Autoantibodies produce pain in Complex Regional Pain Syndrome by sensitizing nociceptors

Cuhadar, Ulku

Awarding institution: King’s College London

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Autoantibodies produce pain in Complex Regional Pain Syndrome by sensitizing nociceptors

Thesis submitted for the degree of
Doctor of Philosophy
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Ulku Cuhadar

Wolfson Centre for Age-Related Diseases
Institute of Psychiatry, Psychology & Neuroscience
King’s College London
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Abstract

Complex Regional Pain Syndrome (CRPS) is a severe chronic pain condition that typically develops after a trauma such as bone fracture or surgery. CRPS pain is typically confined to the injured limb and exceeds the intensity and duration that can be expected from the initiating trauma.

The neurophysiological basis of pain in CRPS is unknown and there are no effective therapies or diagnostic tests available. Plasma exchange has been shown to alleviate pain in long-standing CRPS patients in a small trial, indicating involvement of humoral factors.

The aim of my project was to determine the neurophysiological mechanisms responsible for pain and hypersensitivity experienced by CRPS patients using a translational mouse model. In this model, a paw incision is used as a minor experimental trauma in combination with passive transfer of IgG purified from CRPS patients or healthy control subjects.

Administration of IgG from CRPS patients, but not from healthy control subjects, exacerbated and prolonged the mechanical and thermal hypersensitivities produced by a paw incision, but did not alter tactile sensitivity to stimulation with von Frey filaments. Passive transfer of CRPS thus recapitulated the sensory profile observed in quantitative sensory testing studies of patients.

Electrophysiological studies of skin-nerve preparations identified an increased ectopic impulse frequency in CRPS-IgG treated mice, indicative of spontaneous pain or paraesthesias. Single-unit recordings in the same preparations revealed an increased evoked impulse rate in mechano-nociceptors, whereas low threshold mechanosensitive afferents appeared functionally intact. Single-unit recordings were made in saphenous afferents innervating the hairy, dorsal paw skin, to reduce the impact of post-surgical sensitization. The observed sensitization of nociceptors innervating the hairy skin is consistent with the limb-confined pain typical of CRPS.

Overnight incubation of isolated DRG neurons with CRPS-IgG, enhanced the Ca²⁺-response produced by a low concentration of K⁺ in a subpopulation of neurons, suggesting that
autoantibodies directly increased neuronal excitability. Immunohistochemical analysis revealed the presence of human IgG in glabrous skin of CRPS and HC-IgG injected mice, with a more pronounced staining or deposit around the incision. Together, these observations suggest that CRPS autoantibodies may act by binding to novel epitopes presented in the post-traumatic environment and that binding may stimulate release of mediators that diffuse locally. Alternatively, the trauma may trigger CRPS by facilitating extravascularization of autoantibodies, exposing peripheral tissues and nerves to higher local titers. In conclusion, my results demonstrate that passively transferred CRPS pain is caused by sensitization of nociceptors.
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CHAPTER 1 GENERAL INTRODUCTION
1.1 Somatosensation

The five senses distinguished and defined by Aristotle (384-322 BC) in *De Anima*, book II, are taste, vision, touch, olfaction and hearing. Humans use them to respond to stimuli coming from the outside world and from the surrounding environment. They play a critical and primitive role in the living survival, particularly when danger needs to be detected.

Changes in the external and internal environment are detected and the information conveyed to the central nervous system by peripheral sensory neurons. Different forms of noxious and innocuous mechanosensation such as touch, vibration, pressure but also thermosensation are all processed by the somatosensory nervous system. Spinal sensory neurons that participate in somatosensation have their cell bodies located in the dorsal root ganglia and project both to the spinal cord and to peripheral target organs. They are termed primary sensory neuron and are the only peripheral afferent neurons carrying sensory information to the spinal cord. By stimulation of its receptive field in the innervated target organ, e.g. the skin, the information is conducted to the spinal cord where the neuron forms synapses with second order neurons. The second order neuron is located within the spinal cord or brainstem and relays information to a third order neuron within the brain.

1.2 Primary afferent neurons

![Diagram of primary afferent neurons]

**Figure 1–1 Pseudo-unipolar neuron.**

The pseudo-unipolar sensory neuron has its cell body (soma) located inside the DRG from where an axon leaves and splits into two branches, a central branch that goes to the spinal cord level (CNS) to form synapses with other neurons, and a peripheral branch that forms the peripheral nerve terminals at the peripheral organ levels (i.e. skin, joint, muscle). Modified from Monica Schroeder/Science Source.
Primary afferent neurons of the somatosensory system have an unusual anatomy, they are pseudo-unipolar neurons with a T-junction: a single axon leaves the cell soma, which bifurcates and projects to both the spinal cord and the target tissue in the periphery (Figure 1–1). The cell body of the primary sensory neuron is contained within the DRG and within trigeminal ganglia (TG) when they innervate the head, the face and the neck region.

1.2.1 Sensory nerve modalities

The separation of sensory and motor functions in the spinal cord dorsal and ventral roots was a fundamental discovery attributed to Charles Bell (1811) and Magendie (1822). The idea of specificity of cutaneous sensation was formulated by Müller (1840) who established the doctrine of specific nerve energies (Norrsell et al., 1999). Müller suggested that the perception of a sense was not linked to how the sensory system was stimulated, but instead was explained by the specific activated pathway (Norrsell et al., 1999; Perl, 2007).

Later, Blix discovered that stimulation of separate localized points on the skin gave rise to distinct sensations of pressure, warmth, cold, and pain. His findings thus supported that the doctrine of specific nerve energies applies to the different cutaneous senses (Blix, 1884). Other investigators such as Goldscheider and Donaldson confirmed these observations. For instance, a cold spot on the skin would be activated by cold but not by heat and an electrical stimulation of the same cold spot would give a cold sensation and not a heat sensation (Norrsell et al., 1999). Max von Frey’s observations strongly supported this concept who further studied these “spots” by mapping them and measuring their density in various places on the body (Norrsell et al., 1999).

It is today known that different primary afferent neurons possess sensitivity to one or more specific sensory modalities. Nerve fibres are often classified according to the sensory modalities that they transduce and can be broadly categorised as mechanoreceptors, thermoreceptors and nociceptors (Zimmermann et al., 2009).

Transduction of sensory neurons requires activation by different chemical and physical stimuli that depolarize the sensory neurons by stimulating cation channels (such as TRP channels), inhibiting K+-channels (e.g. two-pore potassium channels (K2P)), and anion
channels (e.g. anoctamin 1, a Ca\(^{2+}\)-activated Cl\(^{-}\) channel (Yang et al., 2008)) and G protein-coupled receptors (GPCRs) located in the peripheral terminals (Dubin and Patapoutian, 2010). Some of these channels belong to the transient receptor potential (TRP) channels which are sensitive to temperature, osmolality, pH and wide array of endogenous and exogenous chemicals and metabolites (Julius, 2013). Acid-sensing ion channels (ASICs) are other transduction channels well known for sensing reduction in the extracellular pH (García-Añoveros et al., 2001). Finally, PIEZO2, the mechanosensitive ion channel plays a crucial role in mechanosensation. PIEZO2 knock out mice exhibit a profound loss of touch sensation (Ranade et al., 2014). Constitutive deletion of PIEZO2 leads to perinatal lethality (Dublin et al., 2012); an inducible strategy to delete PIEZO2 in adult mice was therefore used (Ranade et al., 2014). In humans mutations in PIEZO2 can result in selective loss of discriminative touch perception, ataxia and dysmetria (Chesler et al., 2016).

1.2.2 Primary afferent nerve fibres

In 1924, Erlanger and Gasser successfully recorded the compound action potential of a frog sciatic nerve (Erlanger and Gasser, 1930). After the nerve was stimulated electrically, several peaks of voltage were recorded through an oscilloscope. Each peak reflected the existence of fibres with different conduction velocities. They classified the fibres according to the nerve fibre diameter size and myelination level. The extent of myelination of the nerve fibre/axon determines its conduction velocity (Gasser; Purves et al., 2001).

\(A_\alpha\) fibres are very large diameter (15-20\(\mu\)m), thickly myelinated neurons that are responsible for proprioception in human. \(A_\beta\) fibres are large diameter (5-15\(\mu\)m), myelinated fibres that signal innocuous (non-painful) mechanical stimuli. \(A_\beta\) fibres have a fast conduction velocity which can vary across species and is \(~50\text{m/s}\) in humans and \(>10\text{m/s}\) in mice (Knibestöl, 1973; Koltzenburg et al., 1997).

\(A_\delta\) fibres are thinly myelinated, medium diameter (1-5\(\mu\)m) neurons and have a slower conduction velocity than \(A_\beta\) fibres. C-fibres are unmyelinated fibres with a slow conduction velocity \(<1.5\text{m/s}\) and are activated by thermal, chemical and mechanical stimulus (Figure 1–2).
Although, Aδ fibres and unmyelinated C-fibres are commonly described as nociceptors based on their responses to noxious stimuli, a proportion of Aδ and C-fibres, the D-hair and C-low-threshold mechanoreceptors display responses to innocuous stimuli (light touch) (Brown and Iggo, 1967).

Some recent findings however suggest that both D-hair fibres and CLMRs can influence or produce pain (Dawes et al., 2018; Habig et al., 2017; Urien et al., 2017).

Figure 1–2 Diagram illustrating the properties of different classes of primary afferent nerve fibres.

*Figure 1–2 Diagram illustrating the properties of different classes of primary afferent nerve fibres.*

*Ax and Aβ fibres are large diameter nerve fibres involved in proprioception and respond to light touch. Aδ fibres are lightly myelinated fibres and classified as nociceptors (Aδ-nociceptors) or non-nociceptors (D-hairs). C fibres are unmyelinated small diameter fibres qualified as nociceptors. C-low-threshold mechanoreceptors are responsive to light touch unlike other C-fibres. Adapted from Julius and Basbaum, 2001.*

1.2.3 Nociceptive versus non-nociceptive nerve fibres

Max von Frey demonstrated that non-damaging mechanical stimulation of certain spots on the human skin could induce a pricking, unpleasant sensation while at other spots the same stimulation would evoke simply a tactile, non-painful sensation (von Frey, 1894). Pressure and pain spots were carefully mapped and were shown to be different from each other and from cold and warm spots. A spot-like distribution of mechanically and thermally sensitive areas suggested that a specific sensory receptor (organ) existed beneath each spot. von Frey therefore suggested that nociception was not only relying on
the noxious stimulus, but also on the receptor that had to be a nociceptor; a ‘pain spot’ (von Frey, 1894).

Sherrington, using experimental animals (the dog) conducted several physiological experiments by applying different noxious and innocuous mechanical/thermal stimulus on various skin spots. He measured the reflex withdrawal of the animals in response to various stimuli and proposed the existence of the nociceptive neuron, a primary sensory neuron activated by stimuli that threaten or actually damage tissue (Sherrington, 1903, 1920).

Perl, Bessou, Brown and Iggo studied unmyelinated cutaneous nerve fibres in the 1960s. Perl recorded the activities of cutaneous nerve fibres in cats and primates, and identified nerves that were activated by noxious stimuli, but showed little or no activity to innocuous stimuli, the C-fibres (Perl, 1968a). These fibres had a higher mechanical threshold for activation than myelinated fibres, and some were temperature sensitive (Bessou and Perl, 1969; Brown and Iggo, 1967; Perl, 1968a). The reduced conduction velocity and higher activation threshold distinguished C-fibres from known classes of nociceptive and non-nociceptive myelinated fibres.

The different functional classes of sensory afferent nerve fibres have since been characterised in detail in the mouse and in the rat (Cain et al., 2001; Koltzenburg et al., 1997; Leem et al., 1993).

Two major classes of nociceptors have been characterized. The first includes medium diameter myelinated (Aδ) afferents that mediate acute, well-localized “first” or fast pain. These lightly myelinated fibres conduct action potentials at intermediate velocities compared to slow, unmyelinated C- and thickly myelinated non-nociceptive Aβ-fibres. C-fibres include small diameter nociceptors that convey, the “second”, slow pain (Zotterman, 1939). Since C-fibres are slower than Aδ fibres at conducting information, they are responsible for the second pain and the Aδ fibres for the first pain when a painful stimulation is for example applied onto an individual skin (Figure 1–3).
Figure 1–3 Fibres conduction velocity and link to the type of pain.

(A) Compound action potential recording from a peripheral nerve. The more a fibre is myelinated (larger its diameter is), the faster an action potential will be generated. Aα/Aβ fibres are thickly myelinated fibres which conduction velocities are higher than less myelinated Aδ or non-myelinated C-fibres. (B) Aδ and C-fibres account for fast (first) and slow (slow) pain. Modified from Julius and Basbaum, 2001

Fibres are differentiated based on their conduction velocities and adaptation properties, but in addition, they are associated with specialised endings in the skin that influences their adaptation and transduction properties (Iggo and Andres, 1982; Iggo and Muir, 1969).

Sensory neurons can be categorized functionally according to their mechanosensitivity as low-threshold mechanoreceptors (LTMRs), which respond to innocuous (non-painful) mechanical stimulation, and high-threshold mechanoreceptors (HTMRs). HTMRs respond to more intense, potentially harmful mechanical stimuli.

Mammalian skin can be divided into glabrous (non-hairy) skin and hairy skin (Figure 1–4). The glabrous skin presents four types of mechano-sensory end organs: Pacinian corpuscles, Ruffini endings, Meissner corpuscles and Merkel’s discs (Zimmerman et al., 2014), which play specific roles in mechanical sensation. Merkel cells, for instance, play a modulatory role during transmission of mechanical forces onto associated Aβ slowly adapting fibre endings via activation of PIEZO2 (Woo et al., 2015). Indeed, Woo et al. hypothesized that gentle pressure on the skin or hair deformation opens PIEZO2 in Merkel cells, which causes Merkel cell depolarization and subsequent activation of voltage-gated calcium channels and neurotransmitter release, which contributes to SA fibre discharge (Woo et al., 2015).
In contrast with the glabrous skin, fibres endings are longitudinal lanceolate around hair follicles in the hairy skin (Li et al., 2011) (Figure 1–4).

In both glabrous and hairy skin, noxious stimuli are detected by free nerve endings of Aδ and C-fibres found in the epidermis (Abraira and Ginty, 2013).

Aδ (AM) and C nociceptive fibres are functionally heterogeneous classes of fibres. Most of C-fibres can be activated by noxious mechanical stimulus (M) as well as by heat (H), cold (C) and chemicals because they are activated by different modalities, they are termed polymodal C-fibres (CMH, CMC, CMHC). Electrical search rather than mechanical search for the fibre localization allowed identification of silent fibres which are normally insensitive to mechanical and heat stimulus (CMH), but becomes sensitive to noxious mechanical and heat stimuli after being exposed to inflammatory agents (Meyer et al., 1991; Schmidt et al., 1995). Prato et al. showed that mechanoinsensitive silent nociceptors, characterized by the expression of the nicotinic acetylcholine receptor subunit alpha-3 (CHRNA3), were becoming mechanosensitive following a 24h exposure to NGF (Prato et al., 2017).

The detection of stimuli by nociceptors is based on membrane signalling molecules which convert the stimulus energy into a conformational change, leading to an alteration in ionic permeability and depolarization of the nerve terminal. Most transduction molecules are ion channels that are directly gated by the stimulus or by intracellular messenger systems activated by a variety of chemical substances (Belmonte and Viana, 2007). G-protein-coupled receptors such as the bradykinin receptor (BK2) are also known to mediate nociception. Activation of BK2 by bradykinin, an inflammatory factor, leads to membrane depolarization of the sensory neuron via interaction with voltage-gated ion channels (Burgess et al., 1989; Wang et al., 2006). Channels such as TRP channels or purinergic (P2X2, P2X3 and P2Y1) channels are known to be transduction channels modulating neuronal excitability following activation of the channels by their ligand (Treede, 1999). TRPV1 is a nonselective cation channel with pronounced permeability for Ca²⁺ ions. It is the main molecular substrate for the ability of polymodal nociceptors to respond to acid, heat, and certain pungent chemicals. Other TRP channels (TRPV2, TRPV3, and TRPV4) are also gated by temperature, with thresholds ranging from warm to very hot (>52°C)
temperatures (Julius, 2013). A recent study showed that acute noxious heat sensing in mice was depending on a triad of TRP ion channels: TRPM3, TRPV1, and TRPA1. Trpv1\(^{-/-}\)Trpm3\(^{-/-}\)Trpa1\(^{-/-}\) triple knockout mice lacked the acute withdrawal response to noxious heat that is necessary to avoid burn injury, while showing normal nociceptive responses to cold or mechanical stimuli and a preserved preference for moderate temperatures (Vandewauw et al., 2018).

An additional histamine sensitive C-fibre type that mediate itch sensation has been identified in the human skin (Schmelz et al., 1997). Histamine mediated itch is well treated by antihistamines. Most clinically important and challenging itch is independent of histamine. Although histamine induced itch can be blocked by histamine receptor antagonists, there are many other itch conditions where such blockers are ineffective to stop the itch, as the one arising from renal and liver diseases, atopic dermatitis or caused by some agents (e.g. chloroquine, an antimalarial drug) (Liu et al., 2009). Itch when extreme or prolonged can become unbearable and make patients refuse treatments as it is the case for patients treated for malaria with chloroquine (Mnyika and Kihamia, 1991). However, Mrgprs, a family of G protein-coupled receptors expressed only in peripheral sensory neurons have been shown to function as itch receptors (Liu et al., 2009). Mrgpr gene lacking mice display significant deficit in chloroquine induced itch but not histamine (Liu et al., 2009).

Mrgprs are expressed almost exclusively in neurons that are positive for tropomyosin receptor kinase A (TrkA+), the nerve growth factor (NGF) receptor, and that stained with the plant lectin IB4, a marker of nociceptors (Dong et al., 2001).

Beside electrophysiological characterization of the large class of unmyelinated C-fibres, recent transcriptomics approaches aimed for their molecular characterization. Usoskin et al. thus revealed 11 types of DRG neurons, three distinct low-threshold mechanoreceptive neurons, two proprioceptive, and 6 principal types of thermosensitive, itch sensitive, type C low-threshold mechanosensitive and nociceptive neurons with markedly different molecular and operational properties (Usoskin et al., 2015). The classification Usoskin et al. did not contradict a polymodal nature of C-fibre nociceptors, but it formally shows that discrete transcriptional states in different classes of unmyelinated polymodal neurons underlie distinct stimulus response (Usoskin et al., 2015). In vivo GCaMP (a genetically-
encoded fluorescent Ca$^{2+}$ indicator) optical imaging approach of sensory neurons was used to explore polymodal C-fibres in mice DRGs in response to painful stimuli by Emery et al. but also by Chisholm et al. Interestingly, Chisholm et al. demonstrate that the majority of primary afferents are polymodal with between 50–80% of thermally sensitive DRG neurons also respond to noxious mechanical stimulation (Chisholm et al., 2018) which is in contrast with Emery et al. finding demonstrating that neurons responsive to both mechanical and heat stimuli are restricted to ~10% of the DRG neurons (Emery et al., 2016).

The current scheme proposes that proprioception, C-LTMR, TrpM8-dependent cold detection, some forms of pruritus, and probably acute mechanical pain may be tuned to specific neuronal types relatively selectively, whereas other types of sensation are encoded by a broader integration of ensembles of neurons with different transcriptional states (Usoskin et al., 2015). A similar study using retrograde labelling of gut sensory neurons, transcriptomic profiling and unsupervised clustering of 314 colonic sensory neurons, revealed seven subtypes of colonic neurons (Hockley et al., 2019).

Further details about the primary sensory nerve fibres electrophysiological properties will be described in the results chapter.
Figure 1–4 The organisation of cutaneous mechanoreceptors in the skin.
(A) The glabrous skin contains nerve terminals which mediate touch by four types of mechanoreceptors. Merkel cells make synapse-like associations with enlarge nerve terminals branching from a single A\textsuperscript{β} fibre. A\textsuperscript{β} fibre end organs can be Pacinian corpuscles, Ruffini endings or Meissner corpuscles. Each end organ is specialised in sensing a different type of mechanical touch (i.e. stretching, stroke, vibration). The glabrous skin also has free nerve endings (A\textsuperscript{δ}-mechanoreceptor and C-fibres). (B) The hairy skin contains hair follicles involved in stimuli transduction. In the hairy skin LTMRs have longitudinal lanceolate endings around the hair follicles. A\textsuperscript{δ}-LTMRs (D-hair fibres) and C-LTMRs form lanceolate on zigzag and awl hairs, which are thin hairs. The longest hair type, guard hairs, is associated with touch domes at the apex and A\textsuperscript{β}-LTMR longitudinal lanceolate endings at the base. In contrast, HTMRs free nerve endings have their endings situated in the epidermis layer (Abraira and Ginty, 2013).

1.2.4 Central projections of primary afferent neurons

Sherrington, who suggested the existence of the nociceptor neuron, proposed a distinction between nociception and pain by conducting experiments using a decerebrate animal preparation. In these preparations, animal’s brain structures were removed, and the brain no longer received direct neuronal input from the spinal cord or brainstem trigeminal structures. Woodworth and Sherrington measured the pain behaviour in these preparations and suggested the essential contribution of the cortex to the perception of pain (Woodworth and Sherrington, 1904).

Indeed, the sensory neuron innervates the target organ, but also projects to the spinal cord to form connections with neurons contained within the spinal cord. Different fibre types project to different locations within the dorsal horn (Figure 1–5). The low threshold, rapidly conducting A\textsuperscript{β} afferents, which respond to light touch, project to deep laminae
(III, IV, and V). Aδ nociceptors project to lamina I as well as to deeper dorsal horn (lamina V). By contrast, C nociceptors project more superficially to laminae I and II (Basbaum et al., 2009).

Some responses to pain stimuli operate at the spinal cord level without requiring the brain input, it is referred as spinal reflex response. Nociceptors can synapse with interneurons which in turn synapse onto a motor neuron, all within the spinal cord and activate a motor reflex following e.g. an intense heat stimulus (Le Bars et al., 2018).

A more detailed study of C-fibre projection has been conducted and distinct projection patterns have been identified. Most peptidergic (CGRP/TrkA-expressing) C-fibres terminate within lamina I and the most dorsal part of lamina II, whereas IB4-binding and Ret-expressing DRG neurons, sensitive to the glial cell line-derived neurotrophic factor (GDNF), project most heavily to the inner aspect of lamina II (Snider and McMahon, 1998). These two types of neurons constitute two major classes of nociceptors.

![Figure 1–5 Central projection patterns of primary afferent fibres.](image)

**Figure 1–5 Central projection patterns of primary afferent fibres.** All primary afferent neurons have their cell body within the DRG or TG and axons innervating target organs (fibre endings) but each fibre type projects and forms synapses in the spinal cord at various level. Peptidergic C-fibre central projections occur at level I and II in the dorsal horn and nonpeptidergic C-fibres at level II only. Aδ fibres synapse at level I and IV within the dorsal horn in the spinal cord whereas Aβ fibres synapse with neurons at level III, IV and V with the dorsal horn.

### 1.3 Inhibition of pain transmission

Patrick Wall and Ronald Melzack were the first to propose the concept of “the gate control of pain” in 1965. They suggested that physical pain was not the only result of activation of nociceptive fibres, but rather the perception of pain was modulated by interaction
between excitatory and inhibitory interneurons in the dorsal horn (Melzack and Wall, 1965).

Indeed, pain seems to be lessened when the injured (noxious) area is rubbed (innocuous). Fast innocuous signals reach the dorsal horn before noxious signals (both non-nociceptive and nociceptive are likely activated by most noxious mechanical stimuli), the fast afferent may synapse onto inhibitory interneurons which in turn synapse on projections from nociceptors, thereby inhibiting input from to projection neurons (Melzack and Wall, 1965). A more recent study conducted by Arcourt et al. explores the role of LTMRs and nociceptors in pain signalling. Using optogenetics they reveal that tactile sensory input via LTMRs has an analgesic effect on A-fibre nociceptors mediated acute pain (Acourt et al., 2017).

Prolonged noxious stimuli can induce pain hypersensitivity by central sensitization. Central sensitization represents an enhancement in the function of neurons and circuits in nociceptive pathways caused by increases in membrane excitability and synaptic strength as well as to reduced inhibition (inhibitory neurons). Central sensitization produces pain hypersensitivity by changing the sensory response elicited by normal inputs (Latremoliere and Woolf, 2009; Woolf, 1983, 2011; Woolf et al., 1988). More recent studies used single-cell RNA sequencing to classify neurons and non-neuronal cells throughout the nervous system. According to unbiased principle component analysis, the dorsal horn contains 15 inhibitory and 15 excitatory subtypes of neurons that can be defined by their molecular expression patterns. The existence of these neurons subtype were confirmed in situ in the spinal cord (Häring et al., 2018).

The periaqueductal grey (PAG) in the midbrain and the rostral ventromedial medulla (RVM) are two important areas of the brain involved in descending inhibitory modulation, capable of activating a powerful analgesic effect. Both these centres contain high concentrations of opioid receptors and endogenous opioids (Morgan et al., 1992). Descending pathways project to the dorsal horn and inhibit pain transmission. These regions engage descending noradrenergic and serotonergic systems that can enhance or attenuate nociceptive inputs at the spinal level (Ossipov et al., 2014). Loss of descending inhibition may explain chronification of pain in certain patient (Ossipov et al., 2014).
1.4 Inflammatory, neuropathic and nociplastic pain

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (International Association for the Study of Pain). As defined by the IASP, pain can have different origins and is different from nociception. Nociception refers to the peripheral and central nervous system (CNS) processing of information about the internal or external environment, as generated by the activation of nociceptors (Animals, 2009). IASP defines nociception as the neural process of encoding noxious stimuli. The consequences of encoding may be autonomic or behavioural (motor withdrawal reflex or more complex nocifensive behaviour). Pain sensation is then not necessarily implied (International Association for the Study of Pain).

Also, the terms nociceptive and neuropathic pain are used to differentiate the type of pain. Nociceptive pain is described as pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors with a normally functioning somatosensory nervous system, such as the pain arising from a damaged skin (e.g. burn), muscle (e.g. strain) or bone (e.g. bone fracture). In contrast, neuropathic pain is described as pain caused by a lesion or disease of the somatosensory nervous system. Neuropathic pain can be central when the pain is caused by a lesion or disease of the central somatosensory nervous system or peripheral (IASP).

