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Effects of Spinal TLR3 Activation in Non-Neuronal Cells of Rats on Pain Processing

A Thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Biology by Hamid Ehsani-Nia

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2011
The Thesis of Hamid Ehsani-Nia is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2011
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ABSTRACT OF THE THESIS

Effects of Spinal TLR3 Activation in Non-Neuronal Cells of Rats on Pain Processing

by

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Master of Science in Biology

University of California, San Diego, 2011

Professor Tony Yaksh, Chair

Toll-Like Receptor 3 (TLR3) is a receptor of the innate immune system. TLR3 exists within spinal glial cells which are known to play an important role in facilitating spinal pain processing. We hypothesized that activation of these spinal TLR3 would cause glial activation and initiate an enhanced response to noxious stimuli. Using rats with chronic lumbar intrathecal (IT) catheters these studies demonstrated the following observations. 1) IT Polyinosine–polycytidylic acid (Poly I:C, 100µg) resulted in a fall in the tactile threshold (tactile allodynia: TA) with no change in thermal escape by 24 hrs. The TA lasted at least though 4 days. 2) Rats receiving IT minocycline, pentoxifylline, ketorolac and valdecoxib prior to IT Poly I:C showed significant decreases in the
persistent pain state. 3) Rats receiving IT Poly I:C and sacrificed at day 1 showed increases in astrocyte and microglia activation in the superficial lamina and deep lamina of the dorsal horn as well as in the ventral horn. 4) Activation of astrocytes and microglia diminished at day 4. 5) Rats receiving intrathecal pretreatments of glial inhibiting drugs (minocycline and pentoxifylline) as well as COX blocking drugs (ketorolac and valdecoxib) prior to intrathecal Poly I:C and sacrificed at day 1 showed normal levels astrocyte and microglia activation, indicating an inhibition of the nociceptive cascade. 6) IT Poly I:C produced no change in nocturnal behavior. These results indicate that activation of TLR3 can initiate a persistent cascade of facilitated pain processing, mediated at least in part by a glially related cascade.
I. INTRODUCTION

Pathological pain is a debilitating physiological condition that affects millions of people worldwide. The causes of chronic pain are diverse, and range from diseases like AIDS, diabetes, and cancer to nerve injury in the periphery and spinal cord (Tsuda et al, 2005; Cao and Zhang, 2008). Widely prescribed medication for chronic pain bears a burden to society as the potential for abuse and addiction is high. In 2009, over 1.8 million people in the United States were either dependent on or abused otherwise legal pain medication (Substance Abuse and Mental Health Services Administration, 2010). Advances in the field of pain processing over the last decade have widened our understanding of the mechanisms for which the persistent pain state is induced, and may lead to better therapy in the future.

Somatosensory Anatomy and Acute Pain

Under physiologically normal conditions, the somatosensory system provides a protective role, preventing tissue damage from external environmental conditions. The somatosensory system provides afferent input into the CNS. The four somatic inputs are touch, temperature, nociception, and proprioception. External stimuli cause activation of molecules including transient receptor potential cation channel (TRPV1, TRPM8), Purinoreceptor P2X3, and DEG (degenerin), which transducer sensory input to dendrites. These transduction molecules produce an action potential in the peripheral nociceptive Aδ or C neuronal fibers (which sense high threshold stimuli), which carry the signal into
the ipsilateral dorsal horn of the spinal cord at the level for which it innervates (Cao and Zhang, 2008; Bennett, 1993).

Large sensory axons carry information initiated by low threshold mechanical stimuli. They enter the spinal cord and form synapses with neurons in the neck of the dorsal horn (Rexed Lamina III-V). Small, lightly myelinated or unmyelinated sensory neurons typically express transducer proteins which are activated by high intensity stimulus (e.g. >48°C; <18°C, or physical tissue distortion) (Lang and Hope, 1994; Story et al, 2003). Accordingly, these afferents respond to high threshold stimulus information (temperature and mechanical) and terminate in the superficial dorsal horn (Rexed Lamina I-II) (Carr and Goudas, 1999; Milligan and Watkins, 2009).

Under normal physiological conditions over the range of stimulus intensities to which the afferent is sensitive (e.g. low or high threshold), the afferent axons discharge with a frequency proportional to stimulus intensity. This frequency dependency is mirrored in the frequency of evoked activation of the second order neurons in response to a given stimulus. These second order neurons are efferent to the synapse and carry neuronal signals to higher brain centers in the CNS. For the high threshold axons, the more intense the stimulus the higher the frequency of firing of the second order neuron will be. Accordingly, events which lead to an enhanced responsiveness of the second order neurons will mimic the output produced by a more intense stimulus.

**Central Sensitization and Pathological Pain**

As opposed to acute pain, pathological pain (or chronic pain) is often defined as being persistent (e.g. outlasting the initiating stimulus) and causes pain behavior which is
exaggerated relative to that behavioral response produced by a given stimulus. There are two sources of pathological pain: nerve injury (neuropathic) and persistent tissue injury (inflammatory). Neuropathic pain arises from damage in the central or peripheral nervous system. Inflammatory pain, on the other hand, arises from peripheral tissue damage and local inflammation (Bennett, 1993).

**Glial Role in Central Sensitization**

Pathological pain states arising from tissue or nerve injury are thought to represent not only changes in peripheral function of the afferent neurons but to arise from a change in spinal processing which causes sensory neurons to be hyperexcitable in the transmission of signal in the CNS (e.g. a spinal sensitization) (Ji and Woolf, 2001). A number of mechanisms have been proposed for this sensitization, however in this work we wish to focus on the likely contribution of spinal glial cells.

The classical notion of the role of glial cells in the CNS was for trophic maintenance and reaction to tissue injury. Glial cells are known to constantly survey the CNS in search of changes in the microenvironment that can be indicative of pathogen invasion (Hanisch and Kettenmann, 2007). However, it has been shown that activation of glial cells in the spinal cord can modulate dorsal horn response to afferent input, and may mediate pathological pain. (Hain and Waxman, 2006; Milligan et al, 2003; Raghavendra and DeLeo, 2003; Raghavendra et al, 2003).

Upon their activation, glial cells release cytokines and chemokines like interleukins, tumor-necrosis factor, and prostaglandins at the level of the first order synapse in a manner that results in signal amplification. This process, noted above as
“central sensitization,” causes presynaptic signals to elicit an enhanced postsynaptic response. The result is the initiation of a pain state by otherwise non-noxious stimuli (Fellin et al, 2006; Parpura et al, 1994; Woolf and Mannion, 1999; Milligan and Twining, 2003; Sommer and Kress, 2004). The two major families of glial cells focused on in this study are astrocytes and microglia.

**Astrocyte and Microglia Activation**

In spinal cord, astrocytes reside in close proximity to the soma and synapses of the neuron (Porter and McCarthy, 1997). Microglia, on the other hand, are produced in the bone marrow and invade the CNS during development. In addition, circulating macrophages may later gain access to the CNS during the course of local inflammatory responses (Hickey and Kimura, 1988; Lassmann et al, 1993). Both astrocytes and microglia have been shown to express a variety of neurotransmitter receptors on the cell membrane which can lead to their activation. Such an organization suggests that afferent input is a possible mechanism of their activation and form the basis behind their contributions in the face of peripheral inflammation and nerve injury (Haydon, 2001).

Astrocytes and microglia have been implicated in the regulation of nociceptive processing. Chronic inflammatory states and nerve injury have been shown to activate both astrocytes and microglia. A stimulus from injury or inflammation causes expression of ligand-gated ion-channels like P2X4 and P2X7 as well as G-protein coupled receptors like CX3CR1, CCR2, and P2Y. This causes an influx of calcium ions which activates cascades sensitive to calcium concentration, namely p38 mitogen activated protein kinase (MAPK) and nuclear transcription factor-κB (NF-κB) (Tsuda et al, 2005; Deleo et al,
P38 MAPK activates various proinflammatory mediators such as prostaglandin E2 (PGE2) and cyclooxygenase (COX) (Kumar et al, 2003; Ji and Suter, 2007; Cao and Zhang, 2008; Fitzsimmons et al, 2010). Furthermore, many cytokines which mediate central sensitization are produced, including TNF, IL-1β, and, IL-6 (DeLeo et al, 1997; Hashizume et al, 2000; Milligan et al, 2001; Raghavendra et al, 2004).

Such activation is evidenced by the increased expression of epitopes such as glial fibrillary acidic protein (GFAP) in astrocytes and OX42 in microglia. An important element in defining the contribution of these cells is that several agents such as minocycline and pentoxifylline which can reduce activation of astrocytes and microglia. The spinal delivery of these agents in a variety of models of hyperalgesia after tissue and nerve injury has been shown to reduce the hyperalgesic state (Hua et al, 2005; Yrjänheikki et al, 1998; Tikka et al, 2001; Krady et al, 2005; Semmler et al, 1993; Zabel et al, 1993; Han et al, 1990; Sweitzer et al, 2001).

