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TITLE: Exploration of a Novel Persistent Reversal of Pathological Pain: Mechanisms and Mediators

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<b>14. ABSTRACT</b> Neuropathic pain, resulting from nerve injury or inflammation, affects approximately 4 million people in the USA alone (1) and remains poorly managed by currently available therapeutics. Most of these therapeutics specifically target neurons. However, it is now known that spinal glia (astrocytes and microglia) play an important role in facilitating and maintaining neuropathic pain in animal models (2). We have identified a novel therapeutic target in adenosine 2A receptors that modulate the immune cells within the CNS such that they switch from a classically pro-inflammatory state to an alternatively activated IL-10 generating state. The behavioral outcome of such a phenotypic switch results in a reversal of allodynia induced by neuropathic injury in rats for at least 4 wks from a SINGLE bolus administration. The purpose of this grant is to provide further evidence that this remarkable therapeutic effect can be translated to numerous animal models of neuropathic pain and to elucidate the underlying mechanisms that result in the production of IL-10 and subsequent reversal of the allodynia.					
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**\*We have submitted a revised statement of work that has been approved for a one year no-cost extension by the DoD. Tasks 8, 10, 11, 12 will be completed during this no-cost extension period as outlined in the most current approved statement of work.**

## **Introduction**

Neuropathic pain, resulting from nerve injury or inflammation, affects approximately 4 million people in the USA alone (1) and remains poorly managed by currently available therapeutics. Most of these therapeutics specifically target neurons. However, it is now known that spinal glia (astrocytes and microglia) play an important role in facilitating and maintaining neuropathic pain in animal models (2). We have identified a novel therapeutic target in adenosine 2<sub>A</sub> receptors that modulate the immune cells within the CNS such that they switch from a classically pro-inflammatory state to an alternatively activated IL-10 generating state. The behavioral outcome of such a phenotypic switch results in a reversal of allodynia induced by neuropathic injury in rats for at least 4 wks from a SINGLE bolus administration. The purpose of this grant is to provide further evidence that this remarkable therapeutic effect can be translated to numerous animal models of neuropathic pain and to elucidate the underlying mechanisms that result in the production of IL-10 and subsequent reversal of the allodynia.

## **Body**

**Task 1. Obtain approval from the University of Colorado Institutional Animal Care & Use Committee (IACUC) for all animal work in the proposal (Timeframe: 2-4 months).**

Task 1 has been completed and animal research has been conducted.

**Milestone 1:** Animal protocol is approved to allow funding to be received on or before January 1, 2011, and to allow the project to start.

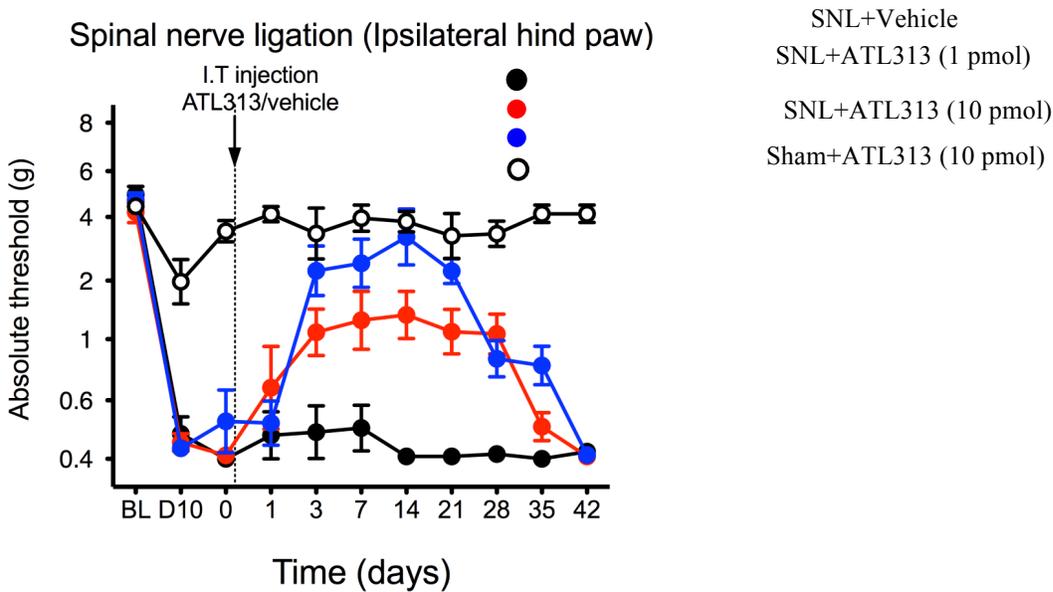
Milestone 1 has been completed.

**Task 2. Aim IA1. Spinal Nerve Ligation (SNL): reversal of acute and chronic traumatic neuropathic pain by intrathecal (IT) ATL313.**

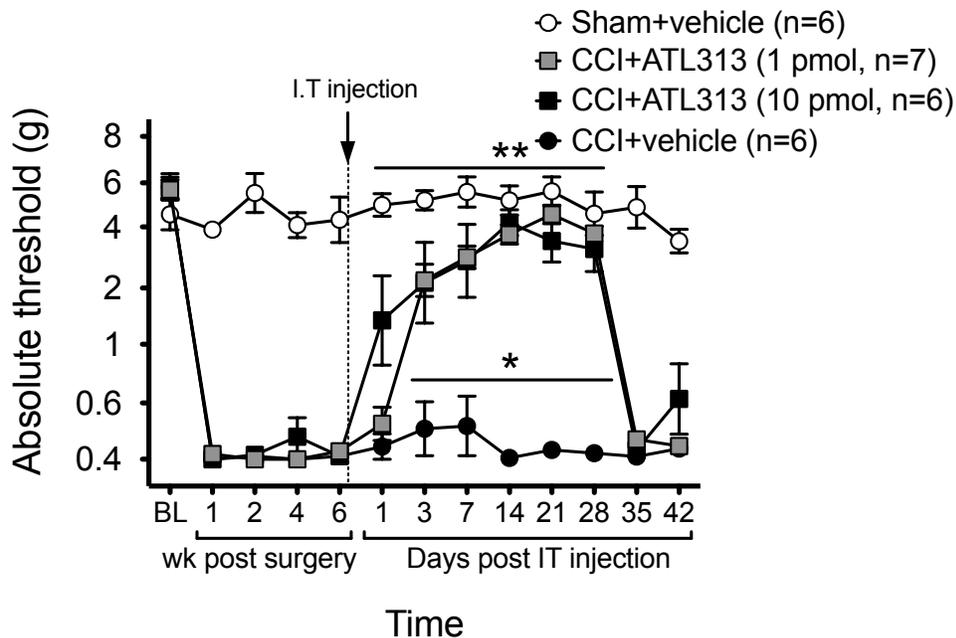
Task 2 has been completed.

We have shown that a single intrathecal administration of ATL313 2 wk after spinal nerve ligation is able to reverse the neuropathic allodynia as evident by Figure 1 below. We have tested 2 doses of ATL313 and found that a higher dose (10 pmol) than that required for chronic constriction injury (1 pmol) reverses the allodynia for 4 wk. We also proposed to evaluate ATL313 administration several weeks after neuropathic pain had been established in the spinal nerve ligation model; however, given evidence of increased duration of action of ATL313 in the chronic constriction injury model relative to the spinal nerve ligation model, we have completed the proposed experiment using the chronic constriction injury model. The results show that ATL313 is as effective in established neuropathic pain as it is in acute neuropathic pain, as

presented in Figure 2 below. These data show that the enduring effects of ATL313 on pain reversal are consistent across different pain models and that ATL313 is equally effective when administered shortly after the induction of pain as well as when administered many weeks after the onset of chronic neuropathic pain. These data are particularly clinically relevant, as many pain patients do not seek medical interventions until pain has endured for weeks or even months.



**Figure 1.** Spinal nerve ligation injury was induced at the L5 spinal level. Two weeks after surgery, a single intrathecal dose of ATL313 (0.5 ng or 5 ng) or vehicle was given. Mechanical allodynia was tested on the ipsilateral hind paw before surgery, before and after intrathecal drug delivery, and for 6 wks post-injection. ATL313 reversed the allodynia induced by chronic constriction injury from 3-28 days after drug administration ( $P < 0.05$ , 2-way-repeated measures ANOVA).



**Figure 2.** Chronic constriction injury was induced in the left sciatic nerve at the level of the mid thigh. 6 weeks after surgery, a single intrathecal dose of ATL313 (1 pmol or 10 pmol) or vehicle was given. Mechanical allodynia was tested on the ipsilateral hind paw before surgery, before and after intrathecal drug delivery and for 6 wks post-injection. ATL313 significantly reversed the allodynia induced by chronic constriction injury from 3-28 days after drug administration ( $P < 0.05$ , 2-way-repeated measures ANOVA).

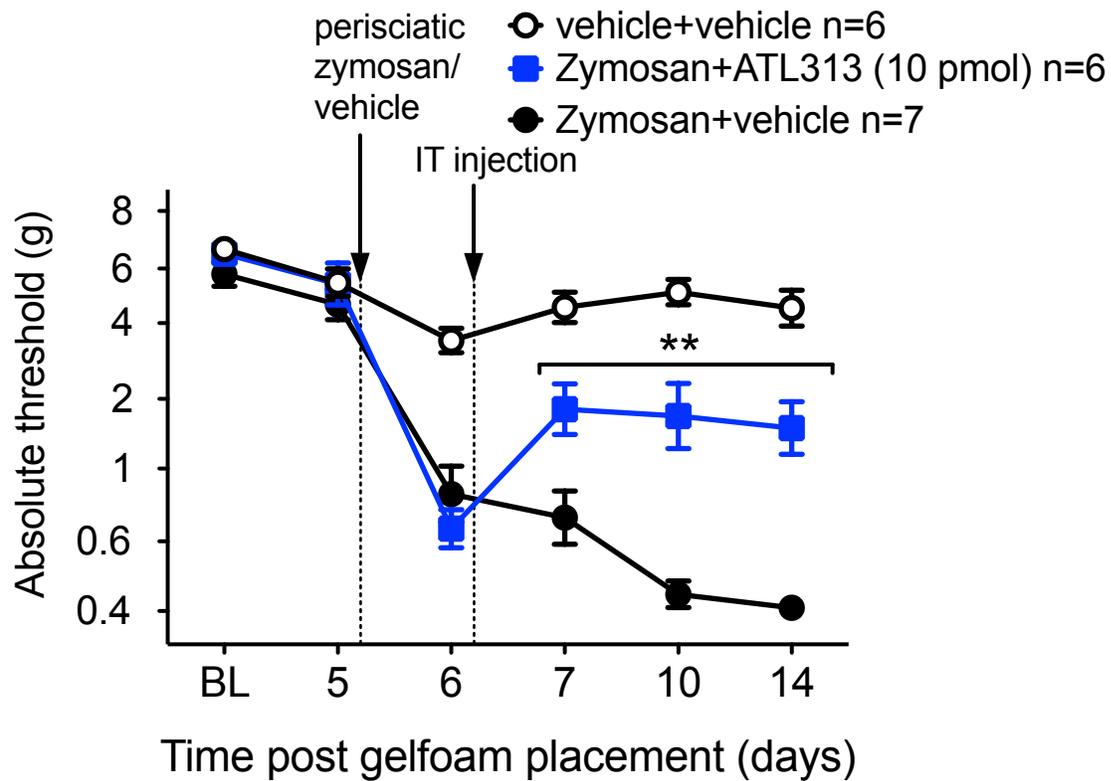
**Milestone 2:** Definition of optimal IT ATL313 dose for use in tasks 7, 9, and 11. Completed analysis of the ability of a *single* IT ATL313 dose to reverse neuropathic pain from traumatic peripheral neuropathy.

Milestone 2 is complete. We determined that 1 pmol ATL313 is optimal for tasks 7, 9, and 11. A single IT dose of ATL313 reverses neuropathic pain from traumatic peripheral nerve injury in both the spinal nerve ligation (SNL) and chronic constriction injury (CCI) pain models for ~ 4 wk.