A third and more recent type of pain has been added to the IASP taxonomy in 2017, the term “nociplastic pain”. The term has not reached wide acceptance, since it is essentially not used in the literature yet. Nociplastic pain refers to pain that arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing the pain. This term can help describe the pain underlying different chronic pain conditions that cannot be covered by the terms "nociceptive pain" or "neuropathic pain (e.g. chronic low back pain or irritable bowel syndrome).

1.4.1 Inflammatory pain

Pain is frequently a consequence of inflammation, which is a localized reaction that produces redness, warmth, swelling as a result of infection (bacterial or viral), irritation (UV-light, chemicals, etc), or injury. Surgery, bone fracture, cuts or burns generate
inflammatory nociceptive pain that can last for up to 6 months. If the pain persists for more than 6 months, it is referred as chronic pain.

Inflammation around the damaged area causes sensitisation of peripheral nerve afferents. Nerve endings find themselves exposed to products of tissue damage and inflammation, sometimes referred as “inflammatory soup” (Figure 1–6). Important mediators or factors of this inflammatory soup include extracellular protons, arachidonic acid, lipid metabolites, serotonin, bradykinin, nucleotides and NGF, which may sensitize sensory nerve endings by acting at receptors or on ion channels (Figure 1–6) (Julius and Basbaum, 2001; Basbaum et al., 2009; Yam et al., 2018).

The inflammatory mediators can be exogenous or endogenous. LPS of gram-negative bacteria (endotoxin) acts as an exogenous inflammatory mediator and initiates an inflammatory response in infected tissues via binding to the TLR4 receptor and activation of monocytes, dendritic cells, macrophages and B cells, which mediate secretion of pro-inflammatory cytokines (Abbas et al. 2012). Although mostly expressed on immune cells, TLR4 receptor is also expressed on human and murine non-immune pancreatic β-cells (Garay-Malpartida et al., 2011). Interestingly, LPS has been also shown to trigger strong defensive responses in the mice airways. LPS mediates an immediate intracellular Ca²⁺ in airway epithelial cells via a TLR4-independent but TRPV4-dependent mechanism (Alpizar et al., 2017).

In contrast, endogenous mediators of inflammation are not produced from foreign exterior factors (e.g. bacteria), but are instead secreted from immune cells (e.g. mast cells) or other non-immune cells (e.g. ATP release from keratinocytes (Burrell et al., 2005; Moehring et al., 2018)).

Bradykinin and prostaglandin are two examples of endogenous inflammatory mediators, often used to study nociception and signalling in vivo and in vitro.

Bradykinin, physiologically released by mast cells, when applied to primary sensory nerve terminals or cultured sensory neurons, produces immediate membrane depolarization as well as sensitization to other noxious, or even innocuous stimuli by activating G-protein-coupled (BK2) receptors that in turn interact with voltage-gated and ligand-gated ion channels (Burgess et al., 1989; Wang et al., 2006).
Prostaglandin E2 (PGE$_2$) is produced by cyclooxygenase (COX) enzymes and with the action of microsomal (mPGES-1, mPGES-2) and cytosolic PGE (cPGES) synthases in response to inflammation both in peripheral inflamed tissues and in the spinal cord (Zeilhofer, 2005).

COX-2 is induced in inflammatory cells by pro-inflammatory, cytokines and growth factors (Nakanishi and Rosenberg, 2013) and contributes to the synthesis of PGE$_2$ and peripheral sensitization by binding to G-protein-coupled receptors (PGE2Rs) (Sugimoto and Narumiya, 2007). There are four subtypes of PGE2 receptors, EP$_1$, EP$_2$, EP$_3$ and EP$_4$. All four subtypes of PGE$_2$ receptors belong to a large superfamily of G-protein-coupled, seven-trans-membrane domain receptors (GPCRs) (Kawabata, 2011). EP$_1$ receptors are coupled to heterotrimeric $G_{q/11}$ protein, and the agonist stimulation of EP$_1$ receptors causes activation of phospholipase C, which in turn produces inositol trisphosphate (IP$_3$) and diacylglycerol (DG), followed by cytosolic calcium mobilization and activation of protein kinase C (PKC) (Kawabata, 2011). EP$_2$ and EP$_4$ receptors are coupled to $G_s$ protein, and the agonist stimulation of those receptors activates adenylyl cyclase, which generates cyclic AMP (cAMP) (Sugimoto and Narumiya, 2007; Kawabata, 2011). Finally, EP$_3$ receptors are coupled to $G_i$ protein (Kawabata, 2011). PGE$_2$ signalling can modulate both excitatory and inhibitory neurotransmission by increasing the responsiveness of peripheral nociceptors that generate excitatory glutamatergic transmission, and by disinhibition of dorsal horn neurons that are relieved from inhibitory glycineergic transmission (Zeilhofer, 2005). Nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g. aspirin and ibuprofen) inhibit the activity of COX enzymes and are administered to reduce pain in patients.

Nociceptive nerve fibres can release neurotransmitters, i.e. substance P, CGRP from their peripheral terminals when activated by noxious stimuli (Maggi, 1995). Substance P and CGRP release from the peripheral terminal induce vasodilation and plasma extravasation (leakage of proteins and fluid from postcapillary venules), as well as activation of many non-neuronal cells, including mast cells and neutrophils and vascular tissue. This phenomenon is known as neurogenic inflammation. In the central nervous system, loss of spinal neurons that possess the substance P receptor (SPR) attenuate pain and hyperalgesia produced by capsaicin, inflammation, and nerve injury (Khasabov et al., 2002; Li et al., 2015).
Inflammation at site of injury.

The illustration shows inflammatory mediators released at the site of tissue injury, which activate response in primary afferent nociceptor. Some of the main “inflammatory soup” are shown, including peptides (bradykinin), lipids (prostaglandins), neurotransmitters (serotonin (5-HT) and ATP) and neurotrophins (NGF). These factors sensitize or excite the terminals of the nociceptor by interacting with receptors expressed at the neuron surface. Activation of the nociceptor not only transmit afferent messages to CNS (the spinal cord dorsal horn and then the brain), but also initiates the process of neurogenic inflammation. This is an efferent function of the nociceptor whereby release of neurotransmitters, SP and CGRP, from the peripheral terminal induces vasodilatation and plasma extravasation of non-neuronal cells (mast cells and neutrophils). Modified from Julius & Basbaum, 2001; Yam et al., 2018.

1.4.2 Neuropathic pain – general definition

The word "neuropathy", used since 1827, refers to nerve dysfunction/damage and abnormal nerve activity. Neuropathy can lead to chronic pain or to loss of sensation. Neuropathic pain is a direct consequence of a lesion or disease affecting the somatosensory system. Neuropathic pain is one of the highly debilitating chronic pain conditions, for which, currently, there is no therapeutic treatment. According to a large-scale European study, 19% of 46,394 participants were suffering from chronic pain (>6 months) (Breivik et al., 2006) and according to the International Association for the Study of Pain (IASP), 7–10% adults suffer from chronic neuropathic pain. Often it is described as a shooting, burning, stabbing pain or “pins and needles” feeling. Patients often experience spontaneous pain, evoked-pain such as allodynia if non-painful stimuli (e.g. light touch)
provoke pain and/or hyperalgesia if a stimulus that normally provokes pain is inducing an intensified pain feeling.

1.4.2.1 Nerve injury

Nerve damage can occur as a consequence of a traumatic injury or can be initiated by particular infections, metabolic diseases, and by certain pharmacological agents (e.g., chemotherapy).

A major consequence of nerve damage is the onset of neuropathic pain characterized by allodynia and hypersensitivity in partially denervated regions (Gilron et al., 2015).

Wallerian degeneration refers to the process by which axons distal to the site of injury are broken down and reabsorbed by immune cells. This process is associated with the release of inflammatory mediators, many of which act on afferents to modulate ion channel properties and afferent excitability (Wu et al., 2001).

Patients with neuropathic pain are naturally heterogeneous in etiology, pathophysiology, and clinical appearance (Baron et al., 2017). They exhibit a variety of pain-related sensory symptoms and signs (sensory profile), which might indicate different classes of neurobiological mechanisms and require different type of approaches for treatment (Baron et al., 2017). Using 13 quantitative sensory testing parameters, the authors subgrouped a large group of patient with neuropathic pain of different etiologies (Baron et al., 2017). Three distinct subgroups with characteristic sensory profiles were identified and replicated. Cluster 1 (sensory loss, 42%) showed a loss of small and large fibre function in combination with paradoxical heat sensations. Cluster 2 (thermal hyperalgesia, 33%) was characterized by preserved sensory functions in combination with heat and cold hyperalgesia and mild dynamic mechanical allodynia. Cluster 3 (mechanical hyperalgesia, 24%) was characterized by a loss of small fibre function in combination with pinprick hyperalgesia and dynamic mechanical allodynia (Baron et al., 2017).

Various animal models of peripheral nerve injury have been established in order to study the underlying mechanism for neuropathic pain. Among them, there is the spared nerve injury (SNI) model, a classic partial denervation model (Decosterd and Woolf, 2000a) which produces robust mechanical allodynia within one postoperative day that lasts at least 28 days (Howard et al., 2005) and that is used extensively to explore the mechanisms
responsible for neuropathic pain. In the SNI model the spared nerve injury model, a lesion of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) is involved, leaving the remaining sural nerve intact (Decosterd and Woolf, 2000). The SNI model resulted in early (<24 h), prolonged (>6 months) and robust behavioural modifications. The mechanical sensitivity to von Frey hairs and thermal (hot and cold) responsiveness was increased in the ipsilateral sural and to a lesser extent saphenous territories, without any change in heat thermal thresholds (Decosterd and Woolf, 2000b). Many other animal models of nerve injury have been developed. They all differ in the anatomical position of the inflicted nerve damage, but also in the symptoms and sensory change they cause. Each model has advantages and disadvantages. They are known as the partial sciatic nerve injury (PNI) (Seltzer et al., 1990), the spinal nerve ligation (SNL) (Ho and Mo, 1992) and the sciatic nerve chronic constriction injury (CCI) (Bennett and Xie, 1988).

1.4.2.2 Diabetic peripheral neuropathy (DPN)

Diabetes is one of the largest global health-care problems of the 21st century. The number of people with diabetes worldwide is predicted to double between 2000 and 2030, reaching a pandemic level of 366 million people (Hossain et al., 2007; Zimmet et al., 2014). Diabetic neuropathy is one of the most common diabetic complication (Vinik et al., 2013). It is a leading cause for disability due to subsequent foot ulceration and amputation (Volmer-Thole and Lobmann, 2016).

Diabetic peripheral neuropathy is a well-known example of peripheral neuropathy associated with pain, hyposensitivity or hypoesthesia and sometimes with spontaneous pain. Between 25% and 50% of patients with peripheral diabetic neuropathy develop neuropathic pain (Juster-Switlyk and Smith, 2016; Themistocleous et al., 2016). Patients describe a range of sensory symptoms, which may include loss of pain sensation or tingling, “pins and needles” sensation, burning, “electric shocks”, allodynia, or hyperalgesia (Juster-Switlyk and Smith, 2016). Sensory loss is a major clinical problem, since it may lead to ulcers, foot complications and amputations. The pathogenesis of diabetic peripheral neuropathy is complex and is marked by both metabolic and vascular factors (Cameron et al., 2001). Hyperglycemia is one of the metabolic events known to cause axonal and microvascular injury. Themistocleous et al. showed that no correlation was found between intraepidermal nerve fibre density and
severity of pain in the cohort of patient they studied (Themistocleous et al., 2016). The pathophysiological basis of neuropathic pain in diabetes is complex and poorly understood.

Diabetic mice models have been induced in order to study the mechanisms of the disease. Injection of streptozotocin (STZ) to mice leads to destruction of insulin producing cells in the mice and to generation of diabetic mice. The sensory nerve fibre properties showed an impaired nociception characterized by hyposensitivity to noxious mechanical and thermal stimuli with impaired C-fibre and A-fibre response in single-fibre recording experiments (Lennertz et al., 2011). However, STZ generates peroxynitrite, which produces sensory abnormalities by stimulating TRPA1 directly (Andersson et al., 2015). As a consequence, sensory loss is established within an hour of STZ administration, 2-3 days before the mice become diabetic. Therefore, more refined models, like the Ins2+/Akita mouse should be used for studies of sensory neuropathy. The Ins2+/Akita mouse model, a hereditary model of diabetes, was used by Vastani et al. in order to investigate sensory neuropathy in diabetes. Akita mice exhibited hyposensitivity to noxious mechanical and heat stimulation in vivo and electrophysiological recordings of single nerve fibres showed a reduced activity in mechanonociceptors while low-threshold A-fibres activity were unaffected (Vastani et al., 2018).

1.4.2.3 Human immunodeficiency virus (HIV)-induced neuropathy

Distal symmetric neuropathy (DSP) characterized by axonal degeneration is one of the main neurologic complications in individuals living with HIV. HIV patients can experience pain that starts in the toes and spreads proximally; the pain frequently is described as burning or aching and is worse on the soles.

HIV acts on sensory neurons in an indirect way by infecting macrophages and glial cells (Moss et al., 2015). Gp120 is a glycoprotein expressed on the surface of the HIV envelope. Application of culture media from gp120-exposed bone marrow derived macrophages induced a significant reduction in DRG neurite outgrowth. In addition, gp120 significantly increased the expression of 25 cytokine-related genes in primary bone marrow derived macrophages, some of which have been implicated in other painful polyneuropathies. An interaction between gp120 and the C-C chemokine receptor type 5 (CCR5) expressed on macrophages seemed to exist as inhibition of the CCR5 receptor with a pharmacological
antagonist (maraviroc) inhibited gp120-induced tumour necrosis factor-alpha gene expression (Moss et al., 2015). Gp120 has been shown to induce peripheral neuropathic pain when put in contact with the rat sciatic nerve (Herzberg and Sagen, 2001). Treatment of rat sciatic nerve with gp120 induced a persistent mechanical hypersensitivity with a decreased intraepidermal nerve fibre density and macrophage infiltration (Wallace et al., 2007). Keswani et al. designed a model where a transgenic mouse expressing the HIV coat protein gp120 under a GFAP promoter is used. Expression of gp120 in these mice is under the control of GFAP promoter. As astrocytes and Schwann cells are the main cells expressing GFAP, gp120 is thus expressed and released by astrocytes in the CNS and Schwann cells in the peripheral nervous system (PNS). The rodents presented peripheral neuropathy characterized by distal degeneration of unmyelinated C-fibres (Keswani et al., 2006).

1.4.2.4 Chemistry induced neuropathic pain

Chemotherapy-induced peripheral neuropathy (CIPN) is a common side effect of some chemotherapeutic agents. Patients usually present with sensory disturbances, which range from mild tingling sensations to spontaneous burning pain and hypersensitivity to stimuli (Park, 2014). The pain induced by chemotherapeutic drugs may be severe enough for the patients to decline treatment or to limit the therapeutic dose that can be administered. The prevalence of CIPN is found to be 68.1% within the first month of chemotherapy treatment, 60.0% at 3 months, and 30.0% at 6 months (Seretny et al., 2014). In the cancer cell, vincristine prevents microtubule aggregation, whereas paclitaxel prevents microtubule disaggregation, an effect leading to cancer cell division arrest and cell death. Oxaliplatin and cisplatin bind to nuclear DNA (deoxyribonucleic acid) of cancer cells, causing disruption of DNA replication and RNA (ribonucleic acid) transcription and subsequent arrest of cancer cell division (Starobova and Vetter, 2017). However, the mechanisms that lead to the development of neuropathic pain induced by chemotherapeutic drugs are unknown. Hence, chemotherapeutic drugs such as oxaliplatin, cisplatin, taxanes or vincristine have been used to establish translational rodent model of CIPN for mechanistic studies.
1.5 Autoimmune pain

For many years, pain has been broadly categorized into two main classes, neuropathic and nociceptive pain (Millan, 1999). However, it is becoming increasingly clear that some pain conditions cannot be readily placed in either of these categories (Klein et al., 2012; Wigerblad et al., 2016). Pain caused by autoimmune processes has recently attracted attention from clinicians and scientists since recent studies have demonstrated that some chronic pain conditions such as rheumatoid arthritis and neuromyotonia are associated with autoimmunity, but not with classical signs of inflammation or neuropathy (Klein et al., 2012; Wigerblad et al., 2016).

The recent discovery of antibodies directed against citrullinated proteins (ACPA) in rheumatoid arthritis (RA) or the identification of autoantibodies targeting voltage-gated potassium channel (VGKC) complexes in patient with neuromyotonia lead to the emergence of the notion of “autoimmune pain” in the pain field.

In rheumatoid arthritis (RA) e.g., an absence of discernible nerve injury, persistence of pain even when inflammation is well controlled and the chronic nature of the pain in certain patients indicated a distinct pathophysiological basis for pain explained by the presence of ACPA (Wigerblad et al., 2016).

Some patients with neuromyotonia experience pain as a clinical feature while others experience myotonia or epilepsy. Recent findings identified autoantibodies to leucine rich glioma inactivated 1 (LGI1) and contactin-associated protein-like 2 (CASPR2), both associated with VGKC complexes, in patients with neuromyotonia. Klein et al. showed that CASPR2-IgG significantly associates with pain and further in vivo and in vitro studies demonstrated the mechanisms of pain induced by LGI1 and CASPR2 autoantibodies (Dawes et al., 2018; Watanabe, 2016).

In this section, autoimmune pain and autoimmune conditions associated with chronic pain will be introduced together with autoimmune pain animal models and discussion of potential mechanisms responsible for pain.

Several autoimmune diseases are reproducible in rats or mice by human patient autoimmune IgG administration, passive transfer, to the animal. Human autoantibodies recognize the mouse or rat epitope as a result of the high antigen epitope homology between humans and other mammalian species. Thus, the first identification of an
autoantibody mediated pathology using this approach, demonstrated that myasthenia
gravis could be passively transferred from patient to mouse (Toyka et al., 1975).

1.5.1 Autoantibody mediated pain

1.5.1.1 Rheumatoid arthritis (RA)

In rheumatology, assays for the detection of ACPA are commonly used for the diagnosis of rheumatoid arthritis (RA) (Aggarwal et al., 2009). One of the most dominant symptoms of RA in patients is chronic joint pain. Joint pain (arthralgia) regularly develops before the signs of joint inflammation (swelling joints) and persists during clinical treatment of RA patients using anti-inflammatory/anti-rheumatic drugs (Bos et al., 2010; Steenbergen et al., 2015; Wigerblad et al., 2016). Therefore, it seems that these patients have pain that is independent of inflammation.

Wigerblad et al. suggested that ACPA are actually responsible for the induction of pain in RA patients (Wigerblad et al., 2016). To determine the role of ACPA in RA, mice were injected with human ACPA purified from RA patients, with murinised ACPA or human healthy donor antibodies. Both human and murinised ACPA induced long-lasting (28 days) pronounced pain, without signs of inflammation, whereas non-ACPA human IgG/control monoclonal IgG were without effect. In parallel, *in vitro* mouse osteoclast were cultured and treated with antibodies and the supernatant analyzed. ACPA mediated activation of osteoclasts and release of the nociceptive chemokine CXCL1. Protein citrullination by peptidylarginine deaminases (PADs) is essential for osteoclasts differentiation. Citrullination is a post-translation modification that involves the enzymatic conversion (deimination) of protein-contained arginine residues. Although citrulline is a common metabolite present throughout the human body, it is a non-standard amino acid, which means that it cannot be incorporated into proteins during protein synthesis (Vossenaar et al., 2003). Citrulline-containing proteins can only be generated through post-translational modification of arginine residues, a reaction that is catalysed by peptidylarginine deiminase (PAD) enzymes. The loss of a positive charge may cause structural or functional alterations and while the modification has been linked to several diseases including RA, its physiological or pathophysiological roles remain largely unclear.
Activated osteoclasts release the pronociceptive chemokine CXCL1 (mouse analogue of the human IL-8) that also promotes osteoclast activation, thereby establishing an autocrine positive feedback loop (Krishnamurthy et al., 2016). IL8-dependent osteoclast activation may constitute an early event in the initiation of the joint specific inflammation in ACPA-positive patients via enhanced osteoclast activity, but IL-8 also produces pain and hypersensitivity by acting directly on nociceptors (Krishnamurthy et al., 2016; Wigerblad et al., 2016). RA inflammation-independent pain is initiated by ACPA before the disease onset and is later followed by joint/bone destruction (Krishnamurthy et al., 2016).

1.5.1.2 Neuromyotonia

Patients with neuromyotonia present with clinical and electrophysiological evidence of excessive motor unit activity due to enhanced motor axon excitability which causes spontaneous muscle activity. A proportion of patients with neuromyotonia also present with the pathophysiological symptoms of Morvan’s syndrome, insomnia, epilepsy, myotonia and pain. The pathology is produced by autoantibodies that bind to VGKC or to associated protein that directly complex with shaker-related, voltage gated potassium channels (Kv1) themselves, i.e. LGI1 or CASPR2 (Irani et al., 2010; Klein et al., 2012; Watanabe, 2016). A common feature described in patients seropositive for anti-CASPR2 antibodies is the presence of pain. CASPR2 is required for trafficking or membrane insertion of fast potassium channels (Kv1). Dawes et al. showed that immune or genetic mediated ablation of CASPR2 induces and enhances pain behavior in mice as well as excitability of DRG neurons through regulation of Kv1 channel expression at the cell membrane (Dawes et al., 2018). Thus, autoantibody mediated internalization of CASPR2 is thought to lead to hyperexcitability. Patient anti-CASPR2 autoantibodies bind in vivo but do not cause overt inflammation or substantial damage to the nervous system. In summary, anti-CASPR2 antibodies produce pain without inducing any inflammation or nerve damage, a novel pathophysiological mechanism responsible for pain.
Introduction to Complex Regional Pain Syndrome (CRPS)

1.6.1 CRPS – A historical view

Complex Regional Pain Syndrome (CRPS) is a painful condition characterized by limb-confined sensory, autonomic, motor and trophic impairment that develops after a traumatic event i.e. bone fracture (Goebel, 2011; Marinus et al., 2011). The hallmark of CRPS is regional pain, which is disproportionate to the expected clinical course of the original trauma or lesion (Harden et al., 2007).

Alexander Denmark, a British surgeon was the first physician to describe CRPS by publishing a case report of a soldier wounded by a bullet in his upper arm during the siege of Badajoz in 1812. Denmark reports that “the wound soon healed” but also writes: “I always found him with the forearm bent and in supine position and supported by the firm grasp of the other hand” (Denmark, 1813).

He describes in detail his observations and the type of pain the patient experiences, which is reported as: “It was a burning nature, [...], and so violent as to cause a continual perspiration from his face”.

A few decades later, during the American Civil War, Silas Weir Mitchell reports many other cases of gunshot wounds and limb nerve injuries. In 1864, George Morehouse, William Keen, and Weir Mitchell, wrote and published “Gunshot Wounds and Other Injuries”. The authors describe the signs and symptoms of causalgia (CRPS) in great detail (Mitchell et al., 1982). The symptoms observed in the patients led Mitchell to coin the term causalgia from the Greek, “Kausos” (heat) and “algos” (pain). Many individual cases were reported. One example is the case of a 27 year old soldier shot in his left arm. Two years after his injury, the wound had healed completely, but Mitchell reported that skin of the palm was eczematous, thin, red, and shining. The second and third phalanges of the fingers were flexed and stiff; whereas the first was extended. The finger nails on the hand of the affected arm were extraordinarily curved, laterally and longitudinally, except that of the thumb. Pain was still present in the median distribution, but much less prominent than in the ulnar tract, where it was extreme. The pain is a “burning” type of pain and can be described as the symptoms of what is today called CRPS (Mitchell et al., 1982).
Later, other clinicians such as Paul Sudeck, Rene Leriche and James A. Evans studied CRPS and contributed to its definition based on clinical signs and symptoms (Sudeck, 1900; Leriche, 1916; Evans, 1946). Evans coined the term “reflex sympathetic dystrophy (RSD)”, which has been the most common name to describe this medical condition during the late 20th century before the term was replaced by CRPS.

In 1994, IASP consensus conference agreed to abandon several widely used names for the condition in favour of CRPS (Harden et al., 2010). Previously, CRPS was referred to as RSD, Sudeck’s atrophy, algodystrophy, algoneurodystrophy, causalgia, shoulder-hand syndrome and Steinbrocker syndrome (terminology used in the 50s) (Iolascon et al., 2015).

The term RSD was considered problematic as it refers to a “reflex” impairment, which if involved is complicated and not fully characterized. The assumed “sympathetic”/autonomic changes may not be causative pain component and may not be physiologically involved throughout the entire course of the condition in every patient. Finally, the actual “dystrophy” is only present in ~15% of cases (Harden et al., 2013).

1.6.2 CRPS today

1.6.2.1 Diagnostic criteria

The pathophysiological mechanisms responsible for CRPS, and other pain syndromes, are not fully understood. The diagnosis of CRPS is based purely on clinical signs and symptoms. In 1994, IASP produced consensual agreement on the first diagnostic criteria of CRPS.

CRPS is subdivided into two types, CRPS I and CRPS II. CRPS II is associated with nerve injury while patients with CRPS I do not exhibit demonstrable nerve lesions (Table 1-1) and both present similar diagnostic features. Sensory profiles suggest that CRPS I and CRPS II may represent one disease continuum with comparable underlying mechanisms (Gierthmühlen et al., 2012).

Although, CRPS I excludes neuropathic mechanisms, Oaklander et al. tested the hypothesis that CRPS I was associated with distal degeneration of small-diameter axons.
18 adults with IASP-defined CRPS I were studied for quantitative sensory testing (QST) and quantification of epidermal neurite densities in skin biopsies from affected ipsilateral and contralateral limbs. Axonal densities were diminished at the CRPS-affected sites of 17/18 subjects, on average by 29% \((p<0.001)\) (Oaklander et al., 2006).

