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Administration of anti-GFAP antibodies increases CGRP expression and increases pain hypersensitivity in spinal cord injured animals

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Georgene W Hergenroeder[1], Samuel T Molina[1] and Juan J Herrera[2]

Abstract

Background: Spinal cord injury (SCI) results in a multitude of cellular and pathological changes including neuronal loss, axonal damage, gliosis, and loss of motor and sensory function. In 40%–70% of patients, SCI can also trigger the development of neuropathic pain. Our previous study demonstrated that SCI patients who developed autoantibodies to glial fibrillary acidic protein (GFAP) were at increased risk for the subsequent development of neuropathic pain. However, whether GFAP autoantibodies (GFAPab) contribute to the development of neuropathic pain after SCI had yet to be examined.

Objective: Using a mid-thoracic contusion model of SCI in male Sprague-Dawley rats, we examined the effect of exogenous anti-GFAP antibodies on SCI pathology, pain-associated molecular changes, and behavior.

Methods: Anti-GFAP or IgG was administered at 7- and 14-days post-injury. Immunohistochemistry was performed to measure the relative levels of calcitonin gene-related peptide (CGRP), and inflammatory proteins in dorsal horn tissue. To assess the development of neuropathic pain, the von Frey test and the Mechanical Conflict-Avoidance Paradigm (MCAP) were performed.

Results: CGRP immunoreactivity was significantly higher in the anti-GFAP-treated injured rats compared to control SCI IgG-treated rats. As anticipated, SCI rats had a lower pain threshold at 1- and 2-months post-injury compared to laminectomy-only controls. However, pain withdrawal threshold was not significantly affected by post-injury administration of the anti-GFAP. Operant testing revealed that SCI rats treated with the anti-GFAP had a trending increase in pain sensitivity.

Conclusion: Taken together, these data suggest that autoantibodies to GFAP following SCI may contribute to developing pain states following SCI.

Keywords

autoantibodies/immunology, calcitonin gene-related peptide, glial fibrillary acidic protein/immunology, neuralgia/etiology, spinal cord injuries/complications

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[1]Vivian L. Smith Department of Neurosurgery, McGovern Medical School at the University of Texas Health Science Center at Houston, Houston, TX, USA
[2]Department of Diagnostic and Interventional Imaging, McGovern Medical School at the University of Texas Health Science Center at Houston, Houston, TX, USA

Corresponding authors:
Georgene W Hergenroeder, Vivian L. Smith Department of Neurosurgery, McGovern Medical School at the University of Texas Health Science Center at Houston, 6431 Fannin Street, MSB 7.156, Houston, TX 77030-1503, USA.
Email: Georgene.W.Hergenroeder@uth.tmc.edu

Juan J Herrera, Department of Diagnostic and Interventional Imaging, McGovern Medical School at the University of Texas Health Science Center at Houston, 6431 Fannin Street, MSE-R166, Houston, TX 77030-1503, USA.
Email: Juan.Herrera@uth.tmc.edu

Introduction

Neuropathic pain is a debilitating secondary consequence of spinal cord injury (SCI) occurring in 40%–70% of patients.[1–5] Unmitigated pain limits physical activity, negatively impacts rehabilitation, infringes on work and social activities, and reduces quality of life.[6] The majority of those with SCI-induced neuropathic pain report that it is a more significant problem than their loss of motor function.[7,8] A growing amount of evidence indicates that autoantibodies to central nervous system proteins are generated after SCI.[9] While SCI triggered autoantibody production has been linked to modulations in electrophysiological function and synaptic transmission,[10–18] their contributions to SCI pathology, especially their role in neuropathic pain, remain largely unknown.[9] In our previous studies, we identified the presence of circulating GFAPab in human SCI patients, the levels of which were highest in SCI patients who subsequently developed chronic neuropathic pain.[15] While these and other studies have implicated GFAPab in the clinical symptomology associated with nervous system injury and disease,[19–21] the causal relationship between elevated GFAPab on secondary pathology and outcome has not been investigated. To investigate the pathological consequence(s) of GFAPab, we utilized a rat mid-thoracic spinal contusion model to examine whether exogenous administration of rat anti-GFAP antibody alters tissue pathology and/or influences development of mechanical allodynia.

Methods

Experimental design

To understand the effect of circulating anti-GFAPab on tissue pathology in the context of spinal cord injury, we conducted a randomized, blinded study of the effects of administration of exogenous rat anti-GFAP antibodies to rats. We administered rat anti-GFAP or rat IgG after midthoracic spinal contusion to reproduce the levels of circulating antibodies previously observed in human SCI patients. Adult male Sprague-Dawley rats were randomized and treated in three groups ($n=12$/group): (1) SCI anti-GFAP-treated (anti-GFAP), (2) SCI isotype control IgG (rat IgG1) (IgG), and (3) surgical sham control (laminectomy only) rats receiving equivalent volume of normal saline (NS) (sham). Rats were observed for 60 days. Immunohistochemistry measured tissue expression of markers of pain, inflammation, and immune responses in spinal cord dorsal horn tissue. To determine which animals developed pain-like behaviors, we performed tactile sensitivity (sensory) and motivational (affective) behavioral assays. The Mechanical Conflict-Avoidance Paradigm (MCAP) measuring motivational behaviors was defined as the primary outcome measure.

Experimental animals

All experiments were in strict compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston (Approval no. AWC22-0008 1/24/22-12/31/24; AWC21-0144 1/19/22-12/31/2024). Males represent 79% of human acute spinal cord injuries.[22] In adult human studies, we did not detect a difference between male and female levels of circulating anti-GFAPab, therefore adult male Sprague-Dawley rats (~225 g, 6–8 weeks old) (Envigo, Inc.) were used for this study. To model SCI, we performed an established thoracic level 10 (T10) contusion injury.[23] Rats were deeply anesthetized (inhaled Isoflurane 4%–5% for induction and 1.5%–2.5% for maintenance) for sham laminectomy, or SCI with a moderate controlled contusion injury (150 kdynes of force, 1 s dwell time) delivered to the exposed cord using an Infinite Horizon Impactor (Precision Systems and Instrumentation, LLC, Lexington, KY). The laminectomy only procedure does not cause injury to the spinal cord. Post-operative care included manual urinary bladder expression, buprenorphine (0.02–0.05 mg/kg) for acute post-operative pain control, and antibiotics (2.5 mg/kg enrofloxacin twice daily for 10 days) to prevent infection. Food and water were available ad libitum. Animals that lost greater than 20% of their original body weight were excluded from the study. No animals lost greater than 20% of their original body weight. Any animal with a Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale score (>1) at 24 h after injury was excluded from the study.[24] Any animals that did not regain weight bearing stepping were excluded from the study. Any animal that did not regain bladder voiding

were excluded from the study. No animals observed were excluded from the study.