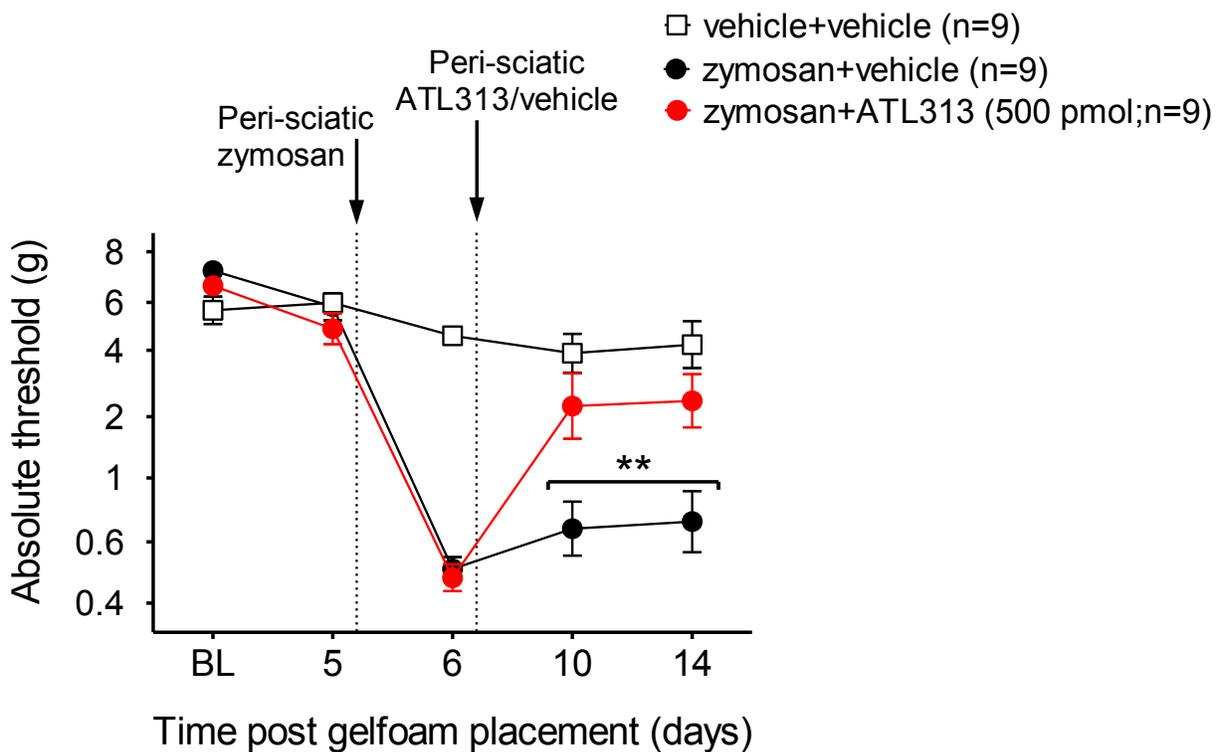
**Task 3. Aim IB1. Sciatic Inflammatory Neuropathy (SIN): reversal of inflammatory neuropathic pain by IT vs. peri-sciatic nerve ATL313.**

Task 3 has been completed.

Figures 3 and 4 below show reversal of SIN induced allodynia when ATL313 is delivered either peri-sciatically or intrathecally 24 h after the first dose of zymosan. We have established that chronic allodynia can be maintained by suspending the zymosan in saline as opposed to the conventional incomplete Freund's adjuvant, and all further studies will use saline. These data show the ability of ATL313 to reverse pain not only in a different peripheral pain model, but also its ability to reverse pain using different administration routes (intrathecal and peri-sciatic). Again the clinical relevance of this important in that every pain patient is different and may not be able to or want to use certain routes of administration, and thus being able to use different routes and get the same efficacy is desirable.



**Figure 3.** Gel foam was placed perisciatically to allow for zymosan delivery around the sciatic nerve at the mid thigh level. The rats were allowed to recover for 5 days from the gel foam placement surgery before zymosan was delivered. 5 days after surgery, 160  $\mu\text{g}$  zymosan in 50  $\mu\text{l}$  incomplete Freund's adjuvant was delivered through the catheter to the gel foam surrounding the sciatic nerve. Mechanical thresholds of the ipsilateral hind paw were tested before surgery, before zymosan delivery and 24 h after zymosan delivery. In rats displaying allodynia from the zymosan, rats were injected intrathecally with ATL313 or vehicle. Zymosan administration was continued every alternate day in order to maintain mechanical allodynia for 8 days. Intrathecal ATL313 significantly reversed the mechanical allodynia induced by zymosan for the duration of the experiment ( $P < 0.05$ , 2-way repeated measures ANOVA).

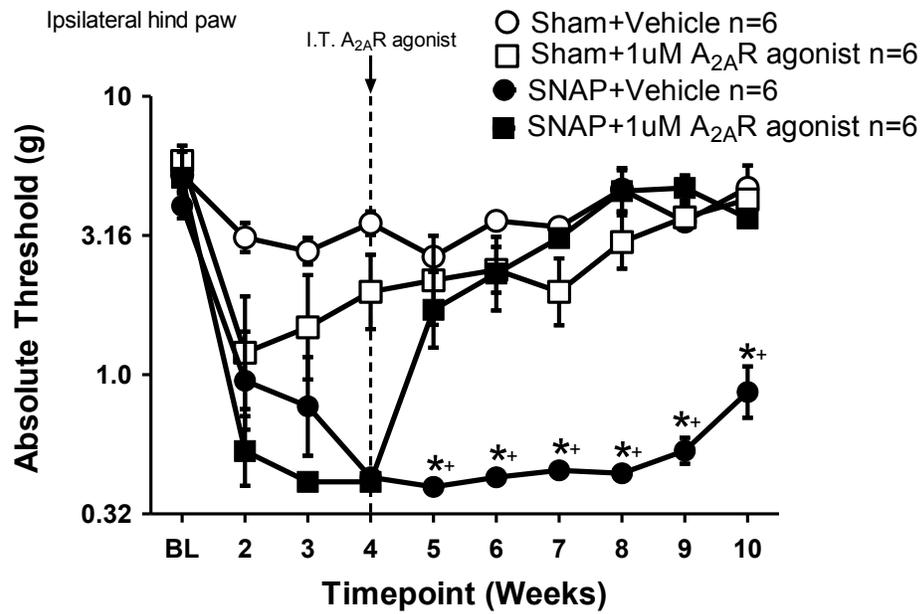


**Figure 4.** Baseline measures on the von Frey test for mechanical allodynia were performed before surgery. Sciatic inflammatory neuropathy (SIN) surgery was performed and rats were allowed to recover for five days. At five days post-surgery, following von Frey testing, zymosan (3.2 ug/ul) or saline vehicle was injected peri-sciatically. Twenty four hours later, again following von Frey testing, ATL313 (500 pmol) or DMSO vehicle was injected peri-sciatically. Peri-sciatic zymosan or saline vehicle was administered every other day for 7 days in order to maintain allodynia. Rats were tested on von Frey for mechanical allodynia at 10 and 14 days post-surgery. Peri-sciatic ATL313 significantly reversed zymosan-induced mechanical allodynia ( $P < 0.0001$ , 2-way repeated measures ANOVA).

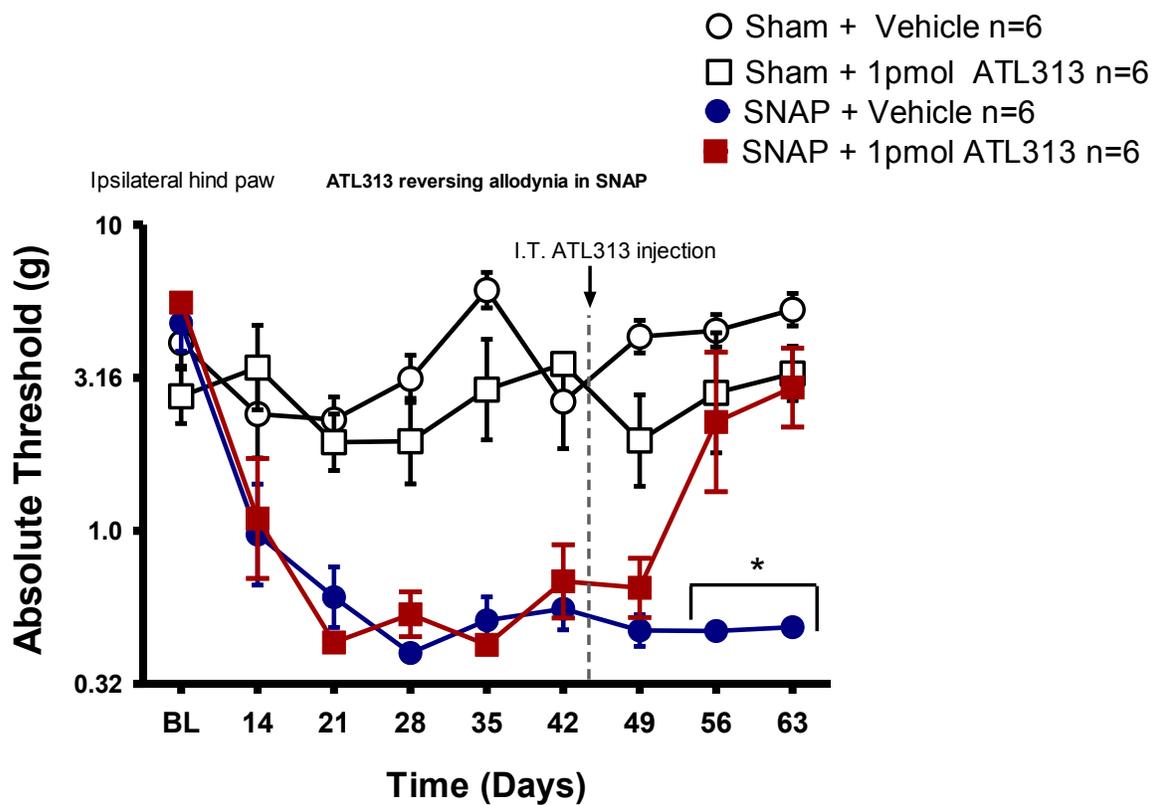
**Task 4. Aim IB2. Spinal Cord Injury (SCI): reversal of acute and chronic central neuropathic pain by IT ATL313.**

Task 4 has been completed.

We have shown that a single IT administration of an  $A_{2A}R$  agonist (1 uM) 4 wk and 7 wk after T13/L1 spinal avulsion injury, what we have termed spinal neuropathic avulsion pain (SNAP), is able to completely reverse neuropathic allodynia as evident by Figures 5 and 6 below. What is remarkable about this is that SNAP spinal cord injury (SCI) is a *central* neuropathic pain model, whereas all of the previous tasks in this grant were on peripheral neuropathic pain models. Neuropathic pain can be from central or peripheral origin, or both, so it is important to develop treatments that are effective in both types of neuropathic pain. These data are also interesting in that  $A_{2A}R$  agonism still reverses *established* central neuropathic pain (7 wks of robust, stable allodynia), which is again clinically important since neuropathic pain patients often do not seek treatment until after they have had the pain for weeks to months.



**Figure 5.** Unilateral T13/L1 avulsion induces mechanical allodynia as assessed by von Frey testing. An A<sub>2A</sub>R agonist given as a single IT injection at 4 wk post-surgery reverses SCI-induced mechanical allodynia for at least 6 wk after administration ( $p < 0.05$ ). A<sub>2A</sub>R agonist had no effect on sham-operated rats. Data are presented as mean  $\pm$  SEM and analyzed using two-way repeated measures ANOVA. \* $p < 0.05$  SCI plus Vehicle compared to SCI plus A<sub>2A</sub>R agonist; + $p < 0.05$  SCI plus vehicle compared to Sham plus vehicle and Sham plus A<sub>2A</sub>R agonist.



