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# Pioglitazone rapidly reduces neuropathic pain through astrocyte and non-genomic PPARγ mechanisms

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## Abstract

Repeated administration of peroxisome proliferator-activated receptor gamma (PPARγ) agonists reduces neuropathic pain-like behavior and associated changes in glial activation in the spinal cord dorsal horn. As PPARγ is a nuclear receptor, sustained changes in gene expression are widely believed to be the mechanism of pain reduction. However, we recently reported that a single intrathecal injection of pioglitazone, a PPARγ agonist, reduced hyperalgesia within 30 minutes, a time frame that is typically less than that required for genomic mechanisms. To determine the very rapid anti-hyperalgesic actions of PPARγ activation we administered pioglitazone to rats with spared nerve injury (SNI) and evaluated hyperalgesia. Pioglitazone inhibited hyperalgesia within 5 min of injection, consistent with a non-genomic mechanism. Systemic or intrathecal administration of GW9662, a PPARγ antagonist, inhibited the anti-hyperalgesic actions of intraperitoneal or intrathecal pioglitazone, suggesting a spinal PPARγ-dependent mechanism. To further address the contribution of non-genomic mechanisms, we blocked new protein synthesis in the spinal cord with anisomycin. When co-administered intrathecally, anisomycin did not change pioglitazone anti-hyperalgesia at an early 7.5 min timepoint, further supporting a rapid non-genomic mechanism. At later timepoints anisomycin reduced pioglitazone anti-hyperalgesia, suggesting a delayed recruitment of genomic mechanisms. Pioglitazone reduction of SNI-induced increases in GFAP expression occurred more rapidly than expected, within 60 min. We are the first to show that activation of spinal PPARγ rapidly reduces neuropathic pain independent from

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canonical genomic activity. We conclude that acute pioglitazone inhibits neuropathic pain in part by reducing astrocyte activation, and via both genomic and non-genomic PPARγ mechanisms.

**Keywords**

neuropathic; pain; PPAR gamma; astrocyte; pioglitazone; non-genomic

## INTRODUCTION

Peroxisome proliferator-activated receptor gamma (PPARγ) is activated by thiazolidinedione (TZD) drugs such as pioglitazone [74] that cross the blood brain barrier [55]. TZDs reduce molecular and behavioral sequelae of many neurological diseases [see [23;42;54;93]]. It is widely assumed that genomic PPARγ activity mediates reduction of inflammatory [59;64] and neuropathic [53;60] pain after repeated TZD dosing. However, agonists for PPARγ and other nuclear receptors (e.g. estrogen, glucocorticoid, PPARα) can modulate neuronal excitability [24;41;46] and pain [13;22;48;95] within minutes. Thus, the anti-hyperalgesic effects of nuclear receptor agonists may occur through multiple mechanisms including: 1) genomic, receptor-dependent transcription/translation, 2) non-genomic, receptor-dependent activation of membrane-bound receptors [41;50;73], 3) receptor-independent “off-target” effects at GPCRs [56;61;62].

Traumatic nerve injury produces changes in the spinal cord such as astrocyte activation [88;94], increased post-synaptic responses to glutamate [91], and long-term potentiation [30] resulting in central sensitization [77]. During chronic pain states, activated spinal astrocytes release pro-nociceptive mediators [63;90] such as TNFα that facilitate pain sensitization [for review see [27]]; therefore, pharmacotherapeutic approaches that decrease astrocyte activation may reduce chronic pain. TZDs agonize PPARγ expressed in spinal cord [58] and brain [17] astrocytes to reduce activation [6] and GFAP upregulation [34]. Dosing over several weeks produced anti-hyperalgesia that was associated with reductions in spinal GFAP [39;53;60], injury-induced pro-inflammatory cytokine expression [40], and *in vitro* astrocyte TNFα release [31;82]. A single injection of PPARγ agonist reduces neuropathic pain [13]; however, an important gap is whether there are acute effects on spinal astrocytes.

Here, we characterized the effects of PPARγ activation in neuropathic (spared nerve injury; SNI) and acute nociceptive (capsaicin) conditions after acute drug administration. We hypothesized that rapid inhibition of pain by PPARγ agonists is mediated by receptor-dependent, spinal mechanisms and is independent of translation (i.e. non-genomic). We used systemic and spinal agonist/antagonist administration to determine the site of PPARγ anti-hyperalgesia. We investigated pain-like hypersensitivity as early as 5 min after intrathecal injection of pioglitazone in the presence or absence of anisomycin, an inhibitor of protein synthesis [25;29;76]. Finally, we tested whether a single pioglitazone injection, as opposed to repeated administration [60], would reduce astrocyte activation in the form of GFAP overexpression after SNI.

## MATERIALS & METHODS

### Animals

Male Sprague-Dawley rats (CD-IGS, Charles River Laboratories, Inc., Wilmington, MA) weighing 300–450g at the time of behavioral procedures were housed 2 per cage on a 12-hour light/dark cycle (7am lights on / 7pm lights off) in a temperature (68–72 °F) and humidity controlled room with food and water provided *ad libitum*. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, in accordance with the International Association for the Study of Pain and the National Institutes of Health Office of Laboratory Animal Welfare Guide for the Care and Use of Laboratory Animals. All behavioral procedures were performed between 8am–6pm (lights on) and approved by an Institutional Animal Care and Use Committee (IACUC) protocol. Behavioral measurements and immunohistochemistry quantification were performed by an observer blinded to experimental treatments (e.g. injury and/or drug).

### Spared Nerve Injury (SNI) surgery

Surgical anesthesia was achieved with isoflurane (5% induction and 1.5% maintenance diluted in oxygen). As previously described [18;85], the skin was incised on the left hindlimb over the sciatic nerve trifurcation. The overlying muscles were retracted to expose the common peroneal, tibial, and sural nerves. The common peroneal and tibial nerves were ligated with 6-0 silk (Ethicon, Somerville, NJ) and transected 1mm proximal and 1mm distal to the ligation. The ligation knot and adjacent nerve were removed. Sural nerve perturbations were avoided. The muscle and skin were closed with loosely tied 5-0 absorbable sutures (Ethicon) and 9mm stainless steel wound clips, respectively. During sham surgeries, all steps were performed except ligation and transection of the common peroneal and tibial nerves. SNI or sham surgery day is referred to as day 0.

### Pain-like behavior

Animals were acclimated in individual Plexiglas boxes (4″ × 8″ × 4″) on top of a raised stainless steel mesh grid (mechanical and cold) or Plexiglas floor (Hargreaves) for 1 h. Fluctuations in noise, vibrations, temperature, and other distractors in the behavioral testing room were avoided to optimize reliable measurements between cohorts of animals tested on different days.

Mechanical hypersensitivity was assessed using von Frey filaments (Stoelting, Inc., Wooddale, IL). The lateral aspect of the hindpaw plantar surface (sural receptive field) was stimulated with an incremental series of 8 monofilaments of logarithmic stiffness using a modified up-down method [10;20]. Each filament was applied to the sural receptive field three times at proximal, intermediate, and distal locations with respect to the heel. Testing began by applying an intermediate von Frey monofilament (number 4.31, exerts 2.0 g of force) perpendicular to the glabrous skin, causing a slight bending. In the case of a positive response (withdraw of the paw) a filament exerting less force was applied. In the case of a negative response, a filament exerting greater force was applied. The calculated 50% withdraw threshold is reported.

Cold hypersensitivity was assessed after application of a drop of acetone to the sural receptive field. We used a 3 mL syringe attached to an 8 cm length of PE-10 tubing flared to a diameter of 3.5 mm at the distal end. Surface tension maintained the volume of the drop to 10–12 μl. The amount of time the animal lifted, shook, or licked the affected hindpaw was recorded with a cutoff of 30 s after each acetone application. The average of three trials per subject at each timepoint is reported.

Heat hypersensitivity was assessed by recording paw withdraw latencies using the Hargreaves method and apparatus [32]. An adjustable infrared heat source (8 V, 50 W lamp, Ugo Basile, Italy) was positioned under the Plexiglas floor directly beneath the uninjured hindpaw. Prior to each behavioral testing session, intensity was adjusted so that the average latency to paw withdraw was 9 ± 2 s. If an animal did not respond within 20 s, the radiant beam was shut off to avoid tissue damage. The average of three trials per subject at each timepoint is reported.

### Motor coordination

Motor coordination was assessed by placing the animals on an accelerating rotarod (Stoelting, Wood Dale, IL). Beginning at 2 revolutions per minute (rpm), the rotarod machine was programed to accelerate 0.5 rpm every 5 s until reaching 60 rpm. Animals were acclimated to the rotarod and subjected to one training session per d for 2 consecutive d prior to drug administration. During training and testing, the rats were placed on the rotating bar at 2 rpm, the acceleration program was started, and the time spent on the rotarod prior to fall was recorded. During the first training session, the animals were repeatedly placed on the rotarod until they performed 3 consecutive trials ≥ 150 s or reached a predetermined cutoff of 20 trials, whichever occurred first. By the second day of training, all rats successfully completed 3 consecutive trials ≥ 150 s prior to reaching 20 trials. Baseline measurement of time spent on the rotarod was recorded 7 d after SNI followed by reassessment at 1, 2, and 3 h after i.p. or i.t. drug administration. Three trials per timepoint were averaged.

