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14. ABSTRACT

The overarching goal of this proposal is to test the hypothesis that mutations in the Snf5 gene (also called SMARCB1) contribute to the intractable pain experienced by patients with schwannomatosis. We previously found that Schwann cells with Snf5 mutations secrete factors the induce the TRPV1 capsaicin receptor in sensory neurons. During the second year of the project, we have confirmed that Schwann cells with Snf5 mutations secrete the chemokine CCL2, and that CCL2 induces TRPV1 expression in sensory neurons linked to pain signaling. We have also found that TRPV1 becomes elevated in sensory neurons through a mechanism that does not involve increased TRPV1 gene transcription. These findings have resulted in a refocusing of the project to confirm the roles of CCL2 in schwannomatosis pain and in the induction of the calcitonin gene-related peptide (CGRP) in the pain phenotypes of SNf5-mutant mice.

15. SUBJECT TERMS
schwannomatosis, SNF5, SMARCB1, Schwann cells, TRPV1, CCL2, pain

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1. INTRODUCTION

Schwannomatosis is a clinically and genetically distinct form of neurofibromatosis that affects 1 in 40,000 individuals worldwide. The disease is characterized by multiple peripheral nerve tumors, called schwannomas, and a predisposition to other nervous system tumors including meningiomas. Unlike other forms of neurofibromatosis, patients with schwannomatosis overwhelmingly present with intractable pain. Clinical findings suggest that the pain afflicting schwannomatosis patients is not strictly linked to tumor growth or mechanical nerve compression by schwannomas. Mutations in the *SNF5* (also called *SMARCB1*) gene as well as the neurofibromatosis 2 (*NF2)* gene are linked to schwannomatosis. To test the role of *SNF5* loss in schwannomatosis, we generated and characterized mice in which the *Snf5* gene was inducibly and conditionally knocked-out in Schwann cells. We found that these mice had increased capsaicin sensitivity and elevated levels of the pain mediators, TRPV1 and calcitonin gene-related peptide (CGRP) in sensory neurons. These phenotypes are induced by a factor or factors released by *Snf5*-mutant Schwann cells. The original goals of this study were to fully characterize the cells induced to express ectopic TRPV1 and CGRP, identify the factor or factors that induce TRPV1 and CGRP in sensory neurons, determine how TRPV1 activity and elevated CGRP are related and their contributions to schwannomatosis pain, and whether altering the activities of factors released from Snf5 mutant Schwann cells can reverse pain phenotypes in Snf5-mutant mice. All together, these studies will help determine whether *SNF5*/*SMARCB1* mutations in Schwann cells lead to pain, and have the potential to define potential targets for the treatment of schwannomatosis pain.

2. KEYWORDS:

Schwannomatosis, Schwann cells, Snf5, SMARCB1, TRPV1, pain, calcitonin gene-related peptide, proteomics, CCL2

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Our original specific aims were:

1. *To identify factors secreted by Snf5-/- Schwann cells that induce increased TRPV1 and CGRP expression in sensory neurons.* In years 1 and 2, we characterized the effects of *Snf5*-/- Schwann cell conditioned medium (CM) on TRPV1 and CGRP expression in sensory neurons by defining which cells express TRPV1 and CGRP and determining the time it takes for TRPV1 and CGRP expression to increase. We also determined the profile of secreted proteins in Snf5-/- vs. Snf5+/+ Schwann cell CM using a novel and sensitive proteomic strategy. Finally, we are determining if factors identified in our proteomic and earlier DNA microarray screens are expressed by human schwannomatosis patient schwannoma cells *in vitro* and *in situ*, and whether these factors are sufficient to induce TRPV1 and CGRP expression in sensory neurons.
2. *To test if TRPV1 elevation is sufficient to induce increased pain sensitivity in mice with Schwann cell-targeted Snf5 mutations*. We had originally aimed to test whether elevated TRPV1 expression is necessary and sufficient for the elevated pain sensitivity in our mice by crossing our B6.Cg-Tg(Plp1-cre/ERT)3Pop/J +/-; Snf-fl/fl mice into a *Trpv1*-null background and performing a battery of pain sensitivity assays. However, because we have now identified a specific target upstream of TRPV1 activation, we have decided to

focus on testing the efficacy of using agents that block these new targets for treating pain (outlined below).

3. *To test how CGRP influences pain sensitivity in mice with Schwann cell targeted loss of Snf5.* New reagents have been made available to us to test the precise role of CGRP in our model. We will use these agents and a pharmacological inhibitor of CGRP receptors to determine the contribution of CGRP to pain phenotypes in our *Snf5* mutant mice under both basal and inflammatory conditions (see below).