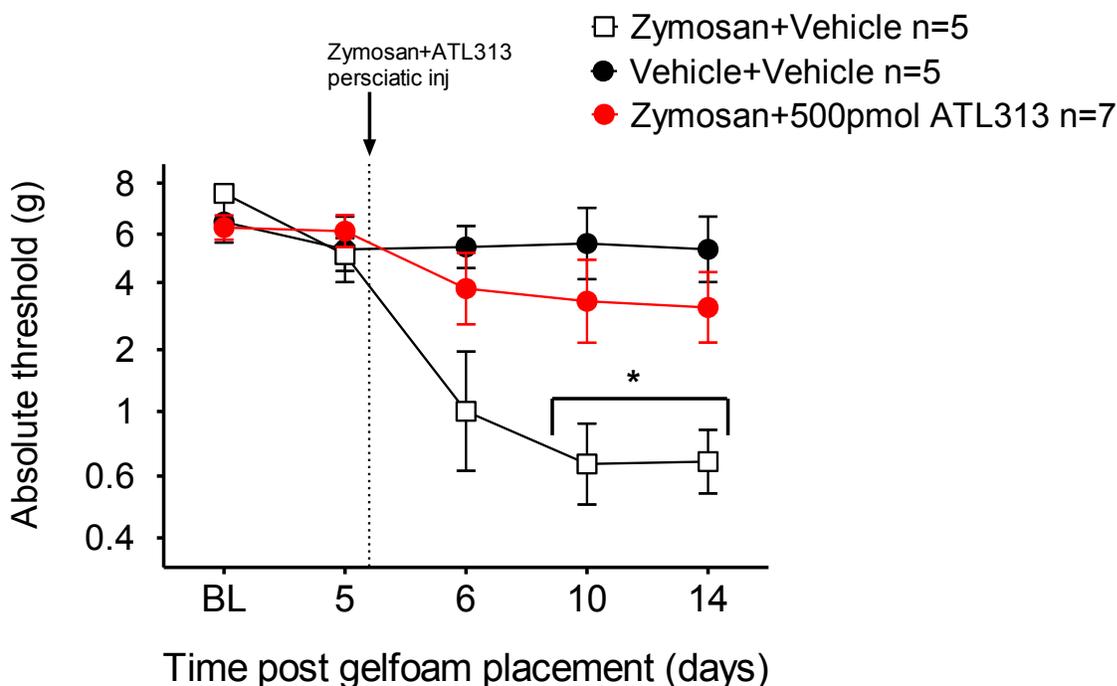
**Figure 6.** Unilateral T13/L1 avulsion SNAP or sham surgery with dura suture was performed and rats were allowed to recover for two weeks. Mechanical thresholds of the ipsilateral hind paw were tested before surgery. At seven weeks post-surgery, a single bolus injection of 1 pmol ATL313 or DMSO vehicle was administered intrathecally. Beginning two weeks post-surgery (one week post-ATL313 administration), rats were tested for mechanical allodynia weekly for nine weeks, at which point allodynia from the SNAP surgery is resolved. Intrathecal ATL313 significantly reversed the mechanical allodynia induced by SNAP surgery ( $P < 0.0001$ , 2-way-repeated measures ANOVA).

**Task 5. AimIB3i. SIN: Prevention of inflammatory neuropathic pain by IT vs. peri-sciatic ATL313.**

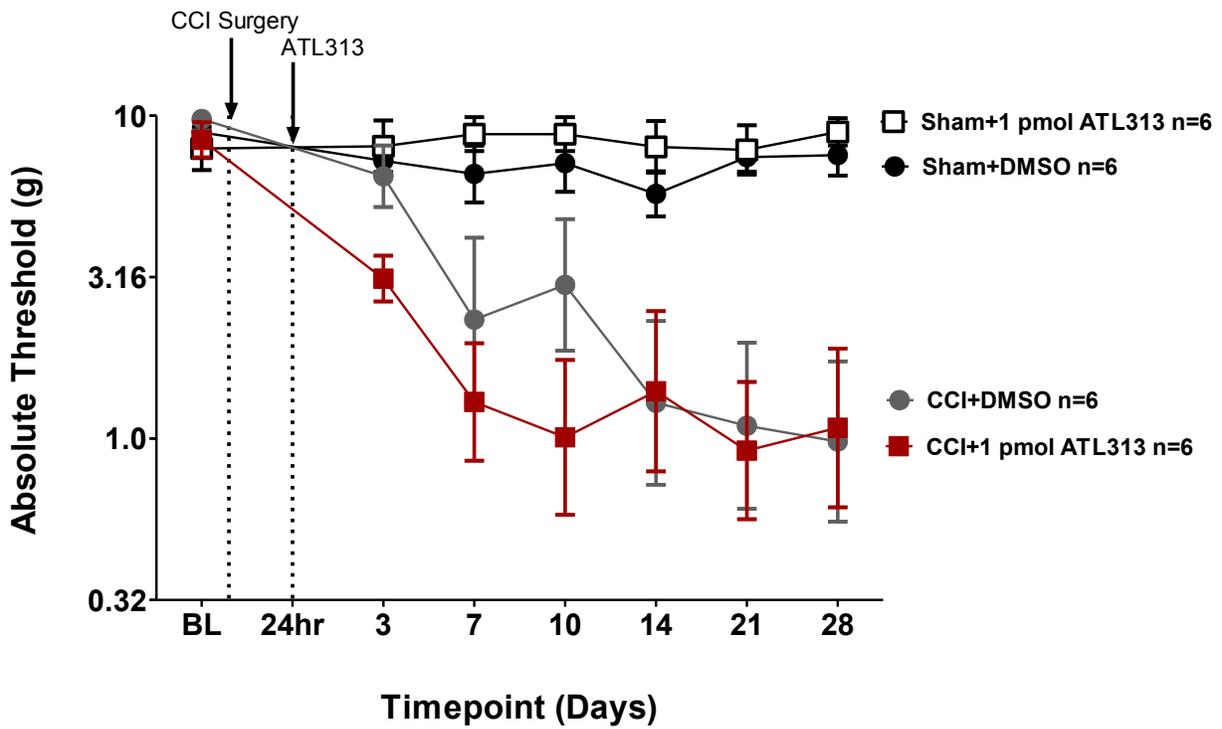
Task 5 has been completed.

We know from Task 3 that ATL313 reverses zymosan-induced allodynia when it is administered both intrathecally and peri-sciatically. Here we co-administered zymosan and ATL313 on the same day to see if it would prevent the induction of allodynia. Our hypothesis was that neither peri-sciatic nor IT ATL313 would prevent neuropathic pain since we believe that A2AR agonists are only able to exert their anti-inflammatory effects if administered into a chronic proinflammatory environment (we know that ATL313 has no effect on sham operated rats). Figure 7 below shows that peri-sciatically administered ATL313 significantly prevents zymosan-induced allodynia compared to vehicle controls, which was the opposite of what we had predicted. We think that this result is explained by the nature of the SIN model paradigm we

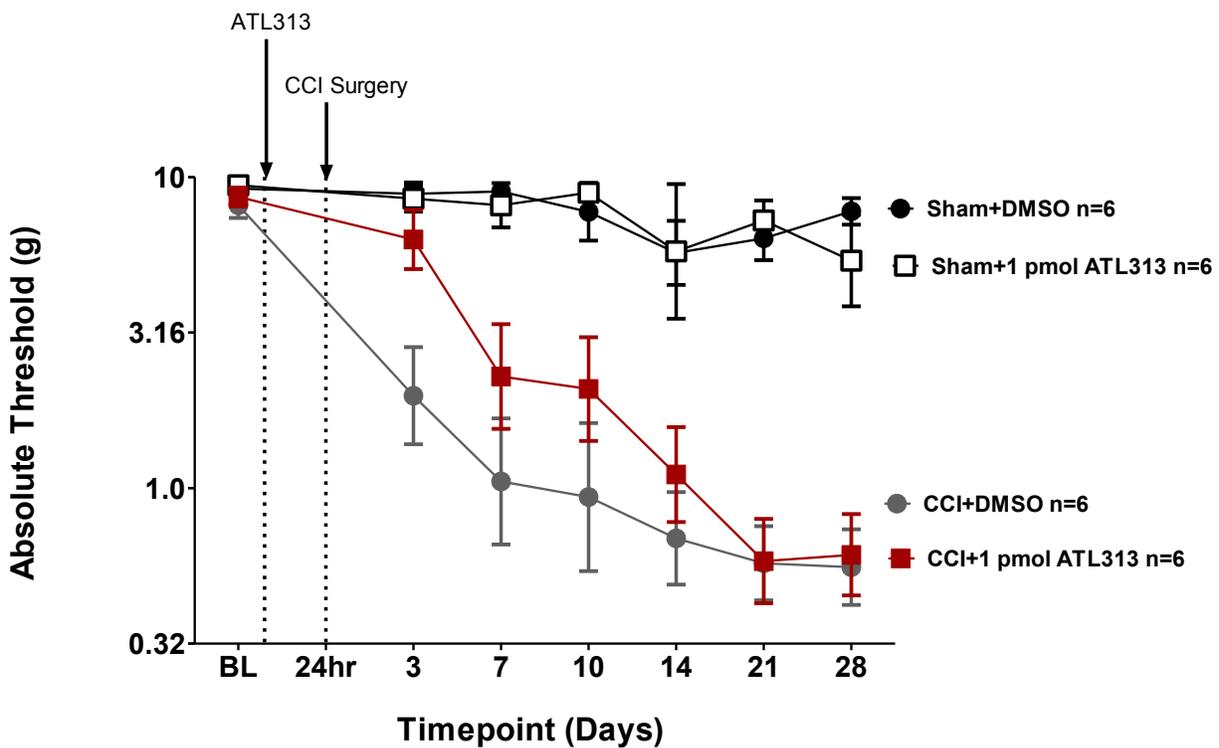
used here. We co-administered the peri-sciatic zymosan and ATL313 5 days after the sciatic nerve gel foam wraps were implanted. Although the rats were not allodynic during the behavioral test on Day 5 prior to the injection, this does not rule out inflammation at the site of the gel foam sciatic nerve wrap. Gazda et. al (3) show that there is a significant recruitment of inflammatory cells to the gel foam wraps 3 and 24 hr after implantation. Since we injected the ATL313 peri-sciatically, we believe that perhaps implanting the gel foam sciatic wraps on their own was enough to induce a chronic proinflammatory environment for ATL313 to be able to exert its anti-inflammatory effects. We also examined whether or not a single intrathecal (I.T.) injection of ATL313 could prevent CCI pain if it was given 24 hrs pre- or post-surgery, before neuropathic pain had developed. We chose to use CCI for the IT prevention part of this task because it is the most reliable and robust pain model we have for examining the effects of IT ATL313 on neuropathic pain. Figures 8 and 9 below show that IT ATL313 was not able to prevent CCI-induced mechanical allodynia compared to vehicle controls when it was administered either 24 hr before or after CCI, which was what we predicted. This supports our hypothesis that there must be chronic on-going inflammation in order for ATL313 to exert its anti-inflammatory effects.



**Figure 7.** Baseline measures on the von Frey test for mechanical allodynia were performed before surgery. Sciatic inflammatory neuropathy (SIN) surgery was performed and rats were allowed to recover for five days. At five days post-surgery, following von Frey testing, zymosan (3.2 ug/ul) or saline vehicle and ATL313 (500pmol) or DMSO vehicle was injected peri-sciatically. Peri-sciatic zymosan or saline vehicle was administered every other day for 7 days in order to maintain allodynia. Rats were tested on von Frey for mechanical allodynia at 6, 10, and 14 days post-surgery. Peri-sciatic ATL313 significantly prevented zymosan-induced mechanical allodynia ( $P < 0.001$ , 2-way repeated measures ANOVA).



**Figure 8.** Baseline measures on the von Frey test for mechanical allodynia were performed before surgery. Chronic constriction injury (CCI) surgery was performed. Twenty-four hours post-surgery, a single intrathecal injection of 1 pmol ATL313 or 0.01% DMSO vehicle was given. Rats were tested on von Frey for mechanical allodynia at 3, 7, 10, 14, 21, and 28 days post-surgery. Intrathecal ATL313 did not prevent CCI-induced mechanical allodynia ( $P > 0.05$ , 2-way repeated measures ANOVA).