### Intracerebroventricular cannulation

Surgery was performed one week before injury to allow for 5 μl of drug or vehicle injection directly into the ventricle. Surgical anesthesia was achieved with isoflurane (5% for induction, 1.5% for maintenance in oxygen). Rats were placed in a stereotaxic apparatus fitted with blunt ear bars (Stoelting, Kiel, WI). After an incision to expose the cranium, the dorsal surface of the skull was leveled by zeroing the dorsoventral coordinate at lambda and bregma. A 26Ga stainless steel guide cannula (Plastics One, Roanoke, VA) was lowered to the right lateral brain ventricle using the following stereotaxic coordinates: 0.7 mm posterior to bregma, 1.5 mm lateral from midline and 3.3–4.0 mm below the skull surface [70]. Correct placement was indicated by the observation of the movement of 1μl sterile saline from a piece of PE-10 tubing attached to the cannula into the ventricle [86]. The cannula was fixed to the skull with 3 small screws and dental cement and after suturing the incision a 30 Ga stylet (Plastics One) was secured within the guide cannula.

### Drugs

Pioglitazone potassium salt (10028, Cayman Chemical, Ann Arbor, MI) was dissolved in a 0.9 % saline slurry. GW9662 (70785, Cayman Chemical) was dissolved in a mixture of ethanol, ethoxylated castor oil, and saline (2:2:6) for i.t. administration and 50% DMSO in saline for i.p. administration. Neither vehicle solution altered pain-like behavior (see Fig 2). Anisomycin (A9789, Sigma-Aldrich, St. Louis, MO) was first dissolved in a small volume of 1 M HCl and then an equal volume of 0.9% saline was added. Next, the solution was titrated with NaOH to obtain a physiological pH of 7.0–7.4. Finally, the anisomycin solution was further diluted in saline to a final concentration of 20 mg/ml. Capsaicin (M2028, Sigma-Aldrich) was first dissolved in 100% ethanol (5% final v/v) followed by addition of a Tween 80 (P1754, Sigma-Aldrich) in saline (8% v/v) solution to a final capsaicin concentration of 50 mg/ml (w/v). Capsaicin (50 μl) was injected into the subdermal space between the 2$^{nd}$ and 3$^{rd}$ digits on the ventral plantar surface using a 30 Ga ½” needle.

### Drug Injections

Rats were anesthetized with isoflurane. Vehicle or drug solution (10–20 μl) was injected into the subarachnoid space using a 27 Ga 1″ needle inserted into a stretch of PE-20 tubing and attached to a Hamilton micro syringe [57]. We confirmed needle placement by both visualization of cerebrospinal fluid aspiration within the tubing and/or a reflexive tail/hindpaw flick. For systemic pioglitazone studies, i.p. (2 or 10 mg/kg) or i.t. GW9662 (300 μg) was administered 15 min prior to pioglitazone. For intrathecal pioglitazone studies, GW9662 (300 μg) or anisomycin (0–200 μg) was co-administered with pioglitazone to avoid multiple i.t. injections.

### Capsaicin-induced nociception

Intrathecal vehicle (saline), pioglitazone (0–300 μg), or anisomycin (0–200 μg) were injected 20 min before intraplantar (i.pl.) capsaicin (50 μg in 50 μl). Behavior was recorded for 0–2 min following i.pl. capsaicin. The number of flinches and the time spent licking or lifting (s) the affected hindpaw were combined and reported as the number of nociceptive responses, where 1 s of licking/lifting is equal to one flinch response [1;49;78]. Sham injections consisted of capsaicin vehicle as a control. Rats were perfused 60 min following i.pl. capsaicin for Fos immunohistochemistry.

### Immunohistochemical quantification of Fos and GFAP in the dorsal horn

Rats were anesthetized with pentobarbital (Fatal Plus, Med-Vet International, Mettawa, IL) and perfused through the left ventricle with 250 ml of room temperature 0.1 M phosphate buffered saline (PBS) with heparin (10,000 USP units/L) followed by 250 ml of ice-cold fixative (10% phosphate buffered formalin). The lumbar spinal cord was removed and post-fixed overnight in 10% phosphate buffered formalin and then cryoprotected in 30% sucrose in 0.1 M PBS for several days. Transverse sections (30 μm) from L4-L5 were cut on a freezing microtome and collected in 0.1 M PBS. The sections were washed three times in 0.1 M PBS and then pretreated with blocking solution (3% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS) for 1 h. Sections were incubated overnight at room temperature in blocking solution containing either rabbit anti-Fos (1:500, SC-52, Santa Cruz

Biotechnology, Santa Cruz, CA) for capsaicin studies or anti-GFAP (1:1000, ab7779, Abcam, Cambridge, MA) for SNI studies. The slices were washed three times in 0.1 M PBS, incubated in goat anti-rabbit (1:800, Alexa 488 or 568, Molecular Probes, Grand Island, NY) for 90 min, washed in 0.1 M PBS then 0.01 M PB, mounted onto Superfrost Plus slides, air dried, and cover-slipped using Prolong Gold with DAPI mounting medium (Molecular Probes, Grand Island, NY).

All images were captured and analyzed on a Nikon Eclipse TE2000-E microscope using 4× or 10× objectives using NIS-Elements Advanced Research software. In capsaicin studies we quantified the number of Fos+ cell profiles in lamina I-V of the dorsal horn. In SNI studies the dorsal horn was separated into lamina I-II, lamina III-IV, and lamina V as well as medial, central, and lateral regions of interest (ROI). Medial-lateral ROI correspond to afferent input from the tibial (medial), common peroneal (central), and sural (lateral) receptive fields as previously described [15]. Each of the six ROI were quantified and analyzed separately. For GFAP, pixel intensity values were summed, normalized to ROI area, and background subtracted. Background intensity was determined by selecting a ROI in the devoid of GFAP+ cells. This method accounts for changes in both the number and the fluorescence intensity of GFAP+ cells. Slices were analyzed by an observer blinded to treatment. Quantification of 4–6 slices per subject was averaged for each ROI and n=3–4 rats per group are reported.

### Western blotting of lumbar spinal GFAP

Western blot analyses were performed on lumbar spinal cord quadrants that were sonicated on ice in 50 mM Tris buffer containing 100 mM 6-amino-n-caproic acid, 1 mM EDTA, 5 mM benzamidine, 0.2 mM phenylmethyl sulfonyl fluoride (in 100% ethanol), and protease inhibitors. After extraction, proteins were subjected to NuPAGE Bis–Tris (4–12%) gel electrophoresis under reducing conditions (Invitrogen, Carlsbad, CA) and then transferred to nitrocellulose membranes electrophoretically (Invitrogen, Carlsbad, CA). Nonspecific binding sites on the membrane were blocked with Odyssey Blocking Buffer (50%; LI-COR Biosciences, Lincoln, NE) in TBS containing 0.1% Tween-20, 0.05% Tris-Chloride, and 0.03% 5 M NaCl for 1 h at 22–24°C. Membranes were subsequently incubated with primary antibodies in Odyssey Blocking buffer containing 0.1% Tween-20 overnight at 4°C. The membranes were then washed with PBS containing 0.1% Tween-20, and probed with appropriate IRDye secondary antibodies (LI-COR Biosciences) in Odyssey Blocking buffer containing 0.1% Tween-20 for 1 h at 22–24°C, protected from light. Following washing with PBS containing 0.1% Tween-20, membranes were scanned on an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Primary antibodies and dilution ratios used were rabbit GFAP (1:1000, z0334, Dako, Carpinteria, CA), and mouse β actin (1:100,000, A5316, Sigma-Aldrich, St. Loius, MO). Secondary antibodies used were goat anti-mouse IRDye 680RD (1:15,000, LI-COR Biosciences), goat anti-rabbit IRDye 800CW (1:15,000, LI-COR Biosciences). Bands were quantified using Image Studio (LI-COR Biosciences).

### Statistical analysis

For analysis of behavioral data and westerns, multiple groups were compared for significant differences over time using repeated measures two-way ANOVA or between groups using a standard two-way ANOVA followed by Holm-Sidak multiple comparison correction. Area-under-the-curve (AUC) was calculated using the trapezoidal method and analyzed using a one-way ANOVA with Holm-Sidak multiple comparison correction or an unpaired, two-tailed t-test. Ipsilateral versus contralateral (paired) or pioglitazone versus saline (unpaired) comparisons of GFAP immunohistochemistry ROIs (6 total analyses) or GFAP western quadrants were analyzed using a two-tailed t-test. An alpha value of $\alpha$=0.05 was used to determine statistical significance. All data were analyzed and graphed using Prism 6.0 (GraphPad, La Jolla, CA) and are presented as mean ± SEM.

