Autoantibodies produce pain in Complex Regional Pain Syndrome by sensitizing nociceptors

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Running Title: Passive transfer of CRPS pain.

33 pages

Number of Figures: 7

Number of tables: 2

ABSTRACT

Complex regional pain syndrome (CRPS) is a post-traumatic pain condition with an incompletely understood pathophysiological basis. Here, we have examined the cellular basis of pain in CRPS using behavioral and electrophysiological methods in mice treated with IgG from CRPS patients, in combination with a paw incision. Mice were subjected to a hind paw skin-muscle incision alone, or in combination with administration of IgG purified from either healthy control subjects (HC) or patients with persistent CRPS. Nociceptive function was examined behaviorally *in vivo*, and electrophysiologically *in vitro* using skin-nerve preparations to study the major classes of mechanosensitive single units. Administration of IgG from CRPS patients exacerbated and prolonged the post-surgical hypersensitivity to noxious mechanical, cold and heat stimulation, but did not influence tactile sensitivity following a paw incision. Studies of IgG preparations pooled from patient cohorts (n=26-27) show that pathological autoantibodies are present in the wider population of patients with persistent CRPS, and that patients with more severe pain have higher effective autoantibody titre than patients with moderate pain intensity. Electrophysiological investigation of skin-nerve preparations from mice treated with CRPS IgG from a single patient identified both a significantly increased evoked impulse activity in A- and C-nociceptors, and an increased spontaneous impulse rate in the intact saphenous nerve. Our results show that painful hypersensitivity in persistent CRPS is maintained by autoantibodies, which act by sensitizing A- and C-nociceptors.

Introduction

Complex regional pain syndrome (CRPS) is a post-traumatic condition typically confined to a single limb. CRPS is characterized by pain of disproportionate intensity and duration compared to that expected from the clinical time course of the initial injury or trauma, but the etiology and pathophysiological basis of CRPS are incompletely understood[6,18,30,32,45]. Distal limb fractures are the most common triggers of CRPS, but the severity of CRPS is independent of the initiating trauma, and apparently trivial insults can lead to CRPS[36,52]. The affected limb typically exhibits several characteristic abnormalities, including increased sensitivity to both normally innocuous and painful stimuli, skin discoloration, fluctuating temperature asymmetry (compared to the uninjured limb), swelling, sudomotor alterations, and trophic changes to skin, hair and nails. Pain is, however, the dominant symptom and CRPS is not associated with tissue destruction[9,45]. No diagnostic tests exists for CRPS, and patients are diagnosed based on clinical symptoms and signs[10,19,24]. Most patients recover spontaneously, but 15-20% of patients develop persistent severe pain, which may last for life and is associated with an exceptionally low quality of life, even when compared to other chronic pain conditions[33,50]. The characteristic autonomic abnormalities seen early in the course of CRPS typically normalize in patients with long-standing disease, whereas pain, sensory andmotor abnormalities may remain. This has led to suggestions that persistent CRPS is sustained by abnormal central nervous system plasticity[39].

Earlier clinical and experimental investigations suggested a role for humoral factors[2] including autoantibodies[46] in the genesis of CRPS pain. In mice, passive transfer of IgG, but not immunoglobulins of other isotypes, purified from CRPS patients exacerbated and prolonged

mechanical hyperalgesia and edema produced by a paw incision[46]. Importantly, the underlying neuronal mechanisms and sites of action responsible for pain have not been identified.

In this study, we have performed a detailed behavioral and electrophysiological investigation of painful sensory abnormalities in passively transferred CRPS (tCRPS) and identified peripheral sensitization of nociceptors as a major mechanism by which autoantibodies produce pain in CRPS.

Materials and Methods

Study Design

We examined the impact of a paw incision in combination with administration of serum IgG purified from healthy control subjects or CRPS patients on mouse nociception both *in vivo and ex vivo*.

Research subjects

The main immunoglobulin donor was a 40 year old female who had CRPS of 9 years' duration in a lower limb and a high average pain (9-10/10 on a 11-point numeric rating scale (NRS) with 10=the worst pain imaginable) and had been offered clinical plasma exchange treatment on compassionate grounds[2,22]. She had signs in all four Budapest diagnostic categories[24] and alternative causes for her pain had been excluded by a pain specialist, a consultant rheumatologist and a consultant neurologist. This patient clinically exhibited strong pain to mild pressure over the painful area (mechanical hyperalgesia, the most common sensory abnormality in persistent CRPS[19,25]), whereas she had little or no pain to light touch; she reported that

ambient temperatures below room temperature and also temperatures above approximately 24°C would increase her pain. Waste plasma from the first exchange treatment was secured with her consent and was stored frozen – the local Ethics committee confirmed that the use of human waste tissue did not require ethical permission. She received three plasma exchange treatments over 5 days through a central venous line, but unfortunately the treatment had to be ceased thereafter as a consequence of clotting around the tip of the line. She reported no pain relief following this treatment; in line with earlier observations indicating that 7-8 exchange treatments over 3 weeks may be required before meaningful pain relief occurs in this condition[2,22,42].