Anti-GFAP administration

A rat anti-GFAP IgG antibody was generated in rats using a synthesized peptide (Thermo Scientific Pierce Antibodies). A synthetic 15-mer peptide from the C-terminus of GFAP was used to inoculate rats and the antibody was generated in vivo according to the vendor's approved protocol. ELISA titers were performed by the vendor, and antibody reactivity and specificity were tested by the authors using capillary western blot on initial and final crude sera. The final antibody was affinity purified. This affinity purified anti-GFAP antibody was reported by the vendor as "excellent" on immunohistochemistry. Authors independently confirmed antibody sensitivity and specificity. (Figure 1) IgG isotype control IgG (Rat IgG1 isotype control (Thermo Scientific Pierce Antibodies)) was used as the control agent as human data indicated it is the most prevalent isotype and it lacks specificity for the GFAP target. The specificity and sensitivity of antibodies was verified using immunohistochemistry compared to commercial controls, and using Western blot against both purified recombinant GFAP protein (OriGene, Rockville, MD) and brain tissue. The custom antibody used for therapeutic intervention came from multiple batches, prepared by Pierce Custom Antibodies. Consistency between batches was assessed using ELISA and Western blot and was found to be specific for GFAP protein.

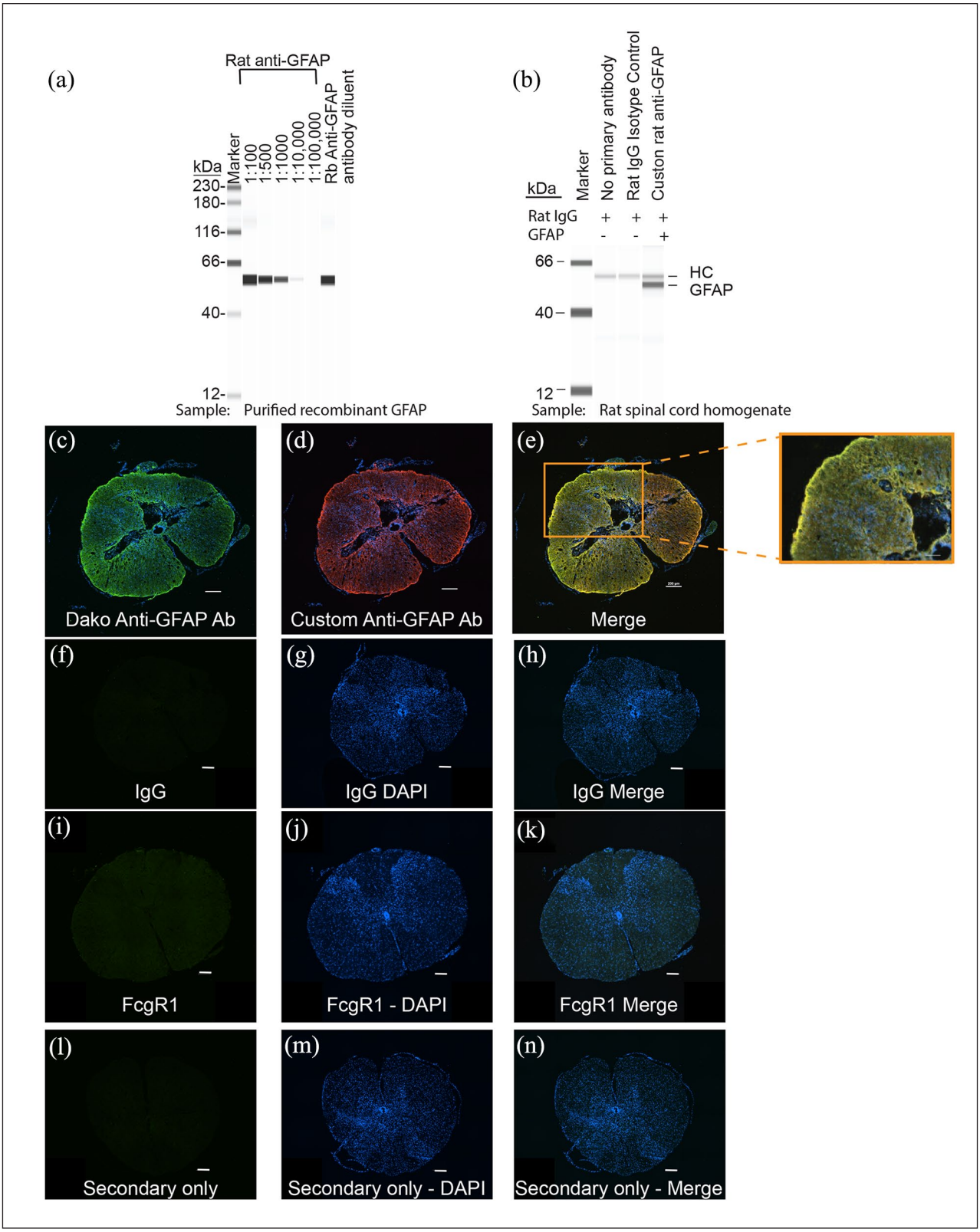
Adult male Sprague-Dawley rats were randomized into one of the following three groups ($n = 12$/group): SCI anti-GFAP-treated group (anti-GFAP), SCI control IgG in normal saline (IgG), and laminectomy sham control rats receiving NS (Sham). An injured saline treated group ($n = 6$) was added for behavioral assessment taken from a separate unpublished study. The animals for this group were age matched and underwent the same surgical procedures, but received a saline IP injection. Randomization to groups was performed using GraphPad random number generator (GraphPad Software, San Diego, CA). The anti-GFAP (0.12 mg/kg) or IgG isotype (0.12 mg/kg) control was administered intravenously via tail vein at 7 days post-injury with booster dose (0.06 mg/kg) at 14 days to mirror the previously observed level of GFAP autoantibodies in humans[15] and the 5–8-day half-life of IgG in rats.[25] Due to weight-based dosing, volumes infused of the study agent ranged from 274.8 to 326.4 ul for the first dose and 157.8 to 186 ul for the booster dose. Investigators who administered the study agent, performed the behavioral analysis, and assessed the tissue immunoreactivity were blinded to group assignment by use of coded agents.

Plasma analysis

Plasma was analyzed for immunoreactivity to GFAP at baseline (pre-SCI), day 7 post-SCI pre-study antibody administration, day 14 (7 days after antibody administration and pre-booster), day 28 (7 days post-booster), day 42, and day 56 in anti-GFAP and IgG treated rats ($n = 12$/group). Levels were measured by capillary western blot and antigen competition studies confirmed specificity of immunoreactivity to GFAP protein.[15] Capillary westerns were conducted using a JESS system, essentially as described by the vendor (ProteinSimple, San Jose, CA). Purified recombinant GFAP protein (142 ng/µl) was denatured in a lithium dodecyl sulfate (LDS)-containing buffer including fluorescent standards to control for differences in separation across capillaries. Rat plasma samples were diluted 1–100 and used as the primary antibody. Each assay included positive controls consisting of a commercial anti-GFAP antibody and a negative control in which the primary antibody was not added. A standard curve was generated by serial dilution of purified recombinant protein for between-assay comparison. Peak detection, area under the curve (AUC) and the signal-to-noise ratio were calculated using Compass software (ProteinSimple, San Jose, CA). The measurements of AUC, expressed in GFAP ng/ul, was used for comparison across groups.