## RESULTS

### Systemic pioglitazone reduces mechanical and cold hypersensitivity

To determine whether PPARγ activation reduces hyperalgesia, we evaluated behavioral indices of neuropathic pain after a single i.p. injection of pioglitazone. Spared nerve injury (SNI) decreased mechanical [time; $F(1, 37) = 1453$; $P < 0.0001$] and cold [time; $F(1, 37) = 86$; $P < 0.0001$] sensitivities at 14 d after injury. As illustrated in Fig 1A–B, systemic pioglitazone attenuated mechanical [dose × time; $F(21, 259) = 5.756$; $P < 0.0001$] and cold hypersensitivity [$F(21, 259) = 3.485$; $P < 0.0001$]. Area under the curve (AUC) analyses in Fig 1C–D indicate that pioglitazone dose-dependently attenuated mechanical [dose; $F(3, 37) = 6.747$; $P = 0.001$] and cold hypersensitivity [$F(3, 37) = 3.575$; $P = 0.023$].

### PPARγ in the spinal cord mediates the anti-hyperalgesic actions of systemic pioglitazone

Churi *et al.* reported that a single intrathecal injection of the PPARγ agonist rosiglitazone produced anti-hyperalgesia within one hour [13]. However, the authors did not test for PPARγ-dependency. To address this gap and test the hypothesis that PPARγ mediates the acute anti-hyperalgesic effects of i.p. pioglitazone, we pretreated SNI rats with GW9662, an irreversible PPARγ antagonist [12;45]. As illustrated in Fig 2A–D, systemic GW9662 (i.p.) prevented pioglitazone reductions in mechanical [AUC; $F(2, 25) = 13.14$; $P = 0.0001$] and cold [AUC; $F(2, 27) = 3.992$; $P = 0.03$] hypersensitivity.

Next we sought to determine whether the anti-hyperalgesic effects of systemic pioglitazone are mediated through activation of spinal PPARγ. As illustrated in Fig 2E–H, intrathecal (i.t.) GW9662 attenuated pioglitazone reduction of mechanical [AUC; $F(3, 23) = 18.55$; $P < 0.0001$] and cold [AUC; $F(3, 23) = 12.85$; $P < 0.0001$] hypersensitivity. Both systemic and spinal administration of GW9226 attenuated the reduction of mechanical and cold hypersensitivity by administration of systemic pioglitazone.

### PPARγ in the spinal cord mediates the anti-hyperalgesic actions of intrathecal pioglitazone

To support the PPARγ antagonist experiment above in identifying the spinal cord as a key site of PPARγ anti-hyperalgesic action, we administered pioglitazone and/or GW9662 by the intrathecal or intracerebroventricular route. Fig 3A demonstrates that i.t. pioglitazone

attenuated mechanical hyperalgesia [dose × time; F (18, 162) = 2.951; P = 0.0001] as rapid as 30 min after injection in a dose-dependent manner. To account for possible supraspinal activation of PPARγ after i.t. pioglitazone via the cerebrospinal fluid circulation, we injected the same doses via the intracerebroventricular (i.c.v.) route. We found no anti-hyperalgesic effect of pioglitazone at these doses [F (3, 21) = 1.959; P = 0.15]. Fig 3B illustrates that co-administration of the PPARγ antagonist GW9662 completely blocked the anti-hyperalgesic actions of i.t. pioglitazone [drug × time; F (18, 198) = 16.26; P < 0.0001].

### PPARγ activation does not produce motor deficits or analgesia

We determined whether the anti-hyperalgesic actions of pioglitazone might be confounded by off-target effects on motor systems or transient reflexive pain [84]. Fig 4A–B illustrates that a relatively high dose of pioglitazone did not change rotarod performance after i.p. [p = 0.81] or i.t. [p = 0.35] injection. Because von Frey mechanical withdraw thresholds are maximal in uninjured rats (e.g. 15 g), we assessed response latencies to noxious heat to determine if pioglitazone altered acute nociception in sham rats. Intrathecal pioglitazone did not change heat response latencies [p = 0.5] suggesting that the effects of PPARγ activation are not analgesic. Together with our previous reports [13;59;60], we conclude that reduction of mechanical hyperalgesia by PPARγ agonists is not secondary to adverse effects on motor coordination or normal sensory thresholds.

### PPARγ agonists rapidly reduce nociceptive and neuropathic pain-like behavior

The above studies illustrate that i.t. administration of pioglitazone reduces tactile hypersensitivity within 30 min of administration in SNI rats. Consistent with this finding, intrathecal pioglitazone, compared to saline, reduced nociceptive behavior when given 20 min prior to intraplantar capsaicin (nociceptive responses; 16.3 ± 2.3 vs. 62.2 ± 16.1; n = 5–6) [p = 0.013]. To test the hypothesis that PPARγ anti-hyperalgesic mechanisms occur rapidly after traumatic nerve injury, we extended our temporal analysis of pain-like behavior in the SNI model to earlier time points. Fig 5A–B demonstrates that the PPARγ agonists rosiglitazone [F (1, 9) = 5.942; P = 0.0375] and pioglitazone [F (1, 8) = 28.24; P = 0.0007] attenuated SNI-associated mechanical hypersensitivity within 5 min of injection. We repeated the study with 7.5 min as the first behavioral timepoint to allow full recovery from isoflurane anesthesia, as evidenced by the slight increase in von Frey thresholds in the saline control group in Fig 5B. Fig 5C illustrates a robust anti-hyperalgesic effect of pioglitazone [F (1, 14) =26.62; P = 0.0001] and at the 7.5 minute timepoint [p < 0.0001] in the absence of a residual anesthetic effect [p = 0.9998]. Our findings that rosiglitazone and pioglitazone produce anti-hyperalgesia and anti-nociception within 5–20 min of administration suggest that non-genomic PPARγ mechanisms mediate rapid reduction of pain-like behavior.

### In vivo blockade of protein translation in the dorsal horn by intrathecal anisomycin

To examine whether a translation-independent (i.e. non-genomic) mechanism mediates the rapid anti-hyperalgesic effects of PPARγ, we administered intrathecal anisomycin to block protein synthesis in the dorsal horn *in vivo* at a dose that did not produce confounding alterations in nociception [43]. To achieve this, we evaluated the effect of multiple doses of anisomycin, based on previously used doses of 25 to 125 μg [2;7;69;76], on capsaicin-

induced protein (i.e. Fos) expression. Capsaicin activates TRPV1-positive primary afferent nociceptors [8] resulting in spontaneous, dose-dependent pain in humans [79]. Intraplantar injection of capsaicin in rodents elicits spontaneous nociceptive behavior [80] and induces Fos expression in the superficial dorsal horn within 30 to 90 min [3;35], providing a suitable tool for evaluating effects of anisomycin on new protein synthesis.

As depicted in Fig 6A, we administered intrathecal pioglitazone and/or anisomycin twenty minutes prior to evaluation of capsaicin-evoked nociceptive behavior. Sixty minutes after capsaicin injection, we euthanized animals for quantification of Fos expression in the L4–L5 dorsal horn. As illustrated in Fig 6B, combinations of capsaicin, pioglitazone, and anisomycin administration significantly altered Fos expression in laminae I-V [group; $F(7, 17) = 9.38$; $P < 0.0001$]. Compared to vehicle injection, intraplantar capsaicin increased Fos expression [$p = 0.007$], an effect that was dose-dependently inhibited by pretreatment with 100–200 μg doses of anisomycin [$p < 0.05$] but not 50 μg [$p = 0.064$]. Anisomycin (200 μg) also reduced Fos expression when co-administered with pioglitazone [$p = 0.0006$]. Pioglitazone did not alter capsaicin-evoked Fos [$p = 0.74$] when administered alone.

We offer two explanations for the lack of effect of pioglitazone on spinal Fos expression even though nociceptive behavior was reduced after intraplantar capsaicin injection. First, Fos and behavior do not always positively correlate: drug-induced reduction of pain-like behavior can occur in the absence of [14;26;33] or with increases in [65] Fos expression. Second, the target of pioglitazone, PPARγ, directly activates *c-fos* transcription [71]. If pioglitazone directly increases Fos expression in inhibitory interneurons, which can account for a significant population of total cells expressing Fos [87], then this would confound the ability of our Fos method to reflect noxious stimulus-evoked spinal neuron activation.

### Anisomycin does not alter the anti-nociceptive effects of intrathecal pioglitazone

Having validated that anisomycin reduces protein translation (i.e. Fos) in the dorsal horn, we used this method to explore the anti-nociceptive and translation-independent actions of PPARγ activation in the capsaicin model. As illustrated in Fig 6C, combinations of capsaicin, pioglitazone, and anisomycin administration significantly altered nociceptive behavior [group; $F(7, 35) = 5.476$; $P = 0.0003$]. Intraplantar capsaicin markedly increased nociceptive behavior when compared to vehicle injection [$p = 0.0055$]. Anisomycin alone (50–200 μg) did not alter capsaicin-evoked nociceptive behavior [$p > 0.05$]. Pioglitazone reduced nociceptive behavior when administered alone [$p = 0.0029$] or in combination with the 200 μg dose of anisomycin [$p = 0.0029$]. These results indicate that anisomycin blocks genomic activity (i.e. Fos expression) in the dorsal horn without altering nociceptive behavior or pioglitazone anti-nociception, providing proof of principle for this method.