For experiments on pooled IgG, serum samples were randomly selected, stratified according to either moderate (NRS 5-7) or high (NRS 7.5-9) baseline pain intensity, from frozen samples (-80C) available from participants in the recently-completed LIPS-trial[21]. A research technician (SS) used an anonymized list of all patients' sera (n=111) created by the trial statistician. The LIPS study-inclusion criteria were patients with persistent CRPS of between 1 and 5 years' duration, fulfilling international research criteria for the diagnosis of CRPS[24], who had an average pain intensity at baseline of at least 5/10. Ethical permission and individual consent for the use of these sera for the purpose of autoantibody research is available (12/EE/0164, East of England).

IgG purification

IgG was purified as described previously[46], using protein G beads (Sigma-Aldrich, Gillingham, UK). Briefly, serum was diluted 1:3 with Hartmann's solution, passed through a protein G column, and the bound IgG was eluted using 100 mM glycine pH 2.3, the pH was adjusted to 7.4 using 1

M Tris pH 8. The preparation was then dialyzed overnight at 4°C in Hartmann's solution using a 10 kDa dialysis membrane (Fisher Scientific, Loughborough, UK). The concentration of IgG present after dialysis was determined using a modified Lowry assay (DC protein assay, BioRad, Hemel Hempstead, UK) and adjusted by dilution with Hartmann's solution or by dialysis against a sucrose solution (Sigma-Aldrich). Finally, the IgG solution was sterile filtered using syringe-driven 0.2 µm filter units (Millipore, Watford, UK), stored at 4°C and used within 3 months.

Animals

Behavioral experiments were carried out according to the U.K. Home Office Animal Procedures (1986) Act. All procedures were approved by the King's College London Animal Welfare and Ethical Review Body and conducted under the UK Home Office Project License PPL 70/7510. Experiments were performed on female C57Bl/6J mice (8– 10 weeks old) obtained from Envigo UK Ltd., Bicester, UK, housed in a temperature-controlled environment with a 12h light/dark cycle and with access to food and water *ad libitum*. Mice were injected intraperitoneally with 0.8-16mg of IgG in Hartmann's solution from either healthy control subjects or CRPS patients on 4 consecutive days, or as indicated.

Plantar Incision

On the day of the second IgG injection, a 5 mm long midline incision was made through the plantar skin fascia starting 2 mm from the heel and extending towards the toes, using aseptic techniques. The underlying plantar muscle was elevated with curved forceps and incised

longitudinally, leaving the muscle origin and insertion intact[8]. The skin incision was then closed using sutures (Mersilk 7/0) and the animals were housed on paper bedding for the first 3 days post-surgery.

Behavioral studies

Behavioral experiments were carried out according to the U.K. Home Office Animal Procedures (1986) Act. All procedures were approved by the King's College London Animal Welfare and Ethical Review Body and were conducted under the U.K. Home Office Project License PPL 70/7510. Before any nociceptive testing, mice were kept in their holding cages to acclimatize (10-15 min) to the experimental room. Mice were randomized between cages and the experimenter blinded to their treatment.

The Randall-Selitto paw-pressure test was performed using an Analgesymeter (Ugo-Basile, Italy). The experimenter lightly restrained the mouse and applied a constantly increasing pressure stimulus to the dorsal surface of the hind paw using a blunt conical probe. The nociceptive threshold was defined as the force in grams at which the mouse withdrew its paw[37]. A force cut-off value of 150 g was used to avoid tissue injury.

Tactile sensitivity was assessed using von Frey filaments (0.008-2 g) according to Chaplan's up-down method[12]. Animals were placed in a Perspex chamber with a metal grid floor allowing access to their plantar surface and allowed to acclimatize prior to the start of the experiment. The von Frey filaments were applied to the plantar surface of the hind paw with enough force to allow the filament to bend, and held static for approximately 2-3 s. The stimulus was repeated up to 5 times at intervals of several seconds, allowing for resolution of any behavioral responses

to previous stimuli. A positive response was noted if the paw was sharply withdrawn in response to filament application or if the mouse flinched upon removal of the filament. Any movement of the mouse, such as walking or grooming, was deemed an unclear response, and in such cases the stimulus was repeated. If no response was noted a higher force hair was tested and the filament producing a positive response recorded as the threshold.