### Table 1-1 IASP CRPS Diagnostic Criteria

<table>
<thead>
<tr>
<th>CRPS I</th>
<th>CRPS II</th>
</tr>
</thead>
<tbody>
<tr>
<td>With 2,3, and 4 being mandatory:</td>
<td>All of the following:</td>
</tr>
<tr>
<td>1. The presence of an initiating noxious event, or a cause of immobilization.</td>
<td>1. The presence of continuing pain, allodynia, or hyperalgesia after a nerve injury, not necessarily limited to the distribution of the injured nerve.</td>
</tr>
<tr>
<td>2. Persistent pain, allodynia, or hyperalgesia, which is disproportionate to the initial event.</td>
<td>2. Evidence at some time of oedema, changes in skin blood flow, or abnormal sudomotor activity in the region of the pain.</td>
</tr>
<tr>
<td>3. Evidence at some time of oedema, changes in skin blood flow, or abnormal sudomotor activity in the region of the pain.</td>
<td>3. This diagnosis is excluded by the existence of conditions that would otherwise account for the degree of pain and dysfunction.</td>
</tr>
<tr>
<td>4. This diagnosis is excluded by the existence of conditions that would otherwise account for the degree of pain and dysfunction.</td>
<td></td>
</tr>
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</table>

The terminology “warm” and “cold” CRPS are also used in clinics and refer to CRPS based on symptoms, \(i.e.\) skin temperature difference between the affected and unaffected limb (Bruehl et al., 2016). To classify patients into presumed warm and cold subtypes, clinicians only use temperature asymmetry in the limbs. Bruehl et al. studied patients with warm and cold CRPS in order to confirm the existence of these two CRPS subtypes and suggested unbiased statistical evidence in a large clinical sample for warm CRPS and cold CRPS subtypes (Bruehl et al., 2016). The results indicated that skin color, skin temperature, and edema indicated a much greater role for inflammatory mechanisms in warm CRPS. Overall, inflammatory characteristics were significantly elevated in the warm CRPS subtype only at initial assessment within approximately 5 months of CRPS onset, diminishing significantly over the subsequent 3 months to levels statistically indistinguishable from cold CRPS (Bruehl et al., 2016; Harden et al., 2010). In addition, it
seemed that the median pain duration was significantly (p<0.001) shorter in the warm CRPS (4.7 months) than in the cold CRPS subtype (20 months), with no difference in the pain intensity (Bruehl et al., 2016).

Although the 1994 IASP diagnostic criteria improved the diagnosis of CRPS, they were extremely sensitive and lacked specificity, which was potentially leading to over-diagnosis (Harden et al., 2010). Hence non-CRPS neuropathic conditions were in some cases identified as CRPS. Therefore, another IASP consensus meeting was held in Budapest in 2003 in order to discuss the existing issues and improve the IASP CRPS Diagnostic Criteria. The resulting improved diagnostic consensus criteria were adopted and referred to as the “Budapest Criteria” (Table 1-2) and are those currently used clinically. The Budapest Criteria improved diagnostic consistency between clinicians and resulted in higher specificity (less frequent misdiagnosis of CRPS) (Bruehl et al., 2016).

Table 1-2 Current IASP clinical diagnostic criteria for CRPS also called “Budapest Criteria”

| 1) Continuing pain, which is disproportionate to any inciting event. |
| 2) Must report at least one symptom in three of the four following categories: |
| • Sensory: Reports of hyperalgesia and/or allodynia. |
| • Vasomotor: Reports of temperature asymmetry and/or skin color changes and/or skin color asymmetry. |
| • Sudomotor/Edema: Reports of edema and/or sweating changes and/or sweating asymmetry. |
| • Motor/Trophic: Reports of decreased range of motion and/or motor dysfunction (weakness, tremor, dystonia) and/or trophic changes (hair, nails, skin). |
| 3) Must display at least one sign* at time of evaluation in two or more of the following categories: |
| • Sensory: Evidence of hyperalgesia (to pinprick) and/or allodynia (to light touch and/or deep somatic pressure and/or joint movement). |
| • Vasomotor: Evidence of temperature asymmetry and/or skin color changes and/or asymmetry. |
| • Sudomotor/Edema: Evidence of edema and/or sweating changes and/or sweating asymmetry. |
| • Motor/Trophic: Evidence of decreased range of motion and/or motor dysfunction (weakness, tremor, dystonia) and/or trophic changes (hair, nails, skin). |
| 4) There is no other diagnosis that better explains the signs and symptoms. |

*A sign is counted only if it is observed at time of diagnosis.
1.6.2.2 Sensory abnormalities in CRPS

Quantitative sensory testing (QST) is a non-invasive method to assess and quantify sensory nerve function. It is used by clinicians for diagnosing peripheral nervous system disorders such as neuropathies and other chronic pain conditions and provides important clinical measures for translational mechanistic studies. This technique determines the perception threshold of light touch or pressure, vibration, cold and warm temperatures and pain (Siao and Cros, 2003). The German Research Network on Neuropathic Pain (DFNS) has developed a standardized QST battery that investigates different aspects of sensory afferent nerve fibre function. Gierthmühlen et al. conducted a study according to the DFNS protocol to determine the patterns of sensory signs in CRPS using QST. Quantitative sensory testing of CRPS patients, has identified a markedly increased sensitivity to noxious mechanical pressure in combination with an increased sensitivity to noxious cold and heat (Gierthmühlen et al., 2012). In another QST study, acute CRPS I patients presented warm and cold hyperalgesia with significant change in heat and cold, pain and detection, thresholds. Patients with chronic CRPS showed deteriorated cold and heat detection thresholds (Huge et al., 2008). An observational cross-sectional multicentre study, the Pain in Neuropathy Study (PiNS) aimed for the neurological examination, QST, nerve conduction studies, and skin biopsy for intraepidermal nerve fibre density assessment of 191 patients with diabetic neuropathic pain (Themistocleous et al., 2016). Also, it seems that the sensory phenotype of patient with diabetic peripheral neuropathy is characterised by hyposensitivity to applied stimuli that is more marked in the moderate/severe neuropathic pain group than in the mild neuropathic or no neuropathic pain groups. Loss of sensitivity in CRPS is a symptom shared with small-fibre neuropathies (Themistocleous et al., 2016).

1.6.2.3 Acute versus chronic CRPS

In most cases, CRPS is triggered by a limb injury such as fracture or surgery (Goh et al., 2017). The injury or trauma leads to inflammation, which is part of the expected physiological response. In CRPS, an exaggerated inflammation induces a persistent oedema, vasodilation and temperature changes in the affected limb. The evolution of the clinical signs over time defines the chronic or acute nature of CRPS. Indeed, in most of the patients the clinical signs as well as the pain resolve within 6 to 13 months of onset (Bean
et al., 2014; Grieve et al., 2017). However, in a subset of CRPS patients (15-20%), patients fail to recover, and individuals will develop a long-term disability, negatively affecting their quality of life. It is referred as chronic or long-standing CRPS. In this case, the sudomotor and vasomotor symptoms associated with CRPS such as oedema and vasodilatations typically resolve, whereas the pain remains persistent and becomes chronic (Grieve et al., 2017). The reasons why CRPS manifests as an acute or chronic condition are not understood. The lack of clear evidence of tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing the pain in chronic CRPS I suggest that chronic CRPS I pain can be described as nociplastic pain (IASP).

1.6.2.4 Epidemiology

Epidemiological studies about the incidence of CRPS in the general populations are limited. An American population-based study quantified the prevalence of CRPS I in the Olmsted County, Minnesota using IASP criteria for diagnosing CRPS. The incidence was rated as 5.46 per 100,000 people. Females were affected four times more than males, with a median age of 46 years at onset (Sandroni et al., 2003).

A European epidemiological study conducted in the Netherlands, indicated that the incidence of CRPS was 26 for 100,000 people. Females seemed to be affected at least three times more often than males (ratio: 3.4:1) (de Mos et al., 2007).

In both American and European studies, females were more affected and postmenopausal woman appeared to have higher risks of developing CRPS. In addition, both studies showed that upper limbs were affected more frequently than lower limbs, and limb fracture was the most common triggering event (de Mos et al., 2007; Sandroni et al., 2003).

1.6.2.5 Treatments

The pharmacological approach used to treat CRPS patients is mainly symptomatic, including analgesics such as ketamine (Russo and Santarelli, 2016; Sheehy et al., 2015), glucocorticoids (Atalay et al., 2014; Munts et al., 2010) and bisphosphonates but there is not sufficient evidence to recommend their use in practice (Brunner et al., 2009). Non-
steroidal anti-inflammatory drugs (NSAIDs), showed no value in treating patients with CRPS (Breuer et al., 2014). Physical therapy is also employed in preventing atrophy and contractures in order to reduce the need for analgesic therapy and to facilitate rehabilitation. Hydrotherapy pools seem to have a positive pain relief effect on CRPS patients (Schencking et al., 2009). Case reports of more invasive approaches include stimulation of the spinal cord (Crapanzano et al., 2017; Herschkowitz and Kubias, 2018) and insertion of peripheral nerve catheters with anaesthetics such as ketamine (Zhao et al., 2018). In the most severe cases and mostly on patient demand, as no treatment is efficient for pain relief, limb amputations are performed to improve patient function (Kashy et al., 2015). However, a systematic review conducted by Bodde et al. about amputation in CRPS I patients highlighted that studies did not clearly delineate the beneficial and adverse effects of an amputation (Bodde et al., 2011).

Clinical outcomes are very heterogeneous and pharmacological treatments do not provide satisfactory relief for most patients, making CRPS pain a particularly challenging pain condition to manage clinically (Resmini et al., 2015). There is a critical lack of high-quality evidence for the effectiveness of most therapies for CRPS. A study conducted on a population of well-established chronic CRPS patients suggested low resolution rates even with specialty pain care (Schwartzman RJ et al., 2009). In one large (n=102) retrospective longitudinal study of patients over an average six-year follow-up period, 30% of patients reported resolution of chronic CRPS, 16% reported progressive deterioration, and the remaining 54% reported stable symptoms (De Mos M et al. 2009).

1.6.2.6 CRPS an autoimmune pain condition?

Plasma exchange (PE) therapy of CRPS patients

Plasma exchange (PE) therapy (Figure 1–7) or plasmapheresis was first explored in CRPS patients in a retrospective study (Aradillas et al., 2015) and in a series of case reports (Goebel et al., 2014). Interestingly, 30 of the 33 patients demonstrated significant (p<0.01) median pain reduction of 64% following the initial series of PE. The success of PE on long standing chronic CRPS patients hints at a humoral component inducing pain in those patients.
The origin of CRPS is unknown but previous studies suggested an autoimmune contribution to its development. Goebel et al. hypothesised that some CRPS cases may have a post infectious and/or autoimmune basis (Goebel et al., 2005).

Figure 1–7 Plasma exchange therapy. A patient blood is extracted from one arm using a blood pump and mixed with anticoagulant. The plasma separator separates the plasma from the whole blood. In one hand the plasma is pumped out (plasma pump) and on the other hand, it is replaced with a substitution fluid (i.e. saline solution or isotonic human albumin solution) before being returned to the patient. The waste contains the plasma proteins such as albumin, immunoglobulin, enzymes, ions, etc).

Earlier animal studies of CRPS were based on tibial fracture of rat limb (Guo et al., 2004; Wei et al., 2016a). The fracture and immobilization of the injured paw induces phenotype changes such as hind paw allodynia, warmth, oedema, which were associated with expression changes in neuropeptide signalling molecule substance P (SP), SP receptor, inflammatory mediators TNFα, IL-1β, IL-6 and nerve growth factor (NGF) within the first 4 weeks following the injury in hindpaw skin.

Using the fracture/cast CRPS model, Guo et al. suggested that serum antibodies from fracture mice can induce regionally restricted pain behaviours in B-cell deficient fracture mice. Serum IgM antibodies from wild type (WT) fracture mice had pro nociceptive effects in the fractured limb when injected into B-cells and antibodies lacking µMT fracture mice (Guo et al., 2017).

This model does not use any CRPS patient IgG and focuses on rat IgM antibodies. Therefore, the fracture/cast model lacks the translational value that present model of human IgG transfer.
Finally, Tékus et al. showed that some symptoms of CRPS such as mechanical hypersensitivity, swelling and redness on the injured limb area observed in CRPS patients could be passively transferred to mice (Tékus et al., 2014). However, sensitivity to noxious heat and cold were not different between CRPS and control groups. In their study, Tékus et al. purified IgGs from plasma obtained after plasma exchange of a CRPS patient (Figure 1–7). The rest of the plasma which contained other immunoglobulins (IgA, IgM and IgE) and plasma proteins, but not IgG, did not induce hypersensitivity in mice (Tékus et al., 2014). Only IgG produced a detectable pain in the mice.

**Intravenous immunoglobulin (IVIG) treatment**

IVIG treatment involves administration of a highly purified globulin preparation obtained from the pooled plasma of thousands of healthy donors. Although initially given as replacement therapy for patients with primary and secondary immunodeficiency states, IVIG has proven to be effective in the treatment of various autoimmune and inflammatory disorders (Dalakas, 2004; Lünemann et al., 2016; Sherer et al., 2002; Zuercher et al., 2016). Several hypotheses have been proposed to explain the underlying biological mechanism of the treatment.

Preliminary works suggested that low-dose IVIG treatment to CRPS patients ameliorates their condition. Indeed, a study conducted in a small group of 12 patients sample showed that the average pain intensity was 1.55 units lower (95% CI, 1.29 to 1.82; P < 0.001) after IVIG treatment than after saline. In 3 patients, pain intensity after IVIG was less than after saline by 50% or more (Goebel et al., 2002, 2010).

Fc region neutralization of the autoantibodies by the variable region of the injected IgGs is one possible explanation for this phenomenon (Figure 1–8). The Fc region can trigger inflammation via binding the Fc receptor on immune cells (Fridman, 1991), leading to sensitization/activation of intact nociceptors by inflammatory mediators (Ren and Dubner, 2010).

IVIG has been shown to be effective in various autoimmune diseases such as in Guillain-Barre polyneuropathy, an autoimmune disease where the myelin sheath that surrounds the axons is degraded (Arnson et al., 2009; Kaveri et al., 1991).
A more recent randomised placebo-controlled Phase III multicentre trial, in which patients with long-standing complex regional pain syndrome (the LIPS trial) received IVIG treatment, demonstrated that the therapy was not effective in relieving pain in patients with moderate to severe CRPS of 1–5 years’ duration (Goebel et al., 2017).

The lack of efficacy of IVIG treatment in long-standing CRPS patients might suggest that the mechanism of action of CRPS autoantibody is Fc region independent. The reason that CRPS autoantibodies may act without causing tissue destruction is not known, but it is possible that they cause internalisation of target proteins, as is thought to be the case for CASPR2 antibodies (Dawes et al., 2018).

Figure 1–8 Structure of immunoglobulin G (IgG) and mode of action of the IVIG.
(A) Schematic representation of an IgG. The IgG has a non-variable region, which is the Fc region. The Fc region binds to its receptor once an immune-complex (IgG-antigen complex) is formed to initiate innate immune reaction. Fc receptors are expressed on the surface of immune cells, including mast cells, neutrophils and macrophages. (B) Patient IgG neutralisation at its Fc region by the IVIG.

The specificity of pathological autoantibodies in CRPS has not yet been determined and the molecular targets for CRPS autoantibodies remain unknown. However, some previous work suggested neural surface marker specificity of the CRPS autoantigens (Kohr et al., 2009) whereas a more recent study suggests the presence of activating autoantibodies against alpha1a adrenoceptors in longstanding CRPS patients (Dubuis et al., 2014). Nevertheless, both studies have been conducted either in in vitro cultured cardiomyocytes or in SH-SY5Y cells, which restrict the epitopes only to the one present on these cells. A more rigorous, unbiased method may be required for an accurate
identification of the possible epitopes recognized by the CRPS patients IgG. Indeed, identification of the autoantibodies responsible for the pathology would allow better informed, more refined strategies to be employed in the study of CRPS and would facilitate identification of new therapies.

1.7 Aims and objectives

Despite the highly debilitating nature of CRPS, this pathology remains an enigmatic pain disorder with unknown neuropathophysiological mechanisms. The aim of the study was to identify the neurophysiological mechanisms of CRPS pain using a translational mouse model of CRPS.

The first goal of this study was to establish a translational and relevant mouse model of CRPS. In order to do that, CRPS-IgG was transferred to a paw skin-muscle incision trauma mouse model (Brennan, 1999; Tékus et al., 2014). CRPS-IgG injected and control mice were studied for their behavioural response to various modalities (Chapter 3). We hypothesised that CRPS-IgG transfer from CRPS patient to mouse would induce sensitization of the mouse to noxious stimuli.

Following the in vivo studies and validation of the model, the in vitro skin-saphenous nerve recording technique was used to determine the origin of CRPS pain at the nerve level. Single-unit nerve fibre properties were studied in order to identify neurophysiological abnormalities responsible for, or caused by, CRPS (Chapters 4 & 5). We hypothesised that CRPS-IgG would affect the properties of at least one class of nociceptor fibre.

In addition, to determine whether IgG act by directly affecting the excitability of sensory neurons, the effect of IgG on depolarization induced [Ca^{2+}]-responses was examined (Chapter 6).
CHAPTER 2  MATERIAL AND METHODS
2.1 Animals

Experiments were performed on female C57Bl/6J mice (8-10 weeks old). Most of the experiments conducted in our laboratory and in the field are done on C57Bl/6J young adult (8-10 weeks old) mice. IgG donor patients (section 2.2) were mainly females we therefore used female mice. Mice were obtained from Envigo UK Ltd., Bicester, UK, and were housed in a temperature-controlled environment on a 12h light/dark cycle with access to food and water ad libitum. 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. No animals have been excluded from analysis. n=6/group was used after an a priori sample size calculation. Behavioral studies described in section 2.4 (except for 2.4.3) were conducted by Clive Gentry.

2.2 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. She had signs in all four Budapest diagnostic categories (Harden et al., 2010) 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 (Huge et al., 2008; Gierthmühlen et al., 2012), 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 (15/NW/0467, North West – Haydock Research Ethics Committee (see Appendices)). She received three plasma exchange treatments over 5 days through a central venous line, but unfortunately then developed line-related complications and
treatment was stopped. 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 (Aradillas et al., 2015; Goebel et al., 2014; Schwartz et al., 2016).

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 (-80°C) available from participants in the recently-completed LIPS-trial (Goebel et al., 2014). A research technician (Serena Sensi) 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, 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 (see Appendices)).

Serum does not contain human cells and is therefore not considered as “relevant material”, as defined by the Human Tissue Act (2004).

2.3 IgG purification

Purified IgG were provided by Andreas Goebel (Liverpool, UK). As previously described (Tékus et al., 2014), IgG fractions were prepared using protein G beads (Sigma-Aldrich, Gillingham, UK). Briefly, serum was diluted 1:3 with phosphate-buffered saline (PBS), passed through a protein G column, and the non-bound fraction (‘flow-through’) was collected and retained. The bound IgG was eluted using 100 mM glycine pH 2.3, the pH was adjusted to 7.4 using Tris pH 8 and then dialysed overnight at 4°C in PBS 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 to 8–9 mg/mL, either by dilution with PBS or by further dialysis against a sucrose solution (Sigma-Aldrich). The purified IgG was sterile filtered using syringe-driven 0.2μM filter units (Millipore, Watford, UK), stored at 4°C and used within 3 months.
2.4 Mouse injection and incision

Mice were injected intraperitoneally with 0.8-16mg of IgG from healthy control subjects or CRPS patients on 4 consecutive days at day -1 (1 day before the incision), day 0 (the day of incision) and on day 1 and 2. Using aseptic techniques, a midline incision 0.5 cm long is made through the plantar skin fascia starting 0.2 cm from the heel and extending towards the toes. The underlying plantar muscle is elevated with curved forceps, leaving the muscle origin and insertion intact and incised longitudinally. The skin incision is then closed using sutures and the animals housed on paper bedding for the first 3 days post-surgery.

2.5 Behavioural studies

2.5.1 Cold and heat sensitivity

Before any nociceptive testing, the mice were kept in their holding cages to acclimatize (10-15 min) to the experimental room. Thermal sensitivity was assessed using a commercially available 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 and each hind paw in turn was placed onto the surface of the plate. The end point was taken as the withdrawal of the paw and recorded as the withdrawal latency for the ipsilateral and the contralateral paw. A maximum cut-off of 30 seconds was used for each paw.

2.5.2 Mechanical sensitivity

2.5.2.1 von Frey

Tactile allodynia was assessed by measuring withdrawal thresholds to calibrated von Frey hairs (0.008–2g). Animals were placed into a Perspex chamber with a metal grid floor, giving access to the underside of their paws and allowed to acclimatize before the start of the experiment. von Frey hairs were applied perpendicular to the mid plantar surface of the hind paw with sufficient force to cause slight bending against the paw and held for a few seconds. This was repeated 5 times at intervals of 1–2s. A positive response was noted if the paw was sharply withdrawn or there was flinching upon removal of the hair.
If no response was noted a higher force hair was tested and the filament producing a positive response recorded as the threshold.

2.5.2.2  Randall-Selitto/paw pressure

Paw withdrawal thresholds (PWT) were determined in the hind paws of both ipsilateral and contralateral hind limbs using the Randall-Selitto paw pressure technique (Randall and Selitto 1957). The analgesymeter (Ugo Basile, Italy) employs a conical shaped probe. Cut-off was set at 150 grams and the end point is taken as withdrawal of the hind paw. Data are expressed as withdrawal thresholds in grams.

2.5.3  Advanced Dynamic Weight Bearing (ADWB)

The distribution of body weight between the paws was monitored using the ADWB instrument designed by Bioseb (Vitrolles, France). The test allows the evaluation of the behavioural impact of CRPS-IgG on freely behaving animals. The instrument consists in a box with an embedded matrix at the bottom that comprises 2000 high precision force sensors and a camera at the top. To acquire data, the mouse is put in the box where the animal is free to move as it pleases. The force sensors measure the weight distribution on each of the four paws of the animal in grams. The animal is filmed from above using a camera. The video feed is analyzed in real-time later thanks to a tracking software allowing a precise analysis of the animal’s posture. The weight the mice put on their hind paws was expressed as weight ratio (ipsilateral/contralateral).

2.6  Assessment of post-surgical-recovery

Photos were taken of the injured ipsilateral hind paw from CRPS-IgG injected (n=5) and incision-only (n=7) mice after behavioural reading (paw pressure test on ipsilateral and contralateral paws), using a camera. Mice were scored blindly by seven individuals for the healing level of the paw around the incision level. Participants were asked to use the following codes. 0: looks normal (the incision has healed, no redness), 1: can see the incision, not fully healed, 2: redness associated with a bad healing. The scores and the difference in the paw pressure reading (contralateral-ipsilateral) were correlated using a linear correlation function.
2.7 Skin-nerve recording

The mammalian skin nerve preparation was developed by Peter Reeh in the 1980’s (Reeh, 1986) and later adapted for mice (Koltzenburg et al., 1997). In comparison to other widely used techniques, such as recording from single units in vivo or human microneurography, the technique allows experimental control over the external variables which could interfere with the physiology of the receptors. Using the in vitro skin nerve preparation allows the receptive fields of individual units to be directly stimulated by electrical, mechanical, thermal and chemical stimuli in a non-invasive, controlled manner. In addition to this, there are no obvious time related changes in respect to excitability and spontaneous activity and the preparation can be used for recordings up to 8 hours from the time of dissection. Many fibres are spontaneously active immediately after the dissection and therefore the preparation should be left in the organ bath for at least one hour before carrying out recordings, by which time no spontaneous activity remains.

2.7.1 Skin-Saphenous nerve dissection

Mice were sacrificed by cervical dislocation. The hind paw was shaved prior to dissection. 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% O₂ and 5% CO₂) prewarmed synthetic interstitial fluid (SIF buffer): 108mM NaCl, 3.5mM KCl, 0.7mM MgSO₄.7H₂O, 26.2mM NaCO₃, 1.65mM NaH₂PO₄.2H₂O, 1.53mM CaCl₂, 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 from the organ bath through a small gap to an adjacent recording chamber on a mirror platform. Desheathed and filamented saphenous nerve was covered with paraffin oil for electrical isolation and fine filaments were placed on a fine gold wire-recording electrode using a microscope (Figure 2–1).
2.7.2 Determining conduction velocity of individual fibres

Mechanically sensitive units were characterized by their conduction velocity, calculated by dividing the conduction distance over the electrical latency for the spike (Figure 2–2). According to the conduction velocity obtained from the recording, the fibre was identified as an Aβ (velocity>10m/s), an Aδ (1.2<velocity<10m/s) or a C-fibre (0<velocity<1.2m/s). Electrical latency for each single-fibre was obtained using a stimulator microelectrode placed perpendicularly onto the most sensitive spot of the receptive field. Once in contact with the receptive field of the fibre, a constant current stimulator (Digitimer DS2) delivered a square-wave electrical pulse of 1ms duration, every 2 or 4 seconds (depending on whether an A or C-fibre was being stimulated).
Figure 2–2 Electrical stimulation of fibre. The fibre receives an electrical stimulus before to generate action potential. The response time is defined as latency and measured in millisecond (ms).

2.7.3 Mechanical stimulation

The stimulating probe, equipped with a force transducer, was placed onto the most sensitive spot within the receptive field. The stimulator was controlled by Spike2 program from Cambridge Electronic Design (CED), the force was entered in mV in Spike2, which was automatically delivered by the stimulator. Stimulus response function for the fibre was obtained by following a mechanical stimulation protocol.

2.7.3.1 Mechanical threshold

The mechanical threshold for each unit was determined by applying a 2s mechanical stimulation with a step waveform onto the most sensitive spot of the receptive field. Increasing and decreasing levels of force (g) were applied iteratively until the mechanical threshold could be identified. The threshold was determined as that force required to evoke at least two action potentials.

2.7.3.2 Step protocol

A 10s long mechanical stimulation comprised between 0 and 20g of amplitude was applied in order to characterize more precisely the type of fibre according to the adaptation property stimulation response shape. Increasing force levels of 0.5g, 1g, 2g, 4g, 5g, 10g, 15g and 20g were applied with a 2 minute gap before each new step to let the fibre recover with minimal tachyphylaxis. Forces between 0.5g-20g cover a range of...
forces that are innocuous and noxious, i.e. forces capable of stimulating low threshold mechanoreceptors (LTMRs) and high threshold mechanoreceptors (HTMRs).

