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Perispinal injection of a TNF blocker directed to the brain of rats alleviates the sensory and affective components of chronic constriction injury-induced neuropathic pain

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ABSTRACT

Neuropathic pain is chronic pain that follows nerve injury, mediated in the brain by elevated levels of the inflammatory protein tumor necrosis factor-alpha (TNF). We have shown that peripheral nerve injury increases TNF in the hippocampus/pain perception region, which regulates neuropathic pain symptoms. In this study we assessed pain sensation and perception subsequent to specific targeting of brain-TNF (via TNF antibody) administered through a novel subcutaneous perispinal route. Neuropathic pain was induced in Sprague-Dawley rats via chronic constriction injury (CCI), and thermal hyperalgesia was monitored for 10 days post-surgery. On day 8 following CCI and sensory pain behavior testing, rats were randomized to receive perispinal injection of TNF antibody or control IgG isotype antibody. Pain perception was assessed using conditioned place preference (CPP) to the analgesic, amitriptyline. CCI-rats receiving the perispinal injection of TNF antibody had significantly decreased CCI-induced thermal hyperalgesia the following day, and did not form an amitriptyline-induced CPP, whereas CCI-rats receiving perispinal IgG antibody experienced pain alleviation only in conjunction with i.p. amitriptyline and did form an amitriptyline-induced CPP. The specific targeting of brain TNF via perispinal delivery alleviates thermal hyperalgesia and positively influences the affective component of pain. *Perspective:* This study presents a novel route of drug administration to target central TNF for treatment of neuropathic pain. Targeting central TNF through perispinal drug delivery could potentially be a more efficient and sustained method to treat patients with neuropathic pain.

1. Introduction

According to the American Academy of Pain Medicine, pain affects more individuals than diabetes, heart disease and cancer combined. Chronic pain afflicts over 1.5 billion people worldwide and approximately 3–4.5% of the global population suffers from neuropathic pain, with incidence rates increasing with age (Global Industry Analysts, Inc. Report, January 10, 2011. http://www.prweb.com/pdfdownload/8052240.pdf). Chronic pain is a widespread medical need for which current treatments are inadequate.

Neuropathic pain is a complex experience comprised of peripheral and central components that involve higher brain centers responsible for learning, cognition, and emotional processes (Campbell and Meyer, 2006; Nickel et al., 2012; Woolf and Mannion, 1999; Zimmermann, 2001). Neuropathic pain, which manifests as chronic pain following an injury to neuronal tissue, develops in part due to an immune response orchestrated by macrophages (Shubayev et al., 2006). These immune effector cells release pro-inflammatory cytokines, notably the proximal pleiotropic cytokine Tumor Necrosis Factor-alpha (TNF), at the site of injury. TNF rapidly accumulates at various points along the neuraxis causing dysregulation of neurotransmission and leads to the sensitization of nociceptors (Covey et al., 2000; Ignatowski et al., 1999; Leung

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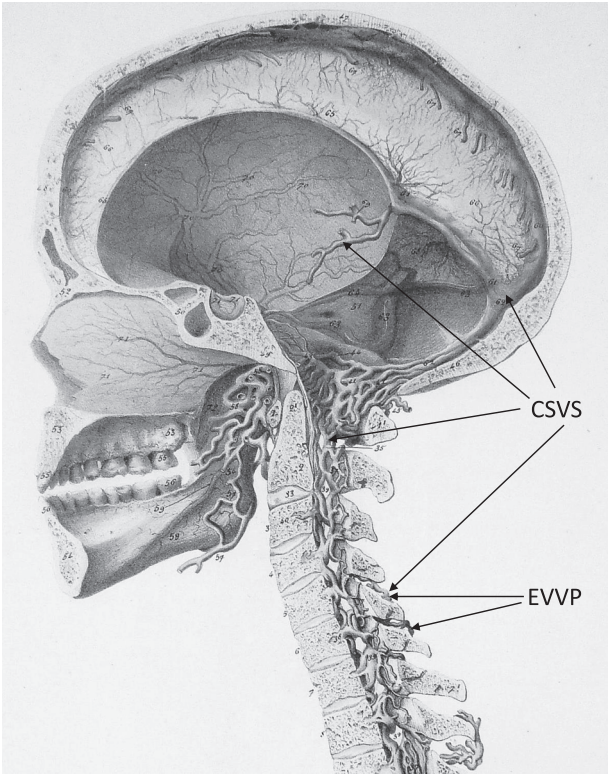

Fig. 1. The cerebrospinal venous system. Detail of plate 5 from Breschet G, Recherches anatomiques physiologiques et pathologiques sur le systaeme veineux. Paris: Rouen frareres; 1829. Courtesy of the Sidney Tobinick Collection. Adapted from Edward Lewis Tobinick, *Perispinal Delivery of CNS Drugs,* CNS Drugs. 2016 Jun;30(6):469–480, Copyright 2016, Edward Tobinick, used in accordance with Creative Commons License, https://creativecommons.org/licenses/by/4.0/legalcode. CSVS, cerebrospinal venous system; EVVP, external vertebral venous plexus.

and Cahill, 2010). The negative effect of over-expression of TNF, particularly over-expression within the brain, elicits clinical presentation of symptoms associated with neuropathic pain, such as thermal hyperalgesia, which is defined as an exacerbated response to a noxious thermal stimulus (Fasick et al., 2015).

The chronic constriction injury (CCI) rodent model of neuropathic pain induces thermal hyperalgesia through upregulation of peripheral and central TNF expression. Specifically, TNF expression in the hippocampus, a region linked to the limbic system and responsible for learning and memory formation, is a key molecular mechanism that directs neuropathic pain and the associated symptoms (Covey et al., 2000; Gerard et al., 2015; Ignatowski et al., 1999).

The perispinal route of administration is a novel and negligibly invasive technique currently being used clinically to deliver etanercept, a biopharmaceutical agent that selectively neutralizes TNF, to the central nervous system (CNS), via the cerebrospinal venous system (CSVS), in order to treat selected neuroinflammatory disorders, including chronic neurological dysfunction and/or pain after stroke, brain injury, or disc herniation (Ignatowski et al., 2014; Tobinick, 2010, 2011, 2012, 2016; Tobinick et al., 2012, 2014). Fig. 1 depicts the CSVS, which provides a direct vascular pathway to the brain. Additional illustration of the connections between the spinal and cerebral venous systems show that the spinal and cerebral venous plexuses are in anatomic and physiologic continuity (Tobinick, 2010; Tubbs et al., 2018). The perispinal injection utilizes the valveless anatomical feature of the CSVS, which upon Trendelenburg positioning or inversion of the patient, allows treatment to be delivered to the brain through the choroid plexus. This route of administration allows an increased amount of biologically active drug to reach the brain by avoiding the systemic metabolism of the drug prior to reaching the blood-brain-barrier (Tobinick, 2010).

In the present study, we hypothesized that CCI-induced pain could be alleviated by targeting brain TNF with subcutaneous, perispinal injection (into the anatomic region that drains into the CSVS via its superficial component, the external vertebral venous plexus, or EVVP; Fig. 1) of a monoclonal TNF antibody. In addition to alleviation of thermal hyperalgesia, we predicted that blocking activity of TNF selectively in the brain would have an influence on the affective component of non-evoked neuropathic pain. In order to test this, we observed the antinociceptive effect of a single perispinal antibody injection, and used the conditioned place preference (CPP) paradigm to assess affective ongoing pain.

2. Methods

2.1. Animals

Male Sprague-Dawley rats (Envigo-Harlan Sprague-Dawley, Indianapolis, IN) initially weighing 175–250 g were used for the majority of experiments. Female Sprague-Dawley rats weighing 175–250 g were used for some experiments. Same sex rats were housed in groups of 2–3 animals per cage at 23 ± 1 °C in Laboratory Animal Facility – accredited pathogen-free quarters with access to food and water *ad libitum*. The animals were maintained on a 12- hour light/dark cycle with the lights on from 0600 to 1800 h. Rats were acclimated to the animal facilities for 3–5 days before initiation of behavioral testing. All experiments were in accordance with the guidelines for the ethical treatment of animals established by the National Institutes of Health and the Committee for Research and Ethical Issues for IASP (Zimmermann, 1983). Also, procedures performed were in accordance with the Institutional Animal Care and Use Committee (IACUC) of The University at Buffalo. All efforts were made to minimize animal suffering and to use the minimal number of animals to generate statistical results.

2.2. Experimental model

The experimental paradigm used for these studies is illustrated schematically in Fig. 2.

In *Experiment 1*, male rats receiving CCI (n = 8) or sham (n = 7) surgery were tested for peripheral hypersensitivity and were randomized into i.p. saline (CCI + saline, n = 3; Sham + saline, n = 4) or i.p. amitriptyline (CCI + amitriptyline, n = 5; Sham + amitriptyline, n = 3) treatment groups to test amitriptyline-induced antinociception and CPP. These results were compared to CCI rats receiving perispinal (p.s.) TNF antibody (n = 4) or IgG control antibody (n = 4) injection. In *Experiment 2*, nine female CCI rats were randomized to receive either p.s. TNF antibody (n = 3), p.s. IgG isotype control antibody (n = 3), or no p.s. injection (n = 3) to determine if blocking TNF in the brain was antinociceptive for female rats. In *Experiment 3*, three naïve male rats were administered fluorophore-labeled TNF antibody by p.s. injection and brain tissue harvested after one hour for image analysis. In *Experiment 4* male CCI (n = 18) and Sham (n = 12) rats were randomized into groups receiving p.s. or intrathecal (i.t.) injection of either saline, TNF antibody, or IgG isotype control antibody to determine the antinociceptive efficacy of p.s. and i.t. delivery of TNF antibody treatment. In each experiment, rats were weighed before and on the day of CCI and sham surgery, and every other day thereafter before nociceptive behavioral measurements were taken. Weight was matched before CCI and sham surgery, and rats in all groups gained an equivalent amount of weight during the course of the study. Following surgery, animals were blindly randomized to receive a p.s./i.t. injection of either TNF antibody, IgG isotype control antibody, or received no p.s. injection (control group). The experimenter performing behavior testing of the rats was blinded to the treatment. Rats injected (p.s. or i.t.) with the TNF antibodies did not experience any difference in weight gain over