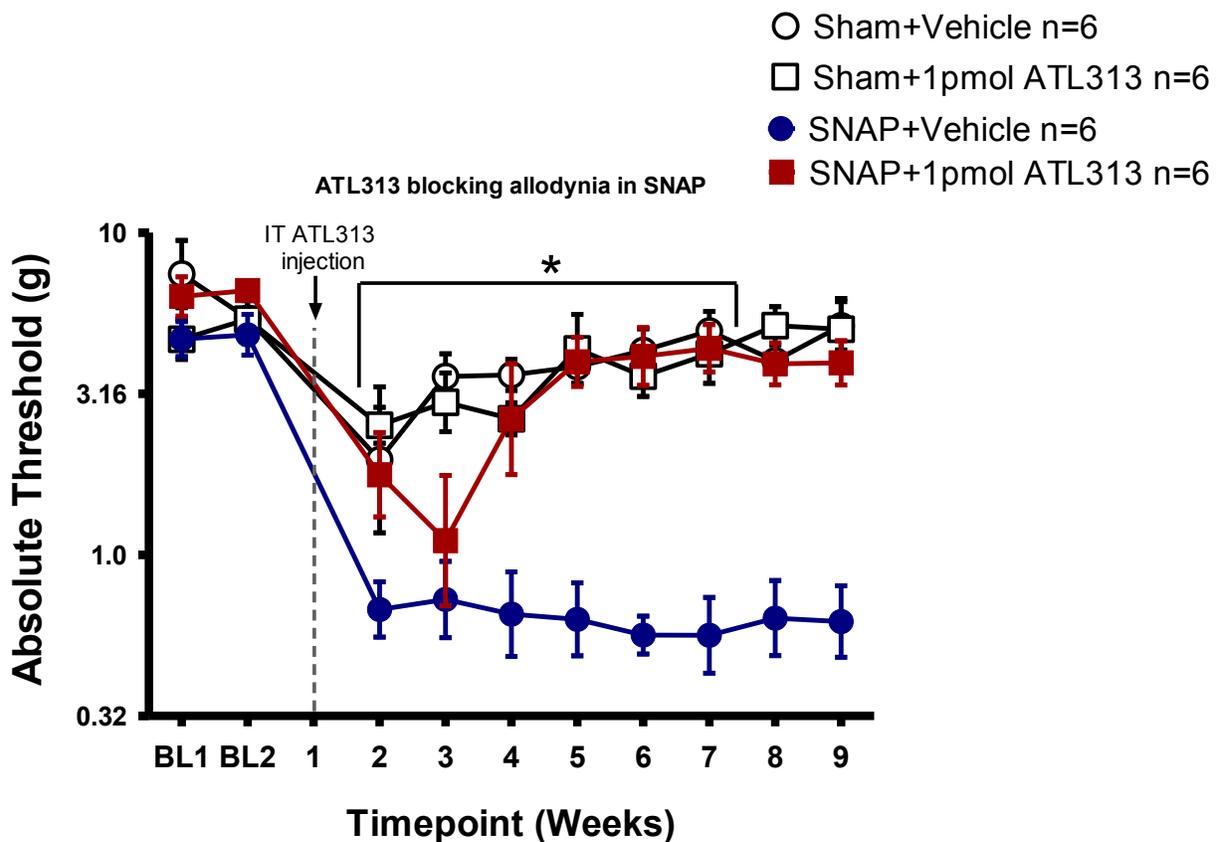


**Figure 9.** Baseline measures on the von Frey test for mechanical allodynia were performed before the ATL313 injection. A single intrathecal injection of 1 pmol ATL313 or 0.01% DMSO vehicle was given. Chronic constriction injury (CCI) surgery was performed twenty-four hours post-ATL313. Rats were tested on von Frey for mechanical allodynia at 3, 7, 10, 14, 21, and 28 days post-surgery. Intrathecal ATL313 did not prevent CCI-induced mechanical allodynia ( $P>0.05$ , 2-way repeated measures ANOVA).

**Task 6. Aim IB3ii. SCI: prevention of central neuropathic pain by ATL313.**

Task 6 has been completed.

In Task 4 we showed that  $A_{2A}R$  agonism is able to reverse both acute and chronic central neuropathic pain. Here we are able to show that  $A_{2A}R$  agonism is also able to attenuate the induction of allodynia when administered 1 wk post-SCI SNAP surgery as seen in Figure 10 below. Although ATL313 was not able to completely prevent allodynia, it was still able to significantly decrease pain thresholds compared to controls. We know that SNAP induces massive inflammation beginning 24 hr post-surgery, thus when ATL313 is administered at 1 wk post-surgery the proinflammatory environment is optimal for allowing ATL313 to exert its anti-inflammatory effects similar to what we saw with SIN in Task 5.



**Figure 10.** Unilateral T13/L1 avulsion SNAP or sham surgery with dura suture was performed and rats were allowed to recover for one week before ATL313 administration. Mechanical thresholds of the ipsilateral hind paw were tested before surgery. At one week post-surgery, a single bolus injection of 1 pmol ATL313 or DMSO vehicle was administered intrathecally. Rats were not tested for mechanical allodynia before ATL313 administration as reliable behavior is not obtained in SNAP rats until two weeks post-surgery after all spinal cord swelling has been resolved. Beginning two weeks post-surgery (one week post-ATL313 administration) rats were tested for mechanical allodynia weekly for seven weeks, at which point allodynia from the SNAP surgery is resolved. Intrathecal ATL313 significantly reversed the mechanical allodynia induced by SNAP surgery from week 2 through week 7 post-surgery (week 1 through week 7 post-ATL313) ( $P < 0.05$ , 2-way repeated measures ANOVA).

**Milestone 3:** Definition of optimal IT and peri-sciatic ATL313 doses for use in Tasks 8, 10, and 12. Completed analysis of a *single* IT ATL313 dose to: prevent and reverse acute and chronic central neuropathic pain from traumatic spinal cord injury; to prevent and reverse neuropathic pain from inflammatory peripheral neuropathy following either IT or peri-sciatic (peripheral) injections.

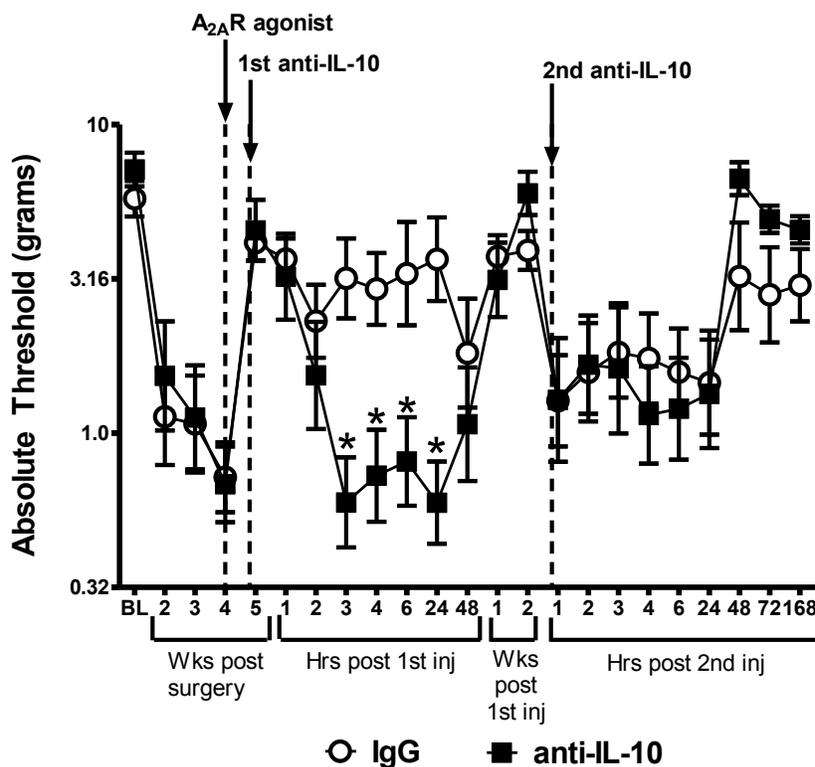
Milestone 3 is complete. We know that a single IT dose of ATL313 is able to reverse acute and chronic central neuropathic pain (CNP) from traumatic spinal cord injury (SCI) and that a single dose of IT ATL313 can somewhat prevent CNP from SCI. We also know that a single IT dose of ATL313 can reverse neuropathic pain from inflammatory peripheral neuropathy following either IT or peri-sciatic (peripheral) injections. We also know that ATL313 does not prevent CCI-induced neuropathic pain. We determined that 1 pmol ATL313 is the optimal IT dose and 500 pmol is the optimal peri-sciatic dose. However, these doses might change slightly because we have begun to combine two previously separate surgical procedures (sciatic nerve chronic

constriction injury [CCI] and peri-sciatic catheter implantation) in an effort to reduce subject attrition from the procedures originally proposed. The originally proposed procedures used a peri-sciatic catheter both to maintain allodynia/inflammation and for a drug delivery system to determine the location of action of drugs of interest. In the original procedures, a pro-inflammatory agent, zymosan, was injected every two days through the peri-sciatic catheter to maintain stable allodynia/inflammation. These procedures proved to be difficult because the peri-sciatic catheter frequently lost patency before the end of the study, causing the subject to be removed from the study. We thus proposed to use CCI surgery to maintain allodynia/inflammation (in lieu of peri-sciatic zymosan injections) and use the peri-sciatic catheter only as a drug delivery system. These procedures were approved in our most recent no-cost extension by the Department of Defense and by our IACUC. CCI surgery is performed first followed by peri-sciatic catheter implantation during the same surgical session. We are currently conducting a pilot experiment to determine if the level of allodynia produced by CCI is altered by the presence of a peri-sciatic catheter. This experiment will inform future experiments using these procedures, which will ultimately lead to completion of the experiments proposed in Tasks 8, 10, and 12 in the Statement of Work.

**Task 7. Aim IIA1. SNL: characterizing the involvement of interleukin(IL)-10 across the timecourse of effect.**

Task 7 has been completed.

Given the evidence of increased duration of action of ATL313 in the SCI SNAP model relative to the spinal nerve ligation model, we have conducted the proposed experiment in the SCI SNAP model. Figure 9 below shows that anti-IL-10 treatment 1 wk after  $A_{2A}R$  agonist administration significantly abolishes the pain-relieving effects of the agonist. This indicates that the anti-inflammatory cytokine IL-10 is critically involved in the mechanism by which  $A_{2A}R$  agonists exert their anti-allodynic effects, at least in the first 1-2 wks post-agonist administration. A second injection of anti-IL-10 two weeks after the first anti-IL-10 injection did not significantly abolish the anti-allodynic effects of the  $A_{2A}R$  agonist, indicating that IL-10 may not be as critically involved in the  $A_{2A}R$  agonist mechanism further out in time after the agonist injection.



**Figure 11.** Unilateral T13/L1 avulsion SNAP surgery with dura suture was performed and rats were allowed to recover for two weeks. Mechanical thresholds of the ipsilateral hind paw were tested before surgery. Beginning two weeks post-surgery, rats were tested for mechanical allodynia weekly. At four weeks post-surgery, a single bolus injection of a 1uM  $A_{2A}R$  agonist was administered intrathecally to all rats. One week later rats received a single injection of sheep anti-rat neutralizing IL-10 IgG antibodies (0.2 ug/ml; 10 ul) or equivolume and equidose sheep IgG (0.2 ug/ml; 10 ul) and behavior was tested hourly for 6 hrs and then again at 24 and 48 hrs. Rats were then tested weekly for 3 wks and again injected with either IgG or anti-IL-10 and tested hourly for 6 hrs and then again at 24, 48, 72, and 168 hrs. Anti-IL-10 significantly abolished the effects of the  $A_{2A}R$  agonist beginning 4 hrs after the injection and this effect lasted for 48 hrs (interaction,  $F_{(6,72)}=4.808$ ,  $p<0.001$ ;  $n=9$  per group). Behavior returned to pre-anti-IL-10 or IgG levels 2 wk later. IgG had no effect on behavior. A second injection of either anti-IL-10 or IgG administered 3 wk later did not have a significant effect on allodynia behavior (interaction,  $F_{(7,84)}=1.007$ ,  $p>0.05$ ;  $n=9$  per group). Data are presented as mean  $\pm$  SEM and analyzed using two-way repeated measures ANOVA. \* $p<0.05$  SCI plus IgG compared to SCI plus anti-IL-10.

**Task 9. Aim IIB1. SNL: protein kinase (PK) C/PKA involvement with acute and chronic peripheral neuropathic pain.**

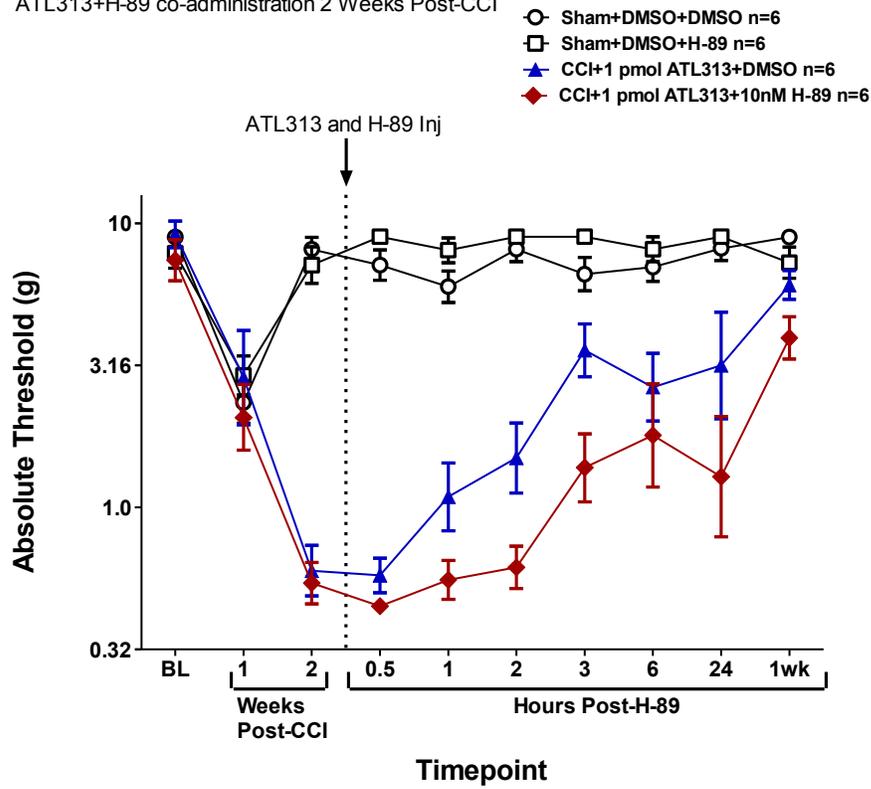
Task 9 has been completed.

Given the evidence of increased duration of action of ATL313 in the chronic constriction injury model relative to the spinal nerve ligation model, we have conducted the proposed experiment in the chronic constriction injury model. Here we administered a protein kinase A (PKA) inhibitor to determine the involvement of PKA in the mechanism by which  $A_{2A}R$  agonists exert their anti-allodynic effects. There is some controversy in the literature as to whether or not PKA inhibitors are proinflammatory or anti-inflammatory when administered *in vivo*. Based on Dr. Lisa Loram's

findings with PKA and PKC, we chose only to examine PKA *in vivo* because of the complexity of the PKC signaling cascade, which makes the results of PKC inhibition *in vivo* difficult to interpret. Figure 12 shows intrathecal co-administration of ATL313 and H-89 (PKA inhibitor) when injected intrathecally either 2 weeks or 6 weeks post-CCI surgery. ATL313 is able to reverse CCI-induced allodynia even in the presence of the PKA inhibitor, although the 2 week timepoint shows attenuation in this reversal in the group that received H-89, suggesting that PKA is playing a role. However, at the 6 week timepoint there does not seem to be an effect of PKA. Figure 13 shows administration of intrathecal ATL313 either 2 or 6 weeks post-CCI followed by intrathecal H-89 1 week later. Here we see that PKA is involved when ATL313 is administered 2 weeks post-CCI, but is not involved when ATL313 is administered after sustained (6 wk) CCI. Figure 14 shows administration of intrathecal ATL313 2 and 6 weeks post-CCI followed by intrathecal H-89 5 weeks later. This suggests that PKA may play a small role in the mechanism by which ATL313 reverses allodynia, but only during the acute pain phase (ie. 2 wk post CCI-surgery). These studies will be expanded on in task 10, which will give a more complete analysis of the role of PKA in ATL313-induced reversal of allodynia. We also know from task 13 below that administering PKA and PKC inhibitors to cultured glial cells had little or no effect on TNF and IL10 production, suggesting that there are other mechanisms that are involved in this phenomenon. One potential mechanism is alternative activation, which will also be studied furthered in tasks 11 and 12.

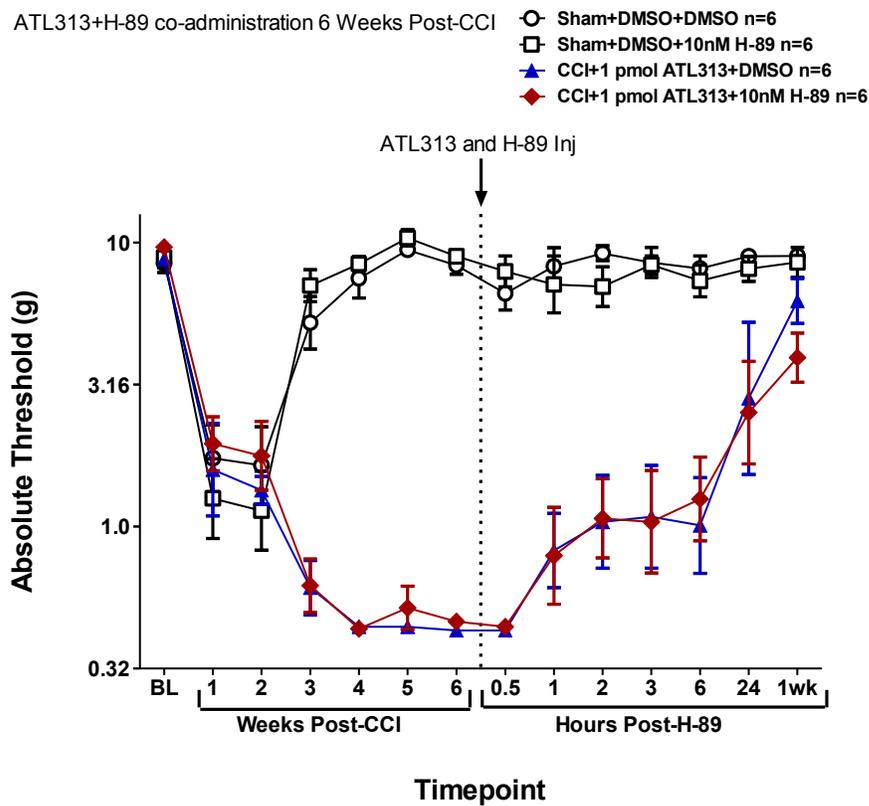
A.

ATL313+H-89 co-administration 2 Weeks Post-CCI



B.

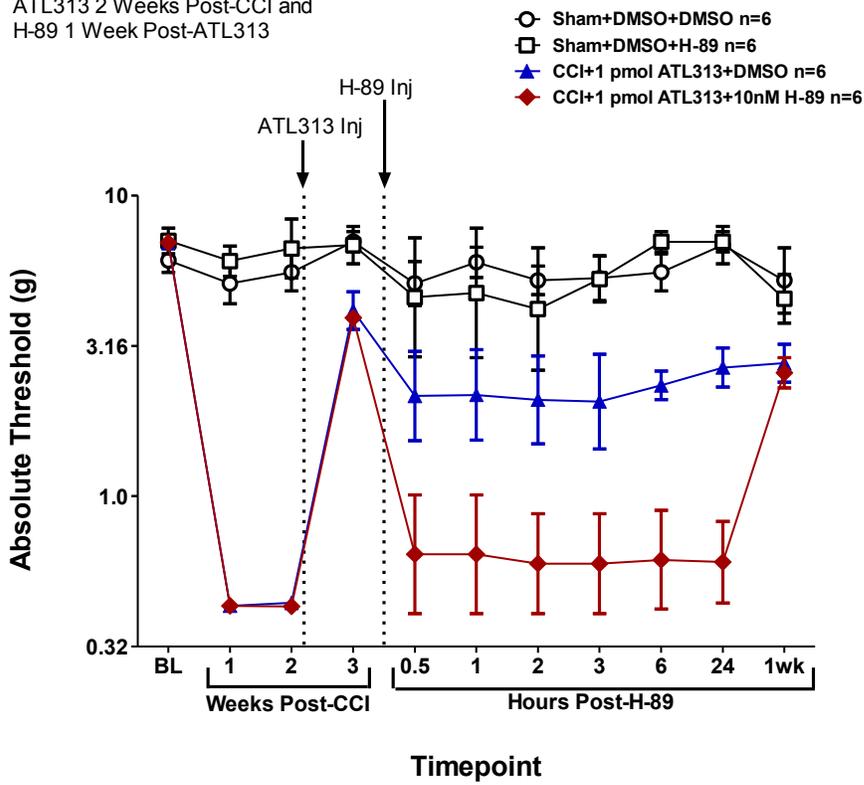
ATL313+H-89 co-administration 6 Weeks Post-CCI



**Figure 12.** Unilateral chronic constriction injury (CCI) or sham surgery was performed and rats were allowed to recover for one week. Mechanical thresholds of the ipsilateral hind paw were tested before surgery. At either 2 (**A**) or 6 (**B**) weeks post-surgery, after behavioral verification of allodynia, a single bolus injection of 1 pmol ATL313 or DMSO vehicle and 10 nM H-89 (PKA inhibitor) was co-administered intrathecally. Rats were tested across a timecourse beginning 30 min after the injection. ATL313 is able to reverse CCI-induced allodynia even in the presence of the PKA inhibitor, although the 2 week timepoint shows an attenuation in this reversal in the group that received H-89, suggesting that PKA is playing a role. However, at the 6 week timepoint there does not seem to be an effect of PKA.

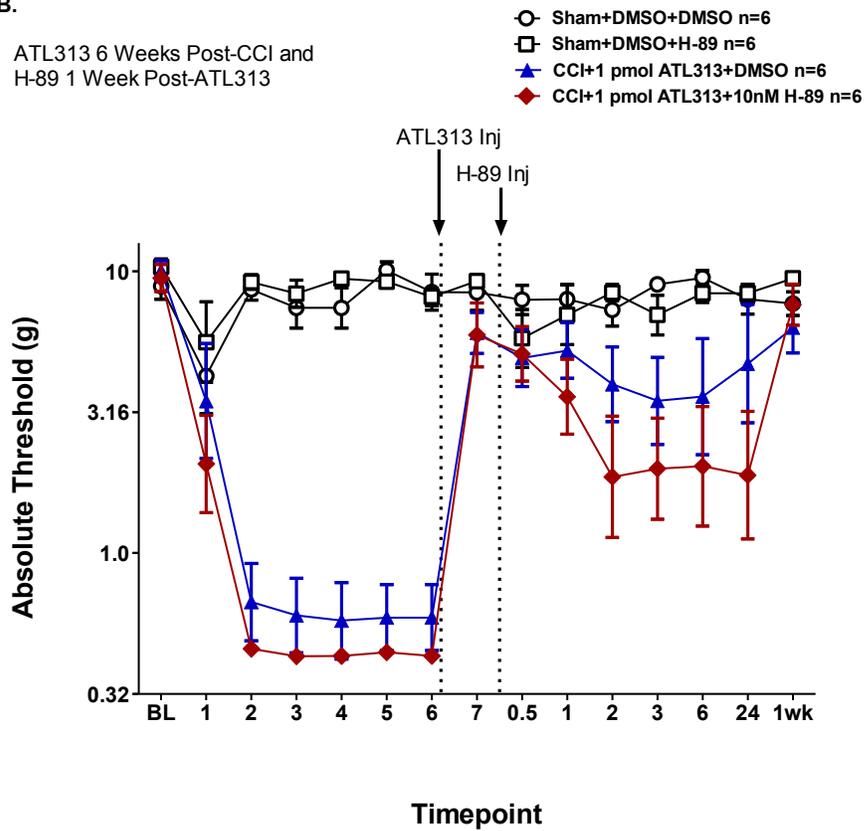
A.

