

The role of spinal thrombin through protease-activated receptor 1 in hyperalgesia after neural injury

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OBJECTIVE Painful neuropathic injuries induce blood–spinal cord barrier (BSCB) breakdown, allowing pro-inflammatory serum molecules to cross the BSCB, which contributes to nociception. The goal of these studies was to determine whether the blood-borne serine protease thrombin also crosses a permeable BSCB, contributing to nociception through its activation of protease-activated receptor-1 (PAR1).

METHODS A 15-minute C-7 nerve root compression, which induces BSCB breakdown and painful behaviors by Day 1, was administered in the rat (n = 10); sham operation (n = 11) and a 3-minute compression (n = 10) that does not induce sensitivity were administered as controls. At Day 1 after root compression, spinal cord tissue was co-immunolabeled for fibrin/fibrinogen, the enzymatic product of thrombin, and IgG, a serum protein, to determine whether thrombin acts in areas of BSCB breakdown. To determine whether spinal thrombin and PAR1 contribute to hyperalgesia after compression, the thrombin inhibitor hirudin and the PAR1 antagonist SCH79797, were separately administered intrathecally before compression injuries (n = 5–7 per group). Rat thrombin was also administered intrathecally with and without SCH79797 (n = 6 per group) to determine whether spinal thrombin induces hypersensitivity in naïve rats through PAR1.

RESULTS Spinal fibrin(ogen) was elevated at Day 1 after root compression in regions localized to BSCB breakdown and decreased in those regions by Day 7. Blocking either spinal thrombin or PAR1 completely prevented compression-induced hyperalgesia for 7 days. Intrathecal thrombin induced transient pain that was prevented by blocking spinal PAR1 before its injection.

CONCLUSIONS The findings of this study suggest a potent role for spinal thrombin and its activation of PAR1 in pain onset following neuropathic injury.

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KEY WORDS thrombin; blood–spinal cord barrier; pain; radicular injury; protease-activated receptor-1

CHRONIC pain impacts 112 million adults in the United States annually, which is more than the number of people affected by cancer, heart disease, and diabetes combined.^{23,51} Despite the relatively high prevalence of painful disorders, current treatments are not effective at treating all of the presenting symptoms.^{1,4,13,17} Many treatments target only one neuronal aspect in the central nervous system (CNS) that maintains neuropathic pain,^{4,41,57,58} despite a host of biochemical cascades in both neuronal and nonneuronal cells contributing to pain.^{32,41,55} Mechanical injury to either a peripheral nerve or nerve root transiently increases the permeability of the blood–spinal cord barrier (BSCB),^{5,14,20,25} which facilitates transmission of serum molecules into the spinal cord, where they activate

resident neuronal and glial cells.^{2,40,56} We recently showed that BSCB breakdown occurs exclusively after nerve root compression that also induces pain and that blocking early BSCB breakdown prevents pain.⁴⁷ Yet, the serum proteins that extravasate into the CNS during increased BSCB permeability and enhance nociception are not defined.

Thrombin is a bioactive blood-borne enzyme restricted from the CNS under normal healthy conditions.^{33,38} When introduced into the CNS, certain concentrations of thrombin induce cellular cascades that promote nociception when applied for an extended duration.^{19,36,37} Of those spinal effects, astrocyte activation has been shown to occur in parallel with pain-related behaviors induced by a variety of neuropathic insults in animal models.^{21,41,42} Exogenous

ABBREVIATIONS BSCB = blood–spinal cord barrier; CNS = central nervous system; DMSO = dimethyl sulfoxide; IgG = immunoglobulin G; PAR1 = protease-activated receptor-1; PBS = phosphate-buffered saline.

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thrombin administered to the spinal cord produces tactile allodynia in mice and blocking spinal thrombin activity partially attenuates pain from a sciatic nerve ligation.³³ These studies suggest that spinal thrombin may contribute to the pain-related behaviors that develop after neural injury. Yet, it is not known whether thrombin enters the CNS after injury or whether it contributes to the development of pain.

Although thrombin controls coagulation by cleaving fibrinogen into fibrin, it also initiates many cellular signaling cascades by activating cell receptors.^{11,12} Mammalian thrombin preferentially activates protease-activated receptor-1 (PAR1), which is expressed in multiple CNS cell types.^{45,49,53} PAR1 activation in the CNS is implicated in glial activation and related inflammatory responses.^{6,16,35,49} Activating PAR1 in the striatum of mice induces local astrocyte activation and proliferation.³⁵ Blocking PAR1 activation with the small-molecule inhibitor SCH79797 inhibits thrombin-induced astrocytic activation as well as astrocytic production of pro-inflammatory proteins.^{9,44} Despite PAR1's implication in glial-controlled inflammatory processes and the well-established role of gliosis in pain-related behaviors,^{31,32,52,55} no study has defined the contribution of thrombin's central activation of PAR1 to pain. We hypothesize that thrombin extravasates into the spinal parenchyma in areas that undergo BSCB breakdown after painful nerve root injury, inducing behavioral sensitivity through its enzymatic activation of PAR1.

Methods

Study Design

Defining the Influence of Compression-Induced Spinal Thrombin on Hyperalgesia

To determine whether endogenous thrombin is enzymatically active in the spinal cord after neural injury, immunohistochemical techniques were used to label spinal fibrin(ogen) expression after different durations of nerve root compression that induce varied levels of behavioral hypersensitivity.⁴¹ A 15-minute (15 min, $n = 10$), 3-minute (3 min, $n = 10$), or 0-minute (sham, $n = 11$) compression was separately applied to the C-7 nerve root via a microvascular clip using previously described surgical procedures on Day 0 of the study.⁴¹ Mechanical hyperalgesia was measured using an ascending series of von Frey filaments (1.4–26 g) in the forepaw ipsilateral to the compression in rats on Day 0 before surgery (baseline) and on Days 1, 3, 5, and 7 after surgery (details described in the *Specific Detailed Methods* section below). Time-dependent differences in paw withdrawal threshold between 15 min ($n = 5$), 3 min ($n = 5$), and sham ($n = 6$) were determined using a 2-way repeated measures ANOVA (group \times day) with Tukey's honestly significant difference test.

