A CRPS-IgG-transfer-trauma model reproducing inflammatory and positive sensory signs associated with complex regional pain syndrome

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ABSTRACT

The aetiology of complex regional pain syndrome (CRPS), a highly painful, usually post-traumatic condition affecting the limbs, is unknown, but recent results have suggested an autoimmune contribution. To confirm a role for pathogenic autoantibodies, we established a passive-transfer trauma model. Prior to undergoing incision of hind limb plantar skin and muscle, mice were injected either with serum IgG obtained from chronic CRPS patients or matched healthy volunteers, or with saline. Unilateral hind limb plantar skin and muscle incision was performed to induce typical, mild tissue injury. Mechanical hyperalgesia, paw swelling, heat and cold sensitivity, weight-bearing ability, locomotor activity, motor coordination, paw temperature, and body weight were investigated for 8 days. After sacrifice, proinflammatory sensory neuropeptides and cytokines were measured in paw tissues. CRPS patient IgG treatment significantly increased hind limb mechanical hyperalgesia and oedema in the incised paw compared with IgG from healthy subjects or saline. Plantar incision induced a remarkable elevation of substance P immunoreactivity on day 8, which was significantly increased by CRPS-IgG. In this IgG-transfer-trauma model for CRPS, serum IgG from chronic CRPS patients induced clinical and laboratory features resembling the human disease. These results support the hypothesis that autoantibodies may contribute to the pathophysiology of CRPS, and that autoantibody-removing therapies may be effective treatments for long-standing CRPS.

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1. Introduction

Complex regional pain syndrome (CRPS) is usually a limb-confined chronic pain syndrome arising after trauma. It is associated with sensory, motor, autonomic, bone, and skin changes, but the leading symptom is pain. Its causes are unknown, but recent evidence indicates that the condition is associated with autoimmunologic autoantibodies, and the finding that some patients respond to immunoglobulin treatment suggests the possible involvement of autoantibody-mediated autoimmunity [12,13,22,25].

Diseases arising from pathogenic autoantibodies can sometimes be transferred to rodents by intraperitoneal injection of serum IgG from patients (‘passive transfer’). This has been shown to occur for myasthenia gravis and pemphigus [2,37], where pertinent cell-surface epitopes are structurally preserved between species. Successful passive-transfer experiments of this type can thus directly establish autoantibody involvement in a disease [31] and can provide a model for future mechanistic and therapeutic studies. We have previously shown that the passive transfer of serum IgG from patients with long-standing CRPS decreases exploratory behaviour in normal mice, but IgG treatment fails to induce hyperalgesia or oedema in normal mice [14,16]. CRPS is usually a post-traumatic
condition, and successful passive transfer might depend on the induction of trauma; for example, trauma-induced regional inflammation may be required to allow the binding of circulating autoantibodies (and the bound IgG may in turn then enhance that inflammation). We therefore hypothesized that the passive transfer of patient serum IgG to mice, followed by limb trauma, would induce pertinent features of CRPS.

The activities of the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) are abnormally high in CRPS-affected skin, and concentrations of both tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) are raised [20,38] in affected skin blister fluid. In a postfracture rodent model of CRPS, interleukin 1 (IL-1) was raised in affected skin, likely induced by enhanced SP signalling [17,21]. Since these mediators have been suggested to contribute to the ‘neurogenic inflammation’ [38] that is a characteristic of the clinical presentation, we determined the concentrations of these molecules in this passive-transfer model of CRPS.

2. Patients and methods

2.1. Patients and preparation of samples

For internal pilot experiments, after obtaining written informed consent, we first obtained 150 mL of blood (preintervention) from an unaffected limb of 2 patients with long-standing CRPS who were participating in an open clinical trial of low-dose immunoglobulin maintenance treatment for CRPS (ISRCTN63226217), and from 2 healthy volunteers [15]. Blood samples were centrifuged, and the serum stored frozen at −20°C for later use.