ATL313 2 Weeks Post-CCI and  
H-89 1 Week Post-ATL313



B.

ATL313 6 Weeks Post-CCI and  
H-89 1 Week Post-ATL313

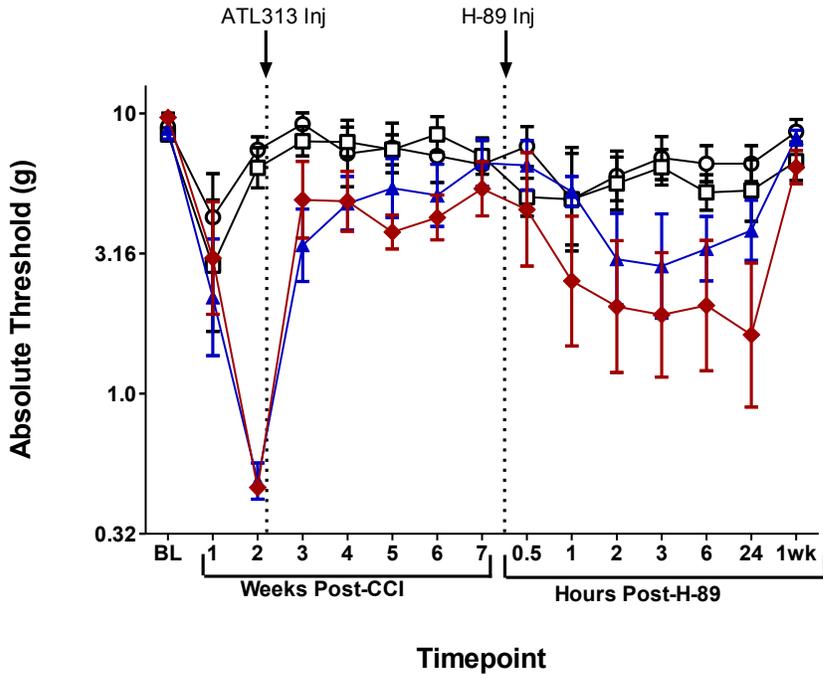


**Figure 13.** Unilateral chronic constriction injury (CCI) or sham surgery was performed and rats were allowed to recover for one week. Mechanical thresholds of the ipsilateral hind paw were tested before surgery. At either 2 (**A**) or 6 (**B**) weeks post-surgery, after behavioral verification of allodynia, a single bolus injection of 1 pmol ATL313 or DMSO vehicle was administered intrathecally. One week later, a single bolus injection of 10 nM H-89 or DMSO was administered intrathecally. Rats were tested across a timecourse beginning 30min after the injection. ATL313 reversed CCI-induced allodynia as expected. H-89 is able to attenuate the ATL313-induced reversal we see, but only when ATL313 is administered 2 weeks post-CCI. H-89 did not attenuate the ATL313-induced reversal when ATL313 was administered after sustained (6 wk) CCI.

A.

ATL313 2wk Post-CCI + H-89 5wk Post-ATL313

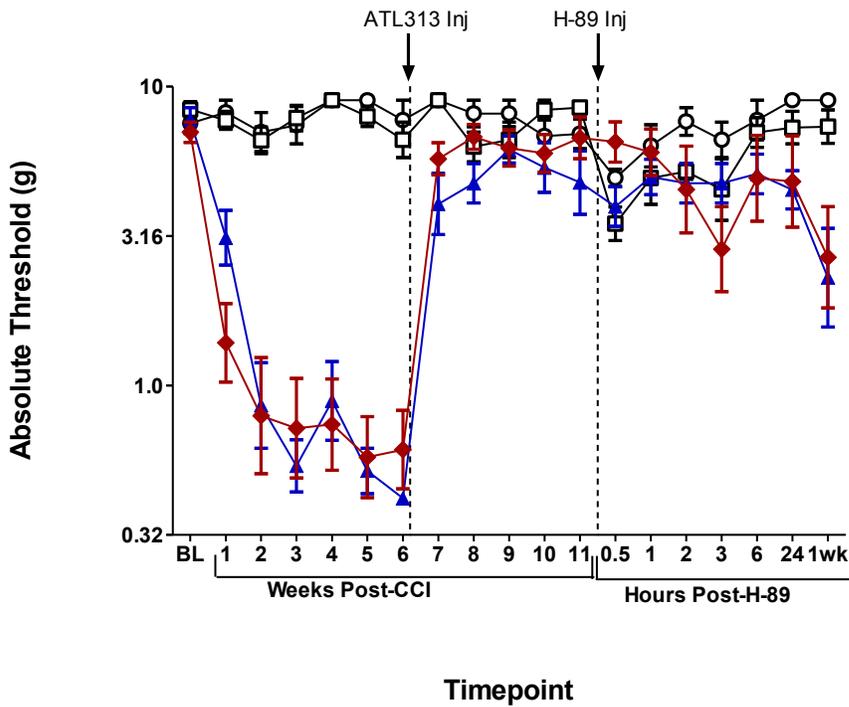
- Sham+DMSO+DMSO n=6
- Sham+DMSO+10nM H-89 n=6
- ▲ CCI+1 pmol ATL313+DMSO n=6
- ◆ CCI+1 pmol ATL313+10nM H-89 n=6



B.

ATL313 6wk Post-CCI + H-89 5wk Post-ATL313

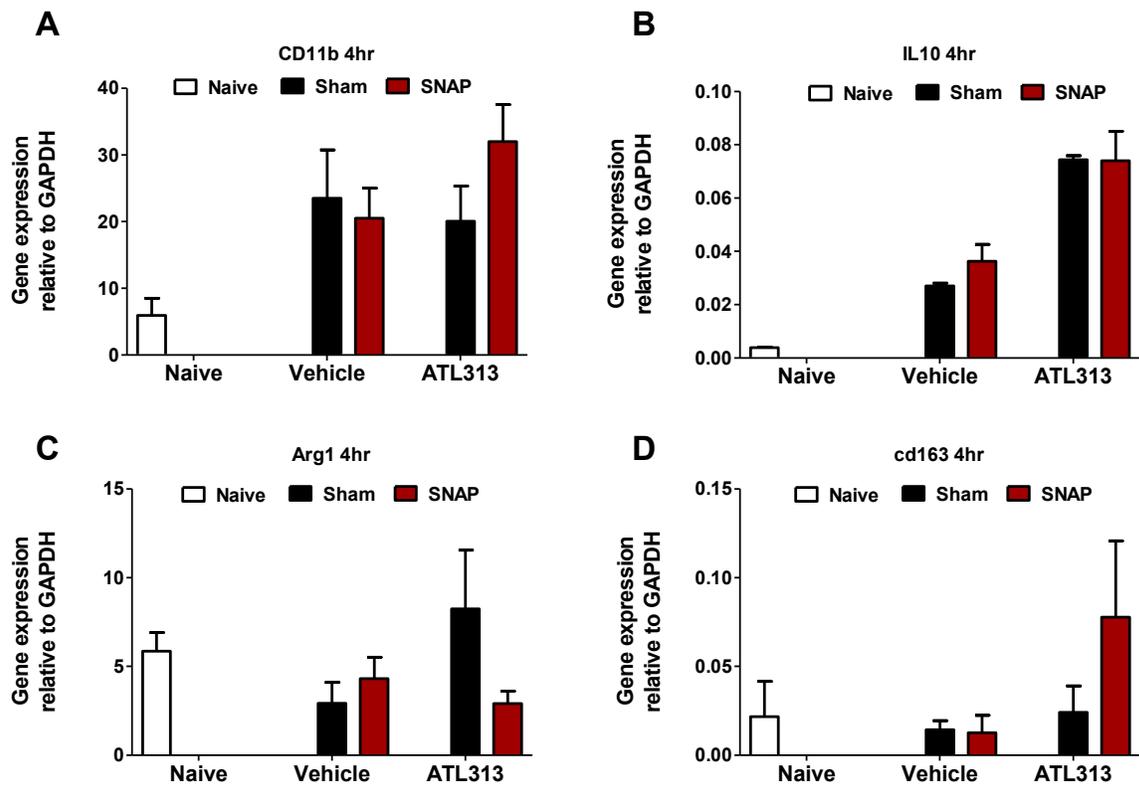
- Sham+DMSO+DMSO n=6
- Sham+DMSO+H-89 n=6
- ▲ CCI+1 pmolATL313+DMSO n=6
- ◆ CCI+1 pmolATL313+H-89 n=6



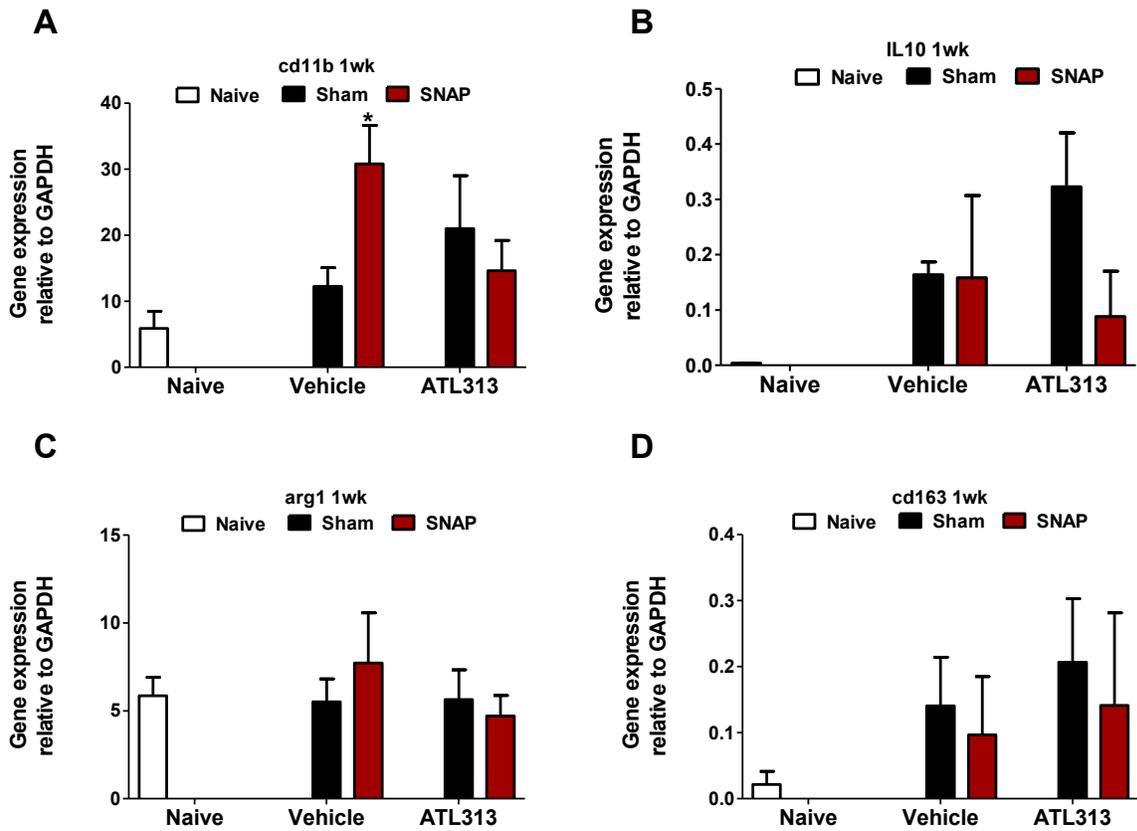
**Figure 14.** Unilateral chronic constriction injury (CCI) or sham surgery was performed and rats were allowed to recover for one week. Mechanical thresholds of the ipsilateral hind paw were tested before surgery. At 2 (A) or (B) 6 weeks post-surgery, after behavioral verification of allodynia, a single bolus injection of 1 pmol ATL313 or DMSO vehicle was administered intrathecally. Five weeks later, a single bolus injection of 10nM H-89 or DMSO was administered intrathecally. Rats were tested across a timecourse beginning 30min after the injection. ATL313 reversed CCI-induced allodynia as expected. H-89 is able to attenuate the ATL313-induced reversal only when ATL313 was administered at 2 weeks post-CCI; there was no effect of H-89 when ATL313 was administered 6 weeks post-CCI.