Thermal sensitivity was assessed using a hot- and cold-plate (Ugo Basile, Milan). Paw withdrawal latencies were determined with the plate set at a chosen temperature (50 °C for hot-plate and 10 °C for cold-plate tests). The animals were lightly restrained (scruffed) and each hind paw in turn was placed onto the surface of the plate[1,17]. The latency to withdrawal of the paw was taken as the endpoint and recorded for the ipsilateral and the contralateral paw. A maximum cut-off of 30 seconds was used for each paw.

Human IgG ELISA

Human IgG ELISA Kit ab195215 (Abcam, Cambridge, UK) was used to measure the transferred human-IgG concentration in mouse plasma. Plasma was prepared from whole blood using heparin treated tubes and stored at -20°C until use according to the provided instructions from the manufacturer.

Skin-nerve recording

Mice were killed by cervical dislocation and the hind paw was shaved prior to dissection of the isolated skin-nerve preparation. The saphenous nerve and the shaved skin of the hind limb were placed in a recording chamber at 32 °C. The chamber was perfused with a gassed (95% O_2 and 5% CO_2) prewarmed synthetic interstitial fluid (SIF): 108mM NaCl, 3.5mM KCl, 0.7mM $MgSO_4$,

26.2mM $NaCO_3$, 1.65mM NaH_2PO_4, 1.53mM $CaCl_2$, 9.6mM sodium gluconate, 5.55mM glucose and 7.6mM sucrose. The skin was placed inside up (corium side up) and pinned down using insect pins (0.2 mm diameter) in the organ bath to allow access to the receptive fields. The saphenous nerve was placed through a small gap from the organ bath to an adjacent recording chamber on a mirror platform. The desheathed saphenous nerve was covered with paraffin oil for electrical isolation and either whole nerve or dissected fine nerve filaments were placed on a fine gold wire-recording electrode using a microscope[38,54].

Conduction velocity

The saphenous nerve was divided into progressively thinner filaments until a single unit could be isolated in response to mechanical stimulation of the receptive field with a glass rod. Identified units were electrically stimulated (Digitimer DS2, Digitimer Ltd, Welwyn Garden City, UK) and the action potential latency was used to determine the conduction velocity and to categorize units as Aβ (velocity>10m/s), Aδ (1.2<velocity<10m/s) or C-fibers (0<velocity<1.2m/s).

Mechanical stimulation

A computer-controlled stimulating probe, equipped with a force transducer, was used to deliver mechanical stimuli to the most sensitive spot of a receptive field (Axolent UG, Erlangen, Germany). The mechanical threshold for each unit was then determined iteratively by applying a series of 2s mechanical force steps. The mechanical threshold was determined as the force required to elicit at least two action potentials. The adaptation properties of single units were characterized by stimulation with 10s force step challenges in the range 0.5-20g (allowing a 2 min recovery period between challenges). Aβ units that fired briefly during the application and

removal of the force were considered rapidly adapting (RA), and those that sustained firing throughout the challenge were classified as slowly adapting (SA). Low-threshold Aδ units with a transient action potential discharge pattern (on and off) were identified as D-hair fibers. The coding properties of Aδ and C-fibers were additionally examined using a series of force ramp stimuli (0.5-20g, 15s duration, with a 2 min recovery period between challenges). Recording and analysis were done using Spike 2 (Cambridge Electronic Design, Cambridge, UK).

Statistical analysis

Data are expressed as mean ± SEM of the number of animals or nerve fibers indicated (n). Behavioral data were analyzed using unpaired t-test, Mann-Whitney U test (when Levene's test of equality of variances $p<0.05$) or ANOVA (followed by Tukey's, Dunnett's or Holm-Sidak's) as appropriate. Statistical tests were performed in SPSS 24 (IBM), Statistica (Tibco), SigmaPlot 14, or Excel 2016 (Microsoft).