### Spinal anisomycin does not change the rapid anti-hyperalgesic effects of pioglitazone

Next we extended spinal anisomycin blockade of translation in the dorsal horn to the SNI model of neuropathic pain. We began with a dose of anisomycin that, when administered into the brain, inhibits conditioned taste aversion [72], auditory fear conditioning [68;69;76], and incorporation of radioactive methionine into nascent proteins [69;72]. We found that the 200 μg dose of anisomycin abolished capsaicin-evoked Fos, but also produced a

confounding attenuation of SNI-induced mechanical hypersensitivity. By lowering the anisomycin dose to 100 μg, we obtained a significant reduction in Fos without altering SNI-induced hypersensitivity. As illustrated in Fig 7A, combinations of intrathecal vehicle, pioglitazone, or anisomycin changed mechanical sensitivity in SNI rats when analyzed across the entire 240 min time course [drug × time; $F(24, 168) = 3.416$; $P < 0.0001$]. Further analysis of the 7.5 – 120 min timepoints revealed that pioglitazone attenuated mechanical hypersensitivity when injected alone [vs. Vehicle + Saline; $F(1, 9) = 29.41$; $P = 0.004$] or with 100 μg anisomycin [vs. Vehicle + Saline; $F(1, 11) = 5.098$; $P = 0.045$]. At this dose, anisomycin alone did not inhibit mechanical hypersensitivity [$p = 0.54$]. To compare the effect of anisomycin on pioglitazone anti-hyperalgesia at early versus delayed timepoints, we analyzed the data at each of four timepoints (Fig 7B): before intrathecal injections (pre-drug), during early pioglitazone anti-hyperalgesia (7.5 min), during late pioglitazone anti-hyperalgesia (60 min), and after anti-hyperalgesia resolved (180 min). Compared to intrathecal vehicle, pioglitazone [$p = 0.013$] and pioglitazone plus anisomycin [$p = 0.026$] attenuated hypersensitivity at 7.5 min when compared to intrathecal vehicle only [drug; $F(3, 20) = 5.173$; $P = 0.008$]. By contrast, the delayed anti-hyperalgesic actions of pioglitazone at 60 min [$p = 0.003$ vs. Vehicle + Saline] were significantly reduced by anisomycin [$p = 0.021$ vs. Vehicle + Pio].

## Acute pioglitazone reduces expression of GFAP after nerve injury

Neuropathic pain is associated with astrocyte activation after nerve injury [94]. We previously reported that repeated administration of pioglitazone reduced not only established neuropathic pain, but also astrocyte activation in the dorsal horn of SNI rats [60]. To determine whether a single administration of pioglitazone also reduces astrocyte activation after SNI, we evaluated the protein expression of GFAP in the lumbar dorsal horn using both immunohistochemistry and western blot as soon as 60 min.

Similar to previous reports [94], Fig 8A–B illustrates that SNI produced a unilateral increase in GFAP expression in the ipsilateral dorsal horn 14d after injury [ipsi vs. contra; $F(1, 12) = 8.55$; $P = 0.0127$]. This occurred in both injured (tibial and common peroneal) and uninjured (sural) innervation territories [t-test; $p < 0.05$]. Fig 8C–D illustrates that pioglitazone reduced GFAP expression in the contralateral and ipsilateral dorsal horn within 60 min of injection [drug; $F(1, 8) = 74.93$; $P < 0.0001$].

To confirm that the acute reduction of GFAP expression elicited by pioglitazone (Fig 8) did not result from structural changes that mask the GFAP antibody epitope in fixed tissue, we performed denaturing western blots. At 14 d after sham or SNI surgery, we administered i.p. pioglitazone, measured behavioral anti-hyperalgesia, and harvested L4-5 spinal cord quadrants 90 min later. As illustrated in Fig 9A, pioglitazone but not saline reduced mechanical hyperalgesia in SNI animals [drug; $F(1, 10) = 11.17$; $P = 0.0075$] at 60 [$p = 0.023$] and 90 [$p < 0.0001$] min after administration. Western blot analysis of the four lumbar quadrants is reported in Fig 9B (dorsal) and Fig 9C (ventral). Neither injury [$p = 0.58$] nor drug [$p = 0.33$] treatment changed GFAP expression in the contralateral dorsal horn. By contrast, we found a significant injury × drug interaction in the ipsilateral dorsal horn [$F(1, 20) = 11.39$; $P = 0.003$]. Post-hoc tests revealed that SNI increased GFAP

expression when compared to sham animals treated with saline [p = 0.0014]. This increase was reduced by pioglitazone in the ipsilateral [p = 0.0026] but not contralateral [p = 0.54] dorsal horn of SNI animals (Fig 9B). In the contralateral ventral horn, neither injury [p = 0.14] nor pioglitazone [p = 0.40] changed GFAP expression. Injury [p = 0.41] or pioglitazone [p = 0.84] did not change GFAP expression in the ipsilateral ventral horn.

## DISCUSSION

### Anisomycin to assess translation-independent activity of nuclear receptors in the spinal cord

Here we used *in vivo* administration of anisomycin to dissect genomic versus non-genomic mechanisms of a nuclear receptor in the spinal cord. Previous studies using anisomycin suggested that protein translation is necessary for late phase LTP in the brain [25;44], hyperalgesic priming in the peripheral nervous system [2], ongoing pain transmission in the spinal cord [43], and reduction of established hyperalgesia during pain memory reconsolidation in the spinal cord [7]. As these studies did not confirm inhibition of translation, we found it important to demonstrate that intrathecal anisomycin inhibits spinal protein expression. We found that anisomycin, at a dose of 100 μg, decreased expression of capsaicin-evoked Fos without changing SNI-induced hyperalgesia. This provides proof of principle for using this approach to determine the contribution of non-genomic PPARγ mechanisms to the rapid and delayed phases of pioglitazone anti-hyperalgesia.

### Non-genomic PPARγ activity mediates the early anti-hyperalgesic effect of pioglitazone

Based on our finding that the anti-hyperalgesic effect of pioglitazone occurred very rapidly (7.5 min) and this was maintained in the presence of 100 μg anisomycin, we suggest the involvement of a non-genomic PPARγ mechanism. Non-genomic mechanisms in the dorsal horn might also explain the rapid anti-hyperalgesic effects observed after interruption of other nuclear receptors including estrogen and PPARα receptors. For example, *in vivo* administration of 17β-estradiol rapidly (within 15 min) enhanced bradykinin-induced hyperalgesia [73] and inhibited opioid receptor-like 1 anti-nociception [81]: these were unaffected by pretreatment with anisomycin or conjugation of 17β-estradiol to membrane-impermeable BSA [73;81] suggesting that membrane estrogen receptors contribute to rapid pain modulation. Also, the inhibition of neuropathic pain (within 30 min) by the PPARα agonists PEA and GW7647 [16] was abrogated in PPARα knockout mice or by pharmacological blockade of calcium-activated potassium channels [16;47;48] suggesting both rapid and PPARα-dependent analgesic actions. We speculate that, in a similar manner, the rapid anti-hyperalgesic effect of pioglitazone is mediated by membrane PPARγ that is linked to ion channel activity, neuronal excitability, and/or central sensitization mechanisms.

### Genomic PPARγ activity mediates the late anti-hyperalgesic effects of pioglitazone

We found that anisomycin significantly reduced the anti-hyperalgesic effects of pioglitazone at later time points (60 minutes), supporting a contribution of classical genomic mechanisms involving transcription and translation. This is consistent with several reports indicating that repeated pioglitazone administration produced sustained anti-hyperalgesic actions persisting for days to weeks after cessation of drug administration [60;83], beyond the time necessary

for drug clearance [55]. Similarly, our current results and previous studies [13;59;67] indicate that a single TZD injection produces anti-hyperalgesia lasting for several hours, much longer than the half-life of pioglitazone [55]. In summary, our results suggest pioglitazone anti-hyperalgesia transitions from a rapid, anisomycin–resistant mechanism to a delayed, anisomycin-sensitive mechanism within approximately 60 minutes.

### The anti-hyperalgesic effect of pioglitazone is mediated by spinal PPARγ

PPARγ agonists reduce tactile hypersensitivity after nerve injury, but the site(s) of action and selectivity of these agents remain elusive. Here, systemic or spinal pioglitazone decreased nerve injury-induced tactile hypersensitivity, and this effect was lost when given in the presence of intrathecal GW9662. We conclude that PPARγ in the dorsal horn of the spinal cord contributes to the anti-hyperalgesic effects of pioglitazone. This is consistent with previous studies showing that: 1) PPARγ is expressed in the lumbar spinal cord [13;53]; 2) hyperalgesia after SNI was exacerbated by intrathecal administration of the PPARγ antagonist BADGE [22]; and 3) intrathecal GW9662 inhibited the anti-hyperalgesic effect of both repeated systemic or single intrathecal administration of rosiglitazone or 15d-$PGJ_2$ [13;60]. It is unlikely that brain PPARγ mediates anti-hyperalgesia after intrathecal pioglitazone, because i.c.v. administration of comparable doses had no effect; this is consistent with previous results showing that low i.c.v. doses of rosiglitazone or 15d-PGJ2 [13] did not change tactile hypersensitivity.