2.7.3.3 Ramp protocol

Unlike LTMRs, AM- (Aδ-) and CM-HTMRs fibres encode the applied force with a linear increase in the evoked impulse rate. Therefore, ramp stimuli were also applied on AM and CM fibres after the series of step stimuli. 0.5g, 1g, 2g, 4g, 5g, 10g, 15g and 20g ramps of 15s were applied. The number of action potentials and the temporal profile were analysed in order to characterize the fibre adaptation and discharge properties to increasing force stimulus.

All the recordings and analysis were performed using the Spike2 CED.

2.7.4 Data acquisition

Action potentials were recorded using a WPI (World Precision Instrument) low-noise head stage DAM80 AC differential amplifier with a ground connection which amplified the signal (gain of $10^4$). The signal was filtered with a low pass (300Hz) and a high pass (10kHz) filter. The differential amplifier was connected to a CED 1401 data acquisition board. The output of the signal was visualised on an oscilloscope, recorded on a PC and made audible via a speaker. All recordings were performed using the Spike2 software from Cambridge Electronic Design.

2.7.5 Data analysis

Raw data were analysed using Spike2, which was also used during the data acquisition phase. A template was defined manually by choosing one single spike among all the recorded spikes. The entire recording was scanned with the template to identify and select matching waveforms. The identified waveforms were displayed simultaneously in a separate event-viewer window (Figure 2–3). To finish, results were displayed in in a separate channel to allow curation and validation of the selected events as shown Figure 2–3.
Figure 2–3 Template settings. A spike is defined as a template and helps spikes discrimination.

To determine and visualise discharge rates, the channel was duplicated, and the channel draw mode was changed for number of event/second (Hertz) to visualize discharge rates as histogram (Figure 2–4).

Figure 2–4 Force steps and responses. In green are the fibre responses to the applied forces, shown as action potentials. In blue are the spikes corresponding to the action potentials matching to the template after screening. Histograms represent fibre impulse frequency (events/sec or Hertz).
2.7.6 **Analysis of mechanical response**

To identify action potentials, the period during which mechanical stimuli were applied was scanned for events. To do so, two vertical cursors were placed to determine the time frame of the recording to analyse. Cursor (1) was placed a few seconds before the start of the mechanical stimulus and the other cursor (2) was placed a few seconds after the end of the stimulus (Figure 2–5). The selected period was scanned for events matching the templated defined for the select unit. The force output was exported as numerical text data into an Excel spreadsheet. Cursor (1) corresponded to the start time and cursor (2) to the end time (seconds). The output sample rate was set as 10Hz and the waveform interpolation method was “as drawn”.

Using an existing script, Spike2 generated an output window, which indicated the number of impulses occurring during each second as 1-second bins (Figure 2–5). The duration of a step stimulus was 10s and of ramp stimulus 15s but respectively 11s- and 16s-time windows were selected in order to include action potentials occurring just at the end of a stimulus.

The output was exported to a Microsoft Excel spreadsheet for basic analysis, and graphs were plotted using PRISM GraphPad. Statistics were done using GraphPad and SPSS. Data were tested for normality and analysed accordingly with Mann-Whitney or two-sided t-test (mechanical threshold).
Figure 2–5 Analysis of mechanical responses using Spike2.
The first upper channel shows the spikes of interest (in blue). The second channel is a representation of spikes as events/s. The first channel shows the force applied onto the receptive field of the fibre. The last channel shows the original fibre activity recording. The right window indicates the number of impulses occurring during each second as 1-second bins. The cursors (1) and (2) on the left window determine the period scanned for events.

2.8 Human-IgG staining

Mouse hind paw glabrous skin was removed and immersion-fixed with paraformaldehyde for 24h. The tissue was then processed to paraffin wax; 6μm thick sections were cut and mounted onto Superfrost Plus slides. Sections were deparaffinised and rehydrated to water before blocking endogenous peroxidases. After rinsing in buffer, sections were incubated overnight at room temperature with biotinylated goat polyclonal antibody to human IgG (dilution 1:5000, Abcam, ab98561). One slide was incubated in buffer containing no antibody, as negative control. After further buffer rinses, sections were incubated with streptavidin Biotin- HRP Complex (VectorlabsPK6100). Sites of Antigen-antibody interaction were visualised using in-house DAB preparations. Nuclei were counterstained using Gill's No. 1 Haemalum before slides were dehydrated, cleared in xylene and coverslipped using DPX mountant.
Slide images were captured using a Zeiss Axioskop bright-field microscope and Zeiss Axiovision software.
2.9 ELISA

Human IgG ELISA Kit ab195215 (Abcam) was used to measure mouse plasma IgG concentration. Plasma was prepared from whole blood of mice that had been injected intraperitoneally with 8mg human IgG daily on four consecutive days, using heparin treated tubes. The collected plasma was frozen at -20°C before use according to the provided instructions from the manufacturer.

2.10 Dorsal root ganglion (DRG) neuron culture and imaging

Dorsal root ganglion neurons were prepared from adult C57Bl/6J mice. Animals were killed by cervical dislocation, as approved by the United Kingdom Home Office, and DRGs were removed from all levels of the spinal cord using aseptic methods. DRGs were incubated in 0.25% collagenase in serum-free MEM (Invitrogen, Paisley, UK) containing 1% penicillin and streptomycin for 3 hours at 37°C in a humidified incubator gassed with 5% CO₂ in air. This was followed by 20-minute incubation with 0.25% trypsin in MEM. The DRGs were then dissociated mechanically via trituration with flame polished Pasteur pipettes to obtain a suspension of single cells. Trypsin was removed by addition of 10ml MEM (containing 10% FBS) followed by centrifugation at ~168 x g (1000 revolutions min⁻¹) for 10 minutes. The pellet, containing the DRGs, was re-suspended in MEM containing 1% penicillin and streptomycin, 10% FBS and 0.05% DNase. The cell suspension was then centrifuged through a 2ml cushion of sterile 15% bovine albumin in MEM at ~168 x g (1000 revolutions/min) for 10 minutes. The pellet, containing the neurons, was then re-suspended in an appropriate volume of MEM containing 10% FBS, 50ng/ml NGF and 10μM cytosine arabinoside to prevent/reduce the growth of non-neuronal cells. The neurons were then plated at a high density, ~80% of confluency, onto the centre of sterile 13mm glass coverslips previously coated with 10μg/ml poly-D-lysine. Cultures were maintained at 37°C in a humidified incubator gassed with 5% CO₂ and cells were studied 12 to 24 hours after dissociation.
2.11 Calcium imaging

2.11.1 Imaging of intracellular calcium ion levels

2.11.1.1 Fura-2

Fura-2 (Invitrogen), a UV excitable ratiometric calcium indicator dye was used to measure changes in \([\text{Ca}^{2+}]_i\). The excitation spectrum for Fura-2 changes upon binding calcium ions, emission measured at >510nm increases when the dye is excited at 340nm and decreases at 380nm excitation. Cells were loaded with the acetoxyethyl (AM) ester version of the dye which allows Fura-2 to pass across cell membranes by passive diffusion. Intracellular esterases then cleave the ester bonds once the dye is inside the cell, yielding a relatively membrane-impermeant acidic form of the dye. For all calcium imaging experiments the cells or neurons were loaded with Fura-2 for 1-2h prior to experimentation. Fura-2 was loaded in physiological extracellular solution supplemented with 0.01% pluronic acid and 1mM probenecid. Pluronic acid is included to promote solubilization of Fura-2, and probenecid prevents Fura-2 being extruded from the cell by inhibiting organic-anion transporters in the plasma membrane.

2.11.1.2 Microscope-based imaging of intracellular calcium ion levels

Neurons were plated onto 13mm glass cover slips which formed the base of the perfusion chamber (volume~0.5ml). The chamber was mounted on to the stage of an inverted microscope (Nikon Diaphot) and cells were visualized using a 10x Fluor objective with a numerical aperture of 0.5. Test solutions were applied to cells by local microperfusion of solution through a fine tube placed very close to the cells being studied. The temperature of the superfusate was controlled using a Peltier device connected to a feedback regulated power supply (Marlow Industries, model SE5010) with the temperature measured at the orifice of the inflow tube. Neurons were perfused with solutions (gravity fed) supplied from one of 8 reservoirs. Neurons were distinguished from non-neuronal cells by a final depolarising challenge with a solution containing 50mM KCl, which evoked a calcium ion influx through voltage gated calcium channels.
Fura-2 signals were measured using RatioMaster Fluorescence Microscopy System (PTI). Cells were excited by light generated by a xenon-arc lamp which was passed alternately through one of two monochromonators (DeltaRam high speed monochromator, PTI) to transmit light of the pre-selected wavelengths (340nm and 380nm, ± 2nm). The emitted light was filtered by a long pass optical filter (>510nm) and captured by a cooled CCD camera (PTI CoolOne). Exposure length was equal for each excitation wavelength and determined by the user to ensure adequate signal without saturation of the camera (typically 100-400msec).

PTI ImageMaster software served as the user interface during the experiments to monitor the fluorescence emission intensity ratios at 340nm/380nm excitation and was also used to select individual neurons of interest for analysis. The ratio time-base data was exported to Microsoft Excel (Microsoft) for further analysis and then into Origin (Origin Pro, version 9.1) for graphical representation of the results.

2.11.1.3 Extracellular solution (ECS)

ECS was prepared with 140mM NaCl, 5mM KCl, 10mM glucose, 10mM HEPES, 2mM CaCl₂, and 1mM MgCl₂ and buffered to a pH of 7.4 using (NaOH).

2.11.1.4 Statistical tests

Chi² (χ²) test were performed in Microsoft Excel. Kruskal-Wallis test (comparison of non-normally distributed multiple groups) was followed by Dunn’s multiple comparison test using GraphPad.

2.12 Statistical tests

Data are expressed as mean ± SEM of the number of animals (Chapter 3), nerve fibres indicated (n) (Chapter 4 & 5) or number of neurons (n) (Chapter 6). Behavioural data were analysed using unpaired t-test or ANOVA (followed by Tukey’s) as appropriate. Electrophysiological data (Chapter 5) were analysed by Mann-Whitney U test, since the mechanical response thresholds and impulse frequencies are not normally distributed. Intracellular calcium imaging data were analysed by Mann-Whitney U test, since neuronal responses are not normally distributed and by Dunn’s multiple comparison test when
more than two groups (non-normally distributed) were tested. A \( \chi^2 \) test was used to test the number of cold responders. Statistical tests were performed in SPSS 24 (IBM).

An \textit{a priori} sample size calculation has been done to determine the number of animals required for the behavioural study. Data from previous experiments using the paw pressure test in other experimental pain models, indicate that the minimum reduction in paw pressure test required for meaningful results is 15g. Assuming that naïve mice respond to paw-pressure with an average threshold of 105g and an SD of 6.6, a minimum of 5 mice is required to detect a reduction of 15g with a statistical power of 0.9 and allowing for a type I error rate of 5%.
CHAPTER 3  PASSIVE TRANSFER OF CRPS PAIN FROM HUMAN TO MOUSE
3.1 Introduction

CRPS is a chronic disease condition with unknown cause and pathophysiological basis. There are no well-established experimental models of CRPS, but a small number of studies have explored the mechanisms responsible for a CRPS-like condition in a tibial-fracture model. In these studies, the rat right distal tibial bone was fractured and the hind limb fixed in a cast for 4 weeks before subjecting mice to behavioural nociceptive tests (Guo et al., 2004). Although in this model mice developed painful hypersensitivities in their injured limb, it is unclear whether the fracture model shares a mechanistic basis with CRPS I in patients, only a small proportion of patients that have suffered a fracture develop CRPS (Beerthuizen et al., 2012). Tékus et al. demonstrated for the first time the transferability of CRPS from human to mice via IgG administration to incised mice (Tékus et al., 2014).

3.2 Aim

The aim of this chapter was to establish and characterize the passive-trauma-IgG-transfer model of CRPS in our laboratory. Furthermore, the behavioural phenotype observed in this model was compared with sensory abnormalities observed in quantitative sensory testing of CRPS patients, to determine whether symptoms were faithfully translated into a behavioural phenotype.

3.3 Methods

Female C57BL/6J mice were used throughout the entire study, since we worked with female patient/donors. Mice with a hind paw skin-muscle incision (Brennan, 1999) were either injected with IgG purified from CRPS patients or IgG purified from healthy control (HC) subjects. A third group of control mice received no IgG, but were subjected to paw incision alone. Mice were tested by Clive Gentry for tactile allodynia with von Frey filaments, for mechanical hyperalgesia with the Randall-Selitto paw-pressure test and for thermal hyperalgesia with a plate set at 50°C for hot-plate and 10°C for cold-plate tests. Weight distribution on the mice hind paw was assessed with the dynamic weight-bearing instrument as described in the Chapter 2. The severity of the incision was correlated with the healing phenotype of the paw. Immunostaining on the mouse paw skin tissues was performed in order to investigate the distribution of human IgG on both ipsilateral and contralateral paws of both CRPS and HC mice.
3.4 Results

Three groups of mice (n=6/group) were used in this experiment. Groups of mice were injected on four consecutive days (day -1, 0, 1 and 2) with either IgG from healthy control subjects (HC) or with IgG from a CRPS patient. All the groups were subjected to incision on their ipsilateral paw either alone or in combination with intraperitoneal IgG injection and the threshold of the paw withdrawal was measured on both paws, ipsilateral and contralateral, using either von Frey hairs or the Randall-Selitto test (as described in the methods).

3.4.1 CRPS-IgG transfer to mice exacerbated and prolongs mechanical hypersensitivity produced by paw incision

All groups displayed a significant hypersensitivity to stimulation with von Frey filaments on day 1 after the incision in the ipsilateral paw when compared to baseline (day -1) (Figure 3–1, Dunnett’s test). Three days after the paw incision, all groups displayed a reduced hypersensitivity to von Frey stimulation, but had not yet recovered fully to the naive level. We observed no significant difference between the CRPS-IgG versus HC-IgG injected mice and the incision-only mice (one-way ANOVA test). No difference was observed in the mechanical sensitivity in the mice contralateral paw, which is in good agreement with earlier studies of paw incision as a model of post-surgical pain (Brennan, 1999) (Figure 3–1B).

Similar to the observations with the von Frey test, all treatment groups developed hypersensitivity to paw pressure on the day following the incision. The mean paw withdrawal threshold was reduced from about 105g to around 80g in the HC-IgG injected mice, the CRPS-IgG injected mice and the incision only mice groups (Figure 3–1C). A slight but significant difference between the two groups was detectable already on day 1 after the incision (Figure 3–1C). While on day 2 post-incision, the control group’s paw withdrawal threshold to mechanical pressure started to recover; the CRPS-IgG injected mice maintained mechanical hypersensitivity, which was statistically significant. On day 3 after the incision, the HC-IgG injected mice and incision-only groups recovered whereas the CRPS group showed a significant and statistically reduced threshold, with no sign of recovery (Figure 3–1C).
The uninjured contralateral paw showed no change over time in mice injected with HC-IgG or incision-only mice, while the CRPS mice group displayed a slight, but significant increased sensitivity to mechanical pressure in this experiment (Figure 3–1D).

This first behavioural study suggested that administration of CRPS patient IgG, but not HC-IgG, exacerbates the hypersensitivity to mechanical pressure in the incised ipsilateral paw. In addition, these results indicated a slight systemic sensitising effect of CRPS-IgG, since the contralateral paw withdrawal threshold was reduced slightly.
Figure 3–1 Serum IgG from CRPS patient, but not healthy control subject, exacerbates and prolongs the mechanical hypersensitivity produced by a paw incision.

Mice were injected intraperitoneally with 8 mg of healthy control (HC)-IgG or CRPS-IgG (8 mg daily) on 4 consecutive days. von Frey ipsilateral (A) and contralateral paw (B). Randall–Selitto ipsilateral (C) and contralateral paw (D). n=6/group. One-way ANOVA, comparison with HC-IgG group using Tukey’s HSD test. *p < 0.05, **p < 0.01, ***p < 0.001. †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.
3.4.2 CRPS-IgG transfer to mice exacerbated and prolongs thermal hypersensitivity produced by paw incision

In addition to exhibiting mechanical hypersensitivity, CRPS patients regularly display hypersensitivity to heat and cold temperatures (Gierthmühlen et al., 2012; Huge et al., 2008). Therefore, we wanted to assess mouse sensitivity to cold and heat. We examined the influence of IgG on thermal nociception using a modified 10°C cold plate (Figure 3–2A-B) and a 50°C hot plate assays (Andersson et al., 2009; Gentry et al., 2010) (Figure 3–2C-D).

Both groups, CRPS-IgG and HC-IgG treated mice, displayed a slight non-significant increased sensitivity to both cold and heat stimulation 1 day after the incision in the ipsilateral paw (Figure 3–2A; C). However, on the following days, while the postsurgical hypersensitivities to cold and heat were progressively lost in control groups, mice treated with CRPS-IgG showed a significantly increased hypersensitivity to cold and noxious heat stimulus (Figure 3–2A; C). A significant increase of contralateral cold hypersensitivity was also observed in the CRPS mice group on day 2 and 3 post-incision (Figure 3–2B). In addition, CRPS mice developed sensitivity to heat on their contralateral paw, but only on day 3 post-incision (Figure 3–2D).
Figure 3–2 Serum IgG from CRPS patient, but not healthy control subject, elicits cold and heat hypersensitivity following a paw incision.

Mice were injected intraperitoneally with IgG from healthy control subject (8mg) or CRPS patient (8mg on 4 consecutive days). Ipsilateral (A) and contralateral (B) paw withdrawal latencies to a cold (10°C) stimulus. Ipsilateral (C) and contralateral (D) paw withdrawal latencies to a heat (50°C) stimulus. n=6/group. One-way ANOVA, comparison with HC-IgG group using Tukey’s HSD test. *p < 0.05, ** p < 0.01, ***p < 0.001. †p<0.05, ††p<0.01, †††p<0.001. One-way ANOVA, compared to naive values before surgery and injection, Dunnett’s post-hoc test.
3.4.3 CRPS patient IgG administration to mice induces sustained hypersensitivity to mechanical stimuli

The previous behavioural results raised several questions; we wanted to evaluate the IgG dose regimen required to induce behavioural hypersensitivity, and to establish a dose-response relationship for CRPS-IgG (Figure 3–3). We also sought to determine the time course of the exacerbated hypersensitivity produced by IgG from CRPS patients. To do so, three different doses (0.8mg, 4mg and 8mg) of IgG from the same CRPS patient-were administered to three groups of mice (n=6/group). As in the previous study, IgG was injected on four consecutive days, and mice were subjected to a paw incision on the day of the second injection. The paw withdrawal threshold to mechanical pressure was monitored for 2 weeks (Figure 3–3A-B). A separate group of mice received only two injections of 8 mg (Figure 3–3A-B). The group that received four injections of 8mg CRPS-IgG displayed a sustained reduction of the paw withdrawal threshold to mechanical pressure (Figure 3–3A). In fact, the increased sensitivity of these mice remained throughout the whole experiment and for mice treated with 4x8mg of CRPS-IgG this phenotype persisted throughout the 2-week time course when compared to the HC-IgG injected mice. In contrast, injection of two doses instead of four, one on the day before the incision and one on the day of incision, was not sufficient to produce the behavioural changes observed in the reference group (Figure 3–3A). In addition, injections of lower doses of CRPS-IgG (4mg/day or 0.8mg/day) induced no, or markedly reduced behavioural abnormalities compared to the group receiving 8mg/day (Figure 3–3A).

Unlike the injured side, the modest behavioural sensitization to noxious cold or mechanical stimulation of the contralateral paw resolved fully by day 6 (Figure 3–3B-C).
Figure 3–3 CRPS-IgG administration induced sustained hypersensitivity to paw pressure and noxious cold in correlation with the dose of administered IgG.

(A) Effect of serum IgG from CRPS patient and HC on ipsilateral and (B) contralateral paw withdrawal thresholds to mechanical pressure. (C) Ipsilateral and (D) contralateral paw withdrawal latencies to noxious cold (10°C). n=6/group. ANOVA with Tukey’s post-hoc analysis: *p < 0.05, **p < 0.01, ***p < 0.001, vs HC-IgG group.
3.4.4 CRPS patient IgG administration to mice induces sustained hypersensitivity to noxious cold

We also examined mice for their sensitivity to noxious cold (+10°C) using a modified cold plate assay, in which a hind paw is placed in contact with plate surface and the latency to paw withdrawal is measured in seconds (Andersson et al., 2009; Gentry et al., 2010). Cold allodynia and hyperalgesia are characteristic symptoms of chronic CRPS patients (Grothusen et al., 2014; Finch et al., 2009). In good agreement with the clinical situation, mice treated with 8mg of CRPS-IgG/day displayed a significantly increased sensitivity to cold stimulation, which was maintained for at least 2 weeks (Figure 3–3C). Interestingly, the group that received half the dose of CRPS-IgG (4mg/day) had a relatively long and persistent hypersensitivity to cold from day 1 to day 13 post-incision (Figure 3–3C). Indeed, although this group response to mechanical stimulus was not significantly different from the control group, it shows here a significant sensitivity to cold stimulus, suggesting that IgG from CRPS patients is particularly associated with cold allodynia or paraesthesia.

As seen in Figure 3–3A, a very slight, transient sensitization to mechanical stimulus could be detected in the contralateral paw. Similarly, the contralateral paw of the mice injected with the highest dose of CRPS-IgG displayed a modest, but significant sensitivity to cold stimulus on day 3 post-incision (Figure 3–3D). However, this sensitivity was later lost, after day 6 post-incision. The contralateral phenotype indicates that IgG from this patient may produce sensitization of nociceptors in the absence of an initiating trauma or injury. Alternatively, it is possible that the heightened hypersensitivity from the injured paw, sensitized the contralateral side through a central, spinal mechanism, as it has been described in neuropathic conditions (Hatashita et al., 2008).

Interestingly, the mechanical and cold hypersensitivity observed in mice treated with IgG from CRPS patients were maintained even 10 days after the last IgG injection and 13 days post-incision.

To determine whether the circulating human IgG concentration correlated with the observed behavioural phenotype, we measured the plasma concentration of human IgG in mice treated with HC-IgG or CRPS-IgG on days 3, 8 and 13 after incision with a human IgG ELISA (Figure 3–4).
Perhaps not surprisingly, the concentration of human IgG at day 3 was high, similar to that seen in human subjects (19±9 mg/ml). In contrast, the concentrations of circulating human IgG at days 8 and 13 were negligible (2.3±0.3 ng/ml).

Figure 3–4 Anti-human IgG ELISA results in human-IgG injected mice plasma at day 3, 8 and 13.

Mice were injected intraperitoneally with HC or CRPS-IgG on 4 consecutive days (day -1, 0, 1 and 2). Circulating human IgG concentration was measured with an ELISA kit using mice plasma from different time course (day 3, 8 and 13).

3.4.5 Hypersensitivity to paw pressure in CRPS-IgG treated mice is correlated with the patient pain score

To determine whether the sensory phenotype observed in mice correlated with the donor-patients’ pain intensities, we next examined the activity of IgG pooled from randomly selected patients with either high or moderate baseline pain from the LIPS trial (Goebel et al., 2017) (high: n=27, mean pain intensity 8.3±0.4 (SD), range 7.3-9.5 on a 11-point numerical rating scale (NRS); moderate: n=26, 6.0±0.8 (SD), range 5.0-7.0NRS). IgG (4x8mg) from patients with severe pain, but not from patients with moderate pain, produced significant mechanical hypersensitivity (Figure 3–5A), indicating that the degree of sensory abnormalities in CRPS mice reflect the donor patients’ spontaneous pain intensities. The discrepant results observed with IgG from patients with high or moderate pain, may suggest that target heterogeneity or autoantibody titre influences symptom severity, as
it is the case in patients with autoantibodies against the voltage-gated potassium channel complex constituents LGI1 and CASPR2 (Klein et al., 2012).

Next, we therefore examined whether larger doses of IgG (16mg/day) from patients with moderate pain intensity influenced mechanical nociception. This larger dose of IgG (from patients with moderate pain) produced a significant hypersensitivity compared to HC, which was more transient than that observed in mice treated with IgG (8mg) from patients with higher pain intensity (Figure 3–5B). Our results thus strongly suggest that autoantibodies are responsible for maintaining pain in patients with persistent CRPS.

Figure 3–5 Hypersensitivity to paw pressure in CRPS-IgG treated mice is correlated with patient pain score.
(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.
3.4.6 CRPS-IgG is responsible for the mechanical hypersensitivity independently of the incision

CRPS-IgG injected (n=5) and incision-only (n=7) mice ipsilateral paws were pictured after the behavioural reading, using a camera. Both groups of mice were scored blindly by seven individuals for the healing level of the paw around the incision. 0: looks normal (the incision is healed, no redness), 1: can see the incision, not fully healed, 2: redness associated with a bad healing. Both CRPS-IgG injected (R²=0.4816) and incision only (R²=0.1866) mice did not show a strong correlation between the mechanical sensitivity of the animal to paw pressure (Δ paw pressure vs healing score) and the healing level of the incision (Figure 3–6).

Figure 3–6 CRPS mice hypersensitivity to mechanical pressure did not strongly correlate with impaired healing of the incision.

Correlation between mechanical sensitivity and healing in CRPS (A) versus HC mice (B). Δ paw pressure is the difference between contralateral and ipsilateral mouse paw withdrawal to mechanical pressure (g). Healing was assessed visually by different subjects and averaged. CRPS n=5 mice, Incision-only n=7 mice.

Since the healing did not differ between the groups, it is not likely that the rate of healing is directly responsible for pain. Since the healing was scored similarly in the two groups of mice, it is unlikely that the hypersensitivity observed is caused by the incision per se. Instead, the CRPS-IgG seems to be responsible for all of the mechanical hypersensitivity (Figure 3–6). In addition, the n number for both groups is too low to conclude about potential correlation between an impaired or delayed healing and the mechanical...
hypersensitivity to paw pressure induced by CRPS-IgG however a trend is observed for CRPS group.

3.4.7 CRPS-IgG did not affect mice ipsilateral vs. contralateral weight distribution

A last behavioural experiment was conducted in order to assess the behavioural impact of passively transferred CRPS. A device that measures in a dynamic setting where mice could move freely, the weight distribution on both hind paws of CRPS and control mice was used in these experiments. The weight distribution a mouse uses on its paws to move is considered a measure that integrates hypersensitivity, spontaneous pain and impaired movement due to injury (Figure 3–7).

