacology experiments. She conducted majority of screening experiments and analyzed data for many of these experiments. She is also being trained to conduct behavioral assays for rodent pain models.

Funding Support: NA

Name: Samuel Espino
Project Role: Postdoctoral Fellow
Researcher Identifier: u0611775
Nearest Person month worked: 2.5
Contribution to Project: Dr. Espino is trained in constellation pharmacology and purification of marine natural products. He is involved in the screening of C.geographus venom and C.gloriamaris venom.

Project 3:

Name: Eric W. Schmidt

Project Role:	Principal Investigator of Project 3
Researcher Identifier:	00301927
Nearest person month worked:	0.22
Contribution to Project:	Dr. Schmidt 15 years of experience in organic and marine natural products chemistry, microbiology and biosynthesis. Expertise in biodiversity, drug discovery, and synthetic biology. Responsible for design and synthesis of peptidomimetic compound libraries.
Funding Support:	Dr. Schmidt is also supported by funding from an NIH grant.

Name:	Zhenjian Lin
Project Role:	Research Associate Professor
Research Identifier:	00676503
Nearest person month worked:	3.96
Contribution to Project:	Dr. Lin is performing natural products identification, and analysis.
Funding Support:	Dr. Lin is also supported by funding from an NIH grant.

Name:	Changshan Niu
Project Role:	Post Doc Student
Research Identifier:	06014292
Nearest person month worked:	7.50
Contribution to Project:	Dr. Niu is performing natural products identification, and analysis.
Funding Support:	Dr. Niu is also supported by funding from an NIH grant.

Name:	Noemi Paguigan
Project Role:	Post Doc Student
Research Identifier:	06015596
Nearest person month worked:	7.50
Contribution to Project:	Dr. Paguigan is performing natural products identification, and analysis.
Funding Support:	Dr. Paguigan is also supported by funding from an NIH grant.

Project 4:

Name:	Christopher Reilly
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Project Role:	Principal Investigator of Project 4
Research Identifier:	00163406
Nearest person month worked:	1.45
Contribution to Project:	Leading project 4, Dr. Reilly is an expert in TRP channel pharmacology who led several studies to elucidate roles of TRP channels in inflammatory pathways linked to pathologies such as glaucoma, pain, acute lung injury and asthma, as well as the discovery of novel agonists and antagonists of TRP channels (A1, M8, V1, V4) from commercial, synthetic, and natural product libraries. Experienced with lead optimization strategies and the design/execution of animal and human studies. Additional expertise in mass spectrometry, molecular biology/pharmacology, ADME and toxicology.
Funding Support:	Dr. Reilly is also supported by funding from an NIH grant.

Name:	Katherine L. Burrell
Project Role:	PhD Student
Research Identifier:	01075829
Nearest person month worked:	2.04
Contribution to Project:	Katherine is performing primary and secondary screening for compounds obtained from Schmidt's Group (Project 3) and neurometabolomics efforts in Project 4 which modulate TRPV3 activity.
Funding Support:	Katherine Burrell is also supported by funding from an NIH grant.

Name:	John Gregory Lamb
Project Role:	Research Associate
Research Identifier:	00035288
Nearest person month worked:	1.99
Contribution to Project:	Expert in molecular biology, pre-clinical drug screening, management of animal and human studies. Experience with TRP screening assays, pharmacological mechanisms determination, analytical methods development, and ADME/Toxicology studies.

Funding Support: Dr. Lamb is also supported by funding from an NIH grant.

Name: Tosifa Memon
Project Role: Post-Doctoral Research Associate
Research Identifier: 00706464
Nearest person month worked: 3.96
Contribution to Project: Dr. Memon performs primary and secondary screening for compounds obtained from Schmidt's Group (Project 3) and neurometabolomics efforts in Project 4 which modulate TRPA1 activity. She also assists in coordinating tissue procurement from Light (RPM Core), Olivera (Project 2) Groups and processing of tissues for neurometabolome and transcriptome studies.

Funding Support: Dr. Memon is also supported by funding from an NIH grant.

Name: Nam D. Nguyen
Project Role: Undergraduate Student
Research Identifier: 00991837
Nearest person month worked: 1.99
Contribution to Project: Nam Nguyen performs primary and secondary screening for compounds obtained from Schmidt's Group (Project 3) and neurometabolomics efforts in Project 4 which modulate TRPM8 activity. She also develops new screening assays.

Funding Support: Nam Nguyen is also supported by funding from an NIH grant.