IgG fractions were prepared using protein G beads (Sigma-Aldrich, Gillingham, UK) and adjusted to 8–9 mg/mL, either by dilution with PBS or by further dialysis against a sucrose solution (Sigma-Aldrich). The non-IgG flowthrough serum fractions were concentrated back to their original concentrations, and their levels of IgM, IgG, and IgA were determined using an immunoturbidimetric assay (Fortress Diagnostics, Antrim, UK) to confirm the removal of IgG but retention of IgM/IgA. Both IgG and some flowthrough fractions were sterile filtered using syringe-driven 0.2 M filter units (Millipore, Watford, UK), stored at 4°C and used within 3 months.

After the completion of internal pilot experiments with these 4 IgG preparations, and after obtaining separate ethical permission (12/NW/0126) and individual written informed consents, 150 mL of blood was obtained from the unaffected limbs of an additional 4 patients and from 4 healthy volunteers. For inclusion, patients fulfilled the 2012 International Association for the Study of Pain (IASP) CRPS diagnostic criteria (clinical criteria [18]). They had a disease duration of >1 year; a pain intensity of ≥5 on an 11-point (0–10) numerical rating scale; clinical evidence for static mechanical hyperalgesia (pain to light pressure in the affected limb, the most frequent CRPS-associated positive sensory sign [11]); and no other significant pain or medical disorders. Healthy volunteers were age matched (±10 years) and gender matched to the patients, and had no chronic pain problems; their first-degree relatives had no known history of autoimmune disorders. These 8 blood samples were processed as described earlier.

2.2. Animals

Experiments were performed on female C57Bl/6 mice (8–10 weeks old, weighing 18–23 g). The original breeding pairs were purchased from Jackson Laboratories (USA) through Charles River Hungary. The animals were bred and kept in the animal house of the University of Pécs under standard conditions at 24–5°C, provided with rodent chow and water ad libitum.

2.3. Experimental design

Mice (5–7 per group) were treated with the IgG fractions obtained from the 6 CRPS patients and the 6 healthy volunteers. Flowthrough- and saline-treated groups served as controls (Fig. 1). After acclimatization and conditioning, 3 control measurements were performed in the week prior the first injection day (days −4, −3, and −2, with day 0 defined as the day of the limb injury; Fig. 1). Mice were treated intraperitoneally with serum IgG, flowthrough or saline in the mornings of days −1 and 0, and the injections were repeated on days 5 and 6. We chose these reinjection times as we assumed metabolism of the injected proteins, with some loss of activity, from day 5 or 6 [16]. Each mouse received a total injected volume of 6 mL (1 mL in the morning and an additional 0.5 mL in the afternoon of each injection day).

We incised the plantar skin and muscle of 1 hind limb under general anaesthesia (see Section 2.4) in the afternoon of day 0. Measurements were taken from day 1 to day 8 (or day 10 in one experiment). The animals were sacrificed after the last measurement and their paws were stored frozen at −80°C for later analysis of tissue neuropeptides and cytokines (further detail on the excision method is given in Section 2.5.9). The operative procedure, as well as each measurement, was performed by the same investigators, who were blinded to the treatment condition.

2.4. Plantar skin and muscle incision

We used the hind paw plantar incision model that was originally developed in rats [7] and subsequently adapted for use in mice [30]. The model evokes a significant decline of the mechano-nociceptive threshold, with a maximum 1 day after surgery, which persists for 7–8 days. Mice were anaesthetized with a combination of ketamine (Richter Gedeon, Hungary, 100 mg/kg intraperitoneally) and xylazine (Eurvet Animal Health BV, Netherlands, 5 mg/kg intraperitoneally) on day 0, and a midline incision 0.5 cm long was performed starting 0.2 cm from the heel, involving skin, fascia and muscle. The wound was closed by a suture with sterile 5/0 silk thread, and was treated locally with povidone-iodine. Suture removal (by the animals) usually occurred within 1 day of the operation [10]. Because the silk suture itself might be inflammatory, we recorded the time of suture removal to exclude any potential confounding effects from a difference in removal times between experimental groups. All operations were performed by the same blinded operator.