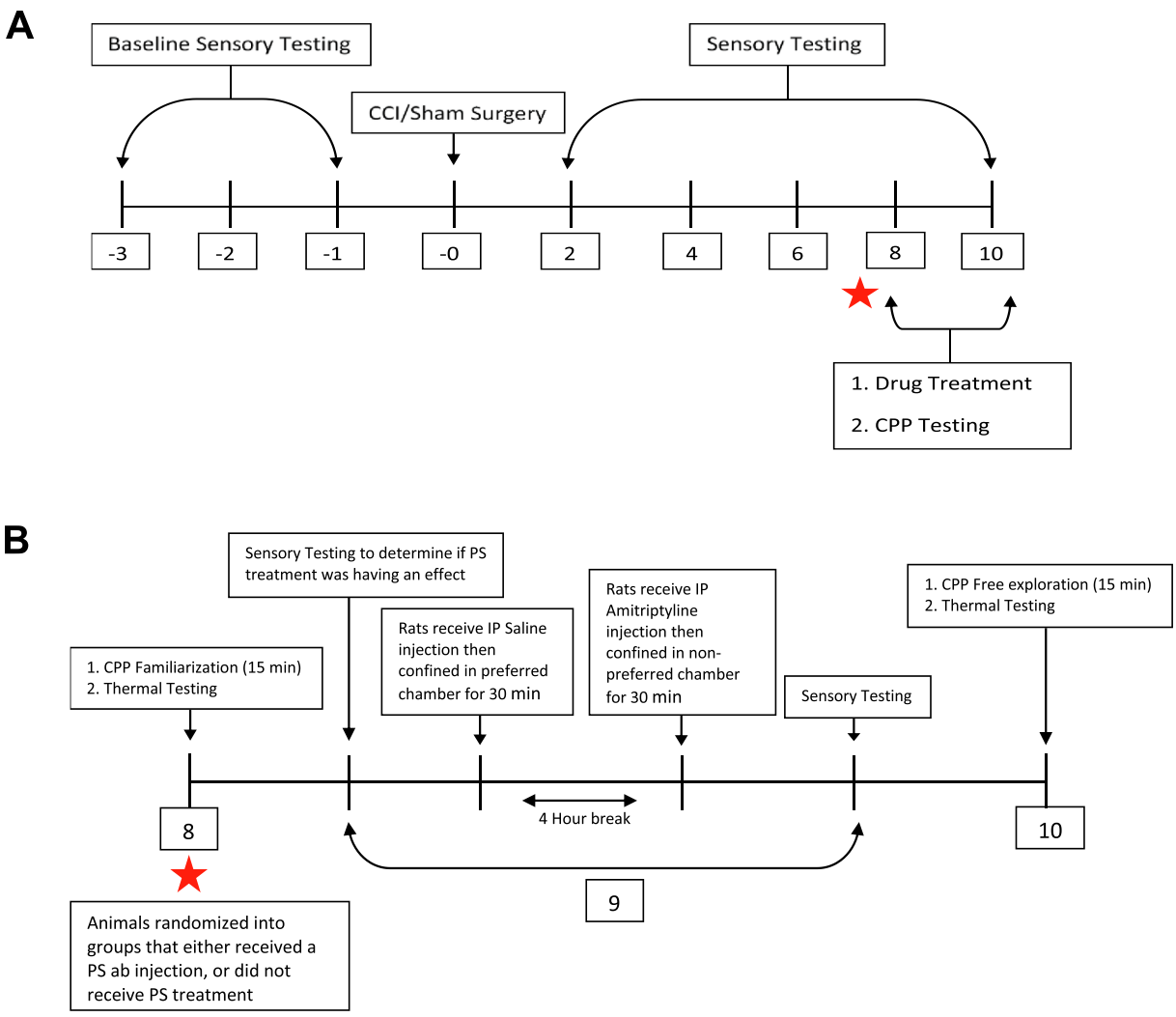

Fig. 2. Schematic depicting the protocol used to evaluate sensory (thermal hyperalgesia) and affective (CPP) components of pain induced by CCI and following perispinal (PS) injection of TNF antibody. (A) Experimental timeline showing the thermal hyperalgesia testing days pre- and post-surgery. (B) Detailed thermal hyperalgesia and CPP testing during treatment days 8 and 9 and on CPP test day 10. CPP = conditioned place preference, CCI = chronic constriction injury.

time (2–6 days) as compared with the control animals (data not shown). All rats exhibited similar grooming behaviors throughout the study. Sample size was determined based on power analysis of previous experiments (see Section 2.10 Statistics).

2.3. Chronic constriction injury

Loose ligatures were applied around the common sciatic nerve of the right hind paw according to described methods (Bennett and Xie, 1988). Briefly, rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) intraperitoneally (i.p.) prior to surgery. The sciatic nerve was exposed unilaterally, and four ligatures (4–0 chromic gut, Roboz Surgical Instrument Co., Inc., Rockville, MD) were placed around the nerve, ~1 mm apart, proximal to the trifurcation. Ligatures were tied such that constriction to the diameter of the nerve was barely discernable, allowing for uninterrupted circulation through the epineural vasculature. In sham procedures, the nerve was similarly exposed and freed of adherent tissue/muscle, but no ligatures were placed. The muscle layer was closed using chromic gut suture followed by skin closure using 9 mm stainless steel wound clips. All surgeries were performed between 0800 and 1200 hr.

2.4. Injection methods

At specified times following CCI surgery, rats were administered drugs or vehicle (sterile saline) via one of three methods: (1) Intraperitoneal (i.p.): Standard i.p. injection of amitriptyline (10 mg/kg) or 1 ml/kg saline; (2) Perispinal (p.s.): Subcutaneous injection at the dorsal cervical spine (C7-T1) level (100 µl, 30-G needle; subcutaneous to depth of ~ 6 mm). Trendelenburg positioning (head down by gentle hand restraint or in restrainer, 6 min) was performed to facilitate delivery of antibodies into the brain through the choroid plexus after injection. (3) Intrathecal (i.t.): Injection was by direct lumbar puncture (of dura) at L4-L6 (10 µl; 30-G needle), which was confirmed by reflexive tail flick or 'S' formation of tail (Fairbanks, 2003). Antibodies for injection (BioLegend, San Diego, CA) were as follows: anti-mouse/rat $TNF\alpha^{PE}$ (0.1 µg/ml) and Armenian hamster IgG^{PE} isotype control antibody (negative control; 0.1 µg/ml).

2.5. Thermal hyperalgesia

At specified times post-CCI, the thermal nociceptive threshold was measured in each hind paw. Hyperalgesia (increased sensitivity to noxious sensory stimuli) was measured by determining changes in paw withdrawal latency (PWL) using a plantar algesia apparatus (model #33, Analgesia Meter, IITC Life Science Instruments, Woodland Hills,

CA) (Hargreaves et al., 1988). A "difference score" generated from subtracting the ipsilateral PWL from the contralateral PWL was used as an index of hyperalgesia. PWL was measured using an intense heat source to stimulate thermal receptors in the sole of the foot. The use of this apparatus is based on the fact that peripheral nerve injury results in increased sensitivity to a sensory (thermal) stimulus. A maximal automatic cut-off latency of 15 s was used to prevent tissue damage. Rats were placed in Plexiglas chambers, on top of a temperature maintained (32 ± 0.1 °C) glass surface. Rats were acclimated to the testing apparatus for 7–10 min (or until exploratory behavior ceased), and measurements of the thermal withdrawal threshold were taken for each hind paw. Baseline latencies were determined before experimental treatment for all animals as the mean of three separate trials, taken 3, 2 and 1 day pre-surgery and/or the day of the surgery (prior to surgery, day 0). Paw withdrawal responses were measured every other day post-surgery for a 10 day period. Only rapid hind paw movements away from the thermal stimulus (with or without licking of hind paw) were considered to be a withdrawal response. Paw movements associated with weight shifting or locomotion were not counted. Each hind paw was measured three times at ≥4 min intervals, and the averaged values for each day were used to compute thermal hyperalgesia (ipsilateral PWL–contralateral PWL). All measurements were recorded between 0700 and 1200 h.

2.6. Conditioned place preference (CPP)

The CPP test was initiated on day 8, when animals freely explored the apparatus for 15 min. The time spent in each chamber was recorded to determine the animals' chamber bias. The left and right chambers, separated by a middle neutral chamber, differed in appearances (vertical vs horizontal stripes) and floor textures (textile vs marbleized). The day after pre-exposure/free chamber exploration, CCI rats injected with saline (1 ml/kg, i.p.) were confined to their preferred chamber for 30 min. Four hours later, the same animal was then administered amitriptyline (10 mg/kg, i.p.) and confined within the opposite chamber for 30 min. The next day the rats were placed in the neutral chamber with free access to all chambers; the time spent in each chamber was recorded for a 15 min period (King et al., 2009).

2.7. TNF bioassay

The lytic effect of TNF upon the WEHI-13VAR fibroblast cell line was used to analyze brain tissue homogenates for the presence of biologically active TNF (Ignatowski and Spengler, 1994; Khabar et al., 1995). Immediately after sacrifice, the right and left hippocampi were harvested on an ice block, snap frozen in liquid nitrogen, and stored at −20 °C until processed. Tissue samples were weighed and homogenized in 3-ml RPMI-1640 supplemented with 1% glutamine and protease inhibitor cocktail (Sigma-Aldrich Chemical, St. Louis, MO; 2.5 µl/50 mg tissue).

Supernatants from homogenates centrifuged at 14,000 × g at 4 °C for 15 min were stored at −80 °C until analyzed for levels of bioactive TNF. Briefly, WEHI-13VAR fibroblast cells, a TNF sensitive cell line derived from a mouse fibrosarcoma (ATCC, Manassas, VA), were grown in RPMI-1640 culture medium containing 2 mM L-glutamine, 10% fetal bovine serum (Invitrogen, Chicago, IL), and 3 µg/ml gentamicin (Sigma-Aldrich Chemical) in T75 flasks at 37 °C, 95% relative humidity (RH), and 5% CO_2. Cells used in the TNF bioassay were cultured to approximately 90% confluency and were always below passage 25 to avoid loss of TNF sensitivity. Cells were prepared for the assay by detaching with 0.25% trypsin and 0.02% EDTA (Sigma-Aldrich Chemical) and resuspending in culture medium supplemented with 1 µg/ml actinomycin D (Calbiochem, La Jolla, CA) to a concentration of 500,000 cells/ml. One hundred microliters of cell suspension were added to each well of a flat-bottom 96-well tissue culture plate containing 100 µl of 2-fold serial dilutions of unknown samples, in duplicate or triplicate, or known concentrations of rat recombinant TNFα standards (R&D Systems, Minneapolis, MN) in diluting medium, RPMI-1640, 2 mM L-glutamine, 1% fetal bovine serum, and 15 mM HEPES (Sigma-Aldrich Chemical). Following 20 h of incubation at 37 °C, 95% RH, 5% CO_2, 10 µl of Cell Proliferation Reagent WST-1 (a solution of the tetrazolium salt, WST-1 (4-[3-(4-iodophenyl)-2-(4nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) and the electron coupling reagent, mPMS (1-methoxy-5-methyl-phenazinium methyl sulfate); Roche Diagnostics, Indianapolis, IN) in diluting medium was added to each well. WST-1 counting solution was used as a cell viability indicator, which is quantified spectrophotometrically (Berridge et al., 2005). After incubating for 4 h at 37 °C, 95% RH, and 5% CO_2, the absorbance at 440 and 700 nm was measured using a SpectraMax 96 microplate reader with SoftMax Pro v.4.0 acquisition and analysis software (MDS Analytical Technologies, Sunnyvale, CA). A standard curve (0.01 pg/ml – 10,000 pg/ml, reverse sigmoid in shape) of the (OD_{440} – OD_{700}) vs log [TNF] was plotted. The [TNF] of each sample was determined from the dilution closest to the inflection point of the standard curve. This assay has a detection limit of approximately 1 pg/ml (Eskandari et al., 1990). The assay is based on the specific cytotoxicity of the WEHI-13VAR cells to TNF in the presence of actinomycin D. Increasing TNF concentration results in increased cell death and, therefore, a reduced absorbance at 440 nm. Results are expressed as the percentage of control values (pg/100 mg tissue weight) for the brain tissue samples.