Spinal cord tissue at the C-7 level was harvested on Day 1 (15 min, $n = 5$; 3 min, $n = 5$; sham, $n = 5$) or Day 7 (15 min, $n = 5$; 3 min, $n = 5$; sham, $n = 6$) in separate groups, to evaluate the temporal spinal fibrin(ogen) immunolabeling extent and pattern. Spinal fibrin(ogen) expression in the spinal dorsal horn ipsilateral to injury was quantified using densitometry^{34,41} and compared with fibrin(ogen) expression in matching spinal tissue from normal naïve rats,

and significant differences were evaluated using a 2-way ANOVA (group \times day) with Tukey's test. The spinal cord tissue taken from the rats undergoing a 15-minute compression was also co-immunolabeled for fibrin(ogen) with the serum component immunoglobulin G (IgG) to determine whether spinal thrombin activity is localized to spinal regions that undergo BSCB breakdown.

In a complementary study, spinal thrombin activity was blocked prior to imposing the 15-minute compression that induces hypersensitivity,⁴¹ in order to assess whether nerve root–induced spinal thrombin is necessary for the development of pain-related behaviors after that injury. Rats received an intrathecal injection of hirudin, a selective thrombin inhibitor, 1 day prior to a nerve root compression (hir+15 min, $n = 6$). Mechanical hyperalgesia was measured in the forepaw before hirudin injection (Day –1), after hirudin injection but before surgery (Day 0), and on alternating days (Days 1, 3, 5, and 7) after injury. As a measure of hyperalgesia, the paw withdrawal threshold was compared between the hir+15 min group and rats receiving only a compression (15 min, $n = 5$). Since hirudin was delivered in sterile phosphate-buffered saline (PBS), which is an inert vehicle, a vehicle compression was not included. Statistical differences in the withdrawal threshold between hir+15 min and 15 min were determined using a 2-way repeated measures ANOVA (group \times day) with Tukey's test.

At Day 7, spinal cord tissue was harvested from rats that were given hirudin intrathecally with a 15-minute compression. An additional group of rats received an intrathecal injection of hirudin before undergoing a 15-minute root compression, and spinal cord tissue was harvested from those rats at Day 1 after injury ($n = 5$). Tissue harvested at Days 1 and 7 was immunolabeled for fibrin(ogen) and IgG. Quantification of fibrin(ogen) expression for hir+15 min was compared with fibrin(ogen) expression in comparable tissue from rats undergoing a 15-minute compression (Day 1, $n = 5$; Day 7, $n = 6$) but not receiving hirudin treatment; spinal fibrin(ogen) expression was normalized to expression in naïve tissue and differences were compared using a 2-way ANOVA (group \times day) with Tukey's test.

Investigating the Effects of Spinal Thrombin and Its Activation of PAR1 on Behavioral Hypersensitivity

To determine whether exogenous thrombin is sufficient to induce pain-related behaviors in the absence of injury, rat thrombin was administered via a single intrathecal injection to naïve rats (RTh, $n = 6$). Mechanical hyperalgesia was measured in the bilateral forepaws on Day 0 before and on Days 1, 3, 5, and 7 after thrombin injection; the left and right paw withdrawal thresholds were averaged for each rat since this treatment is ubiquitous and not expected to have preferential effects on hyperalgesia in the bilateral forepaws. The average paw withdrawal threshold at Days 1, 3, 5, and 7 were compared with the respective baseline (Day 0) thresholds using separate 2-tailed paired *t*-tests to determine whether the threshold on each of those days was changed from baseline in rats dosed intrathecally with thrombin.

A separate complementary study was performed to as-

sess whether spinal thrombin acts through PAR1 to induce behavioral hypersensitivity. Rats received a single injection of rat thrombin (RTh, $n = 6$) at Day 0 and mechanical hyperalgesia was measured in the bilateral forepaws at Day 0 before and at Day 1 after the thrombin injection. In a separate group of rats, the small-molecule PAR1 inhibitor SCH79797 was injected intrathecally 1 day before the rat thrombin administration (SCH+RTh $n = 6$). Mechanical hyperalgesia was measured bilaterally prior to SCH injection (Day -1), after SCH treatment but before rat thrombin injection (Day 0), and on Day 1 after rat thrombin administration. Differences in the average bilateral paw withdrawal threshold between the SCH+RTh and RTh groups were detected using a 2-way repeated measure ANOVA (group \times day) with Tukey's test.

Determining the Effects of Spinal PAR1 Activation on Compression-Induced Behavioral Hypersensitivity

To examine whether PAR1 activation contributes to the development of pain after a compressive nerve root injury, SCH79797 was administered 1 day before a 15-minute nerve root compression (SCH+15 min, $n = 7$). Since the vehicle for SCH79797 contains dimethyl sulfoxide (DMSO), which is not biologically inert,^{18,39} control groups were included in which DMSO was administered intrathecally 1 day before either a 15-minute root compression (DMSO+15 min, $n = 5$) or a sham surgical procedure (DMSO+sham, $n = 5$). Mechanical hyperalgesia was measured in the forepaw ipsilateral to injury on the day before (Day -1) SCH79797 or vehicle injection, on Day 0 after injection but before surgery, and on Days 1, 3, 5, and 7 after surgery. Differences in withdrawal threshold between SCH+15 min, DMSO+15 min, and DMSO+sham were compared with a 2-way repeated measures ANOVA (group \times day) with Tukey's test.