Our studies do not rule out “off-target” effects that might contribute to rapid pioglitazone anti-hyperalgesia. Gras *et al* (2009) reported that rosiglitazone-stimulated calcium mobilization was lost after GPR40 siRNA knockdown, suggesting that TZDs (i.e. PPARγ agonists) may activate GPR40 [28]. Activation of GPR40, which is expressed in brain [51;52] and spinal cord neurons [61], by endogenous (DHA) and exogenous (GW9508) ligands reduced inflammatory pain [61;62]. Thus GPR40 is an intriguing target for future studies.

### Pioglitazone acutely inhibits astrocyte activation

We previously reported that repeated pioglitazone administration spanning several weeks reduced GFAP in the dorsal horn [60]. Here we observed the same result in a timeframe of just 60 minutes following a single dose of pioglitazone, leading us to speculate that astrocytes contribute to rapid anti-hyperalgesia mechanisms. This might be PPARγ-dependent, since markers of astrocyte activation are reduced by other PPARγ agonists [31;82].

Our results are not the first to describe that an acute intervention (i.e. pioglitazone) can alter astrocyte function. For example, increases in GFAP expression occur as soon as 30 min after traumatic nerve injury [96], electrical stimulation of primary afferents [89], and intraplantar injection of CFA [89] or snake venom toxin sPLA2-Asp49 [9]. Second, the astrocyte toxins l-α-aminoadipate and fluorocitrate reduced neuropathic pain and GFAP expression within 30 to 60 min of administration [91;97]. Third, fluorocitrate prevented the early phase of TNFα-induced long term potentiation (LTP) in the *ex vivo* spinal cord [30]. Therefore, based on the current finding that pioglitazone reduced GFAP in the dorsal horn, we propose that

pioglitazone inhibits the astrocytic release of neuron-sensitizing molecules that contribute to LTP and, ultimately, the maintenance of chronic neuropathic pain. Indeed, pioglitazone reduced the release of TNFα from astrocytes in culture [82].

Our results do not rule out a supraspinal component to the anti-hyperalgesic actions of systemically-administered pioglitazone. This hypothesis is suggested by the findings that: 1) in models of CNS injury, pioglitazone reduced mitochondrial dysfunction [36;37;75;93] and promoted glucose metabolism in astrocytes [19;66]; 2) pioglitazone reduced Aβ-induced astrocyte activation in the hippocampus [34]; 3) PPARγ is expressed in cortical astrocytes [17;58]; 4) astrocytes in the anterior cingulate cortex facilitate pain sensitization and inhibition [11;27;38;92]. This provides for the possibility that systemic pioglitazone administration produces anti-hyperalgesia through cortical, in addition to spinal, astrocytic mechanisms.

Investigations of nerve injury-induced activation of astrocytes in areas of the dorsal horn innervated by injured versus uninjured afferents are sparse [4;15;94]. Here, our immunohistochemical quantification method [15] revealed that nerve injury increased GFAP expression in regions of the ipsilateral dorsal horn innervated by both injured tibial afferents (medial) and uninjured sural afferents (lateral). This is consistent with qualitative studies showing that astrocyte activation extends beyond injured territories [4], and suggests that astrocytes induce hyperalgesia by sensitizing intact nociceptive pathways.

Validation of immunohistochemical results with a complementary approach is essential because IHC assays can be misinterpreted due to drug or injury-induced cytotoxic changes in the conformation and/or the formalin sensitivity of the GFAP epitope (i.e. epitope masking) [5;21]. We chose an approach involving a denaturing western blot, and the results indicate that pioglitazone does not change GFAP expression in sham animals, nor on the contralateral side in SNI animals, nor in the ventral horn in sham or SNI animals. This not only suggests that astrocyte viability is preserved after pioglitazone injection, but also that epitope masking does not confound our conclusion that a single injection of pioglitazone exerts anti-hyperalgesic actions in part by reducing astrocyte activation.

### Conclusions

Our results indicate that pioglitazone acts at spinal PPARγ to inhibit astrocyte activation and to produce fast-acting, dose-dependent, and translation-independent inhibition of pain-like hypersensitivity after traumatic nerve injury. The specific mechanisms of rapid pain reduction by PPARγ and the involvement of astrocytes warrant further investigation. Our studies do not rule out actions of PPARγ agonists in the peripheral nervous system [53;83] that would reduce spinal sensitization, but do illustrate an important behavioral and neurobiological role for non-genomic inhibition of pain in the spinal cord by a nuclear receptor.

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## SUMMARY

Pioglitazone activation of spinal PPARγ reduces GFAP and rapidly attenuates hyperalgesia after traumatic nerve injury. PPARγ pain reduction occurs via both non-genomic and genomic mechanisms.

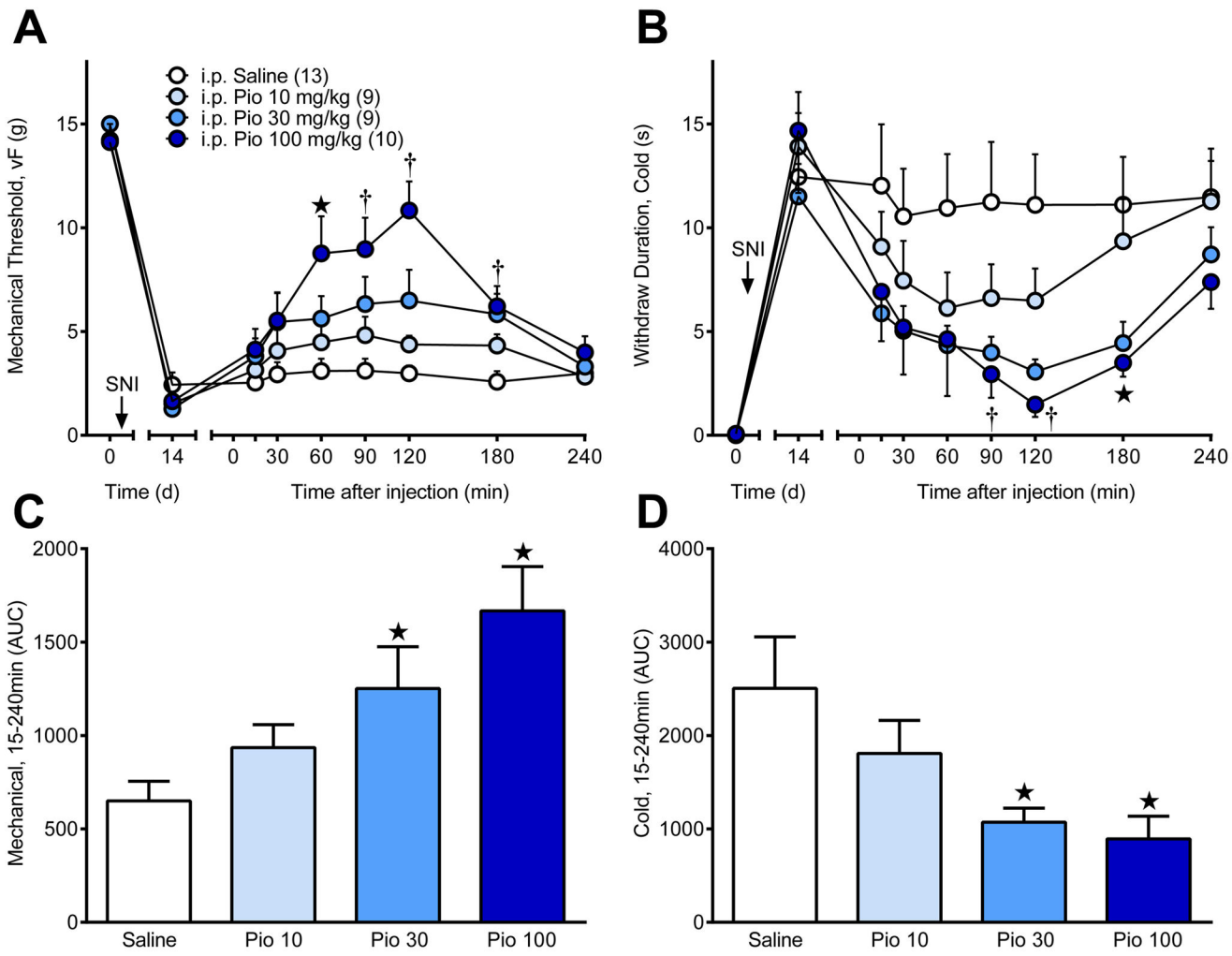


**Figure 1. A single systemic injection of pioglitazone (Pio) dose-dependently reduced behavioral signs of neuropathic pain**

Spared nerve injury (SNI, arrow) resulted in pain-like hypersensitivity. Intraperitoneal (i.p.) pioglitazone dose-dependently reversed **(A)** mechanical and **(B)** cold hypersensitivity 14d after SNI. Pain-related behavioral responses to plantar application of von Frey and acetone stimuli were measured before (0d) and after (14d) SNI and at 15, 30, 60, 90, 120, 180, and 240 min after intraperitoneal (i.p.) injection of pioglitazone (0, 10, 30, 100 mg/kg) in saline vehicle. Area under the curve (AUC) is shown for **(C)** von Frey and **(D)** acetone pain-related behaviors. ★ "i.p. Pio 100 mg/kg" significantly different from "i.p. Saline". † "i.p. Pio 30 mg/kg" and "i.p. Pio 100 mg/kg" significantly different from "i.p. Saline". "n" are shown in parentheses.