**Glial Pattern Recognition and the TLRs**

Glial cells like astrocytes and microglia are immunocompetent, meaning they respond immunologically to molecules associated with pathogen infection or cell necrosis. Being part of the innate immune system, the recognition of these molecular motifs are constant throughout the organism’s life and do not change with pathogenic exposure history, unlike the adaptive immune system. The development of the innate immune system arose to recognize patterns in pathogens such as bacteria and viruses and produce a defensive response (Medzhitov and Janeway, 1997).
The toll like receptors (TLRs) are a group of transmembrane proteins that recognize very specific molecules associated with pathogenic infection. They are a key line of defense in the innate immune system because they reside on innate immune cells. There is wide variety of TLR receptors. Thus far, 15 TLRs have been identified in mammals, 10 of which have been identified in humans. Aside from mammals, TLRs have been found to exist in other vertebrates, as well as plants and insects (they were first identified in drosophila). This broad homology indicates not only how ancient the TLRs, but also the significance of their function (Lemaitre et al, 1996; Beutler and Rehli, 2002). These TLRs have been shown to share a common property in that they are activated by a variety of products which may be released by tissue injury or inflammation or infection. Thus, various TLRs will bind to a variety of products such as non-self single stranded and double stranded DNA and RNA, lipoproteins, and lipopolysaccharides, as well as self signals originating from necrosis. An important element is that many TLRs have been identified in the CNS and have been found to reside in microglia and astrocytes (Bsibsi et al, 2002; Underhill, 2003; Iwasaki and Medzhitov, 2004; Milligan and Watkins, 2009).

**TLR3, Function and Structure**

TLR3 binds to double stranded RNA (dsRNA), single stranded RNA (ssRNA), double stranded DNA (dsDNA) or single stranded DNA (ssDNA) (Bell et al, 2005). However, solely dsRNA activates TLR3. ssRNA, dsDNA and ssDNA only block activation in the presence of dsRNA by binding at an affinity 100x to 1000x weaker than that of dsRNA, indicating the specificity of the ligand (Leonard et al, 2008). dsRNA is produced during the life cycle of many viruses. Because it is not produced anywhere in
mammals, the detection of dsRNA can put in motion an immune response to better defend the organism from attack (Bowie and Haga, 2005).

However, TLR3 has also been shown to bind to endogenous ligands as well. During cell necrosis, ssRNA is released in massive quantities from the dying cell. This ssRNA is thought to be able to form secondary structures and “hairpin” upon itself, producing a mock-dsRNA in a portion of the polynucleotide, which itself is able to bind TLR3. Thus, TLR3 can also initiate an immune response to cell necrosis (Cavessani et al, 2008; Karikó et al, 2004).

Unlike other TLRs which reside on the cell membrane, TLR3 resides in the endosome. The ectodomain protrudes into the lumen of the acidic endosome in the shape of a horse shoe, where it binds to dsRNA (Bell et al, 2005; Bell et al, 2006). Binding of TLR3 to dsRNA is directly dependent on base pair (bp) length, with 40 base pairs being the minimum binding length and greater than 62 base pairs being the length at which 100% of dsRNA bind to TLR3. Furthermore, multiple TLR3s are able to cooperatively bind to a single dsRNA molecule by dimerizing. The minimum dsRNA length to elicit a dimerization is 45-48 base pairs; above which TLR3 binds in multiples of 2. The sequence of dsRNA does not affect binding affinity (Leonard et al, 2008; Bell et al, 2005).

Binding of a ligand to the TLR3 is also pH dependent. The endosome where TLR3 resides has a pH range of 6.0 to 5.5. The minimum acidity for dsRNA to bind to TLR3 is at a pH of 6.5, with relatively no change in affinity between a pH of 6.0 and 5.5, indicating the acidic endosome is an ideal location for TLR3 to bind dsRNA (Cain et al 1989; Leonard et al, 2008).
The route for which dsRNA is brought from the extracellular fluid to the endosome is not completely understood at this point. However, recent studies have suggested that CD14 assists in the uptake and intracellular transportation of dsRNA, which results in activation of TLR3 (Lee et al, 2006).

Synthetic analogues to dsRNA have been shown to be a more practical and cost effective way to study the implications of TLR3 activation. Polyinosine-polycytidylic acid (Poly I:C) has been shown to activate TLR3 and has been used as the standard for TLR3 activation studies (Obata et al, 2008; Leonard et al, 2008; Mei et al, 2011).

**Downstream Cascade of TLR3 Activation**

TLR3 is unique in that it is the only TLR that does not participate in the MyD88 activation cascade, which is shared by every other TLR either with or without the adapter molecule TIRAP. The downstream result of the MyD88 cascade is the activation of NF-κB and AP-1. Furthermore, the activation of TLR3 has been shown to upregulate TLR3 expression, as well as expression of the other TLRs (Ritter et al, 2005; Kawai and Akira, 2006).

TLR3 has only one activation adapter molecule, Trif. TLR4 is the only other TLR that associates with the Trif cascade, which uniquely culminates in IRF3 activation, and also produces downstream signaling molecules which innervate early in the MyD88 pathway. The result is expressing a wide range of NF-κB mediated inflammatory cytokines as well as Type I IFN production mediated jointly by NF-κB, AP-1 and IRF3 (Kawai and Akira, 2006).
The production of these inflammatory cytokines in astrocytes and microglia causes cell activation. Produced pro-inflammatory cytokines include TNFα, IL-1b, and IL-6. All of these cytokines are involved in sensitization of the synapse at the dorsal horn of the spinal cord by amplifying signals in the postsynaptic terminal (Beattie et al, 2002; Stellwagen and Malenka, 2006; Kawasaki et al, 2008; Ozaktay et al, 2006; Besson, 1999).

The role of spinal TLR3 in pain processing has been recently suggested through a variety of experimental approaches. Thus the IT delivery of TLR3 agonists have been reported to produce a hypersensitivity to light touch and diminished hyperpathia after peripheral injury by the spinal delivery of a TLR3 antisense (Obata et al, 2008). Such studies suggest that spinal TLR3 s may play a role in the hyperpathic state that occurs after nerve or tissue injury.

Hypotheses

This thesis focuses on the effects of TLR3 activation in the spinal cord on persistent pain behavior and glial activation. The following hypotheses have been investigated for the purposes of this thesis:

1. Intrathecally delivered Poly I:C produces a persistent pain state which is dose dependent. *Behavioral Analysis:* Tactile and thermal testing.
   *Immunohistochemical Analysis:* astrocyte and microglia activation.

2. The persistent pain state which is induced by intrathecally delivered Poly I:C can be blocked by minocycline pretreatment. *Behavioral Analysis:* Tactile and
thermal testing. *Immunohistochemical Analysis:* astrocyte and microglia activation.

3. The persistent pain state which is induced by intrathecally delivered Poly I:C can be blocked by ketorolac pretreatment. *Behavioral Analysis:* Tactile and thermal testing. *Immunohistochemical Analysis:* astrocyte and microglia activation.

4. The persistent pain state which is induced by intrathecally delivered Poly I:C can be blocked by pentoxifylline pretreatment. *Behavioral Analysis:* Tactile and thermal testing. *Immunohistochemical Analysis:* astrocyte and microglia activation.

5. The persistent pain state which is induced by intrathecally delivered Poly I:C can be blocked by valdecoxib pretreatment. *Behavioral Analysis:* Tactile and thermal testing. *Immunohistochemical Analysis:* astrocyte and microglia activation.
II. METHODS

Animals

Holtzman rats were used in all studies in accordance to the Institutional Animal Care and Use Committee of the University of California, San Diego. Animals were housed at least three days after shipment, before experimentation, and maintained on a 12/12 hour day/night cycle with *ad libitum* access to food and water.

Intrathecal Implantation

Rats were anesthetized using 5% isoflurane and polyethylene-8 catheters were implanted from the base of the skull, down through the intrathecal space, and extending to the third lumbar level of the spinal cord, as previously described (Yaksh and Rudy, 1976; Yaksh and Stevens, 1986). The inner volume of these catheters is 6-8 µL. Rats were given 5-7 days to recover before any experimentation was performed.

Drug Delivery

All IT drugs were delivered in a volume of 10µL followed by a 10µL injection of Saline (0.9%) to clear the catheter. All injections were given to anesthetized rats via a Hamilton syringe.

Treatment groups were injected with an anti-inflammatory agent 30 minutes before being injected with Poly I:C, the TLR3 agonist.

Drugs
The following drugs were used in the study: Poly I:C (Invitrogen, Catalogue No tlrl-picw, Lot No PIW-32-16), Minocycline (Sigma, Catalogue No M9511-250MG, Lot No BCBC2895), Pentoxifylline (Sigma, Catalogue No P1784-10G, Lot No 065K1044), Ketorolac (Sigma, Catalogue No K116-1G, Lot No 101M-1964) and Valdecoxib (Ironwood Pharmaceuticals, Catalogue No 101-1-109, Lot No MM416740 Batch 3). All drugs were obtained in crystalline form and dissolved in water such that the total doses were delivered in 10µL.

Poly I:C was used as the TLR3 agonist and proinflammatory agent. It was injected at 10µg, 30µg, and 100µg for the purposes of creating a dose response curve. Poly I:C was injected only at the 100µg dose when used in conjunction with pretreatments and posttreatments.

Minocycline, Pentoxifylline, Valdecoxib, and Ketorolac were used as pretreatments and posttreatments to Poly I:C. 60µg was the dose used for Minocycline, 120µg was the dose used for Pentoxifylline, 30µg was the dose used for Valdecoxib, and 30µg was the dose used for Ketorolac.