Results

Passive transfer of CRPS pain (tCRPS)

We examined the behavioral phenotype of tCRPS by comparing mice subjected to a paw incision alone with mice that additionally received IgG from a CRPS patient or a healthy control subject (HC) on 4 consecutive days (8mg by intraperitoneal injection), starting the day before paw incision. In these experiments, we used IgG from a 40-year old female patient who had CRPS of 9 years' duration, with high average pain scores (9-10 on a 11 point numerical rating scale), since this may indicate a high titre of pathogenic autoantibodies[15]. Following the paw incision, mice

in all treatment groups developed hypersensitivity to noxious mechanical stimulation in the paw pressure test (Fig. 1, A and B), and to tactile stimulation with von Frey filaments (Fig. 1, C and D). Mice that had been subjected to a paw incision alone or in combination with IgG from control subjects recovered fully from the postsurgical mechanical hyperalgesia measured by paw pressure thresholds within three days, whereas tCRPS mice retained the maximal level of hypersensitivity without signs of recovery during the assessment period. In contrast, all three treatment groups recovered from the postoperative tactile allodynia assessed with von Frey filaments at a similar rate. The mechanical sensitivities of the uninjured, contralateral paws were unaffected, or minimally affected (Fig. 1, B and D), confirming that both circulating CRPS IgG and trauma are required for the onset of tCRPS.

The donor-patient reported increased sensitivities to cold and heat, as is common in CRPS [19,25], and we therefore also assessed thermal nociception using cold and hot plate assays. Mice treated with CRPS IgG progressively developed significant cold and heat hypersensitivities, associated with a significant contralateral sensitization, whereas the other treatment groups displayed less marked changes and recovered quickly (Fig. 1E-H). Our observations indicate that CRPS IgG administration dramatically exacerbates and prolongs painful hypersensitivities produced by a minor surgical trauma and accurately translates the sensory abnormalities observed in the donor patient to mice.

Dose-dependence of CRPS IgG passive transfer

To determine the dose requirements for successful passive transfer of sensory abnormalities, we administered patient IgG at doses between 0.8 to 8 mg on 4 consecutive days, with one group of mice only receiving two injections of 8 mg. In the 4x8 mg group, hypersensitivities to noxious mechanical (Fig. 2A) and cold stimulation (Fig. 2B) were maintained for at least two weeks. Administration of 8 mg CRPS IgG only on the first two days was without effect compared to HC IgG (Fig. 2AB). Lower doses of CRPS-IgG (4x0.8mg, 4x4mg) induced no or only minimal detectable abnormalities to mechanical stimuli (Fig. 2A) whereas administration of 4mg CRPS IgG per day produced an intermediate level of hypersensitivity to noxious cold for the duration of the experiment (Fig. 2B). The contralateral paw displayed a transiently and marginally increased sensitivity to noxious cold or mechanical stimulation in the 4x8mg group, which resolved by day 6 (Fig. 2C, D). We monitored the behavioral profile for a week after the incision but extended this to two weeks for mice treated with 4x8mg CRPS IgG (and the corresponding HC group), since this was the only treatment group that displayed mechanical hypersensitivity.

To determine whether the circulating human IgG concentration was correlated with the observed behavioral phenotype, we measured the plasma concentration of human IgG by ELISA on days 3, 8 and 13 after incision in mice treated with HC-IgG or CRPS-IgG. On the day of the last injection (day 3) the plasma concentration of human IgG was 19±9 mg/ml, similar to that normally found in human subjects (6-16mg/ml). At later time points (day 8 and 13) the circulating human IgG concentration was negligible (2.3±0.3 and 2.6±0.3 ng/ml). Collectively, these results show that induction of the tCRPS sensory phenotype requires doses similar to those used for passive transfer of other autoantibody mediated neurological conditions, such as *myasthenia gravis*[13,47,48]. After induction, the behavioral phenotype of tCRPS is stable for at least two

weeks, a time course that is consistent with the observed terminal half-life of human IgG in mice[41,49].

The tCRPS phenotype reflects the donor patients' pain intensities

To determine whether the sensory phenotype observed in tCRPS mice is related to the donor-patients' pain intensities, we next compared the effects of IgG preparations pooled from patients from the LIPS trial[21]. Patients were stratified according to their baseline pain intensities within moderate (n=26; 6.0±0.8 mean±SD; range 5.0-7.0) and high (n=27; 8.3±0.4; range 7.3-9.5; table 1) ranges on a 11-point numerical rating scale (NRS) [7]. IgG (4x8mg) from patients with severe pain, but not from patients with moderate pain, produced significant mechanical hypersensitivity 7 days after the incision, when the postsurgical hypersensitivity seen in mice that were only subjected to a paw incision had recovered fully (Fig. 3A, see Fig. 2A). This observation indicates that the degree of mechanical hyperalgesia in tCRPS mice reflects the donor patients' spontaneous pain intensities. The different effects of IgG from patients with moderate or high pain may further suggest that target heterogeneity or autoantibody titre influences symptom severity as in other autoantibody-medicated painful conditions[26], or that autoantibodies play no role in the group of patients with only moderate pain. To investigate this further, we examined the effect of a larger dose (16mg/day, Fig. 3B) of IgG from patients with moderate pain intensity and found that this larger dose produced significant hypersensitivity compared to HC IgG (16mg/day). Interestingly, this effect was more transient than that observed in mice treated with IgG (8mg) from patients with higher pain intensity. As the contributions of IgG from individual patients were diluted in these pooled IgG samples (26-27 patients), our results strongly suggest