Behavioral testing—mechanical sensitivity and affective components of pain

Von Frey Assay. To test the development of tactile allodynia (i.e. forepaw and hindpaw sensitivity to mechanical stimulation), animals (sham $n = 6$, IgG $n = 11$, anti-GFAP $n = 11$, saline $n = 6$) were assayed with von Frey monofilaments (electronic Von Frey aesthesiometer, IITC Life Science, Inc, Woodlands Hills, CA) at 1- and 2-months post-injury. Rats'


(a)
Rat anti-GFAP
kDa
Marker
1:100
1:500
1:1000
1:10,000
1:100,000
Rb Anti-GFAP antibody diluent
230-
180-
116-
66-
40-
12-
Sample: Purified recombinant GFAP
(b)
kDa
Marker
No primary antibody
Rat IgG Isotype Control
Custon rat anti-GFAP
Rat IgG + + +
GFAP - - +
66
40
12
HC
GFAP
Sample: Rat spinal cord homogenate
(c)
Dako Anti-GFAP Ab
(d)
Custom Anti-GFAP Ab
(e)
Merge
(f)
IgG
(g)
IgG DAPI
(h)
IgG Merge
(i)
FcgR1
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FcgR1 - DAPI
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Figure 1. (a) Capillary western blot of purified recombinant GFAP protein probed with serial dilutions of the custom rat anti-GFAP antibody and a commercial rabbit anti-GFAP antibody showing immunoreactivity to the 50 kDa GFAP protein and no immunoreactivity when antibody is absent (antibody diluent). (b) When the complex mixture of rat spinal cord homogenate was probed with anti-rat IgG (*H* + *L*) horseradish peroxidase (no primary antibody), as expected a weak band is shown, presumed heavy chain IgG (HC). When the non-targeting rat IgG isotype control was used as the primary antibody, the weak band displays, but no additional band is seen. However, the custom rat anti-GFAP antibody shows a strong GFAP band (GFAP) not seen in the other two lanes. Antibodies were tested on both injured and uninjured (not shown) spinal cord tissue. (c) Representative images of commercial DAKO rabbit anti-GFAP antibody (green). (d) Custom Rat anti-GFAP antibody (red). (e) Merged image (yellow) with magnification. The custom generated anti-GFAP antibody generates the same labeling pattern and intensity as the commercially available antibody. (f–h) The IgG control shows no labeling indicating a control for the study. (i–k) Spinal cord probed with anti-CD64/FcgR1 antibody did not detect Fc gamma receptor 1 (FcγR1) on the spinal cord tissue. Anti-CD64/FcgR1 from two different companies was tested with similar results. (l–n) No signal was detected when no primary, only secondary was used. (DAPI is shown in blue as a histological marker.).

withdrawal thresholds were measured sequentially in the same setting at the same time. The rats were placed on an elevated wire mesh in clear cages and habituated for 20 min before testing. The plantar surface of hind paws was probed with increasing intensity with a von Frey monofilament. The force required for paw withdrawal was recorded. After obtaining the threshold measurement, the next rat was measured to allow a break between measurements for each rat and assure that each rat was poked the same number of times. Five scores for each paw were recorded, the three scores closest to the median for each paw were averaged to arrive at the rat's withdrawal threshold. To determine whether animals are experiencing pain at a given time point, the average of the pressure values were compared to the baseline scores which were recorded prior to injury.[26] Only weight bearing rats were included in analysis.

Mechanical conflict avoidance paradigm (MCAP). The Coy Mechanical Conflict-Avoidance System (Coy Labs, Grass Lake, Michigan) was used to assess both evoked pain and anxiety, depending upon whether sharp (but not injurious) probes were raised in a walkway that the animal traverses to avoid bright light. The Coy box has a sequence of three chambers. A rat was placed in a closed-end chamber containing a bright light (aversive stimuli) that was illuminated 30 s after placement. The chamber door to a long walkway that connects the bright chamber to the dark chamber was opened at the 30 s time point. The rat's movements within the three chambers were recorded for three more minutes. At 2-months post-injury the first introductory trials were run without the probes being raised (0 mm probe height) followed by testing with raised probes (4 mm). Probe heights ranged from 1 mm (not painful) up to 4 mm (painful). Tests were separated by 15–30 min intervals. Decreased time on the probes, decreased crossing of the walkway, and longer latencies to step onto the probes indicate enhancement of evoked pain. Tests were performed only on rats exhibiting adequate plantar placement of their hind paws and stepping behavior.[27] As part of testing for adequate plantar placement, open-field locomotor testing compared to baseline was tested and no differences were noted between treatment groups (data not shown). The number of crosses between the light and dark compartments were recorded. MCAP measuring motivational behaviors was defined as the primary outcome measure, a priori sample size for the assay was based on published studies (sham $n = 8$, IgG $n = 11$, anti-GFAP $n = 11$, saline $n = 6$).[27–30]

Immunohistochemistry—tissue expression of markers of pain, inflammation and immune response

Tissue sections ($n = 12$/animal) from rostral and caudal areas across 1 cm of the cord including the injury epicenter were analyzed. For each animal (sham $n = 3$, IgG $n = 6$, anti-GFAP $n = 6$) the area of the spinal cord lesion was located and the spinal cord was cut approximately 2 mm rostral and 2 mm caudal to the lesion. The tissue was sectioned into 20 µm sections rostral to caudal. Sections were selected at random from across the area representing T9 or T11. The sections were just outside the lesion cavity. The saline animals were not processed since there was no behavioral difference between the IgG treated and saline groups. We double-stained the sections for multiple markers using primary antibodies (Table 1). To visualize non-myelinated primary afferents and active sensory neurons in the dorsal horn

Table 1. Antibodies used.

Antibody name	Catalog number	Company
Complement component 3 (C3)	ab200999	Abcam
Calcitonin gene-related peptide (CGRP)	NB2-88945	Novus biological
FC gamma receptor I (FCGR1/CD64)	10256-RP02 ab203349	Sino biological Abcam
Glial fibrillary acidic protein (GFAP)	MAB360M1	Millipore sigma
Ionized calcium-binding adapter molecule 1 (IBA-1)	NC9288364	Wako chemicals
Neurokinin 1 receptor (NK1R)	NB300101	Novus biological
Signal transducer and activator of transcription 3 (STAT3)	ab119352	Abcam
Alexa Fluor 568 Goat anti-Mouse IgG ($H+L$)	A11031	Invitrogen
Alexa Fluor Plus 488 Goat anti-Rabbit IgG ($H+L$)	A32731	Invitrogen
Goat anti-Mouse secondary HRP	042-205	Protein simple
Goat anti-Rat IgG ($H+L$)	PI-9400-1	Vector
Rat IgG1 kappa Isotype Control	16-4301-81	Invitrogen

lamina we used calcitonin gene-related peptide (CGRP, cat. no. NB036540, Novus Biologicals, Centennial, CO) and neurokinin 1 receptor, a receptor activated by Substance P (NK1R, cat. no. NB300101, Novus Biologicals, Centennial, CO). We visualized microglia and astrocytes using ionized calcium-binding adapter molecule 1 (IBA-1, cat. no. 019-19741, FUJIFILM Wako Chemicals, Richmond, VA) and glial fibrillary acidic protein (GFAP, cat. no. MAB360, Millipore Sigma, Burlington, MA), respectively. Astrocytes were stratified into A1 and A2 subtypes by their expression of complement component 3 (C3, ab200999, Abcam, Cambridge, MA) and signal transducer and activator of transcription 3 (STAT3, ab119352, Abcam, Cambridge, MA), respectively. Primary antibodies were diluted 1:1000 in blocking solution (1X PBS containing 5% goat serum and 0.3% Triton X-100) and incubated overnight at 4°C followed by species-specific secondary antibodies for 2 h at room temperature. The tissue sections were viewed and images were captured using a Nikon Eclipse Ni-E microscope (Nikon Instruments, Melville, NY). No specific signals were observed when the primary antibodies were removed from the incubation mixture. The investigator capturing the images was blinded to rat group assignment. The relative fluorescence intensities of CGRP-, NK1R-, GFAP-, IBA-1-, STAT3-, and C3-labeled tissue were measured in the dorsal horn area. A circular region of interest (ROI) of consistent size was drawn around each dorsal horn for all sections and the fluorescence intensities were quantified as a percent of the ROI using ImagePro Plus software (Media Cybernetics, Inc., Silver Spring, MD). The percentage of the ROI for each image was determined and averaged for each rat's sections. The relative threshold levels were determined from control sections measuring positive staining (excluding background fluorescence) which was maintained across all treatment groups. The fluorescence levels were exported to SigmaPlot 11.0 (SysStat Software, Inc.) for statistical analysis.