Tactile Alldynia Analysis

Rats were placed in individual cells of a raised cage with a wire-mesh floor, and were allowed to acclimate for 1 hour prior to analysis. Plastic von Frey filaments of set pressures (Touch Test® Sensory Evaluators) were used to calculate paw withdrawal thresholds (PWT). 6 pressure readings were taken for each hind paw and PWTs were calculated using the up-down algorithm (Chaplan et al, 1994). Values for PWT for each paw were averaged together for each animal.
**Thermal Hyperalgesia Analysis**

Rats were placed in individual cells on the Hot-Box device with a glass floor, and were allowed to acclimate for 1 hour prior to analysis. Beneath the floor was an incandescent light that was set to heat to 50° Celsius over the course of 20 seconds. The light apparatus was capable of being moved and focused on the hind paw, and also had a component detecting paw withdrawal using a proximity sensor. The time between the initiation of the light and the paw withdrawal was automatically calculated by a Hargreaves type of thermal stimulator (Dirig et al, 1997). 3 paw withdrawal latencies (PWL) were obtained for each paw of each animal and the results from each animal were averaged together.

**Nocturnal Activity Analysis**

Standard rat housing cages were modified to accommodate a motion sensor wired to a computer, which tabulated the presence or absence of motion every 4 seconds. The total motion of each individual cage was summed up every minute giving a minimum value of 0 and a maximum value of 15. Each rat was given one motion value every minute throughout the time course of the experiment. Baseline values were obtained over a two day period, and were rats then separated into treatment groups in a way to minimize the average difference between the groups. The diurnal phase is defined as the time of day 7:00 to 19:00, and the nocturnal phase is defined as the time of day 19:00 to 7:00. The nocturnal phase of 12 hours overlaps 2 calendar days. Rats were injected at day 0 at 15:00, and the following night was the first nocturnal phase.
Perfusion Sacrifice and Tissue Harvest

Prior to immunohistochemical analysis, rats were perfusion sacrificed and spinal cords were harvested. For this procedure, rats were injected with .5mL euthasol into the intraperitoneal space. After loss of consciousness and failing to respond to a tail pinch, large incisions were made transversing the abdomen and thoracic cavity on the ventral side of the rat in order to expose the still-beating heart. The heart was punctured at the apex into the left ventricle with a .5inch, 18-gauge needle which was pinched on to the cardiac tissue with hemostat forceps. .09% NaCl was perfused into the heart and the right atrium was nicked to allow blood to exit the body. A solution of 4% Paraformaldehyde (PFA) and Phosphate Buffered Saline (PBS) was then perfused through the body. The spinal cord was immediately harvested, was allowed to soak in the same 4% PFA and PBS solution for 1 day, and finally, was allowed to soak in 30% Sucrose in PBS for 2 days.

Immunohistochemistry

After the sucrose soak, the cords were frozen in an OCT block, and cut to 30µm sections. Sections were collected in serials of 6 for each rat spinal cord. Immunohistochemistry was done using the free-floating method. Briefly, a removable, 24 well container with a steel-mesh bottom was placed in a tightly fitting block, allowing easy change of tissues between washes and antibody solutions. The total volume of the block was 20mL. Each well corresponded to 1 rat. All washes and antibody applications were done on a rotating plate at room temperature unless otherwise stated.
After 3 10-minute washes of PBS, 3 washes of .2% Triton-X (TX) in PBS, and a one hour block with 5% goat serum, the primary antibody solution was added which consisted of PBS with 1% Bovine Serum Albumin (BSA), .2% TX, GFAP made in mouse at 1:5000 dilution (Millipore, Catalogue №: MAB360, Lot №: LV1634936), and Iba1 made in rabbit at 1:3000 (Wako, Catalogue №: 019-19741, Lot №:). The primary antibody solution was applied for 2 days at 4°C.

After the 2 days of primary antibody application, the tissue was given 3 10-minute washes of PBS before the addition of the secondary antibody solution which consisted of PBS with 1% Bovine Serum Albumin (BSA), .2% TX, 594 goat anti-mouse at 1:1000 dilution (Alexa, Catalogue №: A11032), and 488 goat anti rabbit at 1:1000 dilution (Alexa, Catalogue №: A11037). The primary antibody solution was applied for 1 hour. The sections were then individually placed on slides. 1 to 2 drops of Prolong-Gold + DAPI (Invitrogen, Catalogue № P36935, Lot № 936576) was added to each slide before cover-slips were placed on them.

**Quantification**

All microscopy was done on an Olympus BX51 Microscope, and all quantification was done using Image-Pro 5.1 (Media Cybernetics). The microscope filter was set to emit light at 488nm or 594nm. Images taken for quantification were taken at 20x zoom, and images were taken at higher magnifications for demonstrative purposes. 4 sections were selected at random from each slide. Digital images were taken with white balance set to florescence, and exposures of 4 seconds for 594nm, and .5 seconds for 488nm. Measurements in arbitrary units of intensity were taken from 3 adjacent regions.
in the dorsal horn, 1 region in the deep dorsal horn, 1 region in the motor horn, 1 region in the white matter, and one region in the image void of any tissue.

Statistics

All data are presented as the Mean ± Standard Error of the Mean unless otherwise stated. Statistical calculations were conducted on all data to indicate levels of significance less than $p = .05$.

For data collected with multiple treatments over a period with multiple time points, a two way ANOVA was used in conjunction with a Bonferroni post test to indicate significance for each drug at each time point in comparison to a Saline control. In addition, a hyperalgesic index was used to calculate the change in the Area Under the Curve (AUC) from baseline in the presence of drug treatment. The hyperalgesic index yielded one value per rat, and was subject to a one way ANOVA with a Dunnett post test in comparison to a Saline control. This method was used for the dose response experiments, the pretreatment experiments, and the 24 hour motion experiment.

For data collected from immunohistochemical quantification of activated glial cells, the four data points in each individual region were averaged to yield one data point per region per rat. These data were imputed per treatment group, and were subject to a one way ANOVA with a Bonferroni post test to indicate significance for each drug at each time point in comparison to every other treatment.
III. RESULTS

Poly I:C Effect on Behavior

Baseline levels of tactile allodynia and thermal hyperalgesia were obtained before drug injection at day 0. The average tactile threshold was 15 ± .00 grams and the thermal escape latency was 10.49 ± .37 sec. The intrathecal injection of Saline had no effect on the thermal escape latency or tactile thresholds at any time after delivery. In contrast, the IT Poly I:C (100µg, 10µg and 3µg) elicited a profound dose dependent decrease in PWT at every time point from day 1 through day 7 (Figure 1). None of the doses produced any significant behavioral changes in thermal hyperalgesia on any given time point, and there were no significant changes in the thermal hyperalgesic index in comparison to Saline control (Figure 2).

Poly I:C versus Saline + Poly I:C on Behavior

Intrathecal injection of Poly I:C (100µg) was compared to Saline (10µL) pretreatment 30 minutes prior to Poly I:C (100µg). Tactile and thermal analysis was performed through 11 days. Both groups elicited profound decreases in PWT from day 1 through day 8 when compared to control, and also provided strong increases in tactile hyperalgesic index when compared to control. Furthermore, there was no statistical significance detected between the two groups in tactile alldynia on any given day when analyzed by a two way ANOVA with Bonferroni post test, as well no statistical significance detected in hyperalgesic index when analyzed by a one way ANOVA with Bonferroni post test (Figure 3). Neither of the groups produced significant behavioral
changes in thermal hyperalgesia on any given time point, and there were no significant changes in the thermal hyperalgesic index in comparison to Saline control (Figure 4).

**Poly I:C versus minocycline + Poly I:C**

A minocycline dose of 60µg was used as a pretreatment injected 30 minutes prior to a Poly I:C dose of 100µg.

Baseline levels of tactile allodynia and thermal hyperalgesia were obtained on day 0 before drugs were administered. Behavioral tests were continued from day 1 through day 11. The minocycline pretreatment group elicited a significantly decreased allodynic response in comparison to the Poly I:C control that lasted from day 1 through day 4, in which time there was no statistically significant difference between the minocycline pretreatment and Saline control groups. The tactile response of the minocycline pretreatment group resembled the Poly I:C group from day 6 through day 11 in that there was the same level of statistical significance compared to Saline control on day 6 and 8 and there was no level of statistical significance compared to Saline control on day 10 and 11. The tactile hyperalgesic index indicated that the minocycline group did not elicit an overall significant allodynic response in comparison to the Saline control group (Figure 5). The minocycline pretreatment did not produce significant behavioral changes in thermal hyperalgesia on any given time point, and there were no significant changes in the thermal hyperalgesic index in comparison to Saline control (Figure 6).

**Poly I:C versus Ketorolac + Poly I:C**
A Ketorolac dose of 30µg was used as a pretreatment injected 30 minutes prior to a Poly I:C dose of 100µg.

Baseline levels of tactile allodynia and thermal hyperalgesia were obtained on day 0 before drugs were administered. Behavioral tests were continued from day 1 through day 11. The ketorolac pretreatment group elicited a significantly decreased allodynic response in comparison to the Poly I:C control that lasted throughout the experiment. With the exception of decreased PWT measurements on day 4, there was no statistically significant difference between the Saline control group and the ketorolac pretreatment group on any given day. The tactile hyperalgesic index indicated that the ketorolac group did not elicit an overall significant allodynic response in comparison to the Saline control group (Figure 7). The ketorolac pretreatment did not produce significant behavioral changes in thermal hyperalgesia on any given time point, and there were no significant changes in the thermal hyperalgesic index in comparison to Saline control (Figure 8).

**Poly I:C versus Pentoxifylline + Poly I:C**

A pentoxifylline dose of 120µg was used as a pretreatment injected 30 minutes prior to a Poly I:C dose of 100µg.