2.5. Analytical techniques

2.5.1. Mechanosensitivity

Mechanical hyperalgesia of the plantar surface of the paw was determined using a dynamic plantar aesthesiometer (Ugo Basile 37400, Comerio, Italy), which is a modified electronic von Frey technique. The animals were placed on a metal mesh surface in small boxes and allowed to move freely. After the cessation of exploratory behaviour, the operator placed the stimulator unit under the paw of the animals, using the adjustable angled mirror to position the filament below the target area. The filament was...
positioned to the middle region of the plantar incision. After pressing the ‘start’ key, an electrodynamic actuator advanced a straight metal filament, which touched the plantar surface of the paw and exerted an increasing upward force until the animal removed its paw. The paw withdrawal threshold was numerically displayed in grams on a digital screen. Baseline measurements were performed on both limbs for each group in the week before the experiments (3 measurements, the average of which was the respective group baseline control). The mechanonociceptive threshold was then measured on experimental days 1, 2, 3, 7, and 8 (and additionally on days 9 and 10 in one experiment; Fig. 1), and values were expressed as the percentage change from baseline values. Reduced values (decreased mechanonociceptive threshold) were considered to reflect hyperalgesia [5,35].

2.5.2. Paw volume

Paw volume was measured using a plethysmometer (Ugo Basile 7140). This instrument consists of 2 vertical interconnected water-filled Perspex cells, the larger of which is used to measure volume displacement caused by immersion of the mouse paw. Paws were immersed to the border of the hairy skin. The water level in an interconnected smaller tube, which contains a force transducer, generated a proportional volume measurement of the mouse paw, expressed in cm$^3$. The paw volumes were measured before the passive-transfer experiments (baseline), and on days 1, 2, 3, 7, and 8 of the experimental period (Fig. 1). Oedema was expressed as the percentage change from baseline values. Reduced values (decreased mechanonociceptive threshold) were considered to reflect hyperalgesia [5,35].

2.5.3. Heat and cold sensitivity

The thermoneuroceptive threshold of the paw was measured with an increasing-temperature hot plate (IITC Life Science, Woodland Hills, USA) [1]. The threshold was defined as the lowest temperature that evoked a nocifensive reaction. We tested freely moving mice placed on the plate [5] on days 1, 2, 3, 7, and 8. Cold sensitivity was determined by the withdrawal latency after immersing the affected paw in 0°C water. Mice were gently held by the same technician; the cutoff time was 180 seconds [29]. The test was performed at baseline and on days 3, 7, and 8.

2.5.4. Spontaneous weight bearing

Spontaneous weight bearing on the hind limbs was determined with an incapacitance tester (Linton Instrumentation, Norfolk, UK). Results were expressed as the percentage of weight distributed on the injured hind limb. After habituation and 3 controls to establish a baseline, measurements were performed on days 7 and 8.

2.5.5. Spontaneous locomotor activity (open field test)

Locomotor activity was assessed in a minimally anxiogenic open field test. This test can investigate rodent exploratory behaviour, and intact mice without tissue injury injected with CRPS-IgG had abnormal test results in our earlier experiments [14]. The apparatus comprised a 60 cm $\times$ 40 cm wooden box with the floor divided into 16 equal squares (4 $\times$ 4), on which the mice could move freely. After placing the animal into one of the corners of the box, the behaviour of the mouse was recorded by a video camera over 5 minutes, including the number of squares crossed with all 4 paws, the number of rearings, the time spent moving and grooming, as well as time spent in the central region of the box. The test was performed on day 0 (after the 2 injections, a few hours before limb injury) and day 6.

2.5.6. Motor performance and coordination (Rota-Rod test)

Motor function and coordination were investigated with an accelerating Rota-Rod apparatus (Ugo Basile 37400). Animals were examined for their ability to maintain balance on a rotating wheel, which increased in speed from 4 to 40 revolutions per minute (rpm) over a 5-min period, starting after 10 seconds at a constant 4 rpm. The outcome was the speed at which the mouse fell off the rod, as previously described [14]. Testing was on day 0 (after injection, before limb injury) and day 6.

Fig. 1. Study flow chart. One pair of mice injected with CRPS-IgG or healthy-IgG was sacrificed on day 10 instead of day 8 because abnormal values had not returned to normal on day 8 (details in the results section). i.p., intraperitoneally; N, number of preparations tested.
2.5.7. Paw temperature

Paw temperature was determined with a contact thermometer on day 7 of the experiments.

2.5.8. Body weight

Body weight was measured every day at the same time during the day.

2.5.9. Inflammatory neuropeptides and cytokines in tissue homogenates

After all functional testing had been completed on day 8 (or day 10 in one experiment, Fig. 1), all animals were deeply anaesthetized and then sacrificed by cervical dislocation. The whole paws were excised (including skin and all subdermal tissues), the toes were removed and the weight was measured. The limb parts were snap frozen in liquid nitrogen and kept at −80°C until further processing. The frozen paws were later thawed, chopped into small pieces and then homogenized in 2 mL ice-cold double-distilled water at 0°C. The homogenate was centrifuged at 10,000 g for 10 minutes at 4°C and the supernatant was immediately removed for measuring inflammatory sensory neuropeptides and cytokines.

CGRP- and SP-like immunoreactivities were measured by radioimmunoassay, as previously described [27,28]. For cytokine experiments, we first analysed paw tissues from 2 randomly selected animals per group; where differences between groups were evident, all paws were retested for the mediator. TNF-α, IL-6, and interleukin 1-beta (IL-1β) were simultaneously detected using a Luminex 100 xMAP system (Invitrogen Mouse Inflammatory panel: AthenA Multi-Lyte), according to manufacturer’s instructions. Results were calculated on the basis of mean fluorescent intensity with MasterPlex QT software. For confirmatory experiments, TNF-α was measured with sandwich enzyme-linked immunospecific assay (ELISA) kits (Millipore Chemikine and BD Biosciences). Mediator concentrations were normalized to the wet weight of the tissues and expressed as fmol/mg for the neuropeptides and pg/mg for the cytokines.

2.6. Animal ethics

All experimental procedures were carried out according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988) and complied with the recommendations of the IASP and the Helsinki Declaration. The studies were approved by the Ethics Committee on Animal Research of Pécs University according to the Ethical Codex of Animal Experiments (licence no. BA 02/2000-9-2011). Animals were randomized in all experimental assessments. The examiner taking the measurements was blinded from the treatment the animals received.

2.7. Statistical analysis

Data from the 2 pilot experiments (2 healthy-IgG and 2 CRPS-IgG preparations) were analysed as internal pilots, on the basis of which both the primary and secondary behavioural outcomes (primary outcome: mechanosensitivity on day 7; secondary outcome: limb swelling on day 2) and the number of additional experiments were determined for the study. The protocol submitted to gain ethics committee permission for taking additional blood samples listed these outcomes (see Supplementary data).

For the overall experiments, all experimental results, including those from the 2 pilot experiments and from the additional experiments, were analysed together (6 healthy-IgG and 6 CRPS-IgG preparations). To calculate differences in the overall behavioural results between the CRPS-IgG, healthy-IgG, and saline experiments, the pooled data from all experiments were expressed for each experimental day as means ± SEM, and analysed using two-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni’s post hoc test.

The primary and secondary behavioural outcomes for each of the individual animal groups injected with CRPS-IgG or healthy-IgG (6 healthy-IgG and 6 CRPS-IgG, 5–7 animals per group) were calculated, and the group values were compared between the CRPS-IgG and healthy-IgG groups. For mediator investigations, data from the saline, healthy-IgG, and CRPS-IgG experiments were pooled (separately for injured and noninjured limbs) and analysed using ANOVA and Bonferroni’s post hoc test for each mediator. Bonferroni’s correction for multiple comparisons was then applied to correct for multiple tested mediators. Bonferroni adjusted values of P < 0.05 were regarded as statistically significant (2-tailed tests).

3. Results

3.1. Patients and pilot experiments

The demographic and disease characteristics of the 6 included CRPS patients are given in Table 1, and the study design is shown in Fig. 1.

The first 2 (pilot) experiments, each conducted with 1 pair of healthy-IgG and CRPS-IgG samples (see Section 2.7), indicated that the animals injected with CRPS-IgG had increased limb swelling early (most prominent on day 2) and increased mechanical hyperalgesia later (most prominent on day 7) after trauma, compared with mice injected with healthy-IgG (day 0 was defined as the day of limb trauma). Limb mechanical hyperalgesia in the CRPS group in the first of these 2 pilot experiments did not revert to the control value at the planned final measurement day (day 8; data not shown). In order to explore the maximal duration of this effect, measurements in the second pilot experiment were continued for 2 additional days, at which time limb allodynia had normalized to control values. Pooled results from these 2 pilot experiments are shown in Supplementary Fig. 1. In these 2 pilot experiments, no consistent differences between the groups in-
groups 1 day after surgery, demonstrating an increased pain sensation/nocifensive reaction to mild painful stimuli. In the saline-treated control group, this mechanical hyperalgesia recovered to \(-25.74 \pm 2.30\%\) and \(-16.01 \pm 2.24\%\) on days 2 and 3, and was thereafter maintained at the latter level. There was a main effect of time \(F(5,535) = 245.2, \ P < 0.0001\) and a significant difference between the mechanonociceptive thresholds of the 3 treatment groups \(F(2,107) = 19.98, \ P < 0.001\). There was a significantly reduced mechanonociceptive threshold in the CRPS-IgG mice when compared with the healthy-IgG mice on day 7 (Bonferroni post hoc test, \(P < 0.001\); Fig. 2A), in line with prediction (primary outcome; Fig. 3A). On day 2, the mean paw volume in the CRPS-IgG group \((32.3 \pm 1.8\%\) was 45% increased compared with the healthy-IgG group \((22.3 \pm 2.2\%\); Fig. 3B). The CRPS-IgG groups had larger paw volume increases vs the healthy-IgG groups in each individual experiment. The injection of the flowthroughs did not induce any significant effects compared with the saline-treated control mice. There was <3% change in the paw volumes on the contralateral, noninjured side in either group (individual outcomes, flowthrough, and contralateral data not shown).

3.3. Additional outcomes

3.3.1. Heat hyperalgesia and cold allodynia

The thermonociceptive threshold measured by the increasing-temperature hot plate was tested for the first 2 (pilot) healthy-IgG and CRPS-IgG preparations. We observed only minor changes in response to injury, and there were no significant differences between groups (Fig. 4A); testing was therefore discontinued for the remaining groups.

Cold hyperalgesia was tested for the 4 subsequent healthy-IgG and CRPS-IgG preparations. In the saline-treated group, the mean paw withdrawal latency at 0°C observed before the incision was 157.25 ± 8.26 seconds, which decreased to 52.04 ± 6.45 seconds 3 days after the operation, and the results in CRPS-IgG and healthy-IgG groups were similar. This corresponded to an approximately two-thirds decrease of the nocifensive behaviour latency (cold allodynia), which persisted for all 3 groups throughout the experiment, up to and including day 8, without any significant differences between these groups (Fig. 4B).

3.3.2. Spontaneous weight bearing

Spontaneous weight bearing was tested for the first 2 (pilot) preparations. There was a mild decrease in weight bearing on the injured side in all groups, with a peak on day 3, but no significant differences between the saline, healthy-IgG, and CRPS-IgG mice (Fig. 4C); testing was therefore discontinued for the remaining patient groups.