Statistical analysis

Statistical differences between the plasma levels of immunoreactivity to GFAP in the anti-GFAP, IgG-treated spinal cord injured animals and laminectomy sham groups were tested using a two-way analysis of variance (ANOVA) for repeated measures followed by Pairwise Multiple Comparison Procedures using the Holm-Sidak method. One-way ANOVA using SigmaPlot 11.0 software (Systat Software, Inc, San Jose, CA) was used to compare the three groups' tissue expression of proteins which are displayed as the group mean percentage of ROI. For behavior, repeated measures two-way analysis of variance with Pairwise Multiple Comparison procedures using the Holm-Sidak method was performed for the MCAP test and a one-way ANOVA was performed for the von Frey test. p values less than 0.05 were considered significant.

Results

GFAPab administration increases plasma immunoreactivity to GFAP in a dose-dependent manner

Prior to administration of the GFAPab to SCI rats, we confirmed its specificity using capillary westerns and immunohistochemistry. The results presented in Figure 1(a) show that when purified recombinant GFAP protein was used as the sample source, both the custom anti-rat GFAPab to be used for animal administration and a commercial GFAP

antibody used as a positive control showed strong immunoreactivity to the ~50 kDa GFAP protein. This effect was not seen when normal rat IgG was used as the primary antibody. When the complex mixture of rat spinal cord homogenate was probed with anti-rat IgG ($H+L$) horseradish peroxidase (no primary antibody), as expected, a weak band is shown, presumed heavy chain IgG (HC). When the non-targeting rat IgG isotype control was used as the primary antibody, the weak band displays, but no additional band is seen. However, the custom rat anti-GFAP antibody shows a strong GFAP band (GFAP) not seen in the other two lanes (Figure 1(b)). Likewise, when tested for their ability to recognize GFAP in tissue sections, both the custom anti-rat GFAP antibody and commercial GFAP antibody showed the strong astrocytic staining that is typical of GFAP localization (Figure 1(c–e)). No specific signal was observed when normal rat IgG was used as the primary antibody (Figure 1(f)). No specific staining was observed with Fc gamma receptor 1 (FcγR1, Figure 1(i)) or secondary control (Figure 1(l)).

We next carried out dose finding experiments in order to determine the dose that best reflects the changes in GFAPab levels observed in humans with SCI from our previous study.[15] Groups of SCI rats ($n=6$/group) received either 0.012 or 0.12 mg/kg rat anti-GFAP (or equal amounts of normal rat IgG) administered intravenously at 7 days post-injury. These doses were chosen based on the lower limit of detection of anti-GFAP antibodies identified in plasma of humans with SCI (0.002 mg/kg). An animal equivalent dose (AED) calculated on the basis of body surface area was estimated by the following equation: AED (mg/kg) = human dose (mg/kg) × Km ratio. The Km factor is a correction factor estimated by dividing the average body weight (kg) by body surface area (m^2) (for a 60 kg human Km = 37; for a 0.25 kg rat Km = 6).[31] Maintenance doses of 0.006 mg/kg (for the 0.012 mg/kg group) and 0.06 mg/kg (for the 0.12 mg/kg group) were administered at 14 days post-SCI as the reported half-life for rat IgG in the plasma is ~7 days.[25] The rats tolerated the antibody administration with no apparent study drug-related adverse events such as infection, lack of grooming, loss of appetite, or vocalization to touch. Longitudinal plasma analysis excluded two rodents due to inability to obtain sufficient sample (sham $n=6$, IgG $n=11$, anti-GFAP $n=11$). There was no difference in plasma levels of antibody between the IgG-treated and sham animals. Of note, SCI rats who received control IgG did not demonstrate any increase in immunoreactivity to GFAP at any of the time points or doses tested. While this was not unexpected as our western and immunohistochemistry results indicate that normal rat IgG does not bind GFAP (Figure 1), it does indicate that unlike that seen in humans, the model of SCI used in this study is insufficient to generate elevated endogenous circulating autoantibodies to GFAP. Arevalo-Martin et al. showed SCI patient serum IgG binding to cells expressing GFAP in rat spinal cord tissue sections.[32]

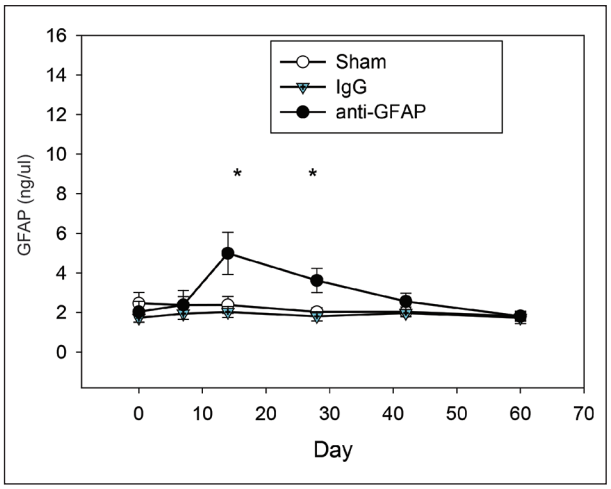

Figure 2. Plasma levels of immunoreactivity to GFAP were significantly higher in the anti-GFAP treated group at day 14 and day 28 compared to IgG-treated and sham saline-treated rats. Those receiving the anti-GFAP showed a mean difference on day 14: 2.97, $t=4.58$, $p<0.001$; and mean difference on day 28: 1.81, $t=2.8$, $p=0.007$ compared to SCI rats receiving normal rat IgG(*). A standard curve was generated by serial dilution of purified recombinant GFAP protein for between-assay comparison, measurements are expressed as ng/ul of GFAP.
GFAP: glial fibrillary acidic protein; IgG: immunoglobulin G.

However, similar to our human subjects plasma immunoreactivity, a dose-dependent increase in GFAP immunoreactivity was observed in rats receiving the anti-GFAP antibody, with those receiving the 0.12 mg/kg dose having a significant and sustained period of GFAP immunoreactivity in their plasma (mean difference on day 14: 2.97, $t=4.58$, $p<0.001$; and mean difference on day 28: 1.81, $t=2.8$, $p=0.007$) compared to SCI rats receiving normal rat IgG (Figure 2). These levels of anti-GFAP immunoreactivity were similar to those seen in our human subjects.[15] The 0.12 mg/kg dose was therefore chosen for all subsequent studies.