Baseline levels of tactile allodynia and thermal hyperalgesia were obtained on day 0 before drugs were administered. Behavioral tests were continued from day 1 through day 11. The pentoxifylline pretreatment group elicited a significantly decreased allodynic response in comparison to the Poly I:C control that lasted throughout the experiment. There was no statistically significant difference between the Saline control group and the pentoxifylline pretreatment group on any given day. The tactile hyperalgesic index
indicated that the pentoxifylline group did not elicit an overall significant allodynic response in comparison to the Saline control group (Figure 9). The pentoxifylline pretreatment did not produce significant behavioral changes in thermal hyperalgesia on any given time point, and there were no significant changes in the thermal hyperalgesic index in comparison to Saline control (Figure 10).

**Poly I:C versus Valdecoxib + Poly I:C**

A valdecoxib dose of 30µg was used as a pretreatment injected 30 minutes prior to a Poly I:C dose of 100µg.

Baseline levels of tactile allodynia and thermal hyperalgesia were obtained on day 0 before drugs were administered. Behavioral tests were continued from day 1 through day 11. The valdecoxib pretreatment group elicited a significantly decreased allodynic response in comparison to the Poly I:C control that lasted throughout the experiment. There was no statistically significant difference between the Saline control group and the valdecoxib pretreatment group on any given day. The tactile hyperalgesic index indicated that the valdecoxib group did not elicit an overall significant allodynic response in comparison to the Saline control group (Figure 11). The valdecoxib pretreatment did not produce significant behavioral changes in thermal hyperalgesia on any given time point, and there were no significant changes in the thermal hyperalgesic index in comparison to Saline control (Figure 12).

**Nocturnal Activity Analysis**
Injections in both the Saline and Poly I:C groups caused a decrease in activity during the first night (Figure 13). Both groups had a nearly identical, steady, increase in activity every night through the 11 day time course. There was no statistically significant difference between the Saline and Poly I:C group at any individual day. Furthermore, there was essentially no difference in the overall hyperalgesic threshold between the two groups.

**Glial Activation**

Groups of rats receiving IT Poly I:C were sacrificed at varying time points to analyze microglia and astrocyte activation motifs. Five groups were used in this experiment: Sham (no injection), Saline sacrificed at day 1, Saline Sacrificed at day 4, 100μg Poly I:C with Saline pretreatment sacrificed at day 1, and 100μg Poly I:C with Saline pretreatment sacrificed at day 4. Additional groups received IT Poly I:C with IT pretreatment of minocycline, pentoxifylline, valdecoxib and ketorolac and sacrificed at 1 day.

*Imaging:* A large visible change in glial morphology is evident in the IT 100μg Poly I:C with Saline pretreatment group sacrificed at day 1. Both astrocytes and microglia appear to increase in density of staining, as well as in the number of profiles showing immunoreactivity of GFAP (indicative of astrocyte activation) and Iba1 (indicative of microglia activation). Astrocytes typically appeared to extend their processes and increase in size. Microglia seem to withdraw their processes and morphologically change into spherical shapes (Figure 15 and 16).
**Time Course:** There were no statistically significant differences between the immunohistochemical signal in Sham, Saline sacrificed at day 1, and Saline sacrificed at day 4 groups in any region quantified. Poly I:C rats sacrificed at day 1 all showed profoundly statistically significant differences in activation compared to Saline rats sacrificed at day 1 with the exception of astrocytes in white matter. Poly I:C rats sacrificed at day 4 showed statistically significant differences in activation compared to Poly I:C rats sacrificed at day 1 with the exception of microglia in the white matter, deep dorsal horn, or ventral horn. Finally, Poly I:C rats sacrificed at day 4 showed statistically significant differences in activation compared to Saline rats sacrificed at day 1 in the SL3 region (Figure 15 and 16).

**Minocycline Pretreatment Quantification:** IT Minocycline pretreatment decreased the activation of microglia and astrocytes to levels profoundly statistically significant compared to Poly I:C treatment alone in every region quantified except the ventral horn. Furthermore, minocycline pretreatment decreased the activation of microglia and astrocytes to levels statistically insignificant compared to Saline control in every region quantified except for astrocytes in white matter (Figure 17).

**Ketorolac Pretreatment Quantification:** IT Ketorolac pretreatment decreased the activation of microglia and astrocytes to levels profoundly statistically significant compared to Poly I:C treatment alone in every region quantified. Furthermore, ketorolac pretreatment decreased the activation of microglia and astrocytes to levels statistically insignificant compared to Saline control in every region quantified except astrocytes in white matter (Figure 17).
**Pentoxifylline Pretreatment Quantification:** IT Pentoxifylline pretreatment decreased the activation of microglia and astrocytes to levels profoundly statistically significant compared to Poly I:C treatment alone in every region quantified except for astrocytes in white matter, deep dorsal horn and ventral horn. Furthermore, ketorolac pretreatment decreased the activation of microglia and astrocytes to levels statistically insignificant compared to Saline control in every region quantified (Figure 17).

**Valdecoxib Pretreatment Quantification:** IT Valdecoxib pretreatment decreased the activation of microglia and astrocytes to levels profoundly statistically significant compared to Poly I:C treatment alone in every region quantified except for astrocytes in white matter and ventral horn. Furthermore, valdecoxib pretreatment decreased the activation of microglia and astrocytes to levels statistically insignificant compared to Saline control in every region quantified (Figure 17).
Figure 1. Poly I:C Tactile Dose Response.

The response to non-noxious mechanical pain stimuli to rats was measured over the course of 7 days. A) At day 0, rats were tested for baseline tactile allodynia, then injected intrathecally with Saline, 100µg Poly I:C, 10µg Poly I:C, or 3µg Poly I:C. Testing continued from day 1 through day 7. The 100µg Poly I:C injection elicited a significantly stronger allodynic response than the other injections at every timepoint after the injection, and was used as the testing dose throughout the other experiments. B) The hyperalgesic index was obtained by calculating the AUC for each rat and comparing it to their individual baselines. The 100µg Poly I:C injection, again, was the only dose to elicit a significant allodynic response, and was used as the testing dose throughout the other experiments.
A

Injection

Day

Paw Withdrawal Threshold (gram)

Baseline 0 1 2 4 6 7

*** *** ***

Injection

Saline 100 µg 10 µg 3 µg

-20

0

20

40

60

*** Poly I:C Poly I:C Poly I:C

Tactile Hyperalgesic Index

B

Saline 100 µg Poly I:C 10 µg Poly I:C 3 µg Poly I:C

-20

0

20

40

60

***

Tactile Hyperalgesic Index
Figure 2. Poly I:C Thermal Dose Response.

The response to non-noxious thermal pain stimuli to rats was measured over the course of 7 days. A) At day 0, rats were tested for baseline tactile allodynia, then injected intrathecally with Saline, 100µg Poly I:C, 10µg Poly I:C, or 3µg Poly I:C. Testing continued from day 1 through day 7. No injection dosage of Poly I:C elicited a significantly strong thermal hyperalgesic response. B) The hyperalgesic index was obtained by calculating the AUC for each rat and comparing it to their individual baselines. Again, no injection dosage of Poly I:C elicited a significantly strong thermal hyperalgesic response.
Figure 3. Tactile, Poly I:C With and Without Saline Pretreatment.

The response to non-noxious mechanical pain stimuli to rats was measured over the course of 11 days to test whether or not a Saline injection prior to Poly I:C injection made a difference in allodynic behavioral response. A) At day 0, rats were tested for baseline tactile allodynia. Then, rats were injected intrathecally either with 10μL Saline followed by 100μg of Poly I:C 30 minutes later or with 100μg of Poly I:C alone. Testing continued from day 1 through day 11. Both the Saline + Poly I:C and the Poly I:C alone groups elicited a significant allodynic response that lasted through day 8. B) The hyperalgesic index was obtained by calculating the AUC for each rat and comparing it to their individual baselines. Both the Saline + Poly I:C and the Poly I:C alone groups elicited a significant allodynic response.
A

Injection

Paw Withdrawal Threshold (gram)

Saline, n=4
Saline + 100µg Poly I:C, n=12 (sig.=*)
Baseline 0 1 2 4 6 8 10 11
Injection
100µg Poly I:C, n=6 (sig.=#)
***##**###
**#
##*
#
Day
Paw Withdrawal Threshold (gram)

B

Tactile Hyperalgesic Index

Saline Saline + PIC PIC
-20 0 20 40 60 80 ** **
Tactile Hyperalgesic Index

Saline Saline + PIC PIC

** **

0

-20
**Figure 4. Thermal, Poly I:C With and Without Saline Pretreatment.**

The response to non-noxious thermal pain stimuli to rats was measured over the course of 11 days to test whether or not a Saline injection prior to Poly I:C injection made a difference in hyperalgesic behavioral response. A) At day 0, rats were tested for baseline tactile allodynia. Then, rats were injected intrathecally either with 10µL Saline followed by 100µg of Poly I:C 30 minutes later or with 100µg of Poly I:C alone. Testing continued from day 1 through day 11. Neither the Saline + Poly I:C nor the Poly I:C alone groups elicited a significant hyperalgesic response at any timepoint. B) The hyperalgesic index was obtained by calculating the AUC for each rat and comparing it to their individual baselines. Neither the Saline + Poly I:C nor the Poly I:C alone groups elicited a significant hyperalgesic response.
A

Injection

Baseline 0 1 2 4 6 8 10 11
Injection

Day

Paw Withdrawal Latency (sec)

Saline, n=4
100µg Poly I:C, n=6
Saline + 100µg Poly I:C, n=12

B

Thermal Hyperalgesic Index

Saline Saline + PIC PIC

-20 0 20 40 60 80

Thermal Hyperalgesic Index

Saline Saline + PIC PIC
Figure 5. Tactile, Minocycline Pretreatment.

