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14. ABSTRACT

We are studying the role of the TNFR2 receptor on the development and resolution of neuropathic pain. We have observed that expression of TNFR2 on Nex^{+} neurons is necessary for CCI-induced pain to resolve. A new TNFR2 specific agonist, mARTOS, is now available to us and will be tested for therapeutic potential against neuropathic pain.

15. SUBJECT TERMS
None listed.

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1. Introduction

Neuroinflammation resulting from injury or disease is a major risk factor for the development of chronic neuropathic pain (CNP). Our previous research demonstrated that the absence of TNFR2 expression prevents recovery from CNP and systemic administration of EHD2-sc-mTNFR2, an agonist activating specifically TNFR2, is therapeutic for CNP in two mouse models (chronic constriction injury and experimental autoimmune encephalomyelitis). Preliminary results suggest that absence of TNFR2 expression in microglia alone prevents recovery from CNP and that TNFR2 signaling is neuroprotective and promotes neurite outgrowth through IRE1α activation.

Our goal is to study the effects of TNFR2 signaling on the recovery process from chronic neuropathic pain as we hypothesize that TNFR2 signaling in microglia and in neurons is therapeutic and promotes the resolution of the pain. These studies will be conducted using mARTOS, a new generation of the mouseTNFR2 agonist for selective activation of TNFR2.

2. Keywords

TNFR2 receptor, TNFR2 agonist ARTOS, neuropathic pain, neuroinflammation, neuron, microglia, pain therapy

3. Accomplishments

Major goals of the project

- **Investigate changes in neuroinflammation in the CNS of TNFR2$^{fl/fl}$ and CX3CR1-CreER^{T2}/TNFR2$^{fl/fl}$ mice**

Milestone on SOW: 10-13 months to extract and analyze the RNA expressed in microglia.
Current status: ongoing (see 5. Changes/Problems).

1) Major activities

The purification and sorting of microglia from cortex and spinal cord was performed, followed by RNA extraction. Obtained next generation murine TNFR2 agonist, ARTOS.

2) Specific objectives

Microglia purification and sorting to extract RNA for RNA sequencing of microglia transcripts to correlate pain development and specific gene expression.

3) Significant results

That part of the project is proving technically challenging. We have been able to purify up to 6972 microglial cells from the cortex and 1844 from the spinal cord of individual animals, yielding 8.4 ng/µl and 14.4 ng/µl respectively. These amounts are too low to perform RNA sequencing with the current methods.

4) Other achievements

Nothing to report.

- **Determine if neuronal TNFR2 activation reduces axonal injury and mitigates chronic neuropathic pain**

Milestone on SOW: 6-24 months to assess the effect of TNFR2 deletion from various neuron populations in both females and males.
Current status: ongoing (results presented below).

1) Major activities

Poster presented at the IASP World Congress on Pain 2022 (Sept. 19-23, 2022, Toronto, Canada)
Title: TNFR2 signaling in Nex^+ (projection) neurons is critical to mitigate chronic neuropathic pain
Authors: Sreejita Arnab, Roman Fischer, John Bethea

2) Specific objectives

Investigate the role of TNR2 signaling in Nex^+ neurons on the development of neuropathic pain and on axonal pathology.

3) Significant results

We have observed that the lack of TNFR2 expression in Nex^+ neurons (glutamatergic projection neurons) prevents the spontaneous recovery that occurs in male and female mice 5-6 weeks following Chronic Constriction Injury (CCI) (fig. 1).

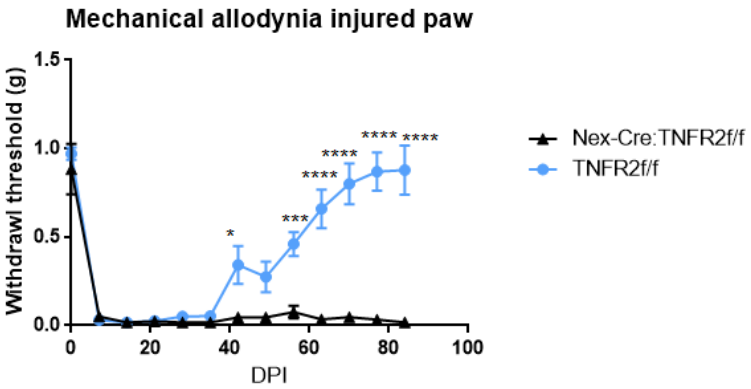

Figure 1. Nex-Cre:TNFR2$^{f/f}$ injured mice never recover from chronic pain. (Two-way ANOVA p= 0.0069**, 0.0001***, <0.00001****)
DPI: days post-injury.