### **Task 11. IT ATL313 induction of alternative activation**

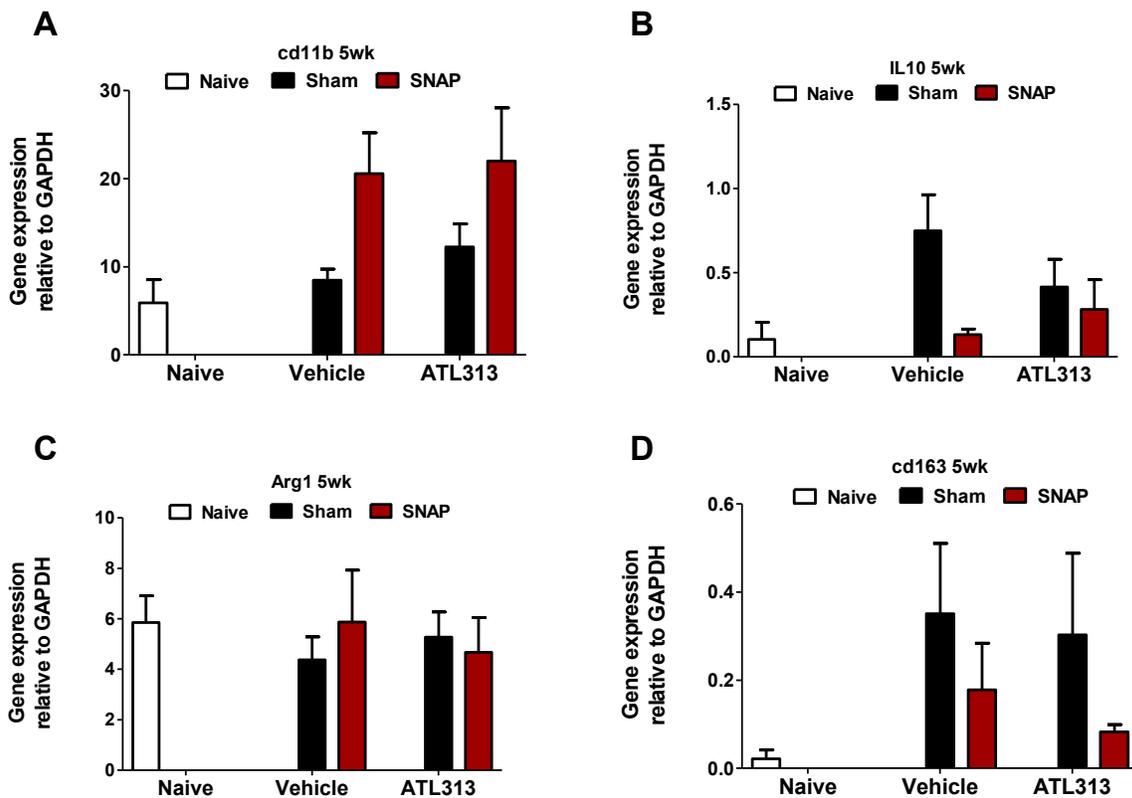
All of the tissue for the task has been collected and is in the process of being analyzed. ATL313 was injected IT into SNAP SCI rats at 2 weeks post-surgery and then rats were overdosed, perfused, and their T13/L1 and L5/L6 spinal cord sections were dissected and collected for PCR and western blot 4 hr, 1 wk, or 5 wk post ATL313 injection. Of note, sham SCI animals are allodynic on the von Frey test for 3 wk post-surgery, which can lead to increased gene expression of proinflammatory markers at these timepoints as well. All of the T13/L1 tissue has been processed for PCR and the data is analyzed, shown in figures 15-17 below. The L5/L6 tissue for PCR has been processed and we are currently analyzing that data. We are not finished processing the tissue for western blot but should have it finished in the next 1-2 quarters. We did not find any significant changes in alternative activation gene expression markers (IL-10, arginase-1 (Arg1), cd163) at any timepoint. We did see that ATL313 significantly decreased cd11b gene expression at the 1 wk post-ATL313 timepoint, although this effect was no longer seen at 5 wk post-ATL313. There was very little total gene expression of both IL-10 and cd163 in the tissue at all of the timepoints, which is consistent with what we have seen in the past with at least IL-10.



**Figure 15.** Unilateral T13/L1 SNAP SCI or sham plus dura suture surgery was performed and rats were allowed to recover for two weeks. At two weeks post-surgery, after behavioral verification of allodynia, a single bolus injection of 1 pmol ATL313 or DMSO vehicle was administered intrathecally. Four hours later, rats were overdosed and perfused and the T13/L1 spinal cord was dissected out for real-time PCR analysis. A surgically naïve group that received no pharmacological manipulation was also included in the analysis. All groups were n=6/group. There were no significant differences in gene expression for the glial activation marker CD11b (A), the anti-inflammatory cytokine IL-10 (B), or the alternative activation markers arginase-1 (C) and cd163 (D).



**Figure 16.** Unilateral T13/L1 SNAP SCI or sham plus dura suture surgery was performed and rats were allowed to recover for two weeks. At two weeks post-surgery, after behavioral verification of allodynia, a single bolus injection of 1 pmol ATL313 or DMSO vehicle was administered intrathecally. One week later, rats were overdosed and perfused and the T13/L1 spinal cord was dissected out for real-time PCR analysis. A surgically naïve group that received no pharmacological manipulation was also included in the analysis. All groups were n=6/group. There was a significant decrease in gene expression for the glial activation marker CD11b (A). ATL313 significantly decreased CD11b gene expression in SNAP rats compared to vehicle. There were no significant differences in gene expression for the anti-inflammatory cytokine IL-10 (B), or the alternative activation markers arginase-1 (C) and cd163 (D). \*p<0.05.



**Figure 17.** Unilateral T13/L1 SNAP SCI or sham plus dura suture surgery was performed and rats were allowed to recover for two weeks. At two weeks post-surgery, after behavioral verification of allodynia, a single bolus injection of 1 pmol ATL313 or DMSO vehicle was administered intrathecally. Five weeks later, rats were overdosed and perfused and the T13/L1 spinal cord was dissected out for real-time PCR analysis. A surgically naïve group that received no pharmacological manipulation was also included in the analysis. All groups were n=6/group. There were no significant differences in gene expression for the glial activation marker CD11b (A), the anti-inflammatory cytokine IL-10 (B), or the alternative activation markers arginase-1 (C) and cd163 (D).

### Task 13. Effect of blocking PKA/PKC on microglial/astrocyte IL-10/alternative activation

Task 13 has been completed.

All of the results from this task are now published in Dr. Lisa Loram's BBI 2013 manuscript. Figure 18 below shows that  $A_{2A}R$  agonism attenuates TNF release by both microglia and astrocytes *in vitro* but does not increase IL-10 release in microglia and decreases IL-10 in astrocytes. In order to determine if ATL313 required a longer incubation period to up-regulate IL-10 release, we repeated the previous experiment with separate glial cultures but incubated the cells for 48 and 72 hrs. Figure 19 below shows that there was a significant attenuation of TNF release from both microglia and astrocytes but no significant change in the effect of ATL313 on IL-10 release in either population. We then tried co-culturing microglia and astrocytes to see if ATL313 would up-regulate IL-10 in this type of environment. Again, ATL313 attenuated TNF release but did not up-regulate IL-10. Lastly we wanted to verify that ATL313 decreases TNF via PKA and/or PKC. Figure 20 below shows that PKA inhibition reversed the ATL313 mediated suppression of TNF in microglia but not in astrocytes, and no effect on IL-10. PKC

inhibition reversed the ATL313 mediated suppression of TNF in both microglia and astrocytes but had no effect on IL-10. It is clear from these data that A2AR agonism attenuates TNF release by microglia and astrocytes and in a PKA-dependent manner in microglia (not astrocytes), suggesting that glia are involved in the mechanism by which A2AR agonism exerts its anti-allodynic effects and it is at least partially via PKA. However, there were no changes in IL-10 release in either microglia or astrocytes. We found in our earlier publication (4) that IL-10 mRNA was significantly increased in the CSF of CCI rats injected with ATL313, but based on the data here we determined that the source of this IL-10 is not from glial cells but is likely coming from immunocompetent cells (mostly macrophages) in the intrathecal space.

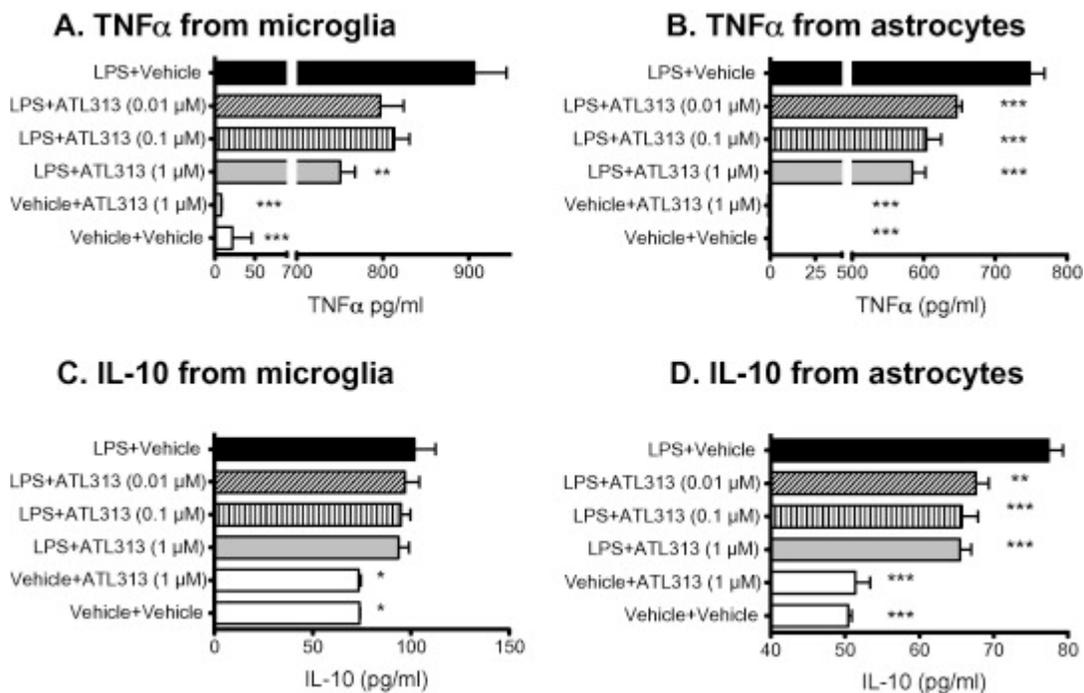


Fig. 18. An A<sub>2</sub>AR agonist downregulates TNF $\alpha$  in central immune cells. (A) TNF $\alpha$  release (pg/ml) from neonatal cortical microglia and (B) astrocytes incubated for 24 h with LPS is attenuated by co-administration of ATL313.  $n = 3/4$  wells/group and the experiment was replicated at least twice. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to LPS + vehicle. IL-10 release (pg/ml) from neonatal cortical microglia (C) and astrocytes (D) incubated for 24 h with LPS is upregulated and maintained by ATL313 but not upregulated by co-administration of ATL313. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to LPS + vehicle.  $n = 3/4$  wells/group and the experiment was replicated at least twice.