The response to non-noxious mechanical pain stimuli to rats was measured over the course of 11 days to test whether or not minocycline pretreatment to Poly I:C injection made a difference in allodynic behavioral response in comparison. A) At day 0, rats were tested for baseline tactile allostynia. Then, rats were injected intrathecally either with 10µL Saline followed by 100µg of Poly I:C 30 minutes later or with 60µg minocycline followed by 100µg of Poly I:C 30 minutes later. Testing continued from day 1 through day 11. The minocycline pretreatment group elicited a significantly decreased allodynic response in comparison to the Poly I:C control that lasted from day 1 through day 4. The tactile response of the minocycline pretreatment group resembled the Poly I:C control group from day 6 through day 11. B) The hyperalgesic index was obtained by calculating the AUC for each rat and comparing it to their individual baselines, and indicated that the minocycline group did not elicit an overall significant allodynic response.
Figure A: Changes in paw withdrawal threshold (gram) over time for different experimental groups. The injection was performed on Day 0.

- **Saline, n=4**
- **Saline + 100µg Poly I:C, n=12**
- **60µg Mino + 100µg Poly I:C, n=8**

Figure B: Tactile hyperalgesic index for each group.

- **Saline**
- **Saline + PIC**
- **Mino**
Figure 6. Thermal, Minocycline Pretreatment.

The response to non-noxious thermal pain stimuli to rats was measured over the course of 11 days to test whether or not minocycline pretreatment to Poly I:C injection made a difference in hyperalgesic behavioral response in comparison. A) At day 0, rats were tested for baseline thermal hyperalgesia. Then, rats were injected intrathecally either with 10µL Saline followed by 100µg of Poly I:C 30 minutes later or with 60µg minocycline followed by 100µg of Poly I:C 30 minutes later. Testing continued from day 1 through day 11. The minocycline pretreatment group did not elicit a significant hyperalgesic response at any timepoint. B) The hyperalgesic index was obtained by calculating the AUC for each rat and comparing it to their individual baselines, and indicated that the minocycline group did not elicit a significant hyperalgesic response.
A

Paw Withdrawal Latency (sec)

- Injection

Saline, n=4
60µg Mino + 100µg Poly I:C, n=8
Saline + 100µg Poly I:C, n=12

B

Thermal Hyperalgesic Index

Saline | Saline + PIC | Mino
Figure 7. Tactile, Ketorolac Pretreatment.

The response to non-noxious mechanical pain stimuli to rats was measured over the course of 11 days to test whether or not ketorolac pretreatment to Poly I:C injection made a difference in allodynic behavioral response in comparison. A) At day 0, rats were tested for baseline tactile allodynia. Then, rats were injected intrathecally either with 10μL Saline followed by 100μg of Poly I:C 30 minutes later or with 30μg ketorolac followed by 100μg of Poly I:C 30 minutes later. Testing continued from day 1 through day 11. The ketorolac pretreatment group elicited a significantly decreased allodynic response in comparison to the Poly I:C control that lasted throughout the experiment, with the exception of decreased PWT measurements on day 4. B) The hyperalgesic index was obtained by calculating the AUC for each rat and comparing it to their individual baselines, and indicated that the ketorolac group did not elicit an overall significant allodynic response.
A

Injection

Saline, n=4
Saline + 100µg Poly I:C, n=12
30µg Keto + 100µg Poly I:C, n=7

Paw Withdrawal Threshold (gram)

Baseline 0 1 2 4 6 8 10 11
#
***
**
*

B

Tactile Hyperalgesic Index

Saline Saline + PIC Keto

-20 0 20 40 60 80 **

**
**Figure 8. Thermal, Ketorolac Pretreatment.**

The response to non-noxious thermal pain stimuli to rats was measured over the course of 11 days to test whether or not ketorolac pretreatment to Poly I:C injection made a difference in hyperalgesic behavioral response in comparison. A) At day 0, rats were tested for baseline thermal hyperalgesia. Then, rats were injected intrathecally either with 10µL Saline followed by 100µg of Poly I:C 30 minutes later or with 30µg ketorolac followed by 100µg of Poly I:C 30 minutes later. Testing continued from day 1 through day 11. The ketorolac pretreatment group did not elicit a significant hyperalgesic response at any timepoint. B) The hyperalgesic index was obtained by calculating the AUC for each rat and comparing it to their individual baselines, and indicated that the ketorolac group did not elicit a significant hyperalgesic response.
A

Injection

Saline, n=4
Saline + 100µg Poly I:C, n=12
30µg Keto + 100µg Poly I:C, n=7

Baseline 0 1 2 4 6 8 10 11
Day

Paw Withdrawal Latency (sec)

-20
0
20
40
60
80
Thermal Hyperalgesic Index

Saline Saline + PIC Keto

B

Saline Saline + PIC Keto

Thermal Hyperalgesic Index
Figure 9. Tactile, Pentoxifylline Pretreatment.

The response to non-noxious mechanical pain stimuli to rats was measured over the course of 11 days to test whether or not pentoxifylline pretreatment to Poly I:C injection made a difference in allodynic behavioral response in comparison. A) At day 0, rats were tested for baseline tactile allodynia. Then, rats were injected intrathecally either with 10µL Saline followed by 100µg of Poly I:C 30 minutes later or with 120µg pentoxifylline followed by 100µg of Poly I:C 30 minutes later. Testing continued from day 1 through day 11. The pentoxifylline pretreatment group elicited a significantly decreased allodynic response in comparison to the Poly I:C control that lasted throughout the experiment. B) The hyperalgesic index was obtained by calculating the AUC for each rat and comparing it to their individual baselines, and indicated that the pentoxifylline group did not elicit an overall significant allodynic response.
A

Injection

Saline, n=4
Saline + 100µg Poly I:C, n=12
120µg Pentox + 100µg Poly I:C, n=7
Injection
Baseline 0 1 2 4 6 8 10 11
***
**
* ***
Day
Paw Withdrawal Threshold (gram)

B

Saline Saline + PIC Pentox
-20
0
20
40
60
80 **
Tactile Hyperalgesic Index

Saline Saline + PIC Pentox

0
-20
0
20
40
60
80
100
Tactile Hyperalgesic Index

A

Injection

Saline, n=4
Saline + 100µg Poly I:C, n=12
120µg Pentox + 100µg Poly I:C, n=7
Injection
Baseline 0 1 2 4 6 8 10 11
***
**
* ***
Day
Paw Withdrawal Threshold (gram)

B

Saline Saline + PIC Pentox
-20
0
20
40
60
80 **
Tactile Hyperalgesic Index

Saline Saline + PIC Pentox

0
-20
0
20
40
60
80
100
Tactile Hyperalgesic Index
Figure 10. Thermal, Pentoxifylline Pretreatment.

The response to non-noxious thermal pain stimuli to rats was measured over the course of 11 days to test whether or not pentoxifylline pretreatment to Poly I:C injection made a difference in hyperalgesic behavioral response in comparison. A) At day 0, rats were tested for baseline thermal hyperalgesia. Then, rats were injected intrathecally either with 10µL Saline followed by 100µg of Poly I:C 30 minutes later or with 120µg pentoxifylline followed by 100µg of Poly I:C 30 minutes later. Testing continued from day 1 through day 11. The pentoxifylline pretreatment group did not elicit a significant hyperalgesic response at any timepoint. B) The hyperalgesic index was obtained by calculating the AUC for each rat and comparing it to their individual baselines, and indicated that the pentoxifylline group did not elicit a significant hyperalgesic response.
A

Injection

Paw Withdrawal Latency (sec)

Saline, n=4
Saline + 100µg Poly I:C, n=12
120µg Pentox + 100µg Poly I:C, n=7

Day

Baseline 0 1 2 4 6 8 10 11

B

Thermal Hyperalgesic Index

Saline Saline + PIC Pentox

-20 0 20 40 60 80

Saline Saline + PIC Pentox
Figure 11. Tactile, Valdecoxib Pretreatment.

The response to non-noxious mechanical pain stimuli to rats was measured over the course of 11 days to test whether or not valdecoxib pretreatment to Poly I:C injection made a difference in allodynic behavioral response in comparison. A) At day 0, rats were tested for baseline tactile allodynia. Then, rats were injected intrathecally either with 10µL Saline followed by 100µg of Poly I:C 30 minutes later or with 30µg valdecoxib followed by 100µg of Poly I:C 30 minutes later. Testing continued from day 1 through day 11. The valdecoxib pretreatment group elicited a significantly decreased allodynic response in comparison to the Poly I:C control that lasted throughout the experiment. B) The hyperalgesic index was obtained by calculating the AUC for each rat and comparing it to their individual baselines, and indicated that the valdecoxib group did not elicit an overall significant allodynic response.
Figure 12. Thermal, Valdecoxib Pretreatment.