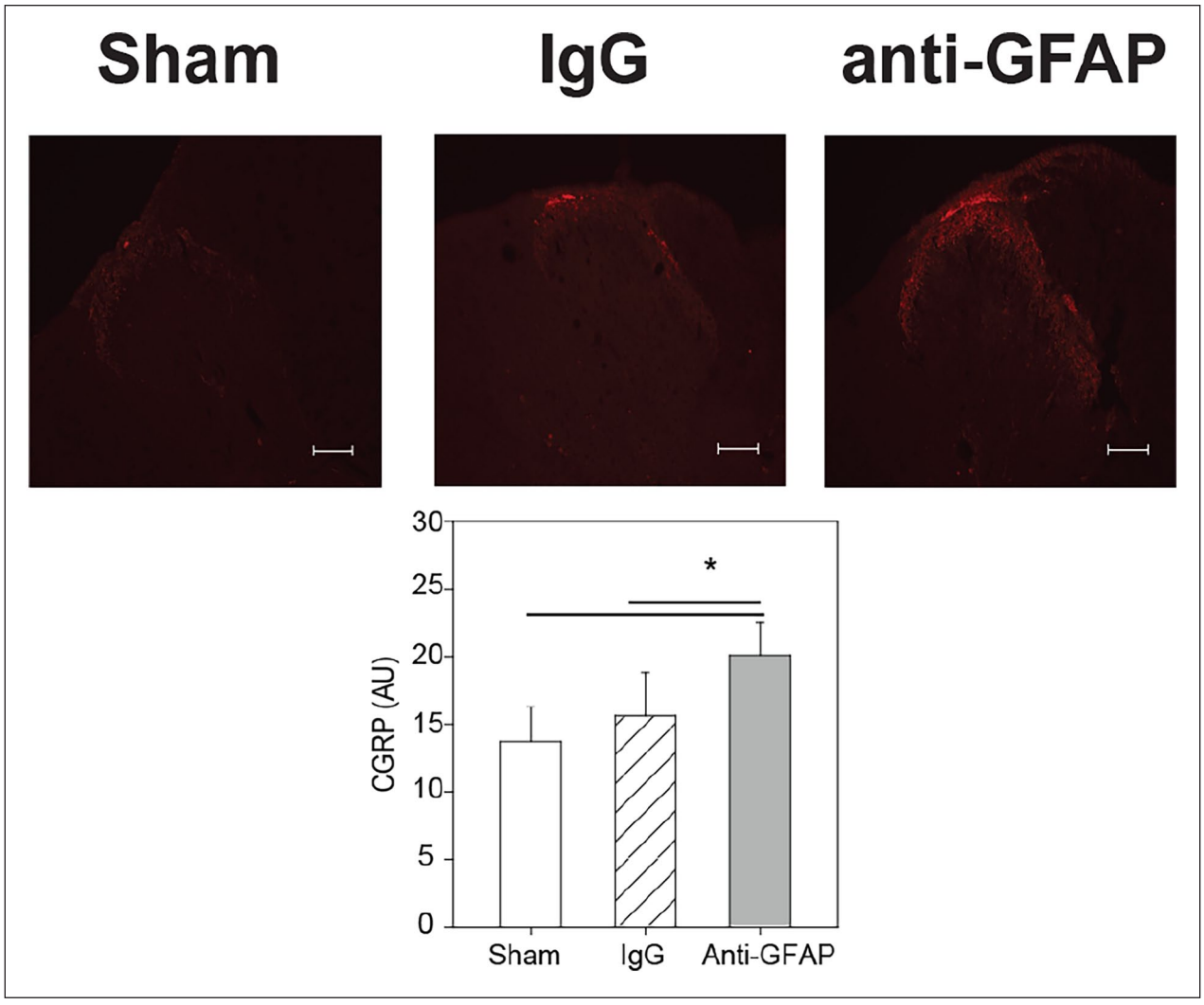

Figure 3. There was a significant difference in mean CGRP expression among the treatment groups ($F(2,12) = 6.519$, $p = 0.012$)(*). Anti-GFAP treated mean CGRP expression ($M = 20.09$, SD = 2.42) was significantly increased compared to both laminectomy-only ($M = 13.71$, SD = 2.555), $p = 0.007$(*), and IgG treated groups ($M = 15.62$, SD = 3.19), $p = 0.017$(*). No significant difference in CGRP expression was determined between the IgG-treated rats and laminectomy-only rats ($p = 0.351$). These results indicate that the anti-GFAP treatment increased expression of CGRP.
CGRP: calcitonin gene-related peptide.

Expression of markers of pain, inflammation, and immune response. There was a significant difference in mean CGRP expression among the treatment groups ($F(2,12) = 6.519$, $p = 0.012$), anti-GFAP treated mean CGRP expression ($M = 20.09$, SD = 2.42) was significantly increased compared to IgG-treated group ($M = 15.62$, SD = 3.19), $p = 0.017$ and laminectomy ($M = 13.71$, SD = 2.56), $p = 0.007$. No significant difference in CGRP expression was determined between the IgG-treated rats and laminectomy-only rats ($p = 0.351$). These results indicate that the anti-GFAP treatment increased expression of CGRP in SCI rats (Figure 3).

No differences were found between anti-GFAP- and IgG-treated SCI rats in the inflammation-related markers STAT3, C3, GFAP, Iba-1, or NK1R (Supplemental Figure 1).

Neuropathic pain assessments. The von Frey test is an assay of mechanical allodynia (i.e. pain threshold) that uses a thin plastic filament applied to the plantar surface of the hind paw in order to determine the force required to stimulate a withdrawal reflex (i.e., the point at which the pressure becomes painful). As previously reported, SCI caused a reduction in pain threshold as indicated by a significant decrease in

the amount of force required to stimulate a withdrawal reflex compared to the laminectomy control rats.[33,34] This was observed at both the 1 and 2 month post-SCI time points (Figure 4(a)). Administration of the rat anti-GFAP antibody did not significantly affect the reduction in pain threshold seen following SCI. We also observed no differences between the anti-GFAP treated and IgG treated SCI rats in the number of ambulatory episodes or total velocity as assessed by computer activity monitoring (data not shown).

The mechanical conflict avoidance paradigm (MCAP) test was conducted at 2-months post-injury. The MCAP tests the cognitive perception of pain by examining the animals' voluntary behavior to remain in an aversive chamber (brightly lit chamber) or to escape to a dark compartment by crossing height-adjustable nociceptive probes.[27] Probes were set at 4 mm and we observed a significant reduction in the number of crosses made by the anti-GFAP treated, but not the IgG-treated SCI rats or the saline-treated SCI rats compared to laminectomy controls, suggesting some pain hypersensitivity in the anti-GFAP treated rats (Figure 4(b)).

Discussion

One of the consequences of damage to the spinal cord is the potential for release of proteins and peptides into the plasma where they can initiate an immune response. Consistent with this, we and others have observed that autoantibodies to human CNS proteins can be found in the plasma of SCI patients, raising the possibility that these autoantibodies may play a role in the progression of disease pathology. Though we have previously reported that elevations in GFAP autoantibodies (GFAPab) are predictive of the subsequent development of neuropathic pain after SCI, whether GFAPab play a causative role in pain development was unknown. GFAP is an intracellular protein, and intracellular autoantigens are generally thought to be inaccessible to autoantibody binding. However, recent studies have begun to demonstrate pathological roles for autoantibodies against intracellular proteins,[35] raising the possibility that GFAPab may contribute to SCI pathology. To address this possibility, anti-GFAP (or control IgG) antibodies were injected systemically into SCI rats in order to reproduce the immunoreactivity levels observed in human patients acutely after injury. We then questioned if enhanced plasma GFAPab levels were associated with histological or behavioral markers of pain. Our study revealed two key findings: (1) anti-GFAP antibody treated SCI rats had elevated expression of CGRP, a marker of pain fibers, in the spinal cord proximal to the injury, and (2) anti-GFAP antibody treated SCI rats displayed a heightened aversion to noxious tactile stimulation compared to the sham rats and a trend toward a difference from the IgG and saline rats, indicating hyperalgesia-like symptomology in response to rat anti-GFAP antibody after SCI compared to the sham rodents.