What was accomplished under these goals?

In our studies from year 1, we confirmed that the vast majority of cells that express elevated TRPV1 in the dorsal root ganglia of *Snf5* mutant mice also express CGRP, and that both small and large diameter neurons were double positive, although a larger proportion of cells that express the marker IB4, which labels nociceptive neurons with smaller diameters were positive. We also confirmed that the effects of *Snf5* null Schwann cell conditioned medium on wild type sensory neuron TRPV1 expression can be observed in as little as an hour following exposure suggesting that the effect may not require transcriptional upregulation of the *TRPV1* gene. We see similar effects when using conditioned media from schwannomatosis patient schwannoma cell CM. In year 2, we analyzed the transcriptional activation of TRPV1 in sensory neurons treated with control and schwannoma CM and found that there was no alteration in TRPV1 transcripts (Fig. 1), confirming the idea that TRPV1 changes in our *Snf5* mutant mice are not at the level of transcription.

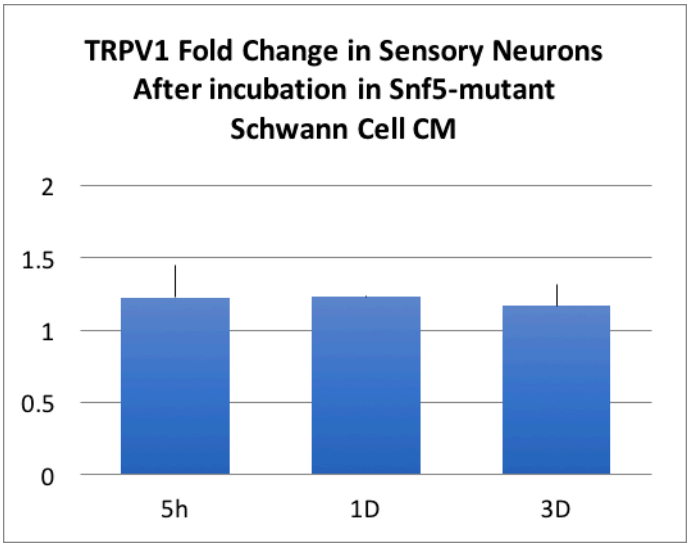

Figure 1: qPCR analysis of TRPV1 mRNA expression in dissociated mouse DRG neuron cultures 5 hours, 1 day, and 3 days following treatment with conditioned media (CM) from Snf5-mutant Schwann cell cultures. A proportion of the cells from these cultures were grown on glass coverslips to confirm elevated TRPV1 expression by immunocytochemistry.

In year 1, we identified several proteins that were elevated in the conditioned media of mouse and human Schwann cells with Snf5 mutations. In year two, we refined our proteomic screen and verified that several proteins were consistently elevated in mutant Schwann cell CM. Two proteins in particular, interleukin-6 and C-C motif ligand 2 (CCL2), were consistently elevated. We attempted to determine if IL-6 had any direct influence on TRPV1 expression in sensory neurons by treating sensory neurons with different concentrations of recombinant IL-6 *in vitro*. However, we did not observe any changes in TRPV1 expression or distribution in IL-6 treated vs. control cultures.

We have therefore focused on CCL2 because it has been implicated in neuropathic pain following peripheral nerve injury, and activation of spinal TRPV1 receptors plays an important role in the modulation of nociceptive signaling induced by CCL2 (Spicarova et al.,

Neuropharmacology. 2014 81:75-84). Furthermore, in year 1 we found that CCL2 mRNA was 2 times higher in Snf5 mutant Schwann cells than in wild type Schwann cells. We have now confirmed that CCL2 protein is elevated in Snf5 mutant Schwann cell CM. Furthermore, we verified that CCL2 induces increased TRPV1 expression in DRG mouse sensory neuron cultures (Fig. 2). All together, these data support the hypothesis that CCL2 is elevated in Snf5-mutant Schwann cells and is necessary and sufficient for the induction of TRPV1 expression in sensory neurons. These data also suggest that CCL2 is a potential target for treating pain in schwannomatosis patients with Snf5/SMARCB1 mutations.