that autoantibodies are universally responsible for maintaining pain in patients with persistent CRPS. The time required for mice to recover from tCRPS appear variable, with full recovery observed within 2 weeks for the pooled IgG preparations (Fig. 3B), but only a partial recovery with the main patient donor over the same period (Fig. 2).

CRPS IgG induces spontaneous impulse generation in skin-saphenous nerve preparations

Ectopic sensory afferent impulse discharge generates spontaneous pain and paraesthesias in rodents and human patients[43,44,51]. Persistent spontaneous pain is a hallmark of CRPS, and we therefore recorded the activity of the intact saphenous nerve, before splitting the nerve into thin filaments for studies of single units described below. In this configuration, IgG from the main patient donor significantly increased the spontaneous ongoing impulse rate (7.1±2.4Hz) compared to preparations from naïve (0.9±0.4Hz), incision only (1.4±0.4Hz) and HC IgG (1.4±0.5Hz) treated mice ($p<0.01$, Kruskal-Wallis test, Fig. 4, preparations harvested 3 days after incision). This demonstration of enhanced ectopic activity in an *in vitro* preparation isolated from the central nervous system suggests that CRPS-IgG exerts a peripheral pathogenic and proalgesic effect.

Electrophysiological investigations of mechanosensitive sensory afferents

Mechanical hypersensitivity is an almost ubiquitous sensory abnormality in patients with persistent CRPS[25], and a prominent characteristic of the tCRPS phenotype (Figs. 1-3), which suggests that CRPS IgG alters the function of mechanosensitive nociceptors. Therefore, we examined the function of mechanosensitive single units in skin-saphenous nerve preparations.

This approach allowed us to quantify the afferent action potential responses evoked by mechanical stimulation of the receptive fields in their intact anatomical and physiological context. We determined the mechanical activation threshold, conduction velocity and temporal response profiles of Aδ- and C-mechanonociceptors (AM and CM), as well as of the low threshold mechanosensitive fiber types, Aβ- (RA and SA), and D-hair Aδ-fibers. We did not observe a significant spontaneous impulse activity in the recorded single units.

A-mechanonociceptors

Aδ-mechanonociceptor (AM) fibers in preparations from tCRPS mice displayed a modestly reduced mechanical force threshold for activation (Levene's $p<0.05$, Mann-Whitney $p=0.056$, Fig. 5A), but an unaltered conduction velocity compared to preparations from mice only subjected to a paw incision (Table 2). Application of mechanical step- or ramp-shaped force stimuli (from 0.5 to 20g, applied for a duration of 10 and 15s, respectively) demonstrated that AM fibers in tCRPS preparations responded with a higher impulse rate throughout the range of forces used (Fig. 5B-G). Analysis of the impulse pattern evoked by a 5g force step challenge, revealed that tCRPS AM fibers responded with a markedly increased impulse rate, but with a very similar temporal profile compared to that observed in preparations from mice that were not treated with IgG (Fig. 5D, F). AM-fibers encode increasing mechanical force with a corresponding linear increase in impulse frequency[29], and our results show that this force-impulse relationship in response to ramp stimuli is significantly steeper in AM fibers from tCRPS mice than in non-IgG treated mice (Fig. 5E, G). The combination of a steeper force-impulse rate relationship, and a relatively minor force threshold reduction suggests that both transduction and excitability processes are sensitized by CRPS IgG in tCRPS mice (Fig. 5A, E). In keeping with the steeper force-impulse relationship

observed in tCRPS AM fibers, the maximal impulse discharge rates evoked by either a 20g force step (Fig. 5H) or a force ramp to 20g (Fig. 5I) were increased significantly, compared to AM fibers in preparations from mice that were only subjected to a paw incision.