male and female TNFR2$^{fl/fl}$ (control) and Nex-CreER^{T2}: TNFR2$^{fl/fl}$ mice were injected with tamoxifen prior to injury and subjected to CCI. Pain (mechanical allodynia) was assessed weekly following the injury using the Von Frey test and cognitive behaviors. As shown on the graph, the animals that do not express TNFR2 in Nex^{+} neurons do not show any pain resolution during the twelve weeks that follow the CCI, whereas the control animals have their sensitivity to the Von Frey probes restored to the levels measured before the injury. Moreover, a Mechanical Avoidance Assay performed at the end of the experiment (twelve weeks post-injury) confirms the difference in mechanical allodynia observed between the two groups (fig. 2).

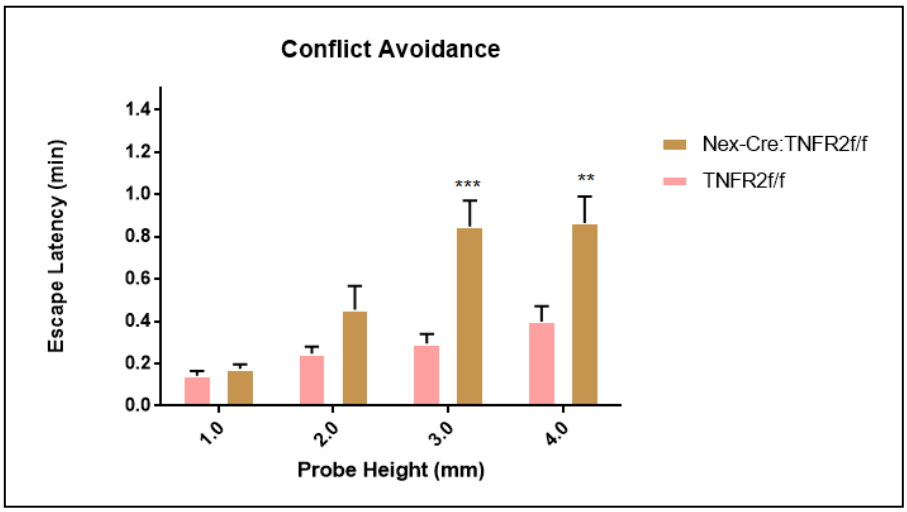

Figure 2. Nex-Cre:TNFR2$^{f/f}$ injured mice take longer to go across nociceptive probes. p= .001***, .01**

The Mechanical Avoidance Assay offers an operant method for pain testing: the mice face a choice and can either escape an aversive (bright light) environment by walking over metal spikes of specified heights to join a dark chamber, or stay in the bright chamber if the pain is too intense during the crossing. The mice whose Nex^{+} neurons do not express TNFR2 take longer to cross to the dark chamber, which correlates directly with their increased mechanical allodynia.

We have also performed preliminary analyses of the tissue (cortex and spinal cord) of TNFR2$^{f/f}$ and Nex-Cre:TNFR2$^{f/f}$ by Western blot. We have observed a decrease in Iba1 expression in the spinal cord of the Nex-

Cre:TNFR2$^{f/f}$ animals compared to the controls, and that decrease is also observed in the cortex but not to significant levels. This could suggest a decreased activation of microglial cells in the animals lacking TNFR2 expression in the neurons. This result is intriguing but needs to be conformed and investigated further. The NeuroFilament protein levels as well as the GFAP levels in both tissues do not show any differences in preliminary studies.

We have also initiated experiments to establish the expression pattern of TNFR2 in Nex^{+} neurons using the RNAscope™ technology to better assess what neuronal populations are involved in the resolution of pain. Figure 3 shows the expression of Nex and TNFR2 in the mouse hippocampus.

