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PRINCIPAL INVESTIGATOR: Distinguished Professor Dr. Linda Watkins

CONTRACTING ORGANIZATION: University of Colorado  
Boulder, CO 80309

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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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# Table of Contents

## Page

<b>Introduction.....</b>	<b>3</b>
<b>Body.....</b>	<b>3</b>
<b>Key Research Accomplishments.....</b>	<b>29</b>
<b>Reportable Outcomes.....</b>	<b>29</b>
<b>Conclusion.....</b>	<b>29</b>
<b>References.....</b>	<b>30</b>

## 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.

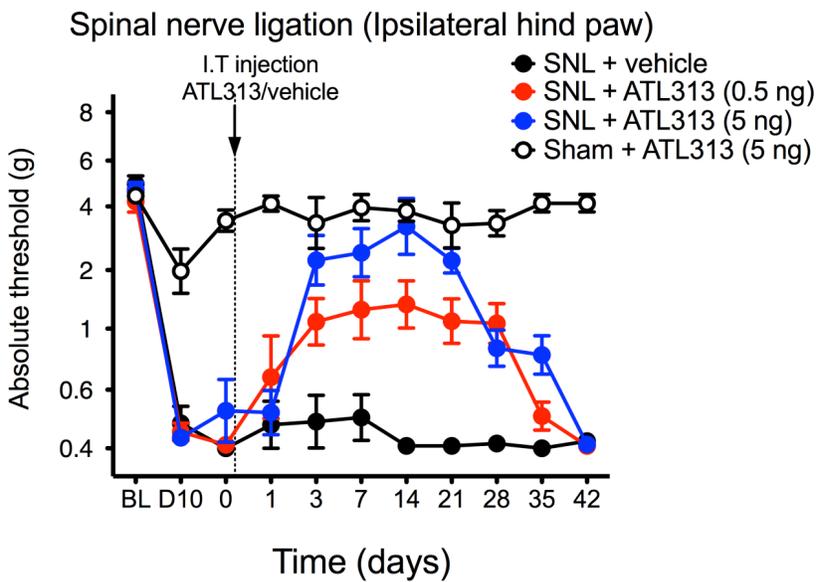
***Milestone 1:*** Animal protocol is approved to allow funding to be received on or before January 1, 2011, and to allow the project to start.

Milestone 1 has been completed.

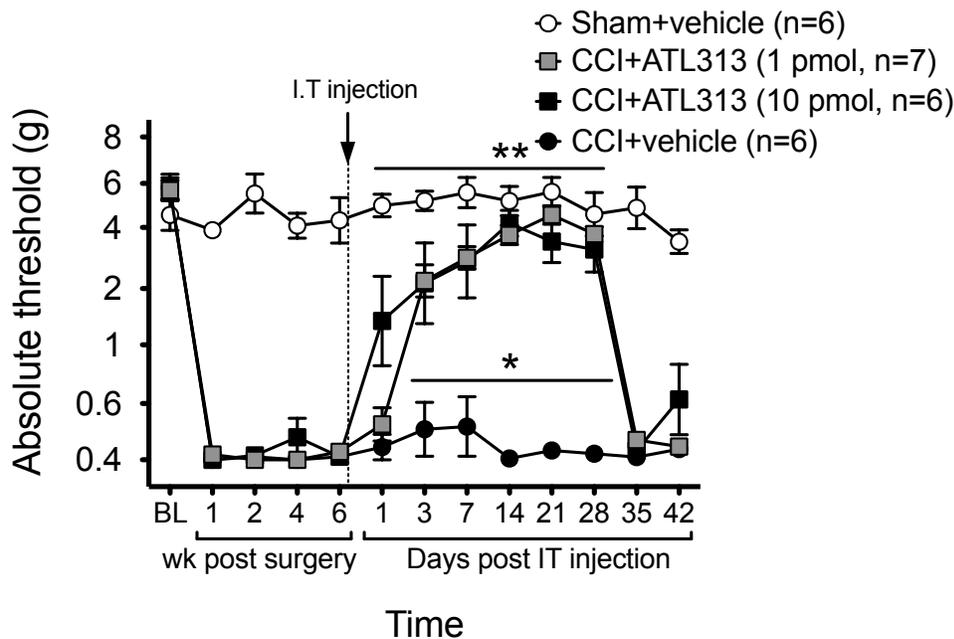
**Task 2. Aim IA1. Spinal Nerve Ligation (SNL): reversal of acute and chronic traumatic neuropathic pain by intrathecal (IT) ATL313.**

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 (5 ng) than that required for chronic constriction injury (0.5 ng) reverses the allodynia for 4 weeks. 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).

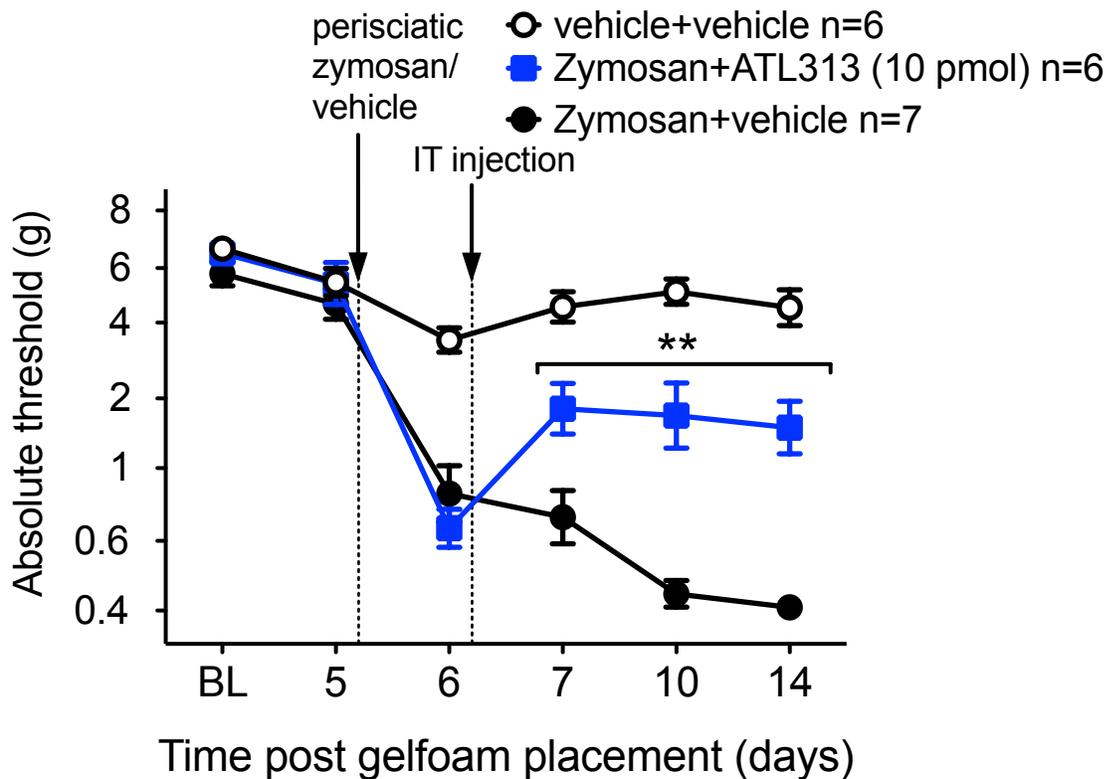
**Milestone 2:** Definition of optimal IT ATL313 dose for use in tasks 7, 9, and 11. Completed analysis of the ability of a *single* IT ATL313 dose to reverse neuropathic pain from traumatic peripheral neuropathy.

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.**

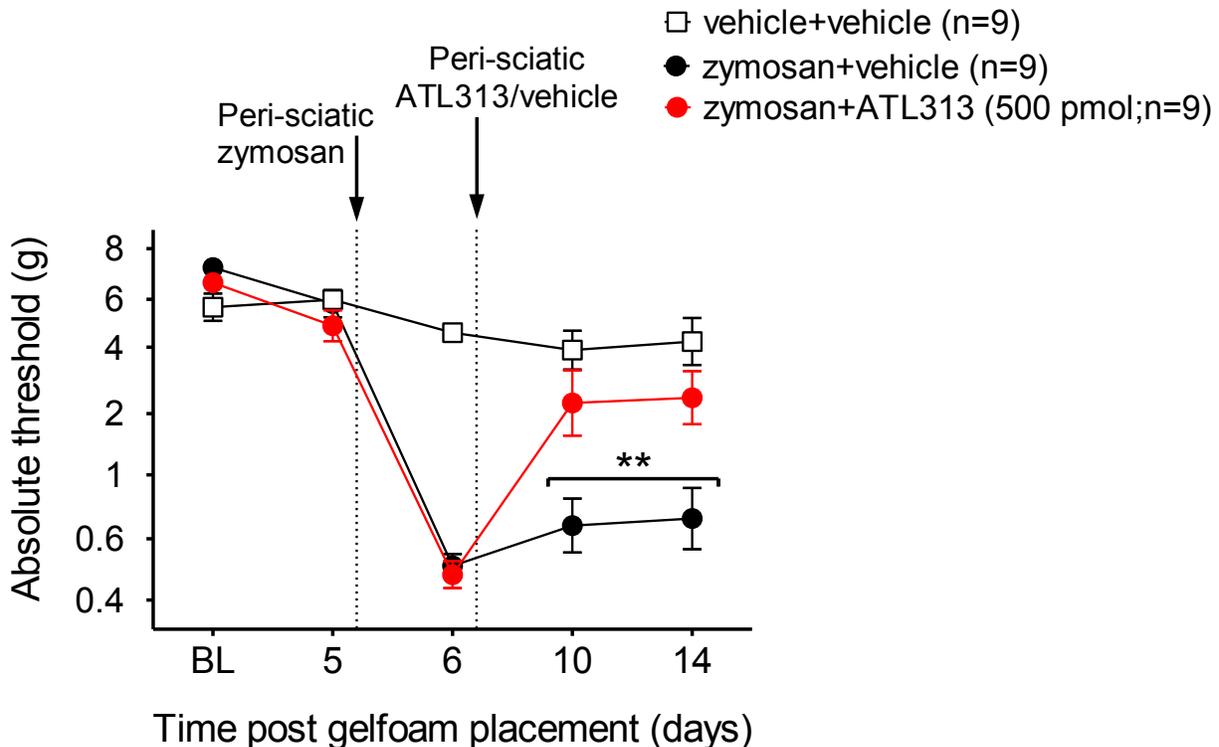
Task 3 has been completed.

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 employing SIN 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 nerve). 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, thus being able to use different routes of administration to get the same efficacy is desirable.



**Figure 3.** Gel foam was placed perisciatic nerve 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).

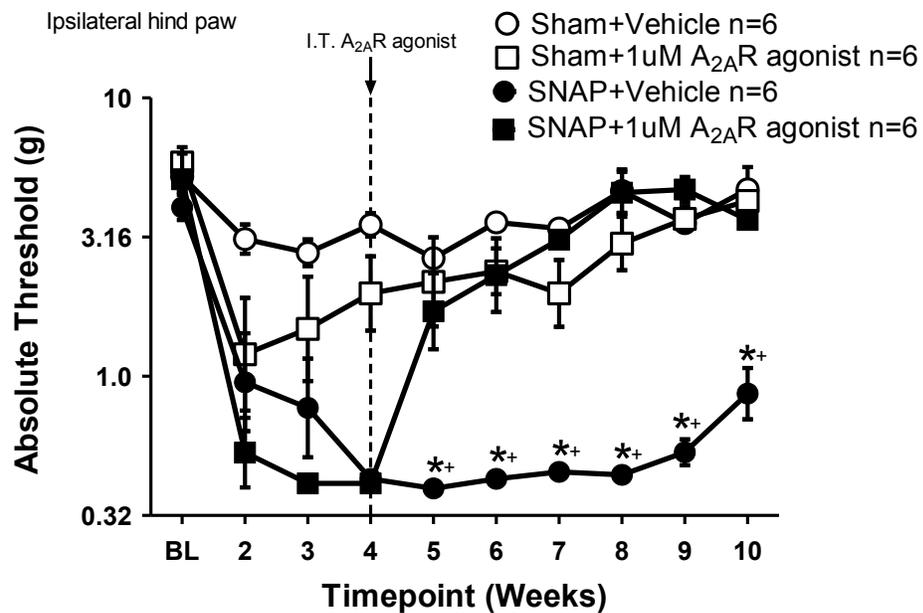


**Figure 4.** Baseline measures on the von Frey test for mechanical allodynia were performed before surgery. Sciatic inflammatory neuropathy (SIN) surgery was performed and rats were allowed to recover for five days. At five days post-surgery, following von Frey testing, zymosan (3.2  $\mu\text{g}/\mu\text{l}$ ) or saline vehicle was injected peri-sciatic nerve. Twenty four hours later, again following von Frey testing, ATL313 (500 pmol) or DMSO vehicle was injected peri-sciatic nerve. Peri-sciatic nerve 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 nerve ATL313 significantly reversed zymosan-induced mechanical allodynia ( $P < 0.0001$ , 2-way repeated measures ANOVA).