Table 1
Demographic and disease characteristics of the study patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>48</td>
<td>43</td>
<td>60</td>
<td>34</td>
<td>37</td>
<td>50</td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>Affected limb</td>
<td>Lower</td>
<td>Both lower</td>
<td>Upper</td>
<td>Lower</td>
<td>Lower</td>
<td>Lower</td>
</tr>
<tr>
<td>Inciting event</td>
<td>Dancing</td>
<td>Minor knock</td>
<td>Operation on the brachial plexus for a benign tumour</td>
<td>Operation for Freiberg’s syndrome, and reoperations</td>
<td>Operation for Morton’s neuroma</td>
<td>Ankle fracture, followed by operations</td>
</tr>
<tr>
<td>CRPS type</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>Not determined</td>
<td>1</td>
</tr>
<tr>
<td>Disease duration, years</td>
<td>6</td>
<td>5.1</td>
<td>7</td>
<td>5–7*</td>
<td>1</td>
<td>8–10*</td>
</tr>
<tr>
<td>Pain intensity</td>
<td>7</td>
<td>7.5</td>
<td>10</td>
<td>7</td>
<td>5.5</td>
<td>8</td>
</tr>
<tr>
<td>Pain to light pressure applied to the skin of the affected limb</td>
<td>None</td>
<td>Past myocardial infarction</td>
<td>Freiberg’s syndrome</td>
<td>Asthma</td>
<td>Diabetes type II; high blood pressure; high cholesterol; overactive thyroid</td>
<td></td>
</tr>
<tr>
<td>Concomitant disease</td>
<td>Psoriatic arthritis; gluten-sensitive enteropathy; pernicious anaemia</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

F, female; M, male; CRPS, complex regional pain syndrome.

*1 = without associated injury, 2 = with associated injury to a major nerve.

a Disease onset followed one of several operations; exact time details were not remembered or recorded.

b Average 24 h pain intensity on the day of enrolment on an 11-point numeric rating scale (0–10); 0 = no pain, 10 = pain as bad as you can imagine.
3.3.3. Spontaneous locomotor activity, motor performance, and coordination

Spontaneous locomotor activity was determined using the open field test on days 0 and 6 in all 6 groups of mice injected with healthy-IgG and in all 6 groups of mice injected with CRPS-IgG by: 1) the number of fields crossed and the number of rearings; 2) the time spent moving and grooming; and 3) the time spent in the central regions. Outcomes did not differ significantly among mice treated with either healthy-IgG or CRPS-IgG compared with saline (rearing results are shown in Fig. 4D). There was also no significant difference in Rota-Rod performance between the groups on day 0. In the first 2 experimental sets we observed generally increased performances in all groups on day 6, possibly attributable to learning, but with no significant differences between groups, and therefore discontinued further testing on day 6.

3.3.4. Paw temperature

There were no differences between the CRPS-IgG, saline, and healthy-IgG groups in either the absolute paw temperature of the injured limbs or in the mean absolute temperature difference between the respective injured and noninjured paws on day 7 upon testing of the first 3 healthy-IgG and CRPS-IgG groups (Fig. 4E). We discontinued temperature testing in the remaining experiments.
3.3.5. Changes in body weight

Body weight was tested in all saline, healthy-IgG (n = 6), and CRPS-IgG (n = 6) groups. Mean body weight was 18.26 ± 0.25 g, 18.95 ± 0.29 g, and 18.53 ± 0.23 g, respectively, on day 0/C0 of the experiment. The weights did not change significantly during the 8 days of the experiment (Fig. 4F).

3.3.6. Concentrations of inflammatory neuropeptides and cytokines in the paw homogenates

These tests were performed in all groups (6 healthy-IgG groups and 6 CRPS-IgG groups). There was no significant difference between the SP immunoreactivity in the intact paw homogenates of mice treated with saline, healthy-IgG, or CRPS-IgG. Incision significantly increased SP concentration in all groups, but the SP concentration was significantly greater (more increased) in mice treated with CRPS-IgG compared with healthy-IgG (Fig. 5A; CRPS: 30.3 ± 9.3 fmol/mg, healthy: 22.9 ± 6 fmol/mg, mean difference 7.5 fmol/mg, 95% CI of the difference: 2.8–12.1 fmol/mg, P < 0.001 one-way ANOVA with Bonferroni post hoc test).