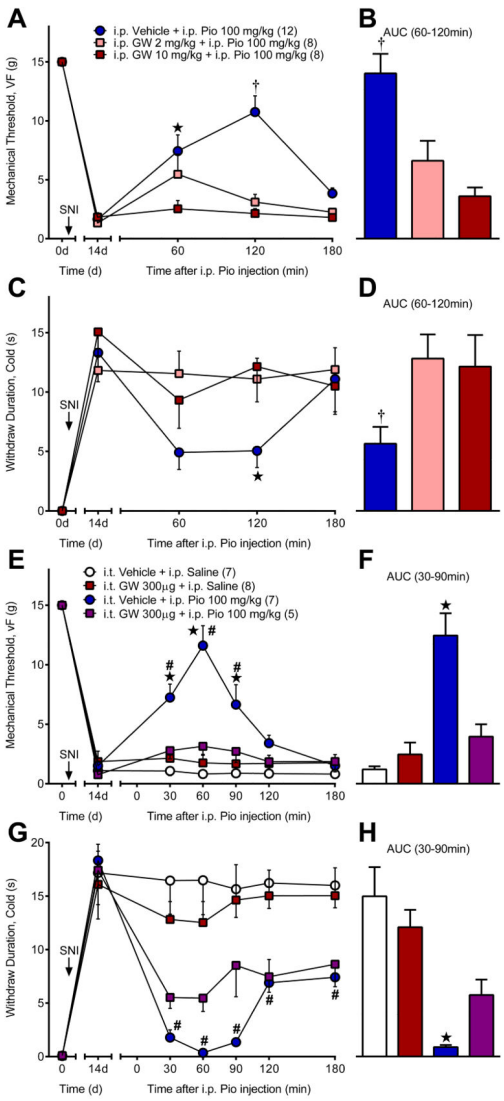


**Figure 2. Anti-hyperalgesic actions of systemic pioglitazone are mediated by spinal PPARγ**

Systemic GW9662 (GW), a PPARγ antagonist, blocked pioglitazone (Pio) reduction of **(A–B)** mechanical and **(C–D)** cold hypersensitivity 14d after SNI. **(E–F)** Spinal GW completely abolished pioglitazone reduction of mechanical hyperalgesia and **(G–H)** partially blocked pioglitazone alleviation of cold hypersensitivity. GW was administered i.p. or i.t. 15 min prior to i.p. pioglitazone. ★ significantly different from “i.p. GW 10 mg/kg” or “i.p. Pio 100 mg/kg + i.t. GW 300μg”. † significantly different from “i.p. GW 2 mg/kg” and “i.p. GW 10 mg/kg”. # significantly different from “i.p. Saline + i.t. Vehicle”. ‘n’ are shown in parentheses.

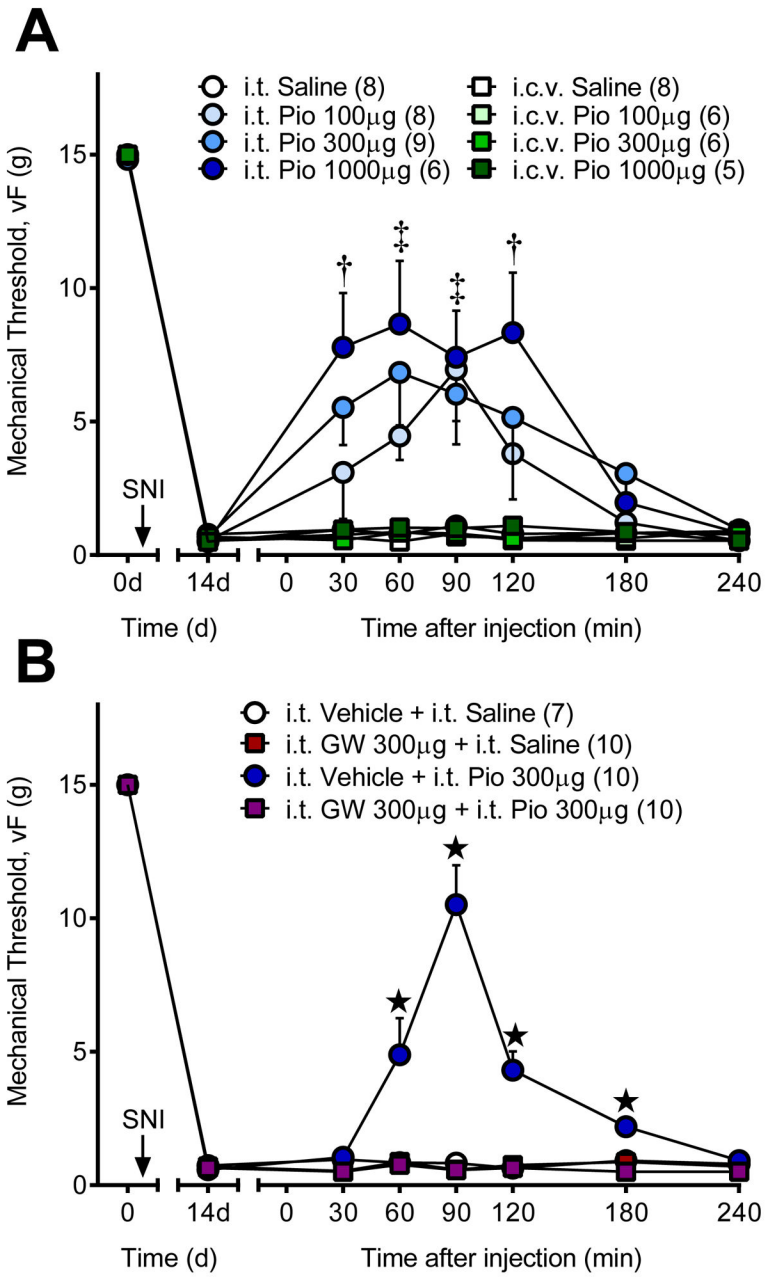


**Figure 3. Spinal PPARγ mediates pioglitazone anti-hyperalgesia**

**(A)** Intrathecal (i.t.) pioglitazone (Pio) dose-dependently reduced mechanical hyperalgesia at doses that have no effect when administered by the intracerebroventricular (i.c.v.) route. **(B)** Intrathecal co-administration of the PPARγ antagonist GW9662 (GW) completely reversed pioglitazone anti-hyperalgesia**.** † high and medium ‡ high, medium, low dose Pio significantly different from “i.t. Saline”. ★ significantly different from “i.t. Saline + i.t. Vehicle”. ‘n’ are shown in parentheses.

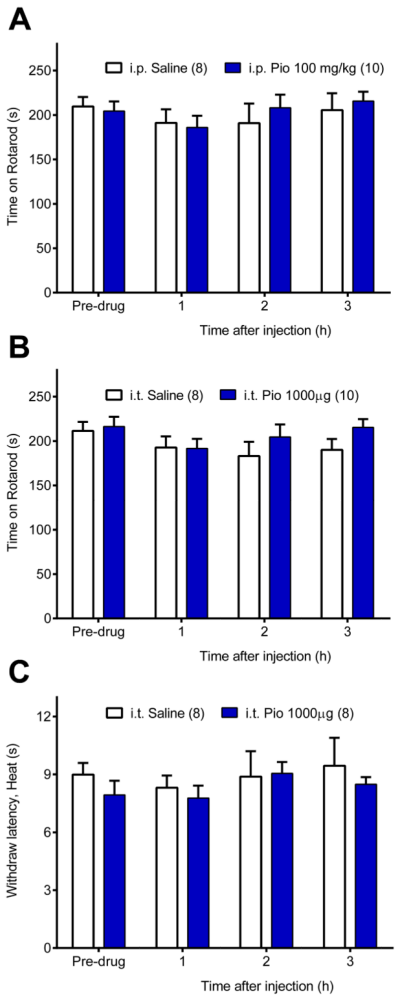


**Figure 4. Pioglitazone did not produce ataxia or changes in transient nociception**

Motor coordination was assessed on an accelerating rotarod before and at 1, 2, and 3 h after high dose pioglitazone (Pio) administration by **(A)** systemic (i.p. 100 mg/kg) or **(B)** spinal (i.t. 1000 μg) routes. Pioglitazone did not change time spent on the accelerating rotarod. The analgesic effects of pioglitazone were tested using the Hargreaves assay before and after intrathecal saline or pioglitazone administration in sham rats **(C).** Pioglitazone did not change paw withdraw latencies to noxious heat. n = 8–10 per group.