Name: Cassandra Deering-Rice
Project Role: Research Associate and Co-Investigator
Research Identifier: 00266993
Nearest person month worked: 1.07
Contribution to Project: Expert at developing and optimizing TRP activity assays in reporter cells and neurons, determining activation mechanisms, and the design and implementation of animal studies including DRG and TG neuron culture. Experience in transcriptomic and bioinformatics

analysis. Have a major hole in the aims 1-3 in the project 4.
Funding Support: Dr. Rice is also supported by funding from an NIH grant.

Name: Emmanuel Reyes Rapp
Project Role: Technician
Research Identifier: 0853677
Nearest person month worked: 1.42
Contribution to Project: Manny coordinates and performs many elements involved in the overall screening effort/workflow between the Reilly Group (Project 4) and personnel in Schmidt (Project 3), Olivera (Project 2) Groups. He also schedules screening assay workflows and documentation and develops new screening assays to support collaborative assays on potential pain targets (TRPs and P2X receptors). Manny is also involved in developing lipid screening and CYP screening assays.
Funding Support: Emmanuel Rapp is also supported by funding from an NIH Grant.

Name: Lili Sun
Project Role: Post-Doctoral Research Associate
Research Identifier: 06024820
Nearest person month worked: 3.0
Contribution to Project: Expert in preparation and high-throughput screening of natural product libraries, lipidomics, compound isolation and characterization, TRP-specific assays for pharmacological mechanisms of action. Also has expertise in animal surgery, genomics, and mass spectrometry. Directly interacts with Project 3 (TRP screening studies) and the pain testing core for performing animal surgeries, and necropsies to obtain tissue to support the lipidomics and TRP screening studies, as well as the metabolomics core facilities. Currently developing CYP models and assays with Ms. Almestica and Mr. Rapp.
Funding Support: Dr. Sun is also supported by funding from an NIH grant.

Project 5:

Name: J. Michael McIntosh
Project Role: Principal Investigator of Project 5
Researcher Identifier: 00031743
Nearest person month worked: 1.0
Contribution to Project: Responsible for the scientific direction of Project 5
Funding Support: NA

Name: Layla Azam
Project Role: Research Assistant Professor
Researcher Identifier: 00310932
Nearest person month worked: 6
Contribution to Project: Dr. Azam has 10 years of experience in nicotinic acetylcholine receptor research. Responsible for gene cloning, receptor binding, electrophysiology and pharmacological profiling assays.
Funding Support: Layla Azam is also supported by funding from an NIH grant.

Name: Pradip Bandyopadhyay
Project Role: Research Associate Professor
Researcher Identifier: 00028304
Nearest person month worked: 6.75
Contribution to Project: Dr. Bandyopadhyay has 15 years of experience working with retrovirus, herpesvirus and picornavirus and expression studies using virus derived vectors. In addition, he has 15 years of experience in Conus molecular biology and expression of conotoxins modification enzymes in insect cells. He will be responsible for developing stable cell lines expressing nicotinic alpha9alpha10 receptor.
Funding Support: NA

Name: Hung-Chieh Chou
Project Role: Research Assistant Professor
Research Identifier: 00982313
Nearest person month worked: 0.49

Contribution to Project: Synthetic chemist with expertise in protein and peptide therapeutics. He is responsible for peptide synthesis with focus on peptide stapling.

Funding Support: Dr. Chou is also supported by funding from JDRF, NIH R35, USTAR grants.

Name: Yixin Fang
Project Role: Post Doc Student
Research Identifier: 06002847
Nearest person month worked: 10
Contribution to Project: Since RglA4 is comprised of 3 circular pieces connected to each other via oxidatively labile disulfide linkages, Yixin overall goal is to dissect the loops of conotoxins into different separate pieces, and to simplify the scaffolds and find much more compact structures. Yixin takes a peptidomimetic, fragment-based approach to design and create drug-like molecular leads for screening. To saturate and refine the chemical space of crucial pharmacophores, Yixin is working on the introduction of a series of well-defined pyridinium-type residues into different single loops originated from RglA4, as well as on the replacement of particular disulfide bridges with N-C macrocyclic bridges. Together, these methods will produce a synthetic library of peptides or peptidomimetic ligands, for the aim to achieve highly potent and selective $\alpha 9\alpha 10$ nAChR antagonists for clinical development and more pharmacologically stable even orally available drugs.

Funding Support: NA

Name: Nan Zhang
Project Role: Post Doc Student
Researcher Identifier: 06012932
Nearest person month worked: 5
Contribution to Project: α -Conotoxins are disulfide-bonded peptides with distinct selectivity for nAChR subtypes and have been identified as potent analgesics in animal models of chronic pain. However, multiple disulfide bonds are inherently unstable in reducing physiological environments and are

susceptible to scrambling, which may induce structural changes that can lead to reduced biological activity. My research with Prof. McIntosh focuses on the development of disulfide bond mimics and chemical synthesis of Rg1A analogs with enhanced stability as well as nAChR subtype selectivity.