Mice were injected with HC or CRPS with 8mg/day during 4 consecutive days as described in the previous sections.

No difference was observed at the baseline measurement (on the day preceding the paw incision). Both groups had a very similar weight distribution. Day 1 after the incision, CRPS-IgG injected mice seemed to put less weight on their ipsilateral, incised, paw. However, no significant difference was observed (Figure 3–7, t-test). Mice treated with CRPS-IgG spared their ipsilateral (incised) paw modestly more than control mice, but not significantly.

Similarly, on day 3 post-incision, CRPS-IgG injected mice seemed to be more sensitive on their ipsilateral paw in comparison to the HC-IgG injected group, again, without any significant difference.

These data suggested that CRPS-IgG or HC-IgG did not have an effect on the mice weight distribution on their hind paws although a slight non-significant effect was observed for CRPS-IgG treated mice.
Figure 3–7 CRPS-IgG did not affect mice weight distribution

Weight (g) mice put on their ipsilateral and contralateral paw was measured using the dynamic weight bearing instrument and presented as weight ratio (ipsilateral weight/contralateral weight) on day before incision (day -1), one day after incision (day 1) and on day 3 post-incision. n=6 mice/group. Tested with t-test.

3.4.8 Human IgG localizes in the mice paw skin

Since CRPS pain seems to be confined to the injured paw, we wanted to stain mouse glabrous skin for the presence of human IgG. CRPS-IgG injected mice and control mice were both assessed for human IgG distribution in the glabrous skin (Figure 3–8).

CRPS-IgG was present around the site of the incision (Figure 3–8A-B). It seemed that more IgG was present in the direct vicinity of the incision, which might be due to blood vessel dilatation and inflammation following paw injury. Mouse paw skin is a highly irrigated tissues (Nebuloni et al., 2014) and IgG was also present in the contralateral paw skin of CRPS mice, suggesting that human IgG are infiltrating surrounding tissues in the mice paw (Figure 3–8C). Patient IgG was found mostly in the very superficial epidermis of the contralateral paw skin (Figure 3–8C). Although, human IgG was found in both ipsilateral and contralateral paw of HC-IgG injected mice, donors IgG seemed to be confined to the dermis area of the skin with less staining on the epidermis (Figure 3–8D-E). No positive staining was observed for incision only mice skin (Figure 3–8F). In contrast, human IgG could not be detected in the control mouse spleen, demonstrating that the human IgG antibody was specific (Figure 3–8G). Human lymph node was also used as a positive control. B cells were positively stained (Figure 3–8H).

Injury and IgG are both required to produce the persistent postsurgical hypersensitivities reported above. The profuse IgG deposits seen in the mouse skin, particularly around the
incision, demonstrate that serum IgG have access to peripheral nerve endings and all dermal layers. The presence of both, CRPS-IgG and an incision, are necessary to induce a pronounced mechanical and temperature hypersensitivity. The link between these two are not well understood but some hypothesis can be generated (see Discussion).
Figure 3–8 Human-IgG localizes in the mouse ipsilateral and contralateral glabrous skin of CRPS and HC-IgG treated mice.

(A-B) CRPS-IgG treated mouse ipsilateral each representative of n=5 CRPS mice and (C) contralateral hind paw glabrous skin stained for human-IgG (representative of n=2 mice) (brown staining). (D) HC-IgG treated mouse ipsilateral and (E) contralateral hind paw glabrous skin stained for human-IgG (brown staining). (F) Incision-only mouse hind paw glabrous skin was negative for human-IgG. (G) Human-IgG was also tested on mouse spleen as negative control. (H) Finally, human lymph node was used as a positive control. B-cells were stained as positive (brown staining). A-10x magnification; B-H-20x magnification.
3.5 Discussion

The results presented in this Chapter, indicate that mice injected with CRPS-IgG exhibited persistent post-traumatic hypersensitivity to stimulation with mechanical pressure, cold and heat, but normal sensitivity to tactile stimulation with calibrated von Frey filaments.

Our results thus demonstrate that administration of CRPS-IgG in combination with paw incision, recapitulate sensory abnormalities observed in patient with CRPS (Gierthmühlen et al., 2012). The behavioural study presented in Chapter 3 was conducted on mice injected with IgG from a single CRPS patient (described Chapter 2 section 2.2) except for the pooled IgG study where mice were injected with IgG from different CRPS patients (also described Chapter 2 section 2.2). Using a single CRPS IgG donor allowed us to compare and correlate the mice behaviour directly to the donor patient clinical observation. Additional experiment with additional CRPS IgG donor would allow us to demonstrate biological reproducibility of the transfer model. However, the sensory abnormalities generated in CRPS mice are correlated with the pain intensity experienced by the donor patients. These experiments demonstrated that IgG from different CRPS patients have additive effects.

Donor IgGs were found to be present in the glabrous skin in both groups at the site of incision but also in the non-incised paw.

Mice injected with donor IgGs demonstrated IgG deposit in both ipsilateral and contralateral paws but with an enriched deposit around the incision.

This strong presence of IgG in the mouse paws can be explained by the doses of IgG we administered and can be due to the highly vascularised nature of the paws. The presence of IgG in both injured (ipsilateral) and non-injured (contralateral) paw when compared with the behavioural findings indicates that neither patient IgG, nor injury is sufficient to induce behavioural change in the mice. Therefore, the incision and the autoantibody present in CRPS serum are required to transfer the sensory abnormalities from patient to mouse. Indeed, since HC-IgG is without any discernible effect compared to paw incision alone, human IgG per se has no effect on nociception, only CRPS-IgG does.

One hypothesis to explain the observed results, is that the skin-muscle incision induces an inflammatory response in the mice with leads to an increased expression of antigens
or to the expression of novel epitopes. Citrullination is a post-translational modification of arginine to citrulline by the enzyme peptidyl arginine deiminase (PAD). Inflammation in the joints induces citrullination of proteins which are targeted by autoantibodies against citrullinated proteins (ACPA). In the ACPA transfer model of RA, Wigerblad et al., found that ACPA targets osteoclasts which release the CXCL1 nociceptor factor in the mice joint (Wigerblad et al., 2016). Hence, the authors described an example of passive transfer model of pain induced by transfer of patient autoantibodies. In our model a similar mechanism can explain the pain behaviour of CRPS mice. CPRS IgGs might target novel epitopes, which are generated after the paw incision. Autoantibodies might as well target existing antigens which expression is increased after the incision. Indeed, gene expression profiling of cutaneous nociceptors in SNI mouse model of neuropathic pain showed an increase of some gene expression in the injured DRG neurons (Berta et al., 2017). Additionally, a recent study revealed 63 differentially expressed genes in the rat dorsal horn between chronic constriction injury (CCI) and control groups (Du et al., 2018). Finally, an increased gene expression of pronociceptive interleukins as well as nerve growth factor genes was also found in a model of post-surgical pain (plantar incision) in the rat. The study showed that there were few changes in gene expression in DRGs while greater changes occurred in muscle and skin tissues (Spofford and Brennan, 2012).

Neuromyotonia with pain, a rare condition, can be associated with other symptoms such as insomnia, epilepsy and myotonia, and is caused by antibodies to voltage-gated K+ channel complexes, CASPR2 and LGI1 (Irani et al., 2010; Klein et al., 2012; Watanabe, 2016). To study the mechanisms of action of these antibodies, human CASPR2 autoantibodies, were transferred to mice, which induced mechanical hypersensitivity in the absence of neural injury (Dawes et al., 2018). In this model, pain is induced indirectly via the action of CASPR2 on the Kv1 channel expression. A similar mechanism of action is possible with CRPS antibodies.

Interestingly, our study showed that CRPS mice were not different from the control groups when tested with von Frey for tactile sensitivity but were hypersensitive to mechanical paw pressure. von Frey and Randall-Selitto (paw pressure) test are used to assay different modes of mechanosensation. Indeed, von Frey filaments apply a very local light pressure on the skin. It can therefore be considered as a test for tactile sensory changes. The Randall-Selitto assay is a paw pressure test that utilizes a more intense
noxious mechanical stimulus. The instruments exert a force increasing at a constant rate on the animal paw, which is placed on a small plinth under a cone-shaped pusher with a rounded tip. When painful the animal withdraws its paw. The weights applied on the animal paws are considerably higher (over 100g) than what is applied with calibrated von Frey filaments (0.008–2g). The Randall-Selitto test is therefore a more complex type of stimulation on the mouse paw. Hairy skin, muscles, joints and glabrous skin are all stimulated. We consider here the paw pressure test as a way of measuring mechanical nociception that undoubtedly integrates input from nociceptive and non-nociceptive fibres (Hogan et al., 2004).

Furthermore, we also showed that the hypersensitivity of the incised mouse paw was long-lasting. Comparing CRPS mice tissues from later stages to CRPS mice tissues prepared on day 3 post-incision might give us a more detailed idea of the changes that occurs over time in the animal. As shown in the results, a slight but significant contralateral effect was observed in CRPS-mice. Regarding this contralateral effect, our hypothesis is that the postsurgical inflammatory environment leads to sensitisation and pain, which is accentuated by CRPS-IgG. It is possible that the high level of nociceptive input to the spinal cord leads to a sensitisation of the contralateral side of the spinal cord via the system of commissural interneurons as in neuropathic pain (Won and Lee, 2015). A study conducted in a rat model of hemi lateral nerve injury outlined the induced contralateral neuropathic pain and neuropathology in dorsal root ganglion and in spinal cord (Hatashita et al., 2008). Another study suggested that activation of spinal microglia in a mouse model of peripheral inflammation-induced a long-lasting contralateral allodynia (Schreiber et al., 2008). This contralateral effect is lost after a brief time, perhaps because of the modest level of input coming from the contralateral paw. It is also possible that a continuous supply of patient IgG is required to generate and maintain the modest sensitisation in the contralateral side, and it is possible that IgG from different patients do not produce identical phenotypic changes. The experiments in the current Chapter have all been produced with IgG from a single patient with very severe CRPS. In addition, injection of CRPS-IgG in the absence of paw injury has been tested in early studies of CRPS-IgG transfer by Goebel et al. The study showed that CRPS-IgG did not induce stimulus-evoked pain or autonomic signs in injected mice (Goebel et al., 2011).

The concentration of human-IgG in injected mice plasma was at negligible levels on day 8
and 13 after incision although behaviourally animals maintained mechanical and cold hypersensitivity. It is possible that the injected IgG are no longer present in the blood circulation, but localized in specific tissues in the mouse. Tissue lysate of various mouse tissue for immunoblotting with anti-human IgG would test this hypothesis. Finally, measurements at plasma levels at the different time points can be repeated.
CHAPTER 4 SINGLE FIBRE RECORDING FROM NAIVE MOUSE SKIN-NERVE PREPARATIONS
4.1 Introduction

The aim of this research project was to investigate the neurophysiological mechanisms of CRPS using a translational mouse model. As described in Chapter 3, CRPS-IgG transfer to mice with paw skin-muscle incision elicited hypersensitivity in the mice ipsilateral paw to mechanical pressure and noxious temperatures. The paw-skin incision model is widely used as a model of post-surgical pain (Brennan, 1999). The skin-muscle incision sensitizes plantar nociceptors in the glabrous skin (Banik and Brennan, 2008). In this Chapter as well as in the following Chapter, we are studying the activity of fibres terminating in the hairy, dorsal paw skin, not immediately juxtaposed to the incision injury. Recording fibres activity from the hairy skin circumvents the potential bias of the incision-induced sensitization of the nerve fibres. Before starting experiments on preparations from incision-only mice and CRPS mice, we examined the properties of saphenous nerve fibres in C57BL/6J female mice to build a control group of the different fibre types (Aβ-SA, -RA; Aδ-DH, -AM and C-fibres). The fibre properties of the hind paw hairy skin have been investigated by Koltzenburg and his team (Koltzenburg et al., 1997) in early studies in mice of both sexes. Recent studies indicate that pain mechanisms differ significantly between male and female mice (Mogil, 2012; Sorge et al., 2015). Since CRPS mostly occurs in female individuals, all studies have therefore been performed in female mice.

4.2 Aim

The aim of the present study was to determine the properties of mechanically sensitive afferent nerve fibre classes in naïve C57BL/6J female mice. The conduction velocity, mechanical activation threshold and the evoked patterns of impulse activity in different fibre types were analysed.

4.3 Methods

Adult female C57BL/6J mice were used for the in vitro electrophysiological analysis, since the results from these studies will primarily serve as a reference for the neurophysiological basis of CRPS, which predominantly affects women. To investigate the properties of fibres innervating the hairy skin, the saphenous-nerve was dissected, and the fibres were characterized using electrical and mechanical stimuli as described in Chapter 2-2.6. Finally, the data were analysed as described in Chapter 2-2.6.4 & 2.6.5.
4.4 Results

A total of n=216 fibres, 75 from naïve C57BL/6J adult female mice, 71 from incision group and 70 from CRPS mice group mechanically sensitive saphenous fibres from the hairy hind paw skin were recorded and classified according to their conduction velocity, mechanical threshold and impulse discharge properties (Zimmermann et al., 2009).

4.4.1 Types of mechanically sensitive fibres in the hairy skin

Table 4-1 Naive mice fibres mechanical threshold and conduction velocity

<table>
<thead>
<tr>
<th>Class</th>
<th>Fibre type</th>
<th>n</th>
<th>Mechanical threshold (g)</th>
<th>Conduction velocity (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SEM</td>
</tr>
<tr>
<td>Aβ</td>
<td>RA</td>
<td>14</td>
<td>0.21</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>12</td>
<td>0.2</td>
<td>0.03</td>
</tr>
<tr>
<td>Aδ</td>
<td>DH</td>
<td>12</td>
<td>0.22</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>AM</td>
<td>20</td>
<td>2.57</td>
<td>0.35</td>
</tr>
<tr>
<td>C</td>
<td>CM</td>
<td>17</td>
<td>4.04</td>
<td>1.21</td>
</tr>
</tbody>
</table>

Fibres in preparations from naïve female C57BL/6J mice were studied as a control group for their properties. Here, we wanted to study the fibre properties in preparations from female mice. Indeed, the overall aim of the study was to assess the effect of CRPS-IgG on the response of the mechanically sensitive fibres. Since CRPS-IgG was from a female patient, we decided to use female mice throughout the study. In addition, previous studies with skin-nerve recordings used male mice whereas our study requires female mice.

N=75 fibres were analysed for their conduction velocity and mechanical threshold in order to establish a reference group for further comparisons. The saphenous nerve from the isolated skin-nerve preparation was dissected into progressively thinner filaments and the receptive field of the fibre innervating the skin spotted mechanically. Once the receptive field was identified, the conduction velocity of the fibre and its mechanical threshold were identified.
Mechanosensitive fibres were categorized into five different classes. Groups of fibres were established according to the conduction velocity of the fibres (Koltzenburg et al., 1997). The fibres were either Aβ fibres (conduction velocity>10m/s), Aδ fibres (1.2<conduction velocity<10m/s) or C-fibres (conduction velocity<1.2m/s). Aβ fibres were then separated into two types, rapidly adapting (RA) and slowly adapting (SA) fibres, according to their response to sustained mechanical stimuli. Aβ fibres that discharged briefly at the beginning and at the end to a constant force stimulus were classified as RA fibres, whereas Aβ fibres that responded with a more sustained pattern of impulses were classified as SA fibres (Table 4-1). Both, RA and SA fibres discharge impulses for very low forces (Table 4-1). The findings were in agreement with previous findings (Koltzenburg et al., 1997; Smith et al., 2013).

Fibres with a conduction velocity between 1.2m/s and 10m/s were classified as Aδ fibres and two distinctly different types were encountered. Aδ fibres with low force threshold for activation were classified as D-hair (DH) and ones with high force threshold for activation were classified as Aδ-mechanoreceptor (AM) fibre (Table 4-1). DH fibres impulse discharge and activation threshold (0.22±0.07g) was very similar to that of RA fibres (0.21±0.03g). DH fibres discharged brief impulses at the very beginning and the end to a constant force stimulus application (Table 4-1). In contrast, AM fibres had a higher mechanical threshold than other classes of A-fibres (2.57±0.35g) (Table 4-1). AM fibres responded throughout the full duration of a constant force application. When the force was increased gradually (ramp stimulation) the fibres displayed increasing firing to increasing force, thereby encoding the stimulus intensity (Figure 4–2). This is in contrast with DH- and Aβ fibres, which do not encode force with a linear increase in the impulse frequency.

Mechanically sensitive C (CM) fibres were also recorded. They had a very low conduction velocity and a high activation threshold in response to mechanical stimulation (Table 4-1). Mechanosensitive C (CM) fibres generally displayed a similar impulse discharge pattern to AM fibres in response to mechanical pressure, faithfully encoding increasing force with a corresponding increase in the impulse discharge rate (Figure 4–2).
Figure 4–1 Example traces of naive low-mechanical threshold fibres response to step stimulus.
(A) Example of an Aβ rapidly adapting (RA) fibre that discharges impulses at the beginning and at the end of the mechanical stimulation without discharging during the duration of the stimulation. (B) Example of an Aβ slowly adapting (SA) fibre, which sustains impulses throughout the whole duration of the mechanical stimulation. (C) Example of an Aδ-D-Hair (DH) fibre that discharges action potentials at the beginning and the end of the mechanical stimulation like RA fibres. DH and RA fibres have similar impulse discharge patterns yet are distinguishable by their conduction velocity.
4.4.2 Conduction velocity of mechanically sensitive fibres

The conduction velocity of each fibre was determined by electrically stimulating the receptive field with a square-wave electrical pulse (duration 1ms, with inter-stimulus intervals of 2 or 4 seconds) as described in Chapter 2. Fibres with a conduction velocity above 10m/s were classified as thickly myelinated Aβ fibres. Fibres that had a conduction velocity between 1.2 and 10m/s were classified as thinly myelinated Aδ fibres. If the conduction velocity of the fibre was below 1.2m/s, the fibre was considered an unmyelinated C-fibre. The mean conduction velocity of Aβ fibres recorded from naïve mice was 15.5±0.74m/s (n=26). The mean conduction velocity of Aδ fibres was 6.1±0.47m/s (n=29) and the mean conduction velocity of C-fibres was 0.65±0.09m/s (n=17).

The distribution of conduction velocities does not appear normally distributed, which is explained by the diverse populations of fibres (Figure 4–3) (Koltzenburg et al., 1997). The distribution of conduction velocities from all fibres, suggested a relatively clear distinction between A and C-fibres, but not between Aβ and Aδ fibres. Conduction velocity is a first good indication of which class of fibre the recorded fibre belongs to but is not enough in itself to distinguish between the two Aβ and Aδ fibres. Therefore, the fibre response

Figure 4–2 AM and CM-fibre impulse discharge response. (A) AM and (C) CM fibres discharge throughout the entire duration of the constant mechanical stimulation (step). Both (B) AM and (D) CM fibres encode an increasing force by discharging more action potentials for higher forces.
properties were further analysed for a clear classification of the fibre, based on their impulse discharge pattern.

Figure 4–3 Bin distribution of the fibres conduction velocity (m/s).
Fibre conduction velocity (CV) varies according to the fibre class. CM fibre are the slowest with a CV between 0.2 and 1.2m/s. A\(\delta\) fibres have an intermediate CV between 2 and 10m/s and A\(\beta\) fibres have the highest CV (>10m/s). The dotted bars indicate a delimitation of the different fibres class according to their CV (Koltzenburg et al., 1997).

4.4.3 Analysis of mechanically sensitive fibres impulse discharge properties

RA, SA and DH fibres are low threshold mechanosensitive fibres (LTMRs), they require lower forces than mechano-nociceptors to be activated. After recording the fibre conduction velocity, fibres were stimulated with mechanical force for 2s and their activation threshold identified iteratively (Chapter 2).

RA fibres had a mean mechanical force threshold of 0.21±0.03g, SA fibres of 0.20±0.03g and DH fibres 0.22±0.07g (Table 4-1 and Figure 4–4A).

In contrast, AM and CM fibres are high threshold mechanosensitive fibre (HTMRs) and require more intense stimulation to evoke action potential discharge. AM fibres had a mean mechanical activation threshold of 2.57±0.35g. CM fibres had a mean mechanical threshold of 4.04±1.21g (Table 4-1 and Figure 4–4B). The HTMRs are nociceptive and need higher forces than the LTMRs to give a response.

The conduction velocity itself might not be enough to identify the fibre class and type with confidence; the activation force threshold is then required to distinguish the different fibres classes and types. Hence, AM and DH fibres have similar conduction velocities but very different responses to mechanical stimulations.
Figure 4–4 Activation threshold of mechanically sensitive hairy-skin nerve fibres in naive mice.
(A) Low threshold mechanical receptors (LTMRs), SA, RA and DH fibres had similar activation threshold. (B) High threshold mechanical receptors (HTMRs), AM and CM fibres, required higher mechanical stimulation for their activation compared with LTMRs. Data are mean ± SEM.
A feedback-controlled mechanical stimulator was used to investigate single unit responses evoked by stimulation of different intensities. Sustained steps of constant force ranging from 0.5-20g were used to study the mechanical adaptation properties of RA, SA, DH, AM and CM fibres.

Since AM and CM fibres encode force with the rate of impulse discharge, these fibres were also stimulated with a mechanical ramp stimulus ranging from 0.5-20g and the evoked impulse discharge pattern and encoding properties analysed.

A typical recording from an SA fibre is displayed Figure 4–5A. As shown in the example (Figure 4–5), low forces (0.5-1g) evoked responses in SA fibres (10-20 imp/s) but forces in the range of 2.5-5g produced a higher impulse rate (25-50 imp/s). The number of impulse discharges decreased for higher forces (10-20g), suggesting that the adaptation rate is markedly increased by forces in the noxious range. The mean response of SA fibres to steps of increasing intensities showed that SA fibres respond to all the forces by discharging impulses throughout the stimulus duration but to a lesser extent for higher forces (Figure 4–6). The discharge rate was higher at the onset of the force application between 0 and 5s of the total 10s stimulation. The average total number of action potentials generated for different step stimuli varied from 25 for the lowest force (0.5g) to around 100 for the highest force (20g) (Figure 4–7A). However, SA fibres generated a number of action potentials that was proportional to forces ranging from 0.5-5g. Observations show that SA fibres can sustain very high impulse rates, with the highest action potential rate recorded for 5g, exceeding 160 total number of action potential (165±30). Higher forces did not induce a higher number of action potential but instead seemed to have an inhibitory effect on the discharge rate of the SA fibre (Figure 4–5A).

RA fibres also belong to the Aβ fibre class but exhibit a different discharge pattern from SA fibres. As shown by the example RA recording (Figure 4–5B), these fibres mostly generate action potentials at the very beginning and end of the step stimulus, which is very characteristic of RA fibres. The mean response rates of RA fibres challenged with stimuli of different intensities revealed a lower peak impulse discharge rate (<20 imp/s) compared to SA fibres (>40 imp/s) (Figure 4–6). In addition, the total number of action potential generated in RA fibres in response to different stimulus intensities is much lower as a consequence of its rapid mechanical adaptation properties (Figure 4–7B). With similar
conduction velocity, these two types of Aβ fibres were distinguished from each other by the distinct adaptation properties of their responses to sustained applications of mechanical force.

Although DH fibres belong to the Aδ fibre class, they display discharge patterns that are reminiscent of RA fibres (Figure 4–6). They responded at high frequencies at the beginning and at the end of the stimulus, but are essentially silent during the remainder of the mechanical challenges. There is no clear correlation between force and the number of action potentials generated for DH fibres (Figure 4–7C).
Figure 4–5 Mechanical response profile of slowly adapting (SA) and rapidly adapting (RA) Aβ fibres to different constant force stimuli. 
(A) SA fibre and (B) RA fibre response to increasing constant force stimuli shown as impulse/s (imp/s) and raw recording (in blue).
Figure 4–6 Mean response of slowly adapting (SA), rapidly adapting (RA) and D-hair (DH) fibres from naïve mice to step stimuli. (A) SA fibres were more responsive to constant force stimulus than (B) RA and (C) DH fibres. SA fibres discharged impulses throughout the stimulation while RA and DH fibres did discharge impulses at the onset and the end of the mechanical stimulation. Data are mean ± SEM.
Figure 4–7 Low mechanical threshold fibre response to mechanical stimulus. (A) Slowly adapting (SA) fibres discharged in average more action potentials (AP) than (B) rapidly adapting (RA) and (C) D-hair (DH) fibres in response to mechanical stimulus. The mean number of AP increased for increasing forces from 0.5 to 5g. The generated number of AP reached its maximum for 5g in SA and RA fibres, and 10g for DH fibres. SA n=12, RA n=12 and DH n=10. Data are mean ± SEM.
Table 4-2 Correlation between force (g) and number of action potential

<table>
<thead>
<tr>
<th>Fibre</th>
<th>Stimulus</th>
<th>R</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>Step</td>
<td>0.3282</td>
<td>0.4274</td>
</tr>
<tr>
<td>RA</td>
<td>Step</td>
<td>0.5864</td>
<td>0.1266</td>
</tr>
<tr>
<td>DH</td>
<td>Step</td>
<td>0.7508</td>
<td>0.0318</td>
</tr>
<tr>
<td>AM</td>
<td>Step</td>
<td>0.994</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Ramp</td>
<td>0.9956</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CM</td>
<td>Step</td>
<td>0.9911</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Ramp</td>
<td>0.9956</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

In contrast to Aβ- and Aδ-LTMRs, AM fibres did not respond to low force stimuli such as 0.5g and 1g (Figure 4–8). They instead responded to higher forces, above 2g. HTMRs, AM and CM fibres, were both stimulated with step stimuli of increasing intensities, which were followed by ramp stimuli as shown on the example Figure 4–8. The mean response of AM fibres shows that they discharged impulses for step stimuli exceeding 4g (Figure 4–9A). AM fibres maintained a constant impulse frequency throughout the step stimulation, in contrast to what was observed for Aβ and DH fibres. In addition, the total number of action potentials and the impulse rate generated were positively correlated with the intensity of the step stimulus (Figure 4–10A). The highest total number of action potentials generated was recorded for the highest force, 20g. Stimulation of AM fibres with a ramp stimulus generated an increasing impulse discharge rate throughout the stimulation period. A maximal impulse discharge rate was reached towards the end of the ramp stimulus (Figure 4–9B). The force intensities applied during ramps were also correlated with the total number of action potentials generated during the stimulation period (Table 4-2).