CGRP is a marker of fine primary afferent fibers, with most being either C-fiber or Aδ-fiber nociceptors. Our histological analysis revealed that post-SCI administration of rat anti-GFAP antibody increased CGRP expression in the dorsal horn of SCI rats compared to SCI rats receiving a non-targeting IgG. Recent evidence indicates that CGRP plays a key role in the development of sensitization, and its upregulation has been observed in conditions associated with inflammation and neuropathic pain.[36,37] For example, SCI studies have shown that the staining density and distribution of CGRP extends deeper (laminae III-IV vs laminae I-II) after SCI in rats with pain, suggesting intraspinal sprouting of afferents.[38,39] Although the mechanism by which anti-GFAP antibody increased CGRP expression is not known at present, anti-GFAP antibody may contribute to an elevated inflammatory state by participating in immune complex formation and/or complement activation that can contribute to enhanced CGRP expression and release.[40,41]

Ankeny et al.[42] injected sera from SCI wild type or B-cell knock out mice, that could not produce antibodies, into the hippocampus of uninjured mice and detected astrocyte hypertrophy and activated microglia in mice receiving SCI wild type sera.[42] They suggested the B-cell dependent pathology after SCI was likely related to an immune complex with neuronal antigens via the activation of complement or microglia/macrophages. Fc receptors on the microglia or macrophages initiate pro-inflammatory pathology. Evidence exists that supports the association of mechanical allodynia induction by IgG binding to the FcγR1 in the setting of experimental nerve injury, rheumatoid arthritis, and cultured DRG neurons.[43–45] A

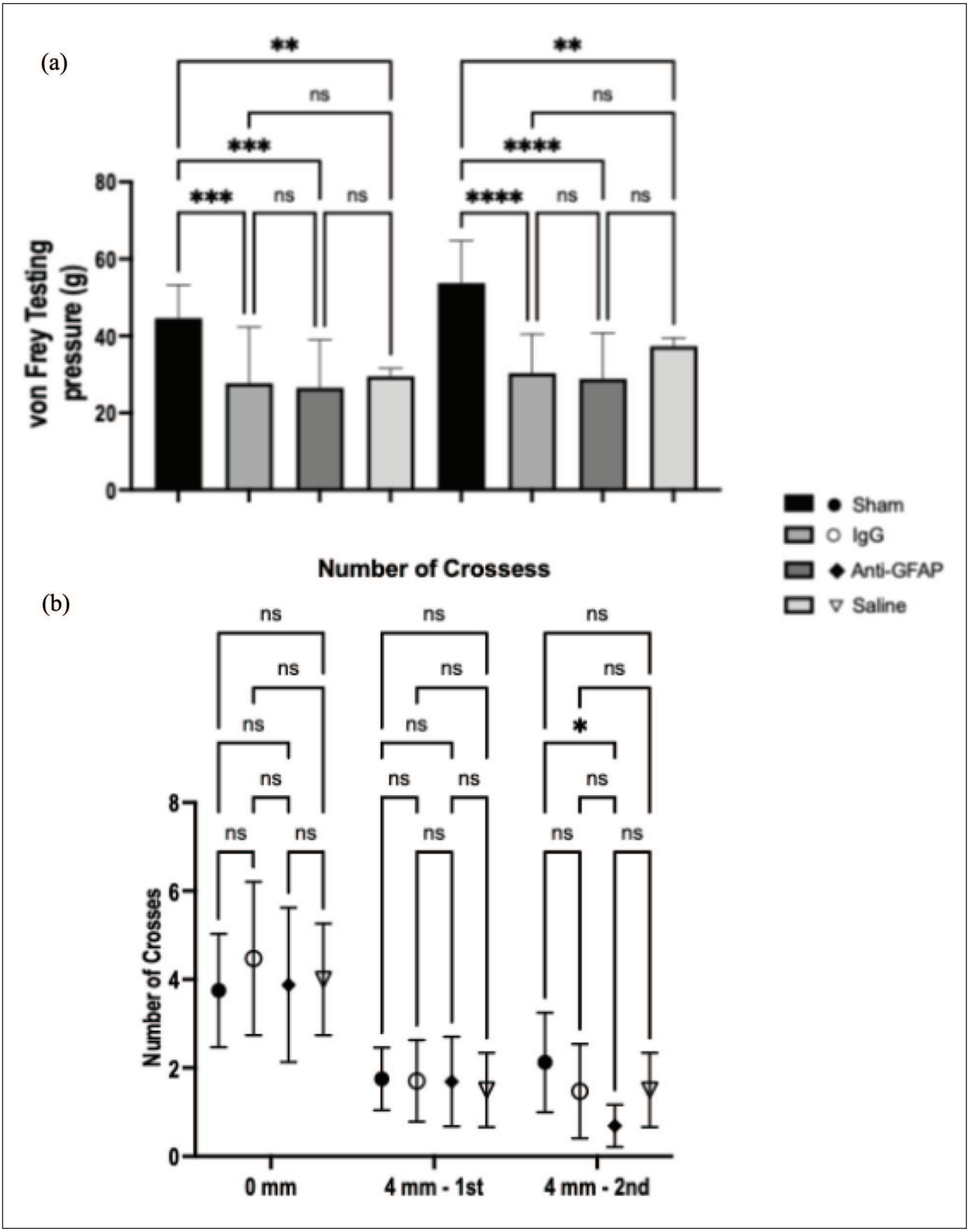

Figure 4. (a) Von Frey assay testing indicated a lower paw withdrawal threshold in the injured groups compared to laminectomy control rats at 1- and 2-month time points. (b) MCAP revealed significantly reduced number of crosses in the anti-GFAP treated rats compared to laminectomy controls at 2 months post-injury (*) indicating avoidance of the crossing, suggesting hyperalgesia. This was not observed in the IgG- or saline-treated animals.

GFAP: glial fibrillary acidic protein; IgG: immunoglobulin G; MCAP: mechanical conflict-avoidance paradigm.

sub-population of small (diameter 10–30 μm) C-type nociceptive DRG neurons that when bathed in an immune complex of IgG bound to an antigen caused an increase in calcium ions, induced depolarization and produced action potentials in some of these small DRG neurons.[44,45] The FcγR1 was shown to be co-expressed with pain-related proteins TRPV1, substance P and CGRP.[45] In addition, a dose-dependent response of plantar-injected immune complex, but not the IgG or antigen alone, induced mechanical hyperalgesia. This mechanical hyperalgesia could be reduced by a high dose (100 ug/ml) of nonspecific rat IgG.[44] While this is a different model, it does provide support for the inability of the small intravenous dose of IgG administered in our study to have had an effect on mechanical allodynia. We cannot eliminate the possibility that an immune complex Fc binding IgG facilitated the development of pain. However, the significant difference in both GFAP and STAT3 immunoreactivity detected on immunohistochemistry (Supplemental Figure 1) in the anti-GFAP rats compared to sham suggests that the anti-GFAP itself induced enough added pro-inflammatory response (higher level of reactive astrocytes) beyond that of the SCI IgG signifying that the FcγR binding was not the only factor. The increase of pro-inflammatory genes accompanying increased levels of GFAP in spinal cord dorsal horn tissue from SCI rats with chronic central neuropathic pain has previously been shown.[46] Astrocytes have been investigated for their contribution to chronic pain states.[47] Furthermore, the increased expression of CGRP into laminae III and IV in the anti-GFAP rats support a pain-related effect of anti-GFAP above that seen in the IgG rats.[38]

We did not detect uninjured rat IgG binding GFAP on rat spinal cord tissue (Figure 1(f)). Arevalo-Martin et al.[32] tested endogenous IgG in intact rat spinal cord tissue and in tissue 1 day post-SCI. Similar to our results, they did not detect endogenous IgG in the intact tissue and no signal was found with rabbit anti-IgG. However, they did detect signal in the injured cord.[32] It is known that IgG deposits into the spinal cord after injury.[48] We utilized rat anti-GFAP IgG to avoid possible interspecies compounding factors. In human SCI, the release of GFAP into the systemic circulation is the proposed event that activates the pathologic immune response that produces autoantibodies to GFAP. The documented relationship between GFAP and pain[46] may be mediated through formation of autoantibody to GFAP generated after the injury and the resultant prolonged inflammatory response. While we did not see differences in microglia and astrocytic responses between the treated groups, we did quantify a significant increase in CGRP expression. This has been shown to correlate with increases in pain states.[38]

Consistent with our human studies, a screen of human SCI and control serum on pooled protein extracts from human spinal cord tissue and rat spinal cord sections by Arevalo-Martin et al.[32] detected autoantibodies to GFAP.[32] They identified IgM and IgG antibodies to GFAP protein in some healthy control, and SCI patient samples as early as 2 days post-injury. They also demonstrated variation from the canonical 5.7–5.8 isoelectric point (pI) and 50 kDa molecular weight of GFAP protein suggesting that the autoantibodies were targeting degraded protein fragments or post-translational modifications of GFAP. After spine trauma, autoantibodies to cellular debris containing GFAP or GFAP breakdown products are likely to have an increase in response to clear that debris.[49,50] Another recent study demonstrated that the plasma from SCI patients binds to dense synaptic areas of spinal cord dorsal horn Rexed laminae II (substantia gelatinosa) and III where CGRP-positive fibers reside.[18] This location of binding suggests there may be a relationship to pain. To test for antibodies to GFAP in plasma, they utilized an indirect immunofluorescent cell-based assay using HEK293 cells transfected with expression plasmid encoding GFAP Homo sapiens transcript variant 1, but they did not detect antibodies against GFAP in serum of those SCI patients. Their inability to detect antibodies to GFAP with their assay may be related to their use of a cell based assay in which normal protein folding occurs, as opposed to our studies (and others) which evaluated immunoreactivity using denatured proteins.[32,50–53] This suggests that proteins in their native conformation may not be useful in the development of autoantibody-based diagnostic assays.

Our direct assessments of pain in response to anti-GFAP antibody exposure after SCI revealed that although pain threshold (determined using the von Frey assay) was not significantly affected by anti-GFAP antibodies, these animals displayed a decreased willingness to encounter noxious mechanical stimuli in the MCAP, suggesting heightened pain hypersensitivity.[29]

The MCAP test is an operant task that reflects the rats' cognitive processing of the effect of

noxious stimuli below the level of injury.[29] The MCAP evaluates mechanical hyperalgesia in SCI rats as measured by the rats' decision to endure noxious stimuli (probes) to cross into the preferred, dark chamber. Probes at a 4 mm height are expected to generate pain in SCI rats experiencing neuropathic pain.[54] At the 4 mm probe height, the anti-GFAP treated rats demonstrated significantly fewer crossings than sham rats, meaning the anti-GFAP treated group chose to endure the aversive stimuli (bright light) rather than traverse over the probes to the dark chamber. The IgG treated rats number of crossings did not differ from those of sham rats. While crossings in our anti-GFAP treated SCI rats did not statistically differ from the IgG-treated SCI rats, we observed a trend toward a decrease between the Anti-GFAP and IgG ($p = 0.12$) treated groups. The difference observed between the Anti-GFAP vs Sham ($p = 0.031$) was enough to determine a significant difference in evoked pain. This suggests the anti-GFAP treatment may have contributed to their hyperalgesia. We speculate that if we had assessed the animals at a more chronic time point we would have observed a statistically significant difference between the three groups. Christensen et al.[55] demonstrated persistent allodynia for 160 days after a T3 spinal cord hemi-section injury using von Frey stimulation that was considered faint (4.41 and 9.41 mN). Other behavioral responses (paw lick, head turns, vocalizations) in response to the mechanical stimuli were more frequent compared to presurgical or sham controls supporting the evidence of paw withdrawals as a pain response.[55] Bedi et al.[56] demonstrated a reduction in threshold for withdrawal for both mechanical and thermal sensitivity that was greater 1 month post-injury and even greater 3–5 months post-injury compared to sham and naïve groups. They identified increased incidence of spontaneous activity in sensory neurons correlated with both thermal and mechanical hypersensitivity. While these studies did not evaluate the MCAP assay, they do indicate persistent pain and heightened sensitivity that increases with time.[56] In a study utilizing IL-10 to reduce mechanical hyperalgesia, rats receiving anti-inflammatory IL-10 spent more time on probes than rats receiving a control vector. When the probes were raised to the 3 mm height, the difference between the groups was more pronounced with half of the vehicle treated rats not crossing at all while all of the IL-10 rats crossed.[29] Those results suggest diminishing neuroimmune activation lessened SCI-related neuropathic pain. Our goal was to test for anti-GFAP antibody contribution to pain, not to test methods of pain reduction. A recent study utilized von Frey testing and the MCAP to evaluate the effects of exercise in reducing pain in SCI rats. They were able to identify a difference in paw withdrawal threshold in SCI rats with and without pain and between exercised rats without pain and both SCI groups. However, they were unable to demonstrate a statistical difference in MCAP average crossing time or escape latency between the SCI and SCI exercised rats.[57]