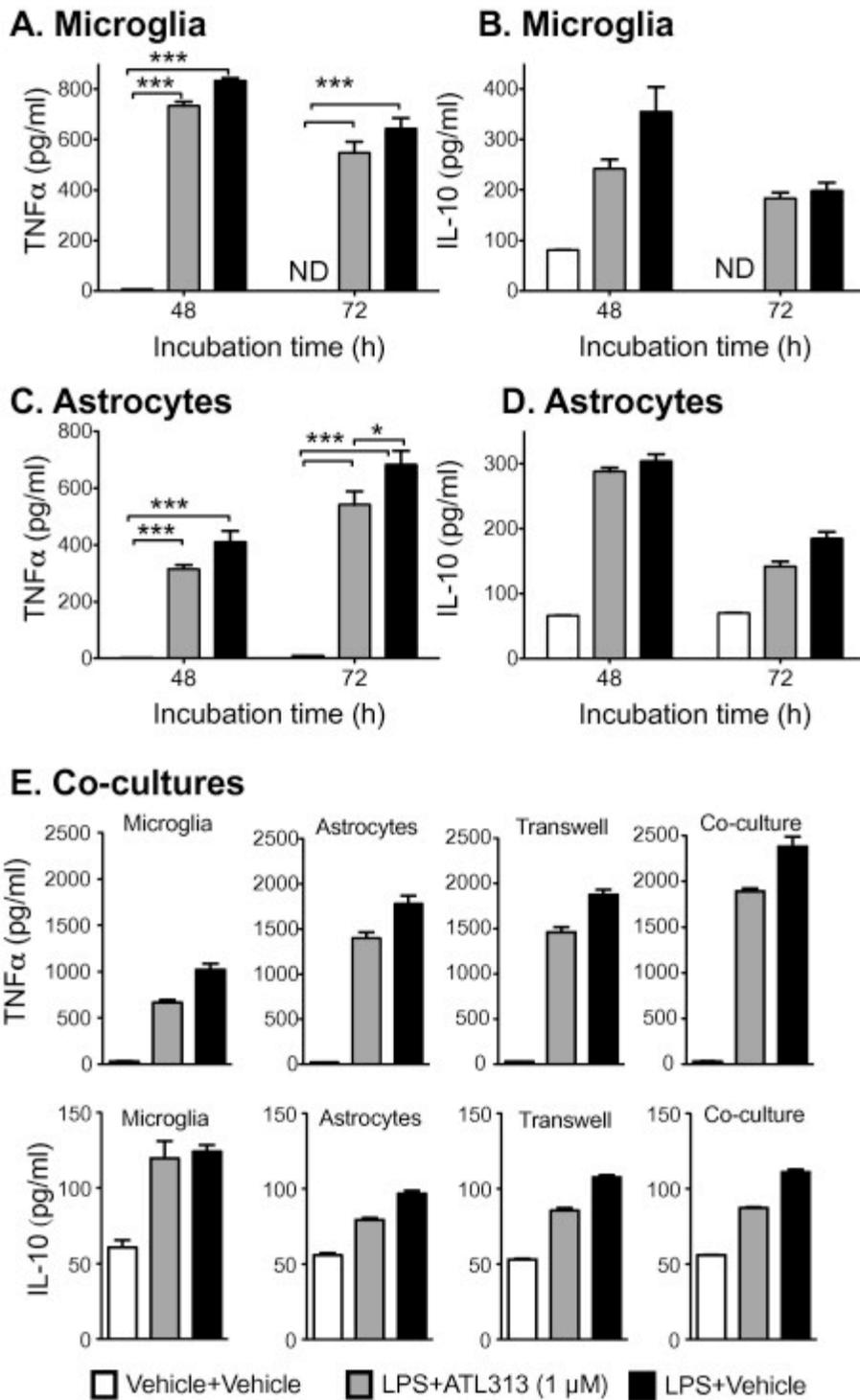


Fig. 19. TNF $\alpha$  and IL-10 produced by glial cells *in vitro* in response to LPS  $\pm$  ATL313 are unaffected by duration of incubation or locality of other glial cells. TNF $\alpha$  release (pg/ml) and IL-10 release (pg/ml) from neonatal cortical microglia incubated for 48 h and 72 h produced comparable results as a 24 h incubation with IL-10 not being elevated by ATL313 beyond that induced by LPS alone (A and B). TNF $\alpha$  release (pg/ml) and IL-10 release (pg/ml) from neonatal cortical astrocytes incubated for 48 h and 72 h with LPS + ATL313 produced comparable results as a 24 h incubation with IL-10 not being elevated beyond that induced by LPS alone (C and D). TNF $\alpha$  and IL-10 release (pg/ml) from neonatal cortical microglia and astrocytes incubated for 24 h either in isolated cell types, co-incubated or with transwell inserts allowing non-contact communication (E). None of the above incubation conditions altered the TNF $\alpha$  or IL-10 release profiles following LPS  $\pm$  ATL313 coadministration. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to LPS + vehicle.  $n = 3/4$  wells/group and the experiment was replicated at least twice. All data are mean  $\pm$  SEM.

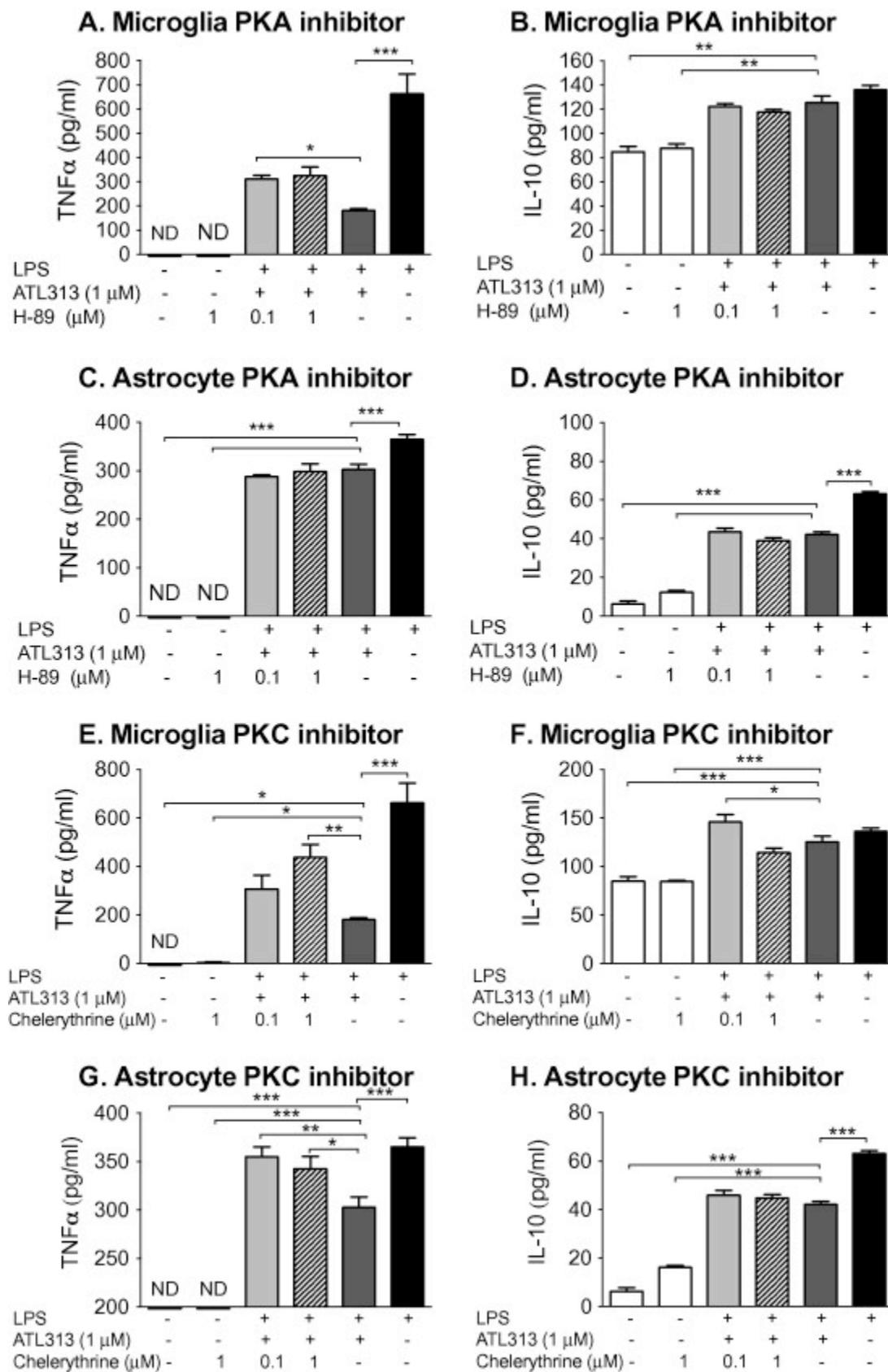


Fig. 20. TNF $\alpha$  release from neonatal microglia (B) and neonatal astrocytes (C) incubated with LPS (100 ng/ml) for 24 h was attenuated by ATL313 (1  $\mu$ M). Administration of H-89 (PKA inhibitor) partially reversed the effects of ATL313 on TNF $\alpha$  production in microglia (A) but not astrocytes (B). IL-10 production induced by LPS was not affected by ATL313 or H-89 in microglia (C and D). Administration of chelerythrine (PKC inhibitor) had no effect on ATL313

mediated effects of TNF $\alpha$  production in microglia (E) but reversed the ATL313 effect in astrocytes (F). IL-10 production induced by LPS was not affected by ATL313 in microglia (G). However, chelerythrine + LPS + ATL313 increased IL-10 compared to LPS + ATL313 + vehicle in microglia (G). Chelerythrine had no effect on IL-10 responses in astrocytes (H). Protein measured by rat-specific ELISA. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to LPS + ATL313 + vehicle;  $n = 4-5$  wells/group. All data are mean  $\pm$  SEM.

## Key Research Accomplishments

- We found that ATL313 reverses and prevents sciatic inflammatory neuropathy (SIN)-induced allodynia and reverses but does not prevent chronic constriction (CCI)-induced allodynia.
- We found that part of the anti-allodynic effects of ATL313 in CCI is through PKA and microglia and astrocytes attenuate TNF release via PKA.
- We found that the alternative activation gene expression markers arginase-1 and cd163 are not up-regulated in our SNAP SCI model
- Since our most reliable and robust effects with ATL313 are in the CCI model, we developed a new peri-sciatic catheter system that can be used with the CCI model. This eliminates the need to use the problematic sciatic inflammatory neuropathy (SIN) model.

## Reportable outcomes

- One manuscript titled *Intrathecal injection of adenosine 2A receptor agonists reversed neuropathic allodynia through protein kinase (PK)A/PKC signaling* was published in *Brain, Behavior, and Immunity* (5)

## Conclusions

We continue to make good progress and have maintained the required outputs and data collection according to the statement of work. ATL313 continues to present as a novel compound producing remarkably long duration of reversal of pain from a single administration. A<sub>2A</sub>R agonism both prevents and reverses acute and chronic neuropathic pain, and does so in neuropathies of both central (SCI SNAP) and peripheral (CCI, SNL, SIN) origin. This is important clinically since many neuropathic pain patients, with both central and peripheral neuropathies, do not seek treatment for weeks or month after the onset of pain. Furthermore, the anti-allodynic effects seen with A<sub>2A</sub>R agonism are consistent with different routes of administration (peri-sciatic, intrathecal). Pain patients are not always comfortable with certain routes of administration, and thus having equal efficacy using different routes is desirable. We have also shown that the anti-inflammatory cytokine IL-10 is initially involved in the mechanism by which A<sub>2A</sub>R agonists exert their pain relieving effects, and likely PKA and PKC, although more work still needs to be done in order to be able to interpret those results. We have also begun to explore possible alternative activation mechanisms that could be involved, and although there is not a clear story yet, we are continuing to pursue these endpoints. Taken together, all of these data suggest that ATL313 would be a successful new neuropathic pain treatment. At the same time, it is important to continue investigating the underlying mechanisms of this remarkable drug compound in order to use it most effectively. We continue to thank the Department of Defense for their continued support of the project and hope they find the outcome of the project to date exciting and novel with potential clinical relevance down the road.

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