The response to non-noxious thermal pain stimuli to rats was measured over the course of 11 days to test whether or not valdecoxib pretreatment to Poly I:C injection made a difference in hyperalgesic behavioral response in comparison. A) At day 0, rats were tested for baseline thermal hyperalgesia. Then, rats were injected intrathecally either with 10µL Saline followed by 100µg of Poly I:C 30 minutes later or with 30µg valdecoxib followed by 100µg of Poly I:C 30 minutes later. Testing continued from day 1 through day 11. The valdecoxib pretreatment group did not elicit a significant hyperalgesic response at any timepoint. B) The hyperalgesic index was obtained by calculating the AUC for each rat and comparing it to their individual baselines, and indicated that the valdecoxib group did not elicit a significant hyperalgesic response.
A

Injection

Saline, n=4
Saline + 100µg Poly I:C, n=12
30µg Vald + 100µg Poly I:C, n=6

Day

Paw Withdrawal Latency (sec)

B

Thermal Hyperalgesic Index

Saline Saline + PIC Vald

-20 0 20 40 60 80
Figure 13. 24 Hour Activity Analysis.

The response total nocturnal activity in rats was measured over the course of 11 days to test whether Poly I:C injection made a difference in overall motion. Activity counts were taken by a computer equipped with motion sensors, which tabulated the presence or absence of motion every 4 seconds. Each rat was given one motion value every minute throughout the time course of the experiment, and the values were totaled each night, which was from 19:00 7:00 the next morning. A) At day 2 and 1, baseline levels of motion were recorded for rats. Then, rats were injected intrathecally either with 10μL Saline or 100μg of Poly I:C at day 0, with Night 1 being the following night. Testing continued from day 1 through day 11. The Saline group did not elicit a significant hyperalgesic response at any timepoint. B) The hyperalgesic index was obtained by calculating the AUC for each rat and comparing it to their individual averaged 2 day baselines, and indicated no significant hyperalgesic response.
**Figure A**

Plot showing nocturnal activity counts over 11 nights for Saline Nocturnal and Poly I:C Nocturnal injections. The x-axis represents the night of the study, ranging from -2 to 11, with a vertical arrow indicating the injection point. The y-axis represents nocturnal activity counts, ranging from 0 to 4000. The plot shows a trend where the activity counts increase after the injection.

**Figure B**

Bar chart comparing Saline Night and Poly I:C Night total activity counts (Night Raw). The x-axis represents the type of night (Saline Night, Poly I:C Night), and the y-axis represents total activity counts ranging from 0 to 1000. The chart shows a higher total activity count for Poly I:C Night compared to Saline Night.
Figure 14. Map of Spinal Lamina and Selected Regions of Spinal Cord.

Illustration of spinal cord cross section at the fourth lumbar. Light gray indicates white matter, dark gray indicates gray matter. Top of figure is dorsal, bottom of illustration is ventral. LEFT) Selected Regions where measurements were taken for quantification. SL1, SL2 and SL3 indicate three regions (adjacent horizontally) of the Superficial Lamina. DD indicates the Deep Dorsal horn. V indicates the ventral horn. WM indicates the White Matter. BG indicates the background noise from outside the specimen. RIGHT) Spinal Lamina I-X are separated by white borders.
Figure 15. Immunohistochemistry, GFAP.

A) Images representative of astrocyte activation in Sham (no injection), Saline at day 1, Saline at day 4, Saline + 100μg Poly I:C (indicated as Poly I:C) at day 1, and Saline + 100μg Poly I:C (indicated as Poly I:C) at day 4. Images of the dorsal horn were taken at 10X and 40X magnification and images of the ventral horn were taken at 10X magnification. B) Quantification, Sham and Time-Course Analysis. Time-course of astrocyte activation one day and four days after Poly I:C injection, as well as activation level of Sham rats (implanted rats receiving no injection). The background noise from each image was subtracted from the selected regions indicated in Figure 15. Indicated are the Mean and SEM, as well as levels of significance.
A
GFAP

Sham

Saline, Day 1

Saline, Day 4

Poly I:C, Day 1

Poly I:C, Day 4

Dorsal (10X)

Dorsal SL (40X)

Ventral (10X)

B

Intensity (AU)

SL1

SL2

SL3

WM

DD

V

Sham, n=4

Saline, n=4 (day1)

Saline, n=4 (day4)

Saline + Poly I:C, n=5 (day1)

Saline + Poly I:C, n=5 (day4)
Figure 16. Immunohistochemistry, Iba1.

A) Images representative of microglia activation in Sham (no injection), Saline at day 1, Saline at day 4, Saline + 100μg Poly I:C (indicated as Poly I:C) at day 1, and Saline + 100μg Poly I:C (indicated as Poly I:C) at day 4. Images of the dorsal horn were taken at 10X and 40X magnification and images of the ventral horn were taken at 10X magnification. B) Quantification, Sham and Time-Course Analysis. Time-course of microglia activation one day and four days after Poly I:C injection, as well as activation level of Sham rats (implanted rats receiving no injection). The background noise from each image was subtracted from the selected regions indicated in Figure 15. Indicated are the Mean and SEM, as well as levels of significance.
A

*Iba1*

Dorsal (10X)  Dorsal SL (40X)  Ventral (10X)

Sham

Saline, Day 1

Saline, Day 4

Poly I:C, Day 1

Poly I:C, Day 4

B

- Sham, n=4
- Saline, n=4 (day1)
- Saline, n=4 (day4)
- Saline + Poly I:C, n=5 (day1)
- Saline + Poly I:C, n=5 (day4)
Figure 17. Quantification, Pretreatment Analysis.

Time-course of astrocyte and microglia activation one day after Pretreatment + Poly I:C injection. The background noise from each image was subtracted from the selected regions indicated in Figure 15. Indicated are the Mean and SEM, as well as levels of significance.
GFAP

A

B

C

D

E

F

Iba1

G

H

I

J

K

L

Saline, n=4
Saline + Poly I:C, n=5
Mino + Poly I:C, n=4
Pentox + Poly I:C, n=6
Keto + Poly I:C, n=4
Vald + Poly I:C, n=4
IV. DISCUSSION

The focus of this study was to activate TLR3 by Poly I:C treatment, study its effects on tactile and thermal thresholds and the concurrent effects on spinal glial activation. Here it shown that after spinal delivery Poly I:C induces a persistent pain state and this effect occurs concurrently with the activation of both spinal astrocytes and microglia. Importantly, these changes were blocked by agents known to attenuate glial activation.

Confirmation of Paradigm

It was important to show that the mere action of injecting a substance into the intrathecal space of a rat did not show significant behavioral or biochemical responses. When testing, a Saline group receiving no drug was used as a control. There were no statistically significant changes in tactile or thermal behavior on any given day when compared to baselines taken before injection. This suggests that the action of intrathecally injecting a substance at 10µL does not cause a behavioral change that skews behavioral readings.

This control testing was taken a step further in immunohistochemical analysis. The antibodies used for analyzing morphology and quantifying activation were GFAP for astrocytes and Iba1 for microglia. Groups of rats receiving no injection were compared with rats receiving Saline injection which were sacrificed at day 1 and day 4. No statistical significance was found between any of these groups in any region tested (with the exception of white matter). This indicates that the act of injecting intrathecally does not produce activation of astrocyte or microglia cells. Furthermore, it indicates that there
is no latent time course for astrocyte or microglia activation solely due to injection into the intrathecal space.

**Actions of IT Poly I:C**

The basis of all of the conclusions of this study rest on the ability of Poly I:C to activate TLR3, and thus activate glial cells in the rat spinal cord. It has been shown that Poly I:C binds saturably and reversibly to TLR3, and it does so in almost identical affinity to equal concentrations of dsRNA (Leonard et al, 2008). There is no data in the literature to support the notion that dsRNA, or Poly I:C, activates any other TLR, and there is very strong evidence that Poly I:C only acts on TLR3. TLR3 knock out and knock down mice failed to produce the inflammatory cytokines and tactile allodynia shown in normal mice (Obata et al, 2008). It can thus be assumed that Poly I:C is acting specifically on TLR3 when administered intrathecally.

An intrathecal dose response study was performed to test the changes in tactile and thermal responses to Poly I:C. The doses used were 10µg, 30µg, and 100µg. Only the 100µg dose provided a tactile response. This intrathecal dose has been shown to provide strong responses in previous studies (Mei et al, 2011; Obata et al, 2008). The 10µg and 30µg doses showed modest numerical decreases in tactile threshold which displayed statistical significance at the highest dose.

No IT Poly I:C dose produced a change in the thermal response latency. This data is consistent with other inflammatory models used in this laboratory, including K/BxN (unpublished data). This pain behavior response has also been shown in other intrathecal inflammatory models, including intrathecal administration of NMDA agonist (Bennett et
al, 2000; Hama et al, 2003) as well as intrathecal delivery of interleukins, a proinflammatory product of glial activation (DeLeo et al, 1996).

The mechanism for this tactile, and not thermal, pain subsequent to intrathecal Poly I:C administration may rely on noxious fiber specificity. The two main types of noxious neuronal fibers providing afferent input from the periphery into the spinal cord are C and Aδ fibers. It has been shown that noxious thermal stimuli are transmitted through C fibers and noxious tactile stimuli through Aδ fibers (Ossipov et al, 1999). With this knowledge, it is possible that the glial activation leading to central sensitization only affects Aδ fiber synapses. This can possibly be due to structural or biochemical differences in the structure of the C and Aδ synapses which causes Aδ to be more sensitive to glial modulation.