CM fibres had a similar firing pattern to AM fibres in response to step and ramp stimuli (Figure 4–9). In comparison to AM fibres, CM fibres displayed a lower peak discharge and a lower total number of action potentials for step stimuli, but a similar number of impulses were evoked by ramps. For forces in the range of 10-20g, CM fibres discharged a similar number of action potentials to AM fibres stimulated with a ramp shaped stimulus, indicating that CM fibres for high forces are able to maintain a high impulse discharge frequency even after long series of stimulations (Figure 4–10). In summary, our observation demonstrates that mechano-nociceptors, both A- and C-type, faithfully encode increasing impulse rates.
Figure 4–8 Example AM fibre encoding force intensity.
(A) Aδ-mechanonociceptive (AM) fibre responses evoked by stimulation with constant force-steps and (B) by force ramps, shown as events/s and raw traces (in blue).
Figure 4–9 Response evoked by step and ramp stimuli in AM and CM fibres in preparations from naïve mice.
(A) AM and CM fibres both discharged continuously during constant step force application and (B) encoded the intensity of the ramp force. Data are mean ± SEM. AM step n=17, AM ramp n=15; CM step n=13, CM ramp n=12.
Figure 4–10 Force-response relationships of AM and CM fibres in preparations from naïve mice.

(A) AM and (C) CM fibres displayed higher number of action potentials (AP) in response to higher forces (g) for both step and (B-D) ramp stimulus. Step stimulus triggered a higher impulse discharge compared to ramp stimulus. AM step n=17, AM ramp n=15; CM step n=13, CM ramp n=12. Data are mean ± SEM.
4.5 Discussion

In this chapter, the properties of myelinated A-fibres and unmyelinated CM fibres from the hairy skin of naïve C57BL/6J female mice were established.

4.5.1 LTMRs

Aβ fibres from hairy skin displayed rapidly adapting (RA) or slowly adapting (SA) properties in response to constant force stimulation of their receptive fields. Both are activated by light touch (LTMRs). Early work conducted in the monkey and human glabrous skin identified the role of these fibres and their properties. These two types of fibres are more abundant in the finger tips when compared to the hand palm and convey very fine spatial information as they have very small receptive fields (Johansson and Vallbo, 1979a, 1979b; LaMotte and Whitehouse, 1986). RA fibres are important for signalling when an object slips from the hand for instance, in the grip of objects (Park et al., 2016). The properties of SA and RA fibres in hairy skin of C57BL/6J female mice were similar to those reported by Koltzenburg and colleagues, who first examined the properties of sensory fibres in skin-nerve preparations from mice (Koltzenburg et al., 1997). Two types of afferents with conduction velocities in the Aδ range; D-hair receptors (DH) and high threshold Aδ mechanoreceptors (AM), were found in the mouse hairy skin.

Zotterman showed that gently stroking the fur of a cat evokes several types of action potentials that travel at different speeds and that strokes of different intensities activate distinct fibre types (Zotterman, 1939). Using single-unit recordings, Brown and Iggo (Brown and Iggo, 1967) confirmed and extended these findings and showed that in cats and rabbits movement of guard hairs preferentially activates thickly myelinated fibres, whereas thinly myelinated A fibres are activated by movement of the smaller zigzag hairs, the D-hair (down hair receptors). Thirty years later, mouse DH properties were studied by Koltzenburg et al. (Koltzenburg et al., 1997) whose findings are in accordance with those presented in this chapter.
4.5.2 HTMRs

4.5.2.1 Aδ-mechanonociceptors (AM)

Myelinated high threshold mechanical receptors (HTMR) that responded to noxious stimuli were first described in cats, monkey and humans (Adriaensen et al., 1983; Burgess and Perl, 1967; Perl, 1968b). Burgess and Perl showed that noxious heat, noxious cold or acid applied to the receptive field and bradykinin injected into the skin did not evoke discharges from these myelinated high threshold mechanical receptors (Burgess and Perl, 1967). The receptive fields of these fibres were described as responsive spots which were separated by unresponsive areas. In 1968, Perl further identified myelinated HTMR in the primate hairy and glabrous skin. Those receptors required strong mechanical stimuli for activation and had thresholds that ranged from moderate to damaging pressures (noxious stimuli) (Perl, 1968a). Due to their thinly myelinated characteristics, intermediate conduction velocities and high threshold for mechanical activation, these HTMRs were referred to as Aδ-mechanonociceptors (AM). In this present study the mechanical threshold and number of action potentials generated for various mechanical stimuli in naive female AM fibres have been described and were in accordance with previous studies conducted in male mice (Koltzenburg et al., 1997; Smith et al., 2013). As described in early rat and mouse studies, AM fibres had a higher activation threshold in comparison to myelinated Aβ fibres and were able to accurately encode the intensity of force stimuli (Koltzenburg et al., 1997; Smith et al., 2013).

4.5.2.2 C-mechanonociceptors (CM)

Unmyelinated C-fibres are divided into two main groups; mechanically sensitive C-fibres (CM) and mechanically insensitive C-fibres. Mechanically sensitive C-fibres are further divided into those with low mechanical thresholds (C-LTMRs) which may primarily have a non-nociceptive function and those with high mechanical thresholds (C-HTMR), thought to be nociceptors. C-LTMRs were first described in the cat (Zotterman, 1939) and were subsequently found in the hairy skin of many species including mouse, rat, primates and humans. C-LTMRs are activated by innocuous mechanical stimuli (Johansson et al., 1988; Kumazawa and Perl, 1977; Leem et al., 1993; Seal et al., 2009; Vallbo et al., 1999). Like DHs (Aδ-LTMRs), C-LTMRs are activated by stimuli that move slowly across their receptive
field and are thus known as ‘caress detectors’. Their role in nociception is controversial although they may play a role for injury induced mechanical hypersensitivity (Seal et al., 2009). Recently, Moqrich and colleagues have defined C-LTMRs using single-cell transcriptomics, and demonstrated a central role for this class of afferents for mechanical allodynia (Reynders et al., 2015). In the present study C-LTMRs have not been encountered due to their anatomical distribution. In the mouse, C-LTMRs are located in the hairy back skin and have not been described as innervating the mouse paw hairy skin (Li et al., 2011).

The present studies on C-fibres have exclusively examined the activity of C-HTMRs. CM fibres exhibited the slowest conduction velocities (CV<1.2m/s) and, similar to AM fibres, had a high mechanical thresholds (>2g) and they encoded the intensity of the mechanical stimulus (Koltzenburg et al., 1997).

AM and CM fibres were stimulated both with constant force stimuli (step) and increasing force stimuli (ramp). AM fibres exhibited a closer correlation between the intensity of the stimulus and the number of action potential in response to mechanical ramp stimulus in comparison with CM fibres. The mean number of action potentials generated by ramp stimuli were lower than for step stimuli in AM fibres. Interestingly, in CM fibres, the mean number of action potential generated was similar for both step and ramp stimuli especially for forces >5g.

Fibre properties of female WT mice hind paw hairy skin were described in this Chapter. The following chapter focus on the comparison of CRPS and control incision mice fibre properties.
CHAPTER 5  CRPS AUTOANTIBODIES SENSITIZES NOCICEPTORS
5.1 Introduction

By administering purified CRPS patient IgG to mice subjected to a minor experimental trauma, such as paw incision, we have been able to transfer pain from human to mice (Chapter 3).

However, the neurophysiological mechanisms and the types of sensory afferents targeted by CRPS patient IgG are unknown. The in vitro skin nerve preparation technique allows the receptive fields of individual units to be directly stimulated by electrical, mechanical and thermal stimuli in a precise, controlled manner. This technique allows the experimenter to study the function of sensory afferents in their anatomical context independently of the CNS (spinal cord and brain).

5.2 Aim

In this chapter the aim was to study spontaneous impulse activity of the desheathed whole saphenous nerve and the evoked activity of single nerve fibres properties from CRPS and control incision-only mice skin-nerve preparations to understand whether or not CRPS-IgG affect the saphenous nerve activity.

5.3 Methods

Preparations from CRPS and incision-only mice ipsilateral hairy paw skin were dissected, and electrophysiological recordings compared. CRPS mice used in this chapter were injected four time (8mg) with IgG from one CRPS patient (described Chapter 2 in section 2.2). Before starting the saphenous-nerve dissection which was required for the single-unit recordings (Chapter 2), the whole skin-nerve activity was recorded in order to assess the spontaneous ectopic impulse discharge activity and quality of the preparation. Ectopic sensory afferent impulse discharge generates spontaneous pain and paraesthesia in both rodents and human patients (Seltzer et al., 1989; Wall et al., 1979).

Single-unit activity was then recorded as described in Chapter 2. Different types of mechanosensitive sensory afferent fibres were characterized using electrical and mechanical stimuli. The data was analysed as described in Chapter 2.
5.4 Results

Table 5-1 CRPS and incision mice fibres details.

<table>
<thead>
<tr>
<th>Class</th>
<th>Type</th>
<th>Mouse</th>
<th>n</th>
<th>Mechanical threshold (g) Mean</th>
<th>SEM</th>
<th>p-value</th>
<th>CV (m/s) Mean</th>
<th>SEM</th>
<th>p-value</th>
</tr>
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<tr>
<td>Aβ</td>
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<td></td>
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<td>0.04</td>
<td></td>
<td>15.44</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>SA</td>
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<td>0.04</td>
<td>0.711</td>
<td>12.88</td>
<td>0.74</td>
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<td></td>
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<tr>
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<td>0.18</td>
<td>0.05</td>
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<td></td>
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<tr>
<td></td>
<td>AM</td>
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</tr>
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<td></td>
<td>6.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>CM</td>
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<td>0.05</td>
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</tr>
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</table>

5.4.1 Comparison of incision group and CRPS group fibres discharge properties

5.4.1.1 Mechanical threshold

The discharge properties of preparations from both incision and CRPS mice (Table 5-1), as it was done for fibres from naive mice preparations (Chapter 4).

The mechanical threshold of SA fibres was not different between the incision group (n=14) and mice treated with CRPS-IgG (n=13) (Figure 5–1A).

RA fibres from the incision group (n=16) and from mice treated with CRPS-IgG (n=12) were compared for their mechanical threshold and no difference was observed (Figure 5–1B).

Similarly, DH fibres from the incision group (n=10) and from mice treated with CRPS-IgG (n=9) were compared for their mechanical threshold and no difference was observed (Figure 5–1C). Conduction velocities of LTMRs were also unaffected (Table 5-1). CRPS-IgG seemed to alter neither the activation threshold of LTMRs nor their conduction velocities.
The mechanical threshold of HTMRs (AM and CM) was determined in the same manner as previously done in preparations from naive mice (Chapter 3). The mean activation thresholds were not different in preparations from incision mice compared to preparations from CRPS-IgG injected mice (p=0.056, Mann-Whitney) (Figure 5–2). C-mechano-sensitive nociceptors (CM) in preparations from mice treated with a paw incision in combination with CRPS patient IgG responded to mechanical stimulation with a reduced force threshold, compared to CM units in preparations from mice with incision only (p=0.035, t-test) (Figure 5–2).

The conduction velocity of both AM and CM fibres did not differ significantly between units studied in mice from the incision group and CRPS-IgG treated mice (Table 5-1).
Figure 5–2 Mechanical threshold of AM was not different between preparations from CRPS-IgG treated and incision only mice but was significantly different for CM fibres. (A) HTMR-AM fibre activation threshold is on average lower in CRPS-IgG treated mice (p=0.056, Mann-Whitney test). (B) HTMR-CM fibre activation threshold is lower than incision-only mice (*p<0.05, t-test). Data are mean ± SEM.

5.4.1.2 Mean number of action potentials (AP)

After identifying individual fibres activation threshold, fibres were stimulated with constant forces for a duration of 10s with a 2-minute recovery period before each force application to avoid tachyphylaxis and allow full recovery from the previous challenge (Figure 5–3).

The observed impulse pattern in SA fibres in preparations from the two treatment groups was similar and their response thresholds and impulse rate were indistinguishable (Figure 5–3A). SA fibres in both groups maintained a sustained and persistent impulse pattern throughout the stimulation (as described in Chapter 4). SA fibres in preparations from CRPS-IgG treated and incision-only mice generated a minimum mean number of action potential for 0.5g, around 50 impulses. The number of impulses generated gradually increased with the increase of the force. Hence, the number of impulses generated for 5g was around 180 in SA fibres from both preparations. The mean number of action potentials generated for 10g, 15g and the highest force 20g, was higher than the mean number of impulses generated for forces between 0.5g and 3g (~120 impulses). However, as described in Chapter 4 for SA fibres in preparations from naïve mice, the SA fibres reach
a plateau and do not generate higher number of impulses for higher forces. Also, the properties of SA fibres are not altered by CRPS-IgG administration to the mice.

Similarly, the observed impulse pattern in RA fibres from the two treatment groups was similar. As observed for SA fibres, the impulse rate was minimal for minimal force application and forces between 5g and 20g induced similar impulse rate. RA fibres impulse rate also plateaued for forces >4g (Figure 5–3B). Both response threshold and impulse rate were indistinguishable for RA fibres in preparations from CRPS-IgG treated and incision-only mice (Figure 5–3B). DH fibres (Aδ-LTMRs) were also identified and their response threshold, as well as their impulse discharge rate, were similar in both treatment groups (Figure 5–3C).

In summary, LTMRs displayed similar responses to mechanical stimulations in both preparation from CRPS-IgG treated mice and incision-only mice groups. Their activation threshold but also their discharge rate in response to forces ranging from low to high (0.5g-20g) were indistinguishable. Therefore, CRPS-IgG seem to have no particular effect on LTMRs. These observations are also consistent with the lack of effect of CRPS-IgG on tactile sensitivity (Chapter 3). An alteration in LTMRs functions can induce painful sensation (Yu et al., 2015), but it appears that saphenous LTMR function is unaffected by CRPS-IgG and plantar incision in mice. Since, we have not studied the plantar mechano-nociceptors, we were unlikely to detect postsurgical sensitization of afferents terminated around this incision.
Figure 5–3 LTMRs discharge rate in response to different forces in incision versus CRPS mice.

(A) SA fibres discharged similar number of action potential (AP) in incision and CRPS mice. (B) RA and (C) DH fibres number of AP generated for different forces was also similar between the two groups. Mann-Whitney test. Data are mean ± SEM.

AM fibres displayed an increased discharge rate in preparations from CRPS-IgG treated mice when compared to mice that had been subjected to a paw incision alone (Figure 5–4). Application of step shaped mechanical stimuli of constant force (from 0.5 to 20g, each stimulus applied for a duration of 10s) demonstrated that AM fibres in preparations from CRPS-IgG treated mice responded with a higher impulse rate throughout the range of forces used (Figure 5–6A, p<0.05, 4 and 5g, Mann Whitney).

Analysis of the impulse pattern evoked by a 5g force step challenge, revealed that AM fibres in preparations from CRPS-IgG treated mice 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 (Figure 5–6C).

Stimulation of the receptive fields with force ramps evoked significantly higher impulse rates in preparations from CRPS-IgG treated mice throughout the range of forces used (p<0.01, Mann-Whitney, Figure 5–6B).

As described in Chapter 4, AM fibres encode increasing mechanical force with a corresponding linear increase in impulse frequency.

In AM fibres in preparations from CRPS-IgG treated mice this force-impulse relationship is significantly steeper in comparison with non-IgG treated mice. The minor reduced response threshold may indicate sensitized transduction mechanisms and the steeper force-impulse rate relationship may indicate that these fibres are hyperexcitable or exhibit a sensitized conduction/transmission in preparations from CRPS-IgG treated mice AM fibres.
In addition, the maximal impulse discharge rates evoked by either a 20g force step (Figure 5–6E) or a force ramp to 20g (Figure 5–6F) were increased significantly in preparations from CRPS-IgG treated mice, compared to AM fibres in preparations from mice that were only subjected to a paw incision, hence affirming the change in fibre properties in CRPS-IgG treated mice.

Figure 5–4 Example traces of AM fibres response to step and ramp stimulations in incision and CRPS mice.

(A) Response evoked by a constant 5g mechanical stimulation in an AM fibre from a mouse treated with CRPS-IgG (red) in combination with a paw incision, or a paw incision alone (black). (B) Response evoked by an increasing 10g ramp stimulus in incision versus CRPS mouse AM fibre. In these examples CRPS AM fibre number of events/s is increased when compared to incision AM fibre response.
Figure 5–5 IgG from CRPS patients sensitizes AM fibres.

(A) Mean number of action potentials (AP) evoked by step or (B) ramp stimulation of AM fibres in preparations from mice only subjected to a paw incision (black) and an incision in combination with CRPS-IgG. (C) The mean impulse pattern of AM fibres during 10s constant force applications (5g) and (D) 15s ascending force of 10g (means calculated from all fibres presented in A, B). (E) Peak firing frequency (events/s) in incision compared to CRPS groups for 5g ramp stimulation and (F) 10g ramp stimulation. *p<0.05, **p<0.001, Mann-Whitney test. Data are mean ± SEM. *P<0.05, **P<0.01; Mann Whitney U-test.
C-fibres constitute a diverse class of fibres. Some C-fibres are only thermosensitive (cold and/or heat sensitive) and other C-fibres are mechanosensitive only. Some C-fibres are both thermosensitive and mechanosensitive (Zimmermann et al., 2009). Mechanosensitive C-fibres are either sensitive to light touch (C-LTMR) (Li et al., 2011) or to mechanical stimuli in the noxious range (C-HTMR). We have not encountered C-LTMR fibres in our preparations and only recorded the activity of mechanosensitive C-HTMR fibres and characterized their response to mechanical stimuli but not to thermal stimuli. Mechanosensitive C-fibres (CM) in preparations from mice treated with CRPS-IgG were analysed with the same testing strategy that was used above for AM fibres and the results compared to CM activities in preparations from incision mice. A series of force steps and force ramps (0.5-20g) were applied. Both type of stimulus, step and ramp, induced an increased discharge rate in preparations from CRPS-IgG treated mice in comparison to the incision-only group, in good agreement with earlier observations that demonstrate that CM fibres encode an increasing force with an increasing impulse rate (Koltzenburg et al., 1997; Smith et al., 2013).

Analysis of the response pattern evoked by a series of force steps identified a significantly increased impulse rate in CRPS CM fibres at the highest forces used (15g and 20g, Figure 5–6A, p<0.05, Mann-Whitney). For the ramp stimulus, the 20g force also induced a significantly increased impulse rate (Figure 5–6B). The temporal distribution of action potentials during force steps appeared unchanged, similar to AM fibres (Figure 5–6C-F).
Figure 5–6 CRPS-IgG sensitized CM fibres.

(A) Mean number of action potentials (AP) evoked by step stimulus and (B) by ramp stimulus in CM fibres in preparations from incision and CRPS-IgG treated mice. (C) The mean impulse pattern of CM fibres during 10s constant 20g force simulation and (D) 15s ascending force of 20g (means calculated from all fibres presented in A, B). (E) Peak firing frequency (events/s) in CM fibres in preparations from incision mice compared to CM fibres in preparations from CRPS-IgG treated mice for 20g ramp stimulation and (F) 20g ramp stimulation. *p<0.05, **p<0.001, Mann-Whitney test. Data are mean ±SEM.
5.4.2 CRPS-IgG induces spontaneous impulse generation in skin-saphenous nerve preparations

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 above. The intact saphenous nerve activity recording in preparations from CRPS-IgG treated mice demonstrated a significantly increased 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, Figure 5–7). Although, whole nerve recording in preparations from CRPS-IgG demonstrated an enhanced ectopic activity, it is not possible to identify which types of units contribute to the spontaneous activity. In contrast with the single-unit recording, the whole nerve recording, does not allow the identification of single fibres which is done by conduction velocity identification and fibres firing properties in response to mechanical stimulus as described above and in Chapter 4.

Nevertheless, the demonstration of enhanced ectopic activity in an in vitro preparation isolated from the central nervous system suggests that CRPS-IgG exerts a peripheral pathogenic effect. The observed heightened spontaneous activity may be consistent with spontaneous pain and paraesthesia observed in CRPS patients.
Figure 5–7 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) Spontaneous activity recorded in the intact saphenous nerve. Each data point represents one preparation/animal. **p < 0.01, Kruskal-Wallis.
5.5 Discussion

Electrophysiological investigations of skin-saphenous nerve preparations from incision-mice model transferred with IgG from a patient-donor with severe, persistent CRPS, demonstrate an increased spontaneous impulse rate in the intact saphenous nerve and a markedly increased stimulus-evoked discharge rate in A- and C-mechanonociceptor single units in combination with a reduced mechanical threshold. Low-threshold mechanoreceptors (Aβ and Aδ-DH fibres) were unaffected, reflecting both the patient’s clinical experience, and the transferred behavioural phenotype in mice, which lasted for at least 2 weeks (see Chapter 3).

Quantitative sensory testing of CRPS patients, has identified a markedly increased sensitivity to noxious mechanical pressure associated with an increased sensitivity to noxious cold and heat in long standing CRPS patients (Gierthmühlen et al., 2012). Similar sensory changes were observed in our CRPS model after the paw incision and IgG injection. Hence the faithful transfer of symptoms from patient to mice constitute an opening for mechanistic studies of the human pathogen in an experimental setting.

In agreement with patient QST data and our behavioural results, LTMRs were not affected whereas nociceptor HTMRs were hypersensitive. However, clinical signs in CRPS patients may vary. In the QST study conducted by Gierthmühlen et al., CRPS patient exhibited a loss of sensitivity as assessed by mechanical detection threshold, which was measured with von Frey hairs. Painful mechanical allodynia or pain in response to light touch (dynamic mechanical allodynia) tested by gentle/light stroking with a cotton wisp (~3mN), a cotton wool tip fixed to an elastic strip (~100mN), and a brush (~200–400mN) was observed in some CRPS patient as frequently as in patient with peripheral nerve injury (Gierthmühlen et al., 2012). Mice treated with CRPS-IgG did not exhibit hypersensitivity to punctate stimulation with calibrated von Frey filaments, which are likely to activate LTMRs or fibres with an activation threshold <1g (since the normal paw withdrawal threshold in the mouse is in the range 0.4-1g). von Frey hairs were applied on the plantar skin whereas LTMRs were recorded from the hairy skin.

In contrast, CRPS mice had hyperalgesia in the injured paw when challenged with the paw pressure (Randall-Selitto) test. A conical probe was used to apply an increasing
mechanical force (g) in the noxious range on the mouse paw (0-150g). In the present study, recordings on CRPS mice hairy skin have shown increased excitability in nociceptive HT-AM and CM fibres in response to high mechanical forces (>4g for AM and >15g for CM). Behavioural and electrophysiological recordings are therefore consistent.

Since thermal pain in CRPS patient is not a very distinct sign (Grothusen et al., 2014) and since the population of fibres that is responsive to thermal stimulation is of low frequency, we focused on mechanosensitive AM and CM fibres in the present study.

In this study, saphenous nerve fibres receptive fields were characterized in the uninjured hind paw hairy 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 (Banik and Brennan, 2008; Brennan, 1999), but are consistent with the more generalized regional pain seen in patients who develop CRPS after injury.

Although, nociceptors are clearly modified functionally by CRPS-IgG it is yet unclear how CRPS-IgG sensitizes nociceptors, and whether the autoantibodies responsible for pain bind directly to sensory neurons.

One possibility is the direct action of CRPS antibodies on nociceptive AM and CM fibres. In this case, CRPS-IgG may act via binding to epitopes expressed on the nerve terminals or be internalized by the nerve endings via endocytosis. CRPS-IgG might act via antibody-epitope interaction extracellularly or intracellularly and affect the signal transduction. This would suggest a Fab-epitope type of interaction and could explain why pain is not sensitive to treatment with steroids or other anti-inflammatory strategies (Barbalinardo et al., 2016).

It is possible that pathological autoantibodies are directed against ion channels expressed on sensory neurons and in this way alter neuronal function independently of inflammation. Hence, antibodies directed against CASPR2 have been associated with pain in seropositive patients (Irani et al., 2012; Klein et al., 2012; Watanabe, 2016). Passive transfer of CASPR2 IgG to mice induced tactile hypersensitivity. Electrophysiological studies of skin-nerve preparations from Cntnap2−/− mice identified an increased excitability to mechanical stimulation of DH fibres (Dawes et al., 2018). In their study,
Dawes and colleagues did not use the IgG transfer model for the electrophysiological recordings, possibly because of the limited availability of patient IgG. Cntnap2<sup>−/−</sup> originally served as a model for autism spectrum disorder (ASD) as the KO mice exhibited many of its characteristics. They display epileptic seizures as well as some core autism related deficits including stereotypic motor movements, behavioural inflexibility, and communication and social abnormalities (Gordon et al., 2016). Gordon et al. using a Caspr2:tau-LacZ knock-in reporter line (Cntnap2<sup>tlacz/tlacz</sup>), showed that CASPR2 is actually expressed in many brain region in the mouse including brain regions involved in different sensory processing (Gordon et al., 2016).

In this Chapter skin-nerve recording data were acquired from a highly translational mouse model of CRPS, transferred with patient IgG. The findings are therefore thought to reflect with higher similarities what occurs in the patient.

Another possibility is sensitization of the neurons via an Fc-FcR type of interaction where the constant region of the antibody, the Fc region, binds to Fc receptors (FcRs), previously shown to be expressed on sensory neurons (Qu, 2012). This type of interaction requires immune-complex (IC) formation with soluble antigen. Qu and co-workers showed that IgG-IC directly excites the primary sensory neurons through neuronal FcγRI. The incision is likely to trigger release of proteins from injured tissues, and it is possible that this may be associated with elevated levels of IgG-IC formation and nociceptor sensitization is possible. A lack of specific expression of the FcγRs would reject the present hypothesis. However, Andoh and Kuraishi have shown that the high affinity IgG receptor FcγRI, but not the low affinity receptors FcγRII and FcγRIII, was expressed especially on small- or medium-sized mouse DRG neurons (Andoh and Kuraishi, 2004). FcγRs expression on the peripheral fibres might however differ.