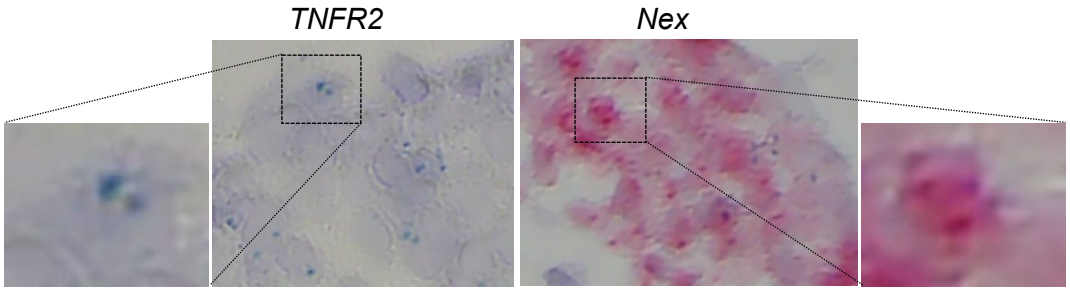

Figure 3. Expression of TNFR2 (left, green) and Nex (right, red) mRNAs in the mouse hippocampus

The picture on the right clearly shows some TNFR2 expression in Nex^{+} neurons, although not in every one of them, raising the interesting question of the possible existence of sub-populations of Nex^{+} neurons involved in the development of pain. One challenge that we are facing is the relative low abundance of the TNFR2 messenger RNA compared to the Nex one. We have obtained probes with opposite colors in the hope that labeling TNFR2 in red will make it more easily distinguishable. Assessing more precisely where TNFR2 is expressed among different neuron populations will provide us with great insight about the role of TNFR2 in pain development and resolution.

4) Other achievements

Nothing to report.

- **Validate the therapeutic efficacy and tolerance of ARTOS in mouse models of chronic neuropathic pain**

Milestone on SOW: 3 months to produce the agonists ARTOS (targeting human receptor) and EHD2-sc-mTNF$_{R2}$

Current status: just obtained mouse ARTOS (see 5. Changes/Problems). These studies are ongoing, and we should have the first indicative results in 2-3 weeks.

1) Major activities

We have recently received a shipment of 2 g of the mouse ARTOS agonist produced by Catalent (Wisconsin). Some of the biochemical characterizations of the compound produced (purity, *in vitro* binding properties) are presented below (figures 4 and 5).