Funding Support:

NA

Constellation Pharmacology Core

Name:

Shrinivasan Raghuraman

Project Role:

Research Assistant Professor

Researcher Identifier:

00745804

Nearest person month worked:

3.0

Contribution to Project:

Dr. Raghuraman is leads and manages all aspects of this drug-discovery core of the DoD grant.

Funding Support:

NA

Name:

Manju Karthikeyan

Project Role:

Laboratory Specialist

Researcher Identifier:

00303661

Nearest person month worked:

12.0

Contribution to Project:

Manju Karthikeyan is conducting drug screening by calcium imaging of mouse DRG neurons to identify natural products that have activity on a variety of plasma-membrane receptors and ion channels. She has conducted many of screening experiments with crude extracts and pure compounds and has analyzed data for many of these experiments. We have obtained many hits from the screening with compounds that have potential as analgesic drugs.

Funding Support:

NA

Name:

Kevin Chase

Project Role:

Statistical Data Analysis

Researcher Identifier:

00031821

Nearest person month worked:

2.7

Contribution to Project:

Kevin Chase performs statistical data analysis for most experiments conducted in this drug-

discovery core. Each experiment generates a large amount of data because the calcium-imaging responses of hundreds of neurons are monitored in each experiment, using various structural and functional markers to classify neurons into different subclasses or cell types. Kevin helps to identify hits that are statistically significant and have potential as analgesic drugs.

Funding Support:

Kevin Chase is also supported by funding from an NIH grant.

Rodent Pain Models Core:

Name:

Alan Right

Project Role:

Leading the RPM Core

Research Identifier:

00320393

Nearest person month worked:

0.76

Contribution to project:

Dr. Light is the director of the animal behavior core, and responsible for all mouse and rat experiments conducted in other locations at the University of Utah. He discusses potential mouse and rat experiments with the 5 other principal investigators and then helps designed the appropriate experiments to test the compounds found by the PI's.

Dr. Light also coordinates the entire personnel from rodent pain models core.

Funding Support:

Dr. Light is also supported by funding from an NIH grant.

Name:

Ronald Hughen

Project Role:

Laboratory Specialist

Research Identifier:

00284353

Nearest person month worked:

1.2

Contribution to project:

Ron is responsible for maintaining the rat and mouse colonies used for some experiments, he is responsible for obtaining all rats and mice for the experiments done by the rodent pain models core.

Ron aids in the preparation and completion of all experiments and surgeries, and helps with initial analysis. He also obtains all supplies needed for these experiments, keeps all equipment in

working order, and teaches personnel how to make and store reagents used in the experiments.
Funding Support: Ron Huguen is also supported by funding from an NIH grant.

Name: Alexi Micinski
Project Role: Laboratory Technician
Research Identifier: 00843132
Nearest person month worked: 4.8
Contribution to project: Alexi is one of the primary personnel that will do the treatments and surgeries of rats and mice that are tested for compounds that may be useful in blocking pain.
Funding Support: NA

Name: Steven Parkin
Project Role: Laboratory Technician
Research Identifier: 00889009
Nearest person month worked: 4.8
Contribution to project: Steven is the other primary person that will do the treatments and surgeries of rats and mice that are tested for the effects of compounds that may be useful in blocking pain.
Funding Support: NA

Name: Jie Zhang
Project Role: Research Assistant Professor
Researcher Identifier: 00092762
Nearest person month worked: 3.6
Contribution to project: Dr. Zhang is an expert in animal models of acute and chronic pain. He teaches the rodent pain models personnel how to do the treatments and surgeries used to create animal models of acute and chronic pain. Dr. Zhang also helps to do the initial analysis of the data obtained with these models. During the past year, he did more than half of the rat and mouse experiments done for the DoD project this year while teaching Jay Jensen (who left in June because he unexpectedly got into Medical School) and Alexi Micinski who was taking over Jay Jensen's position.

Funding Support:

Dr. Zhang is also supported by funding from an NIH grant.

Administrative Core:

Name:

Terry Merritt

Project Role:

Program Manager

Research Identifier:

06016833

Nearest person month worked:

6.0

Contribution to Project:

As program manager, Terry assists in the hiring and onboarding of new personnel, and oversees the review of timecards for all hourly employees. She is responsible for submitting research and personnel amendments to IACUC and ACURO, scheduling meetings with the project leaders of the grant, managing the budget for Projects 1, 2, 5 and CP Core, and assisting in the production and organization of the Annual Report and Animal Report.

Funding Support:

NA

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?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: