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CONTRACTING ORGANIZATION: University of Texas Medical Branch at Galveston

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project is to rigorously evaluate if activating spinal GPR37 (G protein-coupled receptor 37) has a potential of treating and preventing pain chronification by erasing 'spinal pain memory' without posing a risk of abuse. We found that a single intrathecal (i.th.) injection of the putative GPR37 agonists TX14A and protectin D1 (PD1) erases hyperalgesic priming, a form of spinal pain memory, after an acute injury and thus prevent pain chronification upon a subsequent injury. We also found that repeated i.th. administrations of TX14A and PD1 gradually resolve chronic neuropathic pain over time while rapidly but temporarily inhibiting the pain after each injection. These results suggest that activating spinal GPR37 is a promising approach to prevent pain chronification upon recurring acute injury/pain episodes and facilitate the resolution of chronic pain with both rapid onset and long-term effects of pain relief.

15. SUBJECT TERMS

NONE LISTED

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1. Introduction

This project is to rigorously evaluate if activating spinal GPR37 (G protein-coupled receptor 37) has the potential to treat and prevent pain chronification by erasing 'spinal pain memory' without posing a risk of abuse. Specifically in this reporting period, the purpose of experiments is to determine: (1) if a single intrathecal administration of putative GPR37 agonists, either TX14A or protectin D1 (PD1), erases 'hyperalgesic priming', a form spinal pain memory, after an initial injury and thus reduces the risk for pain chronification upon a subsequent injury, and (2) if repeated injections of the agonists facilitate the resolution of chronic neuropathic pain.

2. Keywords

G protein-coupled receptor 37, Spinal pain memory, Pain hypersensitivity, Pain chronification, Pain resolution

3. Accomplishments

3.1. What were the major goals of the project?

The major goals and milestones of the project in this reporting period are:

(1) To examine if a single intrathecal (i.th) injection of GPR37 agonists (TX14A and PD1) erases 'hyperalgesic priming' after an initial injury, thereby preventing the development of more intense/longer than normal pain hypersensitivity upon a subsequent injury (a part of Major Task 2),

(2) To determine if repeated i.th. administrations of the GPR37 agonists facilitate the resolution of chronic neuropathic pain (Major Task 3), and

(3) To test methods to specifically detect GPR37 (for validation of the receptor knockdown in the next research period, a part of Major Task 4).

3.2. What was accomplished under these goals?

(1) Major activities

During this reporting period, we conducted experiments proposed to fulfill the major goals of this project, focusing on the specific objectives described below.

(2) Specific objectives

The specific objectives in this reporting period are:

- To complete acute pain model studies, based on the results we determine the optimum dose of each GPR37 agonist for the chronic pain model study,
- To test the effects of repeated administration of each GPR37 agonist on neuropathic mechanical allodynia, and
- To test the specificity of commercially available GPR37 antibodies.

(3) Significant results

To ensure robust and unbiased results, the experiments described below were performed by researchers blinded as to the experimental nature of animals except for sex and surgery. At least n=7 mice were used for each experimental group. TX14A was dissolved in saline; PD1 was reconstituted in 10% dimethyl sulfoxide (DMSO) after evaporating the packaging solvent in a chamber filled with 100% N_2. These GPR37 agonists (as a <10 μL single bolus) were intrathecally injected by a lumbar puncture (between L5 and L6 vertebrae) method.

- <u>The effects of TX14A and PD1 on hyperalgesic priming.</u>
  Hyperalgesic priming is a phenomenon in which the spinal nociceptive system forms pain memory after an acute injury (interleukin [IL]-6 in this study) and produces greater/longer than normal pain hypersensitivity upon a subsequent injury (prostaglandin E2 [PGE2] in this study). Note that PGE2-produced pain hypersensitivity normally resolves in 24 hours. However, after priming, the same dose of PGE2 produces pain hypersensitivity that persists 24 hours post-injection (see vehicle groups between unprimed and primed animals in **Figure 1-4**). When intrathecally given 2 days before the PGE2 intraplantar injection, the two putative GPR37 agonists TX14A and PD1 erased the hyperalgesic priming in a dose-dependent manner.

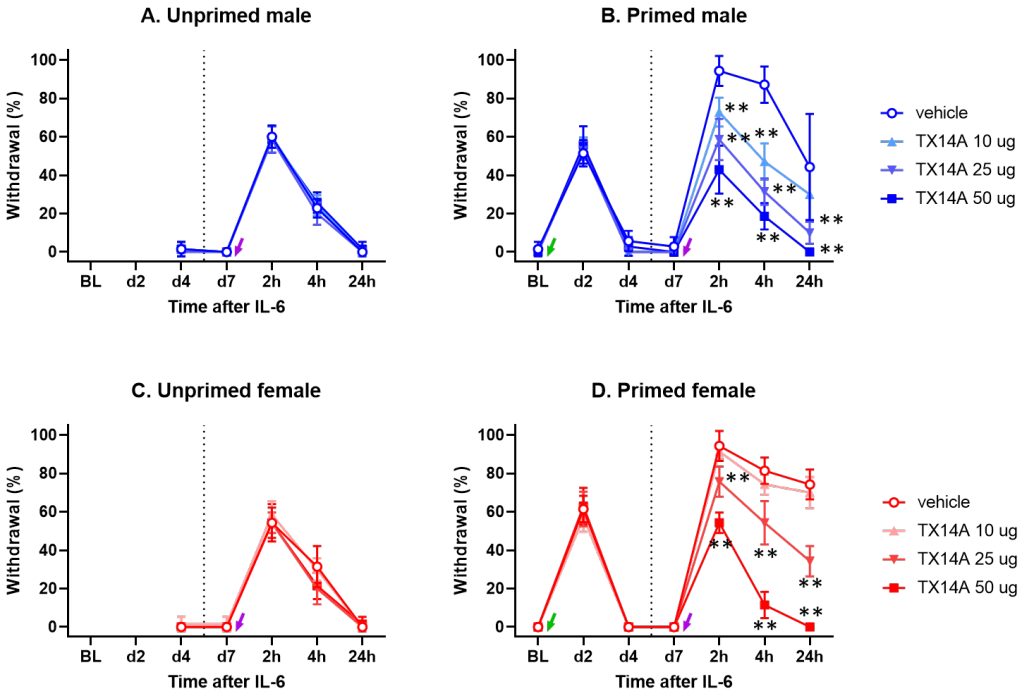

Figure 1. Intrathecally injected GPR37 agonist TX14A dose-dependently erases hyperalgesic priming for mechanical hypersensitivity. TX14A (broken line) was intrathecally injected under anesthesia 2 days before intraplantar PGE2 injection (purple arrow). While it did not affect PGE2-produced mechanical hypersensitivity in unprimed mice, it significantly inhibited the augmentation of the hypersensitivity in mice primed by intraplantar IL-6 (green arrow). Note that the lowest dose (10 ug) was effective in males but not in females, which aligns with our previous finding that TX14A was not as effective on capsaicin-induced secondary mechanical hypersensitivity in females as in males. Data are presented as mean±SD. Statistical comparisons were made by 2-way repeated measures ANOVA followed by Dunnett's t-test (i.e., vs. vehicle at each time point) in each sex. **p<0.01. BL, baseline.

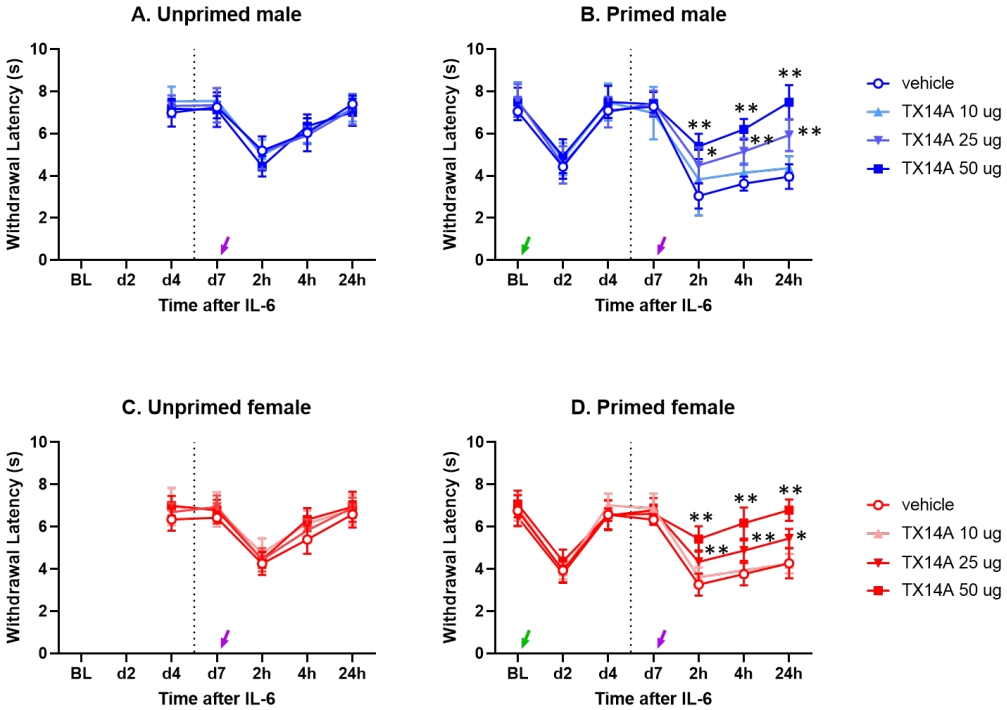

Figure 2. Intrathecally injected GPR37 agonist TX14A dose-dependently erases hyperalgesic priming for thermal hypersensitivity. TX14A (broken line) was intrathecally injected under anesthesia 2 days before PGE2 intraplantar injection (purple arrow). While it did not affect PGE2-produced thermal hypersensitivity in unprimed mice, it significantly inhibited the augmentation of the hypersensitivity in mice primed by intraplantar IL-6 (green arrow). Data are presented as mean±SD. Statistical comparisons were made by 2-way repeated measures ANOVA followed by Dunnett's t-test (i.e., vs. vehicle at each time point) in each sex. *p<0.05, **p<0.01. BL, baseline.

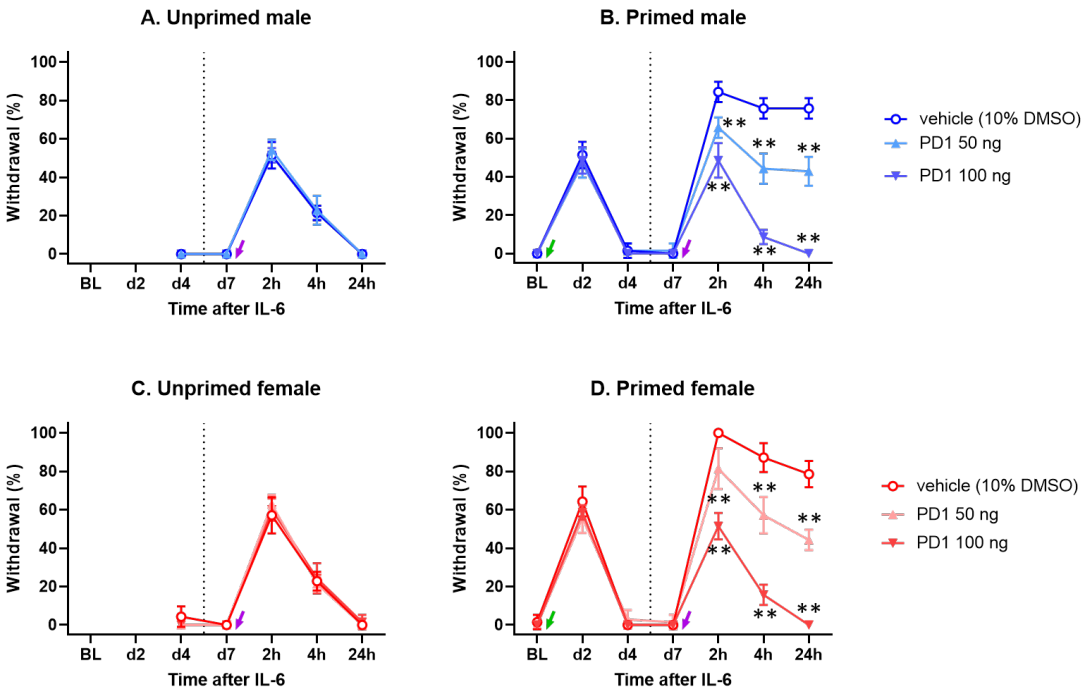

Figure 3. Intrathecally injected GPR37 agonist PD1 dose-dependently erases hyperalgesic priming for mechanical hypersensitivity. PD1 (broken line) was intrathecally injected under anesthesia 2 days before PGE2 intraplantar injection (purple arrow). While it did not affect PGE2-produced mechanical hypersensitivity in unprimed mice, it significantly inhibited the augmentation of the hypersensitivity in mice primed by intraplantar IL-6 (green arrow). Data are presented as mean±SD. Statistical comparisons were made by 2-way repeated measures ANOVA followed by Dunnett's t-test (i.e., vs. vehicle at each time point) in each sex. **p<0.01. BL, baseline.

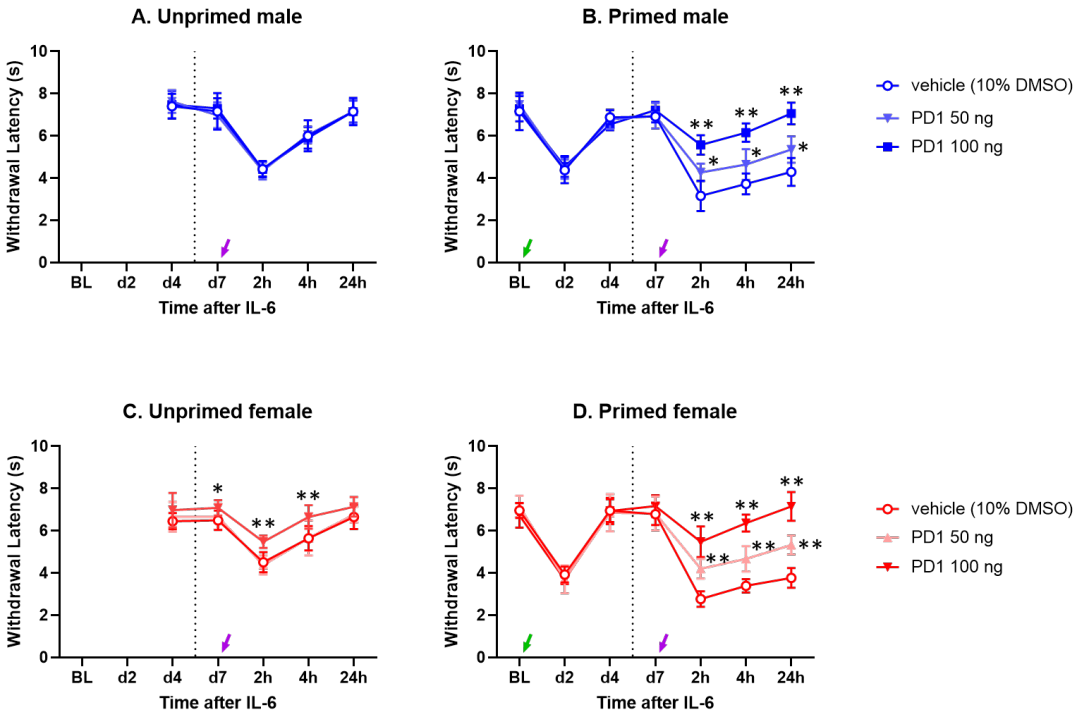

Figure 4. Intrathecally injected GPR37 agonist PD1 dose-dependently erases hyperalgesic priming for thermal hypersensitivity. PD1 (broken line) was intrathecally injected under anesthesia 2 days before PGE2 intraplantar injection (purple arrow). While it did not affect PGE2-produced thermal hypersensitivity in unprimed mice, it significantly inhibited the augmentation of the hypersensitivity in mice primed by intraplantar IL-6 (green arrow). Data are presented as mean±SD. Statistical comparisons were made by 2-way repeated measures ANOVA followed by Dunnett's t-test (i.e., vs. vehicle at each time point) in each sex. *p<0.05, **p<0.01. BL, baseline.

- <u>The effects of repeatedly administered TX14A and PD1 on neuropathic mechanical allodynia</u>

In neuropathic pain conditions, normally innocuous touch causes pain, which is termed mechanical allodynia. We produced traumatic peripheral neuropathy by ligating the L5 spinal nerve (spinal nerve ligation, SNL). This animal model rapidly develops mechanical allodynia after the injury, which persists for weeks. When we repeatedly administered the highest dose of TX14A (50 ug) and PD1 (100 ng) intrathecally at 2-3 days intervals (once daily) up to 6 times, two remarkable effects were observed. First, neuropathic mechanical allodynia was gradually resolved (**Figures 5 & 6**). By day 31 post-SNL, the mice treated with either TX14A or PD1 showed significantly reduced nocifensive withdrawals from von Frey filament probing, which was close to the level of their baseline response.

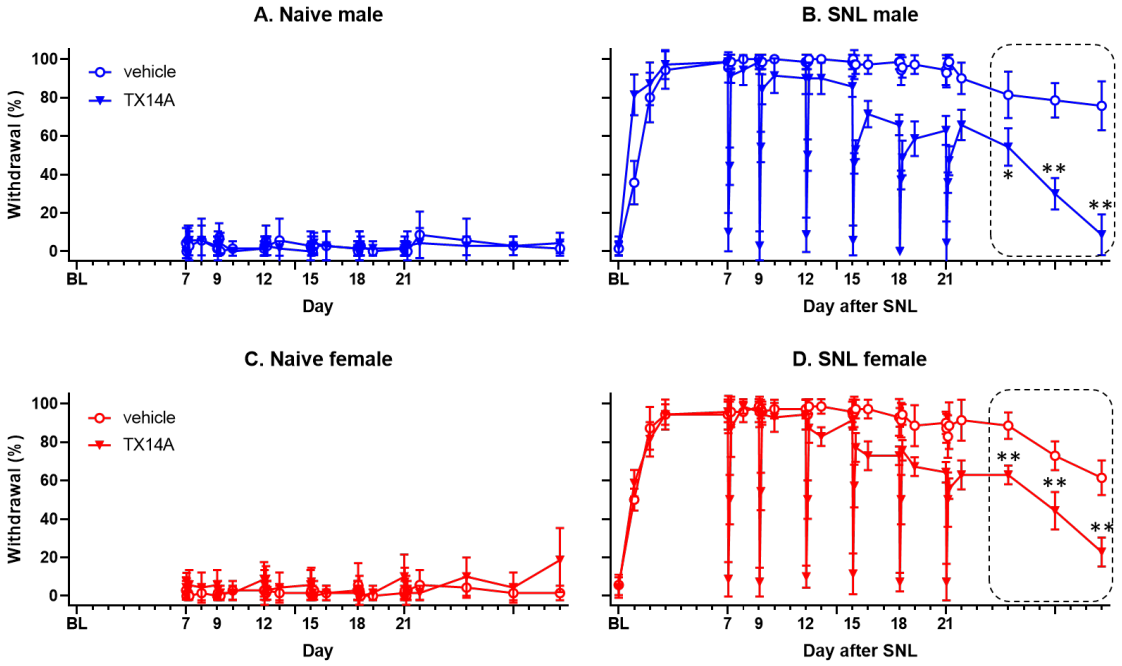

Figure 5. Repeated intrathecal injection of the GPR37 agonist TX14A facilitates the resolution of neuropathic mechanical allodynia. TX14A or saline was repeatedly administered intrathecally on post-SNL days 7, 9, 12, 15, 18, and 21. Each injection produced a significant inhibition of mechanical allodynia on the day of treatment. Notably, a gradual reduction of the allodynia occurred during the 2-week treatment period, which continued afterward without further treatments. Data are presented as mean±SD. Statistical comparisons were made by 2-way repeated measures ANOVA followed by Sidak test in each sex, focusing on the late phase of the gradual resolution on days 25, 28, and 31 post-SNL (broken line box). *p<0.05, **p<0.01. BL, baseline. Note that naïve mice did not undergo SNL, and thus their BL is Day 7.

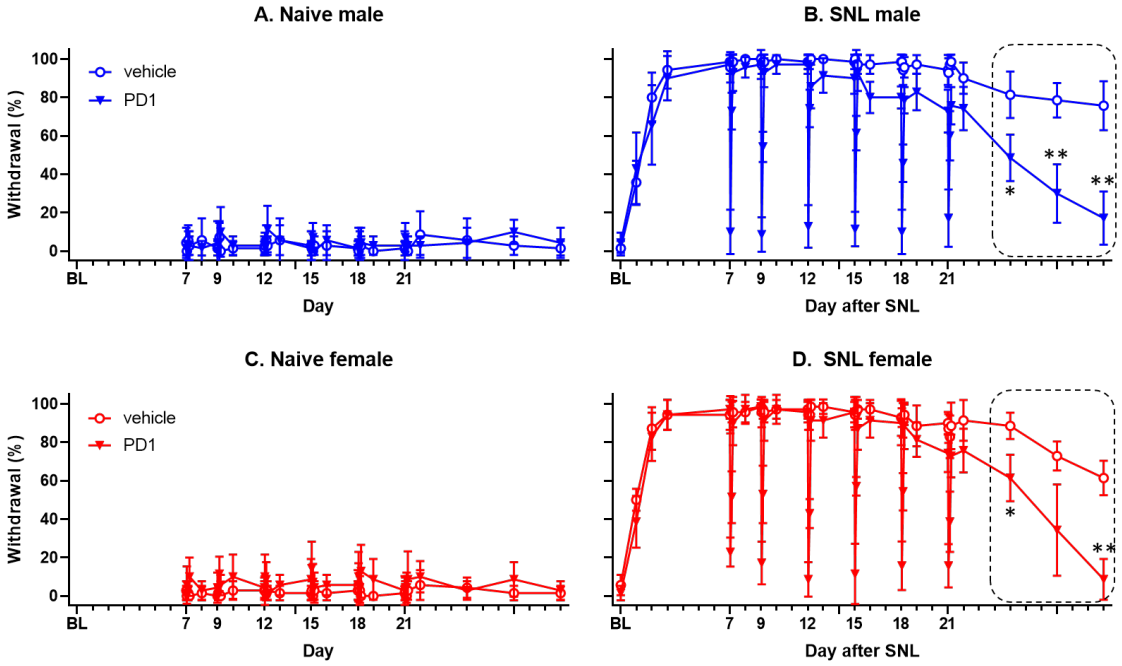

Figure 6. Repeated intrathecal injection of the GPR37 agonist PD1 facilitates the resolution of neuropathic mechanical allodynia. PD1 was repeatedly administered intrathecally on post-SNL days 7, 9, 12, 15, 18, and 21. Each injection produced a significant inhibition of mechanical allodynia on the day of treatment. Notably, a gradual reduction of the allodynia occurred during the 2-week treatment period, which continued afterward without further treatments. Data are presented as mean±SD. Statistical comparisons were made by 2-way repeated measures ANOVA followed by Sidak test in each sex, focusing on the late phase of the gradual resolution on days 25, 28, and 31 post-SNL (broken line box). *p<0.05, **p<0.01. BL, baseline. Note that naïve mice did not undergo SNL, and thus their BL is Day 7.

Second, after each injection, there was a significant alleviation of neuropathic mechanical allodynia on the day of treatment. This effect was of a rapid onset and wore off in 5 hours (**Figure 7**). There was no sign of tolerance development upon the repeated treatments of TX14A or PD1. Importantly, the magnitudes of the rapid effect did not change during the 2-week treatment period. Collectively, these results indicate that the putative GPR37 agonists produce both rapid and long-term pain relief.

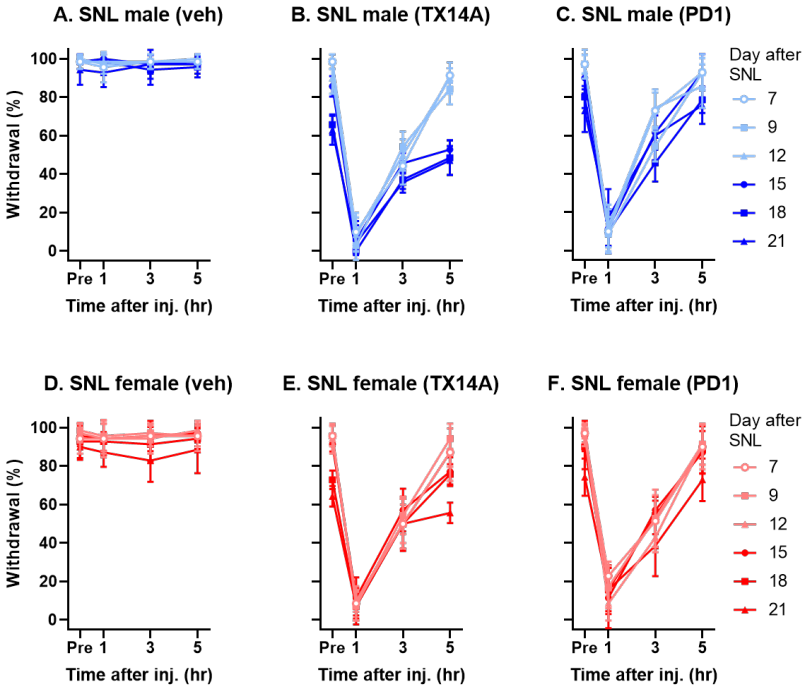

Figure 7. Repeated intrathecal injection of the GPR37 agonists TX14A and PD1 produce a rapid onset relief of neuropathic mechanical allodynia. Either TX14A or PD1 was repeatedly administered intrathecally on post-SNL days 7, 9, 12, 15, 18, and 21. Each injection produced a significant inhibition of mechanical allodynia on the day of treatment. This effect wore off in 5 hours after the injection. No obvious sign of tolerance development was observed during the 2-week treatment period. Pre, pre-drug levels of mechanical allodynia.

- <u>The specificity of commercially available GPR37 antibodies</u>

  In the next research phase, we will knock down spinal GPR37 expression by RNA interference. To validate the knockdown, we will need to quantify the expression levels of GPR37 in the spinal cord. Based on the literature, we tested multiple GPR37 antibodies using GPR37 global knockout (KO) tissue samples as negative control (**Figure 8**). Out of five products, only one (from Cell Signaling D4C8H) showed target-specific but diffuse bands (~45 kDa).

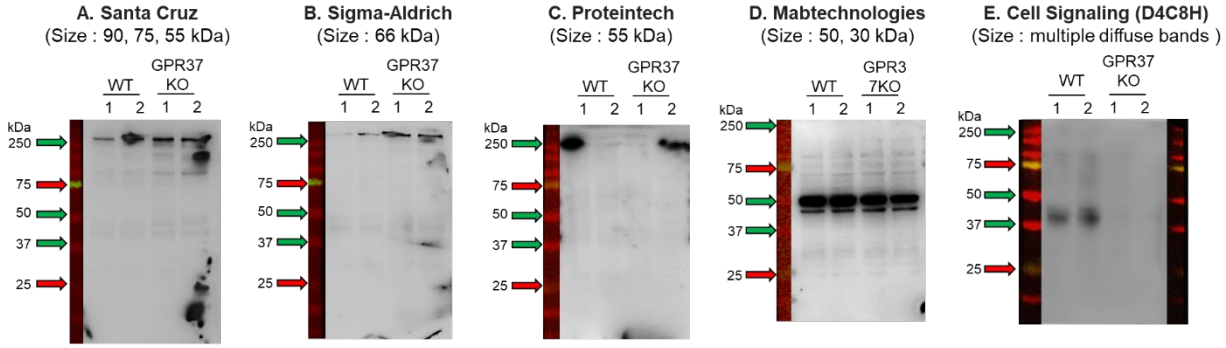

Figure 8. The specificity of commercially available GPR37 antibodies. Out of five tested products, only the Cell Signaling antibody showed specific but diffuse bands (~45 kDa). This product will be used for the validation of our GPR37 knockdown approach in future studies.

- The abuse liability of GPR37 agonism
  While it is planned to be performed in Year 4, we went ahead and collected some preliminary data regarding the abuse liability of GPR37 agonism at the brain level (Major Task 9). Mice received TX14A (50 ug) in the lateral ventricle and subjected to the conditioned place preference tests (3 days of conditioning) with morphine (1 ug) as a reference drug of high abuse liability. The preliminary results suggest that TX14A at the brain level had little to no abuse liability (**Figure 9**).

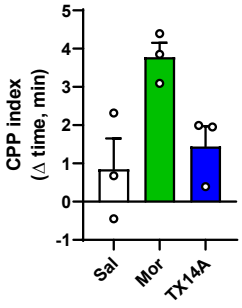

Figure 9. The abuse liability of GPR37 agonism at the brail level. Compared with morphine (Mor)-treated mice, TX14A-treated mice did not develop conditioned place preference (CPP) for the chamber associated with the drug treatment. Saline (Sal)-treated mice received saline in both chambers. A CPP index in the positive direction indicates that the mouse spent more time in the drug-associated chamber after the conditioning than before, which suggests that the mouse had a rewarding experience in the chamber during the conditioning.

(4) Conclusions
The results of experiments conducted in this reporting period demonstrate that the putative GPR37 agonists TX14A and PD1 erase 'hyperalgesic priming' which contributes to pain chronification upon recurring acute injury/pain episodes. Furthermore, the results of repeated administrations of GPR37 agonists show that this therapeutic approach gradually resolves an established chronic neuropathic pain state over time while also providing rapid-onset pain relief after each administration.

3.3. What opportunities for training and professional development has the project provided?
A graduate student (Regan Hammond) joined this project and had the following training opportunities:
(1) Behavioral study training: Regan has been trained to perform the von Frey filament assay and operant behavior tests such as the conditioned place preference test and the conflict avoidance test.
(2) Surgical procedure training: Regan received training for basic aseptic rodent surgery and spinal nerve ligation surgery.
(3) Drug administration training: Regan received training for giving drugs to rodents via various routes such as intraperitoneal, intrathecal, and intraplantar routes.
(4) Western blot training: Regan received training to collect the spinal cord and dorsal root ganglia samples for protein quantification.
(5) Presentations: Regan had opportunities to present her project progress at different meetings (lab meetings, department research forum, scientific session at the department retreat, etc.) to develop visual and oral communication skills.
(6) Participation in this progress report preparation: As a part of training for a grant application, Regan had the opportunity to prepare figures for this progress report and review/revise the contents.

In addition, all research personnel in this project (Dr. Ramesh Pariyar, Dr. Ho Koo, and Regan Hammond) received virtual training for advanced statistical analysis (e.g., generalized linear mixed model analysis) with SPSS.