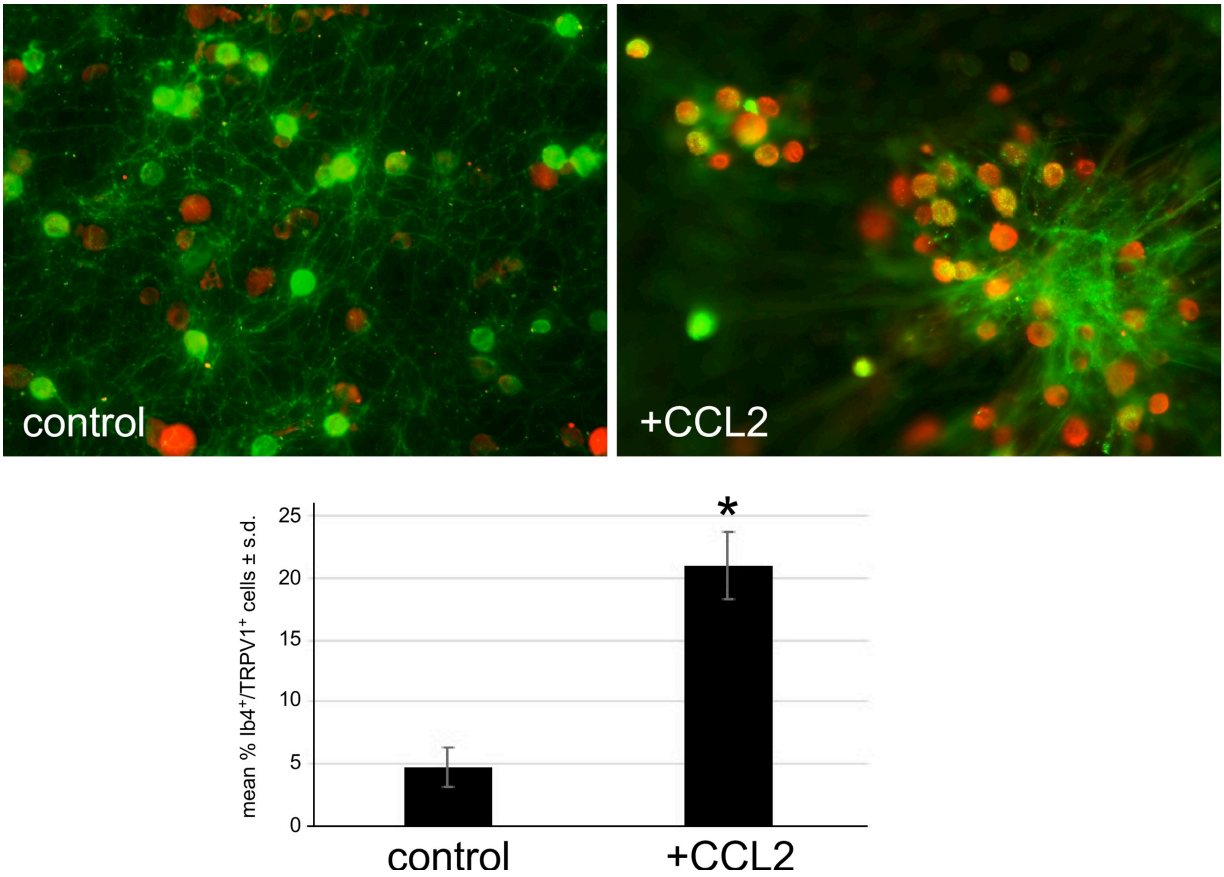

Figure 2: Effects of recombinant CCL2 on mouse DRG sensory neurons *in vitro*. Top panels show sensory neurons stained with IB4 (green; to label small diameter nociceptive cells) and TRPV1 (red) following treatment with CCL2 for 24 hours. Lower panel shows quantification of CCL2+/IB4+ cells. *p<0.001

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

Nothing to Report.

How were the results disseminated to communities of interest?

Some of these data were presented in an oral presentation by Dr. Matsumoto at the 2017 International Neurofibromatosis Conference in Washington, DC and in a poster by Dr. Banine at

the same conference. These data were also presented at the Annual Meeting of the Society for Neuroscience, Oregon Chapter, in 2017.

What do you plan to do during the next reporting period to accomplish the goals?
Because our findings strongly point to CCL2 as a factor released by Snf5-mutant Schwann cells, we will focus our ongoing efforts on (a) testing if CCL2 is elevated in human schwannomatosis schwannomas that have Snf5 mutations; (b) testing if blocking CCL2 activity in CM from Snf5 mutant Schwann cells reverses the induction of TRPV1 and CGRP in sensory neurons; and (c) testing the contribution of CGRP to pain phenotypes in Snf5 mutant mice.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?
To date, this project has demonstrated that the loss of SNF5/SMARCB1 in Schwann cells, a gene that is commonly mutated in the Schwann cells of patients with schwannomatosis, leads to increased transcription and secretion of CCL2, and that CCL2 induces TRPV1 in nociceptive sensory neurons through a mechanism that is independent of elevated TPRV1 transcription. This is potentially highly significant given the known roles of CCL2 in neuropathic pain. The project has also identified a number of other proteins that are elevated in SNF5 mutant Schwann cells.