To determine to what extent the Fab or Fc region of the CRPS autoantibody is crucial in the sensitization of nociceptive nerve fibres, fragmenting the IgG into soluble Fab and Fc fractions would be required. Instead of injecting the mice with intact IgG, they would be injected with either the Fab region or with the Fc region of the IgG. Behavioural investigations would bring a first answer about the role of each antibody region.

The second possible mode of action of CRPS-IgG on nociceptors is via indirect activation of the nociceptors. CRPS-IgG would then not target nerves directly but bind to non-
neuronal cells which would sensitize nociceptors instead. Wigerbald et al. have suggested
a mechanism for pain in rheumatoid arthritis (RA) via the activation of osteoclasts by anti-
citrullinated protein antibodies (ACPA), which by release of nociceptive CXLC1
chemokines sensitizes nociceptors. Patients with RA have elevated levels of ACPA
associated with joint pain that persist even after when inflammation is suppressed with
anti-rheumatic drugs (Wigerblad et al., 2016). A similar mechanism of action is possible in
CRPS-IgG mediated pain where pain persists after resorption of the initial clinical signs of
inflammation especially in long standing chronic CRPS patients.
Taken together, these observations indicate that circulating autoantibodies maintain pain
and painful hypersensitivities in persistent CRPS by sensitizing peripheral nociceptors.
CHAPTER 6  INCUBATION OF CULTURED DRG NEURONS WITH CRPS-IGG INCREASES NEURONAL EXCITABILITY
6.1 Introduction

Analysis of skin-nerve recordings from CRPS-IgG treated mice showed an increased impulse frequency in response to mechanical stimulation in AM and CM fibres. To determine whether this functional abnormality is maintained in vitro, isolated DRG neurons were studied using the intracellular calcium ([Ca$^{2+}$])-sensitive dye fura-2. Using [Ca$^{2+}$]-measurements at modest magnification (10x objective) allows for rapid analysis of large numbers of neurons. Using an imaging technique, we wanted to investigate the effect of patient IgG on isolated DRG neurons. A simple way to measure neuron’s response to various modalities, i.e. chemical or temperature, in cultured neurons, is to measure change in their [Ca$^{2+}$].

6.2 Aim

The aim of the present study was to determine the effect of CRPS patient IgG on neuronal excitability. Specifically, these studies were designed to determine whether CRPS-IgG caused hyperexcitability (assayed as an increased sensitivity to challenges with K$^+$) and cold hypersensitivity in cultured DRG neurons.

6.3 Method

In an effort to assess the excitability of isolated DRG neurons, we used challenges with KCl. Mouse DRGs (all the DRGs) were dissected and DRG neurons cultured as described Chapter 2 (section 2.10). If patient IgG enhances the neuronal excitability, challenges with submaximally effective increases in [K$^+$] would be expected to elicit potentiated [Ca$^{2+}$]-responses.

We used two approaches in order to assess patient IgG effect on neuronal excitability; acute applications of IgG (30min) and extended (overnight ~12h to 24h) incubation in the presence of IgG to examine whether IgG were inducing direct activation/depolarisation or sensitisation of neurons. IgGs were diluted 1:10 for a final concentration of 0.8mg/ml in ECS for the acute application or in MEM when applied for overnight (12h to 24h) incubation with the neurons. IgG treated neurons were stimulated with capsaicin (1µM) before the end of each experiment and followed by 50mM KCl application to identify all viable neurons. DRG neurons were cultured and imaged as described in Chapter 2.
6.4 Results

6.4.1 Acute application of CRPS-IgG had no effect on \([\text{Ca}^{2+}]_i\) in naive DRG neurons

Initially, DRG neurons were challenged with acute application of CRPS or HC-IgG to determine whether IgG could depolarise neurons directly. The \([\text{Ca}^{2+}]_i\), response of DRG neurons to direct incubation with either CRPS or HC-IgG was examined. The neurons were in extracellular solution (ECS) for a minute, followed by addition of CRPS or HC-IgG (concentration of 0.8mg/ml). The \([\text{Ca}^{2+}]_i\) was monitored continuously for 30 minutes, followed by application of capsaicin (1µM) to identify TRPV1 expressing nociceptors and 50mM KCl to identify all viable neurons.

After 30 minutes incubation with HC or CRPS-IgG the \([\text{Ca}^{2+}]_i\) had increased in a subpopulation of DRG neurons (Figure 6–1). After incubation with CRPS-IgG, \([\text{Ca}^{2+}]_i\) had increased in 17% of neurons (56 of 327) and in about 24% of neurons incubated with HC-IgG (56 of 236). The proportion of DRG neurons responding with a \([\text{Ca}^{2+}]_i\)-increase was thus almost significantly larger after incubation with HC-IgG compared to CRPS-IgG (Fisher’s, p=0.06).

Figure 6–1 Example traces of naive mouse DRG neurons incubated with HC or CRPS-IgG for 30 min.

Example traces of DRG neurons incubated for 30 min with either HC (A) or CRPS-IgG (B). Neurons were washed for 2 min after IgG application and stimulated with capsaicin (1µM) followed by KCl (50mM).
6.4.2 EC50 for KCl in IgG incubated neurons

We calculated the equilibrium potential for potassium $K^+$ using the Nernst equation $V_{eq} = \frac{RT}{zF} \ln \left( \frac{[K^+]_{out}}{[K^+]_{in}} \right)$ where $R$ is the universal gas constant and is equal to 8.314 J.K$^{-1}$.mol$^{-1}$ (Joules per Kelvin per mole), $T$ is the temperature in Kelvin ($K=25 \ ^\circ C + 273.15$), $z$ is the valence (+1 for potassium), $F$ is Faraday’s constant and is equal to 96485 C.mol$^{-1}$ (Coulombs per mole), $[K^+]_{out}$ is the concentration of $K^+$ in the extracellular fluid, $[K^+]_{in}$ is the concentration of $K^+$ in the intracellular fluid, which we assumed to be 130mM (Molleman, 2002).

$V_{eq}$ was calculated for $[K^+]_{out}=5$mM and $[K^+]_{out}=25$mM, which respectively gave $V_{eq}$ of -83.7mV and -42.4mV. 5mM is the concentration of potassium in the ECS (see chapter 2).

Increasing the $[K^+]_{out}$ depolarizes neurons by reducing the electrochemical gradient for $K^+$ and thereby the $K^+$ conductance/currents that are responsible for keeping the resting membrane potential at $\sim$-70mV. A depolarization of about 30-40mV (as indicated by the calculations of the impact of increasing $[K^+]_{out}$ by 20mM) will open voltage gated sodium (NaVs) and calcium channels (CaVs). Different neurons will vary with respect to the types of voltage-gated channels expressed, resting membrane potential and therefore also in their response amplitudes.

$[K^+]_{out}$ =25mM would, in contrast with $[K^+]_{out}$=5mM, induce a substantial membrane depolarisation ($\Delta V_{eq}$=41.3mV), thereby activate NaVs and CaVs. The consequent $Ca^{2+}$ influx and increased $[Ca^{2+}]$, can be monitored using Fura-2.
Neuron response to different concentration of KCl.

(A) Naive mouse DRG neurons were incubated with HC or (B) CRPS-IgG before being stimulated with increasing concentrations of KCl of 5mM, 10mM, 15mM and 20mM. Neurons were then stimulated with capsaicin (1μM) followed by KCl (50mM) stimulation.

The effect of different \([K^+]\) increases were tested on DRG neurons in order to identify the half-maximal effective concentration (EC50) that would be amenable for detection of positive and negative modulation of the cellular activity and therefore provide a sensitive assay (Figure 6–3).

\([K^+] \) of 5mM, 10mM, 15mM and 20mM were first used to stimulate the neurons, which were then stimulated with capsaicin (1μM) to identify nociceptive neurons and with a final challenge of 50mM KCl to identify all viable neurons and to determine their maximal \([Ca^{2+}]\) response amplitude. The Δratio (340/380) response was plotted for the different used KCl concentrations. The EC50 was essentially identical for DRG neurons incubated with IgG from HC and CRPS, respectively 18.6±0.06mM (n=529) and 18.9±0.6mM (n=677). We therefore chose a \([K^+] \) of 20mM as an ideal concentration for the following experiments. Next, we examined the effect of incubation with HC- or CRPS-IgG on \([Ca^{2+}]\) responses evoked by 20mM KCl (Figure 6–4).
**Figure 6–3** Dose response curve of DRG neurons incubated overnight with HC or CRPS-IgG to increasing concentration of KCl.

(A) Naive mouse DRG neurons were incubated with HC or (B) CRPS-IgG and stimulated with increasing concentration of KCl. n=3 coverslips for each condition. HC, EC50 18.6 mM ± 0.06, n cells=529; CRPS, EC50 18.9 mM ± 0.6, n cells=677. Data are mean ± SEM.

6.4.3 CRPS-IgG increased $[\text{Ca}^{2+}]_i$ in response to 20mM KCl stimulation

A brief 30 second application of 20mM KCl increased $[\text{Ca}^{2+}]_i$ response in both HC and CRPS-IgG incubated cells (Figure 6–4A-B,D).

Stimulation with 20mM KCl evoked significantly larger $[\text{Ca}^{2+}]_i$-responses in a subset of neurons incubated with IgG from CRPS patients compared to HC-IgG (p<0.001, Mann-Whitney test) (Figure 6–4C). Interestingly, neurons incubated with CRPS-IgG displayed an increased $[\text{Ca}^{2+}]_i$ response (Figure 6–4A) that did not come back to baseline level as observed for neurons incubated with HC-IgG (Figure 6–4B). This observation indicates that the larger $[\text{Ca}^{2+}]_i$-transients evoked by 20mM KCl in a subset of CRPS-IgG treated DRG neurons may be more persistent.

Behavioural experiments demonstrate that passive transfer of CRPS-IgG in combination with a paw incision predominantly, or exclusively affects the ipsilateral, injured paw (Chapter 3). Additionally, analysis of the major classes of mechanosensitive sensory afferent fibres (Chapter 5), showed that low threshold fibres were unaffected by CRPS-IgG and mechano-insensitive fibres were not studied. A large proportion of isolated DRG neurons are thus likely to be unaffected by CRPS patient IgG. To determine whether the $[\text{Ca}^{2+}]_i$-response amplitude was increased in a subset of DRG neurons treated with CRPS-IgG, we compared the distribution of response amplitudes in neurons following IgG treatment. Most of the HC-IgG treated neurons had a relatively low amplitude response,
between 0 and 0.5, whereas CRPS-IgG treated neurons mostly had a response amplitude that was above 0.5 (Figure 6–4E). The distribution of the neuronal response amplitudes indicated that a population of cells became responsive to low potassium stimulation following an overnight (12h-24h) incubation with CRPS-IgG but not with HC-IgG (Figure 6–4E). Neurons with a response amplitude >0.8 (Figure 6–4E) were analysed for their response to capsaicin, in CRPS-IgG treated neurons 79% (total capsaicin responders 47.5%) were capsaicin responders and in HC-IgG treated neurons 82% were capsaicin responders (total capsaicin responders 47.6%). These results indicate that neurons with enhanced sensitivity to low potassium stimulation occurs mostly in capsaicin responders and that CRPS-IgG mostly sensitizes nociceptive neurons. It is also possible that IgG from different patients contain antibodies against a repertoire of targets, not all of which are central for the clinical presentation of CRPS. It is currently unclear whether the effect of the preparation used in Figure 6–4 is representative for patients broadly, or whether the hyperexcitability produced is unique to this patient. A second patient IgG preparation should be used in future investigations.
Figure 6–4 Cells response to 20mM KCl after overnight incubation with HC-IgG or CRPS-IgG

(A) Example traces of neurons incubated with HC and (B) CRPS-IgG that were stimulated with 20mM KCl, capsaicin and 50mM KCl. (C) CRPS-IgG incubated neurons had an increased Δratio (340/380) in comparison with HC-IgG treated neurons (Mann-Whitney, p<0.001). (D) The average cell response curve showed that CRPS-IgG increased [Ca2+]i response in the neurons at least two fold. (E) Bin distribution of CRPS and HC-IgG incubated neurons response amplitude to 20mM KCl. HC-IgG (n=332); CRPS-IgG (n=257); Mann-Whitney test, ***p<0.001.
6.4.4 Reproducibility of KCl-evoked $[Ca^{2+}]$, responses

Next, the neuronal responsiveness to repeated challenges with 20mM KCl following incubation with HC or CRPS-IgG was examined. Reproducible responses to repeated challenges would facilitate pharmacological investigations aimed at identifying the ion channels involved. An altered desensitization to repeated depolarizing challenges may further indicate that IgG from CRPS patients alters the neuronal excitability, or the cellular handling of, or metabolic response to increased Ca$^{2+}$.

Neurons incubated in the presence of either HC or CRPS-IgG were stimulated twice with 20mM KCl for 30 seconds each, with a 2 min recovery in between, followed by challenges with capsaicin 1µM and 50mM KCl (Figure 6–5).

![Graph](image)

**Figure 6–5 Neuronal responses to repeated challenges with 20mM KCl.**

Repeated challenges with 20mM KCl evoked $[Ca^{2+}]$-responses of similar amplitudes, but substantially smaller than responses evoked by capsaicin and 50mM KCl.

Two consecutive challenges with 20mM KCl evoked $[Ca^{2+}]$-responses of similar amplitude in DRG neurons treated with HC-IgG ($p>0.05$, Dunn’s test). In contrast, CRPS-IgG treated neurons exhibited larger $[Ca^{2+}]$-amplitudes ($p<0.001$, Dunn’s multiple comparison test) in response to the first 20mM KCl stimulation compared to the second application (Figure 6–6). The amplitude observed in response to the first KCl challenge in CRPS-IgG treated cells, was significantly higher compared to HC-IgG treated cells ($p<0.001$, Dunn’s test), whereas the second KCl challenge evoked $[Ca^{2+}]$-response of similar amplitudes (Figure
These results indicate that the hypersensitivity observed \textit{in vivo} and the hyperexcitability seen \textit{ex vivo tissue} preparations can be recapitulated by incubating isolated DRG neurons in IgG from CRPS patients.

\textbf{Figure 6–6} CRPS-IgG increased the KCl-evoked $[Ca^{2+}]_i$-response amplitudes. 
\textit{HC} n=408, CRPS=474, n=2 coverslips for each. **$p<0.01$, ***$p<0.001$, Dunn’s multiple comparison test.
6.4.5 Effect of mibebradil on KCl-evoked \([\text{Ca}^{2+}]\)-responses in IgG treated DRG neurons

DRG neurons express a range of voltage-gated \(\text{Ca}^{2+}\) channels (Li et al., 2017; Murali et al., 2015; Watanabe et al., 2015). Earlier studies have demonstrated a surprisingly marked effect of the selective T-type \(\text{Ca}^{2+}\) channel inhibitor mibebradil on CGRP release evoked by KCl (40mM) in synaptosomes from the mouse dorsal horn (Quallo et al., 2015). These results prompted an investigation of the effect of mibebradil on KCl-evoked \([\text{Ca}^{2+}]\) in DRG neurons incubated with HC and CRPS-IgG.

Incubation with CRPS-IgG enhanced the \([\text{Ca}^{2+}]\)-response amplitude evoked by 20mM KCl stimulation. The distribution of \([\text{Ca}^{2+}]\)-response amplitudes (Figure 6–7C) demonstrates the increased responsiveness of DRG neurons incubated with CRPS-IgG compared to HC-IgG (Figure 6–7C).

Responses were not maximal since 20mM is about EC50, which indicated that KCl did not fully activate CaV or did not activate all CaVs. Under conditions that cause a submaximal voltage-gated \(\text{Ca}^{2+}\)-entry, low-voltage activated calcium channels (LVAs) such as T-Type \(\text{Ca}^{2+}\)-channels play an important role, and previous work from the lab identified a marked inhibition of KCl-evoked CGRP release by mibebradil (Quallo et al., 2015). To determine the importance of T-type \(\text{Ca}^{2+}\)-channels for the increased \([\text{Ca}^{2+}]\)-responses in CRPS-IgG treated neurons, we therefore examined the selective CaV inhibitor mibebradil (10µM).

Neurons were pre-treated with 10µM mibebradil and then stimulated with 20mM KCl in the presence of mibebradil (Figure 6–8).
Figure 6–7 Response amplitude and relative amplitude of cells incubated with HC or CRPS-IgG stimulated with 20mM KCl pre-treated or not with mibefradil.

(A) Naive mouse DRG neurons were incubated with HC-IgG or (B) CRPS-IgG. Mean response amplitude of naive neurons (mean ± SEM; n total): No IgG (24.4 ± 1.2; n=386), HC-IgG only (33.1 ± 0.7; n=1330), HC-IgG + mibefradil (23.3 ± 1.1; n=412), CRPS-IgG only (44 ± 0.8; n=1414), CRPS-IgG + mibefradil (19.5 ± 0.8; n=670). Data are shown as dot plot and box plot (percentile 25-75%). Percentage frequency distribution of [Ca^{2+}]-responses amplitudes evoked by 20mM KCl. (C) Incubation of the neurons with CRPS-IgG increased the [Ca^{2+}]-response amplitudes in a subset of neurons. (D) Mibefradil treatment reversed the phenotype induced by CRPS-IgG.
Figure 6–8 Example traces of mibefradil treated neurons.

Neurons were pre-treated with mibefradil 10µM, a T-type CaV blocker, for 1 minute before to be challenged a first time with mibefradil and KCl (20mM), and a second time with KCl (20mM) only. Capsaicin and KCl (50mM) were applied at the end of the experiment.

The amplitude of responses evoked by 20mM KCl in mibefradil treated neurons was compared to non-mibefradil treated neurons and to control neurons that have not been in contact with any IgG (Figure 6–7).

Neurons incubated with HC-IgG exhibited a larger response amplitude to 20mM KCl when compared to naïve neurons (no IgG incubation) (n=386) (p<0.001, Dunn’s multiple comparison test, Figure 6–7A). Furthermore, mibefradil treatment of HC-IgG incubated neurons reduced the neurons response amplitude to 20mM KCl (n=1330 and n=412, p<0.001, Dunn’s multiple comparison test, Figure 6–7A).

Challenges with KCl (20mM) produced responses of significantly larger amplitudes in CRPS-IgG incubated neurons (n=1414) when compared to untreated (no IgG) DRG neurons (n=368) (p<0.001, Dunn’s multiple comparison test, Figure 6–7B).

Mibefradil treatment of CRPS-IgG incubated neurons (n=670) significantly decreased the response amplitude of the highly responsive neurons (p<0.001, Dunn’s multiple comparison test, Figure 6–7B and D). In addition, mibefradil treated neurons displayed a similar response amplitude to non-treated neurons, demonstrating that mibefradil sensitive ion channels are important for the neuronal responsiveness to moderate depolarisations. Mibefradil successfully reversed the increased responsiveness to KCl (20mM) of CRPS-IgG incubated neurons.
6.4.6 CRPS-IgG increased the number of cold sensitive DRG neurons

Cold ramps (from 32 to 10°C), stimulated \([\text{Ca}^{2+}]\)-responses in 4.7% of control naïve DRG neurons, incubation with CRPS-IgG increased the proportion of neurons activated by cooling to 10.2% and incubation with HC-IgG increased the proportion of neurons activated by cooling to 7% (Figure 6–9).

A significantly higher number of neurons responded to cold in CRPS-IgG incubated neurons when compared to naïve neurons (χ² test, p<0.001) and to HC-IgG incubated neurons (χ² test, p<0.01). Surprisingly, incubation with HC-IgG significantly increased the proportion of cold responders (χ² test, p<0.05) (Figure 6–9). The percentage of capsaicin responders among the cold sensitive neuronal population was also analysed (Figure 6–9A). 33% (9/27) of the non-treated cold sensitive neurons, 36% (32/89) of the HC-IgG incubated cold sensitive neurons and 40% (35/89) of the CRPS-IgG treated cold sensitive neurons were sensitive to capsaicin. There was no statistical difference between the groups (χ² test). These data suggest that CRPS-IgG incubation of DRG neurons induces a marked increase in the number of cold responders among the whole population of neurons.

Figure 6–9 Population of cold sensitive neurons in naïve DRG neurons and in neurons incubated with HC or CRPS-IgG.

(A) Example traces of cold sensitive neurons. A cold ramp was applied on the neurons (cooling from 32°C to 10°C) before to be stimulated with capsaicin and KCl. (B) Cold ramps were applied on control naïve neurons, HC-IgG or CRPS-IgG incubated neurons. Neurons with \([\text{Ca}^{2+}]\)-responses were considered as cold sensitive neurons. *p<0.05, **p<0.01, ***p<0.001, χ² test.; HC vs CRPS.
An altered temperature sensitivity *in vivo*, may be generated either by recruiting novel temperature sensitive neurons or by changing the activation threshold or response amplitude of thermosensitive neurons. Analysis of naïve, untreated DRG neurons demonstrate a wide range of cold activation thresholds, in good agreement with previous work (Viana et al., 2002). Incubation of DRG neurons with CRPS-IgG (19.8±0.52°C, n=87 cells) did not change their activation temperature threshold compared to HC-IgG incubated neurons (18.8±0.53°C, n=89 cells) and naïve neurons (22±0.97°C, n=27 cells) (Figure 6–10A, ns, Dunn’s test). The number of naïve neurons was lower than for IgG treated neurons. A higher number of neurons would perhaps give more accurate results.

In contrast to our behavioural investigations *in vivo*, HC-IgG appeared to reduce the average cold activation temperature threshold (Figure 6–10A, p<0.01, Dunn’s test), in addition to increasing the population size of cold sensitive DRG neurons somewhat.
Figure 6–10 Temperature activation threshold of DRG neurons. (A) Naive (no IgG), and HC and CRPS-IgG incubated neuronal response threshold to temperature change. CRPS average temperature threshold 19.8±0.52°C (n=87 cells), HC average temperature threshold 18.8±0.53°C (n=89 cells), No IgG average temperature threshold 22±0.97 (n=27 cells). (B) Data are shown as bin distribution (2°C bin) for naive DRG neurons, (C) HC-IgG incubated neurons and (D) CRPS-IgG incubated neurons. Data are mean±SEM. Dunn’s multiple comparison test, **p<0.01.
For a closer inspection of these results (Figure 6–10A), the data were also plotted as a bin distribution for each group (Figure 6–10B-D). In non-treated naïve neurons, many neurons responded to cold around 26°C (Figure 6–10B) and the rest responded mainly to temperature between 14°C and 24°C.

In contrast, in HC and CRPS-IgG incubated neurons, cold responder neurons seemed to cluster in two groups (Figure 6–10C-D). For HC-IgG incubated neurons, two peak number of responders were observed for 16°C and 24°C (Figure 6–10C). The other responders were distributed in a Gaussian-like fashion.

CRPS-IgG incubated neurons showed a slightly different pattern to HC group with two peaks but one at 20°C and another at 24°C (Figure 6–10D). A less marked scission and a more spread distribution was observed among CRPS-IgG treated cold responders. This observation would be consistent with the novel cold-sensitive neurons, after treatment with IgG, responding at lower temperatures.

The observed increase in the population of cold-sensitive DRG neurons and a reduced mean temperature threshold for activation, suggests that CRPS-IgG may confer cold sensitivity to normally temperature-insensitive neurons. Surprisingly, HC-IgG also increased the proportion of DRG neurons that respond to cooling with a [Ca²⁺]-increase. Future investigations will determine whether these effects were specific to IgG from the donating individuals, or whether the observations hold true for IgG from other patients and control subjects.
6.5 Discussion

The results presented in this chapter demonstrate that an acute 30-minute incubation of the neurons with IgG, did not induce significant change in the number of responders in response to acute application of IgG. In contrast, overnight incubation of freshly dissociated neurons with IgG from CRPS patient increased the neurons excitability to low concentration of KCl (20mM) challenge and doubled the proportion of DRG neurons that respond to cooling. Incubation with CRPS-IgG greatly enhanced the \([\text{Ca}^{2+}]\)-response amplitude evoked by 20mM KCl stimulation.

These findings strongly suggest that longer incubations with CRPS-IgG sensitise DRG neurons directly. The KCl-evoked \([\text{Ca}^{2+}]\)-responses were inhibited by application of the selective T-type CaV blocker, mibefradil. This pharmacological tool is relatively selective for T-type CaV compared to other CaV isoforms, but it does effectively inhibit NaV1.8 and NaV1.9 at the same concentrations as T-type channels (Coste et al., 2007). Mibefradil may thus inhibit KCl evoked \([\text{Ca}^{2+}]\)-responses by acting on these three channels, which are all thought to be important in chronic pain conditions and which may facilitate repetitive firing in DRG neurons.

Although the average activation thresholds of cold sensitive neurons were not different between CRPS and HC-IgG incubated neurons, the fact that a larger population of neurons responded strongly, indicate that CRPS-IgG recruits novel, abnormal cold sensitivity in some neurons. In the present study, neurons were tested for their response to cold ramps. Since the presence of TRPA1 does not influence cold sensitivity of DRG neurons (Bautista et al., 2007) and since the most specific TRPM8 agonist available, icilin, is particularly susceptible to desensitisation (Kühn et al., 2009) and may not identify all TRPM8 expressing neurons in culture, we did not use TRPM8 agonist (e.g. icilin) or TRPA1 agonist (e.g. AITC).

In the present study, a single CRPS patient and healthy donor (HC) were used for IgG purification, the same used for behavioural and skin-nerve preparations presented in the previous chapters. Future investigations using different patients and control subjects IgG are required to determine whether or not the observed effects are representative of the wider patient population.
In the present study, dissociated DRG neurons from naïve mice were exposed to human IgG in an ex vivo setting. In the previous chapters, behavioural and skin-nerve recordings were performed on the CRPS-trauma-transfer model. Mice were subjected to a paw incision and injected intraperitoneally with CRPS patient or control subject IgG. CRPS-IgG induced hypersensitivity to noxious mechanical and cold stimuli in the mice ipsilateral paw. DRG neurons could be harvested from these treated mice in order to study the functional properties of the very neurons responsible for the painful hypersensitivity. Retrograde labelling, the local injection of a dye that is internalised by the peripheral terminals or axons of sensory neurons, followed by transport to the cell soma, can be used to study the functional properties of neurons innervating particular tissues (Rigaud et al., 2008; Schmidt and Rathjen, 2011; da Silva Serra et al., 2016). Neurons that innervate the hind mouse paw have their soma located within the DRGs situated at L3, L4 and L5 levels in C57BL/6 mice (Rigaud et al., 2008). In a study conducted by da Silva Serra and colleagues., it has been shown that only ~6% of the neurons (mostly L3 and L4 neurons) innervate the hind paw skin (da Silva Serra et al., 2016).