Specific Detailed Methods

Animals

All studies were performed using adult male Holtzman rats (Harlan Sprague-Dawley). Rats were housed under conditions approved by the United States Department of Agriculture and the Association for Assessment and Accreditation of Laboratory Animal Care in a temperature- and light-controlled room with free access to water and food. Lighting conditions were maintained as a normal light/dark cycle. All operational and experimental procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and carried out under the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.⁵⁹

Surgical Procedures for Nerve Root Compression

Surgical procedures were performed under inhalation isoflurane anesthesia (4% for induction, 3% for maintenance). Previously reported protocols were followed for administering nerve root compression injury.^{7,34,44} Briefly, rats were placed in a prone position, and a C6-7 hemilaminectomy and partial facetectomy was performed on the right side to expose the right C-7 dorsal nerve root. A small incision was made in the dura over the C-7 root and

a calibrated 10-gf microvascular clip (World Precision Instruments) was applied to the right C-7 dorsal nerve root. The root was compressed for either 3 or 15 minutes, after which the clip was removed and the wound was closed by 3-0 polyester suture and surgical staples. Rats were allowed to recover in room air with continual free access to food and water. Sham-operated rats underwent identical surgical procedures, including opening of the dura, except that they did not undergo nerve root compression.

Preparation and Intrathecal Administration of Pharmacological Agents

Thrombin from rat plasma (Sigma Aldrich) was dissolved in sterile PBS (Mediatech, Inc.) and stored at -80°C until further use, avoiding repeat freeze-thaw cycles. Rat thrombin was administered to rats via intrathecal injection at a final dose of 4 U per rat.³³ Hirudin (Sigma Aldrich) was dissolved in sterile PBS, stored at -20°C , and administered intrathecally at a dose of 4.2 U per rat 1 day prior to any surgical procedures (Day -1).³³ Rat thrombin and hirudin doses were chosen based on a comparable dose of thrombin inducing mechanical allodynia in mice and a comparable pretreatment of hirudin attenuating allodynia when repeatedly administered with a sciatic nerve ligation.³³

SCH79797 dihydrochloride (*N*³-Cyclopropyl-7-[[4-(1-methylethyl)phenyl]methyl]-7*H*-pyrrolo[3,2-*f*]quinazolin-1,3-diamine dihydrochloride; Tocris) was fully dissolved in DMSO (Sigma Aldrich) at a stock concentration of 10 mg/ml and was stored at -20°C . When ready for use, the DMSO SCH79797 solution was diluted with sterile PBS and administered intrathecally (50 $\mu\text{g}/\text{kg}$) 1 day before surgery or rat thrombin administration.^{15,30} This dose was chosen because administering SCH79797 intrathecally at a comparable dose reduced brain edema after surgically induced brain injury.³⁰ A DMSO vehicle control was also included for comparison purposes; DMSO alone was diluted in sterile PBS (1:15) and intrathecally delivered to rats 1 day prior to surgery.

All drug solutions were administered in the intrathecal space between L-4 and L-5 via a lumbar puncture via syringe to rats that were under anesthesia.⁴³ Lumbar puncture volume remained at a constant 30 μl injection for each treatment.

Behavioral Assessment of Mechanical Hyperalgesia

Behavioral sensitivity was defined as the response threshold of the rat forepaw to an applied mechanical stimulus. Mechanical thresholds were measured using previously defined methods applying a series of von Frey filaments of increasing strength to the forepaw and performed blinded to surgical or treatment procedure.^{7,8,29} Experimenters performing the behavioral testing were blinded to the injury and treatment groups. On each testing day, rats were acclimated for 15 minutes to the testing apparatus, which consisted of an elevated mesh-floored cage. A series of calibrated von Frey filaments (1.4, 2, 4, 6, 8, 10, 15, and 26 g) (Stoelting) was applied in ascending order to the plantar surface of the forepaw until a filament induced a positive response, which consisted of the rat withdrawing its forepaw and often was accompanied with shaking

or licking of the paw.^{7,8,29} The filaments were applied for 5 stimulations until a positive response was elicited. If a rat responded to 2 consecutive filaments, the lower-strength filament was recorded as the paw withdrawal threshold. If no filament elicited a response, then the highest-magnitude filament (26 g) was recorded as the threshold. On each testing day, the mechanical threshold was measured 3 times, separated by at least 10 minutes between each testing round, for each forepaw tested and averaged for each rat across the rounds.

For groups that received intrathecal injection(s) but did not undergo surgical procedures (i.e., RTh and SCH+RTh), the forepaw withdrawal threshold was measured in the bilateral forepaws on each testing day. The withdrawal thresholds were then averaged between the left and the right forepaw for each rat on each testing day so that only 1 threshold value was included for each rat for analysis. For groups that underwent surgery to expose the right C-7 nerve root (for either a compression injury or a sham procedure, with or without treatments), the withdrawal threshold was measured only in the forepaw on the side ipsilateral to surgery.

Tissue Harvest, Immunofluorescent Labeling, and Quantification of Fibrin(ogen)

Rats were deeply anesthetized with an overdose of sodium pentobarbital (Oak Pharmaceuticals Inc.) administered intraperitoneally at a dose of 65 mg/kg to prepare for tissue harvest. Rats were transcardially perfused with PBS followed by 4% paraformaldehyde (Sigma). Cervical spinal tissue was post-fixed overnight in 4% paraformaldehyde, transferred to 30% sucrose for 1 week at 4°C, and then embedded in OCT (optimum cutting temperature) medium (Sakura Finetek USA, Inc.) for cryosectioning. Fixed spinal cord tissue was sectioned axially at a 14- μ m thickness along the long-axis and mounted directly onto slides for immunolabeling. Spinal cord tissue was also harvested at the C-7 level from naïve rats ($n = 2$) and included in tissue processing for normal comparison.

Spinal cord sections obtained at the C-7 level were fluorescently co-immunolabeled for fibrin(ogen), as a proxy for thrombin activity,¹⁰ and immunoglobulin G (IgG), as an indicator of BSCB breakdown.^{14,38} Briefly, slide-mounted tissue sections were blocked in 5% normal goat serum (Vector Laboratories) with 0.3% Triton X-100 (Bio-Rad Laboratories) for 1 hour at room temperature. Slides were incubated overnight at 4°C labeled overnight with FITC-labeled rat fibrin(ogen) (1:100, Dako) followed by a 2-hour incubation at room temperature with goat anti-rat IgG Alexa Fluor 568 (1:200, Life Technologies).