2.8. Caliper measurements of sciatic nerve diameter

Sciatic nerve diameter measurements were performed to assess the degree of tissue edema, an indirect measure of inflammation. After decapitation, the sciatic nerves from both legs were excised, placed on ice, and the diameters of the sciatic nerves were measured using a digital caliper (VWR International, West Chester, PA). The ipsilateral sciatic nerves were harvested by cutting the nerves shortly above the location of the ligatures and 1.0 cm distally. Sciatic nerves from the contralateral side, as well as from sham-operated animals, were removed in a similar manner. Three measurements each were recorded for the proximal, ligatured, and distal sections of the ipsilateral nerves, and averaged to give one value for the nerve diameter. Similar measurements were made for the contralateral nerves. The results are expressed as a "difference value" generated by subtracting the average diameter of the contralateral nerve (mm) from the average diameter of the ipsilateral nerve (mm).

2.9. Immunofluorescence imaging

Three naïve male rats were used for brain tissue imaging. Sixty minutes following p.s. anti-mouse/rat $TNF\alpha^{PE}$ injection (n = 3 rats), animals were euthanized via decapitation, and whole brains were harvested and snap-frozen in Tissue-Tek® Optimal Cutting Temperature (OCT) compound (Electron Microscopy Sciences, Hatfield, PA) using liquid nitrogen. Coronal serial sections (8 µm thickness) of the frozen rat brains were prepared by cryostat, and sections were placed on StarFrost glass slides (Mercedes Medical, Sarasota, FL) and stored at −30 °C until stained.

For immunofluorescent labeling of brain tissue sections, slides stored at −30 °C in the dark were brought to room temperature and fixed in acetone for 10 min at room temperature. Slides were completely dried prior to hydration with 3 rinses of 1× PBS, 5 min each, at room temperature. Immunofluorescence staining procedures were followed as previously published (Gerard et al., 2015). Briefly, non-specific immunoglobulin binding was blocked with 10% goat serum for 20 min at room temperature. Slides were blotted without washing to remove serum. Sections were incubated with primary antibody for neurofilament-200, mouse monoclonal NF-200 (1:30,000, Sigma-Aldrich Chemical), which cross-reacts with rat, for 120 min in PBS/1% bovine serum albumin (BSA) (fraction V)/10% goat serum at room

temperature in a humidified chamber in the dark. Slides were washed 3 times, 5 min each, in PBS. Sections were incubated with the secondary antibody, goat anti-mouse IgG1-AlexaFluor 647 (1:2,000, Invitrogen), in PBS/1% BSA for 120 min at room temperature in a humidified chamber in the dark. Slides were washed 5 times, 5 min each, in PBS. Sections were incubated with DAPI nuclear stain (10 μM) for 1 min. Slides were rinsed with PBS for 5 min. Coverglass was mounted on the sections using Fluoromount™ aqueous mounting medium with anti-fade properties (Sigma-Aldrich Chemical). Slides were stored in the dark at 4 °C until analyzed.

Fluorescence images were captured using a Leica DM500B Microscope with Nuance-EX Multispectral Imaging System at 10X magnification. Images were saved as TIFF files with 4 to 6 images taken for each slide, covering the entire brain section. All staining signals were localized to cell nuclei. All samples were analyzed on the same day, with the same exposure settings. Multispectral imaging was used for multiplexing, while at the same time overcoming the effects of autofluorescence on detectability. After a multispectral image set was captured, spectral unmixing was performed to eliminate brain tissue autofluorescence and resolve specific imaging probe fluorescence signals. The individual component images were then combined in a single "composite" image (Levenson and Mansfield, 2006).

2.10. Statistics

Power analysis was performed for sample size estimation, based on data from a pilot study (n = 3/group; 3 groups) of perispinal TNF nanoplex (gold nanorod complexed TNF siRNA) injection for alleviation of CCI-induced pain. Sigma Plot 13 software (SPSS Inc., Chicago, IL) was used to determine the number of animals needed in each group to detect an effect with $\alpha = 0.05$ and a power of 0.8. Thermal hyperalgesia: Residual (Res) MS = 6.66, SD = 2.58, diff in means = 7.5, sample size = 4/group (ANOVA) for antinociception. Similarly, published data from TNF nanoplex silencing of hippocampal TNF levels during CCI (n = 3/group; 6 groups) was used for a priori power analysis (Gerard et al., 2015). Brain TNF levels: Res MS = 2592.163, SD = 50.913, diff in means = 248.5, sample size = 3/group (ANOVA). Data from a published study of amitriptyline-induced antinociception (n = 9 and 13/group; 2 groups) (Ignatowski et al., 2005) determined Res MS = 3.782, SD = 1.945, diff in means = 4.67, sample size = 4/group (ANOVA).

Results from experiments are expressed as mean values ± standard error of the mean (SEM). Data were analyzed using Student's *t*-test, one-way ANOVA, two-way repeated measures ANOVA, or three-way ANOVA. When significant differences were observed, appropriate post-hoc tests were performed as indicated in the figure legends. A difference was accepted as significant when $p < 0.05$.

3. Results

3.1. CCI-induced thermal hyperalgesia

Prior to CCI or sham surgery, male Sprague-Dawley rats had three days of hind paw thermal testing in order to determine the baseline paw withdrawal latencies (PWL) of both hind paws. Table 1 shows the baseline averaged PWLs of each hind paw for all rats prior to surgery, and reports the PWL changes in each hind paw within the sham and CCI groups until day 8 post surgery. There were no differences in withdrawal latencies at baseline between the groups (actual averaged hind paw withdrawal latencies *prior* to surgery: Sham rats (n = 7), ipsilateral 14.18 ± 0.39 s, contralateral 14.14 ± 0.31 s; CCI rats (n = 16), ipsilateral 12.50 ± 0.59 s, contralateral 12.87 ± 0.55 s; ANOVA, NS, $p = 0.271$). Following CCI surgery, thermal hyperalgesia developed in the ipsilateral hind paw as observed at days 2–8 post-surgery, demonstrated as peripheral hypersensitivity to the noxious thermal stimulus as compared to the contralateral hind paw of CCI animals (Days 2–8, $p < 0.001$), as well as compared to ipsilateral hind paws of sham-operated animals (Days 2–8, $p < 0.001$). The significant PWL change over time (from baseline values) in the ipsilateral CCI hind paws ($p < 0.001$ from all days (2–8) post-CCI) was unaccompanied by any changes in PWLs within the contralateral CCI, ipsilateral sham, and contralateral sham hind paws (Table 1). These findings indicate that sham surgery did not evoke thermal hyperalgesia and that contralateral hind paw sensitivity remained unaffected in animals that received CCI surgery.

Fig. 3 shows the comparison of thermal hyperalgesia between sham-operated and CCI animals. The results are expressed as the difference between the averaged ipsilateral and contralateral hind paw withdrawal latencies at each time point, with a negative difference score indicating thermal hyperalgesia. A significant difference in pain responses between CCI and sham-operated animals is observed at day 2 post-surgery and continues through day 8, prior to drug treatment administrations. The sham animals stayed consistent with their baseline values, that is, nociceptive responses to a noxious thermal stimulus did not develop in these animals. The increase in sensitivity to a thermal stimulus following CCI persisted through day 8 in comparison to the baseline difference score, as well as compared to the difference score in sham animals at each time point (CCI Days 2–8 vs baseline, $p < 0.001$; Sham Days 2–8 vs CCI Days 2–8, $p < 0.001$).

3.2. Amitriptyline alleviates CCI-induced thermal hypersensitivity

After development of peripheral hypersensitivity (thermal hyperalgesia), animals received an i.p. injection of either amitriptyline (10 mg/kg) or saline (1 ml/kg) on day 9 post-surgery. Amitriptyline at 30 min after injection did not affect sham-operated rat hind paw sensitivity (Fig. 4A, Day 9), but did have a significant delayed, pronociceptive effect at day 10. In contrast, at day 9 there was a significant decrease in thermal hyperalgesia experienced by CCI rats (Fig. 4B), occurring on the day when amitriptyline (AMI) was administered to the rats, when compared to the same rats on day 8 as well as on day 10 ($p < 0.01$). Since the pain responses returned when tested again the following day, this indicated that the decrease in hyperalgesia was acute or transient (Fig. 4B).

3.3. Amitriptyline-induced CPP forms in CCI rats

In order to assess if amitriptyline elicited an antinociceptive effect centrally in addition to blocking sensory components in the periphery, rats were tested using the CPP paradigm. The results presented in Fig. 5A show the effects of saline and amitriptyline administration on CPP formation in sham-operated animals. The data is presented as the difference score, which is calculated by subtracting the time (sec) spent in the baseline non-preferred chamber from the time spent in the drug-paired chamber on the final CPP testing day. Positive difference scores indicate the animal spent more time in the drug-paired chamber revealing formation of a CPP, while negative difference scores indicate the animal spent less time in the drug-paired chamber after being conditioned. Amitriptyline did not induce a CPP in the sham animals in comparison to the saline control group (Fig. 5A), which is consistent with previously published studies showing that amitriptyline at low doses fails to produce a CPP, but at higher doses (i.p. injection; 10 mg/kg) may produce a conditioned place aversion in naïve animals (Subhan et al., 2000; Tzschentke, 2007). In contrast, amitriptyline elicited antinociceptive effectiveness at the supraspinal level, as CCI rats that had received amitriptyline 24 h prior developed a CPP (Fig. 5B). Saline-paired CCI rats had no change in chamber preference, while the amitriptyline-paired CCI rats spent significantly more time in the drug-paired chamber (Pre vs Post Time $p < 0.001$) (Fig. 5C and D).

Table 1
Development of Thermal Hyperalgesia Following Surgery.

	Baseline	Group	Day 2	Day 4	Day 6	Day 8
Ipsilateral (23)	13.03 ± 0.49	CCI Ipsilateral (16)	9.69 ± 0.45***,###	8.91 ± 0.38***,###	7.96 ± 0.33***,###	8.06 ± 0.36***,###
		CCI Contralateral (16)	13.71 ± 0.46	13.82 ± 0.47	13.72 ± 0.42	13.19 ± 0.41
Contralateral (23)	13.27 ± 0.41	Sham Ipsilateral (7)	13.46 ± 0.77	13.97 ± 0.68	13.89 ± 0.68	13.92 ± 0.67
		Sham Contralateral (7)	13.68 ± 0.59	14.12 ± 0.46	13.88 ± 0.71	13.92 ± 0.40

Statistical significance determined using two-way ANOVA followed by Student-Newman-Keuls (SNK) post hoc analysis. Data are expressed as Mean ± SEM values with rat numbers indicated in parentheses. Day 2 post surgery there was a statistically significant interaction between Side and Surgery $F(1,42) = 9.949$, $p = 0.003$, such that SNK post hoc analysis determined ***$p < 0.001$ from CCI-contralateral, ###$p < 0.001$ from Sham-ipsilateral. Similarly, at Days 4, 6 and 8 post surgery there were statistically significant Side × Surgery interactions: Day 4 $F(1,42) = 19.483$, $p < 0.001$; Day 6 $F(1,42) = 28.767$, $p < 0.001$; and Day 8 $F(1,42) = 28.613$, $p < 0.001$. In each case, SNK analysis indicated side within CCI significance and surgery within ipsilateral significance, whereby ***$p < 0.001$ from CCI-contralateral, ###$p < 0.001$ from Sham-ipsilateral.

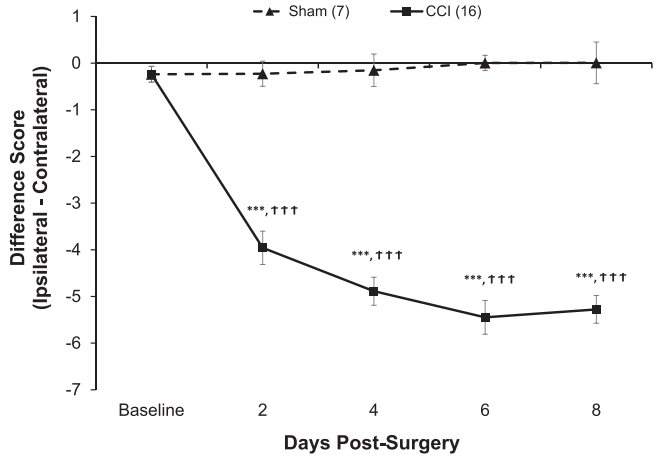

Fig. 3. CCI-induced thermal hyperalgesia. Assessment of thermal hyperalgesia in the CCI model of neuropathic pain. Data is presented as the difference score of ipsilateral/experimental – contralateral/control hind paw withdrawal latency in seconds. Each point is expressed as the mean ± SEM. (number of rats in parentheses). Two-way repeated-measures ANOVA indicated a significant main effect of Surgery [$F(1,84) = 133.38$, $p < 0.001$] and Day post-surgery [$F(4,84) = 22.328$, $p < 0.001$], as well as for the interaction between Surgery and Day [$F(4,84) = 21.132$, $p < 0.001$]. Student-Newman-Keuls post-hoc analysis determined: ***$p < 0.001$, as compared to Sham rats; †††$p < 0.001$, as compared to baseline. CCI, chronic constriction injury; ANOVA, analysis of variance; SEM, standard error of the mean.

3.4. Perispinal anti-TNF antibody injection alleviates thermal hyperalgesia

Fig. 6A shows the effect of p.s. TNF antibody administration on CCI-induced thermal hyperalgesia. In order to perform the p.s. injection with precision, rats were subjected to brief isoflurane anesthesia (administered via inhalation in a bell-jar). Therefore, the antinociceptive effect of the antibodies could not be accurately assessed until after the anesthetic-induced antinociceptive effect had dissipated. Preliminary experiments using this injection method determined that 24 h post-injection is appropriate for assessing pain behaviors. Initial testing of CCI rats at 30 min after receiving the p.s. injection indicated confounding analgesic effects from the isoflurane anesthesia (data not shown). Therefore, rats were injected p.s. with antibodies on day 8 and were then tested for thermal hyperalgesia on day 9, 24 h after the p.s. injection was performed. CCI rats injected p.s. (on day 8) with the TNF antibody showed a significant decrease in the PWL difference score on day 9, and the effect was sustained through the duration of the experiment, ending at day 10 (Fig. 6A). There was no significant change in PWL difference score in the IgG isotype antibody p.s. injection group (Fig. 6A, compare Day 8 to Day Pre-9 bars). In order to assess the effect of p.s. TNF antibody injection on pain perception (the affective pain component; see Section 3.5 for CPP results), the antinociceptive effect of amitriptyline (via i.p. injection) was utilized. There was a significant acute (assessed at 30 min post-i.p. injection) antinociceptive effect (i.e., inhibition of thermal hyperalgesia) following i.p. amitriptyline administration in both groups of antibody injected animals (Fig. 6A, Day Post-9 bars). However, alleviation of thermal hyperalgesia persisted in the p.s. TNF antibody group, while pain behaviors returned in the p.s. IgG antibody group of rats on day 10 (Fig. 6A, Day 10 bars), when the transient antinociceptive amitriptyline effect was no longer evident.

3.5. Perispinal TNF antibody injection elicited antinociceptive effectiveness at the supraspinal level in CCI rats

In order to test the effect of p.s. TNF antibody injection on pain perception in CCI animals, an 'indirect' CPP test was employed. Since p.s. TNF antibody treatment had a sustained antinociceptive effect (Fig. 6A), along with the distinct behavior differences imparted by amitriptyline on CCI rats (experiencing pain) and sham rats (not experiencing pain) (Fig. 5), an 'indirect' CPP method allowed us to investigate whether the p.s. TNF antibody treatment was acting centrally on the affective component of pain. CCI animals were blindly randomized into groups receiving either p.s. IgG isotype antibody treatment with amitriptyline conditioning, or p.s. TNF antibody treatment with amitriptyline conditioning. The TNF antibody or IgG antibody was administered on day 8 following familiarization to the CPP apparatus and thermal nociceptive testing. Fig. 6B and C show that animals receiving p.s. IgG isotype antibody apparently formed a CPP, spending more time in the amitriptyline-paired chamber on the CPP test day (Fig. 6B, Pre vs Post Time, $p = 0.056$). When comparing the time spent in the amitriptyline-paired chamber by CCI rats receiving p.s. TNF antibody, animals spent significantly less time in the amitriptyline-paired chamber on the CPP test day (Fig. 6B and C, Pre vs Post Time, $p < 0.05$). Fig. 6C compares the preference for the amitriptyline-paired chamber in animals that received p.s. IgG and p.s. TNF antibody treatment, reported as a difference score that was calculated by subtracting the time spent in the baseline non-preferred chamber from the time spent in the drug-paired chamber on the final experimental day. Animals receiving the p.s. TNF antibody spent significantly less time in the amitriptyline-paired chamber in comparison to animals that received the p.s. IgG isotype antibody ($p < 0.01$).

3.6. Central TNF levels following perispinal TNF antibody administration

TNF levels were measured in hippocampal (periventricular brain region) tissue homogenates prepared from rats receiving no p.s. injection, p.s. TNF antibody injection, and p.s. IgG isotype control antibody injection. The p.s. injection was performed on day 8 post-CCI, followed by i.p. amitriptyline injection on day 9 post-CCI, and the rats were sacrificed on day 10 post-CCI. These results show that p.s. TNF antibody injection on day-8 post-CCI trended toward reducing the contralateral hippocampal TNF levels on day-10 (Fig. 7).

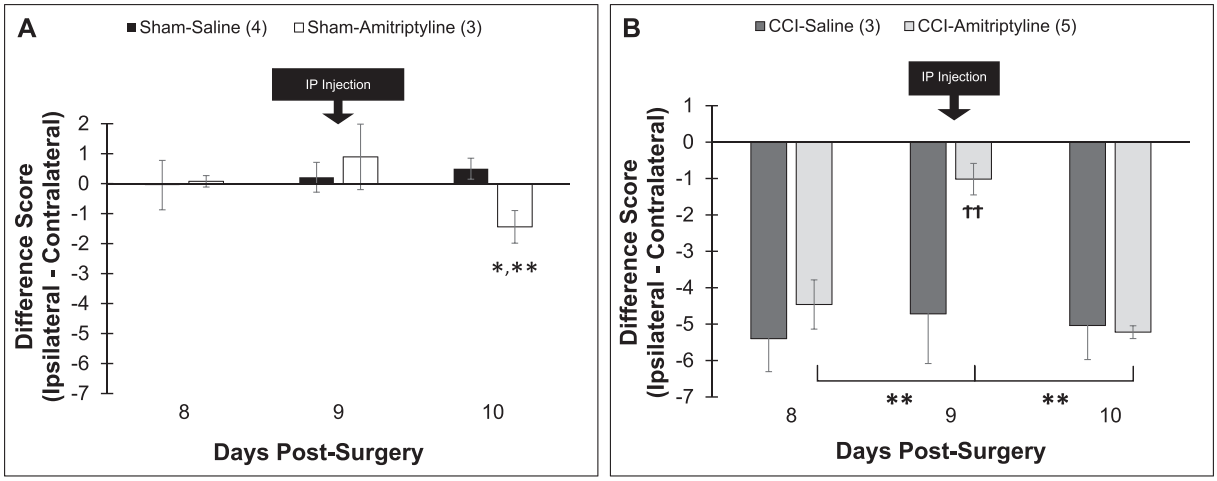

Fig. 4. Amitriptyline transiently alleviates CCI-induced thermal hyperalgesia. Amitriptyline (AMI, 10 mg/kg, i.p.) or saline (1 ml/kg, i.p.) was administered to (A) Sham rats and (B) CCI rats on day-9 post-surgery, and thermal measurements were taken 30 min post-injection. Bars (mean ± SEM) represent difference scores calculated by subtracting contralateral from ipsilateral average paw withdraw latency values at each time point (number of rats in parentheses). Two-way repeated-measures ANOVA indicated that neither main effect was significant for Sham animals (A); Drug: $F(1,10) = 0.222$, $p = 0.657$ and Day: $F(2,10) = 3.495$, $p = 0.071$. However, the interaction between Drug and Day was significant: $F(2,10) = 6.347$, $p = 0.017$, such that Student-Newman-Keuls post-hoc analysis determined $^{*}p < 0.05$ vs AMI on Day 8, and $^{**}p < 0.01$ vs AMI on Day 9. (B) In regards to CCI animals, two-way repeated-measures ANOVA indicated a significant main effect of Day $F(2,12) = 5.799$, $p = 0.017$, but no main Treatment effect $F(1,12) = 2.655$, $p = 0.154$ and only an apparent interaction of Treatment × Day $F(2,12) = 3.541$, $p = 0.062$. Student-Newman-Keuls post hoc analysis determined: $^{**}p < 0.01$ compared to day 9 within the CCI-AMI group. Two-way repeated measures analysis of difference score values on Day 9 post surgery indicated significant main effects for Surgery $F(1,11) = 17.530$, $p < 0.01$ and Drug $F(1,11) = 5.681$, $p < 0.05$, but no significant interaction between Surgery and Drug $F(1,11) = 2.520$, $p = 0.141$. Holm-Sidak post hoc analysis demonstrated that within the Saline-treated rats on day 9, those with CCI had larger negative difference scores (−4.72 ± 0.93 s) compared to Shams (0.21 ± 0.81 s), $p < 0.01$, indicating thermal hyperalgesia. Also, within the CCI group, rats given AMI had significantly reduced difference scores (−1.33 ± 0.72 s) as compared to those given saline (−4.72 ± 0.93 s), $^{\dagger\dagger}p < 0.05$.

3.7. Sciatic nerve edema following surgery

As a measure of edema, caliper measurements of the sciatic nerve, expressed as the difference between averaged ipsilateral right sciatic nerve average diameter (mm) and contralateral left sciatic nerve average diameter (mm) showed significant differences between CCI rats and sham rats. Rats receiving sham surgeries had no change in ipsilateral sciatic nerve diameter compared to their contralateral/left sciatic nerve (Table 2). Rats receiving CCI showed a significant increase in ipsilateral nerve diameter, as compared to their contralateral sciatic nerve (Table 2 and Fig. 8). There was no difference in diameter due to a drug effect in either the sham or CCI groups. However, all CCI groups of animals had significantly increased ipsilateral sciatic nerve diameters as compared to sham-operated animals (Fig. 8). Analysis of proximal, ligatured, and distal regions of sciatic nerves determined that none of the drugs affected the ipsilateral edema induced by CCI (Table 2).

3.8. Perispinal TNF antibody injection elicited antinociceptive effectiveness in female CCI rats

In order to ensure that the antinociceptive effect of p.s. TNF antibody delivery is consistent regardless of gender, *Experiment 2* was designed to test female Sprague-Dawley rats experiencing CCI-induced neuropathic pain. Female CCI rats received the intervention earlier (on Day 4) after sciatic nerve ligature placement in order to assess whether the p.s. TNF antibody injection elicited a sustained antinociceptive effect. Four days following CCI, animals were blindly randomized to receive a p.s. injection of either TNF antibody, IgG isotype control antibody, or received no p.s. injection (control group). CCI induced ipsilateral hind paw hypersensitivity to the noxious thermal stimulus in the female rats, indicating the development of thermal hyperalgesia, which is expressed as a negative difference score (Fig. 9). Animals that received the p.s. TNF antibody demonstrate alleviation of CCI-induced pain, and this antinociceptive effect was sustained until experiment conclusion at day 10 post-CCI (Fig. 9).

3.9. Perispinal injection followed by Trendelenburg positioning delivers TNF antibody to the choroid plexus

In *Experiment 3*, the fluorophore (phycoerythrin, PE)-labeled TNF antibody was used to determine whether p.s. injection of the antibody reached the brain. The location of the PE signal was assessed in coronal brain tissue sections prepared from animals 1 h after receiving p.s. injection of TNF antibody^{PE} and Trendelenburg positioning (Fig. 10). The lateral ventricles contain the cerebrospinal fluid producing choroid plexus, which is hypothesized to be a potential p.s. delivery entry point for agents/drugs into the brain. Tissue sections were labeled with NF-200 antibody using indirect immunofluorescence with secondary antibody conjugated to AF647 to identify neurons and their processes, and DAPI was used to stain nuclei. Multispectral imaging and analysis was used to eliminate brain tissue autofluorescence and to separate the multiple fluorescence signals. Fig. 10 panel A shows the RGB (red–green-blue) representation of the data set. Spectral unmixing resulted in the following signals: Autofluorescence, unmixed AF647 signal, unmixed DAPI signal, and unmixed PE signal (Fig. 10, panels B-E, respectively). As depicted in the composite image (Fig. 10F), the choroid plexus within the lateral ventricle shows signal for the TNF antibody^{PE}, which is labeled green in color.

3.10. Perispinal injection or intrathecal injection for TNF antibody alleviation of thermal hyperalgesia

In *Experiment 4*, antinociceptive effectiveness of p.s. or i.t. administration of TNF antibody was assessed following induction of CCI-induced thermal hyperalgesia. Fig. 11 (panels A and C) shows that neither p.s. nor i.t. TNF antibody injection affected peripheral sensitivity over time in sham-operated rats. Perispinal injection of TNF antibody on day 4 post-CCI elicited antinociception that was apparent at day 6 ($p < 0.07$, compared to p.s. CCI + saline and p.s. CCI + IgG groups) and reached significance on day 7 post-surgery (Fig. 11B). In contrast, TNF antibody administration by i.t. injection on day 4 (under light isoflurane anesthesia similar to p.s. injection) did not induce

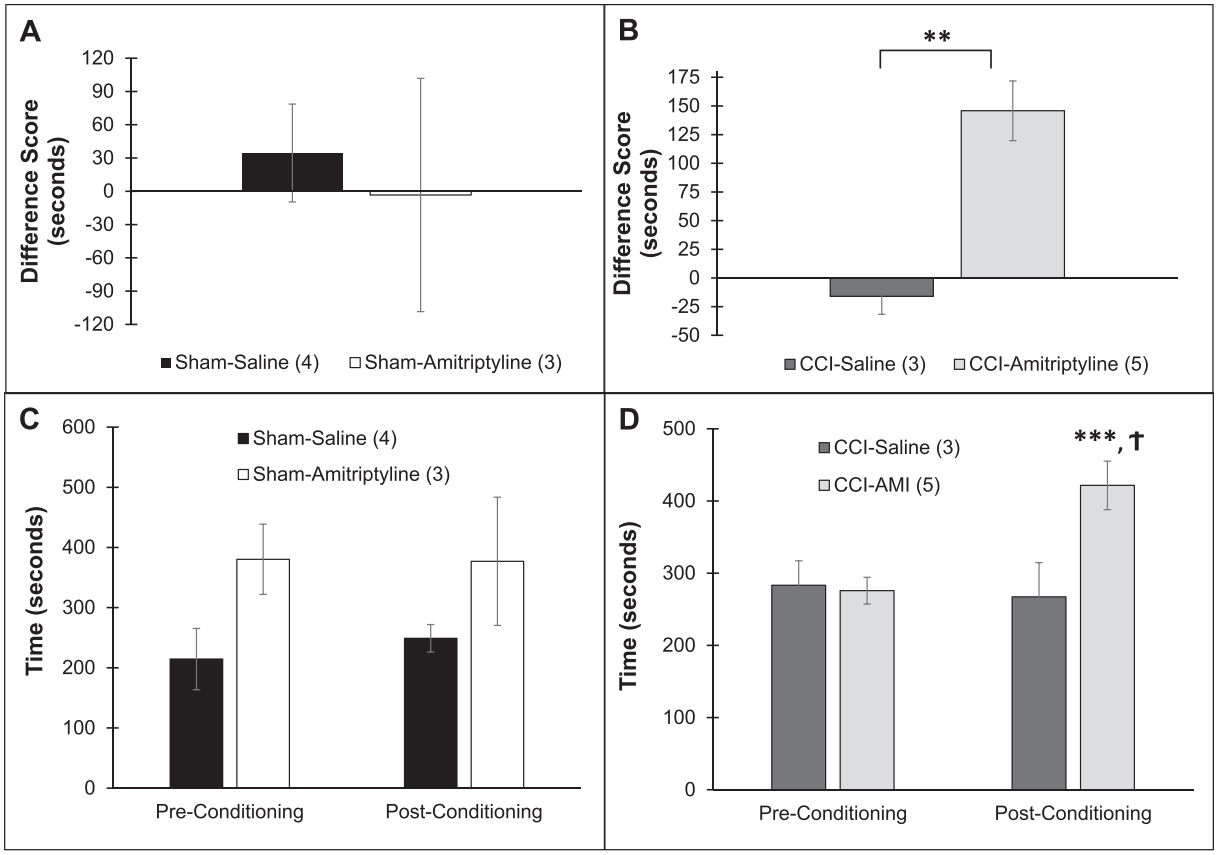

Fig. 5. Amitriptyline-induced conditioned place preference (CPP). Sham (A and C) and CCI (B and D) rats were conditioned with saline (1 ml/kg, i.p.) in their preferred chamber and with amitriptyline (AMI, 10 mg/kg, i.p.) in their non-preferred chamber as determined during the familiarization day. Data represents difference score in seconds of time spent in the amitriptyline-paired chamber on the final CPP testing minus the time spent in the baseline non-preferred chamber for sham rats (A) and CCI rats (B). Two-tailed *t*-test: $^{**}p < 0.01$ (*n* number in parentheses). Actual time in seconds sham (C) and CCI (D) rats spent in the vehicle (saline) and amitriptyline-paired chambers. Statistical significance determined using two-way repeated-measures ANOVA indicated a main effect of Day $F(1,6) = 12.608$, $p = 0.012$ and an interaction between Treatment and Day $F(1,6) = 19.592$, $p = 0.004$, but no main effect for Treatment $F(1,6) = 2.470$, $p = 0.167$. Student-Newman-Keuls post hoc analysis determined: $^{***}p < 0.001$ vs respective pre-conditioning value; $\dagger p < 0.05$ vs CCI-saline post-conditioning.

antinociception (Fig. 11D).

4. Discussion

This study demonstrates that thermal hyperalgesia and affective pain (perception of pain) can be reduced in animals experiencing CCI-induced neuropathic pain by non-invasively decreasing brain TNF using perispinal (p.s.) administration of TNF antibody, thereby establishing proof-of-concept for a minimally invasive means to alleviate pain. TNF overexpression, in response to a nerve injury, drives the development of neuropathic pain, and we demonstrated that CCI-induced hyperalgesia onset is correlated with elevated central TNF levels (Covey et al., 2000; Covey et al., 2002; Ignatowski et al., 1999). We also showed that intracerebroventricular (i.c.v.) infusion of an antibody to target brain TNF alleviated CCI-induced thermal hypersensitivity, whereas i.c.v. infusion of rrTNFα into naïve animals led to the development of peripheral hypersensitivity to a noxious thermal stimulus. TNF was only increased in the brain of naïve rats receiving rrTNFα i.c.v. infusion, with no detectable increase in TNF in blood serum or at the lumbar segment of the spinal cord, which suggested a specific role of brain-TNF in affecting the perception of pain in addition to mediating the sensory component of pain (Ignatowski et al., 1999). However, these preclinical studies used an invasive brain delivery (i.c.v. infusion) of TNF and TNF inhibitors; therefore, a negligibly invasive approach was warranted. We used p.s. injection of the TNF blocker, etanercept, which completely ameliorated the chronic pain of a patient, who years earlier had suffered an acute brain injury (Tobinick et al., 2014). This analgesic effect by off-label etanercept is similar to multi-patient internal-control (before and after assessment) case series (Tobinick, 2012; Tobinick and Davoodifar, 2004; Tobinick and Britschgi-Davoodifar, 2003). Here, we investigated the antinociceptive efficacy of this non-invasive delivery method using a CCI rat model. This is the first controlled, scientific report demonstrating the efficacy of pain relief by specifically and safely delivering an anti-TNF agent to the brain using minimally invasive means. This study establishes proof-of-concept for human use applications.

First we confirmed that CCI, and not sham surgery, induced ipsilateral hind paw thermal hypersensitivity, without any change in sensitivity in the contralateral hind paw. We then determined that the analgesic effect of the antidepressant drug amitriptyline was exerted at both sensory (thermal hyperalgesia) and supraspinal (pain perception) levels. Amitriptyline transiently alleviated thermal hyperalgesia in all CCI rat groups and also elicited antinociceptive effectiveness at the supraspinal level. The formation of amitriptyline-induced CPP in CCI animals experiencing pain indicates that the drug was analgesic and blocked ongoing affective pain (i.e., the perception of pain) (King et al., 2009). The lack of CPP formation in our sham animals indicates that amitriptyline did not have a rewarding effect or produce a CPP in animals not experiencing pain, which is consistent with previous reports on amitriptyline conditioning in naïve animals (Subhan et al., 2000; Tzschentke, 2007). In addition to the known effects of amitriptyline within the CPP paradigm, previous studies have also shown that when administered intraperitoneally to naive rats, amitriptyline increased levels of TNF in several brain regions (Ignatowski et al., 2005; Reynolds

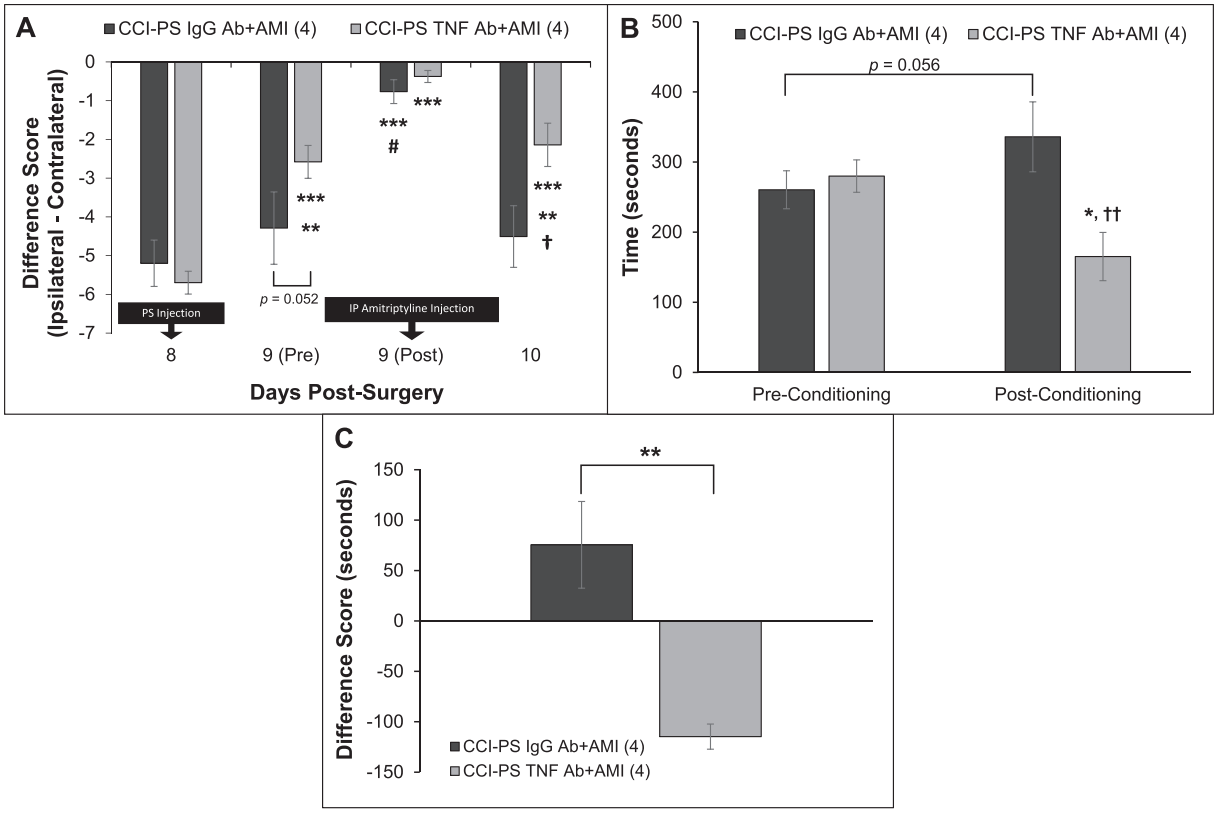

Fig. 6. Perispinal (PS) TNF antibody (ab) injection alleviates (A) thermal hyperalgesia and (B, C) perception of pain in male CCI rats. (A) PS ab injection was on day-8 post-CCI, and thermal measurements were taken the following morning and again following 30 min post-amitriptyline (AMI, 10 mg/kg, i.p.). Difference scores are ipsilateral-contralateral average paw withdraw latency values at each time point. Two-way repeated-measures ANOVA indicated a main effect of Day $F(3,18) = 53.256$, $p < 0.001$ and an interaction between Treatment and Day $F(3,18) = 5.527$, $p = 0.007$, but no main effect for Treatment $F(1,18) = 2.372$, $p = 0.174$. Student-Newman-Keuls post hoc analysis determined: ***$p < 0.001$ compared to Day 8 values within treatment groups; **$p < 0.01$ vs CCI-PS TNF-ab + AMI Day 9(Post); #$p < 0.001$ vs CCI-PS IgG ab + AMI Day 9 (Pre) and Day 10; †$p < 0.05$ vs CCI-PS IgG ab + AMI Day 10. (B) Rats with TNF Ab or IgG control Ab p.s. injection were saline-paired with their preferred chamber and AMI-paired in their non-preferred chamber. Bars represent the time spent in the non-preferred chamber. Two-way repeated-measures ANOVA indicated that while neither main effect was significant: Treatment $F(1,6) = 2.915$, $p = 0.139$ and Day $F(1,6) = 0.754$, $p = 0.419$, there was a significant interaction between Treatment and Day $F(1,6) = 17.717$, $p = 0.006$. Student-Newman-Keuls post hoc analysis determined: *$p < 0.05$ vs respective Pre-Conditioning value; ††$p < 0.01$ vs CCI-PS IgG ab + AMI Post-Conditioning value. (C) Data is difference in time spent in the AMI-paired chamber, pre- and post-AMI pairing. Two-tailed *t*-test: **$p < 0.01$ (number of animals in parentheses).

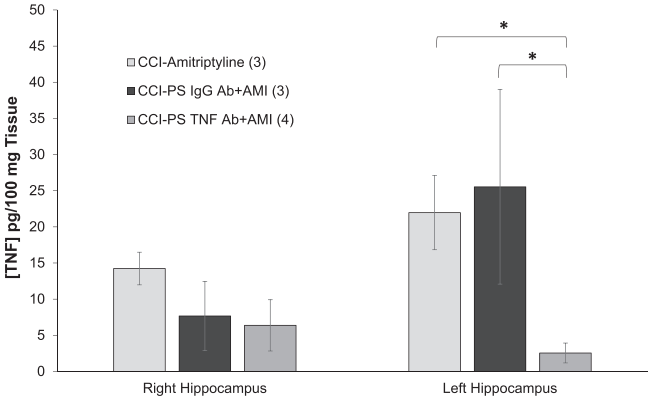

Fig. 7. TNF levels within the right (ipsilateral) and left (contralateral) hippocampi. Comparison of TNF levels in the right and left hippocampus collected following sacrifice on day-10 post surgery. Two-way ANOVA analysis indicated no main effect of Region $F(1,14) = 2.283$, $p = 0.153$ and no interaction between Treatment and Region $F(2,14) = 1.804$, $p = 0.201$. However, there was a non-significant, yet apparent, effect of Treatment $F(2,14) = 3.549$, $p = 0.057$, which with Student-Newman-Keuls post hoc analysis determined: *$p < 0.05$ vs CCI-PS TNF ab + AMI within the left hippocampus. There was also a trend in right hippocampus vs left hippocampus TNF levels within the CCI-PS IgG ab + AMI treated animals ($p = 0.059$) (rat number is in parentheses).

et al., 2004). Since elevated TNF levels in the brain induce the development of thermal hyperalgesia in naïve animals (Ignatowski et al., 1999; Martuscello et al., 2012), taken together, this may explain the slight, albeit significant, amitriptyline-induced nociceptive effect in sham animals noted on day 10 (Fig. 4A).

In order to assess pain perception following p.s. TNF antibody treatment, which was given on day 8 post-surgery, an indirect CPP method was implemented (Navratilova and Porreca, 2014; Navratilova et al., 2013). Amitriptyline was specifically selected as the conditioning drug (injected i.p. on day 9 post-surgery) based on the previously mentioned reports and our data observing the distinct effects amitriptyline has on sham and CCI animals when used as a conditioning agent (Fig. 5). CCI-induced thermal hyperalgesia was significantly decreased in the CCI + p.s. TNF antibody rat group prior to amitriptyline injection, an effect that persisted through day 10 (two days studied Fig. 6A: p.s. TNF ab, Day 8 vs Pre-9; Day 8 vs Day 10). This sustained effect was also observed in female CCI rats, suggesting this treatment is effective for both male and female CCI rats. The p.s. control IgG injection did not affect sensory pain in either male or female CCI rats. CCI rats that received the p.s. IgG only exhibited acute antinociception after receiving amitriptyline on the CPP conditioning day, with pain behaviors returning the next day. Of significance was the finding that CCI rats administered the p.s. TNF antibody did not form an amitriptyline-induced CPP, suggesting that TNF antibody alleviated CCI-induced pain. Conversely, the CCI rats administered p.s. IgG did form a

Table 2
Average caliper measurements of sciatic nerve diameters.

Surgery and Treatment	Sciatic Nerve Averaged Proximal, ligature, and distal values (mm)		Size (mm)		
			Proximal	Ligature	Distal
Sham + Saline (4)	Ipsilateral	1.12 ± 0.04	1.08 ± 0.07	1.15 ± 0.07	1.13 ± 0.07
	Contralateral	1.22 ± 0.04	1.27 ± 0.07	1.17 ± 0.07	1.22 ± 0.07
Sham + Amitriptyline (3)	Ipsilateral	1.07 ± 0.04	1.04 ± 0.08	1.08 ± 0.08	1.10 ± 0.08
	Contralateral	1.16 ± 0.04	1.12 ± 0.08	1.16 ± 0.08	1.19 ± 0.08
CCI + Saline (3)	Ipsilateral	1.50 ± 0.04$^{\#\#\#}$	1.19 ± 0.07	1.71 ± 0.07***	1.61 ± 0.07***
	Contralateral	1.08 ± 0.04	1.09 ± 0.07*	1.10 ± 0.07	1.05 ± 0.07*
CCI + Amitriptyline (5)	Ipsilateral	1.57 ± 0.03$^{\#\#\#}$	1.19 ± 0.06	1.69 ± 0.06***	1.82 ± 0.06***
	Contralateral	1.11 ± 0.03	1.09 ± 0.06**	1.14 ± 0.06	1.11 ± 0.06
CCI + PS TNF-ab + Amitriptyline (4)	Ipsilateral	1.76 ± 0.04$^{\#\#\#}$	1.68 ± 0.07***	1.88 ± 0.07***	1.71 ± 0.07***
	Contralateral	1.26 ± 0.04	1.29 ± 0.07	1.25 ± 0.07	1.24 ± 0.07
CCI + PS IgG-ab + Amitriptyline (4)	Ipsilateral	1.92 ± 0.04$^{\#\#\#}$	1.77 ± 0.07***	2.02 ± 0.07***	1.97 ± 0.07***
	Contralateral	1.18 ± 0.04	1.15 ± 0.07	1.18 ± 0.07	1.20 ± 0.07

Three-way ANOVA with SNK post-hoc analysis: $\#\#\#p < 0.001$ vs Contralateral averaged values, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs Sham + Saline within ipsilateral or contralateral side (proximal, ligatured, or distal).

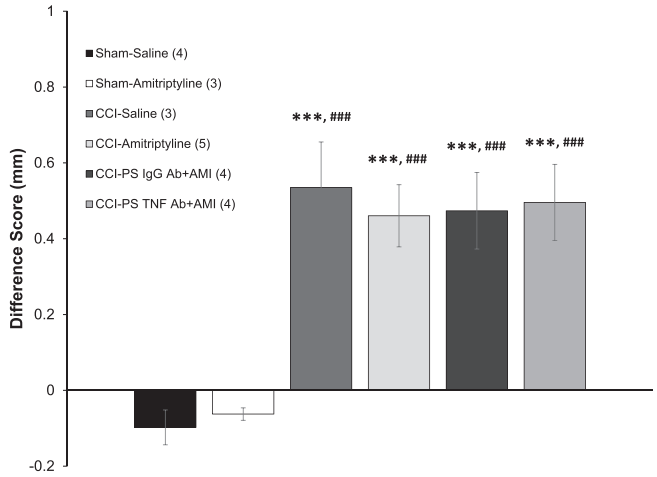

Fig. 8. CCI injury induces edema in the ipsilateral sciatic nerve. Three measurements each were recorded for proximal, ligatured, and distal sections of ipsilateral nerves, and averaged to give one value for nerve diameter; similar measurements were recorded for contralateral nerves. All measurements were on day-10 post-surgery. Data are presented as the difference of averaged ipsilateral (experimental)-averaged contralateral (control) nerve diameter (mm). Each bar is expressed as the mean ± SEM. One-way ANOVA supported statistically significant differences in the mean values among the treatment groups ($p < 0.001$) with Student-Newman-Keuls post hoc analysis revealing: $***p < 0.001$ compared to Sham-Saline animals; $^{\#\#\#}p < 0.001$ compared to Sham-Amitriptyline animals. No statistical significance was found between treatments within the Sham or CCI animals. Note: CCI causes increases in the diameter of the ipsilateral sciatic nerves, which remained unaffected by drug administration. The n for the groups are indicated in parentheses.

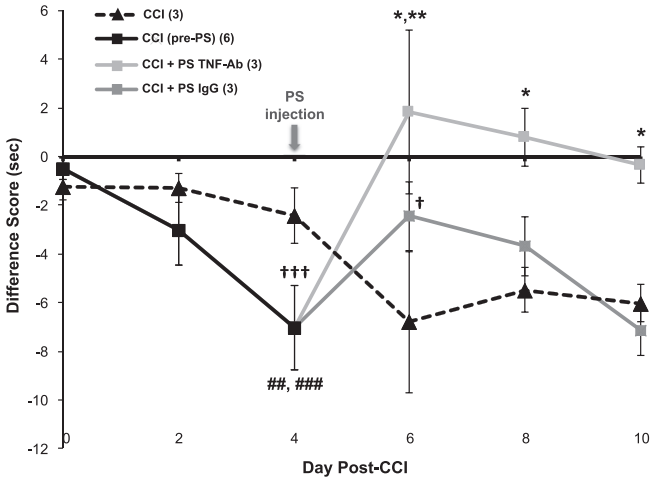

Fig. 9. Perispinal (p.s.) TNF antibody (ab) delivery 4 days following CCI surgery alleviates thermal hyperalgesia in female rats. Onset of thermal hyperalgesia was monitored every other day in female rats that received the CCI injury. Antibodies were administered via p.s. injection on day 4 post-surgery, and thermal measurements continued to be collected until sacrifice on day 10. Difference scores were calculated by subtracting ipsilateral and contralateral average paw withdrawal latency (PWL) values at each time point. Statistical significance was determined using a two-way repeated-measures ANOVA. Prior to p.s. injection on day 4, there was a main effect of Day $F(2,14) = 5.792$, $p = 0.015$, with Student-Newman-Keuls post hoc analysis indicating $^{\#\#}p = 0.01$ vs Day 2 and $^{\#\#\#}p < 0.001$ vs Day 0. In contrast, there was no effect of Group $F(1,14) = 1.546$, $p = 0.254$ and no statistically significant interaction between Group and Day $F(2,14) = 2.658$, $p = 0.105$. After nociceptive behavior testing followed by p.s. injection on day 4, statistical analysis determined there was no significant main effect of Treatment $F(2,18) = 3.686$, $p = 0.090$; in contrast, there was a significant main effect of Day on thermal hyperalgesia $F(3,18) = 5.268$, $p = 0.009$. In addition, a significant interaction was found between the two variables $F(6,18) = 7.634$, $p < 0.001$. Student-Newman-Keuls post hoc analysis determined within the CCI + TNF-Ab group: Day 4, $\dagger\dagger\dagger p < 0.001$ as compared to Days 6, 8, and 10; and within the CCI + IgG group: Day 6, $\dagger p < 0.05$ as compared to Days 4 and 10. Post-hoc analysis for Treatment at Day 6, $**p < 0.01$ CCI + TNF-Ab vs CCI, $*p < 0.05$ CCI + TNF-Ab vs CCI + IgG; Day 8, $*p < 0.05$ CCI + TNF-Ab vs CCI, $p = 0.067$ CCI + TNF-Ab vs CCI + IgG; Day 10, $*p < 0.05$ CCI + TNF-Ab vs CCI and vs CCI + IgG.

preference for the amitriptyline-paired chamber (Fig. 6B and 6C). We previously observed that p.s. injection of etanercept with immediate Trendelenburg positioning completely ameliorated chronic pain of a patient (Tobinick et al., 2014). The present study further demonstrates the sustained antinociceptive effect of p.s. delivery of agents that block central TNF in chronic pain conditions.

It is not surprising that an amitriptyline-induced CPP did not form within the p.s. TNF antibody treated CCI rats, because they were experiencing alleviation of pain (decreased thermal hyperalgesia) prior to amitriptyline conditioning on day 9. In fact, the negative CPP difference score observed for CCI rats treated with p.s. TNF antibody and conditioned with amitriptyline (10 mg/kg) (Fig. 6C) suggests that amitriptyline elicited a conditioned place aversion (CPA). In support, amitriptyline does not produce a CPP in sham animals (Fig. 5A). Both of these findings are consistent with a previous report showing lack of CPP formation in naïve animals (in the absence of pain) conditioned at low doses of amitriptyline (2.5 and 5 mg/kg) and CPA formation occurring at high dose of amitriptyline (10 mg/kg) (Subhan et al., 2000). The additional decrease in stimulus-evoked peripheral hypersensitivity imparted by amitriptyline (Fig. 6A) further indicates a distinction between

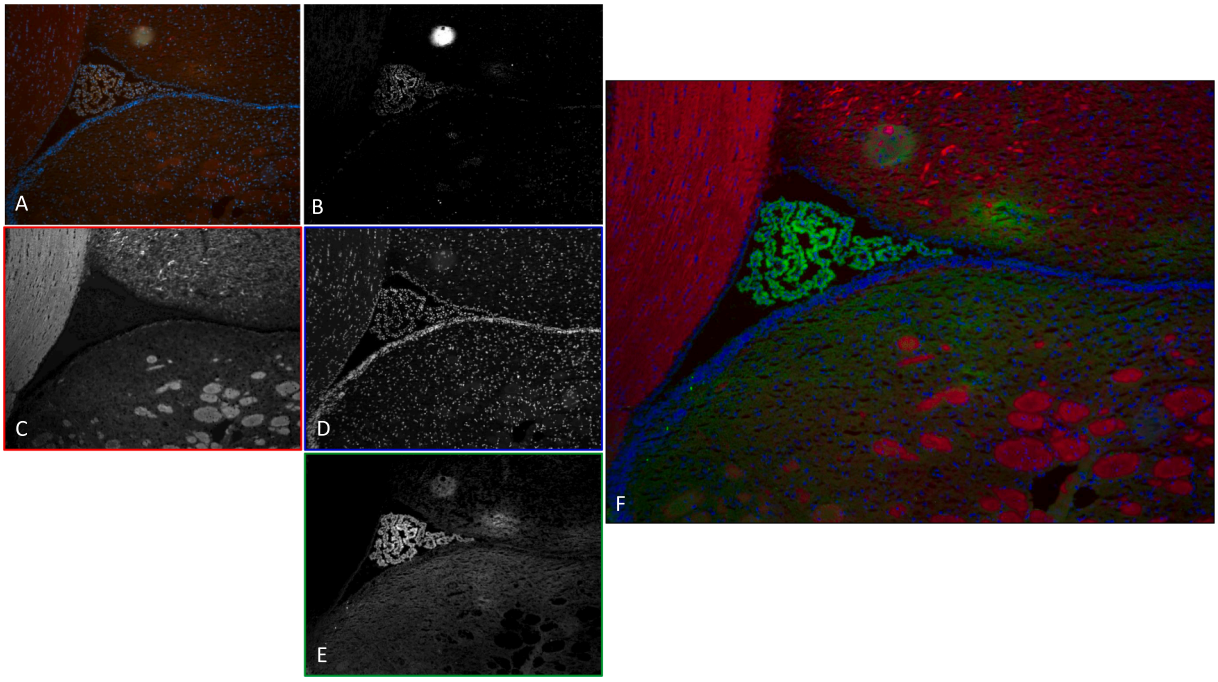

Fig. 10. TNF antibody^{PE} (green) reaches the brain through the choroid plexus after p.s. injection. One hour following p.s. injection, rats were euthanized and brains harvested for imaging. Multispectral imaging of coronal brain section (8 μm, frozen) showing lateral ventricle with choroid plexus from a rat p.s.-injected with TNF antibody^{PE} (green). Panel A shows the RGB (red–green-blue) representation of the data set. Spectral unmixing resulted in the following component signals: (B) Tissue autofluorescence; (C) AF647 signal, which identifies NF-200 labeled neurons and processes; (D) DAPI, which identifies the nuclear signal; and (E) PE signal, which identifies TNF antibody. The border colors of the unmixed images correspond to the pseudocolors used to form the composite image. (F) Triple 10X unmixed composite (C, D, and E): TNF-antibody^{PE} (green), DAPI (blue) and NF-200 (red). Image is representative of three injected rats.

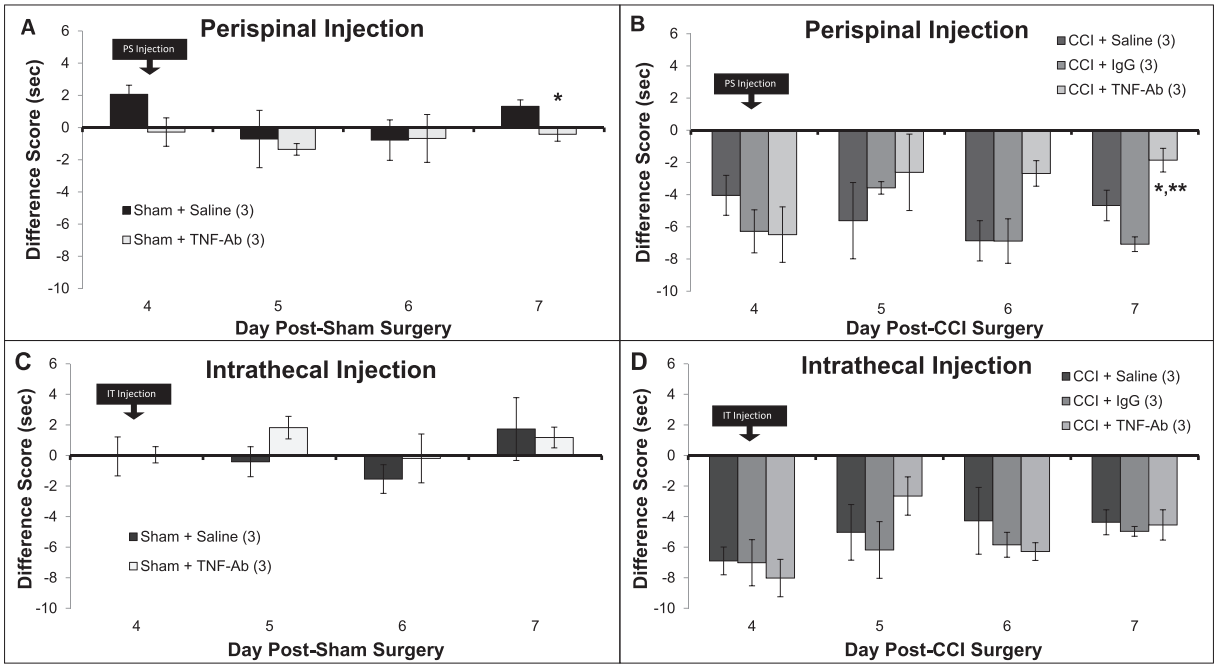

Fig. 11. Perispinal (p.s.), but not intrathecal, TNF antibody (ab) injection alleviates CCI-thermal hyperalgesia. Hyperalgesia on day 4 prior to p.s. (PS) (A, B) or intrathecal (IT) (C, D) injection (on day 4, after pain behavior recording) and on days 5–7, indicated as difference scores of ipsilateral (operated) – contralateral (non-operated) hind paws for saline, control IgG Ab, and TNF-Ab groups. Panels A and C graphs, sham-operated rats; panels B and D graphs, CCI rats. Panel A: One-way ANOVA at each day: *p < 0.05 vs. saline. Panel B: One-way ANOVA at each day: Day 6$F(2,6) = 4.278$, $p = 0.07$; Day 7$F(2,6) = 12.417$, $p = 0.007$. Student-Newman-Keuls post-hoc analysis indicated *$p < 0.05$ vs CCI + saline, **$p < 0.01$ vs. CCI + IgG (number of rats in parentheses). Note: PS injection of TNF-Ab on day 4 in CCI rats appears to trend toward an analgesic effect starting at day 6.

sensory and supraspinal (perception) components of pain, suggesting that by blocking TNF in the brain there was sustained relief of the aversive pain state. A CPP did form in the CCI rats administered p.s. IgG, which was expected since that group was in pain during the conditioning, achieving antinociception only after amitriptyline administration, and then returned back to their pain state on the final experimental day (Fig. 6A).

In response to a nerve injury, TNF overexpression causes dysfunction at regions along the ascending and descending pain pathway, driving the development of peripheral and central sensitization (Christiansen et al., 2018; Dickenson, 2016; Mills et al., 2018). TNF is released at the nerve immediately following the injury, where it contributes to lowering the threshold of nociceptor activation through increased expression of voltage gated sodium channels. The lowered threshold leads to a higher frequency of signals sent to the spinal cord. The combination of increased input from primary afferent fibers and elevated TNF levels leads to increased expression of voltage gated calcium channels at pre-synaptic terminals within the dorsal horn, which allows for increased glutamate release and activation of projection neurons that propagate signals to higher brain centers (Campbell and Meyer, 2006; Christiansen et al., 2018; Dickenson, 2016; Zimmermann, 2001). These initial steps in the development of neuropathic pain promote a state of hyper-excitability and are responsible for peripheral sensitization. High TNF levels are also involved in noradrenergic plasticity sustaining chronic pain (Covey et al., 2000; Ignatowski et al., 1999). The long-term plastic changes and elevated central TNF levels catalyze central sensitization. This is characterized by enhanced facilitative and reduced inhibitory descending input, due to the dysregulation of neurotransmitter systems in brain areas that process afferent nociceptive input and send descending modulatory signals to the spinal cord. This point in the development of neuropathic pain is where the peripheral nervous system displays a “wind-up” mechanism which describes consistent activation of primary afferent fibers sending repetitive excitatory signals to ascending brain regions, while at the same time, the central nervous system operates in a top-down mechanism that amplifies descending excitatory input to the spinal cord and periphery. These two mechanisms work synergistically to create a constant state of hyper-excitability through positive feedback loops that transform persistent pain to chronic pain (Lau and Vaughan, 2014; Millan, 2002; Mills et al., 2018; Staud, 2013; Vanegas and Schaible, 2004; Zhuo, 2017).

There is a general consensus on mechanisms of neuropathic pain states. However, there remains a debate on the ideal treatment and drug target required to restore normal function. We have demonstrated that central TNF levels increase in several brain regions in a time-dependent manner, including in the contralateral hippocampus following CCI (Covey et al., 2000; Covey et al., 2002; Gerard et al., 2015; Ignatowski et al., 1999). We discovered that TNF inhibits norepinephrine release at the level of the hippocampus with enhanced inhibition occurring during neuropathic pain (Covey et al., 2000; Ignatowski et al., 1999; Ignatowski and Spengler, 1994; Ignatowski et al., 2005). Norepinephrine release at higher brain centers activates the descending pain pathway, which culminates in inhibition at the level of the spinal cord of incoming pain signaling. We propose that in pathological pain, the accumulation of TNF in the brain, specifically the hippocampus, causes dysregulation of the normal endogenous analgesic response by preventing activation of the descending inhibitory pain pathway due to TNF-induced inhibition of norepinephrine release. By delivering an anti-TNF antibody to the brain via p.s. injection, we show an apparent decrease in TNF levels in the contralateral hippocampus, which is consistent with the behavioral results showing alleviation of pain and reduction of ongoing, affective pain (Figs. 6 and 7). By blocking TNF activity in the brain, and thus preventing TNF-induced inhibition of norepinephrine release, we predict that the descending pathway is restored. Together with our previous findings, we would expect a return of sympathetic functioning along with re-engagement of descending inhibitory pathway activity (Staud, 2013; Sud et al., 2008). Further studies will need to be conducted to confirm our prediction.

In addition to the favorable effects noted during CCI-induced neuropathic pain following p.s. TNF antibody, another potential benefit of this therapeutic modality is enhanced delivery of the antibody to the brain. It is well-known that CNS delivery of large molecules, such as TNF antibodies, after systemic administration, is largely prevented by the blood–brain barrier. Perispinal delivery, in this model, appears capable of delivering sufficient TNF antibody to the CNS to exert an anti-nociceptive effect and an improvement in pain-related behavior. Further experimentation will be necessary to define the anatomic sites of action. The current experiment suggests that one such site is the hippocampus; a further intriguing possibility is the choroid plexus. To be clear, our data should not be interpreted to imply that the hippocampus is the only anatomic site involved in pain mitigation induced by p.s. injection of an anti-TNF antibody. Corticothalamic and spinothalamic pathways are likely to also be influenced by direct TNF inhibition as a mechanism of pain relief. Further studies investigating these mechanisms are warranted.

These findings establish the antinociceptive effectiveness of a novel and negligibly invasive perispinal TNF antibody injection for both male and female rats, ruling out the possibility of gender specific influences on the ability of this method to alleviate thermal hyperalgesia associated with neuropathic pain. These results also reveal that p.s. targeting of brain TNF rapidly reduces pain and has a sustained antinociceptive effect, successfully and continually blocking peripheral thermal hypersensitivity following a single injection.

Declaration of Competing Interest

The authors have no conflicts of interest to declare, except for Edward Tobinick, who holds multiple U.S. and international patents that cover perispinal administration of etanercept for treatment of selected neurological disorders.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2019.07.036.

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