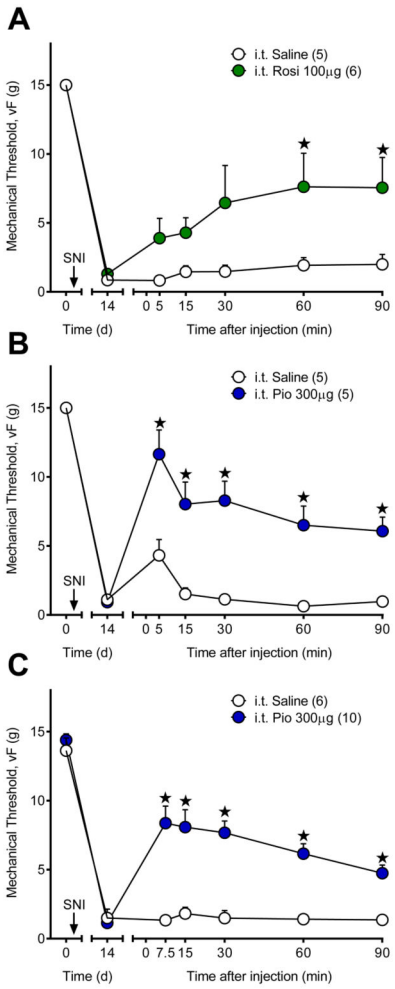


**Figure 5. PPARγ agonists rapidly reduce mechanical hyperalgesia**

Intrathecal injection of the PPARγ ligands **(A)** rosiglitazone (Rosi) or **(B)** pioglitazone (Pio) rapidly (within 5 min) reduced mechanical hypersensitivity 14d after SNI. Intrathecal injections were performed under isoflurane anesthesia, which resulted in a small anti-hyperalgesic effect at 5 min. Therefore, we repeated the experiment with 7.5 min the earliest timepoint tested. **(C)** Pioglitazone rapidly attenuated mechanical hypersensitivity that lasted from 7.5 to 90 min with no anesthesia effect in the saline group. ★ significantly different from “i.t. Saline”. ‘n’ are shown in parentheses.

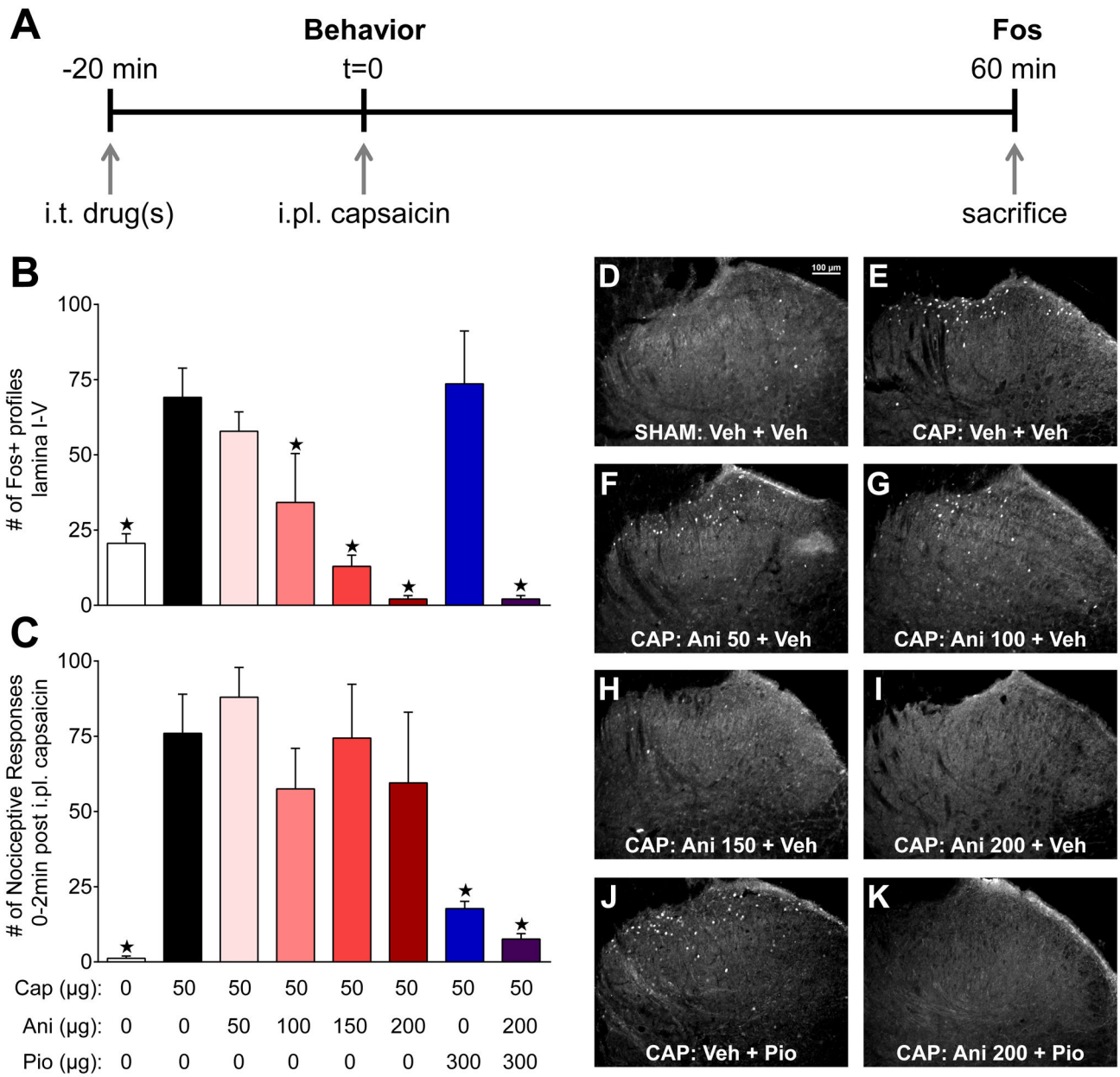


**Figure 6. Anisomycin reduces translation of capsaicin-evoked Fos without blocking the rapid anti-nociceptive actions of pioglitazone**

**(A)** Experimental timeline to test the hypothesis that capsaicin evoked Fos translation is inhibited by intrathecal anisomycin pretreatment. **(B)** Vehicle (0µg Cap), pioglitazone (Pio), and/or anisomycin (Ani) were co-administered i.t. 20 min prior to i.pl. capsaicin (50µg). Capsaicin-induced spontaneous licking, lifting, and flinching nociceptive responses from 0–2 min after injection were almost completely abolished by pioglitazone (300µg) alone or in combination with anisomycin (200µg) pretreatment. Anisomycin (50–200µg) alone did not alter capsaicin nociception. **(C)** Capsaicin-evoked Fos expression was dose-dependently inhibited by anisomycin and unaltered by pioglitazone. **(D)** Sham injection of vehicle produced moderate Fos expression when compared to **(E)** i.t. vehicle injections followed by

i.pl. capsaicin. **(F)** 50μg, **(G)** 100μg, **(H)** 150μg, and **(I)** 200μg anisomycin dose-dependently reduced Fos. (J) Pioglitazone alone had no effect on Fos expression while **(K)** anisomycin completely abolished Fos when co-administered with pioglitazone. ★ significantly different from capsaicin only (black bars). n = 3–9 for behavioral and n = 3–4 for Fos analyses.