The ipsilateral dorsal horn was digitally imaged at 10 \times for 2–6 spinal sections for each rat by an experimenter blinded to the treatment groups. Fibrin(ogen) labeling was quantified in images that were uniformly cropped to include the dorsal horn using a custom densitometry MATLAB script.⁴¹ A pixel intensity threshold was set to include positive fibrin(ogen) labeling in C-7 spinal cord tissue from normal naïve rats; this threshold was kept constant for all analyses. The percentage of the total pixels in the tissue area that was above that threshold of positive labeling for fibrin(ogen) in normal tissue was taken as the percentage

of positive pixels in each tissue section.^{34,42} The percentage positive for fibrin(ogen) was then normalized to labeling in tissue from naïve rats and data were represented as expression relative to normal tissue.

Results

Painful Nerve Root Compression Promotes Extravasation of Spinal Thrombin That Contributes to Hyperalgesia

A 15-minute nerve root compression significantly reduced the withdrawal threshold of the forepaw ipsilateral to injury compared with baseline levels, reflecting an increase in mechanical hyperalgesia, that was induced by Day 1 (3.9 ± 1.3 g, $p < 0.001$) (Fig. 1A). The compression-induced reduction in threshold from baseline was still observed on Day 7 (3.8 ± 1.7 g, $p < 0.001$) (Fig. 1A). At Day 1, the rats undergoing a 15-minute compression also exhibited a significantly lower ($p < 0.001$) withdrawal threshold than those undergoing a corresponding sham procedure (18.1 ± 8.1 g) and this difference was also evident at Day 7 (sham, 18.8 ± 4.5 g, Fig. 1A). In contrast, a 3-minute compression did not induce any change in paw withdrawal threshold from baseline levels at either time point probed (Day 1, 14.0 ± 5.3 g; Day 7, 14.2 ± 9.3 g; Fig. 1A). However, comparison of the overall group responses showed that rats undergoing a 3-minute compression exhibited significantly lower ($p = 0.028$) withdrawal thresholds than those exposed to a sham surgery. The withdrawal threshold did not differ significantly between rats undergoing a 3-minute and those undergoing a 15-minute compression, overall (Fig. 1A).

In parallel with the production of mechanical hyperalgesia at Day 1, a 15-minute compression also induced a marked increase in fibrin(ogen) immunolabeling in the ipsilateral spinal dorsal horn by Day 1 (Fig. 1B). The 3-minute compression induced less spinal fibrin(ogen) labeling than the 15-minute compression and only slightly increased expression over sham, which exhibited virtually no spinal fibrin(ogen) (Fig. 1B). When quantified, a 15-minute compression induced a significant increase ($p < 0.001$) in spinal fibrin(ogen) ($49.7\% \pm 68.6\%$) compared with sham ($5.4\% \pm 6.6\%$) at Day 1 (Fig. 1C). In contrast, the 3-minute compression did not induce a quantifiable increase in spinal fibrin(ogen) expression. The increased spinal fibrin(ogen) that was evident after the 15-minute compression at Day 1 returned to sham levels at Day 7 (Fig. 1B), which was significantly lower ($p = 0.003$) than the levels of fibrin(ogen) at Day 1 (Fig. 1C). Assessment of overall differences between groups showed that spinal fibrin(ogen) expression induced by a 15-minute compression was significantly higher than levels induced by either a 3-minute compression ($p = 0.0015$) or a sham procedure ($p = 0.0001$) (Fig. 1C).

The robust expression of fibrin(ogen) labeling in the ipsilateral spinal cord induced at Day 1 after the 15-minute compression strongly co-localized with IgG labeling (Fig. 1D). The co-localization of fibrin(ogen) with the serum-derived IgG suggests that thrombin likely extravasates into the spinal parenchyma with other serum proteins at times when BSCB breakdown occurs, which happens only after neural injury that also induces pain.