C-mechanonociceptors

The mechanical threshold for activation of C-mechano-nociceptor (CM) fibers was significantly reduced in preparations from tCRPS mice, compared to preparations from mice that had only undergone a paw incision (Fig. 6A). The force thresholds of single units in the control group were distributed through the range 0-7g, whereas all units in tCRPS preparations responded at forces below 3g. The C-fiber conduction velocity did not differ significantly between the treatment groups (table 2). Analysis of the response pattern evoked by a series of force steps and ramps identified a significantly increased impulse rate in tCRPS CM fibers at the highest forces used (Fig. 6B-G, Levene's, $p<0.05$, Mann-Whitney, $p<0.05$). The temporal distribution of action potentials during stimulation with mechanical force appeared unchanged, similar to our observations in AM-fibers (Fig. 6F, G). The maximal impulse discharge rate was increased in preparations from tCRPS mice compared to paw incision alone (Fig. 6H, I).

Low threshold mechanosensitive A-fibers

We assessed the functional impact of tCRPS on the major classes of low-threshold mechanosensitive afferent fibers; slowly adapting Aβ- (SA), rapidly adapting Aβ- (RA) and D-hair (DH) Aδ-fibers. Consistent with the absence of tactile allodynia in tCRPS mice *in vivo* (see Fig. 1C), the conduction velocities and mechanical force activation thresholds of SA, RA and DH fibers

were indistinguishable in preparations from the two treatment groups (Table 2, Fig. 7A-F). In good agreement with earlier characterizations [29], mechanically evoked responses in RA and SA units did not encode forces above 5g with an increasing number of action potentials. The impulse discharge rates of RA and SA fibers did not differ between treatment groups (Fig. 7B, D). Similarly, we could not distinguish between the numbers of action potentials generated in response to force steps in DH fibers in preparations from tCRPS and incision only mice (Fig. 7E, F).

Discussion

Here we demonstrate for the first time that administration of IgG from patients with persistent CRPS, in combination with a minor experimental insult, transfers persistent mechanical and thermal sensory abnormalities from donor patients to mice ('tCRPS'). We further show that the degree of sensory abnormalities correlates both with the transferred IgG dose, and with the donor-patients' subjective pain intensities. Electrophysiological investigations of skin-saphenous nerve preparations from mice treated with IgG from a typical patient-donor demonstrate a markedly increased stimulus-evoked discharge rate in A- and C-mechanonociceptor single units, whereas low-threshold A-fibers were unaffected by CRPS patient IgG. The single nerve fiber properties thus reflect both the patient's clinical experience and the transferred behavioral phenotype. Taken together, these observations indicate that circulating IgG autoantibodies maintain pain and painful hypersensitivities in persistent CRPS by sensitizing peripheral nociceptors. Other autoantibody mediated neurological conditions are typically caused by autoreactive IgG, in good agreement with the results presented here[13]. Intriguingly, mouse autoantibodies of the IgM isotype produce CRPS-like vascular and sensory abnormalities in the

tibial fracture, cast immobilization mouse model of CRPS[23,31]. Our results provide support for the use of immune therapies, such as plasmapheresis[2] or B-cell ablation, to reduce autoantibody titre in persistent CRPS. The variable time required for mice to recover from tCRPS suggests that patients may need to undergo relatively extended treatment periods to benefit from reduced IgG titres, consistent with the available data from trials with therapeutic plasma exchange[22].

An emerging body of evidence indicates that autoantibodies are the primary pathological agents responsible for pain in some painful disorders[14,20]. Autoantibodies that target citrullinated proteins produce pain in rheumatoid arthritis (RA) by stimulating IL-8 release from osteoclasts, even when inflammation is clinically well-controlled [11,53]. Serum-IgG from patients suffering from rare painful neurological autoimmune disorders targeting the voltage-gated potassium channel complex (VGKCC, specifically CASPR2) elicit painful hypersensitivities upon transfer to mice[15]. CASPR2 autoantibodies cause pain by reducing the surface expression of Kv1 channels and enhancing the activity of D-hair Aδ-fibers. These earlier mechanistic investigations thus demonstrate that autoantibodies can cause pain either indirectly, by stimulating release of proalgesic mediators from other cells[11], or by directly affecting the activity of sensory neurons[15]. Importantly, VGKCC autoantibodies have not been observed in CRPS patients[4,26,35], and we did not detect functional abnormalities in D-hair fibers in tCRPS mice, highlighting that CRPS autoantibodies generate pain by acting at different, unidentified targets.