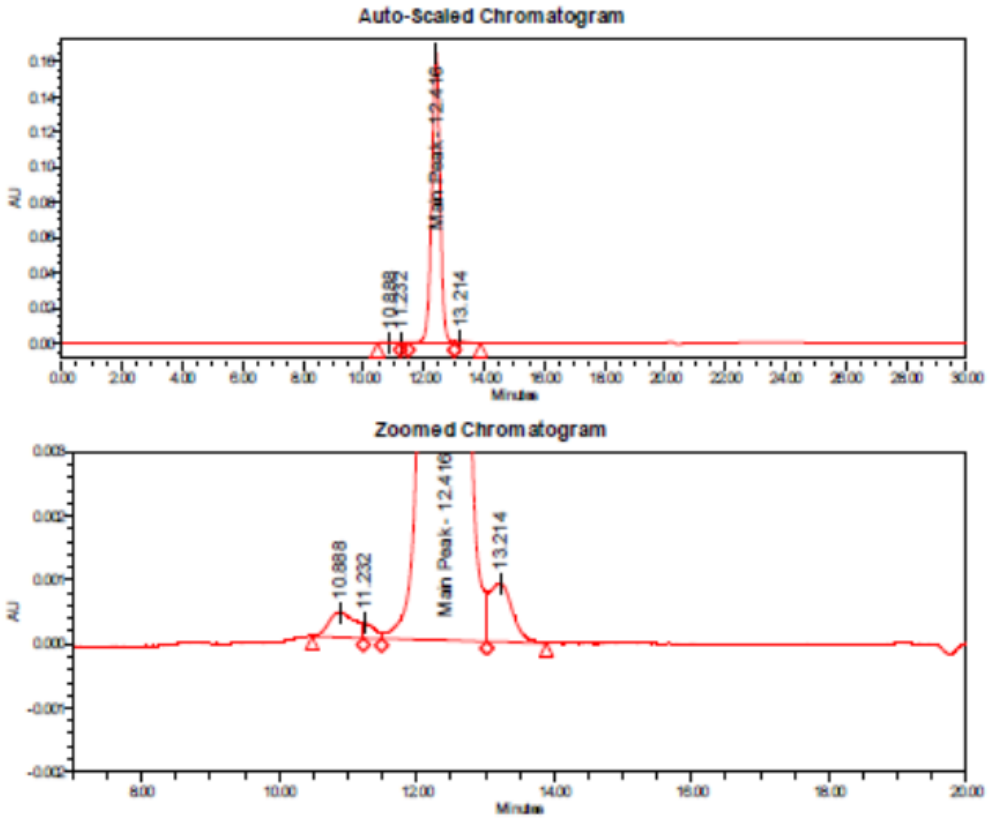

Figure 4. Characterization of murine ARTOS: purity and size exclusion chromatography

Endotoxin	<0.0052 EU/mg		
Size Exclusion HPLC	**HMW (%)**	**Main (%)**	**LMW (%)**
	0.42	98.91	0.67

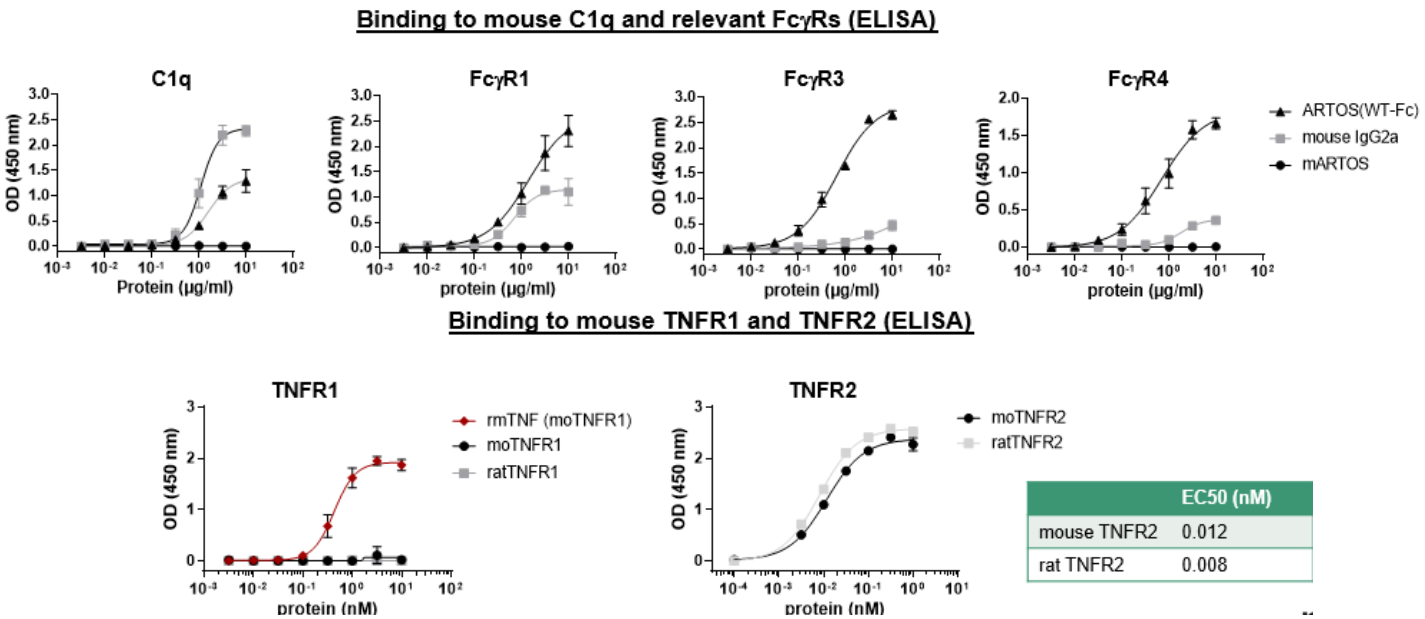

Figure 5. Binding of murine ARTOS to TNF and Fc receptors.