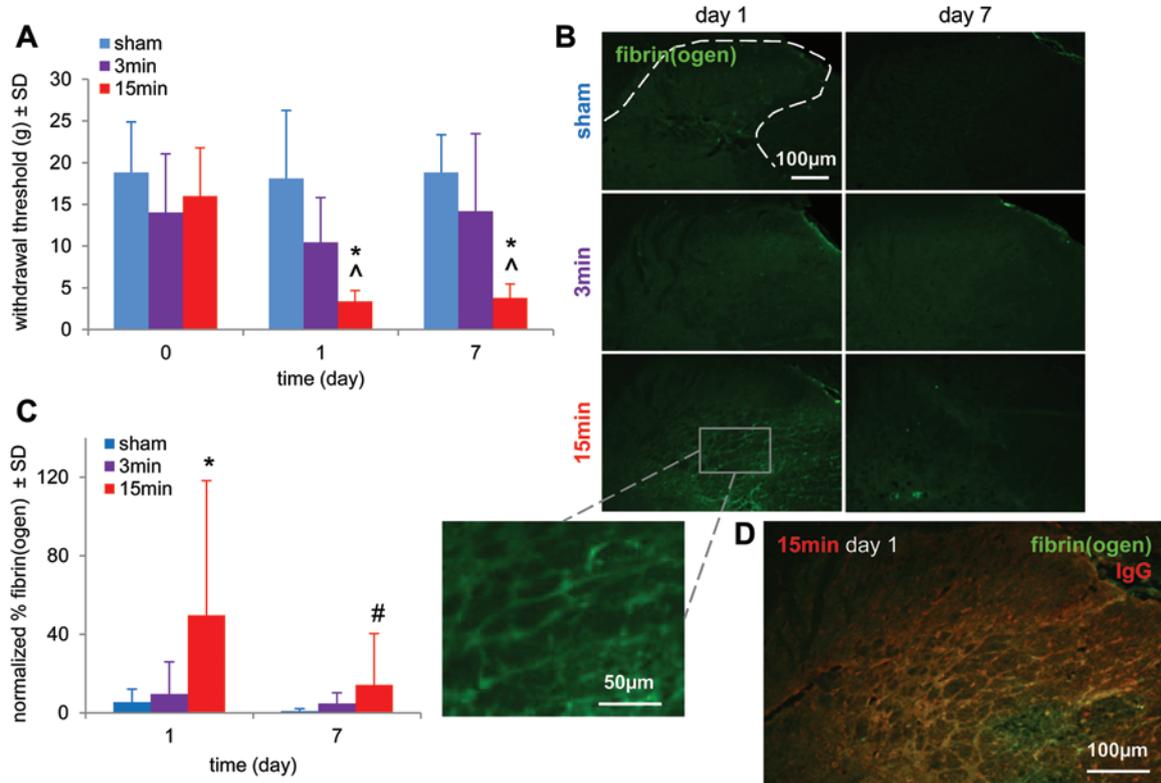


FIG. 1. Spinal fibrin(ogen) expression increases early after nerve root compression that induces hyperalgesia and is localized to areas of BSCB breakdown. **A:** Graph showing differences in withdrawal thresholds on Days 0, 1, and 7 in rats subjected to 15 minutes of compression, 3 minutes of compression, or a sham operation. Data are presented as mean values with SDs (error bars). A 15-minute compression significantly reduces the withdrawal threshold at Days 1 and 7 relative to corresponding responses at Day 0 ($^{\wedge}p < 0.001$) and sham ($^*p < 0.001$) responses at both Day 1 and Day 7. **B:** Photomicrographs of spinal cord sections immunohistochemically stained for fibrin(ogen). Fibrin(ogen) is robustly expressed in the spinal dorsal horn (dotted white outline) in the 15-min specimen, but is minimally expressed in the sham and 3-min specimens. A higher magnification image (inset) is provided to show fibrin(ogen) labeling. **C:** Graph showing the mean normalized percentage of fibrin(ogen) in the 3 groups at Day 1 and Day 7. Error bars indicate SDs. The trends are similar for both days, with 15 min significantly increasing ($^*p < 0.001$) the percent fibrin(ogen) compared with levels after sham at Day 1. By Day 7 after 15 min, percent fibrin(ogen) is significantly reduced ($\#p = 0.003$) compared with Day 1. **D:** Photomicrographs of a spinal cord section from an animal in the 15-min group at Day 1 and stained for fibrin(ogen) and IgG showing that a high degree of co-localization (indicated by yellow). Figure is available in color online only.

Inhibiting Spinal Thrombin Prevents Nerve Root-Induced Hyperalgesia

Spinal fibrin(ogen) expression was elevated by Day 1 after a 15-minute compression; this increase in fibrin(ogen) expression significantly decreased ($p < 0.001$) by Day 7 after that injury (Fig. 2 right). A single intrathecal administration of hirudin substantially reduced the compression-induced increases in fibrin(ogen) and IgG labeling that were evident at Day 1 in the ipsilateral spinal cord (Fig. 2 left). When quantified, hirudin treatment significantly reduced ($p = 0.006$) spinal fibrin(ogen) expression at Day 1 ($5.9\% \pm 6.5\%$) compared with an untreated 15-minute compression ($49.7 \pm 72.2\%$, Fig. 2 right). By Day 7, the spinal fibrin(ogen) expression pattern did not differ significantly between an untreated 15-minute compression and a compression that was pretreated with hirudin (Fig. 2 left). Indeed, the quantified spinal fibrin(ogen) expression did not differ significantly between those 2 groups at Day 7 (Fig. 2 right). These results confirm that hirudin pretreatment of nerve root compression prevented the enzymatic activity of thrombin in the spinal cord early after injury.

The withdrawal threshold in the ipsilateral forepaw was significantly reduced ($p < 0.001$) from the corresponding baseline levels by Day 1 and for all subsequent days tested after a 15-minute nerve root compression (Fig. 3). Pretreating with hirudin 1 day before that compression prevented the compression-induced reduction in the forepaw withdrawal threshold (Fig. 3). Rats undergoing compression alone exhibited a significantly lower ($p < 0.011$) threshold (3.1 ± 1.5 g) than rats receiving hirudin intrathecally (12.3 ± 6.5 g) at Day 1 prior to a 15-minute compression for all days tested (Fig. 3).

Blocking Spinal PAR1 Inhibits Spinal Thrombin-Induced Hyperalgesia

Rats receiving a single intrathecal injection of rat thrombin (4 U per rat) exhibited a significant increase ($p = 0.032$) in mechanical hyperalgesia, as indicated by a decrease in the withdrawal threshold at Day 1 (6.1 ± 2.4 g, Fig. 4 upper). The paw withdrawal threshold remained significantly lower ($p = 0.018$) than baseline levels at Day 3 (9.9 ± 5.2 g) after an injection of rat thrombin, but returned to baseline