The percentage of neurons innervating the hind paw skin constitute a limitation as ipsilateral and contralateral L3-L5 DRGs need to be separated. Indeed, several mice need to be injected with IgG (limited amount) and incised in order to have a sufficient number of retrogradely labelled neurons. ~5% of the neurons were cold responders in our naïve mice. Only 0.3% of ipsilateral L3-L5 neurons would be expected to form hind paw cold receptive afferents in this situation. Retrograde labelling of the neurons innervating the mouse hind paw would be more suitable for patch-clamp experiments. The neuronal excitability could be investigated using the current-clamp configuration in retrogradely labelled neurons. The ionic basis for any hyperexcitability, could thereafter be investigated using voltage-clamp and pharmacological tools in order to identify the type of current and ion channels responsible.

It is yet unclear whether the direct effect of patient IgG on DRG neurons is prevalent among CRPS patients, or a particular feature of the patient IgG we used for this study. It is also unclear whether IgG from CRPS patients in general produce functional modifications of DRG neurons directly, or whether they do so indirectly.
Studies conducted on rat and mouse DRG neurons suggest that neurons express IgG receptors on their membrane, the FcγRs (Andoh and Kuraishi, 2004; Qu et al., 2011). Both studies suggested the direct action of IgG-antigen immune-complex (IC) on FcγRI. Both showed using the calcium imaging technique that the IC, but neither of the antibody (IgG) or antigen alone, produced an increase in intracellular calcium. IC, in addition, induced release of substance P of DRG neurons (Andoh and Kuraishi, 2004) and increased the number of action potentials evoked by a depolarizing current during current-clamp recordings (Qu et al., 2011). IgG need to form immune complex with the antigen and act through its Fc region by binding to the FcγRI on DRG neurons. In our study, the purified IgG that we use does not contain IC, but it is possible that some may be formed after administration in vivo. However, CRPS is not a destructive condition and is unlikely to be caused by conventional Fc-IgG dependent activities. Furthermore, acute applications of IgG did not elicit [Ca^{2+}]-responses in the current study. It then appears highly unlikely that FcγR are involved.

It is more likely that in the present study, CRPS-IgG act via binding to antigens expressed either on the neuron extracellular membrane or to intracellular targets, which is possible when the IgG is internalized by the neurons.

Geis et al., showed that intrathecal application of purified patient anti-amphiphysin IgG induces stiff person syndrome-like symptoms in rats, including stiffness and muscle spasms. They further showed that anti-amphiphysin IgG was internalized into neurons by an epitope-specific mechanism and colocalized in vivo with presynaptic vesicular proteins (Geis et al., 2010).

In contrast, Congdon et al. study showed that antibody uptake into neurons occurs primarily via clathrin-dependant FcγR endocytosis. Excessive Tau aggregates are found in brain neurons of patient with neurodegenerative diseases i.e. Alzheimer disease or Parkinson. Tau immunotherapy can clear Tau in the neurons. Their results showed that Tau antibody uptake correlates with Tau levels, the uptake primarily takes place via clathrin-dependent FcγII/III endocytosis and is required for acute Tau clearance (Congdon et al., 2013).

Dawes and colleagues similarly showed that CASPR2 antibody treatment of mouse DRG neurons induces a reduction in Kv1 channel surface expression by its internalisation...
(Dawes et al., 2018). In that case, CASPR2 antibody contributed to clinical pain by directly targeting neuronal molecules that regulate cell excitability.

The exact mechanism of action of CRPS-IgG on neurons is not fully understood and it is possible that the in vivo mechanism of action of CRPS-IgG might be separate from the in vitro activities described in this chapter. Further investigations will clarify if IgG from other patients with persistent CRPS also causes nociceptor hyperexcitability.
CHAPTER 7  DISCUSSION AND CONCLUSION
CRPS is an extremely painful condition and was reported for the very first time during the 19th century (Iolascon et al., 2015). Acceptance of the pathology as a physiological condition rather than a psychological condition took a long time and it still remains a difficult condition to diagnose (Bruehl et al., 2016; Harden et al., 2010, 2013). Patients with chronic long-standing CRPS experience extreme pain, although the initial injury, autonomic dysfunction and inflammation have resolved. A lack of mechanistic understanding of the disease makes it particularly difficult to treat and therapies mostly fail in alleviating the pain. In most chronic pain conditions, pain deeply affects patient quality of life and mental health (Torrance et al., 2014). Chronic pain patients in general, and CRPS patients in particular, are affected by a larger number of depressions compared to the normal population (Torrance et al., 2014; Brinkers et al., 2018). In the most severe cases CRPS patient ask for limb amputation (Kashy et al., 2015).

Compared to other neurological conditions, our understanding of the mechanisms responsible for chronic pain of different aetiologies is incomplete. Over the last few years, an interest in pain caused by autoantibodies is emerging. Perhaps the two best examples are rheumatoid arthritis (RA) and neuromyotonia. In RA, antibodies directed against citrullinated proteins (ACPAs) are thought to induce pain (Wigerblad et al., 2016). In patients with a rare form of neuromyotonia the pain is caused by autoantibodies directed against VGKC complex (CASPR2) (Dawes et al., 2018; Klein et al., 2012). In both cases, the pathogenic antibody has been identified, and has been used for passive transfer to mice, which resulted in pain behaviour. Passive transfer of human pathology to mice offers very important advantages over other forms of mechanistic, translational models. Unlike other translational approaches, passive transfer enables investigators to examine the pathophysiological consequences of the human pathogen in experimental animals. The translational predictive value of passive transfer models is thus almost unique, because of their excellent face and construct validity.

Using passive transfer of human IgG to mice, we have reproduced the sensory abnormalities observed in CRPS patients in the mouse. In CRPS patients, pain develops after an initial trauma/injury in the affected limb and is confined to the affected limb. Unlike other autoimmune pain condition such as RA, lupus or Sjögren’s syndrome, CRPS pain is in general unilateral.
Here, CRPS patient IgG was transferred to mice that were subjected to a plantar skin-muscle incision in one hind paw. Importantly, this approach accurately reproduced the sensory symptoms presented by CRPS patients. The mice exhibited hypersensitivity to mechanical, cold and heat stimuli, in the injured paw, but not in the uninjured contralateral paw.

We showed that the initial amount of injected IgG was crucial in obtaining a lasting pain behaviour. A dose regimen of 8mg IgG from patients with severe pain on four consecutive days was necessary in inducing significant mechanical and cold hypersensitivity. The transferred painful hypersensitivities appeared persistent and persisted for at least two weeks, in good agreement with the time course of other passively transferred neurological disorders (Wigerblad et al., 2016).

Interestingly, the pain score of CRPS-IgG donors correlated with the hypersensitivity observed in mice injected with IgG pooled from patients with either high pain score or with low/intermediate pain score. Our findings strongly suggest a role for the autoantibody titre in the pain intensity observed in mice, but it is also possible that different CRPS patients have autoantibodies targeting different epitopes or proteins. This would be consistent with findings in other neurological autoantibody mediated conditions, such as those caused by anti-VGKC. Irani et al. measured VGKC-antibody levels in 96 patients and 70 control individuals. The level of VGKC-antibody varied according to the clinical condition and varied among patients with the same pathology. In addition, two distinct proteins complexed with VGKC were found to be targeted by VGKC-antibody, CASPR and LGI1 (Irani et al., 2010). Thereby, two distinct antibodies, CASPR2 and LGI1 were identified. Patients with CASPR2 or LGI1 antibodies shared some similar clinical features such as amnesia, confusion and neuropsychiatric symptoms. However, in patients with CASPR2 autoantibodies, seizures were less common, neuromyotonia, neuropathic pain, insomnia, dysautonomia and weight loss were more frequent than in patients positive for LGI1-autoantibodies (Irani et al., 2010).

Skin-nerve preparations from mice treated with CRPS-IgG exhibited a significantly higher rate of spontaneous activity, which may be consistent with the presence of paraesthesias and spontaneous pain (Chapter 5). However, the dynamic weight bearing assay did not reveal a significant behavioural difference between the two treatment groups. It is possible that the absence of tactile hypersensitivity in these mice (Chapter 3) means that
normal movement is not painful. Future immunohistochemical studies focused on the presence of activity markers such as c-fos, arc, pERK and p38 in DRGs and the dorsal horn may reveal whether mice treated with CRPS-IgG experience spontaneous pain associated with spontaneous input to the dorsal horn (Hunt et al., 1987; Tsuji et al., 2000; Häring et al., 2018).

The goal of this study was to elucidate some of the physiological mechanisms responsible for CRPS pain using a translationally valid mouse model of CRPS. Early animal models of CRPS consisted in tibial fracture and limb immobilization of the hind leg in rats or mice (Guo et al., 2004; Wei et al., 2016). However, it is unclear whether these models share a pathophysiological basis with CRPS. Here we have demonstrated that the passive transfer/trauma mouse model, recapitulates the main sensory signs of CRPS in vivo, and we have examined its impact on the functional properties of all the major classes of mechanosensitive primary afferent neurons, using the ex vivo skin-nerve recording technique.

CRPS-IgG sensitized nociceptors in the injured paw, which displayed an increased discharge rate evoked by mechanical stimuli. In contrast, the properties of low threshold Aβ and Aδ DH fibres were unchanged. Both findings were consistent with QST studies of CRPS patients, which report spontaneous pain, mechanical, cold and heat hypersensitivities, but not tactile hypersensitivity (Gierthmühlen et al., 2012; Huge et al., 2008).

\([\text{Ca}^{2+}]\) imaging of CRPS-IgG incubated naive mouse neurons suggested a sensitization of a population of neurons that were hyperexcitable after incubation with CRPS-IgG, consistent with the electrophysiological findings. Most of the neurons that became hyperexcitable after incubation with CRPS-IgG were TRPV1 positive nociceptor neurons. Together, these results strongly suggest that CRPS pain is caused by IgG autoantibodies, which act by sensitizing nociceptors, at least in part directly.

The results presented in this study were performed with IgG from patients with long standing chronic CRPS. The mice behaviour was monitored for a maximum of 13 days post-incision and in most cases for 3 days post-incision, on which day the skin-saphenous nerve was dissected for fibre activity recording. Although CRPS-IgG treated animals were hypersensitive to mechanical pressure on day 3 post-incision, measuring the fibres activity
at a later stage (*e.g.* day 13 post-incision) might be more representative of the chronic phase of CRPS in the mice. In addition, repeated administration of patient IgG may mimic persistent CRPS, which would allow more valid investigations of anatomical changes (such as loss of IENFs).

*In vitro* skin nerve recordings and [Ca\(^{2+}\)] imaging experiments were performed with IgG from a single CRPS patient IgG. Although, this approach ensures reproducibility and consistency throughout the series of experiments by limiting differences between IgG preparations from different patients, it may mean that heterogeneity in the activity of patients IgG preparations is overlooked. Repeating the experiments with IgG from other CRPS patients with perhaps distinct clinical features would validate the highly translational characteristic of CRPS. Treating the mice with IgG from patient with a more acute form of CRPS or an early stage CRPS patient might provide some insight regarding the pathophysiological mechanisms of CRPS in more acute and early phases versus chronic stages of CRPS. This approach would allow us to assess whether or not sensory abnormalities observed in CRPS patient in early stages is also caused by autoantibodies. However, skin-nerve investigations are time-consuming and laborious compared to behavioural studies. We have therefore examined the activity of IgG from 2 additional individual patients and IgG pooled from a cohort in behavioural experiments to establish whether our findings apply to the wider population of patients. Electrophysiological investigations are suitable for detailed mechanistic studies, but not for repeated characterization of samples.

Additional investigations of the cold and heat sensitivity of A\(\delta\) and C-fibres (Zimmermann et al., 2009) could enhance our understanding of the actions of CRPS IgG and may be an interesting avenue for future investigations. Nerve fibres were identified in the first place based on the fibre response to mechanical stimulus. However, mechanically insensitive fibres do exist in the mouse skin and could be identified and characterized when searched with electrical stimulus. Identification of mechanically insensitive-cold sensitive fibres can be also achieved using cold stimulus and fibres responses analysed. In our study, we focused exclusively on mechano-sensitive fibres, since mechanical hypersensitivity is the most prominent sensory abnormality observed in patients (Gierthmühlen et al., 2012).
Therefore, many other types of fibres were not studied and characterized. To include other these remaining classes of fibres would require more time than was available.

In the present study we used the in vitro intracellular $[\text{Ca}^{2+}]_i$-imaging technique to measure dissociated DRG neurons response to various stimuli following their direct incubation with patient IgG. This technique consists in dissecting DRGs, dissociating the neurons and culturing them in an in vitro setting to measure the neurons sensitivity to various stimuli (e.g. cold). Neuronal properties and quality may vary from experiment to experiment and bias the results. This needs to be taken into consideration although we reduced the risks of variations by treating the neurons which were from the same animal with both control and CRPS IgG on the same day. Some population of neurons especially the large diameter neurons may be underrepresented because of their fragility and low survival rate throughout the culturing process.

An alternative approach to tackle the bias induced by in vitro experiments would be to use the in vivo imaging approach which uses the genetically encoded fluorescent calcium indicator GCaMP to study the activity of DRG neurons in live mice that can be challenged with both noxious and innocuous stimuli (Chisholm et al., 2018; Emery et al., 2016).

In the present study, our findings demonstrate an autoimmune basis of CRPS. However, the proteins targeted by the pathological autoantibodies have not yet been identified. In contrast with previous studies of autoimmune pain conditions, the mice were injected with total IgG as the autoantibody is not yet known. Future efforts should be focused on the identification of target proteins and epitopes.

To improve our understanding of the role of IgG in CRPS, a depletion or fractionation of IgG into subclasses IgG1, IgG2, IgG3 and IgG4 may be useful (Bird et al., 1984). Different subclasses can be administered to mice and behavioural changes studied (Beenhouwer et al., 2007; Dekkers et al., 2017). Identification of the subclass to which the pathogenic IgG of CRPS patients belongs, may bring additional information on the mechanisms involved. Clinical observation of CRPS patients, as well as results from our experimental animal model, indicate that CRPS is a non-inflammatory autoimmune pathology, since pain is typically unaffected by resolution of inflammation. Indeed, there is no tissue damage induced as seen in lupus disease for instance (Nowling and Gilkeson, 2011). CRPS is
associated with exacerbated swelling, but since pain remains after swelling has been resolved, it does not appear to be critical for pain (Goebel, 2011). Importantly, different IgG subclasses are known to be more or less inflammatory (Dekkers et al., 2017). In particular, the IgG4 subclass does not activate the complement system, unlike the other subclasses of IgG and therefore does not induce inflammatory response (Bindon et al., 1988; Bruhns et al., 2009). The lack of effector function of IgG4 antibodies and the absence of inflammatory damage in CRPS patients suggests that an autoimmune IgG4 may be involved in the aetiology of CRPS. Alternatively, it is possible that pathogenic IgG exert their effects by promoting internalisation of its target protein(s), similar to what has been found with anti-CASPR2 IgG (Dawes et al., 2018; Irani et al., 2010).

Identification of the antibodies involved in CRPS pain will facilitate diagnosis of CRPS and may enable prognostic testing before elective surgery. Beyond diagnostic applications, identification of the targeted proteins and epitopes would identify novel therapeutic rationales and development of targeted and specialised treatments to inhibit pain in CRPS patients and avoid limb amputations.
CHAPTER 8 REFERENCES


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using GCaMP. Science advances, 2(11), e1600990.


Expression and Regulation of Cav3.2 T-Type Calcium Channels during Inflammatory Hyperalgesia in Mouse Dorsal Root Ganglion Neurons. PloS One 10, e0127572.


CHAPTER 9  APPENDICES
# ARRIVE Guidelines Checklist

**Animal Research: Reporting In Vivo Experiments**

Carol Kilkenny¹, William J Browne², Innes C Cuthill³, Michael Emerson⁴ and Douglas G Altman⁵

¹The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, ²School of Veterinary Science, University of Bristol, Bristol, UK, ³School of Biological Sciences, University of Bristol, Bristol, UK, ⁴National Heart and Lung Institute, Imperial College London, UK, ⁵Centre for Statistics in Medicine, University of Oxford, Oxford, UK.

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<th>RECOMMENDATION</th>
<th>Section/Paragraph</th>
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<tr>
<td>Title</td>
<td>Provide as accurate and concise a description of the content of the article as possible.</td>
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<tr>
<td>Abstract</td>
<td>Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.</td>
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<tr>
<td>INTRODUCTION</td>
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<td>Background</td>
<td>a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale.</td>
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<td></td>
<td>b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study’s relevance to human biology.</td>
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<tr>
<td>Objectives</td>
<td>Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.</td>
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<td>METHODS</td>
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<tr>
<td>Ethical statement</td>
<td>Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal (Laboratory Procedures) Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.</td>
<td>✓</td>
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<tr>
<td>Study design</td>
<td>For each experiment, give brief details of the study design including: a. The number of experimental and control groups; b. Any steps taken to minimize the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and who). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.</td>
<td>✓</td>
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<tr>
<td>Experimental procedures</td>
<td>For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).</td>
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<tr>
<td>Experimental animals</td>
<td>a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naive, previous procedures, etc.</td>
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<td>Housing and husbandry</td>
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<td>a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish).</td>
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<td>b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish; type of food, access to food and water, environmental enrichment).</td>
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<td>c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.</td>
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<td>Sample size</td>
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<td>a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group.</td>
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<td>b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used.</td>
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<td>c. Indicate the number of independent replications of each experiment, if relevant.</td>
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<td>Allocating animals to experimental groups</td>
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<td>a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done.</td>
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<td></td>
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<td>b. Describe the order in which the animals in the different experimental groups were treated and assessed.</td>
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<td>Experimental outcomes</td>
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<td>Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).</td>
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<td>Statistical methods</td>
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<td>a. Provide details of the statistical methods used for each analysis.</td>
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<td>b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron).</td>
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<td>c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.</td>
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<tr>
<td>RESULTS</td>
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<td>Naïve time points always shown.</td>
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<tr>
<td>Baseline data</td>
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<td>For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naive) prior to treatment or testing. (This information can often be tabulated).</td>
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<td>Numbers analysed</td>
<td>15</td>
<td>a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%).</td>
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<td>b. If any animals or data were not included in the analysis, explain why.</td>
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<tr>
<td>Outcomes and estimation</td>
<td>16</td>
<td>Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).</td>
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<td>We always do.</td>
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<td>Adverse events</td>
<td>17</td>
<td>a. Give details of all important adverse events in each experimental group.</td>
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<td>b. Describe any modifications to the experimental protocols made to reduce adverse events.</td>
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<tr>
<td>DISCUSSION</td>
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<td>No adverse events to report.</td>
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<td>Interpretation/ scientific implications</td>
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<td>a. Interpret the results, taking into account the study objectives and hypotheses; current theory and other relevant studies in the literature.</td>
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<td>b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imperfections associated with the results.</td>
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<td>c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.</td>
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<td>Generalisability/ translation</td>
<td>19</td>
<td>Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.</td>
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<tr>
<td>Funding</td>
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<td>List all funding sources (including grant number) and the role of the funder(s) in the study.</td>
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References:
06 June 2012

Andreas Goebel
University of Liverpool
University Hospital Aintree
Liverpool
L9 7AL

Dear Dr Goebel

Study title: A multi-centre (UK) double-blind randomised parallel
group placebo controlled trial to evaluate the efficacy,
safety, and tolerability of intravenous immunoglobulin
(IVlg) 0.5g/kg plus standard treatment, versus matched
placebo plus standard treatment in patients with
longstanding Complex Regional Pain Syndrome.

REC reference: 12/EE/0164
Protocol number: LIPS
EudraCT number: 2012.00058.73

Thank you for your letter of 02 May 2012, responding to the Committee's request for further
information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair in
consultation with the Vice-Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the
above research on the basis described in the application form, protocol and supporting
documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites listed in the application, subject to
management permission being obtained from the NHS-HSC R&D office prior to the start of
the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment
(SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion
does not therefore apply to any non-NHS site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at [http://www.raforum.nhs.uk](http://www.raforum.nhs.uk).

Where a NHS organisation’s role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Clinical trial authorisation must be obtained from the Medicines and Healthcare products Regulatory Agency (MHRA).

The sponsor is asked to provide the Committee with a copy of the notice from the MHRA, either confirming clinical trial authorisation or giving grounds for non-acceptance, as soon as this is available.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

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<th>Document</th>
<th>Version</th>
<th>Date</th>
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<td>Covering Letter</td>
<td>from Caroline Murphy, King's Clinical Trials Unit</td>
<td>23 March 2012</td>
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<tr>
<td>Investigator CV</td>
<td>Dr Andreas Goebel</td>
<td>16 January 2012</td>
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<tr>
<td>Other: Summary of product characteristics</td>
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<td>23 March 2012</td>
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<td>Dr Gareth Amler</td>
<td>27 April 2012</td>
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<td>Participant Consent Form</td>
<td>1.1</td>
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<td>Participant Information Sheet</td>
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<td>Protocol</td>
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<td>Questionnaire: Limit Stress Scale</td>
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<td>Questionnaire: Work Interference Stamford Presenteeism Scale</td>
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<td>102786/305836/1/181</td>
<td>22 March 2012</td>
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<tr>
<td>Response to Request for Further Information</td>
<td>Caroline Murphy</td>
<td>02 May 2012</td>
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</table>

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document “After ethical review – guidance for researchers” gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review
With the Committee’s best wishes for the success of this project

Yours sincerely

Prof Barry Hunt
Chair

Email: Nicky.Stoney@eoe.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to: Ms Caroline Murphy
          Ms Rebecca McDonald, Walton Centre NHS Foundation Trust
Health Research Authority
National Research Ethics Service

NRES Committee North West - Haydock
3rd Floor - Barrow House
4 Moshal Street
Manchester
M1 3DZ

Telephone: 0161 525 7677
Fax 0161 525 7295

19 August 2015

Dr Andreas Goebel
Pain Consultant
Whiston Centre NHS Foundation Trust
Lower Lane
Runcorn
Liverpool
L8 7LJ

Dear Dr Goebel

Study title: Investigation into the pathogenic role and function of IgG Serum Autoantibodies in Complex Regional Pain Syndrome (CRPS).

REC reference: 15/NW/0467
IRAS project ID: 181615

Thank you for your submission of 29 July 2015 responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair and Dr Ben Johnson.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager, Ms Rachel Katzenellenbogen, nrescommittee.northwest-haydock@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above
research on the basis described in the application form, protocol and supporting documentation
as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the
study.

Management permission or approval must be obtained from each host organisation prior to the
start of the study or the site concerned.

Management permission ('R&D approval') should be sought from all NHS organisations involved
in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research
Application System or at http://www.cfforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential
participants to research sites ('participant identification centre'), guidance should be sought from
the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the
procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on
a publicly accessible database. This should be before the first participant is recruited but no later
than 8 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest
opportunity e.g. when submitting an amendment. We will track the registration details as part of
the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for
non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they
should contact hra.study.registration@nhs.net. The expectation is that all clinical trials will be
registered; however, in exceptional circumstances non registration may be permissible with prior
agreement from NRES. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with
before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites
The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/NHSB R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<table>
<thead>
<tr>
<th>Document</th>
<th>Version</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Covering letter on headed paper [Response to provisional opinion]</td>
<td>1.0</td>
<td>02 June 2015</td>
</tr>
<tr>
<td>GP/consultant information sheets or letters [GP letter patients V1.0 2Jun15]</td>
<td>1.0</td>
<td>02 June 2015</td>
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<tr>
<td>GP/consultant information sheets or letters [GP letter volunteers V1.2/0515]</td>
<td>1.0</td>
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</tr>
<tr>
<td>GP/consultant information sheets or letters [GP letter disease control patients V1.0 2Jun15]</td>
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</tr>
<tr>
<td>Letters of invitation to participant [Recruitment Letter V1.0 2Jun15]</td>
<td>1.0</td>
<td>02 June 2015</td>
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<tr>
<td>Letters of invitation to participant [Recruitment Letter to disease control patients V1.0 2Jun15]</td>
<td>1.0</td>
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</tr>
<tr>
<td>Letters of invitation to participant [Recruitment Letter to healthy volunteers V1.0 2Jul15]</td>
<td>1.0</td>
<td>21 July 2015</td>
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<tr>
<td>Other [Patient response slip V1.0 15/015]</td>
<td>1.0</td>
<td>01 May 2015</td>
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<tr>
<td>Other [Protocol V2 21Jul15 tracked changes]</td>
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<tr>
<td>Other [Independent peer review]</td>
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<tr>
<td>Other [Independent peer review for PhD funding]</td>
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<tr>
<td>Participant consent form [Consent Form V2.0 2Jul15 TC]</td>
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<td>Participant information sheet (PIS) [PIS V2 2Jul15 tracked changes]</td>
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<tr>
<td>Participant information sheet (PIS) [PIS V2 15July15 close]</td>
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<tr>
<td>Participant information sheet (PIS) [Volunteer /PIS V2.0 2Jul15 TC]</td>
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<td>Participant information sheet (PIS) [Volunteer PIS V2.0 2Jul15 TC]</td>
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<tr>
<td>Participant information sheet (PIS) [PIS disease control V2.0 2Jul15 TC]</td>
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<td>Participant information sheet (PIS) [PIS disease control V2.0 2Jul15]</td>
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<td>21 July 2016</td>
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<tr>
<td>REC Application Form [REC Form 04/03/2015]</td>
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<td>04 June 2016</td>
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<tr>
<td>Summary CV for Chief Investigator (C) [Signed CV]</td>
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<td>16 May 2015</td>
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<tr>
<td>Validated questionnaires [Brief Pain Inventory]</td>
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Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review
Reporting requirements

The attached document, "After ethical review – guidance for researchers," gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known, please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/govemance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at
http://www.hra.nhs.uk/hra-training/

15/NW/0467 Please quote this number on all correspondence

With the Committee's best wishes for the success of this project.

Yours sincerely,

Dr Tim S Sprosen
Chair

Email: nrescommittee.northwest@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: Dave Walting, Walton Centre NHS Foundation Trust