3.4. How were the results disseminated to communities of interest?
Nothing to report.

3.5. What do you plan to do during the next reporting period to accomplish the goals?
In the next reporting period, we will complete the experiments testing the effects of the GPR37 agonists on the affective-motivational domain of neuropathic pain (a part of Major Task 3). Using the Cell Signaling GPR37 antibody, we will validate GPR37 knockout and determine if the observed effects of TX14A and

PD1 in acute pain models are mediated by GPR37 or GPR37L1 (Major Tasks 4 & 5). We expect to collect some data from GPR37 KO mice (Major Task 6).

4. Impact

4.1. What was the impact on the development of the principal discipline(s) of the project?
Nothing to report.

4.2. What was the impact on other disciplines?
Nothing to report.

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

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

5. Changes/Problems

(1) The affective-motivational domain of neuropathic pain was originally proposed to be assessed in the conflict avoidance (CA) paradigm. Regan discovered that this behavioral paradigm needs refinement and adjustment. First, the degree of conflict (the balance between natural place preference and mechanical stimulation through bristles on the floor) needs to be optimized by either reducing the natural place preference or increasing the stimulation intensity. Because there is individual variability in the degree of natural place preference, we are currently focusing on changing the intensity of mechanical stimulation. Second, we are considering using a similar but different behavior paradigm – the conditioned place avoidance (CPA) test. In the CPA test, mice will experience unavoidable mechanical stimulation in their naturally preferred chamber for 3 days of conditioning (i.e., confined for 20-30 min in the chamber of the bristle floor). Mice with unresolved neuropathic mechanical allodynia are expected to experience greater discomfort from the stimulation than those with resolved allodynia, avoiding the chamber later when they are allowed to freely choose where to go. The UTMB IACUC approved this procedure, and we are currently awaiting ACURO review.

(2) We are also considering using the GPR37 global KO mice for Major Task 5. We originally proposed to use 2 different types of siRNAs to knockdown (KD) GPR37. Instead, we may use one siRNA together with GPR37 KO mice (i.e., one KD and one KO). Based on our preliminary data, we expect that siRNA-based GPR37 KD will work and the Cell Signaling antibody will validate the KD. However, now we are open to the possibility that the intrathecal siRNA-based KD might not be detectable/validated in the spinal cord (based on personal communication with Dr. Temugin Berta [PMID: 37557960]). In this case, we will solely use GPR37 KO mice for the task (WT vs. heterozygous vs. homozygous). For the same reason, we might develop GPR37L1 KO mice rather than using two different GPR37L1 siRNAs.

6. Products

Nothing to report.

7. Participants & Other collaborating organizations

7.1. What individuals have worked on the project?

Name:	Jun-Ho La
Project Role:	PI
Researcher Identifier (e.g., ORCID ID):	ORCID 0000-0003-4306-0305
Nearest person month worked:	2
Contribution to Project:	Dr. La supervised all research activities in this project.

Name:	Jin Mo Chung
Project Role:	Co-I
Researcher Identifier (e.g., ORCID ID):	ORCID 0000-0003-2601-1720

Nearest person month worked:	1
Contribution to Project:	Dr. Chung supervised the progress of the project and provided an administrative support as a department Chair.

Name:	Jigong Wang
Project Role:	Research Scientist
Researcher Identifier (e.g., ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Dr. Wang performed behavior experiments and coordinated all lab activities (purchasing animals, supplies, etc.) as a lab manager.

Name:	Ramesh Pariyar
Project Role:	Research Scientist
Researcher Identifier (e.g., ORCID ID):	
Nearest person month worked:	8
Contribution to Project:	Dr. Pariyar performed behavior experiments and mouse colony management together with Dr. Koo. He also performed western blot experiments.

Name:	Ho Koo
Project Role:	Research Scientist
Researcher Identifier (e.g., ORCID ID):	
Nearest person month worked:	8
Contribution to Project:	Dr. Koo performed behavior experiments and mouse colony management together with Dr. Pariyar. He also performed the abuse liability test.

Name:	Regan Hammond
Project Role:	Graduate Student
Researcher Identifier (e.g., ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Regan performed behavior experiments. She also helped collecting biological samples for western blot.

Name:	Ye-Eun Oh
Project Role:	Research Assistant (part-time)
Researcher Identifier (e.g., ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Ye-Eun assisted all experiments in the lab.

7.2. 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.

7.3. What other organizations were involved as partners?
Nothing to report.

8. Special reporting requirements

8.1. Quad Chart: an updated Quad Chart is attached to this report