2) Specific objectives

Obtain a substantial amount of the TNFR2 agonist needed to perform the in vivo experiments.

Start testing the therapeutic potential of ARTOS in models of neuropathic pain.

3) Significant results

Nothing to report.

4) Other achievements

Nothing to report.

Opportunities for training and professional development the project has provided

Nothing to report.

Dissemination to communities of interest

Nothing to report.

Goals for next reporting period

Now that we have the mouse ARTOS at our disposal, we expect to test it in the CCI model in both sexes to verify its therapeutic potential against neuropathic pain. It will also be used in animals lacking the expression of TNFR2 in Nex^{+} neurons to evaluate further the role of the receptor during pain resolution in respective cell populations.

We will also pursue the examination of gene expression changes during CCI, with or without treatment with the ARTOS agonist, but through a more focused approach using the NanoString nCounter technology that allows the examination of specific gene expression panels such as “neuroinflammation”.

4. Impact

Nothing to report

5. Changes/Problems

- **Changes in approach and reasons for change**

We were scheduled to obtain EHD2-sc-mTNFR2 from the University of Stuttgart to complete these studies. However, their internal supply was contaminated with LPS and was therefore not suitable for *in vitro* or *in vivo* studies. Therefore, our aim to examine the role of TNFR2 signaling on microglia gene expression during CCI (Aim 1) is facing technical challenges stemming from the collection of low amounts of RNA from purified microglia. That aim has always been focused on analyzing more particularly the process of neuroinflammation and we think it will be more practical to use the NanoString nCounter technology to accomplish that goal. The amount of RNA needed is lower and specific, more restricted gene panels can be examined. We still plan on performing RNA sequencing but on total brain extracts from mice that have TNFR2 expression disrupted in the various neuron populations we are studying. We are proposing to perform NanoString on FACS purified microglia to specifically interrogate changes in neuroinflammatory gene expression in control and ARTOS treated mice. This is because, after several attempts RNA yield from FACS isolated microglia was too low for RNA sequencing. However, we can isolate enough for RNA for NanoString. We will still perform bulk RNAseq cortex and spinal cords of neuropathic mice with and without ARTOS therapy.

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

As stated previously a new murine TNFR2 agonist (ARTOS) had to be generated and purified. It took longer than expected to receive the purified material.

Dr Roman Fischer (expected to complete the work of Aim 3 at "site 2" (University of Stuttgart)) is now working for a German Biotech company. His work was supposed to focus on the characterization of human ARTOS and to evaluate its use for the treatment of neuropathic pain in humanized mice. We will be performing Aim 3 entirely at Drexel. We can provide letters of support demonstrating that we will receive human ARTOS and humanized TNFR2 mice. However, we would like to use the funds requested for Dr. Fischer to hire individual(s) to complete the studies proposed in Aim 3.

- **Changes that had a significant impact on expenditures**

We did not spend a lot on this project this year because we were waiting to receive purified murine ARTOS, the next generation TNFR2 agonist, which we received approximately 3 weeks ago. We have initiated studies with this compound and will be on track to complete this year's goals.

- **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.**

Reduced number of animals used in the first year because of the delay in obtaining murine ARTOS.

- **Significant changes in use of biohazards and/or select agents**

Nothing to report

6. Products

Nothing to report

7. Participants & Other Collaborating Organizations

Name	John R. Bethea

Project Role	Principal Investigator
Researcher Identifier (e.g. ORCID ID)	ORCID ID: 0000-0003-2078-5565
Nearest person month worked	3 months
Contribution to Project	Dr Bethea supervises the project and Ms Arnab. and helps analyze data
Funding Support	none other

Name	Sreejita Arnab
Project Role	Graduate Student
Researcher Identifier (e.g. ORCID ID)	N/A
Nearest person month worked	13 months
Contribution to Project	Sreejita performs the experiments, collects the data and analyzes them
Funding Support	none other

8. Special Reporting Requirements

QUAD CHARTS:

9. Appendices