By contrast, CGRP immunoreactivity did not change either in response to injury or IgG treatments (Fig. 5B). Neuropeptide concentrations in the flowthrough-injected groups did not differ from saline groups (data not shown). IL-1β and IL-6 concentrations were not different between groups (Fig. 5C and D). Although there appeared to be a significant difference in TNF concentration in a limited number of preparations (see Methods section), no differences
were seen when all paw tissues were investigated (Fig. 5E). Following a Bonferroni correction for multiple comparisons for the 5 measured mediators, SP remained significantly raised ($P < 0.005$).

### 3.3.7. Potential confounds

Patient 1 had concomitant psoriatic arthritis (Table 1), which can be painful (although not considered autoantibody mediated). To test for any bias, we recalculated the 3 positive outcomes (mechanical hyperalgesia, paw swelling, and paw SP) excluding patient 1 data; this did not markedly change the results (see Supplementary Fig. 2).

### 4. Discussion

We have developed a novel, IgG-transfer-trauma model, which reproduces key clinical signs of CRPS, including limb swelling and static mechanical hyperalgesia. Typically, patients with CRPS experience both pain and marked limb signs early after the CRPS-triggering trauma; although most patients improve quickly, those whose condition becomes chronic (about 15% [4,9]) have persistent pain, whereas their initial limb signs, such as limb swelling, often improve. The characteristics of the model described in this report resemble this pattern. The mechanisms causing the

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**Fig. 5.** Effects of human IgG treatments on sensory neuropeptide and inflammatory cytokine concentrations in the hind paws. Concentrations of (A) substance P (SP) and (B) calcitonin gene-related peptide (CGRP) were measured by radioimmunoassay in hind paw homogenates excised after sacrifice on day 8. Concentrations of (C) interleukin 1-beta (IL-1β), (D) interleukin-6 (IL-6), and (E) tumour necrosis factor alpha (TNF-α) were measured by Luminex and/or enzyme-linked immunospecific assay from the same samples. Columns show the means ± SEM of either 30–37 mice per group (SP, CGRP, TNF-α), or 6–13 animals per group (IL-1β, IL-6; see Methods section). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs respective intact limbs; ***$P < 0.001$ vs healthy and ++$P < 0.001$ vs saline for the injured side (one-way analysis of variance followed by Bonferroni’s modified post hoc test).
persistent mechanical hyperalgesia in the CRPS-IgG group require further study. These results suggest a possible role for pathogenetic autoantibodies in patients with long-standing CRPS.

The rapid normalization of all behavioural abnormalities by day 8 was similar to that described in earlier, passive-transfer-only experiments, and is likely explained by the rodent’s metabolism of the injected human IgG [16]. Reinjection at day 7 or 8 might have overcome this, but would have required both the availability of larger amounts of sera and the coapplication of cyclophosphamide to suppress serum sickness, with uncertain impact on the unknown immunological mechanisms [8].

It has been suggested that cold allodynia is due to peripheral nerve terminal sensitization, while mechanical hyperalgesia involves central sensitization processes [26]. Our results, showing a disparate outcome for cold allodynia (no difference between groups) vs mechanical hyperalgesia (increased in animals injected with CRPS-IgG), may suggest a contribution of central sensitization to the CRPS-IgG effect on mechanical hyperalgesia, or alternatively a long-lasting flooring effect in the cold allodynia experiments may be responsible (‘central sensitization’, a peripheral trauma-induced change to the response properties of dorsal horn neurons, causing peripheral hypersensitivity [24]). Previous work by others has demonstrated an absence of cold allodynia in the plantar incision mouse model when this was assessed by measuring paw-licking latency on a cold plate at 0°C [33]. The difference in our results, which show generally strong postoperative cold allodynia, when compared with these previously reported results, might be explained through the larger area of contact of the injured paw in the water bath in our experiments. Our findings were unrelated to the treatment group and are therefore not an indication of transfer of a CRPS-related feature.