The protein(s) or epitopes responsible for the pathological impact of CRPS autoantibodies remain unknown, and efforts to identify the targets for pathological autoantibodies are now required to facilitate the development of diagnostic tests, prognostic tests before elective operations, and

novel treatments. Earlier investigations have demonstrated that a subset of CRPS patients produce IgG autoantibodies that recognize epitopes on autonomic neurons and SH-SY5Y cells[28]. Further studies identified specific binding to M2 muscarinic as well as to β2- and α1 adrenoreceptors[16,27], but the relevance of these receptors for CRPS pain is not yet clear. CRPS has very recently been reported to be associated with an expansion and activation of memory T-cells [40] and there is evidence of an altered profile of tissue resident cutaneous T-cells in the limb affected by CRPS[5,34]. It is not yet clear how the initial trauma contributes to the subsequent autoantibody mediated pathology in CRPS [36], but it is possible that the injury induces production of neoantigens, or that the plasma extravasation produced around the trauma facilitates access of IgG to the affected area.

Our investigations of isolated skin-saphenous nerve preparations identify an increased spontaneous impulse rate in the intact saphenous nerve. Although it is not possible to determine which fiber types are responsible for the heightened ectopic activity in our preparations, such ectopic discharge is likely to be responsible for spontaneous pain and paraesthesias[43,44,51]. Furthermore, we identified an increased responsiveness to mechanical stimulation of AM- and CM nociceptors characterized both by a reduced mechanical threshold and by an increased impulse discharge rate in response to supra-maximal stimulation. We studied saphenous single units with receptive fields in the uninjured dorsal hind paw skin, rather than the incised plantar skin. The functional abnormalities observed in these nociceptors are thus not directly explained by the postsurgical hypersensitivity seen in sural and tibial single units in the paw-incision model[3,8], but are consistent with the more generalized regional pain seen in patients who develop CRPS after injury[6,45].

Our results demonstrate that IgG autoantibodies from CRPS patients generate painful hypersensitivities by increasing the activity of peripheral nociceptors and this new information may guide future attempts at identifying targets for interventions.

Acknowledgments: This work was supported by grants awarded by Arthritis Research UK (21544), the Medical Research Council (MR/L010747/1) and the David Hammond Foundation. UC was supported by a PhD studentship from the Pain Relief Foundation, which has also provided support to AG.

Author contributions: U.C., C.G., N.V., and S.S., performed and analyzed experiments. All authors contributed to experimental design and manuscript preparation.

Conflicts of Interest: The authors have no conflicts of interest to report.

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FIGURE LEGENDS

Fig. 1. **CRPS IgG produce polymodal hypersensitivities in mice**. Administration of CRPS patient IgG (8mg, i.p. on days -1 to 2), prolonged and exacerbated the ipsilateral mechanical (**A**), cold (**E**) and heat (**G**) hypersensitivity produced by a paw incision (on day 0) compared to mice treated with IgG from healthy control subjects or no IgG. The sensitivity to stimulation with von Frey filaments was unaffected by CRPS IgG (**C**). The contralateral, uninjured paw displayed a much less marked hypersensitivity in all four tests (**B**, **D**, **F**, **H**). Data are mean ± SEM of n=6. *p < 0.05, **p < 0.01, ***p < 0.001, One-way ANOVA, followed by Tukey's, CRPS compared to HC IgG group. †p<0.05, ††p<0.01, †††p<0.001, One-way ANOVA, compared to naïve values before surgery and injection, Dunnett's post-hoc test.

Fig. 2. CRPS IgG induced dose-dependent and sustained hypersensitivities. Effect of different dose regimens of serum IgG from CRPS patient compared to HC IgG on ipsilateral (A) and contralateral paw (B) withdrawal thresholds to mechanical pressure, and (C) ipsilateral and (D) contralateral paw withdrawal latencies to noxious cold (10°C). IgG was administered on days -1 to 2 (4 injections) or days -1 and 0 (2x8mg), and paw incisions were performed on day 0. Data are mean ± SEM of n=6. Data points from all groups and time points were tested by one-way ANOVA followed by Holm-Sidak's post-hoc: *p < 0.05, **p < 0.01, ***p < 0.001, vs HC IgG.

Figure 3. The phenotype of tCRPS mice is related to the pain intensity of CRPS patient IgG donors. (A) Ipsilateral paw withdrawal thresholds to paw pressure before and 7 days after paw incision (4 days after the last IgG injection), in mice injected with IgG (8mg) pooled from either healthy control subjects (HC), CRPS patients with moderate pain intensities, or CRPS patients with high pain intensities. (B) Paw withdrawal threshold of mice treated with 4x16mg of IgG from HC

IgG or CRPS patients with moderate pain intensities, or 4x8mg of IgG from patients with high pain intensities. n=5-6. ANOVA with Tukey's post-hoc (A, B): *p < 0.05, **p < 0.01, ***p < 0.001, vs HC IgG group. One-way ANOVA, pre and post-surgery comparison using Dunnett's post-hoc test (A). ††† p < 0.001.