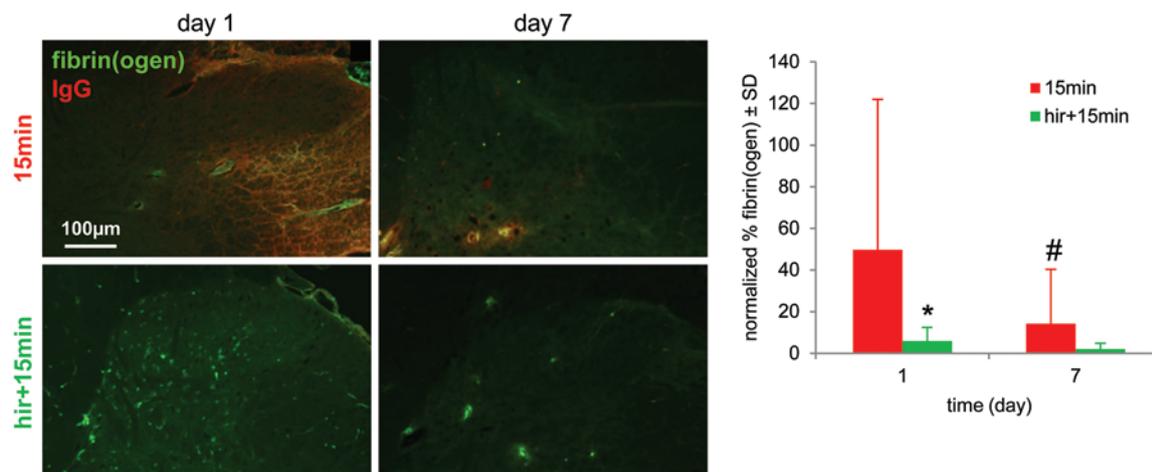


FIG. 2. Intrathecal hirudin pretreatment before a nerve root compression reduces spinal fibrin(ogen). **Left:** Photomicrographs of spinal cord specimens immunostained for spinal fibrin(ogen) and IgG. Spinal fibrin(ogen) and IgG immunolabeling increase at Day 1 after a 15-minute compression (15 min), but then decrease by Day 7. Intrathecal pretreatment with hirudin 1 day before a 15-minute compression (hir+15 min) reduces spinal fibrin(ogen) and IgG expression at Day 1 compared with levels for untreated compression. **Right:** Graph showing the mean normalized percentage of fibrin(ogen) at Days 1 and 7 in the 15-min and hir+15 min groups. Quantification of spinal fibrin(ogen) expression relative to expression in tissue from normal naïve rats shows significantly lower (* $p = 0.006$) levels for hir+15 min compared with 15 min at Day 1 and that fibrin(ogen) expression for 15 min is significantly lower (# $p < 0.001$) by Day 7 than at Day 1. Figure is available in color online only.

levels by Day 5 (Fig. 4 upper). A single intrathecal injection of rat thrombin induced a significant reduction ($p < 0.001$) in the withdrawal threshold on Day 1 compared with the threshold at Day 0 (Fig. 4 lower). However, blocking spinal PAR1 with intrathecal SCH79797 prior to the rat thrombin injection prevented the development of the mechanical hyperalgesia at Day 1. The withdrawal thresholds at Day 1 in rats treated with SCH79797 prior to rat thrombin (9.4 ± 1.4 g) were not different from thresholds before the thrombin administration (at Day 0) (8.5 ± 1.7 g, Fig. 4 lower).

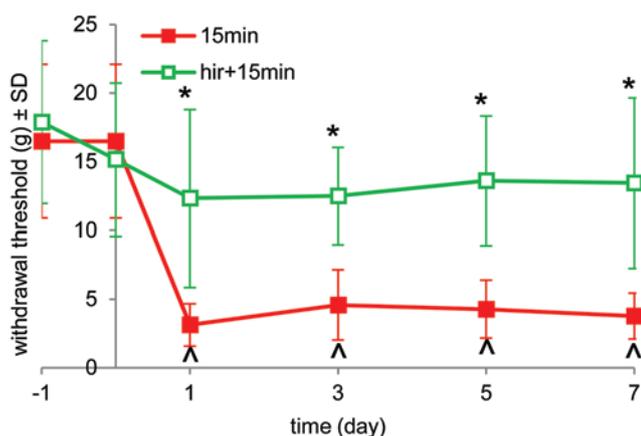


FIG. 3. Intrathecal administration of hirudin prior to a nerve root compression prevents the development of mechanical hyperalgesia. The graph shows the change in the mean withdrawal threshold over time, with SDs indicated by error bars. Mechanical hyperalgesia, measured by the withdrawal threshold of the ipsilateral forepaw, is significantly reduced from baseline by a 15-minute nerve root compression (15 min) at Day 1 lasting for up to 7 days after injury (* $p < 0.001$). Pretreating with hirudin (hir+15 min) at 1 day before injury (Day -1) prevents the compression-induced reduction in withdrawal threshold; the paw withdrawal threshold for hir+15 min was significantly greater (* $p < 0.011$) than for 15 min on all 7 days. Figure is available in color online only.

Blocking PAR1 Activation Inhibits Nerve Root–Induced Hyperalgesia

Intrathecal blocking PAR1 with SCH79797 prior to a 15-minute compression prevented compression-induced mechanical hyperalgesia. By Day 3, SCH79797 treatment significantly prevented ($p < 0.0065$) the drop in withdrawal threshold that was evident with a 15-minute compression after vehicle pretreatment (Veh+15 min, 4.9 ± 0.9 g; SCH+15 min, 12.8 ± 7.4 g), which was sustained for up to 7 days after injury (Fig. 5). Overall, SCH79797 reduced hyperalgesia to sham levels; the same was evident each testing day except for Day 7, when compression plus treatment with SCH79797 resulted in a significantly lower threshold compared than sham compression and vehicle treatment ($p = 0.020$, Fig. 5). Of note, at Day 1, the withdrawal thresholds were not different for rats undergoing root compression with a vehicle treatment and those receiving a sham operation with a vehicle treatment (Fig. 5). In contrast, the respective untreated compression and sham groups at this same time point exhibited a significant difference in mechanical hyperalgesia (Fig. 1A). However, by Day 3 after a compression with a vehicle treatment, the typical developments of a significantly lower ($p < 0.001$) withdrawal threshold than in the sham group were evident (Fig. 5). The increased sensitivity relative to responses in the sham group was maintained until Day 7 (Fig. 5).

Discussion

This is the first study to demonstrate that spinal thrombin activity is elevated after a nerve root compression and is requisite for sustained mechanical hyperalgesia. Thrombin activity is elevated early and transiently in the ipsilateral spinal cord on Day 1 only after a transient nerve root compression that also induces hyperalgesia, and thrombin activity is localized to spinal regions where BSCB break-