Our conclusion that GFAPab may exacerbate pain pathologies after SCI is based on both our observation of elevated CGRP expression, and the presentation of hyperalgesia-like behaviors. However, a couple of caveats must be acknowledged. One, as a comparator, we treated SCI rats with a non-targeting rat IgG as a control. Previous studies have found IgG administration after spinal contusion or clip compression injury can have neuroprotective effects.[58,59] In these reports, the authors attributed the improved locomotor function and tissue sparing to IgG-induced attenuation of the inflammatory response (lower spinal cord concentrations of pro-inflammatory IL-1β, IL-6, and MCP-1 and decreased neutrophil infiltration due to complement C3 reduction). Similarly, in comparison to our 0.12 mg/kg dosing, Brennan et al. administered doses of 0.05–2 g/kg of human IVIg (PRIVIGEN, Behring) to mice in an SCI model.[60] FDA approved human treatment doses for IVIg range from 200 mg/kg (for primary humoral immunodeficiency) to 2 g/kg (for chronic inflammatory demyelinating polyneuropathy) supporting their selected doses for immunomodulatory treatment after SCI.[61] Brennan et al compared laminectomy (sham surgery) to contusive SCI mice and utilized the 1 mg/kg dose in a comparison of diffusion tensor imaging (DTI) to evaluate white matter as a measure of myelin preservation. The higher doses of IVIg improved locomotor function and myelin preservation. They saw no improvement of hind limb locomotor performance compared to vehicle controls at doses of 0.05–0.1 g/kg ($p > 0.12$), they also discounted any effect of low doses of IVIg on myelin content and astrogliosis. While we cannot rule out modest protective effects of IgG administration after SCI, it is worth noting

that the neuroprotective IgG doses used in these previous studies were 4000 times higher than the dose used for our IgG control, and whether these high doses of IgG can reduce chronic pain is currently unknown. Two, we did not observe the generation of GFAPab in rats following SCI as we, and others, have reported for human SCI. While the reason for this is not known, it may be related to the position or magnitude of the injury used in the present study. It is important to recognize that not all SCI patients demonstrate GFAPab, suggesting that some injury modalities/conditions may be more prone to generating central nervous system autoantibodies than others. For instance, patients with complete SCI had lower serum IgG levels, below the lower limit of normal reference ranges, for a week post-injury compared to incomplete and vertebral fracture patients. Those complete patients with level of injury above T4 took longer to recover IgG levels to within the normal reference range and were more susceptible to infection.[62] While that study did not find an association with circulating B-lymphocyte levels and they did not measure circulating levels of anti-GFAP antibody, it does suggest that there is potential for injury level and severity-related differences in ability to generate mature antibodies.[63]

Acute levels of serum and CSF GFAP protein are associated with severity of injury in both TBI and SCI.[64–69] Regarding autoantibodies, in TBI patients, those reporting previous TBI had higher levels of anti-GFAP antibodies.[53] There was no correlation with measures of injury severity, but those with previous TBI and loss of consciousness (more severe injury) had higher anti-GFAP antibody levels than those with TBI without loss of consciousness. In our chronic SCI cohort, the two people with the highest levels of anti-GFAP antibodies were in patients who had recently implanted intrathecal pumps suggesting possible spinal cord disruption during the catheter placement caused some release of GFAP protein. It is possible that these repeat exposures may have (re)stimulated autoantibody production. Our previous work and that of Arevalo-Martin et al. observed no association between anti-GFAP autoantibody levels and SCI injury severity, level of lesion, or complete vs. incomplete injury.[15,32] Early timing of anti-GFAP antibody production (2 days post-SCI) led Arevalo-Martin to suggest that those were the result of previous exposure to the antigen. Schwab et al. report higher autoantibody binding in patients with SCI AIS A or B, and association with gabapentinoids.[18] Gabapentinoids are a first-line treatment for neuropathic pain, but are also used as part of a multimodal pain management after spinal cord injury.[70–72] In our patients, we found an association with neuropathic pain with higher levels of anti-GFAP autoantibodies. Davies et al. also identified a relationship to pain with higher levels of autoantibodies to anti-GM1 ganglioside in SCI patients.[14] These studies indicate production of multiple autoantibodies may be related to pain. A limitation of all of these SCI studies with heterogeneous injuries, is that they have relatively small samples sizes and findings need to be validated in larger cohorts.

While the lack of endogenous GFAPab afforded us the opportunity to randomize our rat study groups and test the consequences of anti-GFAP antibody administration, it did not allow us to test if reducing endogenous GFAPab levels could be used as a strategy to mitigate the development of neuropathic pain. If, in the future, a model of SCI in rats is found to be associated with GFAPab production, the contribution of these autoantibodies to persistent inflammation and the development of neuropathic pain could be tested using techniques like plasmapheresis.

There are several limitations to our study. We tested two doses of anti-GFAP antibody 0.012 and 0.12 mg/kg. We received an elevated immune response with the 0.12 mg/kg dose. We elected to proceed with this dose because we saw the plasma elevation and we wanted to keep the control IgG dose to what we anticipated being below a therapeutic effect. It is possible that a higher dose of the ant-GFAP antibody would have shown a significant difference between the injured groups.

We added a rat SCI saline control group to compare the effect of the SCI itself to anti-GFAP. As expected, the saline and IgG control rats' MCAP and von Frey results were nearly identical. Furthermore, our MCAP number of crosses for the IgG rats are consistent with the SCI-only rats tested in Odem et al.[54] Likewise, Detloff et al.[34] indicated that paw withdrawal thresholds using the Up Down method in SCI rodents ($N=6$) with allodynia were a mean of 25.6 ± 1.93 g and non-allodynic threshold 67.16 ± 1.93 g.[34] We also utilized a surgical sham and did not include a naïve animal. The surgical

sham as a control may introduce potential for inflammation and hyperalgesia-like behaviors from the laminectomy and surgical incision.[54] However, we did not observe increases in pain states with the surgical sham group as these withdrawal thresholds have been observed in naïve animals.[54]

There is a possibility that a test antibody (e.g. anti-GFAP) injected may cause an immune response that could contribute to or cause pain. In order to minimize this possibility a rat antibody was chosen. We utilized an isotype control rat IgG1 to identify any adverse effects that are not attributable to interacting with GFAP. This rat IgG1 isotype control (containing its Fc domain) also has the ability to bind to the FcγR. Because both anti-GFAP and IgG groups received a spinal contusion and have the capacity to bind to FcγR, higher immunoreactive signal or pain behaviors observed in the anti-GFAP group are attributed to the anti-GFAP treatment.

Conclusions

We investigated the consequence of anti-GFAP antibodies following SCI in a mid-thoracic spinal contusion model to examine whether exogenous administration of rat anti-GFAP antibody exacerbates tissue pathology and enhances development of mechanical allodynia in rats. Anti-GFAP antibody administration enhanced chronic pain as demonstrated by a reduced number of probe crossings into the preferred chamber in the MCAP assay by the anti-GFAP compared to sham rats. Accompanying these behavioral differences, we observed an increase in CGRP expression in the dorsal horn in anti-GFAP rats. These data provide support for human findings that the presence of GFAPab at 2 weeks post-injury is associated with the subsequent development of chronic neuropathic pain, and suggest that the presence of GFAPab may exacerbate the pain state. However, GFAPab is unlikely to be the only autoantibody contributing to tissue pathology and the pain state. Testing treatments aimed at reducing the circulating levels of GFAPab or a combination of autoantibodies may result in methods to deter the development of chronic neuropathic pain.

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Author contributions

Design of study (GWH, JJH); Obtaining funding (GWH, JJH); Injury model (JJH); Laboratory and behavioral experiments (JJH, GWH, SM); Analysis and/or interpretation of the data (GWH, JJH, SM) Drafting and revising the manuscript (GWH, JJH, SM).

Declaration of conflicting interests

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Ethical considerations

Ethical approval for this study was obtained from the Animal Welfare Committee of the University of Texas Health Science Center at Houston (AWC22-0008; AWC21-0144).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Animal welfare

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation.

ORCID iDs

Georgene W Hergenroeder https://orcid.org/0000-0002-6170-2191
Samuel T Molina https://orcid.org/0009-0006-3737-7073
Juan J Herrera https://orcid.org/0000-0002-8784-0832

Data availability

Data is available by contacting the corresponding authors

Supplemental material

Supplemental material for this article is available online.

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