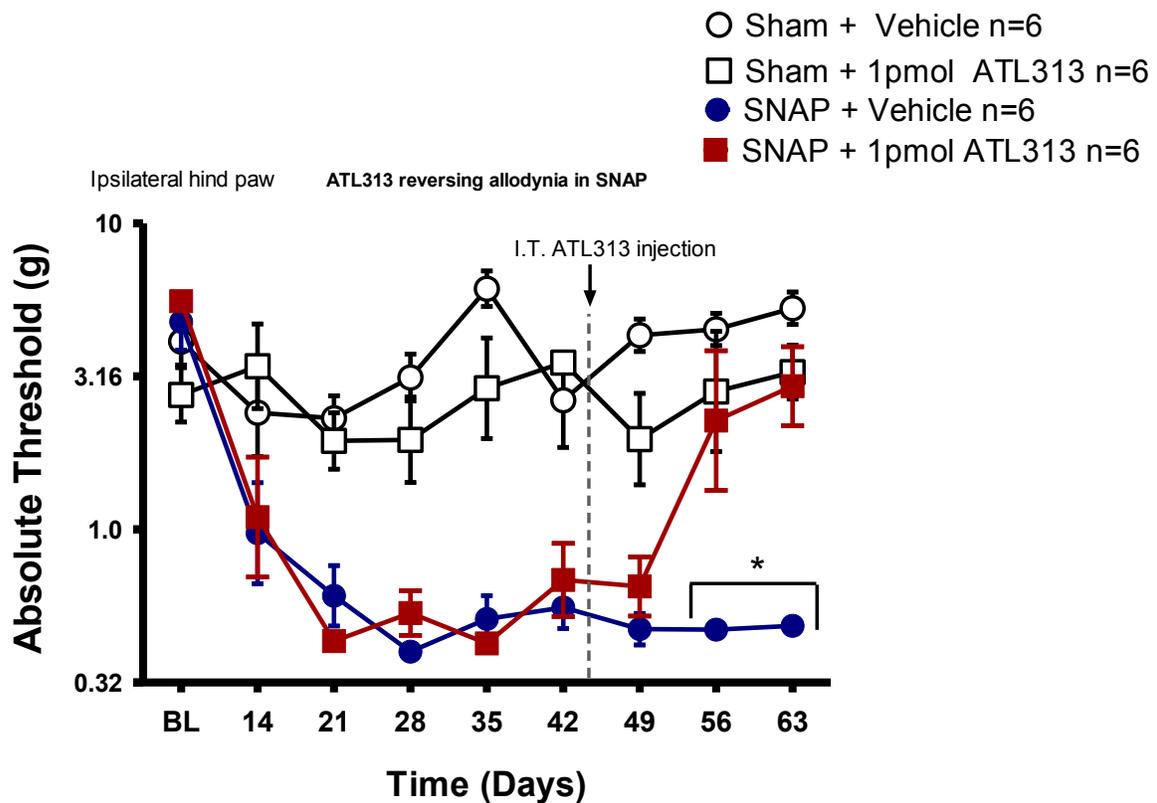
**Task 4. Aim IB2. Spinal Cord Injury (SCI): reversal of acute and chronic central neuropathic pain by IT ATL313.**

Task 4 has been completed.

We have shown that a single IT administration of an A<sub>2A</sub>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<sub>2A</sub>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.



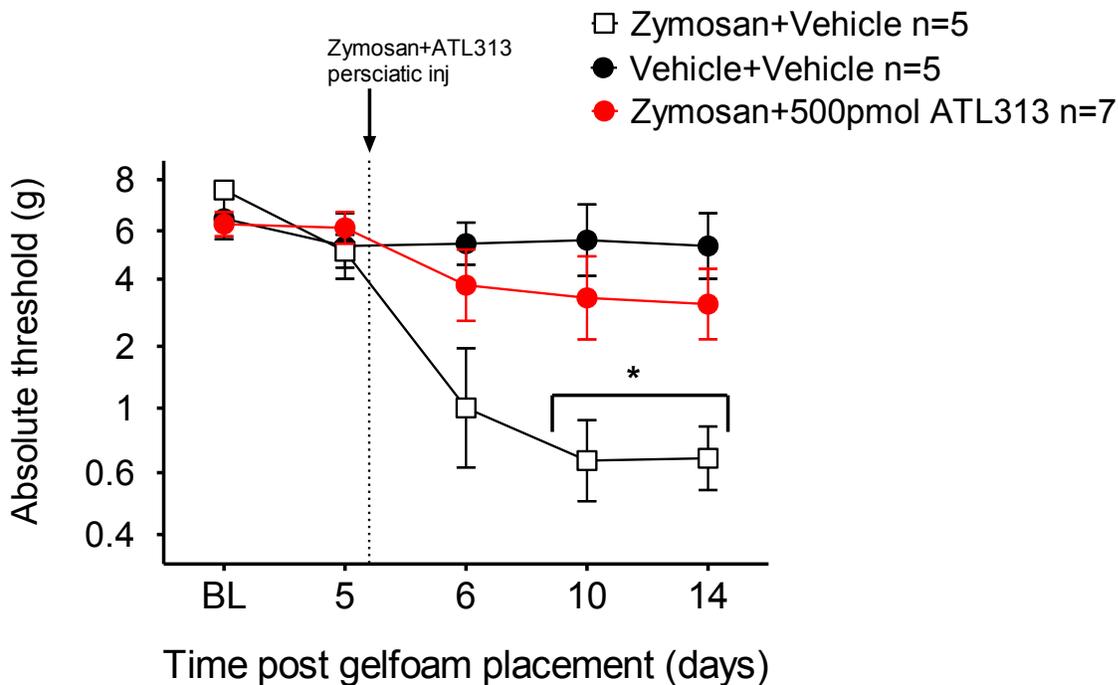
**Figure 6.** Unilateral T13/L1 avulsion SNAP or sham surgery with dura suture was performed and rats were allowed to recover for two weeks. Mechanical thresholds of the ipsilateral hind paw were tested before surgery. At seven weeks post-surgery, a single bolus injection of 1 pmol ATL313 or DMSO vehicle 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.**

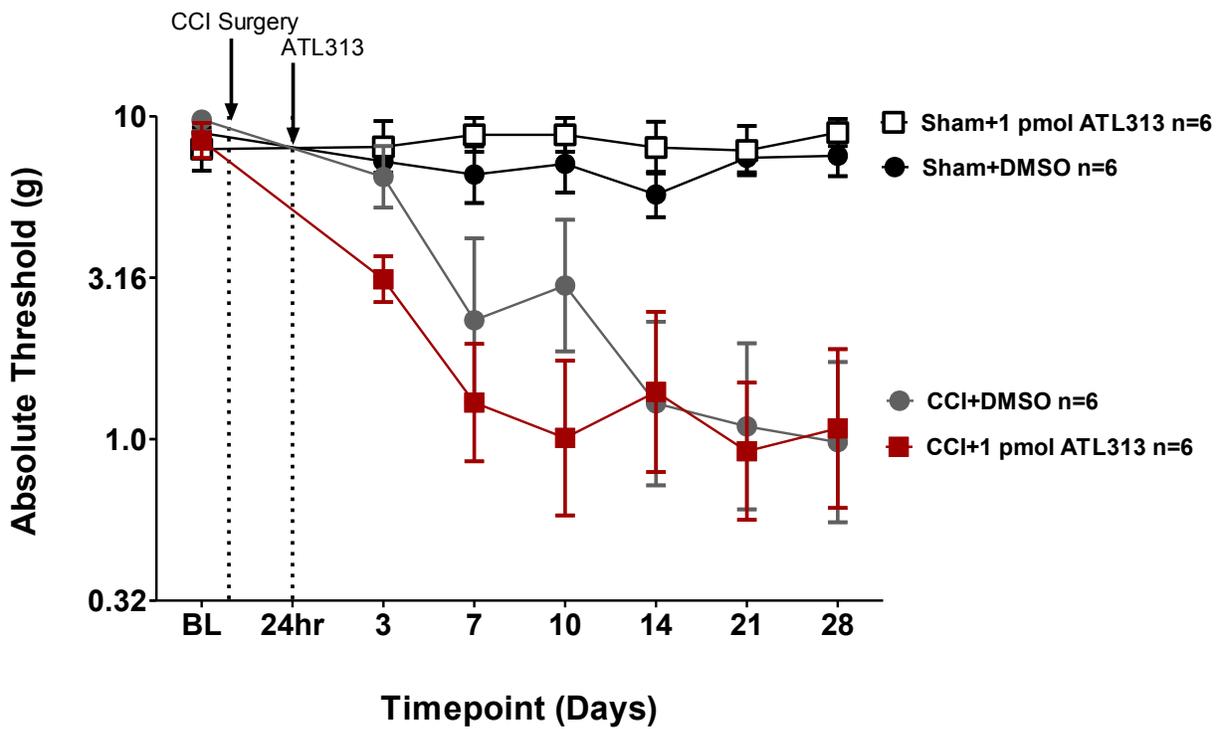
Task 5 has been completed.

We know from Task 3 that ATL313 reverses zymosan-induced allodynia when it is administered both intrathecally and peri-sciatic nerve. 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 nerve 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-sciatic nerve 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

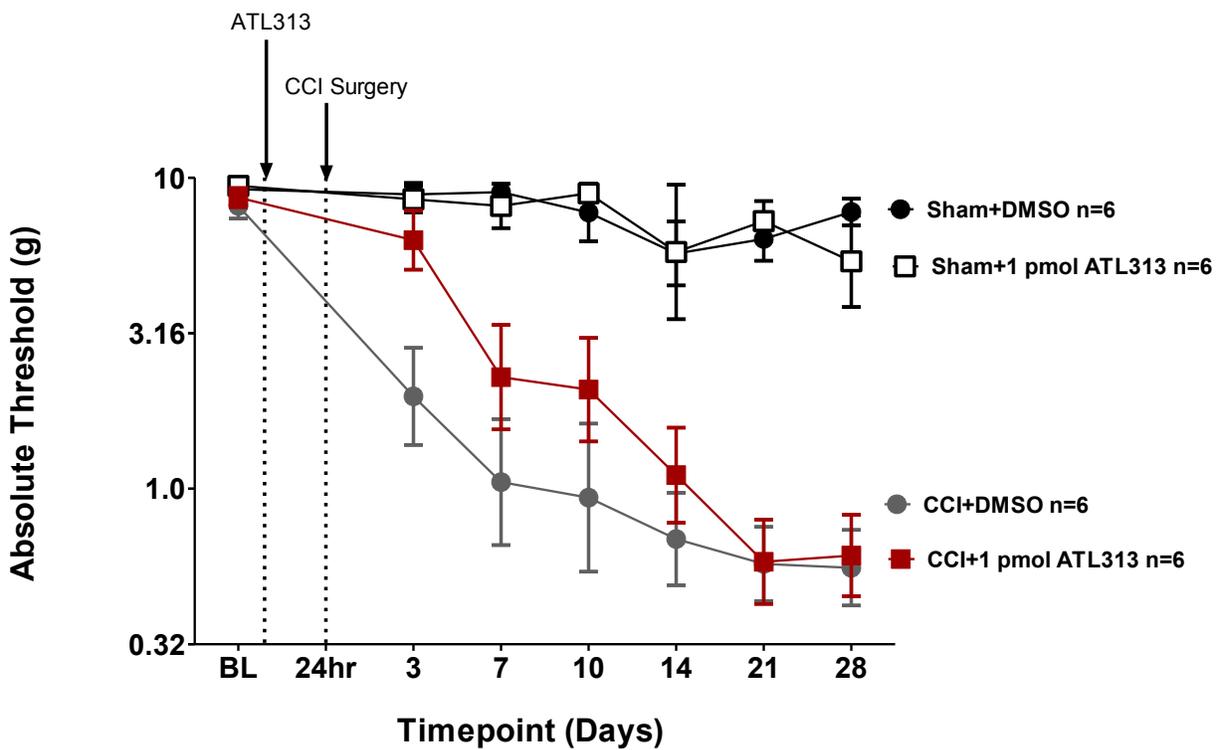
used here. We co-administered the peri-sciatic nerve zymosan and ATL313 5 days after the sciatic nerve gel foam wraps were implanted. Although the rats were not allodynic during the behavioral test on Day 5 prior to the injection, this does not rule out inflammation at the site of the gel foam sciatic 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-sciatic nerve, we believe that perhaps implanting the gel foam sciatic wraps on their own were 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).

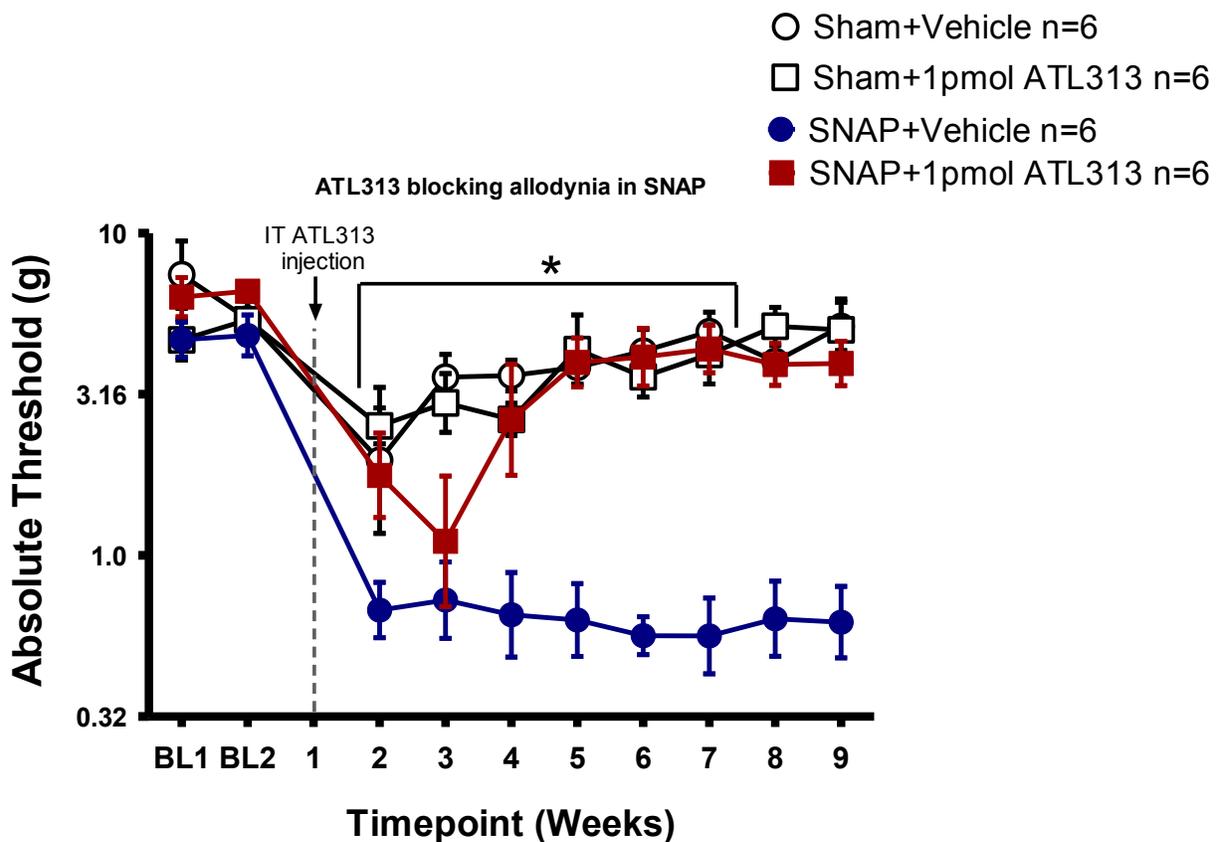


**Figure 9.** Baseline measures on the von Frey test for mechanical allodynia were performed before the ATL313 injection. A single intrathecal injection of 1 pmol ATL313 or 0.01% DMSO vehicle was given. Chronic constriction injury (CCI) surgery was performed twenty-four hours post-ATL313. Rats were tested 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 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).

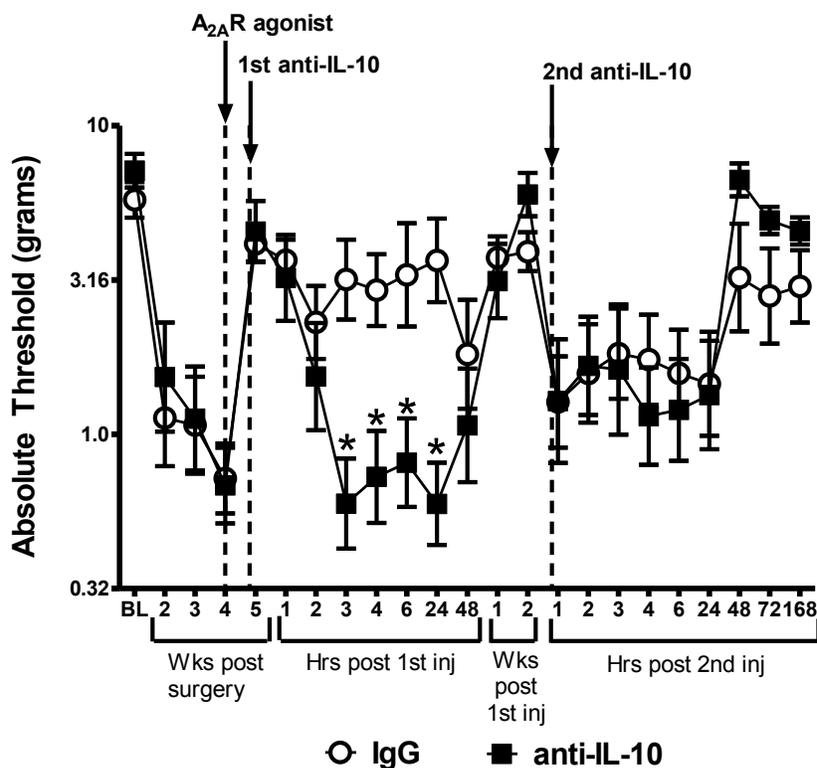
**Milestone 3:** Definition of optimal IT and peri-sciatic nerve ATL313 doses for use in Tasks 8, 10, and 12. Completed analysis of a *single* IT and perisciatic nerve ATL313 dose to: prevent and reverse acute and chronic central neuropathic pain from traumatic spinal cord injury; to prevent and reverse neuropathic pain from inflammatory peripheral neuropathy following either IT or peri-sciatic nerve (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 nerve (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 25 pmol is the optimal peri-sciatic nerve dose.

**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.



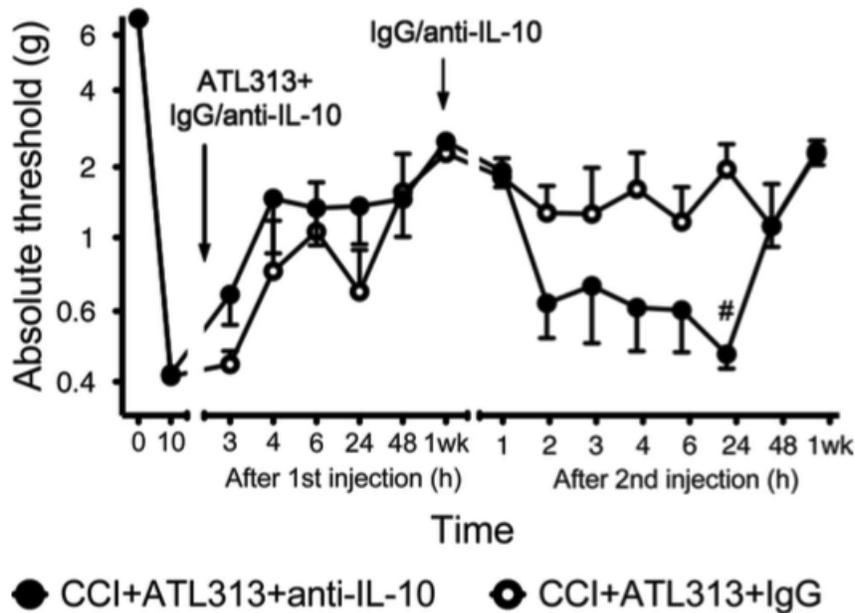
**Figure 11.** Unilateral T13/L1 avulsion SNAP surgery with dura suture was performed and rats were allowed to recover for two weeks. Mechanical thresholds of the ipsilateral hind paw were tested before surgery. Beginning two weeks post-surgery, rats were tested for mechanical allodynia weekly. At four weeks post-surgery, a single bolus injection of a 1  $\mu$ M  $A_{2A}R$  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  $\mu$ g/ml; 10  $\mu$ l) or equivolume and equidose sheep IgG (0.2  $\mu$ g/ml; 10  $\mu$ l) 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 8. Aim IIA2. CCI: characterizing the involvement of IL-10 at spinal cord vs. at sciatic nerve.**

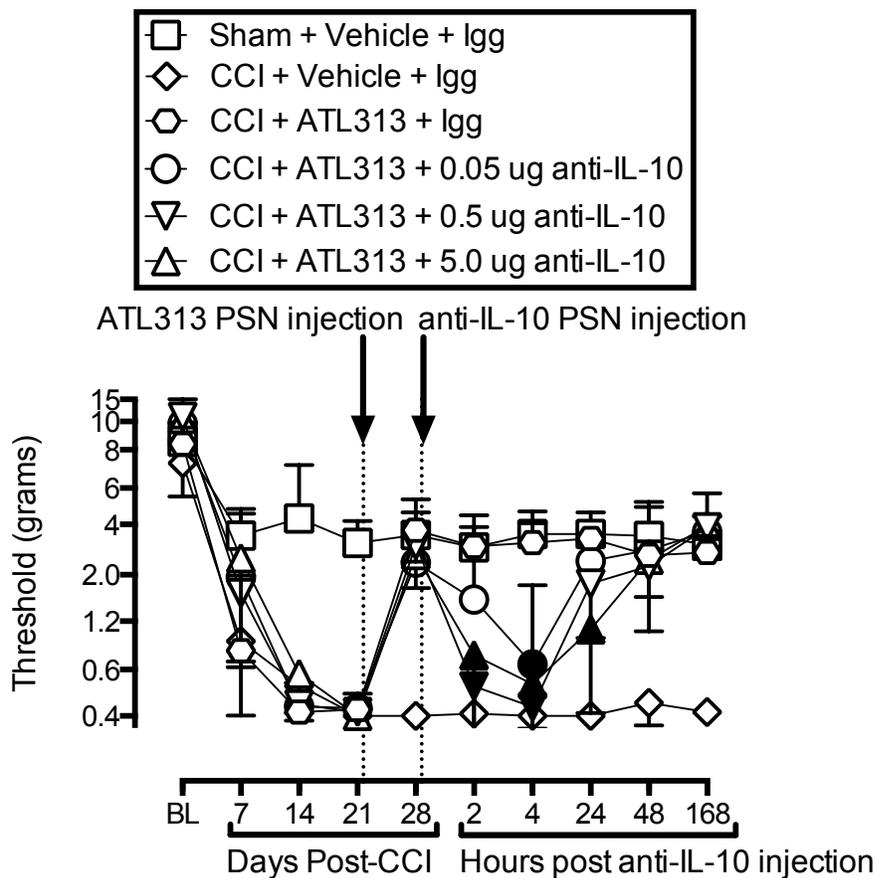
Task 8 has been completed.

Figure 12 below shows that in CCI, IT anti-IL-10 treatment 1 wk after IT  $A_{2A}R$  agonist administration significantly abolishes the pain-relieving effects of the agonist, whereas co-administration of anti-IL-10 and  $A_{2A}R$  agonist does not block the pain-relieving effects. Similarly, Figure 13 below shows that in CCI, perisciatic nerve treatment with an  $A_{2A}R$  agonist produces a pain-relieving effect that is reversed by perisciatic nerve treatment with anti-IL-10. These studies indicate 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 both the level of the sciatic nerve and spinal cord, at least in the first 1 week post-agonist administration.



**Figure 12.** Ten to 14 d after CCI, ATL313 (1  $\mu$ M) was coadministered intrathecally with either IL-10 neutralizing antibodies (0.2  $\mu$ g in 10  $\mu$ l) or equivolume IgG. The behavioral response to mechanical stimuli, assessed by von Frey testing, was measured before surgery, before drug administration, and after drug administration in the ipsilateral hind-paw. No significant effect was found when the drugs were coadministered (main effect of drug,  $p = 0.31$ ,  $F_{(3,20)} = 1.263$ ). Allowing 1 week washout, a second injection of the IL-10 neutralizing antibodies (0.2  $\mu$ g in 10  $\mu$ l) or equivolume IgG was administered. The IL-10 neutralizing antibodies reversed the effect of ATL313 at 24 h but had returned within 48 h to that comparable before neutralizing antibody administration (main effect of drug,  $p < 0.05$ ,  $F_{(1,8)} = 6.339$ ; main effect of time,  $p < 0.0001$ ,

$F_{(8,80)} = 5.921$ ; interaction,  $p < 0.001$ ,  $F_{(8,80)} = 2.975$ ;  $n = 6$  per group). Data are presented as mean  $\pm$  SEM. Solid circle, CCI plus ATL plus IL-10 neutralizing antibodies; open circle, CCI plus ATL plus IgG vehicle. \* $p < 0.05$ , # $p < 0.01$  antagonist against vehicle at the respective time point.



**Figure 13.** 14 days after CCI + perisciatic nerve catheter surgery, ATL313 (25 pmol in 50  $\mu$ l) was perisciatic nerve followed 1 week later by either IL-10 neutralizing antibodies (0.05, 0.5, 5  $\mu$ g in 50  $\mu$ l) or 5  $\mu$ g IgG. The behavioral response to mechanical stimuli, assessed by von Frey testing, was measured before surgery, before drug administration, and after drug administration in the ipsilateral hindpaw. The IL-10 neutralizing antibodies reversed the effect of ATL313 but had returned within 48 h to that comparable before neutralizing antibody administration (main effect of drug,  $p < 0.0001$ ,  $F_{(5,27)} = 26.27$ ; main effect of time,  $p < 0.0001$ ,  $F_{(9,243)} = 105.9$ ; interaction,  $p < 0.0001$ ,  $F_{(45,243)} = 8.377$ ;  $n = 3-7$  per group). Data are presented as mean  $\pm$  SEM. Black-filled symbols represent a significant difference from CCI + ATL313 + Igg at respective time point ( $p < 0.05$ ).

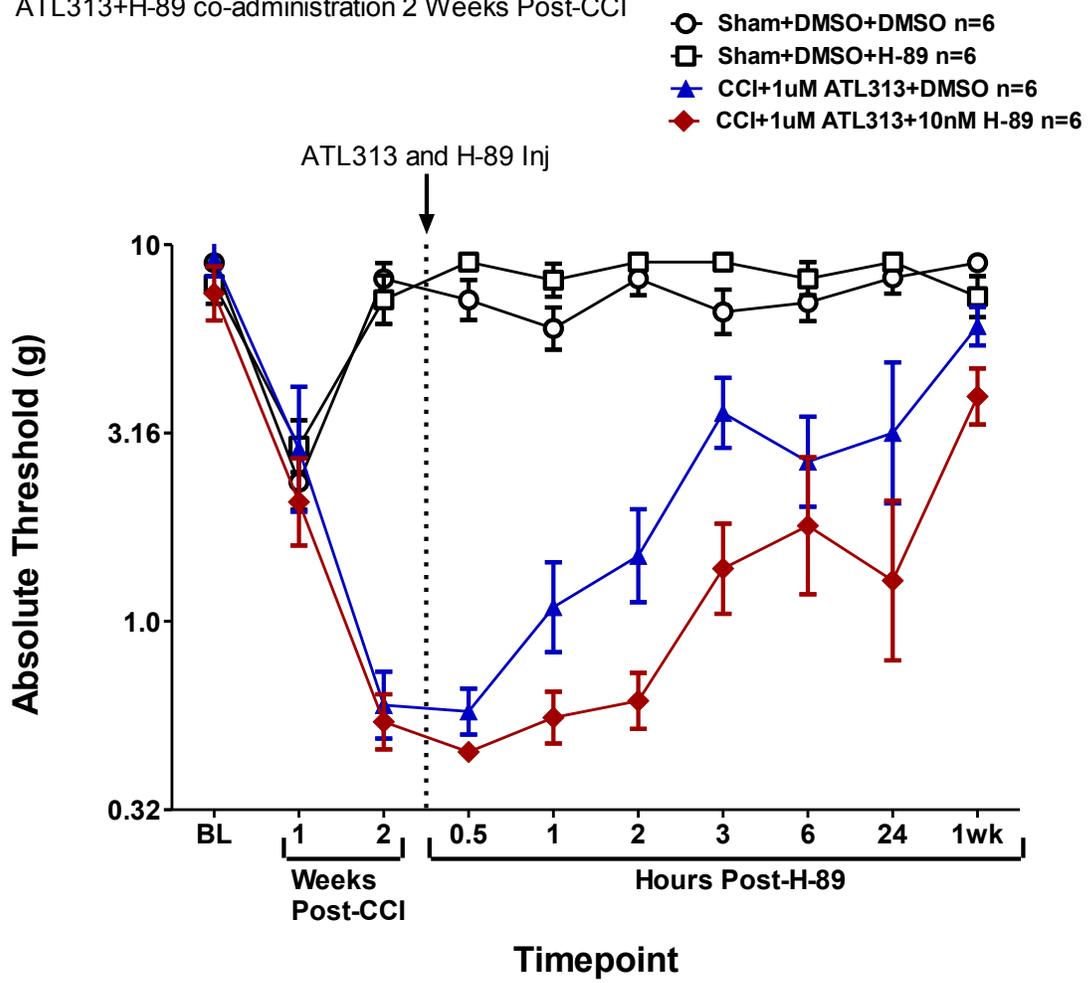
**Task 9. Aim IIB1. SNL: protein kinase (PK) C/PKA involvement with acute and chronic peripheral neuropathic pain.**

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, H-89, to determine the involvement of PKA in the mechanism by which A<sub>2A</sub>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 14 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 15 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 16 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 are expanded on in task 10, which will give a more complete analysis of the role of PKA in ATL313-induced reversal of allodynia at the level of sciatic nerve and spinal cord. We also know from task 13 below that administering PKA and PKC inhibitors to cultured glial cells blocked TNF and but not IL-10 production, suggesting that while PKA and PKC may play a role in A<sub>2A</sub>R agonist effects, there are also other mechanisms involved in this phenomenon.

A.

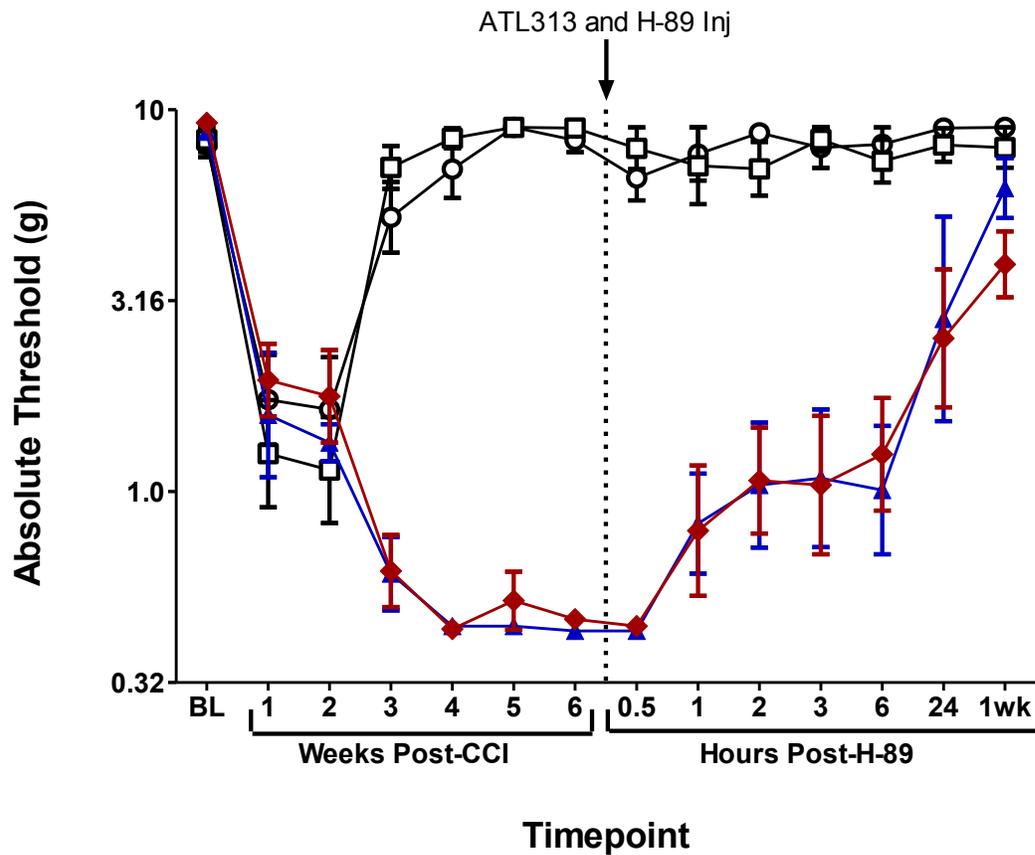
ATL313+H-89 co-administration 2 Weeks Post-CCI



B.

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

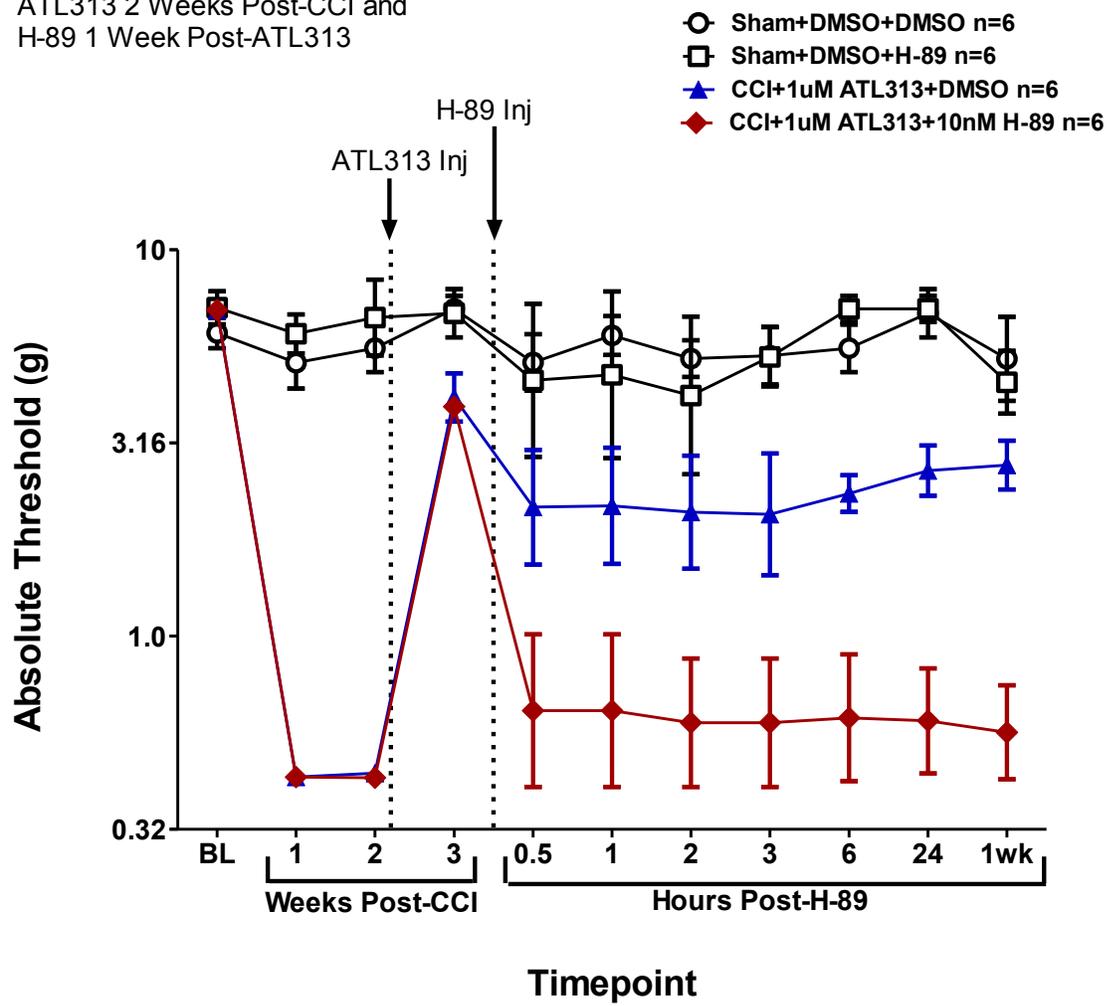
- Sham+DMSO+DMSO n=6
- Sham+DMSO+10nM H-89 n=6
- ▲ CCI+1uM ATL313+DMSO n=6
- ◆ CCI+1uM ATL313+10nM H-89 n=6



**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 either 2 (A) or 6 (B) weeks post-surgery, after behavioral verification of allodynia, a single bolus injection of 1  $\mu$ M 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 presence of the PKA inhibitor, suggesting that PKA is playing a role. However, at the 6 week timepoint there does not seem to be an effect of PKA.

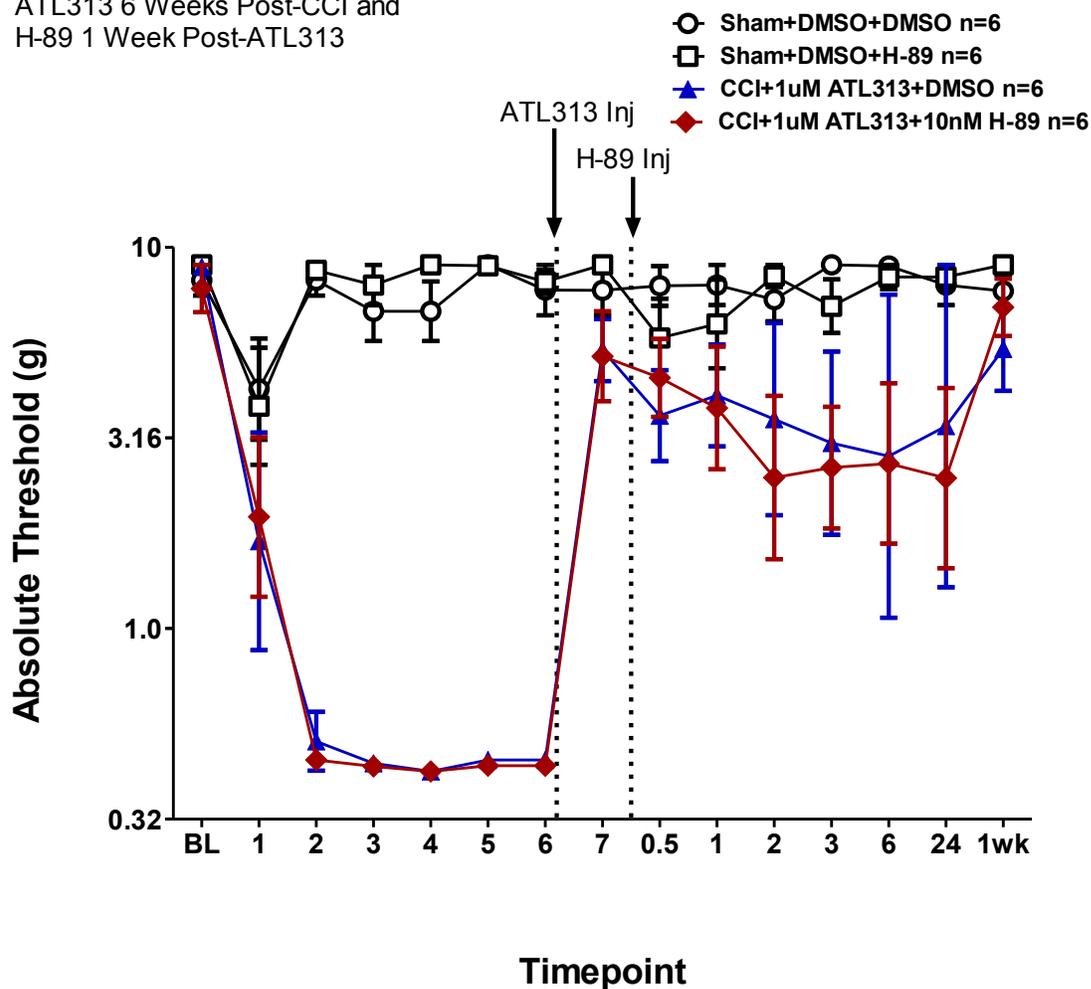
A.

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

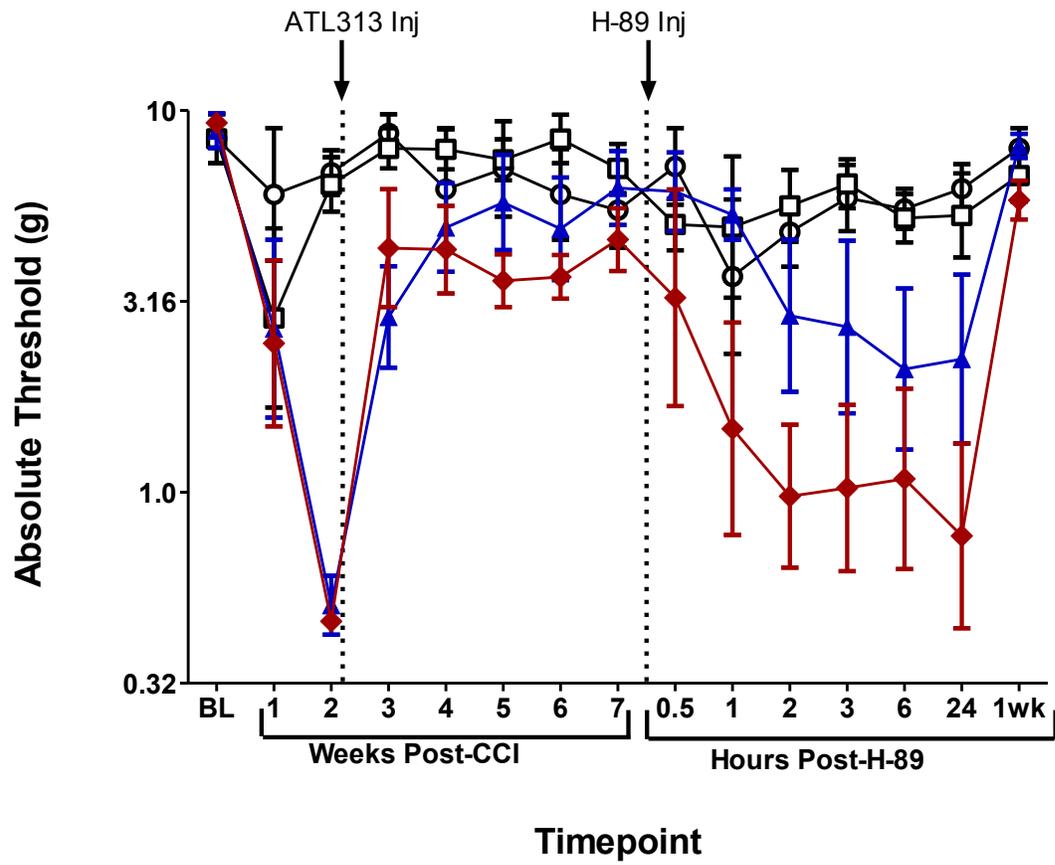


**Figure 15.** Unilateral chronic constriction injury (CCI) or sham surgery was performed and rats were allowed to recover for one week. Mechanical thresholds of the ipsilateral hind paw were tested before surgery. At either 2 (A) or 6 (B) weeks post-surgery, after behavioral verification of allodynia, a single bolus injection of 1  $\mu$ M 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.

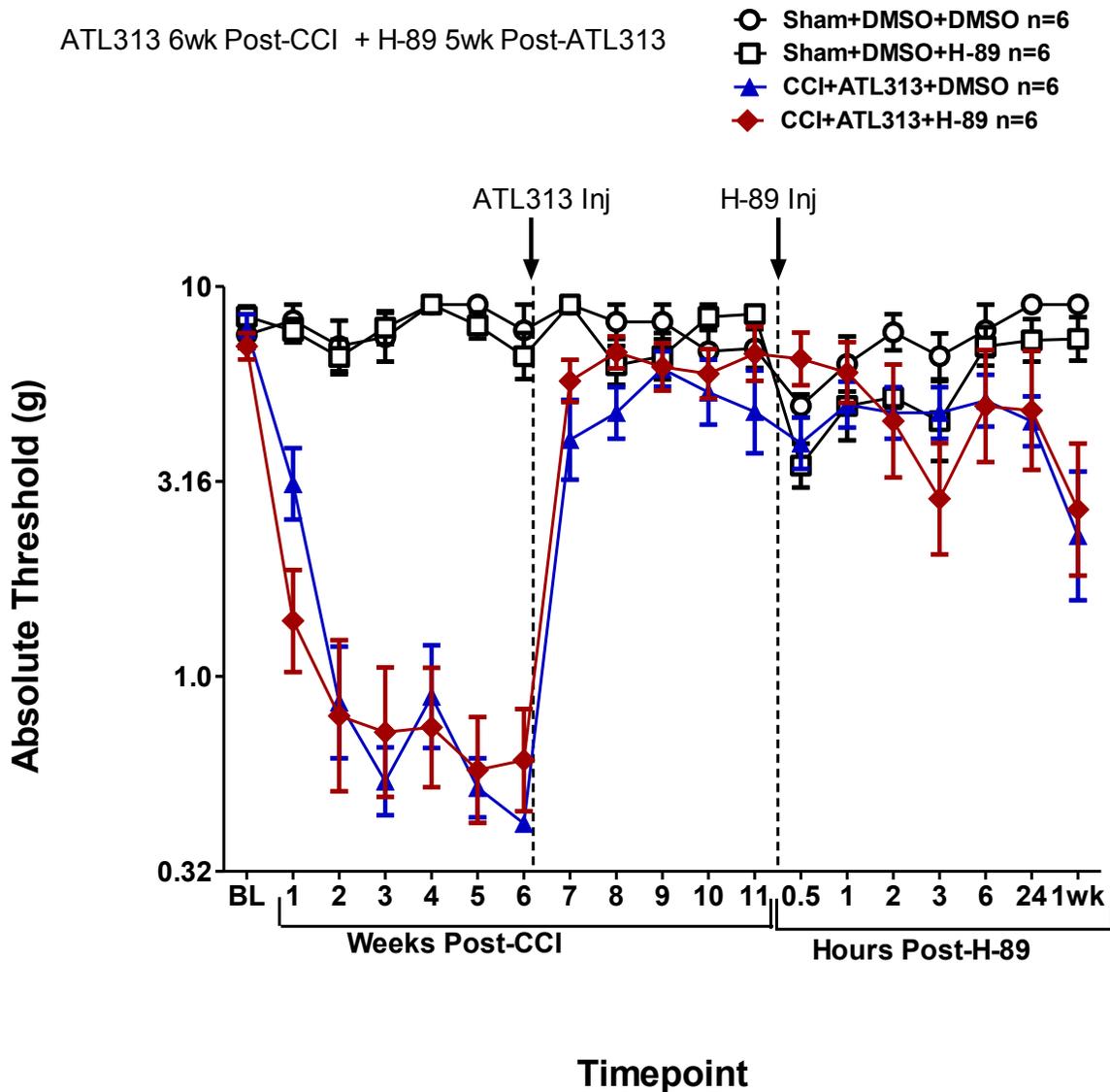
A.

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

- Sham+DMSO+DMSO n=5
- Sham+DMSO+10nM H-89 n=5
- ▲ CCI+1uM ATL313+DMSO n=5
- ◆ CCI+1uM ATL313+10nM H-89 n=5



B.



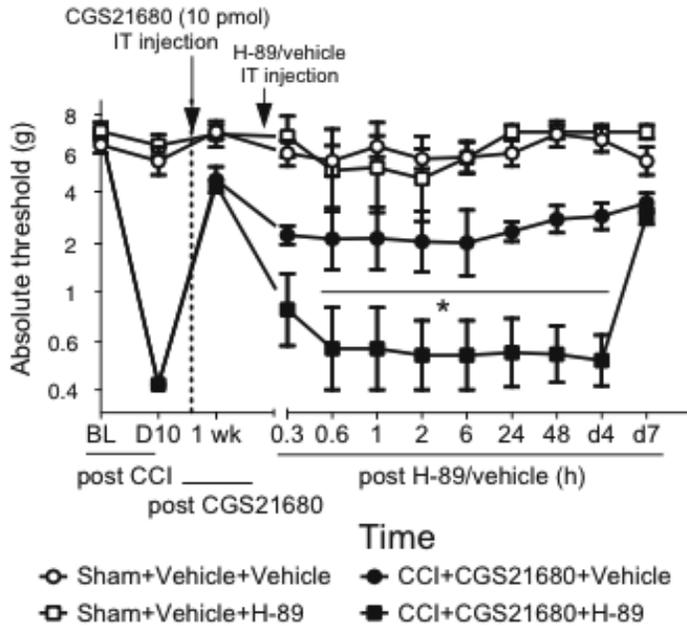
**Figure 16.** Unilateral chronic constriction injury (CCI) or sham surgery was performed and rats were allowed to recover for one week. Mechanical thresholds of the ipsilateral hind paw were tested before surgery. At 2 (A) or (B) 6 weeks post-surgery, after behavioral verification of allodynia, a single bolus injection of 1 $\mu$ M 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 10. Aim IIB2. CCI: PKC/PKA involvement at spinal cord & at sciatic nerve.**

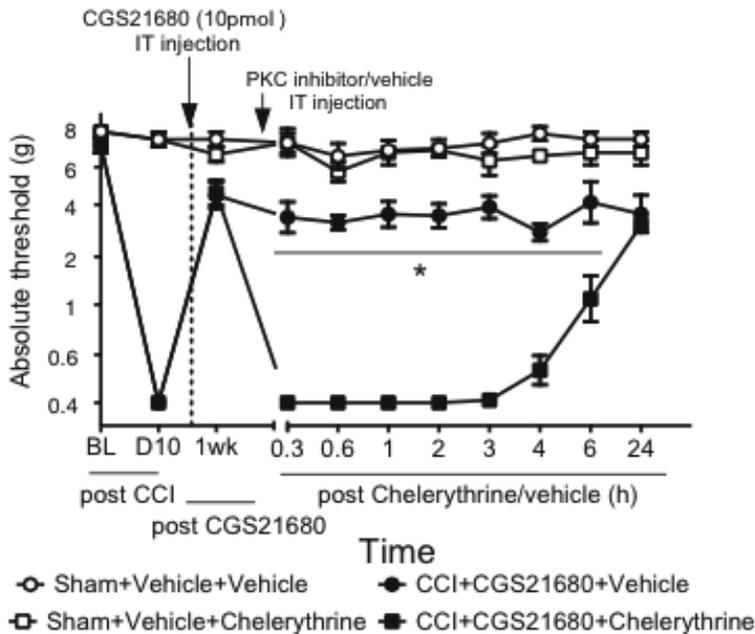
Task 10 is completed.

Figure 17 below shows that in CCI, PKA inhibitor, H-89, and PKC inhibitor, chelerythrine, administered IT 1 wk after IT  $A_{2A}R$  agonist administration significantly abolish the pain-relieving effects of the  $A_{2A}R$  agonist. Similarly, Figure 18 below shows that in CCI, perisciatic nerve treatment with an  $A_{2A}R$  agonist produces a pain-relieving effect that is reversed by perisciatic nerve treatment with H-89 1 wk later. These studies indicate that protein kinase A and C in the spinal cord and protein kinase A in the sciatic nerve are critically involved in the mechanism by which  $A_{2A}R$  agonists exert their anti-allodynic effects, at least in the first 1 week post-agonist administration.

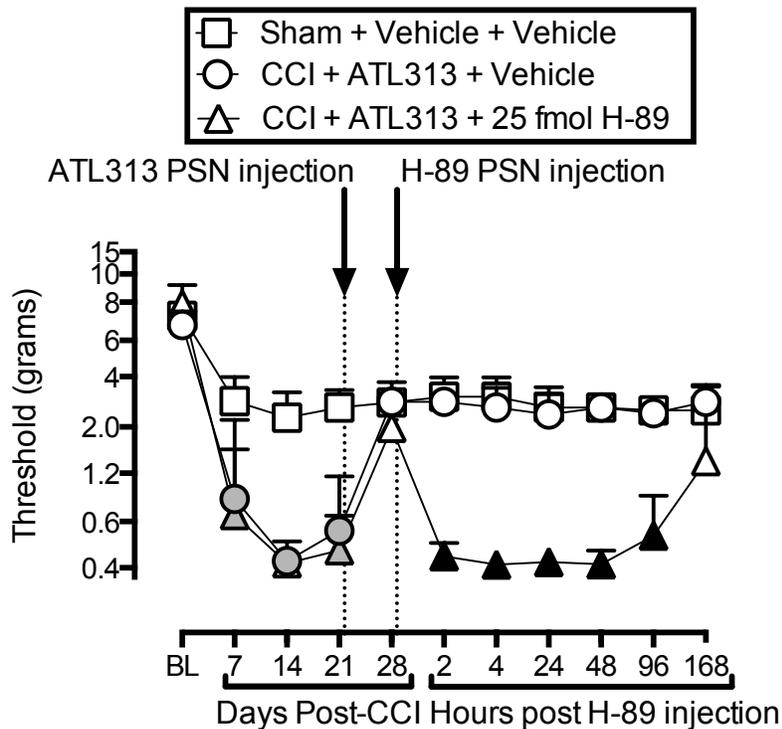
### A. PKA inhibitor



### B. PKC inhibitor



**Figure 17.** An A2AR agonist, CGS21680 attenuates neuropathic allodynia via PKA and PKC. Mechanical allodynia induced by CCI was reversed by CGS21680 (10 pmol administered IT). One week after CGS21680, a PKA inhibitor (H-89, 1 fmol) was administered IT. H-89 reversed the anti-allodynic effects of CGS21680 back to full allodynia for at least 4days (A). One week after CGS21680, a PKC inhibitor (chelerythrine, 60 fmol) was administered IT. Chelerythrine transiently reversed the anti-allodynic effects of CGS21680 back to full allodynia for 6 h (B)  $P < 0.05$  compared to CCI + ATL + vehicle group;  $n = 6$  rats/group.

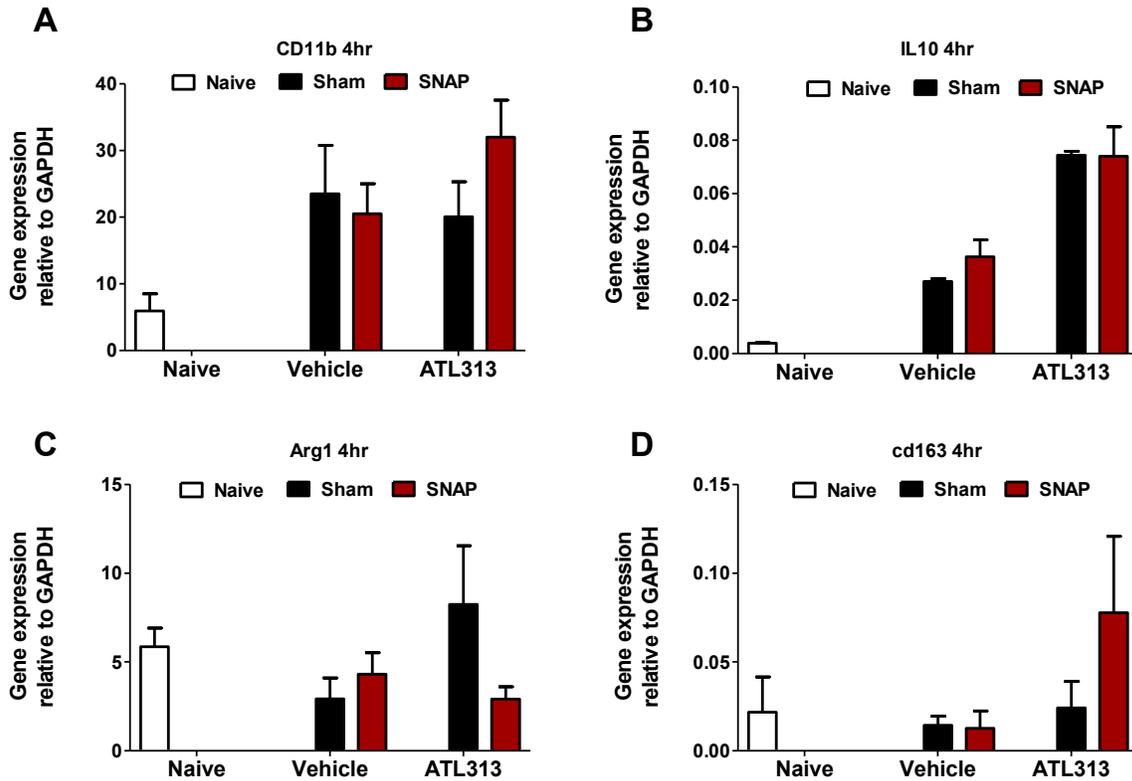


**Figure 18.** 14 days after CCI + perisciatic nerve catheter surgery, ATL313 (25 pmol in 50  $\mu$ l) was perisciatic nerve followed 1 week later by the PKA antagonist H-89 (25 fmol in 50  $\mu$ l) or vehicle. The behavioral response to mechanical stimuli, assessed by von Frey testing, was measured before surgery, before drug administration, and after drug administration in the ipsilateral hindpaw. H-89 reversed the effect of ATL313 but had returned within 1 week to that comparable before H-89 administration (main effect of drug,  $p < 0.001$ ,  $F_{(4,17)} = 8.44$ ; main effect of time,  $p < 0.001$ ,  $F_{(10,70)} = 36.84$ ; interaction,  $p < 0.001$ ,  $F_{(40,170)} = 2.89$ ;  $n = 4-6$  per group). Data are presented as mean  $\pm$  SEM. Black-filled symbols represent a significant difference from CCI + ATL313 + Vehicle at respective time point ( $p < 0.05$ ). Gray-filled symbols represent a significant difference from Sham + Vehicle + Vehicle at respective time point ( $p < 0.05$ ).

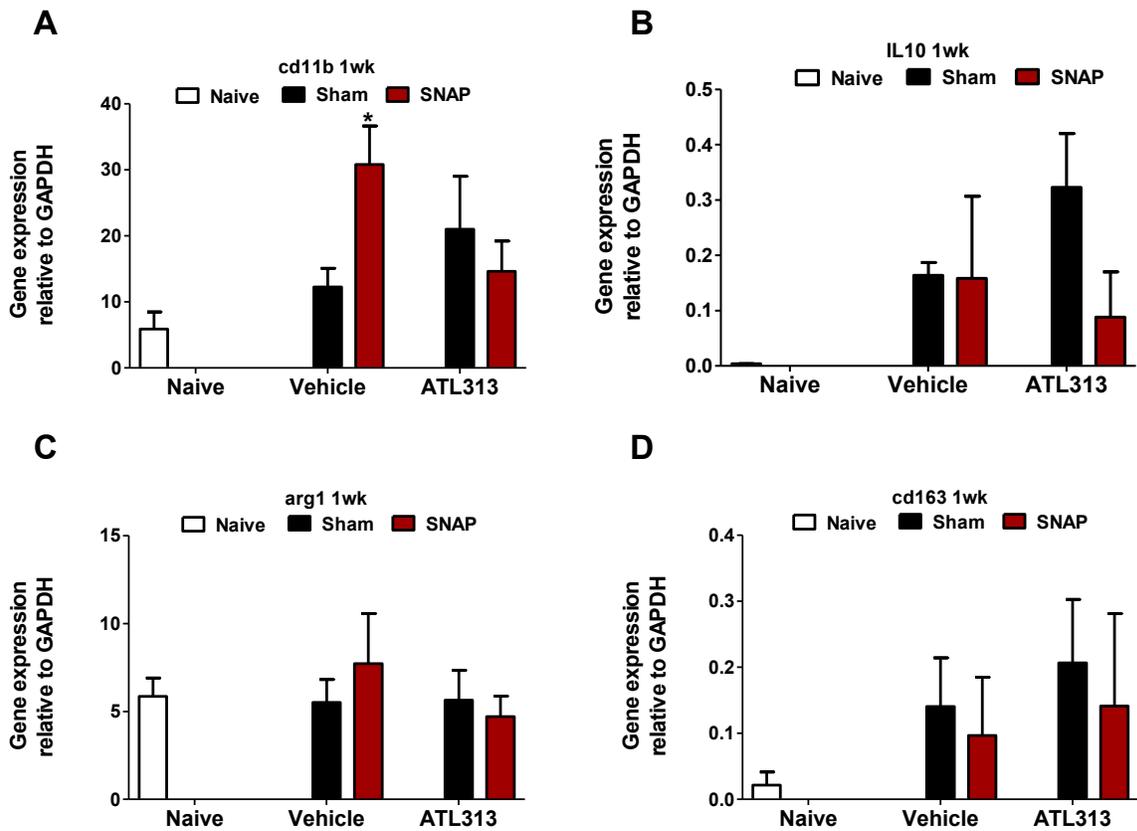
### Task 11. IT ATL313 induction of alternative activation

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

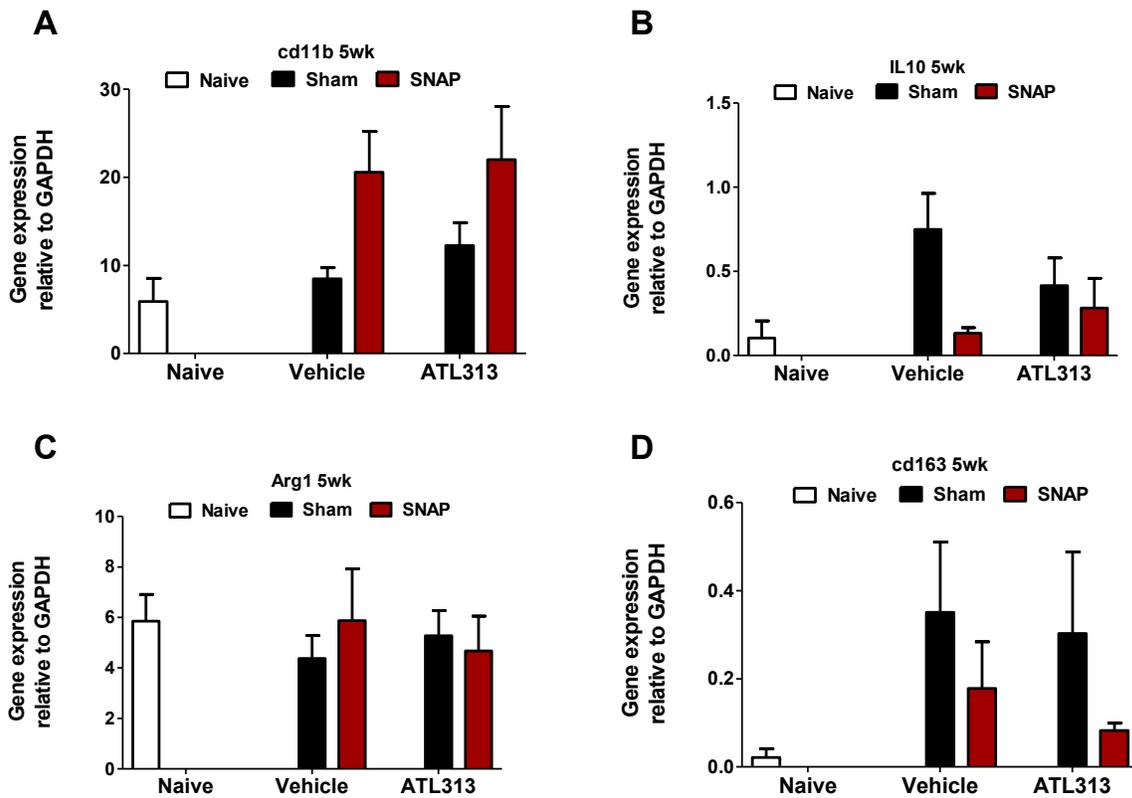
gene expression of proinflammatory markers at these timepoints as well. We did not find any significant changes in alternative activation gene expression markers (IL-10, arginase-1 (Arg1), cd163) at any timepoint or for any tissue type (T13/L1 tissue shown). We also did not detect significant changes in protein expression of these markers when tested by ELISA or western blot for either tissue type. 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 19.** 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  $\mu$ M 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 20.** 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  $\mu$ M 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). \* $p < 0.05$ .

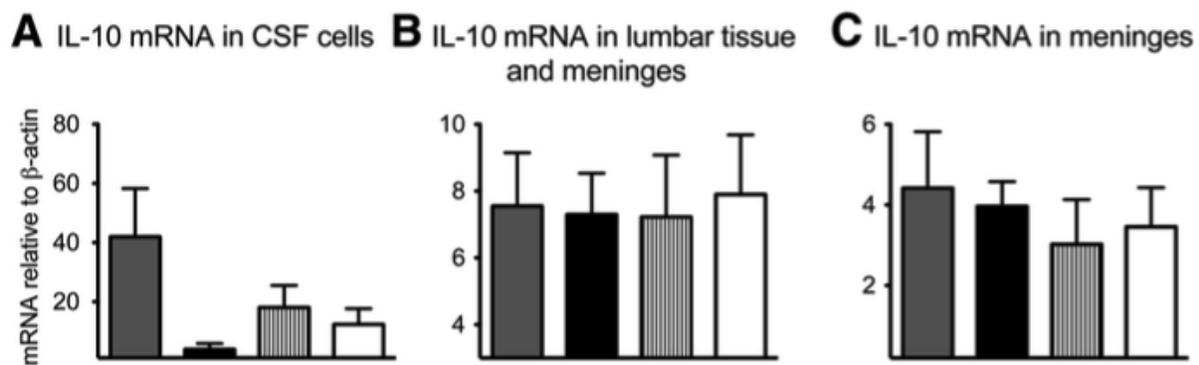


**Figure 21.** Unilateral T13/L1 SNAP SCI or sham plus dura suture surgery was performed and rats were allowed to recover for two weeks. At two weeks post-surgery, after behavioral verification of allodynia, a single bolus injection of 1  $\mu$ M ATL313 or DMSO vehicle was administered intrathecally. 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).

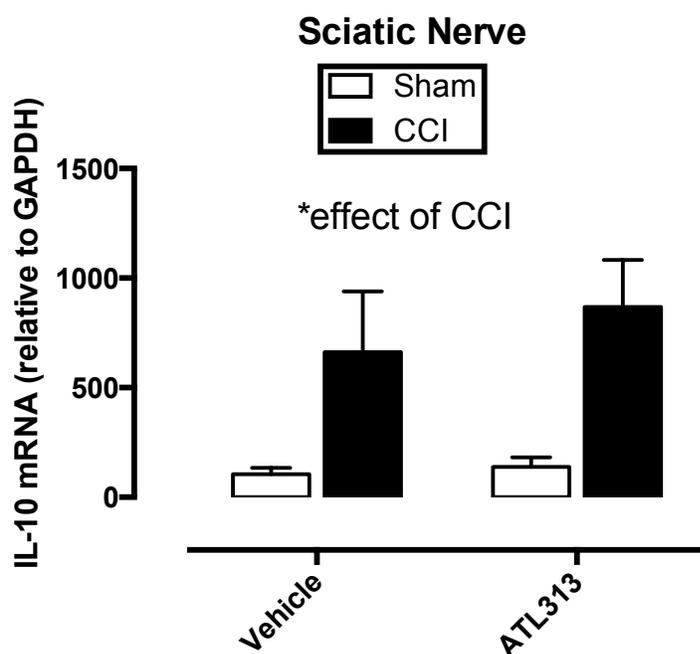
**Task 12. Aim IIC2. CCI: IT vs. peri-sciatic ATL313 induction of alternative activation.**

Task 12 has been completed.

Figure 22 below shows that in CCI, IL-10 mRNA 1 wk after IT  $A_{2A}$ R agonist administration is significantly upregulated in CSF cells, but not in spinal cord tissue (with and without meninges). Figure 23 below shows that in CCI, IL-10 mRNA is significantly upregulated in sciatic nerve but that this IL-10 mRNA is not altered by  $A_{2A}$ R agonist treatment. Contrastingly, Figures 24 and 25 show that in CCI after perisciatic nerve administration of an  $A_{2A}$ R agonist, there are no significant differences in any alternative activation markers measured in either the sciatic nerve or lumbar spinal cord (i.e. cd11b, IL-10, Arginase-1, or cd163). These studies indicate that the alternative activation marker and anti-inflammatory cytokine IL-10 is critically involved in the mechanism by which IT  $A_{2A}$ R agonists exert their anti-allodynic effects at the level of the spinal cord, particularly in CSF cells, at least in the first 1 week post-agonist administration. More work is necessary, however, to determine how perisciatic nerve  $A_{2A}$ R agonists exert their effects.

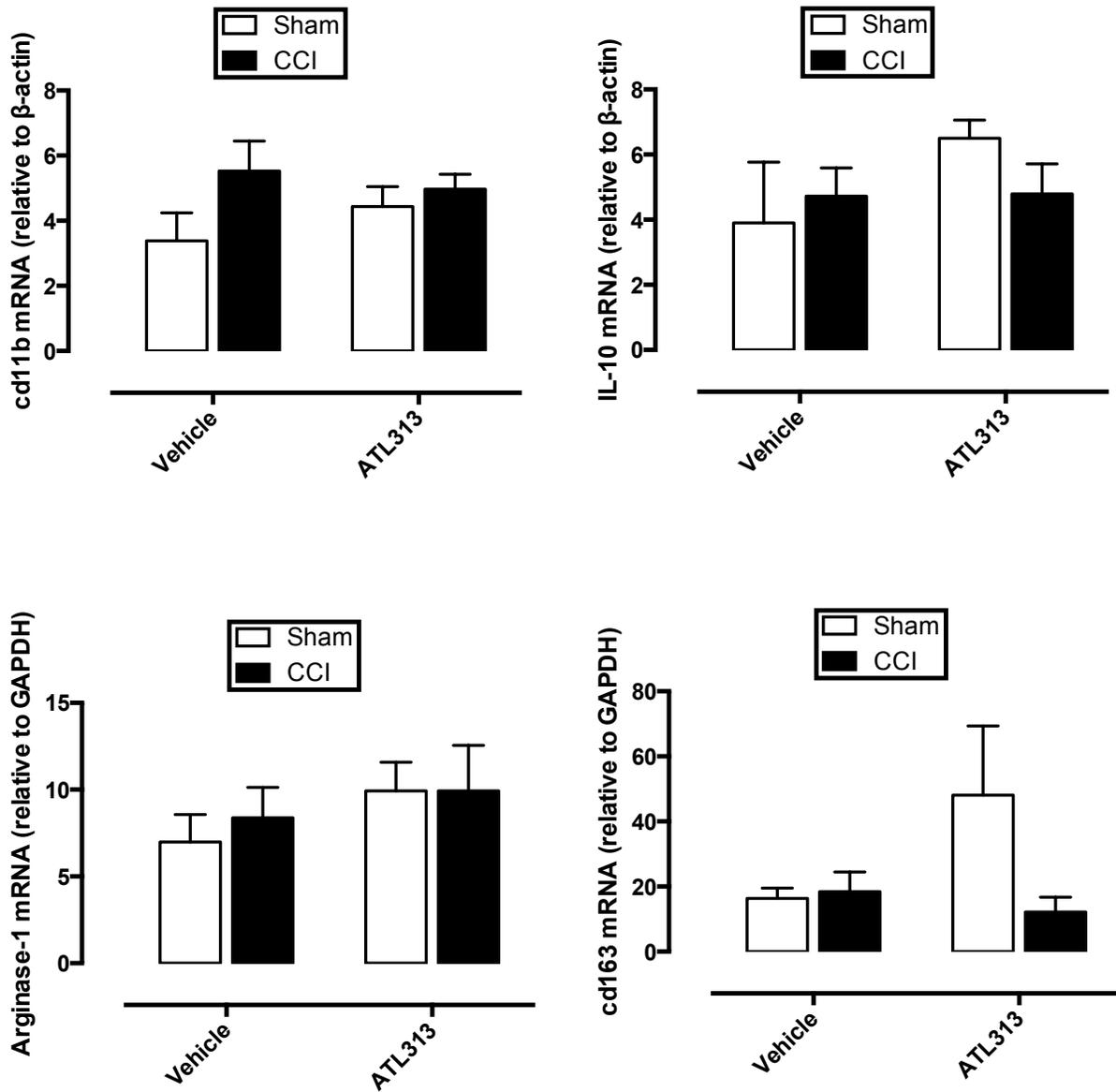


**Figure 22.** Gene expression mRNA expression in tissue collected 1 week after administration of ATL313 (1  $\mu\text{M}$ ) or vehicle in CCI or sham-operated rats. Intrathecal drug administration was 10 days after CCI/sham surgery ( $n = 7\text{--}10/\text{group}$ ). There was a significant elevation in *IL-10* mRNA in CSF cells after ATL313 administration compared with vehicle controls (two-way ANOVA, drug effect,  $p < 0.05$ ,  $F_{(1,21)} = 4.604$ ; surgery effect,  $p < 0.46$ ,  $F_{(1,21)} = 0.57$ ) (A). There was no significant change in *IL-10* mRNA in lumbar and meningeal tissue (two-way ANOVA, drug effect,  $p < 0.898$ ,  $F_{(1,21)} = 0.017$ ; surgery effect,  $p < 0.933$ ,  $F_{(1,21)} = 0.007$ ) (B) or meninges alone (drug effect,  $p < 0.332$ ,  $F_{(1,21)} = 0.976$ ) (C). Data are presented as mean  $\pm$  SEM and analyzed using ANOVA. Gray bar, CCI plus ATL313 (1  $\mu\text{M}$ ); solid bar, CCI plus vehicle; vertical striped bar, sham plus ATL313 (1  $\mu\text{M}$ ); white bar, sham plus vehicle. \* $p < 0.05$ .



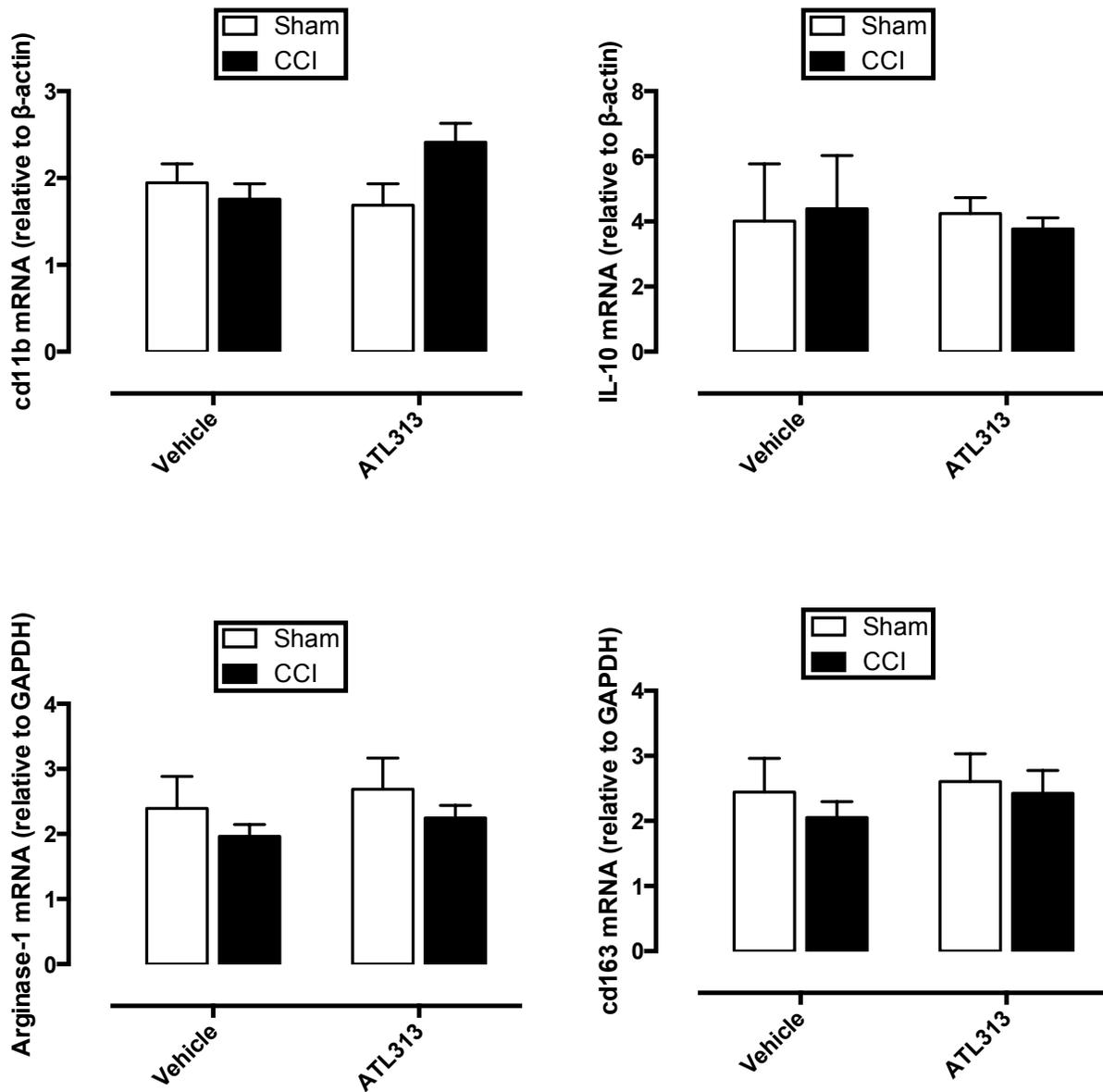
**Figure 23.** Gene expression mRNA expression in tissue collected 1 week after administration of ATL313 (1  $\mu\text{M}$ ) or vehicle in CCI or sham-operated rats. Intrathecal drug administration was 14 days after CCI/sham surgery ( $n = 8/\text{group}$ ). There was a significant elevation in *IL-10* mRNA in after CCI vs. sham controls (two-way ANOVA, surgery effect,  $p < 0.05$ ,  $F_{(1,28)} = 13.14$ ). There was no significant change in *IL-10* mRNA after ATL313 administration. \* $p < 0.001$ .

## Sciatic Nerve



**Figure 24.** Unilateral CCI + perisciatic nerve catheter or sham 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 25 pmol ATL313 or vehicle was administered perisciatic nerve. One week later, rats were overdosed and perfused and the ipsilateral sciatic nerve was dissected out for real-time PCR analysis. All groups were  $n=4-7$ /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).

## Lumbar Spinal Cord



**Figure 25.** Unilateral CCI + perisciatic nerve catheter or sham 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 25 pmol ATL313 or vehicle was administered perisciatic nerve. One week later, rats were overdosed and perfused and the ipsilateral lumbar spinal cord was dissected out for real-time PCR analysis. All groups were n=4-7/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 26 below shows that A<sub>2A</sub>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 27 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 28 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 A<sub>2A</sub>R 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 A<sub>2A</sub>R 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.