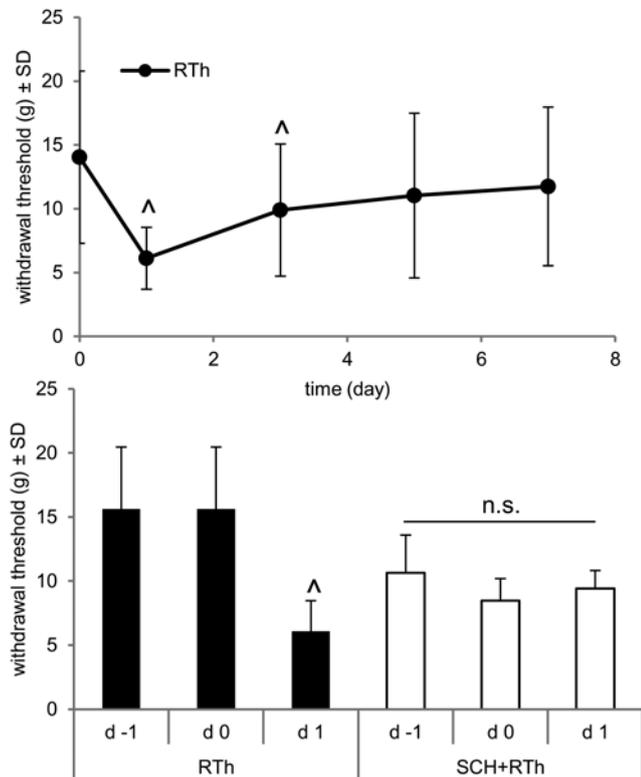


FIG. 4. Intrathecal rat thrombin induces mechanical hyperalgesia, which is inhibited by intrathecal SCH79797. **Upper:** Graph showing significant reduction in bilateral forepaw withdrawal threshold ([^]*p* < 0.032) at Days 1 and 3 after intrathecal administration of rat thrombin (RTh, 4 U/rat). **Lower:** Graph showing comparison of the bilateral withdrawal threshold (in separate groups of rats) at Day 1 after administration of rat thrombin with and without SCH79797 pretreatment. The bilateral withdrawal threshold is significantly reduced ([^]*p* < 0.001) from baseline in the animals that received rat thrombin without pretreatment with SCH79797, whereas the animals that were pretreated with the PAR1 inhibitor SCH79797 one day before receiving rat thrombin (SCH+RTh) showed no significant changes in forepaw withdrawal threshold. Data are presented as mean values with SDs (*error bars*); n.s. = not statistically significant; d = day; d-1 = Day -1.

down also occurs (Fig. 1). Blocking spinal thrombin activity is sufficient to prevent mechanical hyperalgesia after a compression (Fig. 3), suggesting that spinal thrombin is necessary for the onset of pain-related behaviors after nerve root injury. The role of spinal thrombin in hypersensitivity is supported by the results indicating that a single intrathecal injection of rat thrombin is potent enough to produce hyperalgesia in naïve rats, which depends on its activation of PAR1 (Fig. 4). Further supporting the PAR1 pathway in thrombin-mediated hypersensitivity, blocking the activation of spinal PAR1 prior to injury also prevents the development of hyperalgesia (Fig. 5), suggesting that thrombin acts through PAR1 to contribute to pain-like behaviors.

Although thrombin has been hypothesized to cross a compromised BSCB contributing to neuroinflammatory processes,⁴⁸ this is the first *in vivo* study to demonstrate that thrombin enters the CNS in areas of BSCB breakdown induced after peripheral neural injury and is required for the development of pain-related behaviors (Fig. 1). Spinal

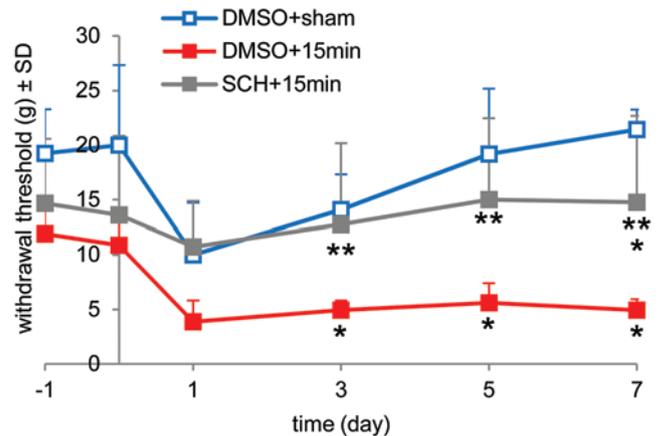


FIG. 5. Blocking spinal PAR1 activation with SCH79797 attenuates compression-induced mechanical hyperalgesia. The graph shows changes in the mean withdrawal threshold over time, with SDs indicated by *error bars*. A 15-minute nerve root compression after pretreatment with DMSO results in a significant decrease (^{*}*p* < 0.001) in ipsilateral paw withdrawal threshold by Day 3 that lasts for up to 7 days compared with vehicle-treated sham (DMSO+sham). SCH79797 (SCH+15 min) prevents that hyperalgesia; the withdrawal threshold for SCH+15 min is significantly higher (^{**}*p* < 0.0065) than DMSO+15 min by Day 3, and that response lasts until Day 7. On Day 7, the withdrawal threshold for SCH+15 min is significantly lower (^{*}*p* = 0.020) than DMSO+sham. Figure is available in color online only.

thrombin activity is elevated along the same time course as BSCB breakdown after a nerve root compression that induces hyperalgesia (Fig. 1).⁴⁷ The co-expression of fibrin(ogen) and IgG in the spinal cord by Day 1 (Fig. 1), supports that thrombin enters the spinal parenchyma from the periphery. Since fibrin(ogen) was used here as a proxy for thrombin activity, it is possible that fibrin or fibrinogen may separately cross the BSCB and accumulate in the spinal parenchyma. Exogenous fibrin administered into the brain does not reduce neuronal health or induce local inflammation,²² suggesting that the elevated level of spinal fibrin(ogen) that is evident after a root compression likely is not responsible for nociception.

The transient elevation in thrombin activity in the spinal cord that occurs early after a root compression (Fig. 1) may contribute to the spinal astrocytic activation that is evident by Day 1 after that injury.⁴² Rat astrocytes express multiple thrombin-activated receptors,^{24,45} and activation of astrocytic PAR1, in particular, induces their activation and proliferation.^{16,35,54} Since thrombin is no longer present in the spinal cord by Day 7 (Fig. 1) when both pain and astrocytic activation persist,^{41,42} it is likely that spinal thrombin activity is an initiator of spinal astrocyte activation as opposed to contributing to their sustained activation.