The lack of difference between groups in the open field tests contrasts with our earlier results [14]; we propose that the limb trauma common to all groups exerted an overriding effect on day 6. Open field rearing is sensitive to a range of confounding factors [36]. The 2–4 weeks higher rodent age in the present experiments, and differing environmental factors in the 2 laboratories, may be responsible for the observed lack of a difference on day 0 in the present study.

In addition to the functional and morphological results, an important finding that parallels human disease [38] is the significantly increased concentration of the inflammatory neuropeptide SP in the injured paws of animals injected with CRPS-IgG. Concentrations of CGRP, as well as the cytokines TNF-α, IL-1, and IL-6, were normal, contrasting with results from investigations in CRPS-affected human skin [20,38]; and animal models after fracture [32,39]. While CGRP is mostly of sensory neuronal origin [6], SP is also localized in immune cells besides the capsaicin-sensitive peptidergic afferents [19]. Furthermore, these 2 peptides are only partially colocalized in the sensory nerve terminals [34]. Of note, behavioural abnormalities had largely resolved in most experiments at the time of tissue harvest on day 8 (Figs 2 and 3). It is possible that the tissue concentrations of these mediators would differ at earlier time points from the concentration on day 8. Previous studies by other groups in a hind paw incision model (without IgG transfer) have established a central role for SP in sustaining hyperalgesia. For example, SP-deficient mice display decreased mechanical hyperalgesia after incision compared with wild-type mice [32]. Thus, it is possible that there is an interaction between the observed CRPS-IgG effects, such that increased tissue SP may result in increased hyperalgesia. Further studies would be required to clarify any such interactions.

The major strength of the study is the delivery of a model in which both limb injury and variant human condition (specific IgG serum autoantibodies) are necessary elements, as in the clinical disease. The model consistently produced behavioural effects in the CRPS-injected groups.

A limitation is that the clinical picture of CRPS was incompletely reproduced. For example, CRPS is associated with an unstable temperature difference between affected and unaffected limbs, which may in part be caused by sympathetic dysfunction [23]. Enhancement of hyperalgesia and oedema alone, as observed in this model, would not be sufficient to merit a CRPS diagnosis under the IASP 2012 CRPS criteria. Although the model does not produce all the features of human CRPS, given that multiple contributing mechanism have been suggested in the literature [12,25], other clinical signs (eg, temperature differences) might derive from unrelated mechanisms. Further, as we have not compared the effects of serum IgG from patients with different clinical phenotypes (eg, patients with or without mechanical hyperalgesia), we cannot draw any conclusion as to whether the clinical signs in patients correlated with the behavioural abnormalities in the rodents. As we analysed only 2 out of 5–7 samples per group for cytokine concentrations, we may have missed significant differences in concentrations, but differences between groups in the tested samples were very small, so that this risk should be small. A pragmatic limitation for the model’s usability is that the time windows for significantly enhanced mechanical allodynia or paw swelling are relatively narrow.

In summary, in this first IgG-transfer-trauma model for CRPS, serum IgG from patients with long-standing CRPS induced clinical and laboratory features of the human disease, indicating a relevant role for IgG autoantibodies. The epitope specificities of the pathogenic autoantibodies remain unknown and require further research. Our results should be relevant to clinical research because they suggest the value of considering autoantibody-removing therapies for long-standing CRPS. The model may provide a novel tool to study both the mechanisms of CRPS and candidate therapies.

Conflict of interest statement

Dr. Goebel declares having received research support, consultancy or speaker honoraria for Pfizer, Axsome, Biotest, Baxter, CSL-Behring, Grifols, BPL. The other authors declare no conflict of interest.

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Appendix A. Supplementary data

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