**Interventional Treatments**

For all the subsequent experiments following the dose response testing, pretreatments were used in conjunction with Poly I:C. Because rats receiving pretreatments were injected with two intrathecal doses, it was important to show that the additional pretreatment injection did not initiate any changes in thresholds. For this, groups of rats receiving Poly I:C were compared to groups receiving a Saline pretreatment before receiving Poly I:C. Both groups elicited a severe drop in paw withdrawal threshold and there was no level of statistical significance between the groups on any given day. Furthermore the time course of the persistent pain state was identical in that both groups experienced tactile allodynia that started from day 1 and persisted through day 8.
These results indicate that the intrathecal, 100µg dose of Poly I:C is sufficient enough to initiate the persistent pain state in rats. It also shows that the pretreatment paradigm is valid for testing the ability of agents to block the effects of Poly I:C induced TLR3 activation.

**Poly I:C Induced Tactile Allodynia Time Course**

The results of tactile allodynia testing with the Saline + Poly I:C group indicated a maximal behavioral effect of TLR3 activation between day 1 and day 4. After this time period, there is a gradual increase of paw withdrawal threshold back to baseline levels in the Saline + Poly I:C group through day 11. These results suggest that activity of the pain producing system begins to be attenuated at day 4. Because the phenomenon of glial activation in the spinal cord modulating dorsal horn response to afferent input has been well documented to mediate pathological pain (Hain and Waxman, 2006; Milligan et al, 2003; Raghavendra and DeLeo, 2003; Raghavendra et al, 2003), it was interesting to correlate the behavioral changes associated with the persistent pain state. For this reason, glial activation motifs subsequent to Poly I:C were analyzed at day 1 and day 4 for astrocyte and microglia activation.

The quantification measurements obtained between the Saline group sacrificed at day 1 and the Saline + Poly I:C group sacrificed at day 1 were statistically significant in both astrocytes and microglia in every region quantified except for the white matter of astrocytes. The profound statistical significance in the superficial lamina corresponds to the synapse of primary to secondary afferent nociceptive neurons. GFAP measurements indicated that astrocytes have a much higher statistical significance in the superficial
lamina region in comparison to the other regions measured, as opposed to microglia which have relatively profound activation throughout the spinal cord. This suggests that a mechanism may be responsible for attracting astrocytes specifically to the dorsal horn which is not present in microglia.

The activation of astrocytes and microglia is also evident by the cell morphology. As opposed to the Saline group, the group subject to Saline + Poly I:C injection had astrocytes which exhibited prolonged processes and microglia which exhibited retracted processes and amoeboid shape. These respective morphologies are highly indicative of cell activation (Stirling et al, 2004; Cao and Zhang, 2008; Yrjänheikki et al, 1998).

The statistically significant differences in quantification between day 1 and day 4 groups of Saline + Poly I:C in the superficial lamina regions of both astrocytes and microglia as well as the microglial white matter, deep dorsal horn and ventral horn which indicate a decrease in overall paw withdrawal threshold correlate with the tactile behavioral findings. In both behavioral and glial analysis, we see a decrease in effect at day 4. Furthermore, we find no difference in morphology between astrocytes and microglia for the Saline group sacrificed at day 1 and the Saline + Poly I:C group sacrificed at day 4. This indicates that the morphology of these glial cells reversed to their resting states.

Astrocytes and microglia have been implicated in the regulation of nociceptive processing because of their ability to cause central sensitization by releasing cytokines, chemokines like interleukins, tumor-necrosis factor, and prostaglandins at the level of the first order synapse in a manner that results in signal amplification; this results in the sensation of a pain state by otherwise non-noxious stimuli (Fellin et al, 2006; Parpura et
Intracellular events associated with activation of astrocytes and microglia include activation of MAPK and NF-κB, (Tsuda et al, 2005; Deleo et al, 2004; Cao and Zhang, 2008), which activates various proinflammatory mediators such as prostaglandin, cyclooxygenase, TNF, IL-1β, and, IL-6. (Kumar et al, 2003; Ji and Suter, 2007; Cao and Zhang, 2008; DeLeo et al, 1997; Hashizume et al, 2000; Milligan et al, 2001; Raghavendra et al, 2004.).

Previous studies with intrathecal Poly I:C administration have shown increased levels of p-p38, Iba1, IL-1b, IL-6, and TNF. The expression of all of these products was prevented by TLR3 knockdown with antisense-oligonucleotide (AS-ODN). The behavior results from this set of experiments correlate with previous studies on intrathecal Poly I:C administration in the absence of a thermal withdrawal latency response as well as a nearly identical paw withdrawal threshold response activation timeline, including maximal effect and overall effect time points. (Obata et al, 2008). The data suggests that the regression of behavior back to baseline levels several days after the administration of Poly I:C is a consequence of astrocyte and microglia activity through the activation of TLR3.

The thermodynamics of dsRNA does not allow it to remain in the system for prolonged periods of time. Longer strands of dsRNA are broken down by nucleases into roughly 22bp strands. Longer dsRNA strands are more stable than shorter ones because the increased number of hydrogen bonds between the strands. Poly I:C, only 1 base pair in length, has a particularly short half life. It has been shown that the plasma degrades
Poly I:C in the matter of minutes (de Clercq et al, 1979; Parrish et al, 2000). The present set of experiments, as well as previous work by Mei and Obata laboratories, have shown that Poly I:C produces a long lasting inflammatory response. This indicates that the ability of Poly I:C to activate TLR3 is very profound and very rapid. It also indicates that the subsequent cascades, and thus glial inflammatory products, are responsible for the persistent pain state rather than continuous TLR3 activation.

Previous studies have suggested that a change in dorsal horn plasticity is the cause of the persistent pain state. Peripheral axotomy has been shown to produce changes in dorsal horn plasticity, Aδ fibers that usually carry non-noxious tactile stimuli sprout from the third lamina higher into the superficial lamina. C fibers, however, have not been shown to do so. The result from peripheral axotomy is tactile allodynia, not thermal hyperalgesia, suggesting that this change in plasticity is somehow correlated with central sensitization (Ossipov et al, 1999; Woolf et al, 1992; Woolf et al, 1995; Lekan et al, 1996). Glial cells have a very important role in this change in dorsal horn plasticity. Microglia and astrocytes in the spinal cord produce nerve growth factor (NGF) after spinal nerve damage and peripheral nerve damage. This product has been proposed be responsible for changing the axonal termination (Heumann et al, 1987; Krenz and Weaver, 2000).

Because both the intrathecal Poly I:C model and the peripheral axotomy model both elicited a tactile, but not thermal, response, change in dorsal horn plasticity may be a possible mechanism in creating the persistent pain state. However, there is time course data which is in disagreement. In the peripheral axotomy model, changes in plasticity are not detectable until 1 week after nerve injury, maximal at 2 weeks, and reverse at 33-35
weeks in rats (Woolf et al, 1995). In an SNL model, corresponding changes in mechanical sensitivity have been shown to be profound from day 1 through day 40 (Ossipov et al, 1999). In the present set of experiments, the maximal behavioral response diminishes after several days, and any semblance of tactile allodynia disappears by day 11. It would be very interesting to compare neuroplasticity results of previous peripheral and central nerve ligation models with the current Poly I:C induced inflammation model. This information would demonstrate whether or not inflammation causes changes in axonal termination of Aδ and C fibers, and it would suggest glial cells have a possible role. If changes in plasticity are indeed demonstrated to occur following Poly I:C treatment, it would also be interesting to see if the reversibility of the axonal termination follows the same time course as other models.

**Nocturnal Behavior**

It is appreciated that pain will suppress spontaneous behavior (Millecamps et al, 2005). In the present work, in spite of the evident persistent decrease in paw withdrawal threshold, there was no change in motion behavior resulting from the intrathecal administration of Poly I:C in comparison to Saline.

In the mouse K/BxN chronic arthritis model, a similar result has been reported. In that model there is an early phase of inflammation and a tactile allodynia which starts early and persists after the resolution of the inflammation. In those animals there is a significant reduction in nocturnal activity during the inflammatory phase which recovers during the later phase where there is an unresolved allodynia (unpublished data). These observations are surprising as they suggest that the tactile allodynia does not represent a
disabling state for the animal. This suggests that the tactile allodynia is not a marker for ongoing nociception, but likely reflects a state that can be tolerated by the animals in manner that does not yield an ongoing motion evoked nociceptive state.

**Block of Glial Activation and IT Poly I:C Evoked TA**

Minocycline, Pentoxifylline, Ketorolac, and Valdecoxib were all used as pretreatments in their respective doses to Poly I:C. To varying extents, all pretreatments showed a decrease in tactile allodynia as well as activation levels of astrocytes and microglia.

**Minocycline**: Minocycline is a very potent, second generation tetracycline. It has been shown to inhibit microglial activation shown in levels of phosphotyrosine immunoreactivity and isolectin B4 binding, as well as morphology. In astrocytes, minocycline inhibits iNOS expression, which is present in all glial cells but primarily in astrocytes. Minocycline has also been shown to decrease levels of Caspase-1 and-3 (which cleave pro-IL-1β into its active form, IL-1β), TNF, and P38-MAPK. (Yrjänheikki et al, 1998; Tikka et al, 2001; Krady et al, 2005). Intrathecal administration of minocycline has been shown to be a potent agent for antinociception in inflammation evoked pain as it reduced flinching from a formalin inflammatory induced model and prevented hyperalgesia in carrageenan model (Hua et al, 2005). In this study, intrathecal administration of minocycline has been shown to reverse the central sensitization caused by TLR-3 activation in the spinal cord. Behaviorally, this was shown in the reversal of the tactile allodynia produced by intrathecal administration of Poly I:C. When intrathecal minocycline was used as a pretreatment, there was a
reversal of allodynia. Immunohistochemical analysis of tissue collected at day 1 indicated that minocycline prevented the activation of astrocytes and microglia in the dorsal horn, as well as microglia in the ventral horn.