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

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

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change
The finding that CCL2 is released from Snf5 mutant Schwann cells and can directly induce TRPV1 expression in wild type sensory neurons, especially small nociceptive cells, is an exciting development. Our original second aim was to focus on confirming that the pain phenotypes related to Snf5 loss in Schwann cells are all linked to elevated TRPV1 expression and not to other changes. However, CCL2 and CCL2 receptors may be better potential therapeutic targets. We will therefore focus our studies in year 3 on (a) testing if CCL2 is elevated in human schwannomatosis schwannomas that have Snf5 mutations; and (b) testing if blocking CCL2 activity in CM from Snf5 mutant Schwann cells reverses the induction of TRPV1 and CGRP in sensory neurons. We will also explore, as time and resources permit, if Snf5 directly regulate CCL2 transcription (e.g. by performing chromatin immunoprecipitation assays to test for interactions between Snf5 and the CCL2 promoter).

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

6. **PRODUCTS:**
Publications, conference papers, and presentations
Journal publications.
Nothing to report

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

Other publications, conference papers, and presentations.
Oral presentation by Dr. Matsumoto at the 2017 International Neurofibromatosis Conference in Washington, DC and in a poster by Dr. Banine at the same conference. These data were also presented at the Annual Meeting of the Society for Neuroscience, Oregon Chapter, in 2017.

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?**

• Name:	• *Larry Sherman*
• Project Role:	• *PI*
• Researcher Identifier (e.g. ORCID ID):	• 0000-0001-6098-6551
• Nearest person month worked:	• *2.4 calendar months*
• Contribution to Project:	• *Dr. Sherman served as PI of the project, coordinating all of the research projects, interpreting data, presenting data at meetings, and authoring reports.*
• Funding Support:	• *NIH, National Multiple Sclerosis Society*

• Name:	• *Steven Matsumoto*
• Project Role:	• *Co-Investigator*
• Researcher Identifier (e.g. ORCID ID):	• 0000–0002–3352–8077
• Nearest person month worked:	• *1.2 calendar months*
• Contribution to Project:	• *Dr. Matsumoto prepared primary cultures of Schwann cells and dorsal root ganglion neurons, and performed and analyzed all immunohistochemistry experiments.*
• Funding Support:	• *National Multiple Sclerosis Society*
• Name:	• *Fatima Banine*
• Project Role:	• Staff Scientist
• Researcher Identifier (e.g. ORCID ID):	•
• Nearest person month worked:	• 7.2 calendar months
• Contribution to Project:	• Dr. Banine performed all of the proteomics experiments and the subsequent data analysis and validation assays (e.g. qPCR)
• Funding Support:	• National Multiple Sclerosis Society
• Name:	• Brian Hammond
• Project Role:	• Research Assistant
• Researcher Identifier (e.g. ORCID ID):	•
• Nearest person month worked:	• 4.2 calendar months
• Contribution to Project:	• Mr Hammond assisted Dr. Sherman, Dr. Matsumoto and Dr. Banine with all aspects of the project. He maintained the mouse colony, arranged for timed matings, prepared reagents, and maintained cell cultures.
• Funding Support:	• NIH, National Multiple Sclerosis Society
• Name:	• Cristina Fernandez-Valle
• Project Role:	• Co-Investigator
• Researcher Identifier (e.g. ORCID ID):	• 0000–0002–6718–1243
• Nearest person month worked:	• 0.6 calendar months
• Contribution to Project:	• Dr. Fernandez-Valle organized the acquisition of human tissues, prepared human schwannoma cell cultures, and arranged for shipments of media and

	tissues to the Sherman lab
• Funding Support:	• DOD, NIH
• Name:	• Stephanie Klingman-Plati
• Project Role:	• Research Assistant
• Researcher Identifier (e.g. ORCID ID):	•
• Nearest person month worked:	• 1.2 calendar months
• Contribution to Project:	• Ms. Klingman-Plati assisted Dr. Fernandez-Valle, maintained cell cultures, and assisted with the preparation of tissues and conditioned media.
• Funding Support:	• DOD, NIH

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
No

What other organizations were involved as partners?
Dr. Fernandez-Valle and Ms. Klingman-Plati are located at the University of Central Florida. Otherwise, no other institutions were involved to date.