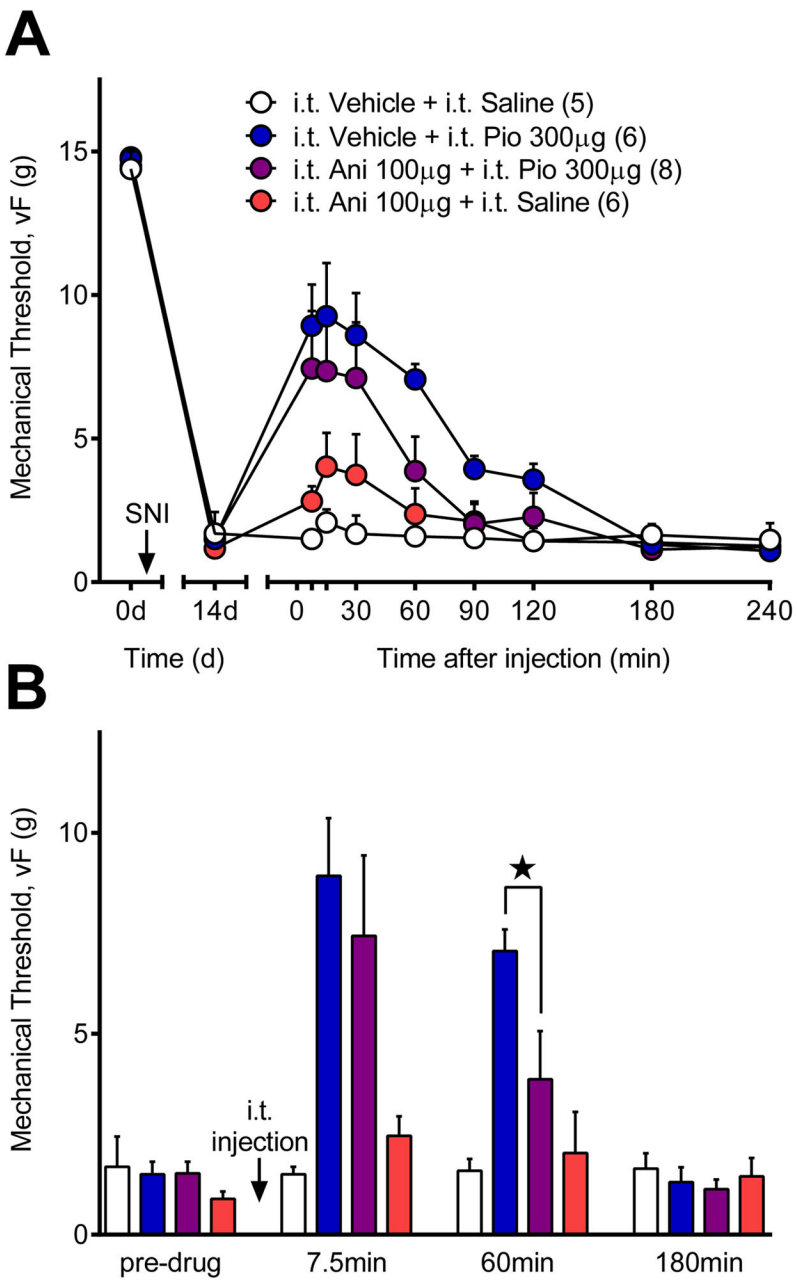


**Figure 7. Early but not late pioglitazone anti-hyperalgesia is independent of translation**

To test whether immediate PPARγ anti-hyperalgesia is dependent on canonical genomic activity, we co-administered pioglitazone with an anisomycin dose that blocks translation but not nociception (100μg; see discussion for details). **(A)** Timecourse of mechanical thresholds after i.t. injection of pioglitazone (Pio) or anisomycin (Ani). **(B)** Analysis of mechanical thresholds at 7.5, 60 and 180 min timepoints. Anisomycin did not change the anti-hyperalgesic effects of pioglitazone at the early 7.5 min period, but reduced pioglitazone anti-hyperalgesia at 60 min. These results suggest that pioglitazone produces its anti-hyperalgesic effects by both translation-independent (7.5 min) and translational-dependent (60 min) mechanisms. ★ significantly different. 'n' are shown in parentheses.

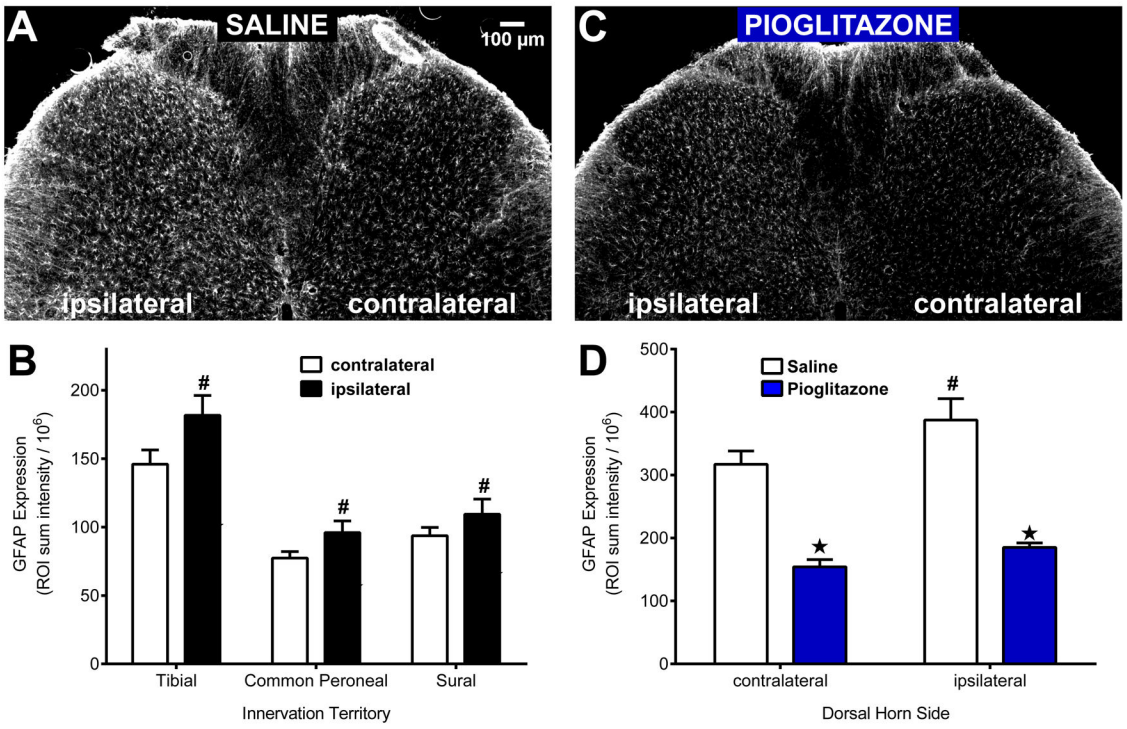


**Figure 8. Acute PPARγ activation reduces GFAP expression**

**(A)** Spared nerve injury produced an increase in GFAP expression on the injured (ipsilateral) side when compared to the uninjured (contralateral) side of the lumbar dorsal horn. **(B)** SNI-induced a GFAP increase in injured (tibial and common peroneal) and uninjured (sural) innervation territories. **(C–D)** Pioglitazone (100 mg/kg i.p.; 1 h prior to perfusion) reduced GFAP expression on both the contralateral and ipsilateral dorsal horn. Each region of interest was analyzed separately using a two-tailed t-test. # significantly different from “contralateral” in the “Saline” group (paired). ★ significantly different from “Saline” (unpaired). n = 3 per group.

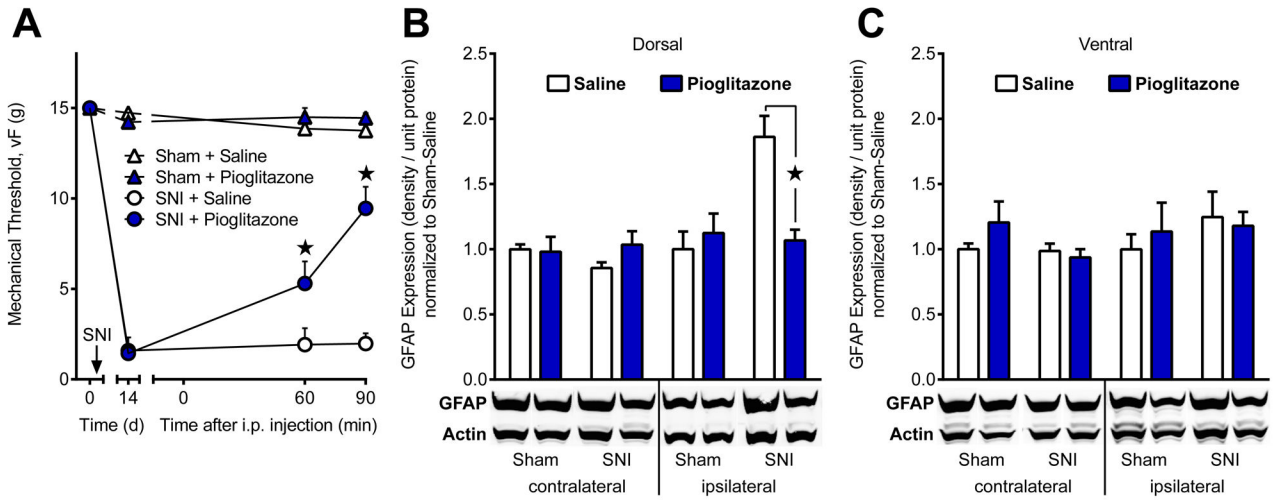


**Figure 9. SNI is required for pioglitazone reduction of pain and astrocyte activation**

To determine whether pioglitazone is toxic to astrocytes and to rule out conformational changes in the GFAP antibody epitope during immunohistochemical analysis we performed a denaturing western blot. Spinal cord quadrants (L4–5) were harvested 90 min after i.p. pioglitazone administration at d14 after sham or spared nerve injury (SNI) surgery. **(A)** Pioglitazone attenuated mechanical hypersensitivity in SNI, but not sham, animals. **(B)** Dorsal and **(C)** Ventral integrated densities normalized to Sham + Saline are shown. There was no effect of injury or drug treatment in the ventral horn or in the contralateral dorsal horn segments. GFAP expression was increased in the ipsilateral dorsal horn of SNI + Saline animals when compared to all other groups. Pioglitazone significantly reduced ipsilateral dorsal horn GFAP expression in SNI, but not sham, animals. This suggests that the anti-hyperalgesic effects of pioglitazone are associated with decreased astrocyte activation after nerve injury. ★ significantly different from “SNI + Saline”. n=6–7 per group.