Figure 4. CRPS IgG increases the spontaneous impulse rate in skin-saphenous nerve preparations. (A) Typical examples of spontaneous activity recorded in skin-nerve preparations from naïve mice, mice only subjected to a paw incision, or subjected to a paw incision in combination with IgG from HC or CRPS patient. (B) Frequency of spontaneous activity recorded in the intact saphenous nerve. Each data point represents one preparation/animal. **p < 0.01, Kruskal-Wallis.

Figure 5. CRPS IgG sensitizes AM fibers. (A) Mechanical response thresholds of AM fibers in preparations from tCRPS mice and mice that only underwent incision. (B) Example traces of AM fiber action potentials evoked by a 5g force step and (C) a force ramp stimulus to 10g. The temporal impulse pattern is displayed in histograms (*lower panel*). (D-E) The mean number of action potentials (AP) evoked by force step (D) and ramp (E) stimuli (0.5g-20g) in preparations from incision only compared to tCRPS mice; the linear regression of the force-impulse relationship with 95% confidence interval is shown in shaded pink/gray. (F) The mean impulse pattern of AM fibers during 10s constant 5g force simulation and (G) 15s ascending force of 10g (means calculated from all fibers presented in D, E). (H) Peak firing frequency (events/s) in incision compared to CRPS groups for 5g ramp stimulation and (I) 10g ramp stimulation. Data are mean±SEM or individual data points. Levene's test p<0.05; Mann-Whitney U-test: *p < 0.05, **p < 0.01, CRPS group comparison with incision group.

Fig. 6. CRPS IgG sensitizes CM fibres. (A) Mechanical response thresholds of CM fibers in preparations from tCRPS mice and mice that only underwent incision. (B-C) Example traces of C-fiber action potentials evoked by a 20g force step (B), and a force ramp (C) stimulus to 20g. The temporal impulse discharge frequencies are shown as histograms (events/s). (D-E) Mean number of action potentials (AP) evoked by (D) step stimuli of (0.5g-20g) and (E) ramp stimuli in incision and CRPS mice. The force-impulse relationship is indicated by linear regression, with the 95% confidence interval shaded. (F-G) Mean pattern of action potential rate in CM-fibers in response to (F) a 10s constant 20g force application and (G) a 15s force ramp challenge to 20g. (H-I) Peak firing frequency (events/s) in incision versus CRPS groups for 20g force step (H) and ramp (I) stimuli. Data are mean±SEM. *$p < 0.05$, ***$p<0.001$, t-test (A, B, H, I) or Mann-Whitney (following Levene's test $p<0.05$), CRPS group comparison with incision group.

Fig. 7. Low threshold mechanosensitive Aβ and DH fibers are unaffected by CRPS IgG. Mechanical response thresholds determined by 2s force step challenges (A, C, E) and mean number of action potentials (AP) evoked by 10s, 0.5g-20g force steps (B, D, F) in preparations from tCRPS mice and mice that were subjected to paw incision alone. Slowly adapting Aβ fibers (A, B), rapidly adapting Aβ fibers (C, D) and DH fibers (E, F). Data are mean ± SEM. Mann-Whitney (following Levene's test $p<0.05$), tCRPS compared to incision only.

Table 1. Characteristics of patient IgG donors.

	Moderate pain (n=26)	**High pain (n=27)**
Age (years, average±SD)	43±14	41±10
CRPS duration (years, median (range))	3 (1-5)	3 (1-5)
% female	54%	77%
Baseline pain intensity (average)	6.0 ±0.8 (SD, range 5.0-6.9)	8.3 ±0.4 (SD, range 7.3-9.5)

Table 2. Conduction velocity of the different fibers from incision only and tCRPS preparations. Data are mean ± SEM. AM, CM and RA-Aβ units were tested with Mann-Whitney, whereas SA-Aβ and D-hair were tested with a t-test.

Fiber type	Incision only conduction velocity (m/s)	tCRPS conduction velocity (m/s)	P values
RA-Aβ	12. 0±0.6 (n=16)	15.7±2.0 (n=12)	0.07
SA-Aβ	12.9±0.7 (n=14)	13.2±0.5 (n=13)	0.85
D-hair	6.3±0.7 (n=10)	7.2±0.8 (n=9)	0.39
AM[1]	7.3±0.7 (n=17)	6.2±0.6 (n=23)	0.28
CM[2]	0.52±0.1 (n=14)	0.78±0.1 (n=13)	0.07

[1]Aδ- mechanonociceptor, [2]C-mechanonociceptor