There are several limitations of this work. Among them is the use of measuring fibrin(ogen) expression as a proxy for thrombin activity. In doing so, the accumulation of thrombin's enzymatic product does not provide information on the dynamic expression of thrombin. Directly measuring the activity of thrombin in the spinal cord prior to Day 1 would provide useful information about the amount of thrombin and the duration of its elevation, which would better inform whether thrombin contributes to astrocytic activation. Moreover, the findings from this study reflect

those of a short-duration nerve root insult and may not fully represent the extent of responses from other clinically relevant causes of cervical radiculopathy. For example, a disc herniation or stenosis imposes sustained compression to the nerve root and may induce longer-lasting BSCB breakdown and/or more robust pain and spinal cord responses than the 15-minute compression used here.

Hirudin prevents the deposition of fibrin(ogen) in the spinal parenchyma at Day 1 when administered before compression (Fig. 2), confirming that it is sufficient to block the enzymatic activity of thrombin. The ability of hirudin to completely inhibit the development of mechanical hyperalgesia and to prevent its onset for up to 1 week after compression (Fig. 3) supports previous findings that intrathecal hirudin prevents mechanical and thermal behavioral sensitivity after a sciatic nerve ligation in the mouse.³³ However, in contrast to that study in which repetitive hirudin dosing only marginally suppressed pain,³³ a single dose of hirudin completely prevents pain development after a root compression (Fig. 3). A sciatic nerve ligation also induces breakdown of the BSCB lasting for up to 30 days,^{5,14} in contrast to a 15-minute nerve root compression, which only induces BSCB breakdown for a single day.⁴⁷ It is possible that a sciatic nerve injury permits thrombin extravasation into the spinal parenchyma for a much longer duration (up to 30 days) than does the transient root compression and hence allows thrombin to exert sustained activity within the spinal cord. Hirudin is relatively stable *in vivo* and retains 60%–80% of its activity after 24 hours.²⁴ Accordingly, administering hirudin 24 hours before compression ensures that it is still active in the spinal cord at the time of injury and likely at the time of BSCB breakdown, which occurs within 24 hours after compression.⁴⁷ This provides support for the effectiveness of just 1 dose of hirudin at preventing pain-related behaviors after a transient nerve root compression.

In contrast to the complete prevention of nerve root–induced hyperalgesia that is seen with hirudin administration, mechanical allodynia is only partially attenuated by inhibiting the action of TNF- α or IL-1 in the spinal cord prior to injury.^{42,43} Thrombin induces glial production of pro-inflammatory cytokines in the CNS.^{9,16,46} Because thrombin is an upstream inducer of pro-inflammatory cytokine production within the CNS,^{46,48} centrally blocking thrombin may also attenuate the elevation of multiple spinal inflammatory cytokines that contribute to nociception, thereby inducing a more robust behavioral response than targeting the actions of only 1 cytokine. Investigating whether blocking spinal thrombin activity reduces spinal glial activation and pro-inflammatory cytokine expression after a nerve root compression would inform this hypothesis. Interestingly, hirudin also reduces nerve root compression–induced BSCB breakdown, as indicated by a reduction in spinal IgG expression on Day 1 compared with an untreated injury (Fig. 2 left). Thrombin is a known activator of endothelial cells and increases vascular permeability.^{11,26} Although we hypothesized that BSCB breakdown is requisite for thrombin extravasation into the spinal parenchyma, it is possible that once in the spinal cord, thrombin further activates vascular endothelial cells, thereby exaggerating the extent of compression-induced BSCB breakdown.

Although a single injection of rat thrombin into the spinal cord induces hyperalgesia for 3 days in the absence of any other injury-induced inputs (Fig. 4), more robust behavioral sensitivity has been reported in mice with mechanical allodynia persisting for up to 10 days after intrathecal thrombin.³³ Since a comparable amount of thrombin (adjusting for the weight difference between mice and rats) was administered in the 2 studies, the disparity in the duration of pain following the central administration of thrombin might depend on the species being treated in comparison with the species in which the exogenous thrombin is derived. Narita et al.³³ did not specify which species of thrombin used in their study; it is possible that thrombin derived from a non-murine species was administered. Administering “non-self” proteins, including thrombin, induces a complex immune response^{3,27,28} and might exacerbate neuroinflammation and contribute to the extended period of mechanical allodynia that was observed in mice in that study.

Blocking spinal PAR1 prior to nerve root injury produces behavioral results that are similar to those obtained by pretreating with hirudin (Figs. 3 and 5); both treatments prevent hyperalgesia for at least 7 days. Yet, in contrast to hirudin, SCH79797 does not alter pain-related outcomes immediately (by Day 1) (Fig. 5). Unlike hirudin, which was administered in an inert saline vehicle, the vehicle used to deliver SCH79797, DMSO, is not biologically inert and has been shown in some cases to be neurotoxic in a dose-dependent manner.^{18,39} In fact, pretreatment with the DMSO vehicle in the sham group even resulted in a slight increase in mechanical hyperalgesia at Day 1 (Fig. 5), which is not typical for sham-operated rats (Fig. 1A). A recent study demonstrated that treating CA1 neurons in hippocampal brain slices with DMSO for up to 5 hours reduces the threshold for action potential firing in response to an electrical stimulus.⁵⁰ In the current study, DMSO also might decrease the threshold for neuronal firing in the spinal cord, thereby contributing to early nociceptive signaling within the spinal cord early after its administration.

Conclusions

This study establishes a novel contribution for spinal thrombin activity in the pain-related behaviors that develop after nerve root injuries. Previous reports stress important roles for the extravasation of pro-inflammatory cytokines and immune cells into the CNS across a compromised BSCB as contributing to neuroinflammation and nociception.^{2,14,56} Our findings extend existing models by demonstrating that thrombin also enters the spinal parenchyma after nerve root injury and is requisite for the onset of mechanical hyperalgesia. Although spinal thrombin induces hyperalgesia through its activation of PAR1 centrally, additional work is needed to define which CNS cell type(s) expressing PAR1 contribute to pathological mechanisms induced by spinal thrombin.

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Disclosures

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Author Contributions

Conception and design: both authors. Acquisition of data: Smith. Analysis and interpretation of data: both authors. Drafting the article: both authors. Critically revising the article: Winkelstein. Reviewed submitted version of manuscript: both authors. Approved the final version of the manuscript on behalf of both authors: Winkelstein. Statistical analysis: Smith. Study supervision: Winkelstein.

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