**Pentoxifylline:** Pentoxifylline is a non specific cytokine inhibitor which modulates products from all glial cells in the spinal cord. IT pentoxifylline decreased formalin and post-nerve injury induced tactile allodynia in rats. The mechanism of pentoxifylline lies in its ability to be a non-specific inactivator of phosphodiesterase, lowering TNFα production and blocking synthesis of IL-3, IL-6, and IL-8 inflammatory cytokines, which cause subsequent inflammatory responses, hyperalgesia, and central sensitization. (Semmler et al, 1993; Sweitzer et al, 2001; Mika et al, 2007; Wordliczek et al, 1999).

In this study, intrathecal administration of pentoxifylline was shown to reverse the central sensitization caused by TLR-3 activation in the spinal cord. Behaviorally, this was shown in the reversal of the tactile allodynia produced by intrathecal administration of Poly I:C. When intrathecal pentoxifylline was used as a pretreatment, there was a reversal of allodynia. Immunohistochemical analysis of tissue collected at day 1 indicated that minocycline prevented the activation of astrocytes and microglia in the dorsal horn, as well as microglia in the ventral horn.

**Ketorolac:** Ketorolac has been identified as a general COX inhibitor that is potent in the spinal cord. Spinal sensitization produces prostaglandins, and NSAIDS like ketorolac can reverse hyperalgesia (Malmberg and Yaksh, 1992; Malmberg and Yaksh, 1993). Intrathecal ketorolac administration has been shown to prevent tactile alldynia in L4/L5 tight nerve ligation model (Uda et al, 1990). Intrathecal administration has also
shown provide antinociceptive in formalin model. (Malmberg and Yaksh, 1993; Lashbrook et al, 1999).

In this study, intrathecal administration of ketorolac reversed the inflammatory central sensitization caused by TLR-3 activation in the spinal cord. Behaviorally, this was shown in the reversal of the tactile allodynia produced by intrathecal administration of Poly I:C. When intrathecal ketorolac was used as a pretreatment, there was a reversal of allodynia. Immunohistochemical analysis of tissue collected at day 1 indicated that minocycline prevented the activation of astrocytes and microglia in the dorsal horn, as well as microglia in the ventral horn.

Valdecoxb: Valdecoxb has been identified as a COX-2 specific inhibitor (Talley et al, 2000). Injection of proinflammatory agents in the periphery, like complete Freund’s adjuvant (CFA), produce upregulation of COX-2 expression in the dorsal horn, but COX-1 expression does not change. (Beiche et al, 1996; Hay et al, 1997; Samad et al, 2001; Zhu et al, 2003). COX-2 has been shown to play a dominant role in the spinal cord for producing inflammatory pain in comparison to COX-1. Intrathecally administered COX-2 specific inhibitors (SC-58125, SC-236 and SC-384) and nonspecific COX inhibitors (ibuprofen) have been shown to block allodynia produced by intrathecal NMDA and Substance-P administration, as well as cause a decrease in PGE2 production. Intrathecal COX-1 specific inhibitors (SC-560), however, have been shown to have no effect on blocking hyperalgesia or prostaglandin production produced by intrathecal NMDA and Substance-P administration. (Yaksh et al, 2001; Zhu et al, 2003).

Conclusions
Several conclusions have been made during the course of the present set of experiments:

1. A dose of 100µg Poly I:C administered intrathecally into the lumbar region of the rat spinal cord results in the persistent pain state, measurable by behavioral experiments. While there was no change in thermal hyperalgesia through a time course of 11 days (measured by thermal paw withdrawal latency), there was a dramatic decrease in tactile threshold (measured by tactile paw withdrawal threshold), measurable at 24 hours lasting at least though 4 days. Because there is no evidence of receptors that bind to dsRNA other than TLR3, it is assumed that activation of TLR3 causes the profound persistent pain state. TLR3 is known to exist in Astrocytes and Microglia in the spinal cord, suggesting that TLR3 activation causes spinal glial activation, which produces inflammatory cytokines and chemokines to be produced, which are known to sensitize the synapse to afferent input.

2. Rats receiving intrathecal pretreatments of glial inhibiting drugs (minocycline and pentoxifylline) as well as COX blocking drugs (ketorolac and valdecoxib) prior to intrathecal Poly I:C showed significant decreases in the persistent pain state. The glial blocking pretreatments attenuating the persistent pain state serves as an indication of the role of glial cell activation in producing tactile allodynia by producing inflammatory agent production act on the synapse. The COX blocking pretreatments attenuating the persistent pain state suggests the role of prostaglandins in producing tactile allodynia. Specifically, the COX 2 inhibitor valdecoxib served to attenuate tactile alldynia to an equal extent as the non
specific COX inhibitor ketorolac. This indicates the role of COX 2 as prominent COX enzyme in producing tactile allodynia.

3. Rats receiving intrathecal Poly I:C and sacrificed at day 1 showed increases in astrocyte and microglia activation in the superficial lamina and deep lamina of the dorsal horn as well as in the ventral horn. Astrocytes and Microglia were shown to increase expression of proteins associated with their activation (GFAP and Iba1, respectively), as well as produce a change in morphology indicative of activation. This provides very strong evidence that dsRNA activates microglia and astrocytes in the CNS through binding to TLR3.

4. Activation of astrocytes and microglia diminished 4 days post intrathecal Poly I:C administration. This indicates that the activation of glial cells by dsRNA is reversible. Furthermore, the activation and deactivation of glial cells subsequent to Poly I:C administration aligns with the timeframe of the maximally induced tactile allodynia. This provides strong evidence that subsequent to activating agents (here, dsRNA), glial cells can be strong mediators of the persistent pain state.

5. Rats receiving intrathecal pretreatments of glial inhibiting drugs (minocycline and pentoxifylline) as well as COX blocking drugs (ketorolac and valdecoxib) prior to intrathecal Poly I:C and sacrificed at day 1 showed normal levels astrocyte and microglia activation, indicating an inhibition of the nociceptive cascade. This correlates with the time course of the behavioral levels of tactile allodynia. The glial blocking drugs used show that preventing the activation of astrocytes and microglia will attenuate the persistent pain state in the presence of dsRNA. The
COX blocking drugs used show that preventing the production of prostaglandins is enough to prevent the activation of astrocytes and microglia and attenuate the persistent pain state in the presence of dsRNA.

**Future Experiments**

These conclusions offer insight in the role of glial activation and attenuation in the persistent pain state, and add to knowledge that has developed on this topic over the last decade. Future experiments on this matter can provide more insight to molecular expression patterns.

1. **Post treatments.** A useful piece of information would be the behavioral analysis of post treatments coupled with analysis and quantification of glial activation motifs. Within the cell, we hypothesize that pretreatments will be more effective than post treatments for both the glial suppressing drugs as well as the COX blocking drugs because it can prevent the TLR3 cascade from activating positive feedback enzymes like p38-MAPK from activating transcription factors known to produce inflammatory cytokines. Post treatments at various time points after intrathecal injection would give insight to the ability of these drugs to modulate glial activation reversibility subsequent to intrathecal Poly I:C administration.

2. **Cytokine and Chemokine Expression.** Studies described in this paper have been performed to test for cytokine and chemokine production subsequent to Poly I:C administration at only one time point. It would be interesting to analyze the cytokine expression motifs at day 1, day 4, and day 11 after intrathecal Poly I:C administration, and correlate specific agents with the rising action, climax, and
falling action of the persistent pain state. It would be of great interest to compare these products to those of other inflammatory models. Furthermore, it would be interesting to identify changes in these expression motifs between various pretreatments of glial and COX blocking drugs.

3. Poly I:C vs dsRNA. In this paper, other laboratories have been described as using Poly I:C as a synthetic analogue to dsRNA. However, it would be interesting to test the validity of the model on all accords, including behavioral analysis of pain thresholds, reduction of the persistent pain state with pre and post treatments of glial and COX blocking drugs, time course of tactile allodynia, and time course of glial activation. Because larger strands of dsRNA are more stable, we hypothesize that the effects of dsRNA on TLR3 activation will be more profound and more prolonged due to a longer half life in the spinal cord.

4. COX 1 Blocker. In this set of experiments, a non specific COX blocker was compared to a COX 2 blocker, and reinforced previous findings described in this paper showing COX 2 is more involved than COX 1 in producing the persistent pain state. It would be interesting to administer a COX 1 blocker intrathecally as a pretreatment to Poly I:C, and compare expression patterns, glial activation patterns, as well as behavioral patterns indicative of the persistent pain state. We hypothesize that COX 1 blockers will not prevent the persistent pain state and will not prevent the activation of glial cells in the spinal cord when used as a pretreatment to Poly I:C.

5. Axonal Plasticity. As described in this paper, it has been previously shown that injury models to nerves in the periphery as well as in the central nervous system
have been shown to alter axonal plasticity in the dorsal horn. It would be very interesting to test if this intrathecal, Poly I:C inflammatory model produces such changes in axonal termination. If so, it would suggest that glial cells are involved in the modulation of axonal plasticity, which is correlated to the persistent pain state. If changes in plasticity do occur, it would also be interesting to analyze their time course as well as reversibility.
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