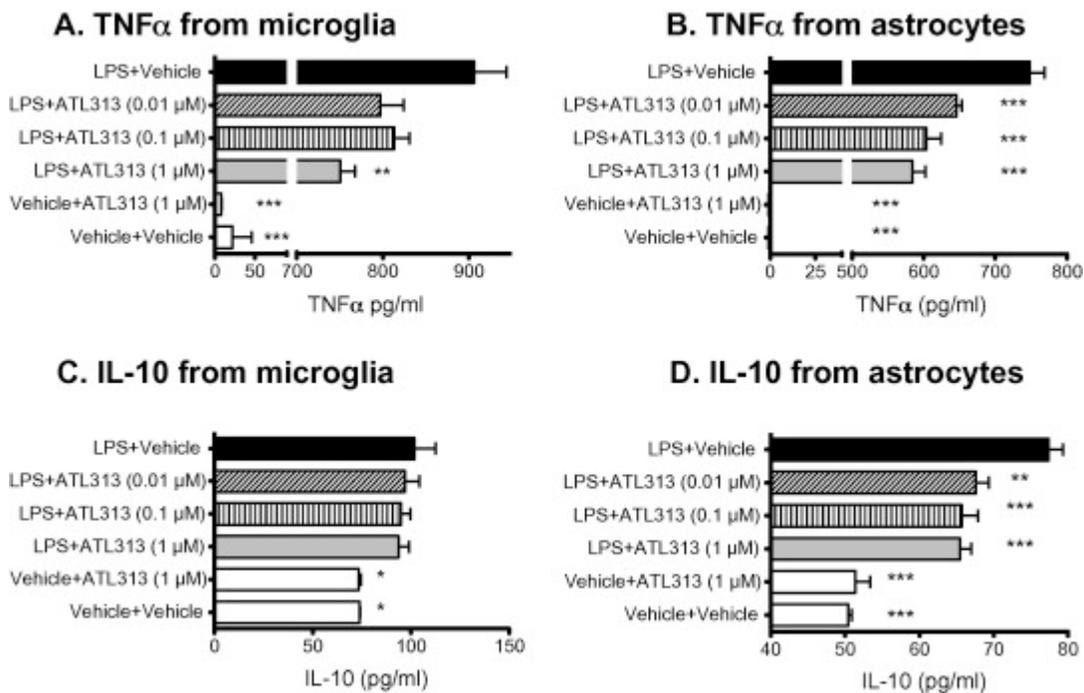


Fig. 26. An A<sub>2A</sub>R agonist downregulates TNF $\alpha$  in central immune cells. (A) TNF $\alpha$  release (pg/ml) from neonatal cortical microglia and (B) astrocytes incubated for 24 h with LPS is attenuated by co-administration of ATL313.  $n = 3/4$  wells/group and the experiment was replicated 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.

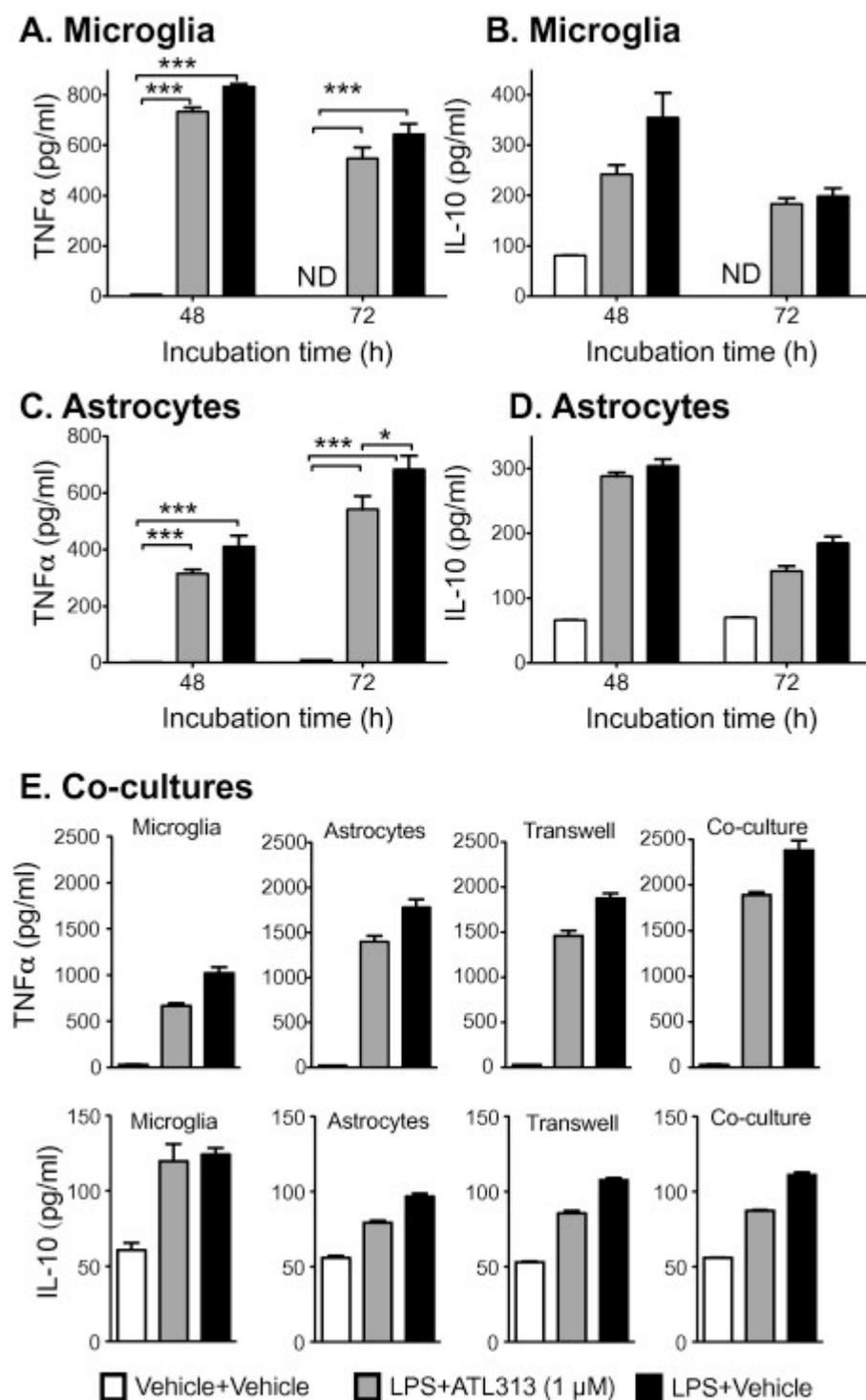


Fig. 27. TNF $\alpha$  and IL-10 produced by glial cells *in vitro* in response to LPS  $\pm$  ATL313 are unaffected by duration of incubation or locality of other glial cells. TNF $\alpha$  release (pg/ml) and IL-10 release (pg/ml) from neonatal cortical microglia incubated for 48 h and 72 h produced 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.

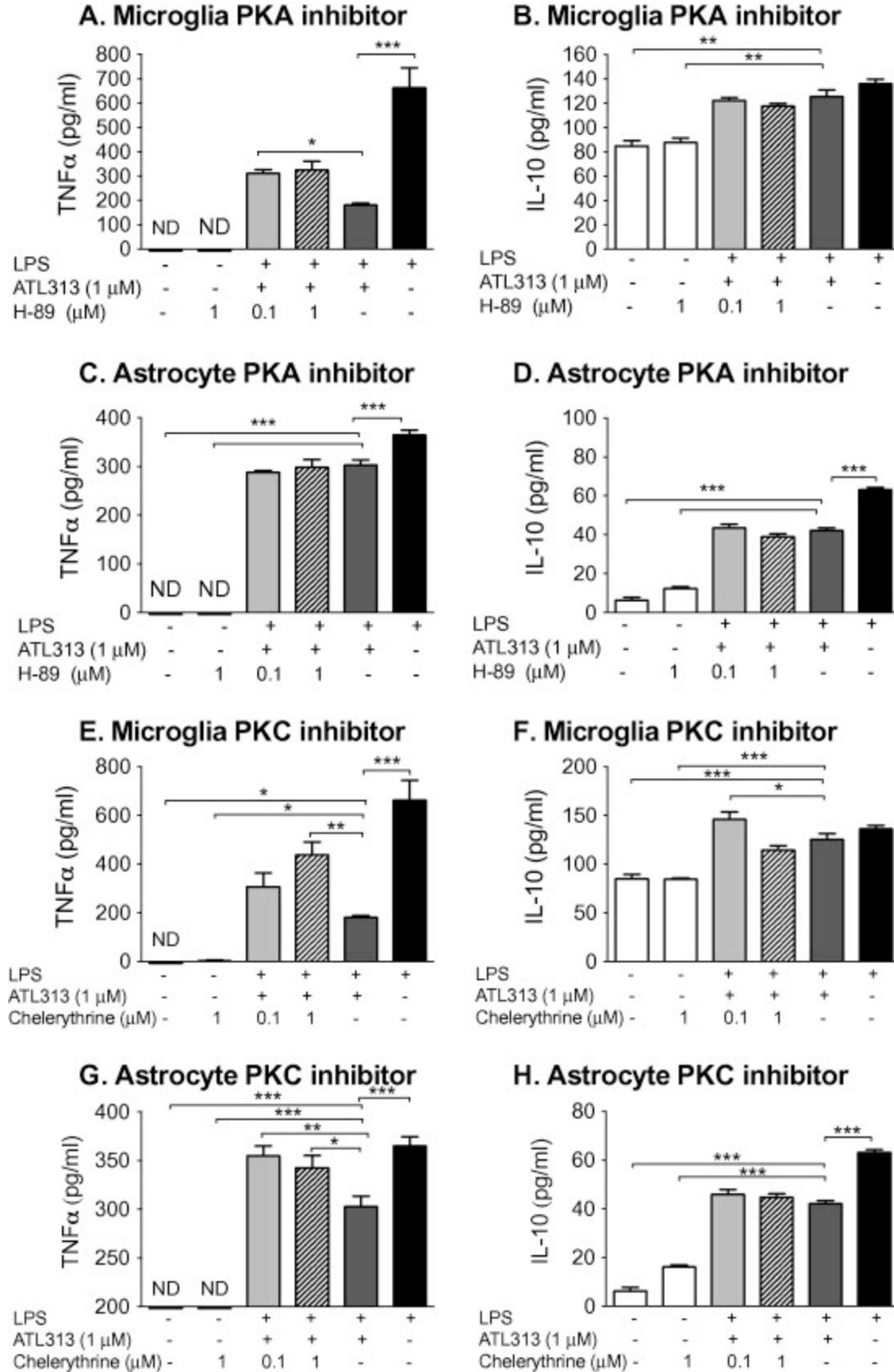


Fig. 28. TNF $\alpha$  release from neonatal microglia (B) and neonatal astrocytes (C) incubated with LPS (100 ng/ml) for 24 h was attenuated by ATL313 (1  $\mu$ M). Administration of H-89 (PKA inhibitor) partially reversed the effects of ATL313 on TNF $\alpha$  production in microglia (A) but not astrocytes (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 intrathecal ATL313 reverses sciatic nerve ligation (SNL)-induced allodynia in the acute phase, as well as after neuropathic pain has been established for several weeks
- We found that intrathecal and perisciatic nerve ATL313 reverses and prevents sciatic inflammatory neuropathy (SIN)-induced allodynia and IT ATL313 reverses but does not prevent chronic constriction (CCI)-induced allodynia.
- 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 SIN model.
- In our spinal neuropathic avulsion pain (SNAP) spinal cord injury (SCI) model, we found that the alternative activation gene expression marker *cd11b* is downregulated at the site of injury (T13/L1) but other alternative activation markers *arginase-1* and *cd163* are not upregulated at the site of injury. In CCI after IT ATL313 treatment, we found IL-10 is upregulated in CSF cells but not spinal cord tissue or sciatic nerve. Lastly, in CCI with perisciatic nerve ATL313 treatment, we found no evidence of alternative activation markers in sciatic nerve or lumbar spinal cord tissue.
- We found that part of the anti-allodynic effects of IT and perisciatic nerve ATL313 in CCI is through PKA and microglia and astrocytes attenuate TNF release via PKA.

## 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)
- One manuscript titled *Perisciatic nerve injection of adenosine 2A receptor agonists reversed neuropathic allodynia through interleukin-10 (IL-10) and protein kinase A (PKA) signaling* is in preparation.
- One manuscript titled *Intrathecal injection of adenosine 2A receptor agonists reverses central neuropathic allodynia in a rat spinal cord avulsion model* is in preparation.

## Conclusions

We have completed all tasks 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 nerve, 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, as well as PKA and PKC. We also were able to show that Iba1 is downregulated as a result of ATL313 treatment, suggesting that alternative activation of microglia/macrophages in the spinal cord likely play a role in the ATL313 effect. Taken together, all of these data suggest that ATL313 would be a successful new neuropathic pain treatment. We thank the Department of Defense for their support for the duration of this project and hope they find the final outcomes of the project exciting and novel with potential clinical relevance.

## References

1. R. S. Taylor, *Pain Pract* 6, 22 (Mar, 2006).
2. L. R. Watkins *et al.*, *Brain, behavior, and immunity* 21, 131 (Feb, 2007).
3. L. S. Gazda *et al.*, *J Peripher Nerv Syst* 6, 111 (Sep, 2001).
4. L. C. Loram *et al.*, *J Neurosci* 29, 14015 (Nov 4, 2009).
5. L. C. Loram *et al.*, *Brain Behav Immun* 33, 112 (Oct, 2013).

## Manuscript Links

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